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The metabotropic glutamate receptor 4 is internalized and desensitized upon protein kinase C activation

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1 The metabotropic glutamate receptor 4 (mGluR4) is a $G\alpha_i$-coupled receptor that modulates glutamatergic neurotransmission. As mGluR4 expression and activation have been implicated in a number of pathological conditions and because the internalization and desensitization properties of this receptor are poorly understood, studies were designed to investigate these aspects of mGluR4 biology.

2 Neither agonist activation by L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) nor L-glutamate caused mGluR4 internalization when cmyc-tagged mGluR4 was expressed in a human embryonic kidney 293 cell line as assessed by cell surface enzyme-linked immunosorbent and immunostaining assays. Instead, a modest increase in mGluR4 surface expression was observed and found to be receptor specific as the competitive antagonist α-cyclopropyl-4-phosphonophenylglycine (CPPG) blocked this effect.

3 In contrast, mGluR4 internalized when the protein kinase C (PKC) pathway was activated either by phorbol-12-myristate-13-acetate (PMA) or by the activation of the $G\alpha_q$-coupled, neurokinin 3 receptor (NK3R) when co-expressed. This process was PKC-dependent as the specific PKC inhibitor GF 109203X inhibited PMA and NK3R-mediated internalization.

4 PKC activation by PMA caused desensitization of mGluR4 as measured by forskolin-stimulated cAMP inhibition, whereas agonist activation had no effect on desensitization.

5 When mGluR4's coupling was redirected from adenylyl cyclase to phospholipase C by coexpression of a chimeric $G\alpha_{qo5}$ protein, mGluR4 both internalized and desensitized in response to its agonists.

6 These findings demonstrate that mGluR4 internalization and desensitization are agonist-independent unless pathways leading to the activation of PKC are induced.
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Abbreviations: AC, adenylyl cyclase; BHK, baby hamster kidney; CPPG, α-cyclopropyl-4-phosphonophenylglycine; D2R, dopamine 2 (long) receptor; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; GF 109203X, (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide); GPCR, G protein-coupled receptor; GRKs, G protein receptor kinases; HBSS, Hank's balanced salt solution; HEK, human embryo kidney; iGluR, ionotropic glutamate receptor; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; mGluR, metabotropic glutamate receptor; NK3R, neurokinin 3 receptor; PBS, phosphate-buffered saline; PHCCC, *N*-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; SPA, scintillation proximity assay

Introduction

The metabotropic glutamate receptors (mGluRs) are G protein coupled, membrane-bound receptors that play an important role in glutamatergic neurotransmission. While fast excitatory neurotransmission is mediated by the ionotropic glutamate receptors (iGluRs), mGluRs regulate neuronal excitability partly through the formation of second messengers. The mGluRs are divided into three groups based on their sequence homology, pharmacology, and G-protein-coupling properties. Group I consists of mGluR1 and 5 and activates phospholipase C (PLC) *via* $G\alpha_q$, whereas Group II (mGluR2 and 3) and Group III (mGluR4, 6, 7 and 8) inhibit adenylyl cyclase (AC) *via* $G\alpha_i$. Whereas Group I mGluRs are primarily postsynaptic and modulate iGluR neurotransmission, Group II and III are generally presynaptic. Group III mGluRs function as autoreceptors and lower the release of glutamate from the presynaptic neuron (Schoepp, 2001). Thus, it is not surprising that these receptors are involved in a number of pathological conditions.

Activation of Group III mGluRs has anticonvulsant effects in animal models of epilepsy (reviewed in Moldrich *et al.*, 2003). In seizure-kindled rats, Group III presynaptic autoreceptor function is altered. The inhibitory effect of Group III agonists on excitatory synaptic transmission is increased in the contralateral amygdala of amygdala-kindled rats (Neugebauer

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et al., 1997) while decreased in the dentate gyrus of hippocampal-kindled rats (Klapstein *et al*., 1999). Furthermore, mGluR4 is upregulated in human temporal lobe epilepsy (Lie *et al*., 2000) and mGluR4 knockout (−/−) mice show resistance to GABA(A) antagonist-induced absence seizures (Snead *et al*., 2000). Collectively, these studies suggest a role for mGluR4 expression and function in normalizing glutamatergic neurotransmission.

Aside from neurotransmission, a role for mGluR4 in cell survival is emerging. A decrease in mGluR4 mRNA levels has been associated with apoptosis of potassium-deprived cerebellar granule cells whereas an increase in mGluR4 expression or Group III receptor activation is associated with neuronal survival (Borodezt & D'Mello, 1998). Moreover, activation of Group III mGluRs is neuroprotective in primary neuronal cultures (Bruno *et al*., 2001). With the identification of the subtype selective mGluR4-positive allosteric modulator PHCCC, it has been possible to demonstrate that mGluR4 activation is partially responsible for the observed neuroprotection in NMDA-induced excitotoxicity and β-amyloid peptide-induced neurotoxicity in mixed cortical cultures (Maj *et al*., 2003).

As G protein-coupled receptor (GPCR) function is clearly linked to receptor membrane expression and trafficking, which are regulated by G-protein receptor and second messenger-dependent protein kinases (Ferguson, 2001), it is important to understand these processes in relation to receptor function *in vivo*. Receptor internalization for Group I mGluRs has been well characterized (Dale *et al*., 2001; Mundell *et al*., 2001; 2002) and is associated with receptor desensitization (Dale *et al*., 2002; Mundell *et al*., 2004a). In contrast, receptor internalization studies for Group III mGluRs and, in particular, mGluR4 are very limited. Owing to the lack of subtype selective ligands activating individual Group III mGluRs, desensitization has only been characterized in terms of the whole group and primarily *in situ* (Macek *et al*., 1998; Cai *et al*., 2001). Given the presence of multiple Group III subtypes in native tissue preparations, expression of receptors in a heterologous expression system has the advantage that the individual mGluR subtype can be studied.

Thus in order to investigate mGluR4 pharmacology, experiments was carried out to examine the effect of mGluR4 activation on receptor internalization and desensitization. Surprisingly, neither activation with the agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) nor L-glutamate caused mGluR4 internalization or desensitization. However, when the protein kinase C (PKC) pathway was activated directly by phorbol-12-myristate-13-acetate (PMA), by activation of a heterologously expressed $G\alpha_q$-coupled receptor, or through homologous activation with a chimeric $G\alpha_{qo5}$ protein, mGluR4 both internalized and desensitized. These findings demonstrate that mGluR4 internalization and desensitization are agonist-independent unless pathways leading to the activation of PKC are activated.

Methods

Materials

All chemicals were purchased from Sigma-Aldrich (Denmark) and all cell culture supplies from Gibco-BRL (Invitrogen, Denmark) unless otherwise noted. The following antibodies were used in these studies: mouse monoclonal anti-cmyc (clone 9E10, Sigma-Aldrich, Denmark), rabbit polyclonal anti-mGluR4 (Zymed, South San Francisco, CA, U.S.A.), Cy2-conjugated sheep anti-mouse and Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, U.K.), and HRP-conjugated goat anti-mouse (Molecular Probes, Leiden, Netherlands). The following mGluR ligands were obtained from Tocris Cookson (U.K.): L-AP4, [^{3}H]L-AP4 (52 Ci mmol^{-1}), α-cyclopropyl-4-phosphonophenylglycine (CPPG), and L-quisqualate. Perkin Elmer Life Sciences (Boston, MA, U.S.A.) supplied [^{35}S]GTPγS (1250 Ci mmol^{-1}) and [^{125}I]-cAMP (2200 Ci mmol^{-1}), while PMA, H-89, and GF 109203X (GFX) were bought from Calbiochem (U.K.).

Plasmid constructs

The cmyc-tagged mGluR4 (cmyc-mGluR4) was generated by introducing the amino-acid sequence TREQKLISEEDLTR between amino acids 22 and 23 in the human wild-type mGluR4. Two overlapping fragments each encoding parts of mGluR4 adjacent to amino acid 22/23 and the cmyc-epitope were generated by PCR with the human mGluR4 (Swiss-Prot no. Q14833) as the template. The human mGluR4 was previously subcloned into pCI-neo (Mathiesen *et al*., 2003) and has a *Nhe*I site located in front of the coding sequence. Fragment 1 that encodes part of the pCI-neo vector, the first 22 amino acids of mGluR4, and the cmyc-epitope insert was amplified using the primer set CTGGGCAGGTAAGTA TCAAGG (sense) and CTCGCTGATCAGCTTCTGCTC TCGCGTTCCCAGGGAGGAAGGCATCCAG (antisense). Fragment 2 that encodes the cmyc-epitope insert and mGluR4 from amino acid 23–304 (including the silent *Age*I site) was amplified using the primer set GCAGAAGCTGATCAGCGA GGAGGACCTGACGCGTAAGCCCAAAGGCCAC (sense) and GAAATGACCGGTCTGGTTGGCCCTTCGTGC (antisense). Fragments 1 and 2 were joined by overlap PCR technique using the outer primers and subsequently subcloned into the original human mGluR4 in the pCI-neo template using the restriction sites *Nhe*I and *Age*I.

The chimeric $G\alpha_{qo5}$ was generated by exchanging the last five amino acids of the human $G\alpha_q$ (EYNLV) with those of $G\alpha_o$ (GCGLY) by PCR. The human $G\alpha_q$ (Swiss-Prot no. P50148) was amplified with the primers GCTAGCGCCACCA TGACTCTGGAGTCCATCATG (sense) and GCGGCCGC TTAGTACAGGCCGCAGCCCTTCAGGTTCAACTGGAG GAT (antisense), which incorporated a *Nhe*I site and a kozak sequence in front of the coding sequence for $G\alpha_q$ and exchanged the last five amino acids of $G\alpha_q$ with those of $G\alpha_o$ and a *Not*I site. The amplified DNA encoding the chimeric $G\alpha_{qo5}$ was subcloned into the pCI-neo vector using the restriction sites *Nhe*I and *Not*I.

The cmyc-tagged mGluR1a was generated as described (Mundell *et al*., 2001) except that the extended cmyc-epitope (TREQKLISEEDLTR) was used instead of the HA-epitope tag and that the human mGluR1a, cloned from cerebellum poly(A)$^+$ mRNA (BD Biosciences, San Jose, CA, U.S.A.) by PCR, was used as a template. The cmyc-mGluR1a was subcloned into pCI-neo.

The dopamine 2 (long) receptor (D2R) (Swiss-Prot no. P14416-1) cDNA was cloned from human pituitary poly(A)$^+$ mRNA (BD Biosciences, U.S.A.) by PCR. For generating the

cmyc-D2R, D2R was flanked by the restrictions sites *Mlu*I and *Not*I in the 5′- and 3′-ends, respectively, by amplification with the primers ACGCGTGATCCACTGAATCTGTCCT GG (sense) and GCGGCCGCTCAGCAGTGAAGGATCTT CAG (antisense). Using the restriction sites *Mlu*I and *Not*I, DR2 was subcloned into an engineered pCI-neo vector, which contained the cmyc-epitope in front of the *Mlu*I site. The neurokinin 3 receptor (NK3R) (Swiss-Prot no. P29371) was constructed as described (Chung *et al*., 1994) and subcloned into pCI-neo and was kindly provided by Dr Soeren M. Nielsen, Department of Molecular Pharmacology, H. Lundbeck A/S. All constructs were sequence verified.

Cell culture and transfection

Baby hamster kidney (BHK) and human embryo kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM from Biological Industries, Israel) containing glutamax I, 10% dialysed fetal calf serum, and 1 mM sodium pyruvate (supplemented DMEM). For transient transfections, BHK or HEK cells were seeded in 35 or 100 mm tissue culture dishes and grown to 80–90% confluency. Transient transfections were carried out for 6 h using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Denmark) after which, the medium was changed to supplemented DMEM. Approximately 0.4 μg of cmyc-mGluR4 or cmyc-mGluR1a DNA was used for 35 mm tissue culture dish transfections. For surface receptor studies, 0.4 μg of cmyc-mGluR4 DNA was supplemented with 0.8 μg of D2R, NK3R or empty vector DNA, whereas 0.8 μg DNA was used for surface receptor studies of cmyc-D2R. Moreover, 0.8 μg of $G\alpha_{qo5}$ DNA was used for transient transfections in stably expressing cmyc-mGluR4 HEK cells. For transfections performed in 100 mm culture dishes, the amount of DNA was increased by eightfold.

Stably expressing, cmyc-mGluR4 HEK cells were generated by dilution cloning in the presence of 5 mg ml^{-1} G418. Clonal cell lines were identified by immunostaining as described below and tested for their ability to inhibit forskolin-stimulated cAMP formation.

Membrane preparation, [^{3}H]L-AP4 radioligand binding and [^{35}S]GTPγS binding

BHK cells were transiently transfected with the cmyc-mGluR4 in 100 mm tissue culture dishes. After 24 h, cells were reseeded in a 245 × 245 × 45 tissue culture dish (Nunc, Denmark) and grown to 90% confluency under selection with 2 mg ml^{-1} G418. Cells were harvested and membranes prepared as described (Mathiesen *et al*., 2003). [^{3}H]L-AP4 radioligand and [^{35}S]GTPγS binding assays were performed on BHK membranes using a scintillation proximity assay (Amersham, U.K.) (Mathiesen *et al*., 2003).

Immunostaining

The day after transfection, cells were seeded in poly-D-lysine coated Lab-Tek 4 well glass slides (Nunc, Roskilde, Denmark) at 120,000 cells per well (HEK) or 65,000 cells per well (BHK). On day 2, cells were drug treated, fixed in 4% paraformaldehyde for 12 min, and incubated in blocking solution (50 mM Tris pH = 7.5, 1 mM $CaCl_2$ and 3% dry milk) for 20 min. Cells were incubated with primary antibodies diluted in blocking solution for 1 h (1 : 1000 for mouse anti-cmyc and 1 : 250 for rabbit anti-mGluR4 antibodies) and then with secondary antibodies (1 : 200) for 45 min. Cells were then washed and mounted with Vectashield (Vector Labs). For visualization of intracellular receptors, the blocking solution contained 0.1% Triton X-100. Cellular localization of the receptors was observed by confocal microscopy using a Nikon Eclipse TE300 microscope equipped with Bio-Rad Radiance 2100 Laser Scanning System.

Cell surface ELISA

Cell surface enzyme-linked immunosorbent assay (ELISA) was performed as described in the immunostaining procedure except that HEK cells were seeded in black poly-D-lysine coated 96-well plates at 31,000 cells per well. The primary antibody used was a mouse anti-cmyc antibody diluted 1 : 1000 in blocking solution followed by a goat anti-mouse HRP-conjugated antibody diluted 1 : 400 in PBS with 1% bovine serum albumin. Cell surface receptors were quantified using the HRP-sensitive substrate Amplex™ Red according to the manufacturer's instructions (A22170, Molecular Probes, Denmark). The amount of substrate initially converted to fluorescent product was proportional to the amount of surface receptors and quantified by determining the fluorescence (Ex = 544 nm/Em = 590 nm) using a Novostar (BML Labtechnologies, Germany) or a M2 Spectramax fluorimeter (Molecular Devices, Sunnyvale, CA, U.S.A.). Fluorescence from pCI-neo transfected HEK cells, which represented non-specific binding of primary and secondary antibodies, was subtracted from each well.

Inhibition of forskolin-stimulated cAMP production

The cAMP assay was done as previously described (Mathiesen *et al*., 2003) with modifications. Stably expressing, cmyc-mGluR4 HEK cells were seeded in poly-D-lysine-coated 96-well plates at 34,000 cells per well. The next day, media was changed to ground buffer (DMEM, 20 mM HEPES and 0.1 mM isobutyl-1-methylxanthine at 37°C, pH = 7.4) and cells received buffer, L-AP4 (100 μM), L-glutamate (100 μM), or PMA (1 μM) for 30 min. Cells were then washed after drug treatment and ground buffer containing 10 μM forskolin and increasing concentrations of L-AP4 were added for 15 min. Assays were subsequently terminated and cAMP measured.

Inositol phosphate accumulation assay

The inositol phosphate (InsP) accumulation assay was performed in the SPA bead format using the positively charged nature of yttrium silicate beads to separate inositol phosphates from inositol as described (Brandish *et al*., 2003) with modifications. HEK cells stably expressing the cmyc-mGluR4 were transiently transfected with $G\alpha_{qo5}$. On day 2, cells were seeded at 34,000 cells per well in poly-D-lysine coated 96-well plates and loaded with 5 μCi *myo*-[2-^{3}H]-Inositol (TRK911, Amersham Biosciences). On day 3, cells were washed in HBSS buffer (containing $CaCl_2$, $MgCl_2$, 10 mM HEPES pH = 7.4) and prestimulated with the respective agonists and/or protein kinase modulators for 30 min at 37°C. After two washes, cells were incubated for 30 min at 37°C with

HBSS buffer containing 5 mM LiCl and varying concentrations of L-AP4. The buffer was aspirated and the reactions terminated by the addition of 50 μl 10 mM ice-cold formic acid. After 30 min on ice, 20 μl lysate aliquots were transferred to white polystyrene 96-well assay plates (Corning, no. 3917) together with 1 mg polylysine-coated YSi SPA beads (RPNQ0010, Amersham Biosciences) suspended in 80 μl ddH_2O. After 1 h of vigorous shaking, plates were centrifuged at 1500 r.p.m. for 5 min and counted in a Topcounter (Packard).

Ca^{2+} mobilization assay

Stably expressing, cmyc-mGluR4 HEK cells were transiently transfected with $G\alpha_{qo5}$ and seeded at 34,000 cells per well in black poly-D-lysine coated 96-well plates the next day. On day 3, cells were washed with assay buffer (HBSS, 2.5 mM probenicid, 20 mM HEPES pH = 7.4) and media replaced with assay buffer containing 1.5 μM Fluo-4 (Molecular Probes). After 15 min, at 37°C, the cells were incubated with either buffer, L-AP4 (100 μM), L-glutamate (100 μM) or PMA (1 μM) for 30 min. Cells were washed and after 5 min, agonist-stimulated intracellular Ca^{2+} was measured on a FLIPR 384 (Molecular Devices, U.S.A.).

Data analysis

Concentration–response and homologous displacement binding curves were analysed by nonlinear regression using GraphPad Prism version 3.0. for Windows (GraphPad Software, San Diego, U.S.A.). Concentration–response curves in the InsP accumulation and Ca^{2+} mobilization assays were normalized to the nonlinear regression fitted maximal response of the agonist in the non-stimulated state. The K_D value of [^{3}H]L-AP4 was estimated using the equation $K_D = IC_{50}–L$, where L is the concentration of the radioactive ligand and IC_{50} values were estimated by nonlinear regression (Motulsky & Neubig, 1997). Changes in cell surface receptors were reported as the percentage of surface receptors at 0 min after subtracting the background fluorescence from pCI-neo transfected HEK cells.

Results

Expression pattern and in vitro *pharmacology of cmyc-tagged mGluR4*

Little is known about the internalization properties for Group III mGluRs and, in particular mGluR4. In order to study this receptor subtype, the human mGluR4 was N-terminally, cmyc-tagged. Insertion of a cmyc-epitope between the signal peptide and the amino terminal domain in the rat mGluR4 does not alter the pharmacological properties of the full-length rat receptor (Han & Hampson, 1999). To ensure that the introduction of an N-terminal cmyc-epitope into the human mGluR4 did not alter its expression pattern, cellular localization of the human cmyc-mGluR4 was compared to that of wild-type mGluR4 in transiently transfected HEK and BHK cells. Immunostaining of wild-type mGluR4 under steady state conditions with an antibody directed against the mGluR4 C-terminus showed both membrane and intracellular localization of mGluR4 in HEK (Figure 1a) and BHK cells

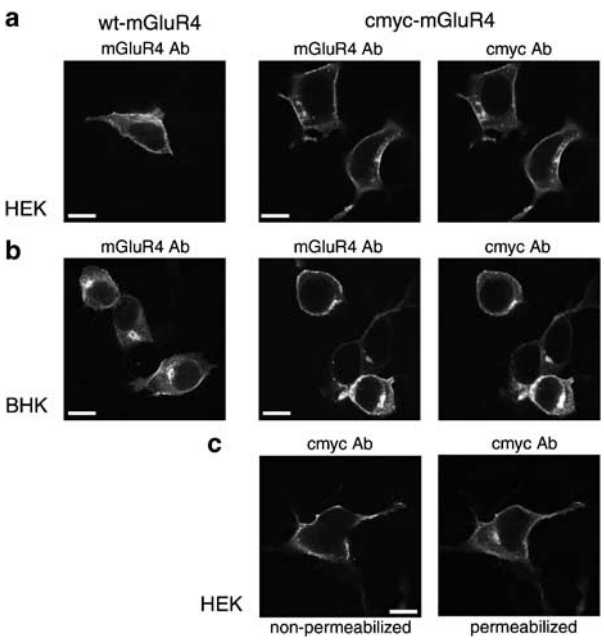

Figure 1 Expression and localization of human wild type (wt) and cmyc-tagged mGluR4 transiently expressed in HEK and BHK cells. Expression patterns of the wt-mGluR4 and cmyc-mGluR4 in permeabilized (a) HEK and (b) BHK cells were examined. The wt-mGluR4 was transiently expressed and visualized with a C-terminally directed, rabbit anti-mGluR4 antibody followed by secondary Cy3-anti-rabbit. The cmyc-mGluR4 was transiently expressed and detected with a C-terminally directed rabbit anti-mGluR4 antibody and an N-terminally directed, mouse anti-cmyc antibody followed by Cy3-anti-rabbit and Cy2-anti-mouse antibodies, respectively. (c) The cmyc-mGluR4 was transiently expressed in HEK cells and detected first with mouse anti-cmyc and Cy2-anti-mouse antibodies under non-permeabilizing conditions, and then with mouse anti-cmyc and Cy3-anti-mouse antibodies under permeabilizing conditions. Immunostained cells were visualized by confocal microscopy. The length of bars is 10 μm.

(Figure 1b). A similar expression pattern was observed for the cmyc-mGluR4 in HEK (Figure 1a) and BHK cells (Figure 1b) when co-stained with antibodies directed against the cmyc-epitope and the mGluR4 C-terminus.

When cmyc-mGluR4 was visualized with the anti-cmyc antibody under non-permeabilized conditions, labeling was restricted to the cell surface, whereas subsequent permeabilization with Triton X-100 revealed the presence of intracellular receptors (Figure 1c). Taken together, the data demonstrate that the anti-cmyc antibody labeled the same sites as the antibody directed against the C-terminus and that cmyc-mGluR4 was expressed similarly to wild-type mGluR4. Furthermore, the anti-cmyc labeling protocol under non-permeabilized conditions could be used to assess cell surface receptors in ELISA studies.

To confirm that the ligand binding and signaling properties of the cmyc-mGluR4 were unchanged, [^{35}S]GTPγS and [3 H] L-AP4 binding assays were performed. The *in vitro* pharmacology of L-AP4 on membranes from BHK cells transiently transfected with the cmyc-mGluR4 was retained (EC_{50} = 0.73 μM, pEC_{50} [6.14 $\pm$ 0.15], $K_D = 0.32 \pm 0.12$ μM) when compared to membranes from BHK cells stably expressing wild-type mGluR4 ($EC_{50} = 0.48$ μM, pEC_{50} [6.32 $\pm$ 0.01], K_D = 0.20 ± 0.03 μM) (Mathiesen *et al.*, 2003). The pharmacology of L-AP4 was also retained in HEK cells stably expressing

Table 1 Efficacies and potencies of L-AP4 on cmyc-mGluR4 when prestimulated with buffer, agonists and/or protein kinase modulators

	Max. response	EC_{50} (nM)	pEC_{50}	n
mGluR4 prestim. 30 min	(% of forskolin cAMP)	L-AP4		
With buffer	49.9±1.4	94	(7.02±0.10)	4
L-AP4 100 μM	48.9±2.0	112	(6.95±0.12)	4
L-Glu 100 μM	43.7±2.0	103	(6.99±0.10)	4
mGluR4 + $G\alpha_{qo5}$ prestim. 30 min	(% of fitted InsP resp.)	L-AP4		
With buffer	100±3.2	192	(6.72±0.11)	4
L-AP4 100 μM	77.3±3.0*	572	(6.24±0.12)	4
L-Glu 100 μM	74.2±2.8*	428	(6.37±0.12)	4
L-AP4 100 μM + GFX 10 μM	91.8±3.9$^{\#}$	247	(6.61±0.15)	4
L-Glu 100 μM + GFX 10 μM	102±4.4$^{\#}$	343	(6.57±0.14)	4
PMA 1 μM	44.9±1.9*	534	(6.27±0.14)	4
GFX 10 μM	97.4±4.5	309	(6.51±0.15)	4
PMA 1 μM + GFX 10 μM	82.8±3.7*$^{,\#}$	307	(6.51±0.16)	4
Forskolin 10 μM	110±3.3	399	(6.40±0.10)	4
H-89 1 μM	101±4.5	249	(6.60±0.15)	4
Forskolin 10 μM + H-89 1 μM	105±5.5	261	(6.58±0.17)	4
mGluR4 + $G\alpha_{qo5}$ prestim. 30 min	(% of fitted Ca^{2+} resp.)	L-AP4		
With buffer	100±1.6	341	(6.48±0.06)	3
L-AP4 100 μM	65.2±1.8*	432	(6.36±0.09)	3
L-Glu 100 μM	51.3±2.1*	901	(6.05±0.11)*	3
mGluR1a prestim. 30 min	(% of fitted Ca^{2+} resp.)	L-quis		
With buffer	100±0.6	122	(6.91±0.02)	6
L-quisqualate 100 μM	77.7±1.2*	193	(6.72±0.06)*	6
L-Glu 100 μM	79.9±1.1*	196	(6.71±0.05)*	6

Data was derived from three different functional assays in cmyc-mGluR4-expressing HEK cells depending on whether $G\alpha_{qo5}$ was present or not. The efficacy and potency of L-quisqualate on cmyc-mGluR1a expressing HEK cells after prestimulation with agonist was also determined. Cells were prestimulated with agonists or modulators for 30 min and agonist concentration–response curves determined as described in the experimental procedures. EC_{50} and pEC_{50} ($-\log EC_{50}$) values of L-AP4 and L-quisqualate (L-quis) were determined by nonlinear regression. The maximal responses of cmyc-mGluR4 in the cAMP assay are given as the percent of inhibition of forskolin-stimulated cAMP production. The maximal responses of cmyc-mGluR4 co-expressed with $G\alpha_{qo5}$ and of cmyc-mGluR1a are given as the percent of maximal stimulated InsP accumulation or Ca^{2+} response in the control buffer-stimulated state. All data are given as mean±s.e.m. from *n* independent experiments performed in quadruplicate (mGluR4 cAMP accumulation), duplicate (mGluR4 InsP accumulation and Ca^{2+} mobilization) or triplicate (mGluR1a Ca^{2+} mobilization). Significant differences from the control nonstimulated state: $^{*}P<0.05$ (ANOVA followed by Dunnett's test). Significant differences from the agonist or PMA stimulated state: $^{\#}P<0.05$ (unpaired *t*-test).

cmyc-mGluR4 when assayed for inhibition of forskolin-stimulated cAMP production, IP_3 accumulation, and Ca^{2+} mobilization (see Table 1 for comparison). Thus, introduction of the cmyc-epitope in the N-terminus did not affect localization, ligand binding, or efficacy of mGluR4.

Lack of mGluR4 agonist induced internalization

In order to investigate the internalization properties of mGluR4, both quantitative cell surface ELISA and qualitative immunostaining studies were carried out. Cell surface ELISAs showed that stimulation of cmyc-mGluR4 transiently transfected in HEK cells with 100 μM L-AP4 did not lead to receptor internalization after 5 min. Even after 60 min of agonist treatment, cmyc-mGluR4 had not internalized (Figure 2a). Instead, L-AP4 increased the amount of surface receptors by a modest 8% after 15 min compared to buffer conditions. The modest increase in cell surface expression was consistently observed and incubation with L-AP4 for up to 6 h also resulted in a similar increase in surface receptors of 11±1% ($P<0.05$).

For comparison, the human $G\alpha_i$-coupled D2 receptor (D2R) and the human $G\alpha_q$-coupled mGluR1a were transiently expressed in HEK cells and examined in the same ELISA experiments. These receptors were also epitope-tagged in the N-terminus as previously described except that the cmyc-epitope was used instead of the FLAG- and HA-epitopes, respectively (Kim *et al*., 2001; Mundell *et al*., 2001). In contrast to cmyc-mGluR4, stimulation of cmyc-D2R with 100 μM dopamine (Figure 2b) and of cmyc-mGluR1a with 100 μM L-quisqualic acid (Figure 2c) caused a rapid and lasting decrease in surface receptors 14±3 and 15±3%, respectively, at 15 min comparable to internalization levels previously reported (Dale *et al*., 2001; Kim *et al*., 2001; 2004; Mundell *et al*., 2001). Treatment of cmyc-D2R with 20 μM of the D2R antagonist haloperidol and of cmyc-mGluR4 with 100 μM of the competitive Group III antagonist CPPG did not cause significant changes in the levels of cell surface expression when compared to the levels of respective buffer conditions (Figure 2a and b).

Immunostaining of cmyc-tagged mGluR4, D2R and mGluR1a supported the ELISA data (Figure 2d). In the non-stimulated state, all receptors showed prominent cell surface expression. cmyc-DR2 and cmyc-mGluR1a were localized almost exclusively to the membrane, whereas cmyc-mGluR4

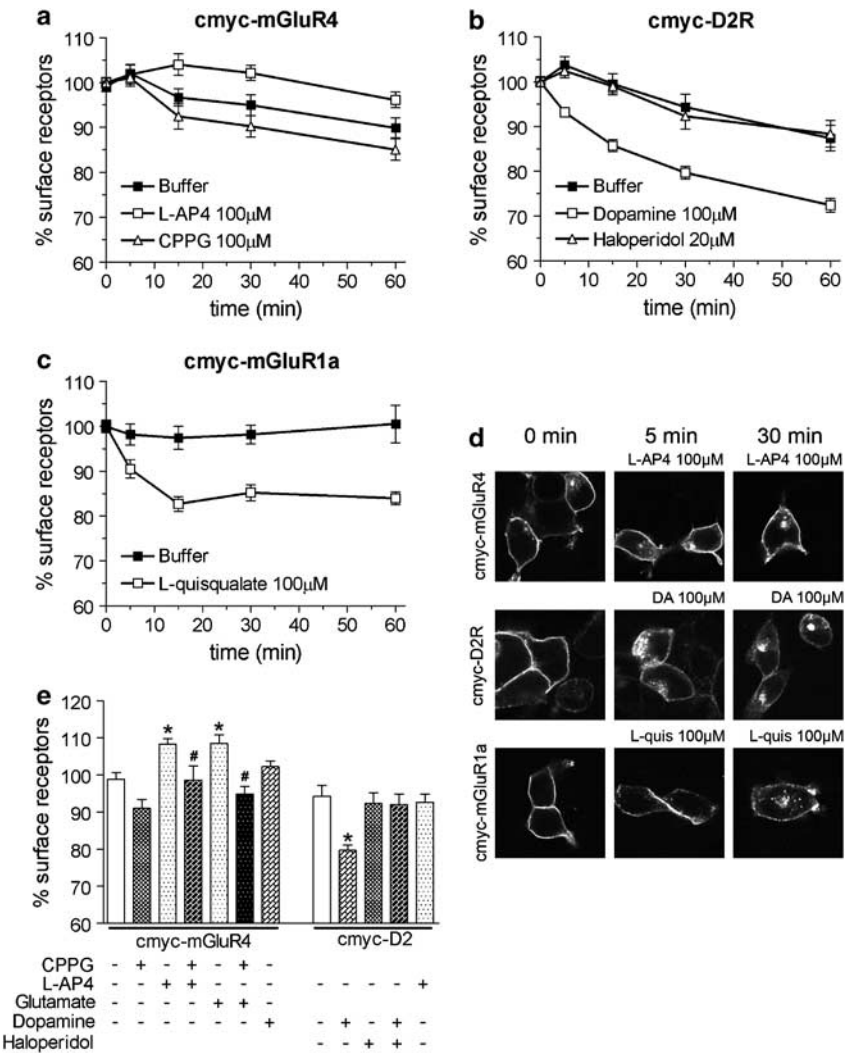

Figure 2 Internalization studies of cmyc-tagged mGluR4, D2R, and mGluR1a surface receptors. HEK cells were transiently transfected with cmyc-mGluR4, cmyc-D2R, or cmyc-mGluR1a and drug treated. The effects of (a) 100 μM L-AP4 and 100 μM CPPG on cmyc-mGluR4, (b) 100 μM dopamine and 20 μM haloperidol on cmyc-D2R, and (c) 100 μM L-quisqualate on cmyc-mGluR1a surface receptors were determined in ELISA assays and compared to buffer. (d) The cellular localization of cmyc-mGluR4 (upper panel), cmyc-D2R (middle panel) and cmyc-mGluR1a (lower panel) was evaluated in their nonstimulated states (0 min) and then at 5 and 30 min stimulation with their respective agonists, L-AP4, dopamine (DA) and L-quisqualate (L-quis). Cells were permeabilized and labeled with primary mouse anti-cmyc followed by secondary Cy2 anti-mouse antibodies and receptor localization determined by confocal microscopy. (e) The effects of various ligands were evaluated on cmyc-mGluR4 and cmyc-D2R surface receptors in transiently transfected HEK cells. After 30 min stimulation with the indicated ligands, surface receptors were quantified by ELISA. Data in (a), (b), (c) and (e) are normalized to the percent of surface receptors detected at time 0 and given as mean $\pm$ s.e.m. from three to six independent experiments each performed in triplicate. Significant differences from the buffer levels in (e): $^{*}P<0.05$. Significant differences from L-AP4 or L-glutamate induced increases in surface receptors: $^{\#}P<0.05$ (ANOVA followed by Dunnett's test).

was also detected intracellularly. In agreement with the ELISA studies, agonist stimulation with 100 μM L-AP4 for 5 and 30 min did not show internalization of cmyc-mGluR4, whereas agonist stimulation of D2R with 100 μM dopamine and mGluR1a with 100 μM L-quisqualate at the same time points lead to the formation of intracellular receptor-containing vesicles (Figure 2d). As cmyc-mGluR1a expressed under the same nonstimulated conditions as the cmyc-mGluR4 showed almost exclusive membrane expression, this suggests that the intracellular localization of cmyc-mGluR4 was not due to constitutive internalization mediated by the presence of ambient glutamate.

In order to confirm that the observed changes in cell surface receptors induced by the mGluR4 and D2R agonists were receptor specific, the effects of antagonists were tested. After a 30 min incubation, 100 μM CPPG antagonized both L-AP4 and L-glutamate-induced increases in cmyc-mGluR4 surface receptors to buffer levels, and 20 μM haloperidol antagonized dopamine-induced internalization of cmyc-D2R (Figure 2e). Furthermore, L-AP4 did not affect cmyc-D2R and dopamine did not affect cmyc-mGluR4 surface receptor levels. Thus, the ELISA and immunostaining studies clearly demonstrate that mGluR4 does not internalize in response to agonist stimulation under these conditions.

Effect of direct and indirect PKC activation on mGluR4 internalization

Signaling mechanisms other than agonist activation have been shown to regulate mGluR surface expression. In the case of mGluR1a, direct and indirect activation of PKC (the former by 1 μM PMA) have been associated with receptor internalization (Mundell *et al*., 2002), while protein kinase A (PKA) activation with 10 μM forskolin has been associated with inhibition of agonist-induced internalization and an increase in surface receptors (Mundell *et al*., 2004b). Initial internalization studies of cmyc-mGluR4 transiently expressed in HEK cells suggested a role for PKC in mGluR4 internalization since stimulation with 1 μM PMA for 30 min caused a 25$\pm$1.1% loss of surface receptors. PKA activation with 10 μM forskolin for 30 min did not alter the level of cmyc-mGluR4 surface receptors compared to buffer, 99.3$\pm$2.1 and 98.7$\pm$1.1%, respectively.

To examine the effect of PKC activation on mGluR4 internalization in detail, changes in cmyc-mGluR4 surface receptors were followed over 60 min in the presence of PMA. Continuous stimulation with 1 μM PMA caused a rapid and persistent loss of cmyc-mGluR4 surface receptors (Figure 3a). To investigate whether the effect of PMA was due to PKC activation, the specific PKC inhibitor GF 109203X (GFX) (Toullec *et al*., 1991) was tested. PMA-induced cmyc-mGluR4 internalization was completely blocked by co-incubation with 10 μM GFX, confirming that PMA-mediated internalization is PKC dependent (Figure 3b).

In order to determine whether activation of a heterologous $G\alpha_q$-coupled receptor could induce mGluR4 internalization, cmyc-mGluR4 was co-expressed with the neurokinin 3 receptor (NK3R). Activation of NK3R with 100 nM neurokinin B (NKB) caused a rapid and persistent internalization of cmyc-mGluR4 (Figure 3c), resembling that of PMA. NKB-induced cmyc-mGluR4 internalization was blocked by 10 μM GFX (Figure 3d), suggesting that NKB-induced internalization of cmyc-mGluR4 was due to downstream PKC activation. NKB had no effect on cmyc-mGluR4 surface receptor levels in the absence of NK3R (Figure 3a). To examine whether mGluR4 internalization could be mediated by activation of a non-$G\alpha_q$-coupled receptor, the cmyc-mGluR4 was co-expressed with the $G\alpha_i$-coupled D2 receptor. Stimulation with 100 μM dopamine did not cause internalization of cmyc-mGluR4 whereas PMA still caused internalization of the cmyc-mGluR4 when co-expressed with the D2 receptor (Figure 3g). These data support a role for $G\alpha_q$-coupling and PKC activation in mGluR4 internalization.

$G\alpha_i$-coupled receptors, including the Group II and III mGluRs, have been shown to activate PLC when co-expressed with chimeric G proteins where the last five amino acids of $G\alpha_q$ have been exchanged with those of $G\alpha_i$ or $G\alpha_o$ (Gomeza *et al*., 1996). To further support the observation that mGluR4 internalization requires the activation of PKC, the cmyc-mGluR4 was transiently co-transfected with the chimeric $G\alpha_{qo5}$ and changes in cell surface receptor levels quantified by ELISA. Stimulation with 100 μM L-AP4 now caused a rapid and persistent loss of cmyc-mGluR4 surface receptors of 17$\pm$2% (Figure 3e) unlike the small increase in surface receptors observed with mGluR4 alone (Figure 2a). Stimulation with PMA also caused cmyc-mGluR4 co-transfected with $G\alpha_{qo5}$ to internalize (Figure 3e). As observed for PMA and

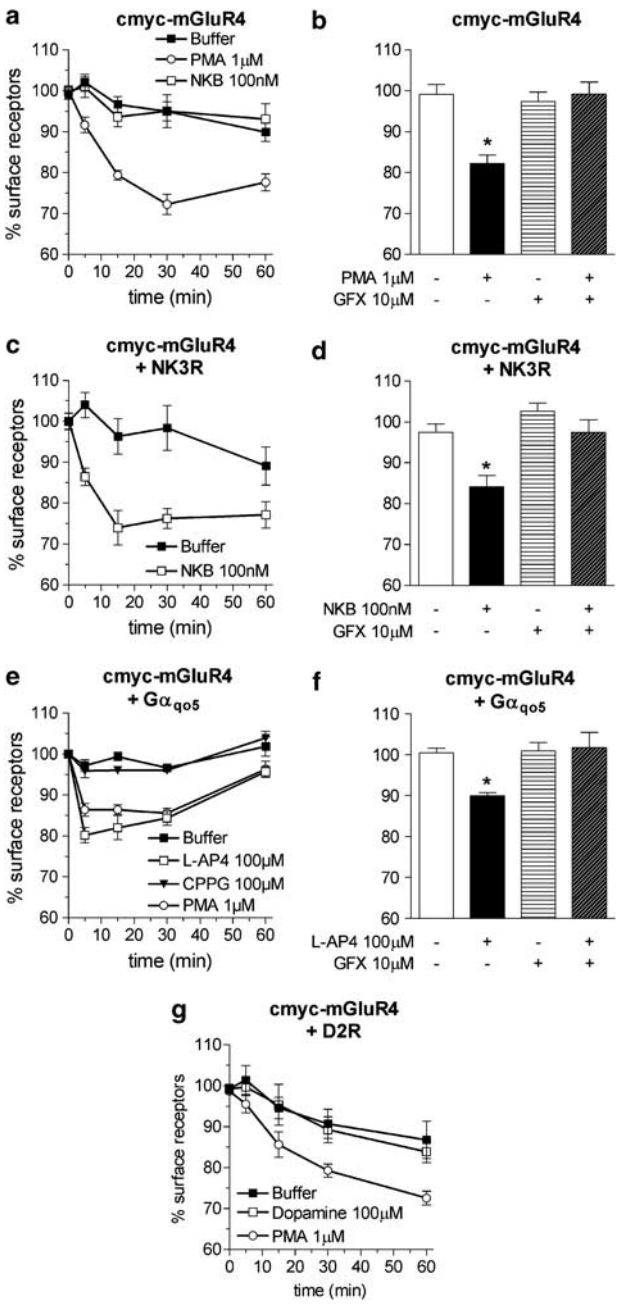

Figure 3 Effect of direct and indirect activation of PKC on mGluR4 internalization. HEK cells transiently transfected with cmyc-mGluR4 alone or together with NK3R, chimeric $G\alpha_{qo5}$ or D2R were drug treated and changes in surface receptors quantified by ELISA. (a) cmyc-mGluR4 surface receptors were quantified over time in the presence of buffer, PMA, or the NK3R agonist NKB or (b) after 30 min in the presence of PMA or GFX alone or in combination. (c) cmyc-mGluR4 surface receptors co-expressed with non-tagged NK3R were quantified over time in the presence of NKB or (d) after 30 min in the presence of NKB or GFX alone or in combination. (e) cmyc-mGluR4 surface receptors co-expressed with chimeric $G\alpha_{qo5}$ were quantified over time in the presence of L-AP4, CPPG, or PMA or (f) after 30 min in the presence of L-AP4 or GFX alone or in combination. (g) cmyc-mGluR4 surface receptors co-expressed with D2R were quantified over time in the presence of dopamine or PMA. All graphs are normalized to the percent of surface receptors detected at time 0 and data given as mean$\pm$s.e.m. from three to six independent experiments each performed in triplicate. Significant differences from the buffer levels in (b), (d) and (f): $^*P<0.05$ (ANOVA followed by Dunnett's test).

NKB-induced cmyc-mGluR4 internalization, GFX blocked L-AP4-induced internalization of the cmyc-mGluR4 co-transfected with $G\alpha_{qo5}$ (Figure 3f). Taken together, these data demonstrate that PKC activation is required for mGluR4 internalization.

Effect of PKC activation and receptor coupling to $G\alpha_{qo5}$ on mGluR4 signaling

As GPCR desensitization has been associated with internalization due to the loss of surface receptors, the effect of agonist PKC and PKA activation on mGluR4 signaling was explored. A stable cmyc-mGluR4 expressing HEK cell line was used for these experiments as the inhibition of forskolin-stimulated cAMP by mGluR4 could not be quantified in transiently transfected cells. In this cell line, L-AP4 and L-glutamate displayed EC_{50} values of 0.1 μM (pEC_{50} [7.0±0.1]) and 3.8 μM (pEC_{50} [5.4±0.1]), respectively, and inhibited forskolin-stimulated cAMP production by 50±1 and 41±2%, respectively (L-AP4 data in Figure 4a).

As agonist activation of cmyc-mGluR4 did not cause receptor internalization, the possibility of a similar lack of agonist-induced receptor desensitization in the cAMP-assay was explored. After a 30 min incubation period with either 100 μM L-AP4 or L-glutamate, the agonist was washed off and cells incubated with increasing concentrations of L-AP4 in the presence of 10 μM forskolin. Interestingly, the potency and efficacy of L-AP4 was not affected by preincubation with either L-AP4 or L-glutamate for 30 min (Figure 4a, Table 1). Importantly, the addition of 10 μM forskolin did not affect the amount of mGluR4 surface receptor levels as described previously, excluding the possibility that the lack of changes in L-AP4 potency and efficacy after agonist prestimulation was compensated by changes in surface expression by forskolin.

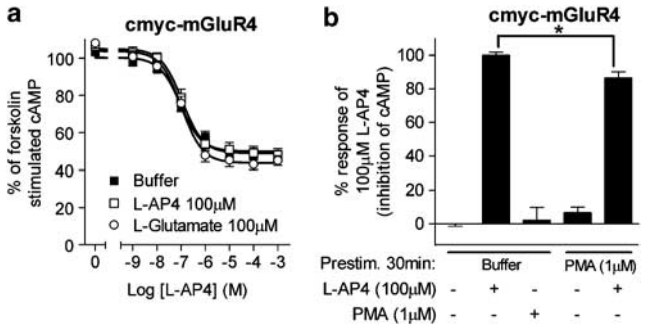

Figure 4 Effect of agonist activation and PMA on mGluR4 desensitization measured by inhibition of forskolin stimulated cAMP production. The experiments were performed on cmyc-mGluR4 stably expressing HEK cells. (a) Cells were preincubated with buffer, 100 μM L-AP4 or 100 μM L-glutamate for 30 min and the effect of agonist prestimulation on mGluR4-mediated inhibition of forskolin-stimulated cAMP was evaluated by generating concentration–response curves of L-AP4. Data are reported as percent inhibition of forskolin-stimulated cAMP production. (b) Cells were preincubated with buffer or PMA for 30 min and assayed for inhibition of forskolin-stimulated cAMP by 100 μM L-AP4. Data are given as the percent of the L-AP4 response in cells preincubated with buffer. Data are represented as mean±s.e.m. from three (a) and four (b) independent experiments each performed in quadruplicate. Significant differences from the buffer levels in (b): $*P<0.01$ (unpaired *t*-test).

As direct PKC activation was shown to induce cmyc-mGluR4 internalization (Figure 3a), the ability of PMA to induce cmyc-mGluR4 desensitization of cAMP signaling was examined. In line with PMA-induced internalization of cmyc-mGluR4, preincubation with PMA for 30 min significantly reduced L-AP4-induced inhibition of forskolin-stimulated cAMP by 14±4% (Figure 4b), suggesting that PMA also contributes to the desensitization of mGluR4. These findings show that mGluR4, with respect to inhibition of forskolin-stimulated cAMP, did not desensitize in response to agonist preactivation. Instead, mGluR4 was desensitized in response to PKC activation by PMA.

As mGluR1a has previously been shown to desensitize upon agonist and PMA stimulation (Francesconi & Duvoisin, 2000), it was used as a control for mGluR4 PMA- and agonist-induced desensitization experiments. Transiently expressing, cmyc-tagged mGluR1a HEK cells were preincubated with PMA, agonist or buffer and assayed for agonist-induced Ca^{2+} mobilization with 100 μM L-quisqualate. Prestimulation with 1 μM PMA significantly reduced the L-quisqualate-induced Ca^{2+} mobilization response by 22±6% as compared to buffer ($P<0.01$, unpaired *t*-test). Prestimulation with either 100 μM L-quisqualate or L-glutamate for 30 min resulted in a significant reduction of the maximal efficacy of L-quisqualate and a rightshift of its EC_{50} value as compared to a 30 min preincubation in buffer (Table 1).

Given that agonist activation caused PKC-dependent mGluR4 internalization when co-transfected with $G\alpha_{qo5}$, the effects of agonist preincubation on mGluR4 signaling in the presence of $G\alpha_{qo5}$ was examined by both InsP accumulation and Ca^{2+} mobilization assays. The EC_{50} values of L-AP4 in the non-prestimulated state were 0.2 μM (95% confidence limits: 0.12–0.32 μM) and 0.3 μM (95% confidence limits: 0.25–0.43 μM), respectively, and closely resembled previously reported values (Gomeza *et al*., 1996; Kowal *et al*., 2003). When cmyc-mGluR4 was transfected with $G\alpha_{qo5}$, preincubation with either 100 μM L-AP4 or L-glutamate resulted in a significant decrease in InsP accumulation of L-AP4 by 22.7±3 and 25.8±3%, respectively (Figure 5a, Table 1). Furthermore, the EC_{50} value of L-AP4 was shifted to the right when preincubated with L-glutamate or L-AP4, albeit not significantly (Table 1). A similar inhibition of cmyc-mGluR4 receptor function by agonist preincubation was observed in the downstream Ca^{2+} mobilization assay, which generates fast transient responses upon receptor activation unlike the InsP accumulation assay (Table 1).

To examine whether agonist-induced desensitization of cmyc-mGluR4 in the presence of $G\alpha_{qo5}$ was dependent on downstream PKC activation, preincubation with 100 μM L-AP4 or L-glutamate was performed in the presence of 10 μM GFX. The agonist-induced decreases in L-AP4 efficacy and affinity were now completely abolished and normalized to the non-prestimulated condition (Figure 5b and Table 1). This rules out that the decrease in efficacy and potency of L-AP4 was due to insufficient removal of agonist after preincubation. Thus, agonist activation of mGluR4 co-expressed with the chimeric $G\alpha_{qo5}$ leads to activation of the PLC pathway with a subsequent increase in intracellular [Ca^{2+}] and PKC-dependent desensitization of mGluR4.

To further support the idea that mGluR4 desensitization like internalization could be regulated heterologously, similar studies measuring InsP accumulation under direct activation of PKC by PMA were performed in stable cmyc-mGluR4

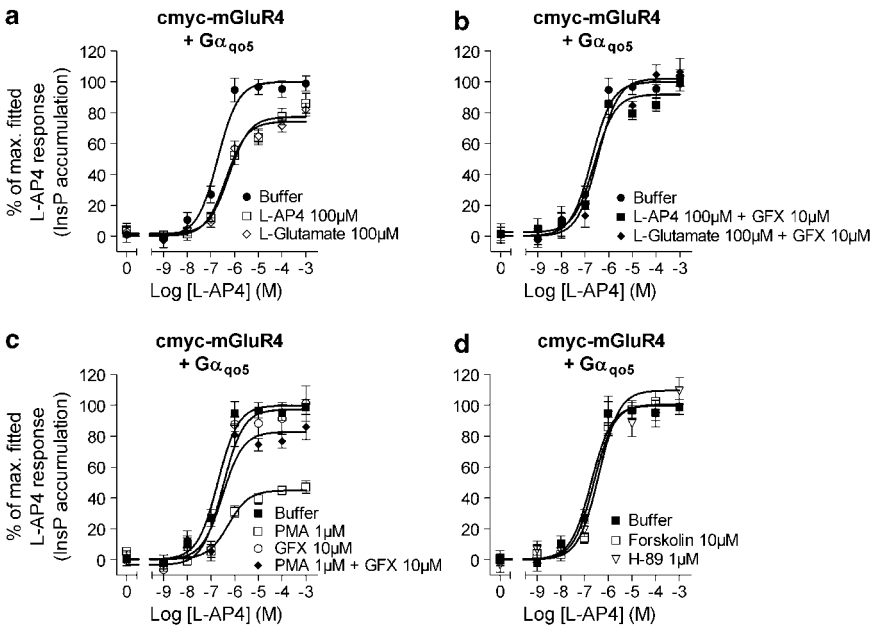

Figure 5 Effect of agonist activation and protein kinase modulators on mGluR4 desensitization when coupled to $G\alpha_{qo5}$. Experiments were performed on stable cmyc-mGluR4 expressing HEK cells that had been transiently transfected with $G\alpha_{qo5}$. The effects of preincubation with buffer or 100 μM L-quisqualate or 100 μM L-glutamate for 30 min in the absence (a) or presence of the PKC inhibitor GFX (b) on mGluR4-mediated InsP accumulation were evaluated by generating concentration–response curves of L-AP4. In the same way, the effects of the PKC modulators PMA and GFX and their combinations (c) and of the PKA modulators forskolin and H-89 (d) were evaluated. Data are given as percent of the L-AP4 response in cells preincubated with buffer by normalizing to the nonlinear regression fitted maximal response of the L-AP4 dose–response curve. Data are represented as mean $\pm$ s.e.m. from four independent experiments each performed in duplicate.

HEK cells co-expressing $G\alpha_{qo5}$. Preincubation with 1 μM PMA for 30 min significantly reduced the efficacy of L-AP4 for cmyc-mGluR4 to 44.9 $\pm$ 1.9% compared to the nonstimulated state, whereas preincubation with 10 μM GFX alone did not change L-AP4 efficacy (Figure 5c, Table 1). In the presence of GFX, however, PMA-mediated desensitization of cmyc-mGluR4 was significantly reduced although not completely (82.9 $\pm$ 3.7% of the control condition), showing that PMA-induced cmyc-mGluR4 desensitization was mainly mediated by PKC activation. As in the cAMP and InsP accumulation assays, preincubation with PMA for 30 min significantly reduced the maximal L-AP4-induced Ca^{2+} response by 18 $\pm$ 6% in the Ca^{2+} mobilization assay as compared to buffer ($P < 0.05$, student's *t*-test).

Previous studies have shown that activation of PKA by forskolin inhibits general Group III mGluR function in the hippocampus, most likely through PKA-mediated receptor phosphorylation (Cai *et al*., 2001). To examine the effect of PKA activation on mGluR4 function, cmyc-mGluR4 was co-expressed with $G\alpha_{qo5}$ and the effect of 30 min prestimulation with forskolin and/or the PKA-specific inhibitor H-89 on InsP accumulation was investigated. Under these conditions, 10 μM forskolin, 1 μM H-89, or their combination had no effect on mGluR4 function as measured by L-AP4 efficacy and affinity (Figure 5d, Table 1), suggesting that PKA activation is not required for internalization and desensitization of mGluR4.

Discussion

Little is known about the mechanisms that regulate mGluR4 membrane expression, trafficking, and desensitization compared to what is known for the Group I mGluRs. As receptor activation and changes in mGluR4 expression have been associated with pathological conditions, investigation of the mechanisms involved in these processes is crucial for understanding the role of mGluR4 activity in disease.

A series of ELISA and immunofluorescent studies were carried out in order to investigate mGluR4 internalization. Our findings clearly demonstrated that the $G\alpha_i$-coupled mGluR4 failed to internalize when activated with L-AP4 or L-glutamate in a heterologous expression system. In contrast, both the $G\alpha_i$-coupled D2R and $G\alpha_q$-coupled mGluR1a internalized upon agonist stimulation in our studies and as previously reported (Mundell *et al*., 2001; Kim *et al*., 2004). Interestingly ELISA studies pointed to a persistent, albeit modest, agonist-induced increase in mGluR4 surface expression that was consistently antagonized by the competitive Group III antagonist CPPG. A similar increase in surface receptor levels has been reported for mGluR1a upon forskolin-mediated PKA activation and has been proposed to be associated with inhibition of constitutive internalization of mGluR1a and 1b (Mundell *et al*., 2004b). As mGluR4 activation is expected to reduce PKA activity by inhibiting AC and because no effect of forskolin on mGluR4 surface expression was seen, the increase in surface mGluR4 is likely PKA independent. Whether such modest increases in mGluR surface expression are physiologically relevant is unknown. Owing to the lack of native cell systems exclusively expressing mGluR4 and potent subtype selective agonists, similar experiments examining mGluR4 internalization in native cells are not feasible.

A recent report focusing on the regulation of mGluR4 signaling by GRK2 showed that a GFP-tagged mGluR4

co-expressed with the EAAC1 glutamate transporter internalized after 5 min stimulation with 100 μM L-AP4. In the continued presence of L-AP4, the membrane expression pattern was returned to that of untreated cells (Iacovelli *et al*., 2004). These findings are in contrast to the data presented here where no internalization of the cmyc-mGluR4 was detected up to 6 h in ELISA studies and up to 30 min in immunostaining studies with the same concentration of L-AP4. The detection methods in this study were sensitive enough to measure fast agonist-induced internalization since stimulation of D2R and mGluR1a by their agonists resulted in rapid receptor internalization by 5 min.

The reason for these differences is unclear although a possible explanation could reside in the tagging of the receptor. We used N-terminal epitope tagging, whereas Iacovelli *et al*. (2004) used a C-terminally GFP-tagged version. Previously C-terminal GFP-tagging of the beta(1)- and beta(2)-adrenoceptors has been shown to result in quantitative differences in receptor trafficking and regulation as compared to the N-terminal epitope-tagged receptors (McLean & Milligan, 2000). It is possible that C-terminal tagging hinders protein-receptor interactions important for mGluR4 membrane stabilization or trafficking. For instance, the extreme C-terminus of mGluR4 has been shown to interact with GRIP and syntenin (Hirbec *et al*., 2002), which are involved in the internalization of membrane proteins (Fialka *et al*., 1999; Hirai, 2001). Importantly, GRIP stabilizes the ionotropic GluR2 at the membrane by a protein-receptor interaction that when disrupted causes internalization of the receptor (Hirai, 2001).

In understanding the mechanisms that regulate mGluR4 membrane expression, the present study provides the first evidence that the human mGluR4 can undergo rapid PKC-dependent internalization. It is well established that the $G\alpha_q$-coupled mGluR1 undergoes direct and indirect PKC-mediated internalization (Dale *et al*., 2001; Mundell *et al*., 2004a) and thus our studies indicate an important role for PKC activation also in mediating internalization of mGluR4. Other GPCRs have also been shown to internalize upon direct and heterologous activation of PKC as exemplified by the delta-opioid receptor (Xiang *et al*., 2001). Interestingly, our studies show that both direct and indirect activation of PKC lead to mGluR4 internalization, the latter exemplified by activation of either the $G\alpha_q$-coupled NK3R or the mGluR4 in the presence of the chimeric $G\alpha_{qo5}$.

This study provides the first evidence of heterologous-mediated internalization and desensitization of mGluR4 specifically. The data presented here support a link between internalization and desensitization of mGluR4 as these processes were found to be dependent on PKC activation. Direct activation of PKC by PMA caused both mGluR4 internalization and desensitization. Additionally, internalization and desensitization were observed upon agonist stimulation when mGluR4 was coupled to PLC *via* $G\alpha_{qo5}$. Importantly, both internalization and desensitization were abolished by GFX, suggesting a dependence on PKC activation for these processes.

In vitro studies of general Group III mGluR presynaptic function support our findings that PKC activation mediates desensitization of mGluR4. Phorbol esters and heterologous activation of the $G\alpha_q$-coupled adenosine A3 receptor, for instance, has been shown to inhibit both Group II and III mGluR presynaptic function in hippocampal slices in a PKC-dependent manner (Macek *et al*., 1998). Based on our findings, it is tempting to speculate that internalization could be an alternative mechanism for *in vitro* PKC-mediated desensitization of Group III mGluRs, besides the uncoupling of the receptors from G-protein as previously suggested (Macek *et al*., 1998). That mGluR4 internalization may mediate mGluR4 desensitization is further supported by the lack of agonist-induced internalization and desensitization when coupled to the $G\alpha_i$-pathway.

With respect to the lack of agonist-induced desensitization of mGluR4 in our studies and the potential involvement of other proteins, it is interesting to note that GRK2 overexpression did not induce mGluR4 desensitization when coupled to AC inhibition (Iacovelli *et al*., 2004). These findings substantiate the lack of homologous desensitization, which is usually promoted by GRKs as seen for mGluR1 (Dale *et al*., 2001). The lack of homologous desensitization and internalization of mGluR4 is in agreement with Group III mGluRs' proposed function as glutamatergic autoreceptors (Schoepp, 2001). As sensors of synaptic glutamate and glutamate spill-over that act to limit glutamate release from the presynaptic neuron, we speculate that glutamate-mediated loss of mGluR4 function due to homologous internalization and/or desensitization would not be favorable.

One of the mechanisms that predisposes a GPCR to internalization is heterologous receptor phosphorylation by PKC (Ferguson, 2001). The underlying mechanisms for PKC-mediated, mGluR4 internalization and desensitization patterns, that is, whether PKC directly phosphorylates mGluR4 or another protein remains to be addressed. Nevertheless, using a web-based phosphorylation prediction server (Blom *et al*., 1999), multiple PKC phosphorylation sites have been predicted in both the C-terminus (Ser-859, Ser-870) and intracellular loops 1 (Thr-615, Ser-621), 2 (Ser-687, Ser-689) and 3 (Thr-774). Interestingly, Ser-859, a predicted PKC phosphorylation site in the mGluR4 C-terminus has been identified as a common consensus site for PKA phosphorylation in all Group III mGluRs (Cai *et al*., 2001).

With regards to the role of PKA in internalization and desensitization, activation of PKA by forskolin did not cause desensitization of mGluR4 in our studies. This may imply a lack of a common PKA-dependent mechanism for desensitization of all Group III mGluR regulation as previously suggested, although a purified GST-mGluR4 C-terminus fusion protein was shown be phosphorylated in an *in vitro* phosphorylation assay (Cai *et al*., 2001). In this respect, it is interesting to note that PKA phosphorylation of the $GABA_B$ receptor C-terminus facilitates rather than inhibits receptor-effector coupling (Couve *et al*., 2002).

Of particular interest is our finding that NK3R activation induces internalization of mGluR4 as NK3R-induced glutamate release in layer V of the entorhinal cortex is dependent on the spontaneous activity of a presynaptic Group III mGluR (Stacey *et al*., 2002). Although Group III mGluR activation has been shown to enhance spontaneous release of glutamate in layer V of the entorhinal cortex as opposed to most other glutamatergic synapses (Schoepp, 2001), it shows that perhaps mGluR4 and NK3R can modulate presynaptic glutamate release from the same neurons.

In summary, mGluR4 surface expression *in vivo* may be regulated by heterologous activation of $G\alpha_q$-coupled receptors and possibly by other routes of activating PKC. This implies that other GPCRs could indirectly serve to regulate and fine-tune glutamatergic neurotransmission at specific synapses. As

upregulation of mGluR4 appears advantageous in certain pathological conditions such as epilepsy and neurotoxicity, antagonism of endogenously coexpressed $G\alpha_q$-coupled GPCRs could serve to improve the therapeutic effect of mGluR4 activation even further.

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