

Complex Involvement of Pertussis Toxin-Sensitive G Proteins in the Regulation of Type 1 α Metabotropic Glutamate Receptor Signaling in Baby Hamster Kidney Cells

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ABSTRACT

Previously, we demonstrated that the coupling of the metabotropic glutamate receptor mGlu1 α to phosphoinositide hydrolysis is enhanced by pertussis toxin (PTX) in stably transfected baby hamster kidney cells (BHK). Here, we show that the PTX effect on agonist-stimulated [3 H]inositol phosphate accumulation can be resolved into two components: an immediate increase in agonist potency, and a more slowly developing increase in the magnitude of the response observed at maximally effective agonist concentrations. Using G $_{q/11}\alpha$ - and G $_{i/o}\alpha$ -selective antibodies to immunoprecipitate [35 S]guanosine-5'-O-(3-thio)triphosphate-bound G α proteins, we also show that agonist stimulation of mGlu1 α in BHK membranes increases specific [35 S]guanosine-5'-O-(3-thio)triphosphate binding to both G $_{q/11}$ and G $_{i/o}$ proteins. Preincubation of BHK-mGlu1 α with L-glutamate (300 μ M) results in a progressive loss (60% in

30 min) of L-quisqualate-induced [3 H]inositol phosphate accumulation (without a change in potency), providing evidence for agonist-induced receptor desensitization. Although such desensitization of mGlu receptor signaling was mimicked by a phorbol ester, agonist-induced phosphorylation of the receptor was not observed and protein kinase C inhibition by Ro 31-8220 did not prevent L-glutamate-mediated desensitization. In contrast, PTX treatment of the cells almost completely prevented L-glutamate-mediated desensitization. Together, these data provide evidence for a multifunctional coupling of mGlu1 α to different types of G proteins, including PTX-sensitive G $_i$ -type G proteins. The latter are involved in the negative control of phospholipase C activity while also influencing the rate of desensitization of the mGlu1 α receptor.

Of the eight mammalian metabotropic glutamate (mGlu) receptors cloned so far, the mGlu1 and mGlu5 receptors constitute a distinct subgroup (group I) sharing a high degree of sequence homology, common G protein-coupling preference, and pharmacological profile (Conn and Pin, 1997). The prototypic mGlu receptor of this class is the type 1 α (or 1a) splice variant (Houamed et al., 1991; Masu et al., 1991), which couples to the stimulation of phosphoinositide turnover via a G protein-mediated activation of phosphoinositide-specific phospholipase C (PLC). However, the nature of the G protein or proteins involved in linking the mGlu1 α receptor to PLC has been the subject of some speculation, with an involvement of both pertussis toxin (PTX)-sensitive and -in-

sensitive proteins being initially implicated in studies in mammalian recombinant systems (Aramori and Nakanishi, 1992; Pickering et al., 1993; Thomsen et al., 1993) and *Xenopus* oocytes (Kasahara and Sugiyama, 1994).

Recently, we demonstrated an apparent dual regulation of PLC- β in baby hamster kidney (BHK) cells expressing the mGlu1 α receptor (BHK-mGlu1 α ; Carruthers et al., 1997). Thus, the finding that the mGlu receptor agonists L-quisqualate and 1-aminocyclopentane-1*S*,3*R*-dicarboxylate exhibit increased potency and efficacy for stimulating phosphoinositide hydrolysis after the inactivation of G $_{i/o}$ -type G proteins led us to speculate that mGlu1 α receptor activation can activate both G $_{q/11}$ and G $_{i/o}$ proteins, which have stimulatory and inhibitory effects on PLC activity, respectively (Carruthers et al., 1997).

In the present study, we examined this interesting dual modulation of PLC activity by a single receptor subtype and demonstrated that G $_{i/o}$ inactivation has two distinct conse-

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ABBREVIATIONS: mGlu, metabotropic glutamate; BHK, baby hamster kidney; PAGE, polyacrylamide gel electrophoresis; GPT, glutamic-pyruvic transaminase; InsP, [3 H]inositol (poly)phosphates; PLC, phospholipase C; Ins(1,4,5)P $_3$, inositol-1,4,5-trisphosphate; KHB, Krebs-Henseleit buffer; mACh, muscarinic acetylcholine; PDBu, phorbol-12,13-dibutyrate; PTX, pertussis toxin; PKC, protein kinase C.

quences for receptor-effector coupling; furthermore, we provide mechanistic information on the $G_{i/o}$ -protein component of the altered phosphoinositide response involving an alteration in the kinetics of mGlu1 α receptor desensitization. In addition, the effects of $G_{i/o}$ -protein inactivation on phosphoinositide responses in BHK cells heterologously expressing either the mGlu1 α or the M₃-muscarinic acetylcholine (mACh) receptor are compared.

Experimental Procedures

Cell Culture. Transfected BHK cells expressing the rat mGlu1 α receptor (BHK-mGlu1 α ; Thomsen et al., 1993) were routinely cultured in Dulbecco's modified Eagle's medium (Glutamax-1) supplemented with 5% dialysed fetal calf serum, 50 IU/ml penicillin, 50 μ g/ml streptomycin, 0.5 mg/ml G418, 50 μ g/ml gentamicin, and 1 μ M methotrexate. Transfected BHK cells expressing the M₃-muscarinic receptor (BHK-m3; Saunders et al., 1998) were grown in the same medium without G418 and methotrexate but with 300 μ g/ml hygromycin. For experiments, both cell lines were seeded onto multiwell plates in the same medium devoid of gentamicin, G418, methotrexate, and hygromycin. For BHK-mGlu1 α , the cell culture medium was supplemented 3 h before any experiment, with glutamate pyruvate transaminase (GPT, 3 U/ml) and pyruvate (3 mM). Treatment of the monolayers with PTX was performed by the addition to the culture medium 24 h before experimentation.

Measurement of [³H]Inositol (Poly)phosphates ([³H]InsP), Inositol-1,4,5-trisphosphate [Ins(1,4,5)P₃], and cAMP Accumulations. For assessment of [³H]InsP, cells grown on 24 multiwell plates were labeled with 1 μ Ci/ml [³H]inositol for 48 h in culture medium. Thereafter, cells were washed three times with 0.5 ml of Krebs-Henseleit buffer (KHB; containing 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 1.2 MgSO₄, 5 mM HEPES, and 10 mM *D*-glucose, pH 7.4) at 37°C. Unless indicated otherwise, 10 mM LiCl was present in the last wash, and the cells were incubated in this buffer for 15 min before the addition of the agonist. Experiments were performed at 37°C in a final volume of 0.5 ml/well and were terminated by the addition of 0.5 ml ice-cold 1 M trichloroacetic acid. After extraction on ice for 20 min, samples (0.8 ml) were collected from the well, mixed with 200 μ l EDTA (10 mM, pH 7.0), and extracted with 1 ml of a 1:1 (v/v) mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. An 800- μ l sample of the aqueous extract was mixed with 50 μ l NaHCO₃ (62.5 mM), and the [³H]InsP fraction (incorporating inositol monophosphates, bisphosphates, and trisphosphates) was recovered by ion-exchange chromatography on Dowex AG1-X8 (formate form) columns as previously described (Challiss et al., 1993).

Ins(1,4,5)P₃ and cAMP production was determined as described previously (Challiss et al., 1993). The preparation of the samples was similar to the protocol described above for [³H]InsP determination, except that [³H]inositol was omitted from the culture medium and no LiCl was added at the incubation stage.

Immunoprecipitation of [³⁵S]GTP γ S-Bound Specific $G\alpha$ Subunits. Membranes were prepared from confluent flasks of BHK-mGlu1 α cells (\pm 20-h incubation with 100 ng/ml PTX) according to Akam et al. (1997). The recovered membranes were stored at -80°C in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, at a concentration of 2 mg protein/ml until assay. [³⁵S]GTP γ S binding/immunoprecipitation was performed as previously reported (Akam et al., 1998; Burford et al., 1998). Membranes (100 μ g protein/tube) were stimulated with 10 μ M L-quisqualate for 2 min in assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, pH 7.4) containing 10 nM [³⁵S]GTP γ S and 1 μ M GDP (for $G_{\alpha_{q11}}$) or 10 μ M GDP (for $G_{\alpha_{i3/o}}$ and $G_{\alpha_{i1/2}}$). Specific $G\alpha$ subunits were then immunoprecipitated with antibodies (1:100) bound to Protein A-Sepharose beads, and the radioactivity was counted by liquid scintillation counting. Nonspecific binding was

determined by incubation with [³⁵S]GTP γ S in the presence of 10 μ M GTP γ S.

Receptor Phosphorylation Studies. Cells grown to preconfluence in 6 multiwell plates were washed three times with phosphate-free KHB and then labeled for 1 h in 1 ml phosphate-free KHB containing 50 μ Ci of [³²P]P_i at 37°C. Thereafter, cells were stimulated by the addition of agonists or phorbol-12,13-dibutyrate (PDBu) in a 50 μ l volume. The buffer vehicle (50 μ l) was added to control wells. Incubations were terminated by aspirating the medium and washing three times with ice-cold phosphate-free KHB. The cells were lysed by the addition of 1 ml solubilization buffer (containing 10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholic acid, 500 mM NaCl, 0.1 mg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After 15 min on ice, solubilized samples were collected and cleared by centrifugation for 3 min at 13,000g. Specific antisera against the mGlu1 α or M₃ receptors were added (1:300) to the supernatant and constantly mixed by rotation for 1 h at 4°C. Thereafter, immune complexes were recovered by addition of 150 μ l of a 1:1 suspension of Protein A-Sepharose and further incubation for 1 h at 4°C with constant rotation. The immunoprecipitate was collected by centrifugation at 13,000g for 30 s and washed twice with solubilization buffer and twice with 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. The pellet was resuspended in 60 μ l of sample buffer containing 125 mM Tris-HCl, 10% glycerol, 50 mM dithiothreitol, and 4% SDS. Samples were heated at 70°C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Concentrations of polyacrylamide in the gels were 3% (stacking gels) and 5 or 8% (running gels) for BHK-mGlu1 α - and BHK-m3-derived samples, respectively. Gels were dried and then exposed to Kodak Biomax film at -80°C for 72 h. Determination of the relative intensities of phosphorylated bands was carried out by gray scale densitometry using a Bio-Rad (Hercules, CA) model GS 670 densitometer.

Immunofluorescent Labeling and Imaging. Cells grown for 20 h on glass coverslips were fixed for 10 min at room temperature in 2% (w/v) paraformaldehyde in PBS. Cells were permeabilized for 30 min with 0.5% Triton X-100 in PBS containing 10% goat serum. After three washes with PBS, the cells were incubated for 2 h with a specific mGlu1 α receptor antibody diluted 1:250 in PBS/goat serum. After washing, the cells were incubated for 90 min with a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody diluted 1:500 in PBS/goat serum. After washing, the cells were mounted on slides and examined using a Bio-Rad 600 laser scanning confocal microscope equipped with a 60 \times objective.

Immunoblotting of Whole-Cell and Cell-Surface Receptor. Detection of cell-surface receptor was achieved as previously described (Mody et al., 1999). Briefly, plated cells were incubated with the cell-impermeable reagent sulfo-NHS biotin [1 mM sulfosuccinimidyl-6-(biotinamido)-hexanoate in PBS], and after solubilization (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%, Nonidet P-40, 0.1% SDS), biotinylated proteins were isolated using streptavidin-agarose beads. Protein were then resuspended in SDS-PAGE sample buffer (125 mM Tris-HCl, 50 mM dithiothreitol, 4% SDS, 20% glycerol, 0.01% bromophenol blue, pH 6.8), heated at 70°C for 5 min, and loaded onto a 5% SDS-polyacrylamide gel. For whole-cell protein analysis, proteins were solubilized (same buffer as indicated above) in the culture plate, and total extract was mixed with SDS-PAGE sample buffer containing 50 mM dithiothreitol, heated at 70°C for 5 min, and loaded onto a 5% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes, and immunoblotting was performed using an mGlu1 α receptor-specific antiserum and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected using enhanced chemiluminescence reagents. Densitometric analysis of the signal detected by autoradiography was performed using an MCID-M4 imaging system (Imaging Research, Ontario, Canada).

Data Analysis. EC_{50} values were determined by nonlinear regression analysis using the software Prism II (GraphPad, San Diego, CA). Data were fitted as sigmoidal concentration-response curves with variable slope and analyzed by a four-parameter logistic equation.

Materials. L-Quisqualic acid was from Tocris-Cookson (Bristol, UK). GPT was obtained from Boehringer (Mannheim, Germany). Triton X-100, PTX, fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody, Protein A-Sepharose, paraformaldehyde, methacholine, PDBu, dithiothreitol, Nonidet P-40, deoxycholic acid, benzamidine, phenylmethylsulfonyl fluoride, streptavidin-agarose beads, and the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody and goat serum were obtained from Sigma Chemical Co. (Poole, UK). Ro 31-8220 was purchased from Calbiochem (Nottingham, UK). myo -[2- 3H]Inositol (70–120 Ci/mmol) and [^{32}P]P $_i$ (carrier free) were obtained from Amersham (Little Chalfont, UK). Sulfo-NHS biotin was purchased from Pierce & Warriner (Chester, UK). Immunoprecipitating $G\alpha$ -specific antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; $G\alpha_{q/11}$) or NEN Life Science Products (Boston, MA; $G\alpha_{i3/o}$, $G\alpha_{11/2}$). The antiserum raised against the C terminus of the rat mGlu1 α was purchased from Chemicon International (Harrow, UK). The antibody raised against the human M $_3$ -muscarinic receptor (number 332) has been characterized previously (Tobin and Nahorski, 1993). All cell culture media and reagents were obtained from Gibco Life Technologies (Paisley, UK). All other reagents were of analytical grade and were obtained from Fisons (Loughborough, UK).

Results

Effects of PTX on L-Quisqualate- and Methacholine-Induced Phosphoinositide Hydrolysis and Adenylyl Cyclase Activation in Transfected BHK Cells. The functional coupling of the mGlu1 α receptor to phosphoinositide hydrolysis in BHK-mGlu1 α cells was initially examined by measuring L-quisqualate-induced [3H]InsP formation in the presence of LiCl. Although BHK-mGlu1 α cells were cultured in glutamate-deficient medium (see *Experimental Procedures*), the release of glutamate from the cells into the medium could not be completely excluded. Therefore, to avoid any interference with L-glutamate, the culture medium was supplemented 3 h before any experiments with GPT (3 U/ml) and pyruvate (3 mM). GPT (1 U/ml) and pyruvate (3 mM) were also present during the incubation with the agonist (except when using L-glutamate as agonist). As shown in Fig. 1A, the addition of L-quisqualate (20 μ M) resulted in a progressive increase in [3H]InsP accumulation that reached a plateau level after 15 min. An analysis of concentration-response curves for L-quisqualate-induced [3H]InsP formation measured after a 5- or 30-min stimulation revealed EC_{50} values close to 1 μ M (Fig. 1B, Table 1). When BHK-mGlu1 α cells were pretreated with PTX (100 ng/ml, 24 h), L-quisqualate-induced [3H]InsP formation was significantly enhanced,

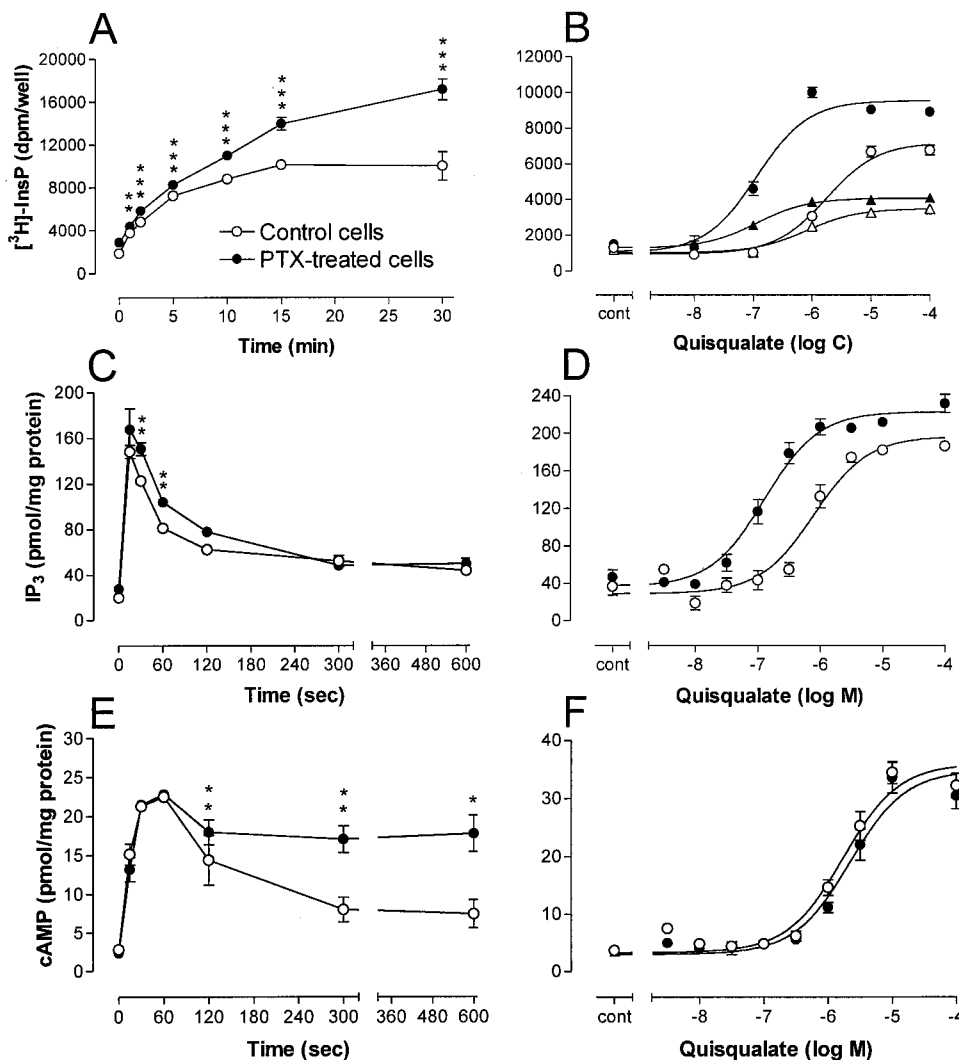


Fig. 1. Effects of PTX pretreatment on the time course and concentration dependence of L-quisqualate-stimulated [3H]InsP (A and B), Ins(1,4,5)P $_3$ (C and D) and cAMP (E and F) accumulations in BHK-mGlu1 α cells. Cells were incubated for 24 h before the experiment with PTX (●, ▲; 100 ng/ml) or vehicle (○). A, C, and E, cells were stimulated with 20 μ M L-quisqualate for the indicated periods of time. B, cells were stimulated for 5 (▲) or 30 (○) min with the indicated concentrations of L-quisqualate. D and F, cells were stimulated for 30 s with the indicated concentrations of L-quisqualate. Data are presented as mean \pm S.E. for at least three experiments performed in duplicate. Significant differences between \pm PTX conditions at specific time points were assessed using Student's t test and are shown as * P < .05, ** P < .01, and *** P < .001.

as indicated by a more sustained increase in [^3H]InsP response to L-quisqualate (Fig. 1A). Furthermore, concentration-response curves for L-quisqualate were left-shifted in PTX-treated cells with ~ 6 - and ~ 17 -fold increases in the potency of L-quisqualate being measured for 5 or 30 min agonist incubations (Fig. 1B, Table 1). These data confirm our previous observations (Carruthers et al., 1997) and further suggest that $G_{i/o}$ inactivation has effects on both the time course and concentration dependence of mGlu1 α receptor-mediated phosphoinositide hydrolysis.

The initial phase of the mGlu1 α receptor-mediated phosphoinositide hydrolysis was also examined by measuring L-quisqualate-induced production of Ins(1,4,5) P_3 in BHK-mGlu1 α cells. As shown in Fig. 1C, Ins(1,4,5) P_3 level peaked (8- to 10-fold above basal) within 15 s after the addition of 20 μM L-quisqualate and then decreased to a low plateau level (approximately 2- to 3-fold above basal) within 5 min. This response was essentially unaltered in cells previously treated with PTX. However, concentration-response curves obtained by measuring the Ins(1,4,5) P_3 level after 30-s stimulation revealed a 6-fold leftward shift in the L-quisqualate concentration-response relationship in PTX-treated cells (Fig. 1D, Table 1). These data confirm those shown in Fig. 1B for 5-min [^3H]InsP stimulations with L-quisqualate.

Stimulation of the BHK-mGlu1 α cells with L-quisqualate (20 μM) resulted in a rapid 8-fold increase in cAMP levels (experiments conducted in the absence of phosphodiesterase inhibitors), reaching a maximum after 30 to 60 s and decreasing thereafter to a sustained plateau (about 2- to 3-fold above basal; Fig. 1E). In PTX-treated cells, L-quisqualate induced a similar maximal increase in cAMP level, but after the initial peak, cAMP decreased only slightly and remained elevated (>6 -fold above basal) for at least 10 min. Examination of the concentration dependence of the L-quisqualate-stimulated response at a time point (30 s) at which a substantial PTX-induced leftward shift is observed with respect to the Ins(1,4,5) P_3 response (Fig. 1D) revealed no significant difference in the potencies observed between cells preincubated in the presence or absence of PTX (see Fig. 1F, Table 1). Therefore, PTX pretreatment affects the later time course but not the initial concentration dependence of mGlu1 α receptor-

mediated cAMP accumulation. These data suggest that mGlu1 α receptors activate adenylyl cyclase, probably through a direct G_s -mediated coupling (Thomsen, 1996), and unlike the situation for PLC regulation by $G_{q/11}/G_{i/o}$, no dual regulation between $G_s/G_{i/o}$ is initially evident. These data further suggest that BHK cells may predominantly express $G_{i/o}$ -insensitive adenylyl cyclase isoforms (Sunahara et al., 1996).

As a comparison, the functional coupling of the M_3 -muscarinic receptor to phosphoinositide hydrolysis was examined in transfected BHK cells. In contrast with the results obtained with BHK-mGlu1 α cells, incubation of the BHK-m3 cells in the presence of 5 μM methacholine resulted in a near-linear increase in [^3H]InsP levels up to 30 min (Fig. 2A), and concentration-response curves revealed similar potencies of methacholine when measured after either 5 or 30 min of stimulation (Fig. 2B, Table 1). After pretreatment of the BHK-m3 cells with PTX, the maximal amplitude of the response to methacholine was slightly increased, but no significant difference in the potency of methacholine was observed compared with untreated cells (Fig. 2, Table 1).

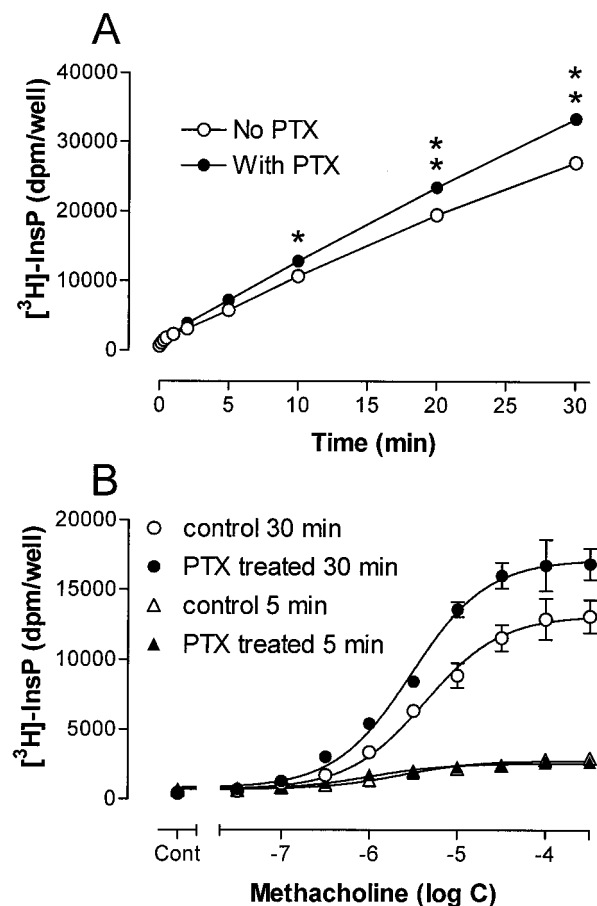


Fig. 2. Effects of PTX pretreatment on the time course and concentration dependence of methacholine-stimulated [^3H]InsP accumulation BHK-m3 cells. Cells were incubated with [^3H]inositol for 48 h, and 24 h before the experiment, PTX (100 ng/ml) or vehicle was added. A, BHK-m3 cells were stimulated for either 5 or 30 min with the indicated concentrations of methacholine. Data are presented as mean \pm S.E. for at least five experiments performed in duplicate. Significant differences between \pm PTX conditions at specific time points were assessed using Student's t test and are shown as * $P < .05$, ** $P < .01$, *** $P < .001$.

TABLE 1

Effect of PTX pretreatment on the potencies of L-quisqualate and methacholine in activating mGlu1 α or M_3 muscarinic receptors in BHK cells

Agonist potencies (presented as pEC_{50} values) in cells preincubated in the absence ($-$ PTX) or presence ($+$ PTX) of PTX were derived from concentration-response curves illustrated in Figs. 1 and 2. Data are shown as mean \pm S.D. for at least three determinations from experiments performed in duplicate.

	Agonist-Induced [^3H]InsP Accumulations			
	5 min		30 min	
	$-$ PTX	$+$ PTX	$-$ PTX	$+$ PTX
BHK-mGlu1 α	6.09 \pm 0.31	6.91 \pm 0.30*	5.80 \pm 0.05	7.02 \pm 0.09*
BHK-m3	5.62 \pm 0.18	5.97 \pm 0.25	5.38 \pm 0.09	5.53 \pm 0.07
	Agonist-Induced Ins(1,4,5) P_3 and cAMP Accumulations in BHK-mGlu1 α Cells			
	$-$ PTX		$+$ PTX	
Ins(1,4,5) P_3	6.11 \pm 0.15		6.90 \pm 0.18*	
cAMP	5.78 \pm 0.20		5.64 \pm 0.21	

Statistically significant differences are indicated, * $P < .05$, for the absence versus presence of PTX.

Effects of PTX on mGlu1 α Receptor-G Protein Coupling Interactions in BHK Cells. To provide more direct evidence for mGlu1 α receptor coupling to both $G_{q/11}$ and $G_{i/o}$ proteins, we used a [35 S]GTP γ S binding/ $G\alpha$ protein-specific immunoprecipitation strategy (Burford et al., 1998) to assess which $G\alpha$ subunits undergo GTP (GTP γ S)/GDP exchange on agonist challenge (Akam et al., 1998). We used $G_{q/11}\alpha$ -, $G_{i1/2}\alpha$ -, and $G_{i3/o}\alpha$ -specific antibodies to immunoprecipitate $G\alpha$ subpopulations after solubilization of $G\alpha$ -[35 S]GTP γ S complexes from BHK-mGlu1 α cell membranes prepared from control and PTX-treated cells and incubated with either agonist (10 μ M L-quisqualate) or vehicle. In membranes prepared from control BHK-mGlu1 α cells, L-quisqualate stimulated an approximate 250% increase in specific [35 S]GTP γ S binding above basal levels (Table 2). In addition, despite the higher levels of basal [35 S]GTP γ S binding seen for $G_{i/o}\alpha$ proteins, L-quisqualate caused significant increases in specific [35 S]GTP γ S binding to $G\alpha$ proteins immunoprecipitated by the $G_{i1/2}\alpha$ - and $G_{i3/o}\alpha$ -specific antibodies, suggesting that under these conditions, mGlu1 α receptors coupled to both $G_{q/11}\alpha$ and $G_{i/o}\alpha$ proteins.

In membranes prepared from PTX-treated BHK-mGlu1 α cells, basal [35 S]GTP γ S binding to $G\alpha$ proteins immunoprecipitated by the $G_{i1/2}\alpha$ - and $G_{i3/o}\alpha$ -specific antibodies was dramatically reduced and L-quisqualate no longer stimulated an increase in specific [35 S]GTP γ S binding over basal (Table 2). In contrast, basal and agonist-stimulated $G_{q/11}\alpha$ -[35 S]GTP γ S binding was essentially unaffected by PTX treatment, with an approximate 250% increase in specific binding seen in the presence of L-quisqualate (Table 2).

Effect of PTX Pretreatment on Localization of mGlu1 α Receptor in Transfected BHK Cells. We have previously shown that PTX treatment of BHK-mGlu1 α cells does not alter whole-cell mGlu1 α receptor expression (Carruthers et al., 1997). This finding has been confirmed (Fig. 3C) and extended by investigating whether PTX alters the subcellular distribution of this receptor. Figure 3, A and B, shows the expression of the mGlu1 α receptor detected by immunofluorescence using a specific antiserum raised against a 20-amino-acid C-terminal region of the receptor. As already reported (Pickering et al., 1993), diffuse immunoreactivity was detected throughout the cytoplasm with high-intensity spots associated with the cell surface. This pattern was not modified by preincubation of the cells with PTX.

Cell-surface expression of the mGlu1 α receptor was further examined using a protein biotinylation method (Mody et al.,

1999). After electrophoresis of biotinylated proteins isolated on streptavidin-agarose beads, mGlu1 α receptor was immunoblotted using a specific antiserum raised against the C terminus of the mGlu1 α receptor. As shown in Fig. 3, C and D, a major immunoreactive band was detected at a relative molecular mass of approximately 165 kDa. The densitometric analysis of this signal revealed no significant difference between cells treated with or without PTX (0.29 ± 0.05 and 0.32 ± 0.06 arbitrary units, respectively; $n = 7$). When the biotinylation reagent was omitted, no immunoreactivity was detected, indicating the absence of contamination of our sample by any nonbiotinylated proteins from intracellular compartments.

Desensitization of mGlu1 α Receptor Signaling in BHK Cells. Time course experiments suggest that the initial rate of agonist-stimulated [3 H]InsP accumulation in BHK-mGlu1 α cells (in the presence of 10 mM Li $^+$) wanes rapidly (see Fig. 1A). Our previous studies have shown that [3 H]inositol phospholipid pools are well maintained even under conditions where high levels of phosphoinositide hydrolysis are stimulated for long periods in BHK cells (Carruthers et al., 1997); therefore, the attenuation of PLC stimulation is unlikely to be due to substrate limitation but rather is consistent with a rapid desensitization of the mGlu1 α receptor. To study the effect of PTX treatment on agonist-induced

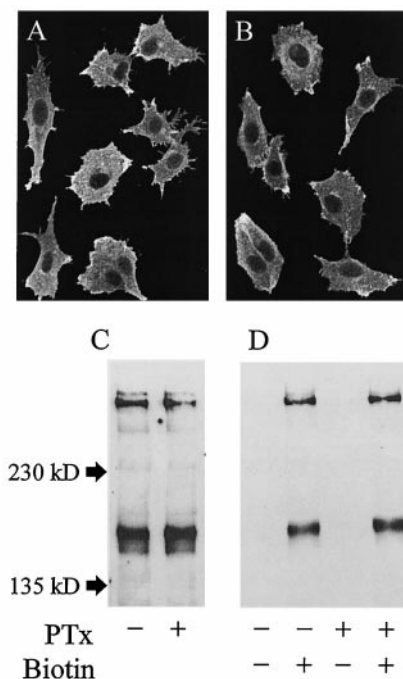


Fig. 3. Localization of the mGlu1 α receptor in transfected BHK cells (A and B). Fluorescence imaging of mGlu1 α receptor immunoreactivity in BHK-mGlu1 α cells preincubated in the absence (A) or presence (B) of PTX for 24 h. Immunostaining was performed as indicated in *Experimental Procedures* using a specific mGlu1 α receptor antiserum and was revealed using a fluorescein isothiocyanate-conjugated secondary antibody. Representative confocal images corresponding to horizontal sections through the middle of cells are shown. C and D, effects of PTX on mGlu1 α receptor cell-surface expression. Representative immunoblots are shown for mGlu1 α receptor immunoreactivity in total cell extracts (C) or on the cell-surface only, detected after treatment of intact cell monolayers to biotinylate cell-surface proteins and analysis of streptavidin-bound material (D). Cells extraction, solubilization, recovery of biotin-labeled proteins, separation on 5% SDS-polyacrylamide running gels, and immunoblotting using a specific mGlu1 α receptor antiserum were performed as described in *Experimental Procedures*.

TABLE 2

Effect of PTX pretreatment on basal and L-quisqualate-stimulated specific [35 S]GTP γ S binding to $G_{q/11}\alpha$, $G_{i3/o}\alpha$, and $G_{i1/2}\alpha$ in BHK-mGlu1 α cell membranes. Data are presented as mean \pm S.E. for either four (–PTX) or three (+PTX) experiments performed in duplicate.

	Specific [35 S]GTP γ S Bound		
	$G_{q/11}\alpha$	$G_{i3/o}\alpha$	$G_{i1/2}\alpha$
	dpm/tube		
–PTX			
Basal	1245 \pm 264	5150 \pm 486	3037 \pm 652
+L-Quisqualate	3020 \pm 665*	6803 \pm 727*	4226 \pm 934*
+PTX			
Basal	950 \pm 110	980 \pm 392	689 \pm 251
+L-Quisqualate	2269 \pm 340*	915 \pm 312	988 \pm 456

Statistical significance (* $P < .05$) was assigned using Student's t test for paired data and tested whether L-quisqualate (10 μ M) caused a significant stimulation above basal of [35 S]GTP γ S specifically bound to $G_{q/11}\alpha$, $G_{i3/o}\alpha$, or $G_{i1/2}\alpha$ in membranes prepared from vehicle or PTX-pretreated BHK-mGlu1 α cells.

desensitization, mGlu1 α receptor signaling was assessed after preincubation of BHK-mGlu1 α cells with 300 μ M L-glutamate and subsequent measure of the L-quisqualate-stimulated [3 H]InsP response. L-Glutamate was chosen for the prestimulation step as it could easily be removed by the washing and addition of GPT/pyruvate. As shown in Fig. 4A, preincubation of cells with L-glutamate for increasing periods of time resulted in a progressive decrease in subsequent L-quisqualate-induced [3 H]InsP accumulation (measured over a 10-min incubation period in the presence of Li $^+$). A maximal decrease of about 60% was approached after a 30-min glutamate pretreatment period; however, no significant change in the potency of L-quisqualate to elicit this response was observed compared with untreated cells (Fig. 4B; pEC $_{50}$ values, 6.37 \pm 0.10 and 6.23 \pm 0.29 M for vehicle and L-glutamate-pretreated cells, respectively). In PTX-treated

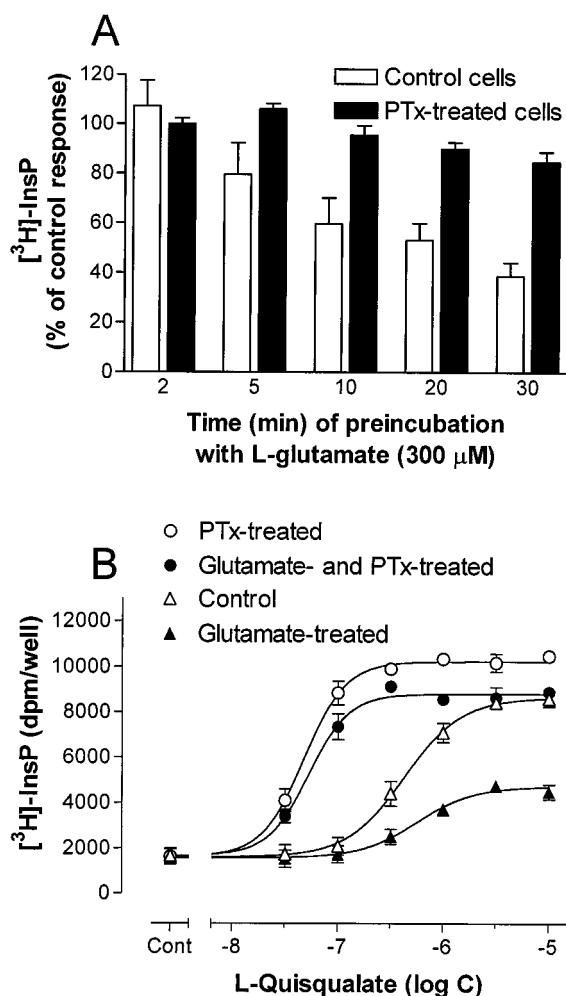


Fig. 4. Effects of preexposure to L-glutamate on subsequent L-quisqualate-mediated [3 H]InsP accumulation in control and PTX-pretreated BHK-mGlu1 α cells. Cells were incubated with [3 H]inositol for 48 h, and 24 h before the experiment, PTX (100 ng/ml) or vehicle was added. A, control and PTX-pretreated BHK-mGlu1 α cells were incubated with L-glutamate (300 μ M) for the indicated periods of time. Thereafter, cells were rapidly washed three times in buffer containing GPT/pyruvate, LiCl was added, and after 5 min, cells were stimulated with 1 μ M L-quisqualate for 10 min. B, cells were preincubated with L-glutamate (300 μ M) for 30 min. Thereafter, cells were rapidly washed as described and rechallenge with the concentrations of L-quisqualate indicated for 10 min. Data are presented as mean \pm S.E. for four experiments performed in duplicate.

cells, prechallenge with L-glutamate (300 μ M, 30 min) resulted in only a small (approximately 15%) decrease in the subsequent [3 H]InsP response to L-quisqualate (Fig. 4A) and no change in the potency of this agonist to cause the response (Fig. 4B; pEC $_{50}$ values, 7.32 \pm 0.15 and 7.26 \pm 0.15 M for vehicle and L-glutamate-pretreated cells, respectively).

Phosphorylation of mGlu1 α and M $_3$ -Muscarinic Receptors in Transfected BHK Cells. Incubation of BHK-mGlu1 α cells with [32 P]orthophosphate followed by solubilization and immunoprecipitation with the specific mGlu1 α receptor antiserum revealed the phosphorylation of a \sim 165-kDa protein that is likely to correspond to the mGlu1 α receptor (Fig. 5) as well as intense labeling of higher-molecular-weight (\sim 300 kDa) unidentified proteins (not shown). Stimulation of the cells with L-quisqualate (10 μ M) for 2 min before solubilization did not enhance the phosphorylation of the receptor, whereas incubation of the cells with PDBu (5 μ M) for 10 min significantly increased (80% above basal) the phosphorylation state of the mGlu1 α receptor (Fig. 5). Similar experiments were conducted on BHK-m3 cells using a specific M $_3$ -mACH receptor antiserum to immunoprecipitate an \sim 100-kDa phosphoprotein corresponding to the receptor (Tobin and Nahorski, 1993). In contrast with the mGlu1 α receptor, phosphorylation of the M $_3$ -mACH receptor was weak in the absence of stimulation and both agonist (methacholine, 5 μ M) and PDBu (5 μ M) significantly increased the phosphorylation state of the M $_3$ -mACH receptor (by \sim 3 and \sim 2.5 fold, respectively).

Effects of PDBu and Ro 31-8220 on mGlu1 α Receptor Signaling in Transfected BHK Cells. The desensitization of L-quisqualate-induced phosphoinositide hydrolysis in BHK-mGlu1 α cells observed after incubation with L-glutamate could be mimicked by preincubating cells with PDBu. Thus, incubation of the cells for 10 min in the presence of 1 μ M PDBu resulted in a 70% decrease in the subsequent

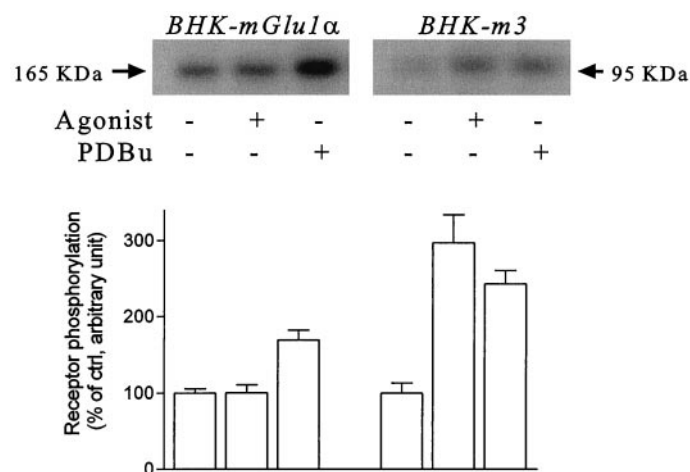


Fig. 5. Effects of L-quisqualate (10 μ M), carbachol (5 μ M), or PDBu (1 μ M) on the phosphorylation state of the mGlu1 α and M $_3$ -muscarinic receptors expressed in transfected BHK-mGlu1 α or BHK-m3 cells. Cells were labeled with [32 P]orthophosphate for 60 min and then incubated in the absence (lanes 1 and 4) or in the presence of agonist for 2 min (lanes 2 and 5) or PDBu for 10 min (lanes 3 and 6). After solubilization, the mGlu1 α or M $_3$ -muscarinic receptors were immunoprecipitated using specific antisera, analyzed by SDS-PAGE, and detected by autoradiography as described in *Experimental Procedures*. Top, typical autoradiograms. Bottom, quantification by gray-scale densitometry of the signal detected by autoradiography as mean \pm S.E. for four (BHK-mGlu1 α) or three (BHK-m3) different experiments performed in duplicate.

[^3H]InsP response elicited by L-quisqualate (Fig. 6). Prior incubation of the cells with the protein kinase C (PKC) inhibitor Ro 31-8220 (10 μM) for 15 min prevented this effect of PDBu. However, under the same conditions, PKC inhibition did not prevent the desensitization induced by L-glutamate.

Discussion

In a previous study, we presented evidence consistent with a dual coupling between recombinant mGlu1 α receptors and PLC in BHK cells mediated by both stimulatory $G_{q/11}$ and inhibitory $G_{i/o}$ proteins (Carruthers et al., 1997). Thus, removal of the $G_{i/o}$ -mediated inhibitory component by PTX resulted in a marked increase in both the potencies and the maximal responses elicited by full and partial agonists of the mGlu1 α receptor (Carruthers et al., 1997). Although the apparent enhancement of mGlu1 α receptor-PLC coupling by PTX contrasts with a number of other reports (Aramori and Nakanishi, 1992; Pickering et al., 1993; Thomsen et al., 1993), the ability of mGlu1 α receptors to couple to multiple G protein partners has been either suggested or demonstrated in a number of studies (Aramori and Nakanishi, 1992; Joly et al., 1995; Thomsen, 1996; Akam et al., 1997; McCool et al., 1998; Kammermeier and Ikeda, 1999). Of particular note is a recent study that reports that recombinant expression of mGlu1 α receptors in sympathetic neurones results in a dual regulation of N-type Ca^{2+} channels by $G_{q/11}\alpha$ - and $\beta\gamma$ -subunits derived from $G_{i/o}$ protein or proteins (Kammermeier and Ikeda, 1999). Further evidence for a functional coupling of mGlu1 α receptors to $G_{i/o}$ proteins comes from experiments exploring the link between the mGlu1 α receptor and extracellular signal-regulated kinase where receptor/extracellular signal-regulated kinase coupling appears to be entirely transduced by PTX-sensitive G proteins (Ferraguti et al., 1999).

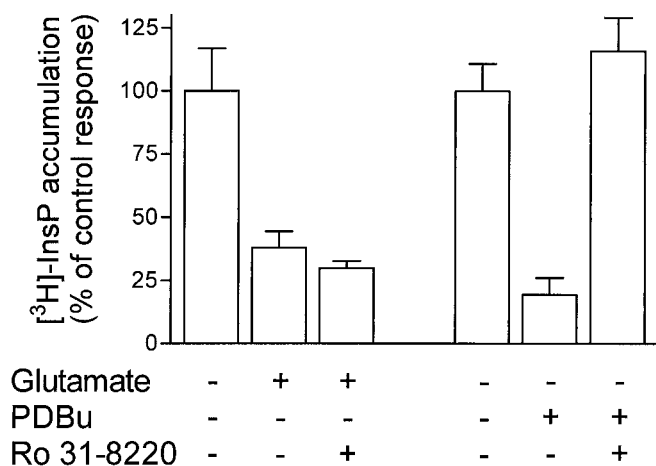


Fig. 6. Effects of activators and inhibitors of PKC on L-quisqualate-induced [^3H]InsP accumulation in BHK-mGlu1 α cells. Cells previously treated with Ro 31-8220 (10 μM) or vehicle for 15 min were incubated with L-glutamate (300 μM , 30 min) or PDBu (1 μM , 10 min). Thereafter, cells were rapidly washed three times in buffer containing GPT/pyruvate, LiCl was added, and after 5 min, cells were stimulated with 1 μM L-quisqualate for 10 min. Data are presented as mean \pm S.E. for three experiments performed in duplicate. For \pm L-glutamate experiments (first three columns), the 100% [^3H]InsP accumulation was $12,472 \pm 1369$ dpm/well (basal, 1990 ± 376), whereas for the \pm PDBu experiments (second three columns), the 100% [^3H]InsP accumulation was 8381 ± 1587 dpm/well (basal, 2344 ± 343).

In this study, we provided direct evidence for the mGlu1 α receptor coupling to both $G_{q/11}$ and $G_{i/o}$ proteins in BHK-mGlu1 α cell membranes using a [^{35}S]GTP γS /immunoprecipitation strategy. Thus, using $G_{q/11}\alpha$ -, $G_{i1/2}\alpha$ -, and $G_{i3/o}\alpha$ -specific antibodies, we have demonstrated agonist-stimulated, PTX-sensitive increases in [^{35}S]GTP γS bound to $G_{i1/2}\alpha$ and $G_{i3/o}\alpha$ proteins and agonist-stimulated, PTX-insensitive [^{35}S]GTP γS bound to $G_{q/11}\alpha$ protein or proteins. Furthermore, PTX treatment does not affect the concentration dependence of L-quisqualate-stimulated [^{35}S]GTP γS binding to $G_{q/11}\alpha$ protein or proteins (J. V. Selkirk, R. A. J. Challiss, G. W. Price, and S. R. Nahorski, unpublished data). These data confirm and extend our previous finding that mGlu1 α receptor stimulation of total specific [^{35}S]GTP γS binding was substantially reduced by PTX treatment (Akam et al., 1997) and emphasize the relative promiscuity of this mGlu receptor subtype. It is interesting to note that dual $G_{q/11}$ and $G_{i2/3}$ coupling has also been reported for the closely related family 3 G protein-coupled Ca^{2+} -sensing receptor (Arthur et al., 1997).

The present data have also shown that $G_{i/o}$ protein inactivation by PTX has at least two distinct effects on mGlu1 α receptor signaling. If the initial phase of receptor-mediated phosphoinositide hydrolysis is measured (either by assessing the initial peak increase in Ins(1,4,5) P_3 mass accumulation or total [^3H]InsP accumulation at an early time point), an ~ 10 -fold increase in agonist potency is evident; however, an increase in maximal agonist responsiveness relative to control is only observed at later time points in PTX-treated BHK-mGlu1 α cells. Thus, although the potency shift is an intrinsic property of PTX-treated cells, the change in maximal responsiveness may be caused by PTX treatment altering (adaptive) processes that occur during ongoing receptor stimulation. This division of the effects of PTX on mGlu1 α receptor signaling is corroborated by experiments using a different PLC-coupled receptor (M_3 -muscarinic) transfected into the same cell background. Thus, in contrast to the situation for BHK-mGlu1 α cells, PTX pretreatment has no effect on the potency of the muscarinic agonist MCh to stimulate [^3H]InsP accumulation in BHK-m3 cells, but a small increase in the maximal responsiveness to MCh is observed if a longer time point (30 min) is examined. Thus, the immediately evident potency effect is receptor-specific, whereas the increased responsiveness seen after PTX treatment may be a more general phenomenon for G protein-coupled receptors expressed in this cell background.

Another notable feature of our data is the effect of $G_{i/o}$ inactivation on the desensitization of the agonist-stimulated [^3H]InsP response. Preincubation of BHK-mGlu1 α with L-glutamate results in a progressive loss of L-quisqualate-induced [^3H]InsP, and this effect is markedly reduced in PTX-treated cells. These data suggest that PTX treatment attenuates the desensitization of the mGlu1 α receptor. Similar PTX-mediated effects have been noted for other G protein-coupled receptors (Woo et al., 1998), and considering the growing literature that suggests an involvement of heterotrimeric G proteins ($G_{i/o}$) in the control of exocytotic/endocytotic processes (Lang et al., 1995; Nurnberg and Ahnert, 1996; Lin et al., 1998), it is possible that $G_{i/o}\alpha\beta\gamma$ inactivation may inhibit endocytotic mechanisms that regulate aspects of mGlu (and perhaps M_3 -muscarinic) receptor desensitization. However, it should be noted that PTX treatment of BHK-

mGlu1 α cells for 20 h does not affect either the total mGlu1 α receptor immunoreactivity or the steady-state cell-surface expression (see Fig. 3).

Phosphorylation by second messenger kinases or G protein-coupled receptor kinases has been proposed to be a near-ubiquitous event linking receptor activation and subsequent desensitization (Tobin, 1997). Accordingly, we and others (Aramori and Nakanishi, 1992; Thomsen et al., 1993) have demonstrated that treatment with the PKC-activating phorbol ester PDBu causes a marked attenuation of the agonist-stimulated [3 H]InsP response and increases mGlu1 α receptor phosphorylation. Therefore, we wanted to investigate whether PTX treatment affects agonist-dependent mGlu1 α receptor phosphorylation because this might provide an alternative explanation for the altered rate of desensitization. However, whereas we could demonstrate robust M $_3$ -muscarinic receptor phosphorylation mediated by both agonist and PDBu in BHK-m3 cells, we could not detect an agonist-stimulated increase in receptor phosphorylation in BHK-mGlu1 α cells. Furthermore, although PKC inhibition prevented the PDBu-evoked attenuation of the [3 H]InsP response, blocking PKC activity failed to affect the decrease in [3 H]InsP response after preexposure to agonist. These data suggest that receptor desensitization may occur independent of receptor phosphorylation and does not involve a PKC-dependent pathway. Such data contrast with the previous report of Alaluf et al. (1995), who used a similar BHK-mGlu1 α cell line. At the present, we have no explanation for the discrepancy between these two datasets.

Finally, how might PLC activity be negatively regulated by a mechanism involving mGlu1 α receptor-G $_{i/o}$ coupling? At the present, the weight of evidence favors the view that PLC- β differs from the prototypic second messenger-generating enzyme adenylyl cyclase in that it is only subject to positive regulation, both by G $_{q/11}$ α -subunits and by $\beta\gamma$ -subunits. Indeed, a number of studies have shown that G $_{i/o}$ -coupled receptors can stimulate PLC- β via the release of $\beta\gamma$ -subunits (Katz et al., 1992; Dickenson and Hill, 1998). However, other studies have provided evidence to suggest that under certain circumstances both G $_{i\alpha}$ - and G $_{i/o}$ -derived $\beta\gamma$ -subunits might exert inhibitory effects on PLC. Thus, reconstitution studies have demonstrated PTX-sensitive inhibition of PLC- β s isolated from pig aortic smooth muscle (Blayney et al., 1996) and $\beta\gamma$ -mediated inhibition of PLC- β 1 from bovine brain (Litosch, 1996). In addition, manipulation of G $_{12\alpha}$ levels in cells has been shown to affect PLC activity consistent with both a basal and an agonist-stimulated inhibitory modulation of PLC (Watkins et al., 1994; Mattera et al., 1998). Thus, although our conclusions concerning the dual (antagonistic) modulation of PLC activity by G $_{q/11}$ and G $_{i/o}$ proteins are controversial, there is growing evidence in the literature to provide some supporting evidence for our hypothesis and to suggest possible mechanisms by which the G $_{i/o}$ -mediated inhibitory action is brought about.

The mGlu1 α receptor subtype is widely distributed in the brain, and its subcellular localization appears to be highly regulated (luján et al., 1996; Stowell and Craig, 1999). Key roles for these receptors in certain forms of synaptic plasticity implicated in learning and memory have been proposed, whereas a pathological role in neuronal damage has also been suggested (Conn and Pin, 1997). Thus, it is possible that the dual regulation observed in this study may offer an

adaptive mechanism by which the ability of mGlu1 α receptors to stimulate phosphoinositide hydrolysis, and thus influence cellular [Ca $^{2+}$] $_i$ and the activities of PKCs, can be increased or decreased, acutely or chronically and independently of mGlu1 α receptor expression. In addition, the fact that such a dual modulatory effect is not observed universally between cell types may reflect cell-specific differences at the level of subcellular mGlu1 α receptor localization, the G protein complement of the cell, and/or the PLC- β isoform expression. Thus, different neuronal populations may differ markedly in the extent to which G $_{i/o}$ protein activation can modulate mGlu1 α receptor-G $_{q/11}$ -PLC coupling and therefore may exhibit different adaptive potentials with respect to modifying the potency (and perhaps efficacy) of L-glutamate to initiate changes in neuronal intracellular Ca $^{2+}$ concentration and PKC activity.

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