0026-895X/04/6506-1507–1516$20.00
MOLECULAR PHARMACOLOGY
Vol. 65, No. 6
3117/1153868

Mol Pharmacol 65:1507–1516, 2004
Printed in U.S.A.

Activation of Cyclic AMP-Dependent Protein Kinase Inhibits the Desensitization and Internalization of Metabotropic Glutamate Receptors 1a and 1b

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Received November 25, 2003; accepted February 18, 2004
This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

In this study, we characterized the effects of activation of cyclic AMP-dependent protein kinase (PKA) on the internalization and functional coupling of the metabotropic glutamate receptor (mGluR1) splice variants mGluR1a and mGluR1b. Using an enzyme-linked immunosorbent assay technique to assess receptor internalization, we found that the glutamate-induced internalization of mGluR1a or mGluR1b transiently expressed in human embryonic kidney (HEK) 293 cells was inhibited by coactivation of endogenous β_2-adrenoceptors with isoprenaline or by direct activation of adenylyl cyclase with forskolin. The PKA inhibitor *N*-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H89) blocked the effects of both isoprenaline and forskolin. The heterologous internalization of the mGluR1 splice variants triggered by carbachol was also inhibited by isoprenaline and forskolin in a PKA-sensitive fashion, whereas the constitutive (agonist-independent) internalization of mGluR1a was inhibited only modestly by PKA activation. Using inositol phosphate (IP) accumulation in cells prelabeled with [^{3}H]inositol to assess receptor coupling, PKA activation increased basal IP accumulation in mGluR1a receptor-expressing cells and also increased glutamate-stimulated IP accumulation in both mGluR1a- and mGluR1b-expressing cells, but only at short times of glutamate addition. Furthermore, PKA activation completely blocked the carbachol-induced heterologous desensitization of glutamate-stimulated IP accumulation in both mGluR1a- and mGluR1b-expressing cells. In coimmunoprecipitation experiments, the ability of glutamate to increase association of GRK2 and arrestin-2 with mGluR1a and mGluR1b was inhibited by PKA activation with forskolin. Together, these results indicate that PKA activation inhibits the agonist-induced internalization and desensitization of mGluR1a and mGluR1b, probably by reducing their interaction with GRK2 and nonvisual arrestins.

Glutamate is the major excitatory neurotransmitter in the central nervous system and acts through both ionotropic and metabotropic (mGluR) receptors. The former drive fast neurotransmission, whereas the stimulation of mGluRs generates slower and longer-lasting changes in the signaling cascades activated in neuronal and glial cells, which may play a critical role in glutamate-mediated neurotransmission and synaptic plasticity events (for reviews, see Pin and Duvoisin, 1995; Schoepp et al., 1999; Hermans and Challis 2001). The mGluRs are members of a distinct G protein-coupled receptor (GPCR) superfamily that shows little similarity with widely studied GPCRs such as β-adrenoceptors. On the basis of their pharmacology, amino acid sequence similarity, and signal transduction mechanisms, mGluRs have been classified into three groups, with the group I mGluR1 and mGluR5 receptors being coupled to G_q and phospholipase C. Five splice variants of the mGluR1 receptor have been described thus far, all of which differ in their length of COOH-terminal tail (Pin et al., 1992; Pin and Duvoisin, 1995; Prezeau et al., 1996).

Upon prolonged agonist addition, most GPCRs undergo decreased agonist responsiveness, a process known as desensitization (reviewed in Krupnick and Benovic, 1998). Mechanisms underlying desensitization are complex and can involve phosphorylation of the receptor, uncoupling from G proteins, internalization, and ultimately intracellular downregulation. Recent studies have shown that the desensitization and internalization of mGluR1a can involve GPCR ki-

This work was supported by the Medical Research Council.

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; PKC, protein kinase C; CaMKII, Ca^{2+} calmodulin-dependent kinase II; PKA, protein kinase A; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; HEK, human embryonic kidney; TBS, Tris-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; H89, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride; TBS-T, Tris-buffered saline-Tween 20; DM-I, deletion mutant I of the metabotropic glutamate receptor 1 (Arg^{847}stop); DM-II, deletion mutant II of the metabotropic glutamate receptor 1 (Arg^{868}stop); GRK, G protein-coupled receptor kinase.

nases (GRKs), and arrestins (Dale et al., 2000; Sallese et al., 2000; Mundell et al., 2001, 2002, 2003; Iacovelli et al., 2003). It is likely that GRK phosphorylation of mGluR1a leads to arrestin association with the receptors, thereby uncoupling the receptors from G-protein. Arrestins also couple many GPCRs to the clathrin-coated pit internalization pathway (Goodman et al., 1996; Ferguson, 2001), and it is clear that mGluR1a can undergo arrestin- and clathrin-dependent internalization (Mundell et al., 2001, 2002). Receptors can then either be dephosphorylated and returned to the cell surface for another round of activation or enter an intracellular degradative pathway (reviewed in Ferguson, 2001).

In recent work, we have begun to identify important roles for second messenger-dependent protein kinases in mGluR1 splice variant regulation. For example, PKC activation contributes to the glutamate-induced desensitization and internalization of the mGluR1a long splice variant but not the mGluR1b short splice variant (Mundell et al., 2002). On the other hand, both PKC and CaMKII activation are required for the $G_{q/11}$-coupled receptor-stimulated heterologous desensitization and internalization of mGluR1a and mGluR1b (Mundell et al., 2002, 2003, 2004). A role for PKA in mGluR trafficking has so far not been reported; however, PKA involvement in the trafficking of other GPCRs, such as the G_s-coupled dopamine D_1 receptor (Mason et al., 2002) and secretin receptor (Walker et al., 1999), has recently been identified. Furthermore, because group II and III mGluR function is regulated by PKA (Schaffhauser et al., 2000; Cai et al., 2001), we thought it possible that PKA also regulates group I mGluRs. Therefore, in the present study, we investigated the role of PKA in the internalization and desensitization of mGluR1a and mGluR1b. Our results indicate that activation of PKA strongly inhibits the agonist-induced internalization of these receptor splice variants and also inhibits both the homologous and heterologous desensitization of mGluR1a and mGluR1b responsiveness. These effects may occur because of the ability of PKA activation to inhibit the agonist-induced association of GRK2 and nonvisual arrestins with the splice variants.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and LipofectAMINE 2000 transfection reagent were obtained from Invitrogen (Paisley, Scotland, UK). Mouse monoclonal anti-HA antibody (3F10) and protein G were from Roche (Lewes, East Sussex, UK), anti-HA-monoclonal antibody (HA-11) and goat anti-mouse fluorescein-conjugated secondary antibody were from Molecular Probes (Eugene, OR). The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, the horseradish peroxidase-conjugated sheep anti-mouse secondary antibody, and [*myo*-^{3}H]inositol (37 MBq/ml) were all from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All other reagents were purchased from Sigma (Poole, Dorset, UK). The anti-GRK2 monoclonal antibody and the anti-arrestin-2 polyclonal antibody were generous gifts from Prof. Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA).

Constructs, Cell Culture, and Transfection. The construction of HA-epitope tagged mGluR1a and mGluR1b subcloned into pcDNA3 has been described previously (Mundell et al., 2001, 2002). The construction of HA epitope tagged DM-I (Arg^{847}stop) and DM-II (Arg^{868}stop) receptor COOH-terminal deletion mutants subcloned into pcDNA3 has also been described previously (Mundell et al., 2003). In all cases, the HA epitope was introduced into the N terminus of the mGluR1 receptor cDNA between amino acids 57 and 58. HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (complete media) at 37°C in a humidified atmosphere of 95% air/5% CO_2. For transient transfections, HEK293 cells were grown in 60- or 100-mm dishes to 80 to 90% confluence and transfected with 5 μg of DNA using LipofectAMINE 2000 following the manufacturer's instructions. Cells were incubated with a DNA/LipofectAMINE mixture for 24 h, the media was replaced, and the cells were used for experimentation 24 h later.

Internalization of mGluR1a and mGluR1b in HEK293 Cells. The agonist-induced cell surface loss of mGluR1a, mGluR1b, DM-I, and DM-II was assessed by ELISA as described previously (Mundell et al., 2001). In brief, cells plated at a density of approximately 6×10^5 cells per 100-mm dish were transiently transfected with mGluR1a, mGluR1b, DM-I, or DM-II. Twenty-four hours after transfection, cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml poly-L-lysine. Another 24 h later, cells were incubated with DMEM (without L-glutamine) at 37°C, to which various combinations of glutamate (10 μM), carbachol (1 mM), isoprenaline (10 μM), forskolin (10 μM), or H89 (10 μM) were added for various times as indicated in the figure legends. Reactions were stopped by removing the medium and fixing the cells with 3.7% formaldehyde in TBS (20 mM Tris, 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 μl of sample was added to 100 μl of 0.4 M NaOH to terminate the reaction, and the samples were read at 405 nm using a microplate reader. Internalization is expressed as percentage loss of surface receptor compared with time 0, with the background signal from pcDNA3-transfected controls subtracted from all receptor-transfected values.

To assess the effects of PKA activation on cell surface levels of mGluR1a and mGluR1b, cells were treated initially as described above before incubation of cells in the presence or absence of forskolin (10 μM) for up to 60 min. Cell surface levels of receptor were compared with surface receptor expression at time 0 (taken as 100%), with the background signal from pcDNA3-transfected controls subtracted from all receptor-transfected values.

For constitutive (agonist-independent) internalization, cells were plated and transfected as described above. For experimentation, culture medium was replaced with fresh complete medium containing 1% BSA and primary antibody (anti-HA monoclonal HA-11; dilution, 1:1000) and incubated for 45 min at 37°C. Cells were then washed once with TBS and incubated at 37°C for up to 30 min more in fresh complete medium containing 1% BSA without primary antibody and in the presence or absence of forskolin (10 μM). The loss of cell surface receptor was then analyzed by ELISA, as described above. Background signal from pcDNA3-transfected controls was subtracted from all receptor-transfected values, and constitutive internalization was calculated by comparison with surface receptor expression at time 0 (taken as 100%).

Immunofluorescence Microscopy. The cellular distribution of mGluR1a and mGluR1b transiently transfected into HEK293 cells was assessed by immunofluorescence microscopy (Mundell et al., 2001, 2002). In brief, HEK293 cells, grown on poly-L-lysine–coated coverslips in six-well plates, were transiently transfected with pcDNA3 containing mGluR1a or mGluR1b. Forty-eight hours after transfection, receptor distribution was assessed using a primary anti-HA monoclonal antibody (HA-11; 1:200) and a goat anti-mouse

fluorescein-conjugated secondary antibody (1:200). Coverslips were mounted using Slow-Fade mounting medium and examined by microscopy on an upright Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with phase-contrast (Leica, Wetzlar, Germany) and a planapochromat 40 × 1.40 numerical aperture oil immersion objective. All images were collected on Leica TCS-NT software for two- and three-dimensional image analysis and processed on Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA).

Inositol Phosphate Determination. This was undertaken as described previously (Mundell and Benovic, 2000). In brief, cells plated at a density of around 6×10^5 cells in each 60-mm dish were transiently transfected with 5 μg of mGluR1a or mGluR1b DNA. Twenty-four hours after transfection, cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml poly-L-lysine. The following day, cells were labeled for 18 to 24 h with [*myo*-^{3}H]inositol (4 μCi/ml of culture medium) in DMEM (high glucose, without inositol). After labeling, cells were washed once in phosphate-buffered saline and incubated in prewarmed DMEM (without L-glutamine) containing 20 mM LiCl. In some experiments, the effect of PKA activation on receptor constitutive activity was examined by incubating cells for 30 min in the presence or absence of forskolin (10 μM). To assess the effects of PKA activation on glutamate-stimulated IP accumulation, cells were incubated in the presence or absence of forskolin (10 μM) or dibutyryl cAMP (1 mM) for 15 min before and during glutamate (10 μM) addition for up to 30 min. In some experiments H89 (10 μM) was included for 15 min before and during dibutyryl cAMP/glutamate addition. For heterologous desensitization experiments, cells were incubated in the presence or absence of H89 for 15 min before and during a further 30-min incubation with carbachol (1 mM) in the presence or absence of forskolin (10 μM). After this, cells were washed before being incubated with glutamate (10 μM) for 30 min. In other experiments, cells transiently transfected with mGluR1a were incubated with or without forskolin (10 μM) for 10 min before and during addition of carbachol (1 mM), ADP (10 μM), or NaF (10 μM) for a further 30 min. In all cases, 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 $KHCO_3$ were added. Tubes were vortexed and centrifuged for 5 min at 14,000 rpm in a microcentrifuge. Inositol phosphates were separated on Dowex AG 1-X8 columns exactly as described previously (Mundell and Benovic, 2000). Total labeled inositol phosphates were determined by liquid scintillation counting. Results are expressed as either fold increase in basal [^{3}H]IP accumulation over that in nontransfected HEK293 cells, as fold increase in total [^{3}H]IP over basal, or as total [^{3}H]IP accumulation divided by the total [^{3}H]inositol content of the cell sample.

Coimmunoprecipitation Experiments. HEK293 cells were transiently transfected with 5 μg of mGluR1a or mGluR1b DNA, along with 2 μg of either empty pcDNA3 or pcDNA3 containing arrestin-2-GFP or GRK2-GFP. After agonist treatment, cells from 60-mm dishes were washed twice with ice-cold phosphate-buffered saline and lysed in 500 μl of ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100 containing the protease inhibitors leupeptin, aprotinin, and phenylmethylsulfonyl fluoride, each at 20 μg/ml). The particulate fraction was removed by microcentrifugation at 14,000 rpm, and 300 μg of supernatant protein was incubated in the presence of anti-HA monoclonal antibody (3F10, 1:500) and 100 μl 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 to 18 h. The beads were subsequently washed three times with immunoprecipitation buffer and solubilized in 2× SDS sample buffer, and the immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Arrestin-2-GFP was detected by immunoblotting with a rabbit polyclonal arrestin-2 antibody (Kim and Benovic, 2002) diluted 1:200, and GRK2-GFP by a monoclonal antibody (Loudon et al., 1996) 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% skim 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% skim milk. The nitrocellulose membranes were then rinsed three times with TBS-T and incubated with ECL Western blotting detection reagents.

Experimental Design and Statistics. Data were analyzed using Prism (GraphPad Software, San Diego, CA). Where appropriate, statistical significance was assessed by Mann-Whitney U test, two-tailed paired *t* test, or two-way analysis of variance.

Results

Effect of PKA Activation on the Internalization of mGluR1a and mGluR1b. In HEK293 cells transiently transfected with HA epitope-tagged mGluR1a, incubation of cells with glutamate (10 μM; 30 min) promoted ~30% internalization of cell surface receptors (Fig. 1A), in agreement with previous studies (Mundell et al., 2001, 2002). Agonist activation of endogenous β_2-adrenoceptors (Mundell and Benovic, 2000) with isoprenaline (10 μM) did not induce internalization of mGluR1a but instead markedly inhibited glutamate-stimulated internalization of mGluR1a (Fig. 1A). In further experiments, either isoprenaline or forskolin (each 10 μM) inhibited the glutamate-induced internalization of both mGluR1a and mGluR1b (Fig. 1, B and C). These effects were mediated by PKA, because the selective PKA inhibitor H89 (10 μM) reversed the effects of isoprenaline and forskolin on glutamate-induced internalization of the mGluR1 splice variants. It is noteworthy that H89 alone did not induce internalization of mGluR1a or mGluR1b or affect glutamate-induced internalization of the splice variants (Fig. 1B). These results were confirmed by immunofluorescent microscopy (Fig. 2). Under basal conditions, mGluR1a and mGluR1b were localized to the cell surface, although some mGluR1a could also be detected intracellularly (Fig. 2, left). After glutamate treatment (10 μM; 30 min) both receptors underwent intracellular redistribution and exhibited a distinct punctate pattern, probably representing internalized receptors in endosomes (Mundell et al., 2001). In the presence of forskolin, glutamate produced little receptor internalization, whereas H89 treatment blocked the inhibitory effect of forskolin such that glutamate was again able to promote mGluR1a and mGluR1b internalization (Fig. 2, right).

To assess the role of the COOH-terminal tail of the mGluR1 splice variants in the PKA effects, the ability of forskolin to inhibit the glutamate-induced internalization of two receptor deletion mutants was tested (Fig. 3). The mutants are Arg^{847} stop and Arg^{868} stop (Mundell et al., 2003), which are in fact deletion mutants of both splice variants, because the splice site is at Ala^{886}. The glutamate-induced internalization of DM-I and DM-II was somewhat reduced compared with mGluR1a and mGluR1b (in agreement with our previous study; Mundell et al., 2003). However, whereas forskolin (10 μM) strongly inhibited the glutamate-induced internalization of mGluR1a (69% inhibition) and mGluR1b (99% inhibition), there was weaker inhibition of the glutamate-induced internalization of DM-I (34% inhibition) and DM-II (28% inhibition) (Fig. 3).

We have recently shown that carbachol activation of muscarinic acetylcholine receptors in HEK293 cells also triggers internalization of mGluR1a and mGluR1b receptors by a

PKC-/CaMKII-dependent process (Mundell et al., 2002). We therefore assessed whether PKA activation would modify carbachol-induced internalization of the splice variants (Fig. 4). In fact, coaddition of isoprenaline or forskolin (each 10 μM) almost abolished the carbachol-induced (1 mM) internalization of each splice variant. This inhibitory effect was mediated by PKA activation, because the effects of isoprenaline and forskolin were completely reversed by H89 (10 μM; Fig. 4).

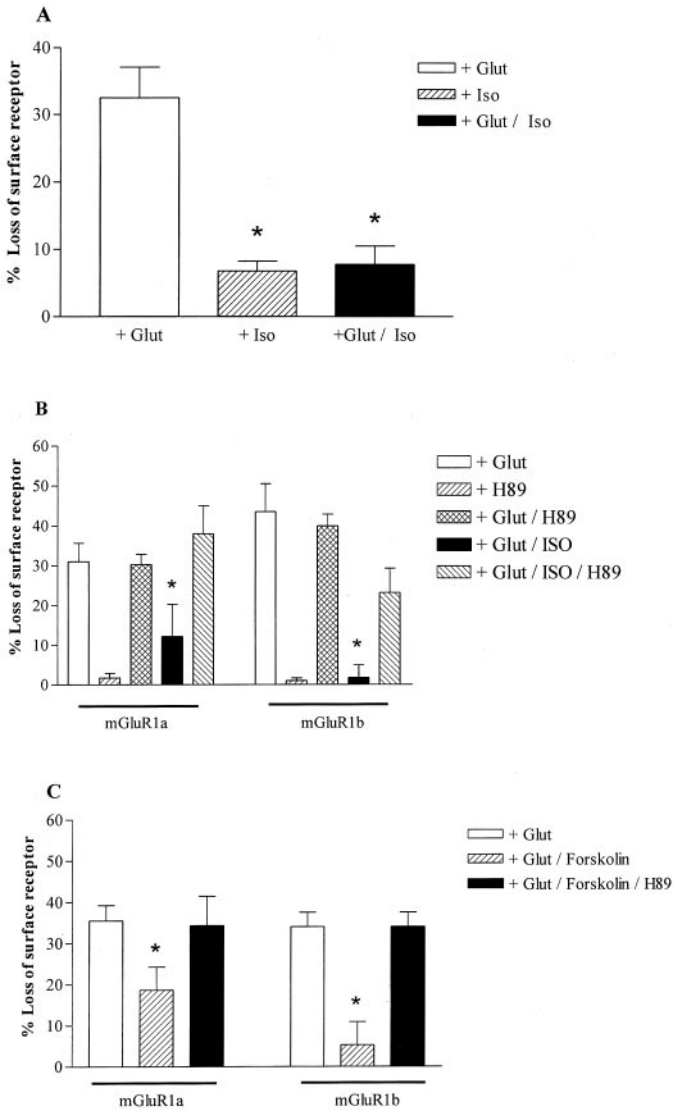

Fig. 1. Glutamate-induced internalization of mGluR1a and mGluR1b is inhibited by PKA activation. A, HEK293 cells were transiently transfected with 5 μg of mGluR1a DNA, and 2 days later cell surface loss of receptor was assessed by ELISA. Cells were challenged for 30 min with either glutamate (10 μM), the β_2-adrenoceptor agonist isoprenaline (10 μM), or a combination of both agonists. B and C, HEK293 cells were transiently transfected with 5 μg of mGluR1a or mGluR1b DNA, and 2 days later cell surface loss of receptor was assessed by ELISA. Cells were incubated in the presence or absence of the PKA inhibitor H89 (10 μM) for 30 min before and during agonist addition. Cells were challenged for 30 min with glutamate (10 μM) in the absence or presence of isoprenaline (10 μM) (B) or forskolin (10 μM) (C). The lack of effect of H89 alone on surface receptor expression, and the inability of H89 to affect glutamate-induced internalization of mGluR1a and mGluR1b is also shown in B. Data are shown relative to unstimulated cells. In each case, the data are mean ± S.E. from four to seven independent experiments. *, $p < 0.05$ compared with respective value for glutamate alone (Mann-Whitney U test).

The constitutive (agonist-independent) internalization of mGluR1a has recently been reported in HEK293 cells (Dale et al., 2001), so we were also interested to assess any possible effects of PKA activation on this form of trafficking. Using a protocol in which mGluR1a and mGluR1b on the cell surface are labeled with antibody at 37°C, we found that mGluR1a internalizes rapidly and extensively (~60% internalization after 30 min; $t_{0.5}$ of <5 min), whereas mGluR1b does not undergo internalization under these conditions (Fig. 5A). Inclusion of forskolin only modestly inhibited (by 10–20%) the constitutive internalization of mGluR1a. To ascertain whether the inhibition of constitutive internalization might affect overall cell surface expression of mGluR1a, we assessed the effect of forskolin (10 μM) on the cell surface expression of mGluR1a and mGluR1b (Fig. 5B). In this experiment, cells were incubated with forskolin before addition of antibody to determine cell surface expression of receptor. Although forskolin had no effect on cell surface expression of mGluR1b, incubation of mGluR1a-expressing cells with forskolin for up to 60 min slightly increased cell surface expression of receptor by 15 to 20%.

Effect of PKA Activation on the Coupling of mGluR1a and mGluR1b. To determine the effect of PKA activation on the coupling and desensitization of mGluR1 splice variants, cells were prelabeled with [^{3}H]inositol and total IP accumulation was subsequently assessed. We first investigated the effect of forskolin (10 μM; 30 min) on basal IP accumulation (Fig. 6). Compared with nontransfected and mGluR1b-expressing HEK293 cells, mGluR1a-expressing cells displayed constitutive activity; basal IP levels were 1.5- to 2-fold greater in mGluR1a-expressing cells than in nontransfected or mGluR1b-transfected HEK293 cells (Fig. 6A). Incubation of mGluR1a-expressing cells with forskolin (10 μM; 30 min) almost doubled basal IP accumulation but had no effect on basal IP accumulation in mGluR1b-expressing cells. Furthermore, H89 blocked the ability of forskolin to increase basal IP accumulation in mGluR1a-expressing cells (Fig. 6A). In initial experiments, we found that glutamate-stimulated (10 μM; 30 min) IP accumulation in mGluR1a- and mGluR1b-expressing cells was unchanged by forskolin (10 μM) treatment (data not shown). However, interpretation of the data for mGluR1a may be challenging; for example, basal IP accumulation changes with forskolin treatment, which could alter stimulated IP accumulation when expressed as fold over basal. To circumvent this, we constructed a time course (0–30 min) of glutamate-stimulated IP accumulation in mGluR1a- and mGluR1b-expressing cells, expressing stimulation in terms of [^{3}H]IP produced over total [^{3}H]inositol label incorporated into the sample (Fig. 6, B and C). Under these conditions, IP accumulation in mGluR1a- or mGluR1b-expressing cells after 5- or 15-min incubation with glutamate was increased by forskolin. However, as observed previously, glutamate-stimulated IP accumulation after 30 min was similar in the presence or absence of forskolin incubation. In further experiments, the PKA activator dibutyryl cAMP (1 mM) was also found to increase basal and glutamate-stimulated (10 μM, 5 min) IP accumulation in mGluR1a-expressing cells, as well as glutamate-stimulated IP accumulation in mGluR1b-expressing cells (Fig. 6D). The dibutyryl cAMP-mediated increase in glutamate responsiveness was blocked by co-inclusion of 10 μM H89 (Fig. 6D). Together, these results indicate that PKA activation in-

creases the constitutive activity of mGluR1a and increases the glutamate-stimulated IP accumulation in both mGluR1a- and mGluR1b-expressing cells when assessed after short periods of agonist incubation.

Carbachol pretreatment not only promotes internalization of mGluR1 splice variants (Mundell et al., 2002), but also triggers the heterologous desensitization of mGluR1a and mGluR1b responsiveness (Mundell et al., 2004). Thus, pretreatment of mGluR1a- or mGluR1b-expressing cells with carbachol (1 mM; 10 min) reduced the subsequent ability of glutamate to stimulate IP accumulation (Fig. 7A). On the other hand, inclusion of forskolin (10 μM) with carbachol completely reversed the latter's ability to desensitize mGluR1a and mGluR1b responsiveness, whereas coaddition of H89 (10 μM) blocked the effect of forskolin such that carbachol was again able to induce mGluR1 desensitization. The effect of forskolin could be caused by inhibition of muscarinic acetylcholine receptor function. To test this, we examined the effect of forskolin on IP accumulation stimulated by carbachol (1 mM), and also the purinergic receptor agonist ADP (10 μM) and the direct activator of the $G_{q/11}$ protein, NaF (Fig. 7B). However, forskolin had no effect on IP accumulation stimulated by these agonists, indicating that PKA activation does not affect carbachol's ability to activate endogenous muscarinic acetylcholine receptors and elevate cellular IP levels.

Effect of PKA Activation on the Association of GRK2 and Arrestin-2 with mGluR1a and mGluR1b. Using a coimmunoprecipitation protocol in cells transfected with mGluR1a or mGluR1b as well as wild-type GRK2 or arrestin-2, we have recently shown that glutamate increases the association of GRK2 and arrestin-2 with the mGluR1 splice variants (Mundell et al., 2004). In the present study, we examined the effects of PKA activation on glutamate's ability to promote the association of GRK2-GFP (Fig. 8) and arrestin-2-GFP (Fig. 9) with mGluR1a and mGluR1b. GFP-tagged proteins were used to avoid confusion of GRK2/arrestin-2 bands with the IgG heavy chain on immunoblots. In the absence of glutamate, both GRK2-GFP and arrestin-2-GFP were to some extent associated with mGluR1a and mGluR1b (Fig. 8A and 9A, lanes 2 and 6). Glutamate stimulation markedly increased the association of GRK2-GFP and arrestin-2-GFP with the receptors (Fig. 8A and 9A, lanes 3 and 7). However, forskolin reversed the glutamate-induced association of GRK2-GFP and arrestin-2-GFP with the mGluR1 splice variants (Fig. 8A and 9A, lanes 4 and 8), back to levels of association observed in the absence of glutamate. On the other hand, coincubation with H89 blocked the inhibitory

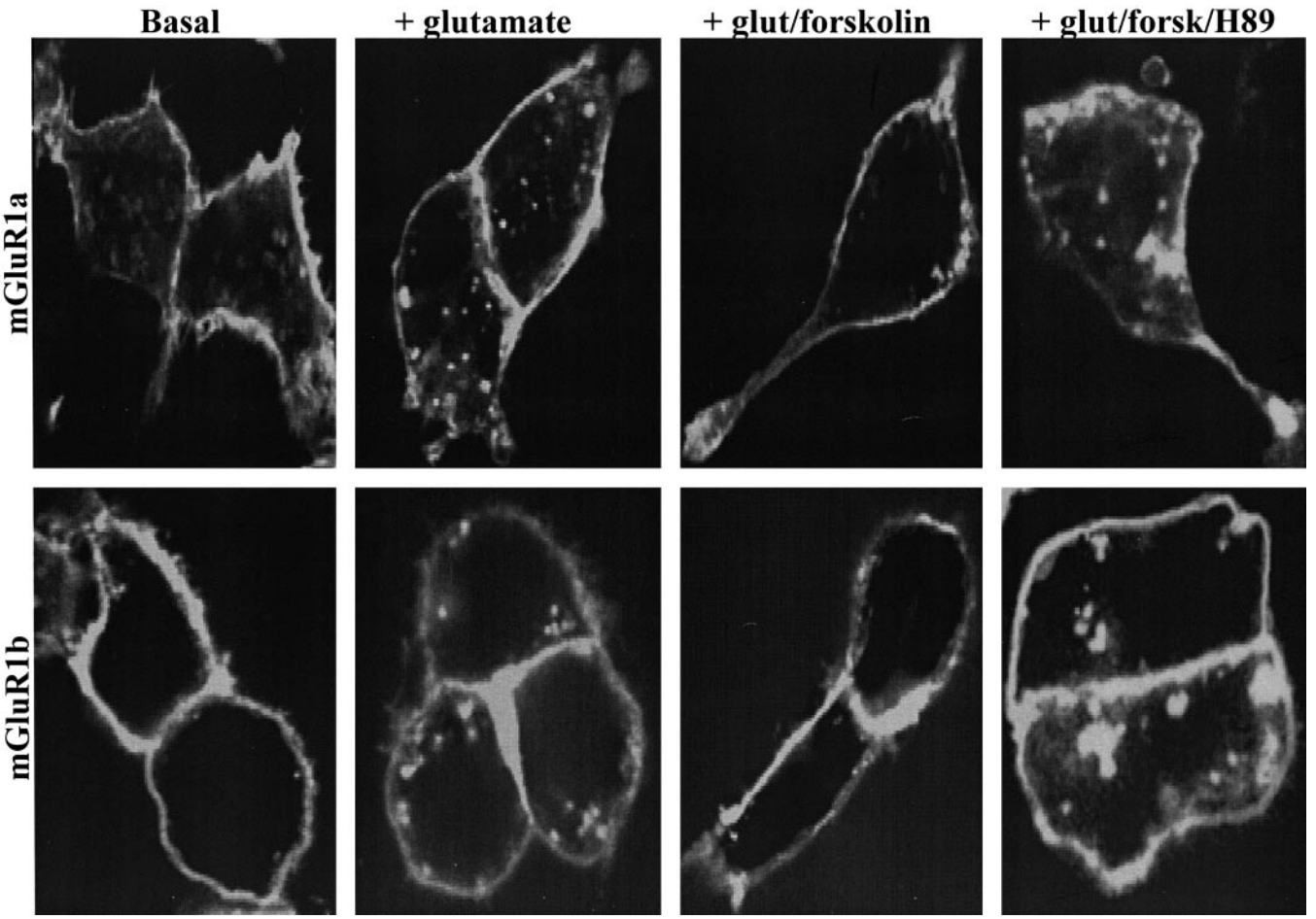

Fig. 2. Imaging of mGluR1 splice variant internalization. HEK293 cells were transiently transfected with 5 μg of mGluR1a (top) or mGluR1b (bottom) and used in experiments 2 days later. Cells were preincubated with an anti-HA antibody at 4°C for 1 h. Subsequently cells were incubated at 37°C for 30 min in the absence (Basal) or presence of 10 μM glutamate (+ glutamate), 10 μM each of glutamate and forskolin (+ glut/forskolin), or 10 μM each of glutamate, forskolin, and H89 (+ glut/forsk/H89). Where appropriate, H89 was added 15 min before forskolin, which was in turn added 15 min before glutamate. Receptor localization was determined by immunofluorescence in fixed cells. The mGluR1 splice variants were visualized using a fluorescein-conjugated secondary antibody.

effect of forskolin on the mGluR1/GRK-2-GFP and mGluR1/arrestin-2-GFP interactions (Fig. 8A and 9A, lanes 5 and 9). H89 alone slightly reduced the basal association of GRK2-GFP and arrestin-2-GFP with mGluR1a; importantly, however, it did not affect the glutamate-induced association of GRK2-GFP or arrestin-2-GFP with this splice variant (Fig. 8C and 9C). Together, these results suggest that PKA inhibits glutamate-induced internalization by reducing the ability of mGluR1a and mGluR1b to associate with GRK2 and arrestin-2.

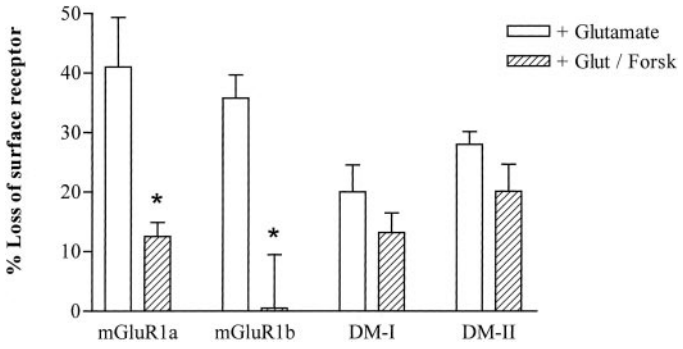

Fig. 3. Truncation of the COOH-terminal tail of mGluR1a and mGluR1b reduces the inhibitory effect of PKA on glutamate-induced internalization. HEK293 cells were transiently transfected with 5 μg of mGluR1a, mGluR1b, DM-I (Arg^{847} stop), or DM-II (Arg^{868} stop) DNA, and 2 days later cell surface receptor loss was assessed by ELISA. Cells were incubated in the absence or presence of forskolin (10 μM) for 30 min before and during glutamate addition (10 μM) for 30 min. Data are shown relative to unstimulated cells. In each case, the data are mean ± S.E. from four to six independent experiments. *, $p < 0.05$ compared with respective value for glutamate alone (Mann-Whitney U test). Forskolin did not significantly reduce the glutamate-induced internalization of DM-I or DM-II. Although the glutamate-induced internalization of DM-I and DM-II was lower than that for mGluR1a and mGluR1b, these reductions did not attain statistical significance.

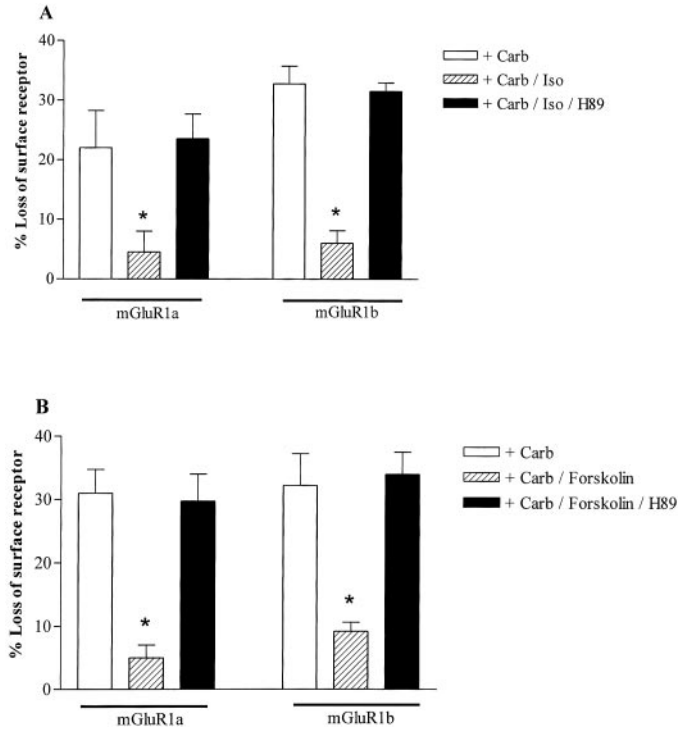

Fig. 4. Carbachol-induced internalization of mGluR1a and mGluR1b is inhibited by PKA activation. HEK293 cells were transiently transfected with 5 μg of mGluR1a or mGluR1b DNA, and 2 days later cell surface loss of receptor was assessed by ELISA. Cells were incubated in the presence or absence of H89 (10 μM) for 30 min before and during agonist addition. Cells were challenged for 30 min with either carbachol (1 mM) alone or a combination of carbachol and isoprenaline (10 μM) (A) or carbachol and forskolin (10 μM) (B) for 30 min. Data are shown relative to unstimulated cells. In each case, the data are mean ± S.E. from four independent experiments. *, $p < 0.05$ compared with respective value for carbachol alone (Mann-Whitney U test).

Discussion

This study identifies PKA as a key regulator of mGluR1 coupling and trafficking and further highlights the complex nature of mGluR1 regulation by protein kinases. In earlier studies, we implicated GRK2, PKC, and CamKII activity in the regulation of mGluR1a and mGluR1b internalization (Mundell et al., 2002, 2003). We now report that PKA has a marked inhibitory effect on the agonist-induced internalization of mGluR1a and mGluR1b. Now, therefore, these mGluR1 splice variants are known to be regulated by at least four different cellular kinases.

Activation of PKA after agonist stimulation of β2-adrenoceptors with isoprenaline or direct activation of adenylyl cyclase with forskolin inhibited the glutamate-induced inter-

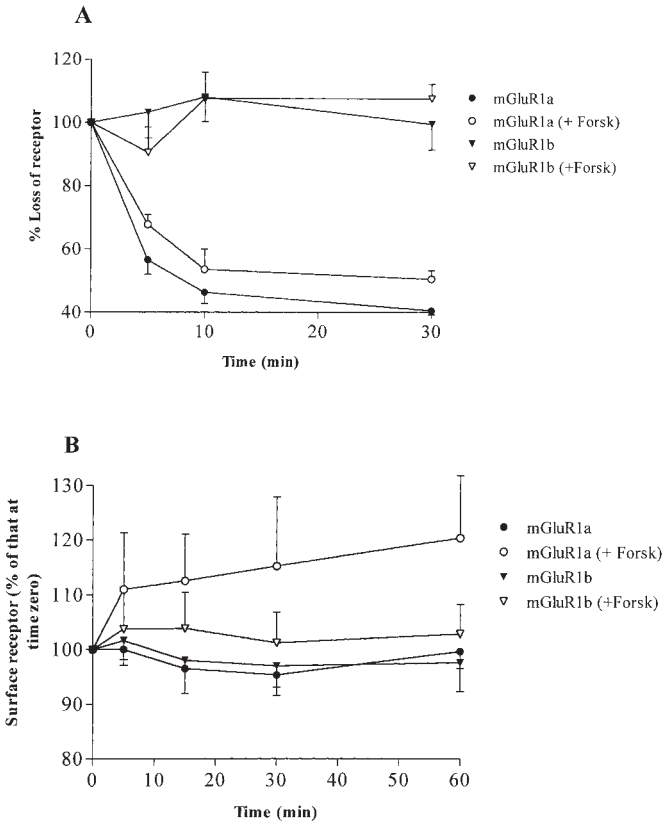

Fig. 5. Effect of PKA activation on the constitutive internalization of mGluR1a. HEK293 cells were transiently transfected with 5 μg of mGluR1a or mGluR1b DNA and used for ELISA 2 days later. A, constitutive (agonist-independent) cell surface loss of mGluR1a and mGluR1b was assessed in cells preincubated with anti-HA antibody. The effects of PKA activation were assessed by incubating cells in the presence or absence of forskolin (10 μM) for up to 30 min. The data are mean ± S.E. from four independent experiments. B, the effect of PKA activation on cell surface levels of mGluR1a and mGluR1b. Cells were incubated in the presence or absence of forskolin (10 μM) for up to 60 min before assessment of total cell surface receptor number. The data are mean ± S.E. from six to seven independent experiments.

nalization of mGluR1a and mGluR1b. These effects are mediated by PKA because H89 reversed the effects of isoprenaline and forskolin. As far as we are aware, this is the first report of PKA-mediated regulation of mGluR trafficking. The effect of isoprenaline and forskolin on glutamate-induced internalization was somewhat greater for mGluR1b than mGluR1a. The reason for this is unclear but may be related to the different mechanisms underlying glutamate-induced internalization of the two splice variants (mGluR1a–GRK2 and PKC, mGluR1b–GRK2 only; Mundell et al., 2003). The PKA-mediated inhibition of internalization was also dependent upon the COOH-terminal tail of mGluR1a and mGluR1b, because forskolin was less effective in inhibiting the glutamate-induced internalization of receptor COOH-terminal tail deletion mutants. However, because the glutamate-induced internalization of the deletion mutants is arrestin-independent (Mundell et al., 2003), PKA activation may be unable to inhibit the arrestin-independent component of internalization of the deletion mutants. We found previously that deletion of the COOH-terminal tails of mGluR1a and mGluR1b up to or beyond Arg^{868} inhibited the GRK2- and PKC-dependent components of mGluR1a internalization, as well as the GRK2-dependent component of mGluR1b internalization (Mundell et al., 2003). Thus, the COOH terminus of these splice variants seems to be critical for regulation of receptor trafficking by multiple kinases.

In addition to glutamate-induced internalization, we have recently found that agonist activation of endogenous muscarinic acetylcholine receptors in HEK293 cells triggers the internalization of agonist-unoccupied mGluR1 splice variants in a PKC- and CaMKII-dependent fashion (Mundell et al., 2002, 2003). In this study, we showed that PKA activation also blocks carbachol-induced internalization, indicating that glutamate occupation of mGluR1a or mGluR1b is not necessary for the action of PKA. We are at present unable to conclude whether PKA directly phosphorylates the mGluR1 splice variants or phosphorylates another protein substrate that then interacts with, or dissociates from, the receptor COOH terminus. In this regard, PKA was recently reported to phosphorylate GRK2 (Cong et al., 2001), which, as we show in the present study, associates with mGluR1a and mGluR1b in an agonist-dependent fashion. However, because PKA phosphorylation is reported to enhance GRK2 activity by increasing recruitment of the GRK2 to the plasma membrane (Cong et al., 2001), then we would expect such an interaction to increase rather than inhibit glutamate-induced internalization. On the contrary, in the present study, coimmunoprecipitation experiments show that PKA activa-

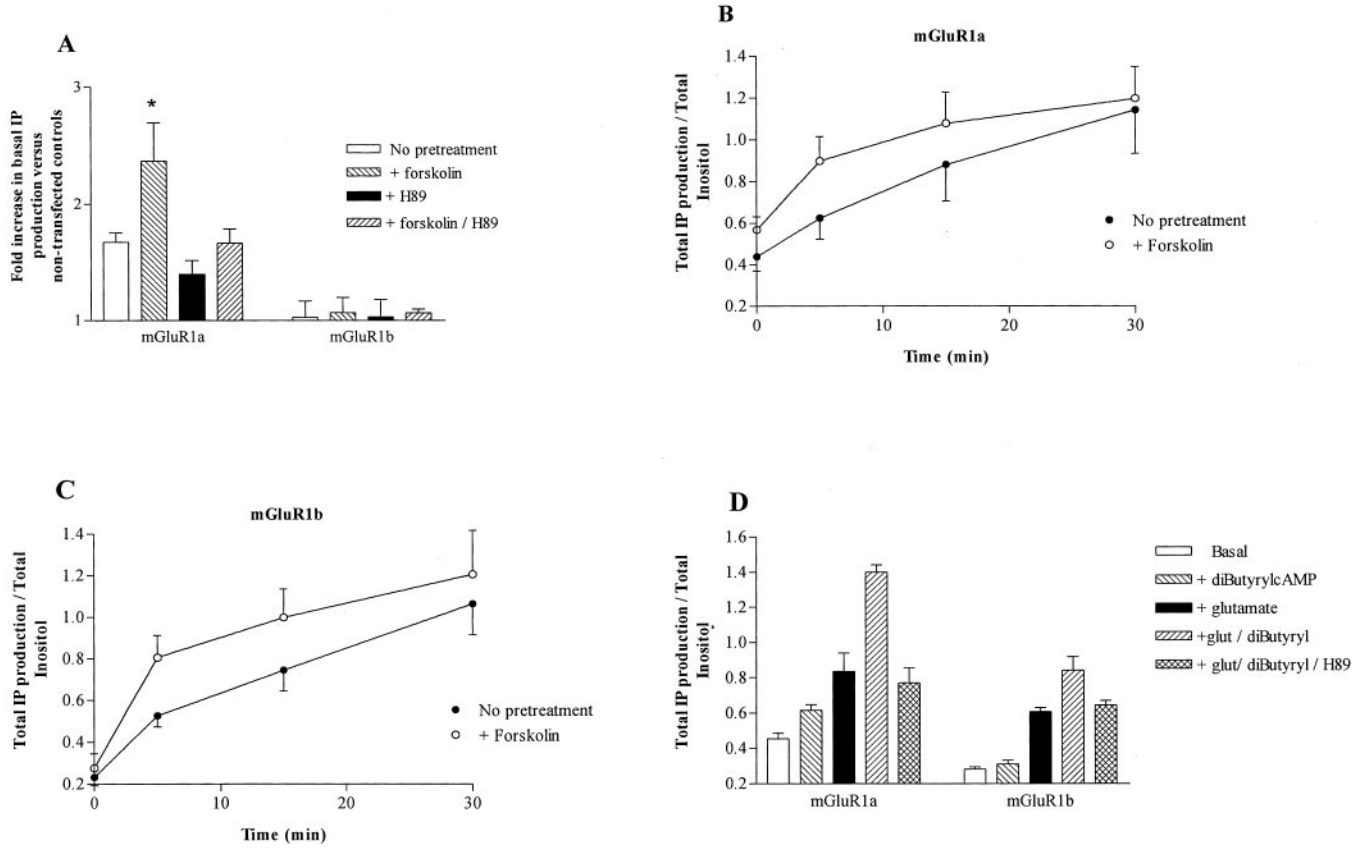

Fig. 6. Activation of PKA increases acute glutamate-stimulated IP accumulation in cells expressing mGluR1a or mGluR1b. Cells transiently transfected with mGluR1a or mGluR1b were used for IP accumulation assays 2 days later. A, basal IP accumulation was measured in the presence or absence of forskolin (10 μM; 30 min). In some cases, cells were incubated in the presence of the PKA inhibitor H89 (10 μM) for 15 min before and during forskolin addition. The data are mean ± S.E. of three independent experiments and are expressed as fold over control, which is IP accumulation in nontransfected HEK293 cells. *, $p < 0.05$ compared with respective value with no pretreatment (Mann-Whitney U test). B and C, effect of forskolin (10 μM) on the time course of glutamate-stimulated (10 μM) total IP accumulation. The data are mean ± S.E. of five independent experiments and are expressed as total IP production/total inositol in the cell sample. Overall, glutamate-stimulated IP accumulation was greater in the presence of forskolin for both mGluR1a- and mGluR1b-expressing cells ($p < 0.05$ by two-way analysis of variance). D, effect of dibutyryl cAMP (1 mM) on basal and glutamate-stimulated (10 μM; 5 min) total IP accumulation. In some cases, 10 μM H89 was added for 15 min before and during dibutyryl cAMP addition. The data are mean ± S.E. of four independent experiments and are expressed as total IP production/total inositol in the cell sample.

tion strongly inhibits the glutamate-induced association of GRK2 and arrestin-2 with mGluR1 splice variants. This therefore represents the likely mechanism whereby PKA blocks glutamate-induced internalization and possibly desensitization.

The constitutive (agonist-independent) internalization of mGluR1a has recently been reported (Dale et al., 2001). Using a protocol in which mGluR1 splice variants were prelabeled with antibody, we also observed extensive constitutive internalization of mGluR1a but none with mGluR1b. In functional experiments measuring the coupling of mGluR1 to IP accumulation, we found that mGluR1a but not mGluR1b display constitutive activity, in line with previous studies (Prezeau et al., 1996). This suggests that the agonist-independent activity of mGluR1a may underlie its ability to undergo internalization in the absence of agonist. However, although PKA activation with forskolin strongly inhibited glutamate- and carbachol-induced internalization of mGluR1, the inhibitory effect of forskolin on constitutive internalization of mGluR1a was only modest. At present, the reason for this is unclear, although it is possible that the mechanism of constitutive internalization is different from agonist-induced internalization, such that the former is less susceptible to PKA regulation or, alternatively, that the different antibody labeling protocols used for the experiments (labeling of surface receptors on fixed cells for agonist-induced internalization versus prelabeling of surface receptors on live cells for constitutive internalization) somehow alters the PKA interaction. Nevertheless, it is possible to account for the modest forskolin-induced inhibition of constitutive mGluR1a internalization by the small increase in total

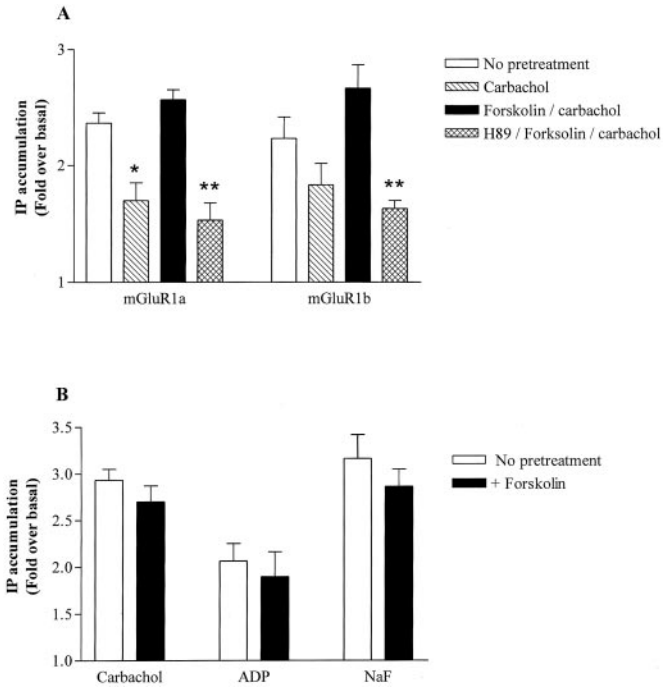

Fig. 7. Activation of PKA blocks the carbachol-mediated desensitization of glutamate-stimulated IP accumulation in cells expressing mGluR1a or mGluR1b. Cells transiently transfected with mGluR1a or mGluR1b were used for IP accumulation assays 2 days later. A, cells were pretreated in the presence or absence of H89 (10 μM) for 40 min before and during glutamate (10 μM) addition for a further 30 min. Some cells were also pretreated with carbachol (1 mM) or carbachol and forskolin (10 μM) for 30 min and washed, before glutamate addition. The data are mean ± S.E. of three independent experiments and results are expressed as fold IP accumulation over basal. *, $p < 0.05$ compared with respective value for glutamate with no pretreatment, **, $p < 0.05$ compared with respective value for glutamate after forskolin/carbachol pretreatment (Mann-Whitney U test). B, cells transiently transfected with mGluR1a were preincubated in the presence or absence of forskolin (10 μM) for 30 min, then washed and IP accumulation measured after addition of carbachol (1 mM; 30 min), ADP (10 μM; 30 min), or NaF (10 mM; 30 min) in the continued absence or presence of forskolin. The data are mean ± S.E. of four independent experiments and expressed as fold IP accumulation over basal.

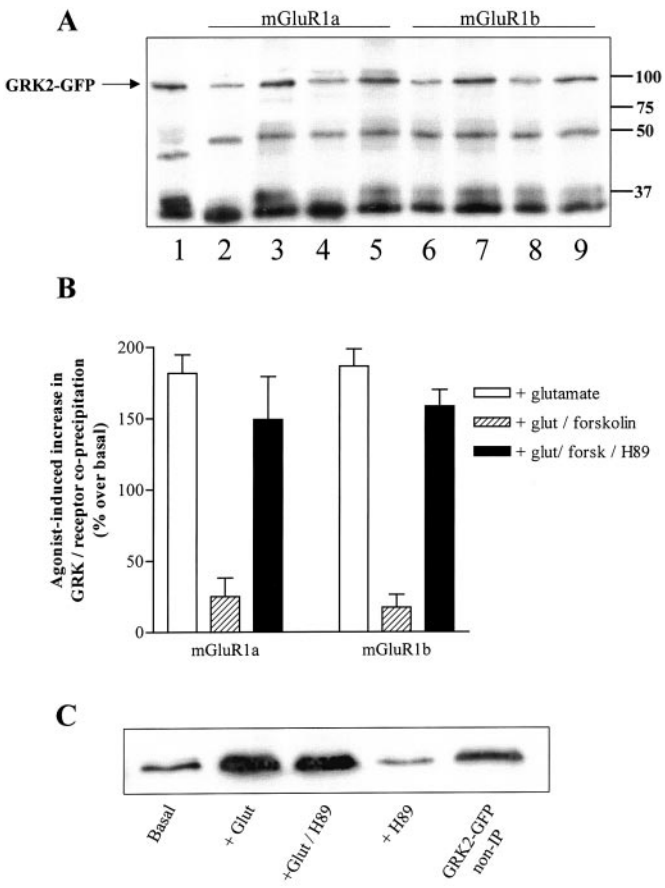

Fig. 8. Activation of PKA inhibits the glutamate-induced association of GRK2 with mGluR1a and mGluR1b. HEK293 cells were transiently transfected with mGluR1a or mGluR1b DNA, and pcDNA3 alone as a control, or pcDNA3-GRK2-GFP and used in coimmunoprecipitation experiments 2 days later. Cells were incubated in the presence or absence of forskolin (10 μM) for 10 min before and during a further 15-min incubation in the presence or absence of glutamate (10 μM). In some cases, H89 (10 μM) was added for 30 min before and during forskolin/glutamate application. In each case, mGluR1a and mGluR1b receptors were immunoprecipitated with an anti-HA monoclonal antibody and associated GRK2-GFP identified with an anti-GRK2 antibody as described under *Experimental Procedures*. A, representative immunoblot. Lane 1 is the cell lysate from a GRK2-GFP-expressing cell; lanes 2 to 5 are for mGluR1a-transfected cells, and lanes 6 to 9 are for mGluR1b-expressing cells. Lanes 2 and 6, no treatment; lanes 3 and 7, glutamate; lanes 4 and 8, forskolin and glutamate; lanes 5 and 9, H89, forskolin, and glutamate. B, densitometric analysis of receptor/GRK2-GFP coimmunoprecipitation. In these experiments, the amount of GRK2-GFP coimmunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of receptor immunoblot). The data are mean ± S.E. of three independent experiments. C, representative immunoblot showing that H89 does not affect the glutamate-stimulated increase in GRK2-GFP association with mGluR1a, although it did slightly reduce basal association of GRK2-GFP with the receptor. This experiment was performed twice, with the same result being obtained on each occasion. The lane on the right is the cell lysate from a GRK2-GFP-expressing cell.

cell surface mGluR1a induced by forskolin (10–20% change in each case).

In functional coupling studies, forskolin treatment increased basal IP accumulation in mGluR1a-expressing cells, in agreement with a previous study (Francesconi and Duvoisin, 2000). Because the forskolin-induced increase in basal IP accumulation (~ 100%) was greater than the forskolin-induced increase in cell surface receptor (10–20%), PKA activation must either increase the fraction of constitutively active receptors in the membrane or increase the constitutive activity of individual mGluR1a. Thus, it is possible that PKA activation stabilizes constitutively active mGluR1a at the cell membrane. It is interesting that PKA activation has recently been reported to increase the cell surface expression and/or membrane stability of the GPCR $GABA_B$ receptor (Couve et al., 2002), as well as the non-GPCR epidermal growth factor receptor (Salazar and Gonzalez, 2002) and the ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (Ehlers, 2000). Thus, PKA activity may play an important role in the regulation of cell surface expression of receptors from very different receptor families.

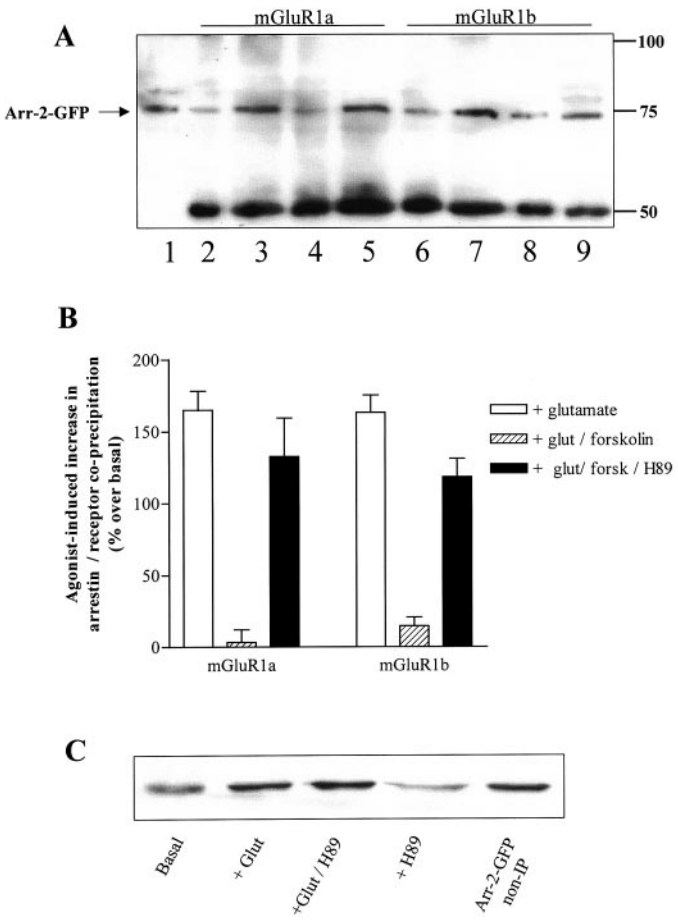

Fig. 9. Activation of PKA inhibits the glutamate-induced association of arrestin-2-GFP with mGluR1a and mGluR1b. HEK293 cells were transiently transfected with mGluR1a or mGluR1b DNA, and pcDNA3 alone as a control, or pcDNA3-arrestin-2-GFP. Cells were used in coimmunoprecipitation experiments 2 days later. Cells were incubated in the presence or absence of forskolin (10 μM) for 10 min before and during a further 15-min incubation in the presence or absence of glutamate (10 μM). In some cases, H89 (10 μM) was added for 30 min before and during forskolin/glutamate application. In each case, mGluR1a and mGluR1b were immunoprecipitated with an anti-HA monoclonal antibody and associated arrestin-2-GFP identified with an anti-arrestin-2 antibody as described under *Experimental Procedure*s. A, representative immunoblot. Lane 1 is the cell lysate from an arrestin-2-GFP-expressing cell; lanes 2 to 5 are for mGluR1a-transfected cells, and lanes 6 to 9 are for mGluR1b-expressing cells. Lanes 2 and 6, no treatment; lanes 3 and 7, glutamate; lanes 4 and 8, forskolin and glutamate; lanes 5 and 9, H89, forskolin and glutamate. B, densitometric analysis of receptor/arrestin-2-GFP coimmunoprecipitation. In these experiments, the amount of arrestin-2-GFP coimmunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of receptor immunoblot). The data are mean ± S.E. of three independent experiments. C, representative immunoblot showing that H89 does not affect the glutamate-stimulated increase in arrestin-2-GFP association with mGluR1a, although it did slightly reduce basal association of arrestin-2-GFP with the receptor. This experiment was performed twice, with the same result being obtained on each occasion. The lane on the right is the cell lysate from an arrestin-2-GFP-expressing cell.

We also assessed the effects of PKA activation on the agonist-induced coupling of the mGluR1 splice variants to IP accumulation. In initial experiments, we were unable to observe any effect of forskolin on glutamate-stimulated IP accumulation after 30 min of agonist addition. However, using shorter periods of agonist addition, we could clearly observe enhanced glutamate-stimulated IP accumulation after forskolin or dibutyryl cAMP treatment. One possibility is that the PKA-induced enhancement of signaling at short time points of glutamate addition is caused by the maintenance of cell surface mGluR1a and mGluR1b number in the presence of forskolin or dibutyryl cAMP. Perhaps other adaptive mechanisms, such as the effects of PKC/GRKs/arrestins (Dale et al., 2000; Sallese et al., 2000; Mundell et al., 2002, 2003; Iacovelli et al., 2003) eventually override the PKA effects on coupling at longer time points.

Activation of PKA also reversed the carbachol-induced heterologous desensitization of mGluR1a and mGluR1b responsiveness, in line with the inhibitory effects of PKA activation on carbachol-induced mGluR1 splice variant internalization. Because forskolin did not affect the ability of carbachol, ADP (via endogenous P2Y receptors), or NaF (via direct activation of $G_{q/11}$) to increase IP accumulation in mGluR1a-expressing cells, then PKA must be acting at a locus downstream of carbachol-mediated IP accumulation, such as the mGluR1 itself. In recent work, we have shown that carbachol increases GRK2/arrestin-2 association with mGluR1a (Mundell et al., 2004). It seems likely that the inhibitory effect of PKA on carbachol-mediated desensitization and internalization of mGluR1a is caused by PKAmediated reduction of GRK2/arrestin association with mGluR1a. However, at present we cannot say whether PKA activation reduces the agonist-induced association of GRK2 and arrestin-2 with mGluR1a after direct PKA-mediated phosphorylation of the receptor or indirectly by modification of some other component of the receptor regulatory machinery. It is intriguing, however, that carbachol does not increase GRK2/arrestin-2 association with mGluR1b (Mundell et al., 2004), so the mechanism whereby PKA inhibits carbachol-mediated regulation of mGluR1b will be an important goal of future studies.

Activation of PKA is known to modulate other mGluR subtypes, although trafficking was not examined. For example, forskolin reverses the mGluR2-mediated inhibition of synaptic transmission at the medial perforant path-dentate gyrus synapse in the hippocampus. The mechanism of this effect probably involves PKA-mediated phosphorylation of the COOH terminus of mGluR2 receptor and consequent uncoupling of receptor and G protein (Schaffhauser et al.,

2000). Furthermore, PKA phosphorylates the COOH terminus of group III mGluRs, which is thought to underlie the ability of cyclic AMP elevation to reverse group III mGluR-mediated inhibition of synaptic transmission at various hippocampal synapses (Cai et al., 2001). Thus, PKA activation after stimulation of neuronal G_s-coupled GPCRs seems to be an important regulator of mGluR function. Indeed, the ability of PKA activation to inhibit presynaptic group II/III mGluR function (Schaffhauser et al., 2000; Cai et al., 2001) coupled with an ability to enhance postsynaptic group I receptor function (present study) could provide a means to efficiently increase overall synaptic activity at glutamatergic synapses.

In summary, we report the novel finding that activation of PKA markedly inhibits the agonist-induced internalization of mGluR1a and mGluR1b. Activation of PKA functionally increases the coupling of mGluR1 splice variants to IP accumulation and blocks the muscarinic acetylcholine receptor-mediated heterologous desensitization of mGluR1a and mGluR1b responsiveness. The mechanism by which PKA activation inhibits the agonist-induced desensitization and internalization of the mGluR1 splice variants probably involves the occlusion of GRK2 and nonvisual arrestin association with the receptors. To our knowledge, this is the first report showing that PKA activity negatively regulates the association of GRK2 and nonvisual arrestins with a GPCR. Future goals will be to determine the molecular mechanism whereby PKA activation mediates this effect and to determine the importance of this mechanism to mGluR1 splice variants expressed in neurons.

Acknowledgments

We thank Professor Jeff Benovic, Thomas Jefferson University, Philadelphia, for generously providing the anti-GRK2 monoclonal antibody and the anti-arrestin-2 polyclonal antibody.

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