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Metabotropic Glutamate Receptor 1 Internalization Induced by Muscarinic Acetylcholine Receptor Activation: Differential Dependency of Internalization of Splice Variants on Nonvisual Arrestins

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ABSTRACT

In this study, we characterized the glutamate- or second-messenger kinase-dependent internalization of the rat metabotropic glutamate receptor 1 (mGluR1) splice variants 1a, 1b, and 1c, and assessed the arrestin and dynamin dependence of these processes. To facilitate this we inserted a hemagglutinin epitope tag in the extracellular N-terminal domain of the splice variants. Quantification of glutamate-induced mGluR1 splice variant internalization provided by enzyme-linked immunosorbent assay and confirmed by immunofluorescent microscopy indicated that each splice variant underwent rapid internalization, which was strongly inhibited by coexpression of dominant-negative mutant (DNM) arrestin or dynamin. In addition glutamate-induced rapid translocation of arrestin-2-green fluorescent protein (GFP) or arrestin-3-GFP from cytosol to membrane was observed in cells expressing mGluR1 splice variants. Glutamate-induced internalization of mGluR1a and mGluR1c was partially blocked by a selective inhibitor of protein kinase C (PKC), 2-[1-(3-dimethylamino-propyl)indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (GF 109203X), whereas mGluR1b internalization was not significantly affected by this inhibitor. Similarly, inositol phosphate production after glutamate-induced activation of mGluR1a and mGluR1c was increased after PKC inhibition, whereas glutamate-induced mGluR1b stimulation was unaffected. Activation by carbachol of endogenously expressed M_1 muscarinic receptors in human embryonic kidney 293 cells, induced the internalization of mGluR1 splice variants, which was partially blocked by pretreatment with inhibitors of either PKC or Ca^{2+} calmodulin-dependent kinase II (CaMKII). Expression of DNM-arrestin with mGluR1a or 1c strongly inhibited carbachol-induced internalization. However, coexpression of DNM-arrestin with mGluR1b was less effective in reducing carbachol-induced receptor internalization. In addition, arrestin-2-GFP or arrestin-3-GFP underwent significant carbachol-induced translocation from cytosol to membrane in cells coexpressing mGluR1a or 1c but not in cells coexpressing mGluR1b. This study demonstrates that the internalization of mGluR1 splice variants is subject to PKC and CaMKII regulation. In addition, regulation by these kinases confers differential arrestin dependence.

Glutamate is the major excitatory neurotransmitter in the central nervous system and acts through ionotropic and metabotropic glutamate receptors (mGluR). Whereas ionotropic receptors drive fast neurotransmission, 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 review, see Schoepp et al., 1999). Sequence analysis shows that mGluRs are members of a distinct G-protein-coupled receptor (GPCR) superfamily that shows little similarity to classic GPCRs such as rhodopsin and β-adrenoceptors. Other members in this subfamily of GPCRs are the γ-aminobutyric acid B receptor, the Ca^{2+}-sensing receptor, and a large number of pheromone receptors. The pharmacology, amino acid sequence

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; HA, hemagglutinin; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; kb, kilobase(s); PCR, polymerase chain reaction; HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay; arr-DNM, arrestin-2 dominant-negative mutant; dyn-DNM, dynamin dominant-negative mutant; PMA, phorbol 12-myristate 13-acetate; TBS, Tris-buffered saline; BSA, bovine serum albumin; GFP, green fluorescent protein; ANOVA, analysis of variance; CaMKII, Ca^{2+}-calmodulin-dependent kinase II; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; GF 109203X, 2-[1-(3-dimethylamino-propyl)indol-3-yl]-3-(1*H*-indol-3-yl)maleimide; IP, inositol phosphate; PKA, protein kinase A; AChR, acetylcholine receptor; KN-93, *N*-(2-[*N*-[4-chlorocinnamyl]-*N*-methylaminomethyl]phenyl)-*N*-(2-hydroxyethyl)-4-methoxybenzenesulfonamide.

similarity, and signal transduction mechanisms of mGluRs have enabled their classification into three groups; mGluR1 and mGluR5, group I mGluRs, are coupled to G_q and phospholipase C. Five splice variants of mGluR1 have been described thus far, each of which differs from the others in the length of its COOH-terminal tail (Pin et al., 1992; Pin and Duvoisin, 1995)

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 down-regulation. Recent studies have shown that desensitization of the mGluR1a is GPCR kinase (GRK)-dependent and that receptor phosphorylation by GRKs probably uncouples the GPCR from G-protein (Dale et al., 2000; Sallese et al., 2000). After uncoupling of receptor and G-protein, many GPCRs are removed from the cell surface by internalization (reviewed in Ferguson, 2001). For some receptors, such as the β_2-adrenoceptor, the binding of arrestin to receptor after GRK-dependent phosphorylation, serves to target the receptor to clathrin-coated pits for internalization (Goodman et al., 1996). Arrestins act as adaptors between the phosphorylated receptor and components of the endocytic machinery, such as clathrin-associated adaptor protein and clathrin, both of which are major components of clathrin-coated pits (Goodman et al., 1996; Krupnick et al., 1997a; Laporte et al., 1999). 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 (Ferguson, 2001).

Compared with other GPCRs, much less is known about the mechanisms of mGluR trafficking, although mGluR1 is known to internalize upon agonist addition (Doherty et al., 1999; Sallese et al., 2000; Mundell et al., 2001). In a recent study, we inserted a hemagglutinin (HA) epitope tag into the N-terminal extracellular domain of the rat mGluR1a and subsequently examined its trafficking (Mundell et al., 2001), demonstrating that agonist-induced internalization of mGluR1a is an arrestin- and dynamin-dependent process. In addition to agonist-specific receptor desensitization, GPCR function can be regulated by agonist-independent heterologous mechanisms. For example, recent studies have demonstrated that protein kinase C (PKC) phosphorylates and regulates the desensitization and internalization of mGluR1a, -1b, and -1c (Ciruela and McIlhinney, 1997; Ciruela et al., 1999).

In the present, more comprehensive study, we first investigated whether glutamate-induced internalization of two other mGluR1 splice variants, mGluR1b and 1c, which have much shorter COOH-terminal tails than mGluR1a (Fig. 1), are arrestin-dependent processes. Second, we determined the involvement of PKC in the glutamate-induced internalization of mGluR1 splice variants. Third, we investigated whether internalization of mGluR1 splice variants can also be regulated by heterologous activation of another G_q-coupled receptor (M_1 muscarinic) and whether such regulation is dependent upon second-messenger kinases and arrestins. Our results indicate that PKC activity and arrestin function play a central role in mGluR1 internalization but that important differences exist in the mechanisms that regulate trafficking of different mGluR1 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). Expand High Fidelity DNA polymerase and rhodamine-conjugated mouse monoclonal anti-HA antibody (12CA5) were from Roche Applied Science (Lewes, E. Sussex, UK). Rneasy RNA isolation kit was from QIAGEN (Crawley, W. Sussex, UK), Moloney murine leukemia virus reverse transcriptase was from Promega (Southampton, UK), and pcDNA3 was from Invitrogen (Paisley, Scotland, UK). Anti-HA-monoclonal antibody (HA-11), goat anti-mouse fluorescein-conjugated secondary antibody, and rhodamine-conjugated transferrin were purchased from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical (Poole, Dorset, UK).

Production of Epitope-Tagged Rat mGluR1a, -1b, and -1c Receptors. An extended HA-epitope (TRMYPYDVPDYA) was introduced into the N terminus of the mGluR1a cDNA between amino acids 57 and 58 and subcloned into pcDNA3 as described previously (Mundell et al., 2001). The HA-tagged mGluR1b splice variant was obtained by substitution of the long COOH-terminal tail of HA-mGluR1a with a synthetic stretch of DNA coding the short COOH-terminal tail of mGluR1b. First, HA-mGluR1a in pcDNA3 was digested with *Psp*AI and *Pvu*I restriction enzymes, and the resultant 3.8-kb band gel was purified. Secondly, pcDNA3 vector was digested with *Not*I and *Pvu*I restriction enzymes, and the resultant 4.0-kb band gel was purified. Two single-strand synthetic DNA molecules, representing a plus (sequence, CCGG GGCAGGGAATGCCAAGAAGAG-GCAGCCAGAATTCTCGCCCAGCAGCCAGTGTCCGTCGGCACATG-CGCAGCTTTGAGC) and a minus strand (sequence, GGCCGCT-CAAAGCTGCGCATGTGCCGACGGACACTGGCTGCTGGGCGAGAA-TTCTGGCTGCCTCTTCTTGGCATTCCCTGCC) of the mGluR1b COOH-terminal tail coding sequence, were purchased from Sigma Chemical. These single-stranded DNA molecules were subsequently annealed into double-stranded DNA and ligated with the 3.8- and 4.0-kb DNA fragments obtained above using T4 DNA-ligase. DH5α *Escherichia coli* bacteria were transformed with the resultant ligation product and recombinant colonies were selected with 100 μg/ml ampicillin. The sequence of pcDNA3-HA-mGluR1b was confirmed by automated DNA sequencing.

The HA-tagged mGluR1c splice variant was obtained by a two-step PCR overlap extension protocol. The product of HA-tagged mGluR1a amplifica-

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KPERNVR**S**AF**TTS**DVVRMHVGDGKLPCR**S**N**T**FLNIFRRKK PGAGNA //

1a

N**S**NGK**S**V**S**W**S**EPGGRQAPKGQHVWQRL**S**VHVK**T**NE**T**ACNQ**T**AVI

KPL**T**K**S**YQG**S**GK**S**L**T**F**S**DA**ST**K**T**LYNVEEEDN**T**P**S**AHF**S**PP**SS**P**S**MVVHR

RGPPVA**TT**PPLPPHL**T**AEE**T**PLFLAD**S**VIPKGLPPPLPQQQPQQPPPQQP

PQQPK**S**LMDQLQGVV**T**NFG**S**GIPDFHAVLAGPG**T**PGN**S**LR**S**LYPPPPPPQ

HLQMLPLHL**ST**FQEE**SIS**PPGEDIDDD**S**ERFKLLQEFVYEREGN**T**EEDEL

EEEEDLP**T**A**S**KL**T**PED**S**PAL**T**PP**S**PFRD**S**VA**S**G**SS**VP**SS**PV**S**E**S**VLC**T**PP

NV**T**YA**S**VILRDYKQ**SSST**L

1b

KKRQPEF**S**P**SS**QCP**S**AHAQL

1c

NFALDRQN**T**VY

Fig. 1. Comparison of the amino acid sequences of the proposed intracellular COOH termini of the mGluR1a, -1b, and -1c splice variants. The top line shows the COOH-terminal amino acids common to all three splice variants. All serine and threonine residues are in bold and underlined.

tion by PCR (forward primer, AAGAAGAAGATCTGCACCCG; reverse primer: GTGTTTTGGCGGTCT AAAGCAAAATTGGCATTCCCTGCCC) was used as a template for a second PCR reaction (forward primer, AAGAAGAAGATCTGCACCCG; reverse primer, TATAGCGGCCGCTCAATAGACAGTGTTTTGGCGGTCTAAAGC). This PCR product was then purified, digested with *Sph*I and *Not*I, and ligated into the pLitmus28 vector, which was subsequently transformed into DH5α *E. coli* bacteria. Positive colonies were selected with 100 μg/ml ampicillin. The sequence of the resultant HA-encoded mGluR1c cDNA was confirmed by automated DNA sequencing and finally subcloned into pcDNA3.

Cell Culture and Transfection. HEK293 and COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 95% air, 5% CO_2. For transient transfections, HEK293 or COS-7 cells were grown in 60- or 100-mm dishes to 80 to 90% confluence and transfected with 0.5 to 10 μg of DNA using LipofectAMINE 2000 following the manufacturer's instructions. Cells were incubated with a DNA/LipofectAMINE mixture for 24 h, the medium was replaced, and the cells were analyzed 24 h later.

Internalization and Immunofluorescence Microscopy of mGluR1a, -1b, and -1c in HEK293 and COS-7 Cells. mGluR1a, -1b, and -1c cell surface loss was assessed by ELISA as described previously (Mundell et al., 2000). Briefly, cells plated at a density of approximately 6×10^5 cells per 60-mm dish were transiently transfected with mGluR1a, -1b, or -1c (10 μg) ± arrestin-2-dominant-negative mutant [arr-DNM, arrestin-2 (319–418), 5 μg] or dynamin-dominant-negative mutant (dyn-DNM, dynamin-K44A, 5 μg). Cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml poly-L-lysine 24 h after transfection; 24 h after that, cells were incubated with DMEM containing 10 μM glutamate, 1 mM carbachol, or 2 μM phorbol 12-myristate 13-acetate (PMA) for 0 to 60 min at 37°C. 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 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 conjugated with alkaline phosphatase, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were 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. Throughout, internalization of the mGluR splice variants were compared against surface receptor expression at time 0. Results are expressed as either the percentage of surface receptor or the percentage loss of surface receptor with the background signal from pcDNA3-transfected control cells subtracted from all receptor-transfected values.

Cellular distribution of mGluR1a, -1b, and -1c transiently transfected into HEK293 cells was assessed by immunofluorescence microscopy (Mundell et al., 2000). Briefly, HEK293 or COS-7 cells, grown on poly-L-lysine coated coverslips in six-well plates, were transiently transfected with mGluR1a, -1b, or -1c (5 μg). Forty-eight hours after transfection, receptor distribution was assessed using a primary anti-HA-monoclonal antibody (HA-11; 1:200) and goat anti-mouse fluorescein-conjugated secondary antibody (1:200). For experiments analyzing transferrin distribution, cells were incubated for 15 min with 200 μg/ml rhodamine-conjugated transferrin. Coverslips were mounted using SlowFade mounting medium (Molecular Probes) and examined by microscopy on an upright TCS-NT confocal laser scanning microscope (Leica, Wetzlar, Germany) attached to a Leica DM IRBE epifluorescence microscope with phase contrast and a Plan-Apo 40 × 1.40 numerical aperture oil immersion objective.

Arrestin-2-green fluorescent protein (GFP) or arrestin-3-GFP redistribution was assessed in HEK293 or COS-7 cells as described previously (Mundell et al., 2000). Briefly, cells were transfected as described above with 5 μg of mGluR1a, -1b, or -1c and 0.5 μg of arrestin-2-GFP or arrestin-3-GFP and grown on poly-L-lysine–coated coverslips. To assess HA-mGluR distribution, cells were incubated for 30 min at 4°C with rhodamine-conjugated mouse monoclonal anti-HA antibody (12CA5; 1:100). Cells were then washed three times with phosphate-buffered saline before imaging and coverslips mounted in a heated imaging chamber through which media and drugs could be added. Cells were examined by microscopy on an inverted Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with phase-contrast and a Plan-Apo 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 essentially as described previously (Mundell and Benovic, 2000). Briefly, cells plated at a density of approximately 6×10^5 cells per 60-mm dish were transiently transfected with mGluR1a, -1b, or -1c (5 μg) ± arrestin-3 (5 μg) or arr-DNM [arrestin-2 (319–418), 5 μg]. Cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml poly-L-lysine 24 h after transfection. On the following day, cells were labeled for 18 to 24 h with [*myo*-^{3}H]inositol (4 μCi/ml) in DMEM (high glucose, without inositol). After labeling, cells were washed once in phosphate-buffered saline and incubated in prewarmed DMEM containing 20 mM LiCl for 10 min at 37°C. Cells were then stimulated with either glutamate (10 μM) or carbachol (1 mM). Reactions were terminated by removing the stimulation medium and adding 0.8 ml of 0.4 M perchloric acid. Samples were harvested in Eppendorf tubes, and 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 then 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.

Experimental Design and Statistics. Data were analyzed by the iterative fitting program Prism (GraphPAD Software, San Diego, CA). Log concentration-effect curves were fitted to logistic expressions for single-site analysis, whereas $t_{0.5}$ values for agonist-induced internalization were obtained by fitting data to single exponential curves. Where appropriate, statistical significance was assessed by Mann-Whitney *U* test or by two-way ANOVA.

Results

Glutamate-Induced Internalization of mGluR1 Splice Variants. In HEK293 cells transiently transfected with HA epitope-tagged mGluR1a, -1b, or -1c, each receptor construct was primarily localized to the cell surface before agonist stimulation (Fig. 2A). In nontransfected cells, there was no surface labeling by antibody (data not shown). After stimulation with glutamate (10 μM, 30 min), mGluR1a, -1b, and -1c underwent intracellular redistribution into a distinct punctate pattern (Fig. 2A). To further characterize the intracellular localization of the mGluR1 splice variants, we used rhodamine-labeled transferrin, which binds to transferrin receptors and labels early endosomes and the endocytic recycling compartment (Cao et al., 1998). After agonist-induced internalization, each splice variant displayed extensive colocalization with rhodamine-labeled transferrin (data not shown). To quantify the kinetics and extent of mGluR1a, -1b, and -1c internalization, each was transiently expressed in HEK293 (Fig. 2B) or COS-7 (Fig. 2C) cells and the time dependence of agonist-induced internalization of the receptor determined by ELISA. Each splice variant underwent inter-

nalization in response to 10 μM glutamate, although the initial rate of mGluR1b and -1c internalization was significantly faster than that of the mGluR1a in both cell lines. After prolonged agonist exposure (30 min), each of the splice variants underwent significant internalization; mGluR1b and -1c displayed more extensive surface loss than mGluR1a.

Recently, we demonstrated that the agonist-induced internalization of mGluR1a is an arrestin- and dynamin-dependent process (Mundell et al., 2001). Therefore, we determined the arrestin and dynamin dependence of agonist-induced internalization of the mGluR1b and -1c splice variants. Cells were cotransfected with each splice variant and either arrestin-2 (319–418) (arr-DNM; Krupnick et al., 1997b) or dynamin-K44A (dyn-DNM; Damke et al., 1994). As shown in Fig. 3A, expression of either the arr-DNM or dyn-DNM strongly inhibited 10 μM glutamate-induced internalization of all three mGluR1 splice variants. Numerous studies have shown that activation of several different GPCRs leads to rapid recruitment of arrestin-2-GFP and arrestin-3-GFP from cytosol to membrane (Barak et al., 1997; Mundell et al., 2000). Therefore, we investigated the effects of mGluR1a, -1b, and -1c activation on redistribution of arrestin-3-GFP in HEK293 cells in which receptor and arrestin-3-GFP were cotransfected (Fig. 3B). Before agonist stimulation of cells transfected with mGluR1a, -1b, or -1c, arrestin-3-GFP displayed a diffuse cytoplasmic distribution. After addition of 10 μM glutamate, a rapid translocation of arrestin-3-GFP from cytosol to membrane was observed with each splice variant. Similar findings were obtained with arrestin-2-GFP (data not shown) and in COS-7 cells cotransfected with mGluR1 splice variants and arrestin-3-GFP (data not shown). No

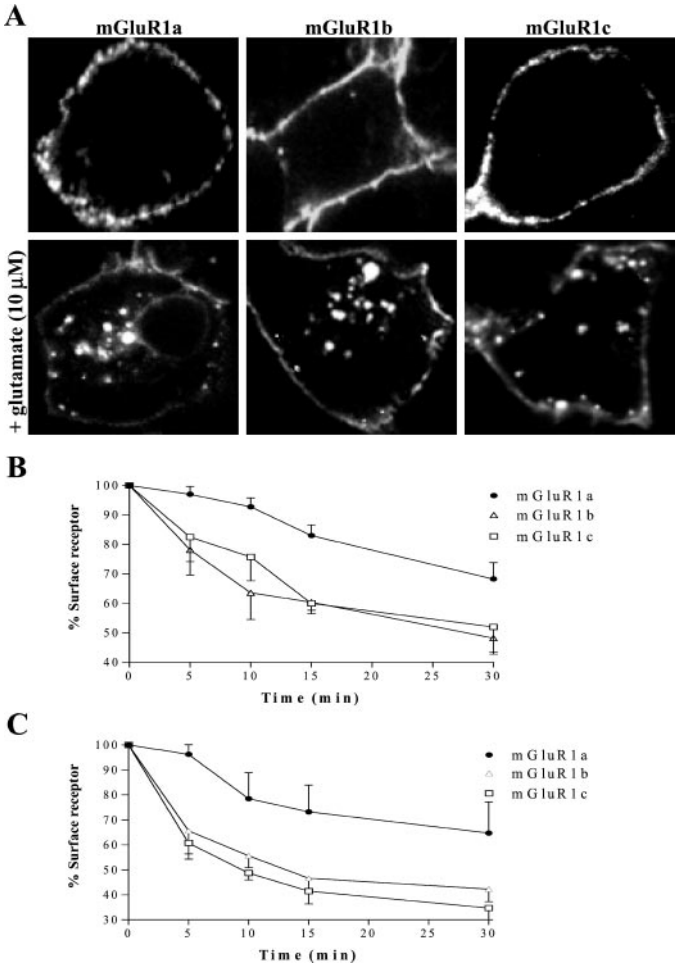

Fig. 2. Glutamate-induced internalization of transiently transfected mGluR1a, -1b, and -1c. HEK293 cells (A and B) and COS-7 cells (C) were transiently transfected with 5 μg of pcDNA3-mGluR1a, -1b, or -1c DNA and used in experiments two days later. A, HEK293 cells were preincubated with an anti-HA antibody for 1 h. Subsequently, cells were incubated at 37°C for 30 min in the absence or presence of agonist (10 μM glutamate). Receptor localization was determined by immunofluorescence. The mGluR1 splice variants were visualized with a fluorescein-conjugated secondary antibody. HEK293 cells (B) or COS-7 cells (C) were challenged with glutamate (10 μM, 0–30 min) and surface receptor loss assessed by ELISA. The data represent means ± S.E.M. of five independent experiments. The rate of mGluR1b and 1c cell surface loss was significantly faster versus mGluR1a in both cell lines. (HEK293 cells, $t_{0.5}$ of 9.7 ± 3.5 and 13.4 ± 2.8 min for 1b and 1c, respectively, were significantly faster versus $t_{0.5}$ of 19.3 ± 3.7 min for 1a, $p < 0.05$ by 2-way ANOVA; COS-7 cells, $t_{0.5}$ of 5.9 ± 2.8 and 6.4 ± 1.8 min for 1b and 1c, respectively, were significantly faster versus $t_{0.5}$ of 12.5 ± 3.7 min for 1a, $p < 0.05$ by two-way ANOVA.)

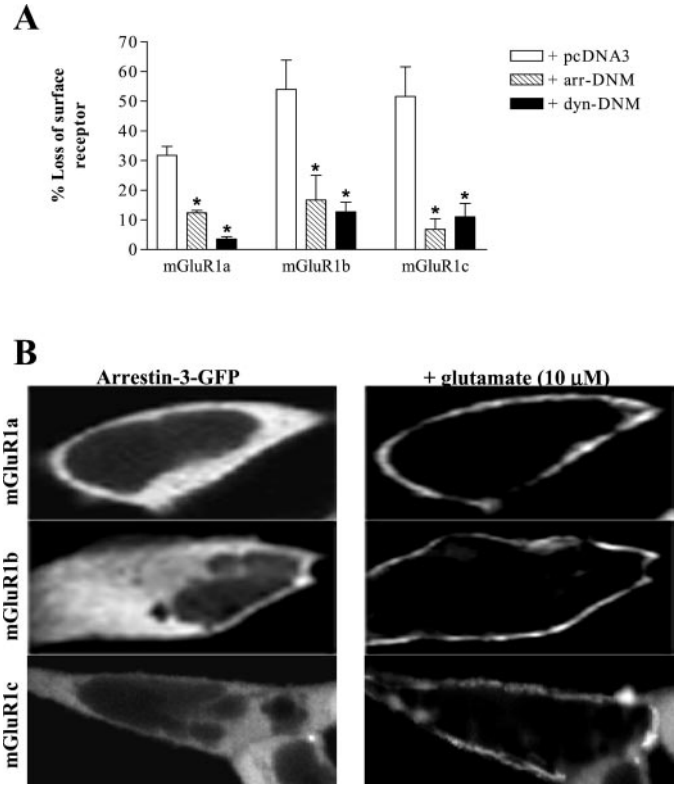

Fig. 3. A, effect of arr-DNM or dyn-DNM on glutamate-induced mGluR1a, -1b, and -1c internalization. HEK293 cells were transiently transfected with 10 μg of pcDNA3-mGluR1a, -1b, or -1c DNA and 5 μg of either pcDNA3 alone, pcDNA3-arrestin-2-(319–418), or pcDNA3-dynamin K44A (overexpression of dominant-negative mutants >10-fold over basal as assessed by immunoblot; data not shown). Cells were subsequently challenged with glutamate (10 μM, 30 min) and surface receptor loss assessed by ELISA. The data represent means ± S.E.M. of five independent experiments. Data presented as percentage loss of cell surface receptor. ⋆, $p < 0.05$ versus pcDNA3 alone (Mann-Whitney U test). B, analysis of agonist-induced translocation of arrestin-3-GFP in HEK293 coexpressing mGluR1a, -1b, and -1c. HEK293 grown on poly-L-lysine coverslips were transiently transfected with 5 μg of pcDNA3-mGluR1a, -1b, or -1c and 0.5 μg of peGFP-N1-arrestin-3-GFP. Membrane expression of mGluR1 splice variants was visualized and confirmed using a rhodamine-conjugated anti-HA antibody (data not shown). Before stimulation and viewing, coverslips were mounted in an imaging chamber at 37°C. The initial diffuse cytoplasmic distribution of arrestin-3-GFP is shown before agonist stimulation (left). Glutamate (10 μM) was added and the redistribution of arrestin-3-GFP was monitored in real time. The images shown were collected at 180 s after agonist addition.

translocation was evident in cells that expressed arrestin-2-GFP or arrestin-3-GFP in the absence of mGluR1 expression (data not shown). Taken together, these observations demonstrate that glutamate-induced internalization of mGluR1a, -1b, and -1c is an arrestin- and dynamin-dependent process.

Involvement of Second-Messenger-Dependent Protein Kinases in Glutamate-Induced Internalization of mGluR1 Splice Variants. Upon agonist stimulation, the G_q-coupled mGluR1 receptors activate PKC and probably also Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). It is possible that these kinases regulate internalization of the receptors, and we determined whether blockade of these kinases by selective inhibitors would modify glutamate-induced internalization of the mGluR1 splice variants. To varying extents, agonist-induced internalization of each of the mGluR1 splice variants was reduced by pretreatment with the PKC inhibitors GF 109203X (2 μM; Fig. 4A). Thus, whereas GF 109203X significantly reduced glutamate-induced internalization of mGluR1a and 1c by more than 50%, it only blocked mGluR1b internalization by approximately 20%. Pretreatment with the CaMKII inhibitor KN-93 (1 μM), although much less effective than GF 109203X, still reduced agonist-induced mGluR1 splice variant internalization by 15 to 30%. The coaddition of both of these kinase inhibitors was nonadditive in terms of blocking glutamate-induced mGluR1 splice variant internalization. Thus, the glutamate-induced internalization of mGluR1 splice variants is partially dependent upon second-messenger–dependent protein kinase activation.

We also sought to determine whether protein kinase A (PKA) could regulate mGluR1 splice variant internalization. Pretreatment with the PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89, 10 μM, 15 min) had no effect on agonist-induced mGluR splice variant internalization (Fig. 4B). Furthermore, treatment with the cAMP analog 8-bromo-cAMP (10 μM, 30 min), which stimulates PKA directly, had no effect on cell surface receptor levels, indicating that this kinase does not regulate the internalization of mGluR1 splice variants.

Heterologous Regulation of mGluR1 Splice Variant Internalization by Muscarinic Acetylcholine Receptor Activation. Because second-messenger–dependent protein kinases are involved in homologous regulation of mGluR1 splice variant internalization, we investigated whether activation of endogenously expressed G_q-coupled M_1 muscarinic receptors (Mundell and Benovic, 2000) or direct activation of PKC with phorbol ester could also promote mGluR1a, -1b, and -1c internalization. After activation of endogenously expressed muscarinic receptors by carbachol (1 mM, 0–60 min), the mGluR1 splice variants underwent extensive internalization (Fig. 5A; $t_{0.5}$ values for carbachol-induced internalization of 28 ± 5, 16 ± 5, and 13 ± 4 min for mGluR1a, -1b, and -1c, respectively). Carbachol-induced internalization was blocked by the muscarinic receptor antagonist atropine (10 μM), indicating that activation of this receptor was responsible for mGluR1 splice variant internalization (treatment with 1 mM carbachol for 30 min induced mGluR1a internalization by 37.8 ± 3.6% and 7.0 ± 3.4% in the absence or the presence of atropine, respectively; $n = 4$). To determine whether release of endogenous glutamate from cells contributes to the observed effects of carbachol, we pretreated cells with 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), a group 1 mGluR antagonist. Pretreatment with 10 μM CPCCOEt had no effect on carbachol-induced mGluR1a receptor internalization, indicating that endogenously released glutamate had no role in this process (treatment with 1 mM carbachol for 30 min induced mGluR1a internalization by 34.8 ± 4.6% and 31.8 ± 3.6% in the absence or presence of CPCCOEt, respectively).

The role of second-messenger dependent kinases in carbachol-dependent mGluR1 splice variant internalization was also studied. Carbachol-induced internalization was partially blocked by pretreatment with either 2 μM GF 109203X or 1 μM KN-93 (Fig. 5B). Coaddition of both kinase inhibitors almost completely abolished carbachol-dependent mGluR1a, -1b, and -1c internalization, indicating that activation of both PKC and CaMKII after muscarinic acetylcholine receptor activation can promote mGluR1 splice variant internalization. These findings were supported by experiments using PMA, an activator of PKC. PMA (1 μM, 30 min) promoted the internalization of all three mGluR1 splice variants (Fig. 5C). In these experiments, pretreatment with the PKC inhibitor GF 109203X completely blocked PMA-induced receptor internalization, whereas KN-93 was ineffective.

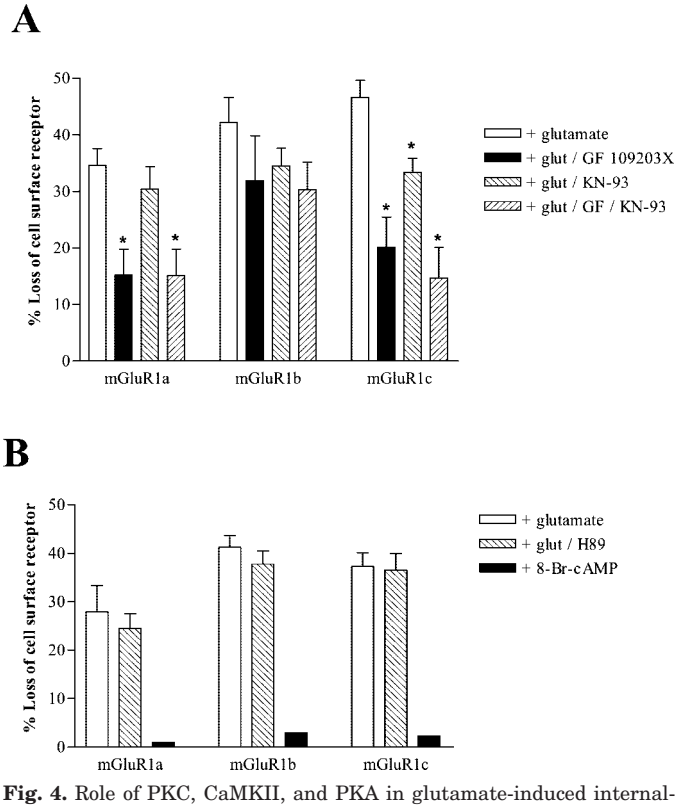

Fig. 4. Role of PKC, CaMKII, and PKA in glutamate-induced internalization of mGluR1a, -1b, and -1c. HEK293 cells were transiently transfected with 5 μg of pcDNA3-mGluR1a, -1b, or -1c DNA, and used 2 days later. A, cells were pretreated with the PKC inhibitor GF 109203X (2 μM, 15 min), CaMKII inhibitor KN-93 (1 μM, 15 min) or both kinase inhibitors and subsequently challenged with glutamate (10 μM, 30 min). B, cells were pretreated with the PKA inhibitor H-89 (10 μM, 15 min) and subsequently challenged with glutamate (10 μM, 30 min), or pretreated with the cAMP analog 8-bromo-cAMP (8-Br-cAMP; 10 μM, 30 min) alone. Surface receptor loss was assessed by ELISA. The data represent means ± S.E.M. of five independent experiments. ⋆, $p < 0.05$ compared with respective glutamate-alone-induced cell surface receptor loss (Mann-Whitney *U* test).

Arrestin and Dynamin Dependence of Muscarinic Acetylcholine Receptor-Mediated mGluR1 Internalization. To determine whether carbachol-induced mGluR1a, -1b, and -1c internalization is an arrestin- and dynamin-dependent process, cells were cotransfected with mGluR1a, -1b, or -1c and either arr-DNM or dyn-DNM (Fig. 6, A and B). Whereas expression of either arr-DNM or dyn-DNM strongly inhibited carbachol-induced (1 mM, 30 min) internalization of mGluR1a and -1c by 50 to 70%, the arr-DNM was much less effective in reducing carbachol-induced mGluR1b receptor (approximately 25% inhibition). However, dyn-DNM expression strongly inhibited carbachol-induced internalization of all three splice variants.

We next investigated whether arrestin-2-GFP or arrestin-3-GFP underwent carbachol-induced translocation from cytosol to membrane in HEK293 cells coexpressing mGluR1a, -1b, or -1c (Fig. 6C). We found that carbachol-dependent arrestin-2-GFP translocation was much more pronounced in cells expressing mGluR1a or mGluR1c than in those expressing mGluR1b. Furthermore, although carbachol addition (1 mM, 10 min) promoted limited arrestin translocation, internalization of the mGluR1b was clearly visible (Fig. 7). Interestingly, subsequent addition of glutamate (10 μM, 1 min)

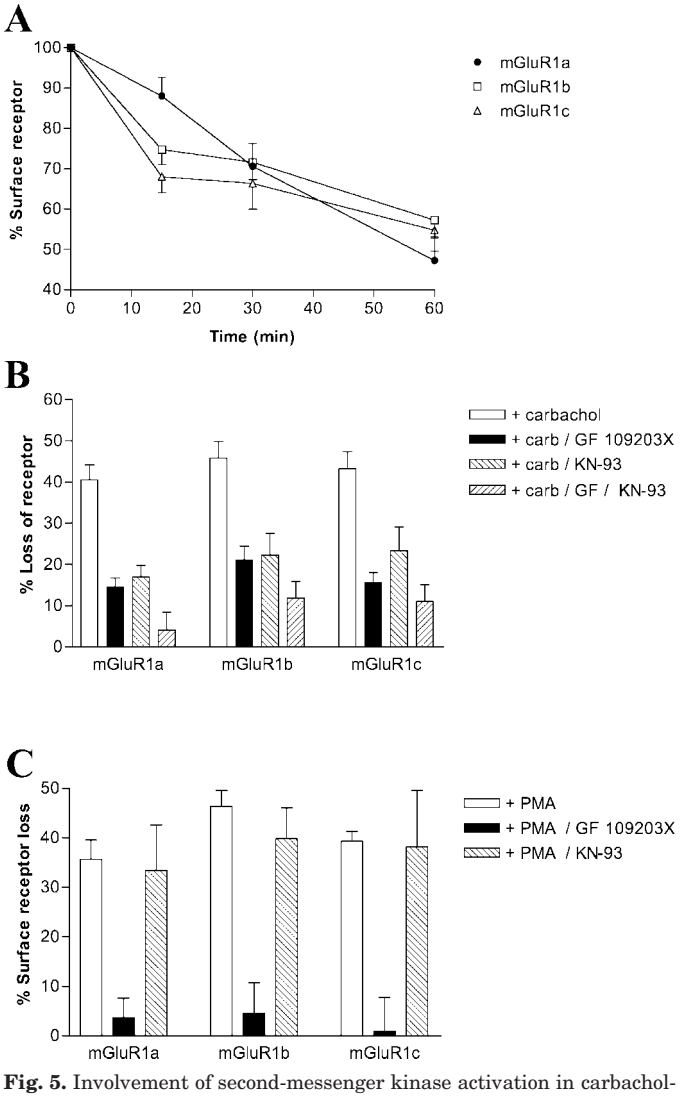

Fig. 5. Involvement of second-messenger kinase activation in carbachol-induced internalization of mGluR1a, -1b, and -1c. HEK293 cells were transiently transfected with 5 μg of pcDNA3-mGluR1a, -1b, or -1c DNA 2 days before assessment of surface receptor loss. A, cells were challenged with carbachol (1 mM, 0–60 min), and surface receptor loss was assessed by ELISA. Internalization of the mGluR splice variants was compared against surface receptor expression at time 0. B and C, cells were pretreated with the PKC inhibitor GF 109203X (2 μM;15 min), the CaMKII inhibitor KN-93 (1 μM, 15 min) or both kinase inhibitors and subsequently challenged with carbachol (1 mM, 30 min) (B) or PMA (1 μM, 30 min) (C). Surface receptor loss was subsequently assessed by ELISA. The data represent means ± S.E.M. of five independent experiments.

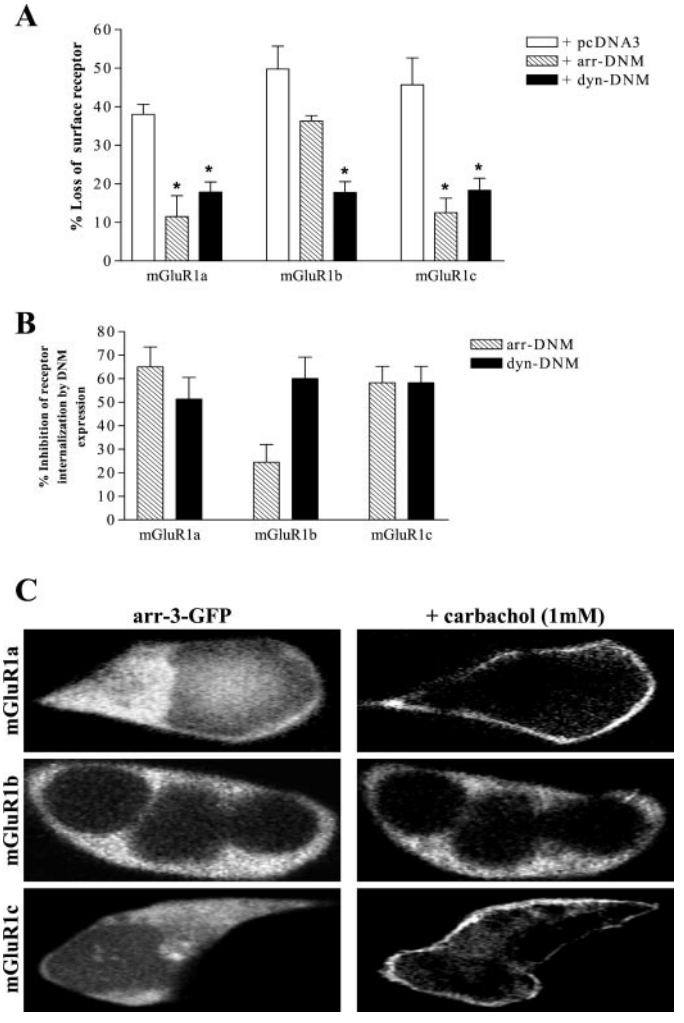

Fig. 6. A and B, effect of arr-DNM or dyn-DNM on carbachol-induced mGluR1a, -1b, and -1c internalization. A and B, HEK293 cells were transiently transfected with 10 μg of pcDNA3-mGluR1a and 5 μg of either pcDNA3 alone, pcDNA3-arrestin-2-(319–418), or pcDNA3-dynamin K44A (overexpression of dominant-negative mutants >10-fold over basal as assessed by immunoblot; data not shown). Cells were challenged with carbachol (1 mM, 30 min) 2 days later, and surface receptor loss was assessed by ELISA. The data represent means ± S.E.M. of five independent experiments. A, data presented as percentage loss of cell surface receptor. $\star$, $p < 0.05$ versus pcDNA3 alone (Mann-Whitney U test). B, same data as in A presented as percentage of inhibition of receptor loss by DNM construct expression. C, carbachol-induced translocation of arrestin-2-GFP in HEK293 coexpressing mGluR1 splice variants. HEK293 cells grown on poly-L-lysine coverslips were transiently transfected with 5 μg of pcDNA3-HA-mGluR1a, -1b, or -1c and 0.5 μg of peGFP-N1-arrestin-2-GFP. The expression of mGluR1 splice variants at the cell membrane was visualized 2 days later and confirmed using a rhodamine-conjugated anti-HA antibody (data not shown). Before carbachol stimulation and viewing, coverslips were mounted in an imaging chamber at 37°C. The initial diffuse cytoplasmic distribution of arrestin-3-GFP is shown before agonist stimulation (left). Carbachol (1 mM) was added and the redistribution of arrestins was monitored in real time. The images shown were collected at 180 s after agonist addition.

promoted extensive arrestin translocation, indicating that agonist-occupation of the mGluR1b is necessary for robust arrestin translocation. Importantly, carbachol-induced arrestin-GFP translocation was absent in cells not cotransfected with mGluR1 constructs (data not shown).

Involvement of Second-Messenger-Dependent Protein Kinases and Arrestins in Glutamate-Induced Signaling of mGluR1 Splice Variants. mGluR-mediated phospholipase C activation was assessed in intact cells by measuring [^{3}H]inositol phosphate (IP) accumulation after addition of agonist (Fig. 8A). We also assessed endogenous M_1 AChR-stimulated IP accumulation with carbachol (1 mM; Fig. 8A). IP accumulation was greater for the endogenously expressed M1 AChR than for any of the transiently transfected mGluR1 splice variants, with IP accumulation for the mGluR1a in turn greater than either the mGluR1b or mGluR1c. Because second-messenger kinases regulated the internalization of mGluR1 splice variants, we determined whether blockade of these kinases would also modify agonist-induced mGluR1 splice variant signaling (Fig. 8B). IP accumulation after glutamate-induced activation of mGluR1a and 1c splice variants was increased by pretreatment with the PKC inhibitors GF 109203X (2 μM) or the CaMKII inhibitor KN-93 (1 μM) with coaddition of both of these kinase inhibitors being additive. Interestingly, inhibition of PKC or CaMKII had no effect on mGluR1b signaling, indicating that these protein kinases selectively regulate the mGluR1a and 1c splice variants. Thus, the homologous desensitization of the mGluR1a and 1c splice variants depends partially upon second-messenger–dependent protein kinase activation.

Because each of the mGluR1 splice variants undergoes arrestin-dependent internalization in response to glutamate, we sought to determine whether arrestins could also regulate mGluR1 IP signaling. Cells were cotransfected with each splice variant and either arrestin-3 or arr-DNM. As shown in Fig. 8C, expression of arrestin-3 reduced IP accumulation after glutamate-induced activation of all three mGluR1 splice variants, indicating that this protein can regulate the functional output of these receptors. Interestingly, expression of the arr-DNM, which lacks the receptor binding region and competes with wild-type arrestin for clathrin binding (Krupnick et al., 1997b) did not affect mGluR1 splice variant

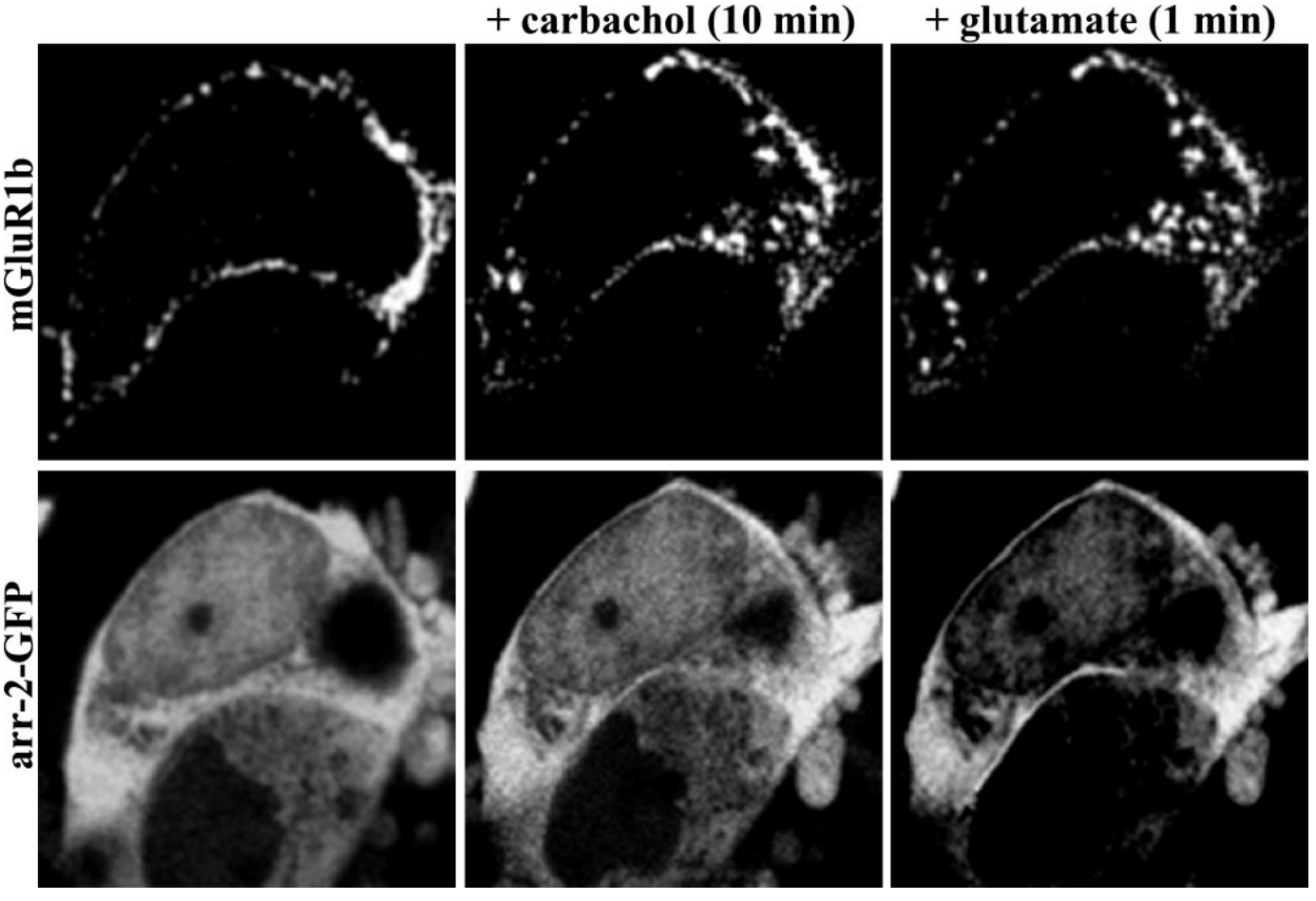

Fig. 7. Carbachol-induced internalization of mGluR1b in the absence of arrestin-2-GFP translocation. HEK293 cells grown on poly-L-lysine coverslips were transiently transfected with 5 μg of pcDNA3-mGluR1b and 0.5 μg of peGFP-N1-arrestin-3-GFP. Two days later, before stimulation and viewing, coverslips were mounted in an imaging chamber at 37°C. mGluR1b was visualized using a rhodamine-conjugated anti-HA antibody (top). The initial predominantly membrane localization of HA-mGluR1b (top left) and the diffuse cytoplasmic distribution of arrestin-3-GFP (bottom left) is shown before agonist stimulation. Carbachol (1 mM) was then added and the redistribution of mGluR1b and arrestin-3-GFP was monitored in real time. The images (center, top and bottom) shown were collected 10 min after carbachol addition. Although the mGluR1b underwent significant internalization (center top), no significant translocation of arrestin was evident (center bottom). Glutamate (10 μM) was subsequently added, and any further redistribution of mGluR1b and arrestin-3-GFP was monitored in real time. The images (right, top and bottom) shown were collected 1 min after glutamate addition. Significant agonist-induced translocation of arrestin-3-GFP is now clearly visible, with little further internalization of mGluR1b.

signaling, suggesting that internalization of mGluR1 splice variants plays no role in their desensitization.

Discussion

In this study of mGluR1 splice variant internalization and signaling, we report a number of novel findings. First, mGluR1a, -1b, and -1c splice variants all undergo glutamate-induced arrestin- and dynamin-dependent internalization, but mGluR1a internalizes more slowly than mGluR1b or -1c. Second, PKC is responsible in part for the glutamate-induced internalization of mGluR1a and -1c. Third, mGluR1 splice variants can all undergo glutamate-independent PKC- and CaMKII-dependent internalization. Fourth, regulation of mGluR1 splice variants by these second-messenger kinases confers differential arrestin dependence; mGluR1a and -1c undergo arrestin-dependent internalization, whereas that of mGluR1b is largely arrestin-independent. Finally, the glutamate-induced homologous desensitization of mGluR1a and 1c, but not 1b, involves in part PKC and CaMKII.

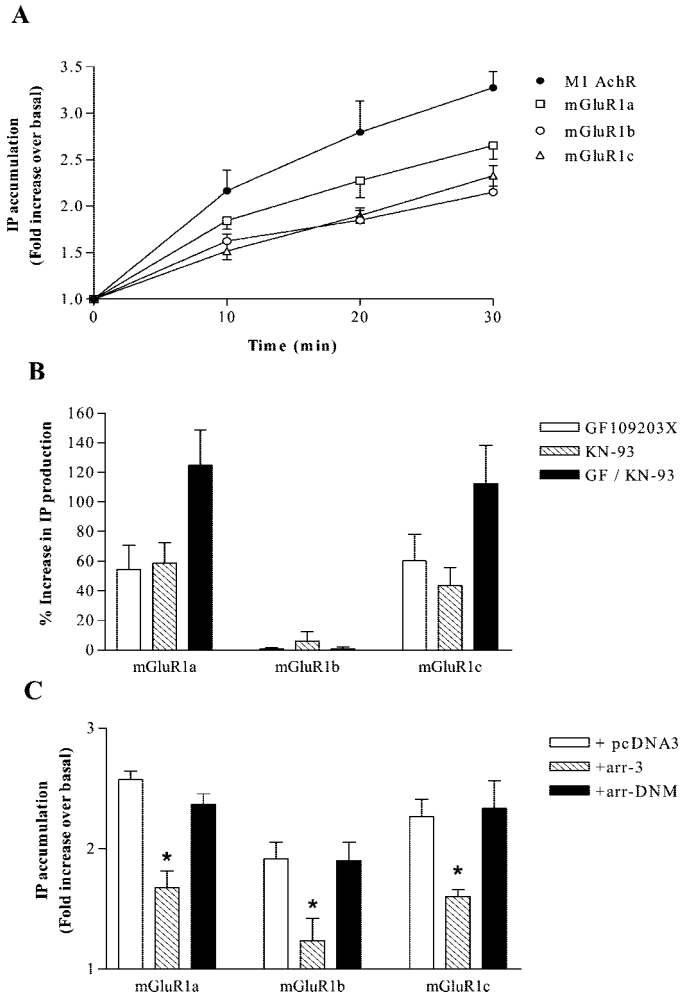

Fig. 8. Involvement of second-messenger kinase activation and arrestins in agonist-induced IP accumulation in HEK293 cells transiently expressing mGluR1 splice variants. HEK293 cells were transiently transfected with 5 μg of pcDNA3-mGluR1a, -1b, or -1c DNA 2 days before assessment of agonist-induced IP accumulation. A, total IP levels were measured at various times after the addition of 10 μM glutamate (for the mGluR splice variants) or 1 mM carbachol (for endogenous M_1 AChR). B, cells were pretreated with the PKC inhibitor GF 109203X (2 μM, 15 min), the CaMKII inhibitor KN-93 (1 μM, 15 min) or both kinase inhibitors and total IP accumulation measured after the addition of glutamate (10 μM, 30 min). C, cells were transiently transfected with receptor and 5 μg of either pcDNA3 alone, pcDNA3-arrestin-3, or pcDNA3-arrestin-2-(319–418) (over-expression of arrestin-3 or dominant-negative mutant >10-fold over basal as assessed by immunoblot, data not shown). Total IP accumulation was measured after the addition of glutamate (10 μM, 30 min). The data represent means ± S.E.M. of five independent experiments and are expressed as fold increase over basal (A and C) or as percentage increase in IP production after pretreatment with protein kinase inhibitor (B). ⋆, $p < 0.05$ versus pcDNA3 alone (Mann-Whitney U test).

The splice variants of mGluR1 have so far been reported to show little difference in their agonist responses, with each exhibiting an activation of phospholipase C (Pin and Duvoisin, 1995). However, expression of the long isoform of mGluR1 (1a) in a heterologous expression system results in an increase in basal phospholipase C activity, which is absent when the short isoforms 1b and 1c are similarly expressed (Prezeau et al., 1996). This has led to the suggestion that the long intracellular tail of the mGluR1a is responsible for constitutive coupling of the receptor to intracellular G-proteins. A recent study showed that interaction of the long COOH-terminal tail of mGluR1a with specific members of the Homer protein family are responsible for this effect (Ango et al., 2001). The present results show that the short-tailed isoforms internalize more rapidly than the longer tailed mGluR1a. This seems to be a general phenomenon, because it was seen in both HEK293 and COS-7 cells. The reason for this difference in rate of internalization remains unclear, but it is possible that the long COOH-terminal tail of mGluR1a either directly inhibits its interaction with some component of the internalization machinery, such as arrestins, or that it promotes interaction with other proteins, such as Homer, which could potentially influence the rate of internalization. However, although the kinetics of glutamate-dependent internalization differs, each of the splice variants undergoes arrestin- and dynamin-dependent internalization. The signaling and internalization of numerous GPCRs are now known to be intimately controlled by arrestins, which bind to the agonist-activated GPCR at the plasma membrane (Goodman et al., 1996; Laporte et al., 1999). Indeed, agonist activation of each of the mGluR1 splice variants induces arrestin translocation to the membrane, a feature common to many GPCRs (Barak et al., 1997; Mundell et al., 2000). A motif necessary for arrestin-GPCR association has yet to be identified, although arrestins seem to interact with GPCRs that have been phosphorylated by GRKs on serine or threonine residues located in the third intracellular loop or COOH-terminal tail of the receptor. There are certainly numerous potential phosphoacceptor residues in the COOH-terminal tails of the splice variants examined in the present study (Fig. 1), but the results make it clear that the short-tailed splice variants are able to interact with arrestins as well as, if not better, than the long-tailed mGluR1a. Recent studies show that mGluR1a is subject to phosphorylation and regulation by GRKs (Dale et al., 2000; Sallese et al., 2000). We are now trying to determine regions of mGluR1a, -1b, and -1c that interact with arrestin.

A number of studies have identified a role of the second-messenger kinase PKC in the regulation of mGluRs. For example, activation of PKC by PMA induces a rapid loss of mGluR1a and -1b from the cell surface in BHK cells (Ciruela and McIlhinney, 1997). In addition, mGluR1c desensitizes in response to PKC phosphorylation (Ciruela et al., 1999). An-

other recent study mapped residue Thr695, in the second intracellular loop (a region critical in G-protein interaction), as a site for phosphorylation by PKC, which proved critical in uncoupling the mGluR1a from $G_{q/11}$ (Francesconi and Duvoisin, 2000). In our study, we show that PKC activation is important for the glutamate-induced internalization of the mGluR1 splice variants, in particular mGluR1a and -1c, where GF 109203X inhibits internalization by approximately 50%. This was surprising because a recent study indicates that PKC plays no role in quisqualate-induced internalization of mGluR1a in cerebellar Purkinje cells (Sallese et al., 2000). Thus, the importance of PKC in mGluR1 internalization may vary among cell types.

Our functional studies with mGluR1 splice variants also discovered an important role for PKC in receptor regulation. We found that the homologous desensitization of only the mGluR1a and -1c splice variants is regulated by PKC, whereas that of the mGluR1b is not. In addition, as with PKC, the desensitization of only mGluR1a and -1c is regulated in part by CaMKII. Unfortunately, the present results do not allow us to conclude that the mGluR1 receptors are targets for direct phosphorylation by PKC, because other components of the internalization and desensitization machinery could also be substrates for PKC action. However, previous studies (Ciruela et al., 1999; Francesconi and Duvoisin, 2000) show that mGluR1 is directly phosphorylated by PKC, making this the most probable means by which PKC regulates mGluR1 splice variant internalization and function. Each splice variant does contain putative PKC phosphorylation consensus sequences, although more complex mutagenesis studies will be required to determine whether these residues are important in altering the phosphorylation status and, subsequently, the internalization and desensitization of mGluR1 splice variants.

We also report the novel finding that heterologous activation of PKC after stimulation of G_q-coupled M_1 muscarinic receptors, or directly with PMA, can induce internalization of agonist-unoccupied mGluR1a, -1b, and -1c. Although PMA-induced internalization of mGluR1a and -1b has been demonstrated previously (Ciruela and McIlhinney, 1997), this is the first report of mGluR1 internalization after activation of another GPCR. The carbachol-induced internalization of the mGluR1 splice variants was markedly dependent on both PKC and CaMKII activity. To our knowledge, regulation of the internalization of a GPCR by CaMKII has not previously been reported. Consensus sequences for CaMKII phosphorylation exist in the COOH-terminal tails of all three splice variants studied here (Thr_{880}; Fig. 1). It will be of great interest to determine whether mGluR1 receptors are targets for direct phosphorylation by CaMKII and whether this activity regulates other receptor mechanisms, such as desensitization and recycling. The CaMKII dependence of glutamate-induced internalization was less than that for carbachol-induced internalization. One possible explanation is that PI hydrolysis (and by inference intracellular Ca^{2+}) in these cells, subsequent to muscarinic receptor-stimulation, is greater than that caused by glutamate receptor stimulation (Fig. 8), thus causing greater activation of CaMKII after carbachol than glutamate addition.

It has recently become apparent that arrestins can interact with non-GRK phosphorylated GPCRs, and a recent study demonstrated that, after PKC phosphorylation, the δ-opioid receptor undergoes internalization via an arrestin- and clathrin-dependent mechanism (Xiang et al., 2001). Another recent report showed that internalization of human parathyroid hormone receptor 1 complex is PKC-dependent and also involves arrestin-3 (Ferrari et al., 1999). In our study, using arrestin-DNM and dynamin-DNM, we show that the mGluR1a and -1c splice variants can also undergo internalization after second-messenger kinase activation, which is arrestin- and dynamin-dependent. Moreover, these mGluR splice variants undergo arrestin-dependent internalization in the absence of the agonist glutamate. A series of elegant studies using the model system of visual arrestin-rhodopsin interaction have demonstrated that arrestin binding has the dual requirement of receptor phosphorylation plus receptor in an active, agonist-bound conformation (Hirsch et al., 1999; Vishnivetskiy et al., 1999). Our study suggests that arrestins can interact with the nonagonist-activated mGluR1a and -1c splice variants; either these receptors have less stringent requirements for arrestin interaction compared with other GPCRs or second-messenger kinase phosphorylation is able to reduce them. Given that mGluR1a displays constitutive agonist-independent activity (Prezeau et al., 1996) it is interesting to speculate that the ability of this receptor to switch to an active conformation plus carbachol-dependent phosphorylation may produce the dual requirements for arrestin binding. In any case, our study is in contrast to the commonly held perception that arrestins will only interact significantly with an agonist-occupied GPCR.

Our findings with the mGluR1b splice variant contrast with those for mGluR1a and -1c. Although the mGluR1b splice variant undergoes PKC- and CaMKII-dependent internalization in response to carbachol, this is largely arrestin-independent. Unlike the other splice variants, extensive arrestin-GFP translocation from cytosol to membrane is not apparent after activation of second-messenger kinases in mGluR1b-expressing cells; however, the receptor still undergoes internalization. In addition, we can promote further extensive agonist-dependent arrestin translocation in mGluR1b-expressing cells subsequent to activation of second-messenger kinases. This indicates that receptor occupancy is essential for significant arrestin interaction with this splice variant, which seems not to be the case for either mGluR1a or -1c. We are unaware of any other report showing that mGluR1 splice variants are regulated by different cellular mechanisms. It will be of interest to determine the mechanism by which mGluR1b internalizes after second-messenger kinase activation, because this is clearly different from the arrestin-dependent mechanism after glutamate activation of the receptor.

In a recent study, mGluR1a was reported to undergo agonist-induced internalization in HEK293 cells only when GRK4 was coexpressed (Sallese et al., 2000). Our findings are at variance with this; we observed extensive mGluR1 internalization in HEK293 cells in the absence of exogenous GRK coexpression. The reason for this difference is unclear. However, the ability of a combination of PKC and CaMKII inhibitors to only partially block glutamate-induced mGluR1 splice variant internalization, coupled with the strong arrestin dependence of glutamate-induced internalization, suggests that other kinases, in particular GRKs, are also involved. Recent reports show that coexpression of GRKs with mGluR1a in HEK293 cells inhibits agonist-stimulated inosi-

tol phosphate formation (Dale et al., 2000; Sallese et al., 2000), suggesting that these kinases can regulate mGluR1 function. Although not addressed in the present study, it is clear that further experiments are warranted to determine the relative involvement of GRKs in mGluR1 function.

In our studies, we found that arrestin over-expression reduced agonist-induced mGluR1 splice variant signaling, indicating that arrestins may play a role in the homologous desensitization of these receptors. Interestingly, we also found that an arr-DNM, which lacks the receptor-binding region and competes with wild-type arrestin for clathrin binding (Krupnick et al., 1997b), does not affect mGluR1 splice variant signaling. These results suggest that although arrestin may uncouple mGluR1 splice variants from G-protein, their subsequent role in directing a receptor for internalization and receptor internalization in general plays no role in homologous desensitization.

In summary, the present study characterizes for the first time the mechanisms of agonist- and second-messenger kinase-dependent internalization and desensitization of the mGluR1 splice variants, representative members of a distinct subclass of GPCRs. Studies are under way to determine the structural elements of mGluR1 splice variants that contribute to these specific pathways of internalization.

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