0026-895X/97/030406-09$3.00/0

MOLECULAR PHARMACOLOGY, **52:**406–414 (1997).

Enhanced Type 1α Metabotropic Glutamate Receptor-Stimulated Phosphoinositide Signaling after Pertussis Toxin Treatment

ALAN M. CARRUTHERS, R. A. JOHN CHALLISS, RAJENDRA MISTRY, RUTH SAUNDERS, CHRISTIAN THOMSEN, and STEFAN R. NAHORSKI

Department of Cell Physiology and Pharmacology, University of Leicester, University Road, Leicester LE1 9HN, UK (A.M.C., R.A.J.C., R.M., R.S., S.R.N.), and Novo Nordisk A/S, Health Care Discovery, 2760 Måløv, Denmark (C.T.)

Received April 11, 1997; Accepted May 7, 1997

SUMMARY

The regulation of phosphoinositide hydrolysis by the type 1α metabotropic glutamate receptor (mGluR1α) was investigated in stably transfected baby hamster kidney (BHK) cells. Incubation of the cells with L-glutamate, quisqualate, and 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid resulted in a marked accumulation of [^{3}H]inositol monophosphate ([^{3}H]InsP$_1$) and inositol-1,4,5-trisphosphate [Ins(1,4,5)P$_3$] mass in a time- and concentration-dependent manner. Pretreatment of BHK-mGluR1α cells with pertussis toxin [100 ng/ml, 24 hr] led to a dramatic 12–16-fold increase in the accumulation of [^{3}H]InsP$_1$ and a 2-fold increase in Ins(1,4,5)P$_3$ in the absence of added agonist. Although only very low levels ($\leq$1 μM) of L-glutamate could be detected in medium taken from control and PTX-treated cell monolayers, the PTX-elicited effect on basal [^{3}H]InsP$_1$ was fully reversed by preincubation of cells in the presence of glutamic-pyruvic transaminase and pyruvate, suggesting that an increased sensitivity to endogenous glutamate was responsible for the apparent agonist-independent activation of phosphoinositidase C (PIC) after PTX treatment. Consistent with this hypothesis, in the presence of glutamic-pyruvic transaminase/pyruvate, the maximal [^{3}H]InsP$_1$ response to quisqualate was increased by $\geq$75%, and the EC$_{50}$ shifted leftward by 65-fold [$-$log EC$_{50}$ values (molar), 7.26 $\pm$ 0.23 versus 5.45 $\pm$ 0.07; n = 4) in PTX-treated compared with control cells. In contrast, antagonist effects on agonist-stimulated [^{3}H]InsP$_1$ responses were similar in control and PTX-treated BHK-mGluR1α cells. These changes in the concentration-effect curves for mGluR agonists are consistent with a model in which the receptor associates with PTX-sensitive inhibitory (G$_{i/o}$) and PTX-insensitive stimulatory (G$_{q/11}$) G proteins that can each influence PIC activity. The present observations are consistent with a dual regulation of mGluR1α-mediated PIC activity that could be fundamental in controlling the output of phosphoinositide-derived messengers.

The recent cloning of eight subtypes of mGluR has not only opened up new avenues for exploration of the central actions of this excitatory neurotransmitter but also expanded the potential to target drugs against specific receptor-mediated actions (1–3). Recently, novel synthetic glutamate analogues have been developed as ligands at different mGluRs, and of particular significance has been the development of competitive antagonists (4–7) and their use in identifying the involvement and roles of different mGluR subtypes in fundamental mechanisms such as long term potentiation and long term depression (8–12).

The mGluRs form a distinct branch of the G protein-coupled receptor superfamily, sharing topological organization but little sequence homology with other G protein-coupled receptors. Sequence homology and pharmacological profiling have allowed three subgroups of mGluRs, termed I, II, and III, to be described (2, 3). Group II mGluRs (types 2 and 3) and group III MGluRs (types 4, 6, 7 and 8) both couple to G proteins of the G$_{i/o}$ family to inhibit adenylyl cyclase or modulate ion channel activities (2, 3); in contrast, group I mGluRs (types 1 and 5) activate PIC with the subsequent generation of the second messengers Ins(1,4,5)P$_3$ and diacylglycerol (2, 3, 13–17). The G protein or proteins responsible for coupling group I mGluRs to PIC have been the subject of some debate. Thus, although the phosphoinositide responses elicited by agonist stimulation of mGluR5 and the mGluR1β splice variant seem to be little affected by PTX treatment (14, 15), the response to mGluR1α activation is substantially

This work was supported by the Wellcome Trust of Great Britain. A.M.C. is the recipient of a Wellcome Trust Toxicology Initiative Studentship, and R.S. holds a Medical Research Council Postgraduate Studentship.

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; PTX, pertussis toxin; BHK, baby hamster kidney; InsP$_1$, inositol monophosphate; Ins(1,4,5)P$_3$, inositol-1,4,5-trisphosphate; PtdIns, phosphatidylinositol; GPT, glutamic-pyruvic transaminase; PIC, phosphoinositidase C; KHB, Krebs-Henseleit buffer; TCA, trichloroacetic acid; 1*S*,3*R*-ACPD, 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid; 4C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

attenuated by PTX when this splice variant is expressed in Chinese hamster ovary cells (13), BHK cells (15, 16), or *Xenopus laevis* oocytes (18). It has been concluded from such studies that mGluR1α can stimulate phosphoinositide hydrolysis via PTX-sensitive ($G_{i/o}$) and -insensitive ($G_{q/11}$) pathways, and it is possible that the distinct pathways may lead to the activation of distinct PIC isozymes.

In the current study, we examined the coupling of recombinant mGluR1α expressed in BHK cells using both [^{3}H]InsP_1 and Ins(1,4,5)P_3 mass accumulations as indices of PIC activity. In contrast to previous reports (13, 15, 16), we do not observe a decrease in agonist-stimulated inositol (poly)phosphate accumulation after PTX pretreatment but rather find a profound increase in the ability of glutamate receptor agonists to increase PIC activity after toxin treatment. These data provide evidence consistent with a dual regulation of PIC by mGluR1α via $G_{q/11}$ and $G_{i/o}$.

Experimental Procedures

Cell culture. BHK cells stably expressing the T45A clone of the rat type 1α mGluR (15, 16) were cultured in Dulbecco's modified Eagle's (Glutamax-1) medium supplemented with 5% dialyzed fetal calf serum, 0.5 mg/ml G418, 50 μg/ml gentamicin, and 1 μM methotrexate. BHK-mGluR1β cells were maintained in a similar culture medium except that methotrexate was omitted (15). Vector control cells (BHK-570) (15) were cultured without G418 or methotrexate but in the presence of neomycin (0.1 mg/ml). Cells were maintained at 37° in a humidified atmosphere (95% air/5% CO_2) and were passaged every 4–5 days.

[^{3}H]InsP_1 and Ins(1,4,5)P_3 determinations. BHK-mGluR cells were seeded onto 16-mm wells (24-well multidishes, Nunc, Naperville, CT) and, where indicated, labeled with 1 μCi/ml [^{3}H]inositol for 48 hr. Treatment of monolayers with PTX was performed by the addition to the culture medium 22–24 hr before experimentation. Cells were washed four times with 1 ml of KHB (containing 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM HEPES, and 10 mM D-glucose, pH 7.4, after equilibration with 95% O_2/5% CO_2) at 37°. Where GPT and pyruvate were added to decrease medium glutamate concentrations, these agents were present for ≥15 min before any other manipulations were performed and ≥30 min before agonist challenge. Where the effects of GPT *per se* were assessed, GPT/pyruvate- and pyruvate-only-treated cells were compared.

Vector or mGluR-expressing BHK cells were incubated with KHB (supplemented with 10 mM LiCl in experiments in which [^{3}H]InsP_1 was to be determined) for 15 min. Additions of mGluR antagonists were made 15 min before agonist challenge. Incubations were continued in the presence of agonist for the times indicated at 37°. Incubations were terminated by rapid aspiration of incubation media followed by the addition of 0.3 ml of ice-cold 0.5 M TCA. TCA was extracted by repeated washing with water-saturated diethylether (four times three volumes). Then, one volume of 60 mM $NaHCO_3$ and one volume of 30 mM EDTA were added to four volumes of the extracted supernatant, and samples were stored at 0–4°C until further analysis.

When inositol phospholipid labeling was assessed, cell monolayers were extracted with acidified chloroform/methanol (40:80:1 volume concentrated HCl) immediately after aspiration of TCA. The recovered [^{3}H]inositol phospholipids were deacylated and the glycerophosphoinositol(phosphates) were resolved by ion exchange chromatography, as previously described (19).

The [^{3}H]InsP_1 fraction was also resolved by ion exchange chromatography on Dowex AG1-X8 formate form columns (200–400 mesh, 1.0-ml bed volume) as previously described (19). Ins(1,4,5)P_3 mass assays were performed using an Ins(1,4,5)P_3-binding protein prepared from bovine adrenal cortex as described (19). Where indicated, cell protein was quantified using the Lowry method to allow [^{3}H]InsP_1 and Ins(1,4,5)P_3 mass data to be presented as dpm/mg of protein or pmol/mg of protein, respectively.

L-Glutamate assay. L-Glutamate in the cell monolayer incubation medium was determined after the addition of TCA (0.5 M final concentration) and extraction with diethylether as described above. A standard curve for known amounts of L-glutamate (0.1–100 μM) was also prepared in a diethylether-extracted-TCA 'buffer-blank' solution. The spectrophotometric assay was performed according to manufacturer's instructions except that assay constituent volumes were adjusted to allow detection of L-glutamate at concentrations ≥0.3 μM.

Preparation of anti-mGluR1α antiserum. A 20-mer peptide (sequence: PNVTYASVILRDYKQSSSTC) corresponding to the final carboxyl-terminal 20 amino acids (residues 1180–1199; C-for-L substitution at position 1199) of the primary sequence of mGluR1α (20) was synthesized and coupled to the carrier protein keyhole limpet hemocyanin using glutaraldehyde. The peptide/keyhole limpet hemocyanin conjugate was purified by gel filtration and antisera against the conjugate raised in New Zealand White rabbits.

Immunoblotting. Membranes were prepared from control and PTX-treated (100 ng/ml, 24 hr) BHK-mGluR1α cells. Membrane proteins were resolved on 7.5% SDS-PAGE minigels and transferred onto nitrocellulose membranes. Blots were blocked in 5% nonfat dry milk/phosphate-buffered saline (blocking solution) overnight and then incubated for 2 hr with a 1:4000 dilution of the mGluR1α antiserum in the blocking solution. Blots were washed with three changes of phosphate-buffered saline for 30 min and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000 dilution in blocking solution) for 1 hr. After washing (30 min), immunoreactive proteins were detected with enhanced chemiluminescence (ECL; Amersham, Little Chalford, UK).

Materials. PTX, quisqualic acid, GPT (EC 2.6.1.2), and methotrexate were from Sigma Chemical (Poole, UK). L-Glutamate was obtained from BDH (Poole, UK), and the L-glutamate colorimetric assay kit was obtained from Boehringer-Mannheim (Mannheim, Germany). 4C3HPG and 1*S*,3*R*-ACPD were purchased from Tocris-Cookson (Bristol, UK). *myo*-[2-^{3}H]inositol (70–120 Ci/mmol) and [^{3}H]Ins(1,4,5)P_3 (30–60 Ci/mmol) were from Amersham. Dowex anion exchange resin AG1-X8 (200–400 mesh, formate form) was from BioRad (Watford, UK). All other chemicals were of analytical grade and were from Fisons (Loughborough, UK). Unless indicated, all cell culture media and reagents were obtained from Gibco Life Technologies (Paisley, UK).

Results

Time- and concentration-dependency of mGluR1α phosphoinositide responses. Receptor coupling to PIC in BHK-mGluR1α cells was investigated either by assessing [^{3}H]InsP_1 accumulation in the presence of 10 mM LiCl in *myo*-[^{3}H]inositol-prelabeled cells or measuring changes in endogenous Ins(1,4,5)P_3 levels. After the addition of 300 μM L-glutamate, [^{3}H]InsP_1 accumulated linearly over the initial 2–30 min of agonist challenge but then plateaued (Fig. 1A). In contrast, Ins(1,4,5)P_3 levels increased to a peak at 30 sec and then declined toward a lower, but still elevated, plateau level (Fig. 1B). Fig. 1, C and D, shows concentration-dependent increases in [^{3}H]InsP_1 (at 30 min) and Ins(1,4,5)P_3 (at 30 sec) accumulations, respectively, stimulated by L-glutamate, quisqualate, and 1*S*,3*R*-ACPD. L-Glutamate and quisqualate caused similar 8–9-fold increases in [^{3}H]InsP_1 accumulation, but the latter agonist was ~10-fold more potent (Table 1). 1*S*,3*R*-ACPD seemed to be a partial agonist, eliciting a maximal response that was ~50% of that stimulated

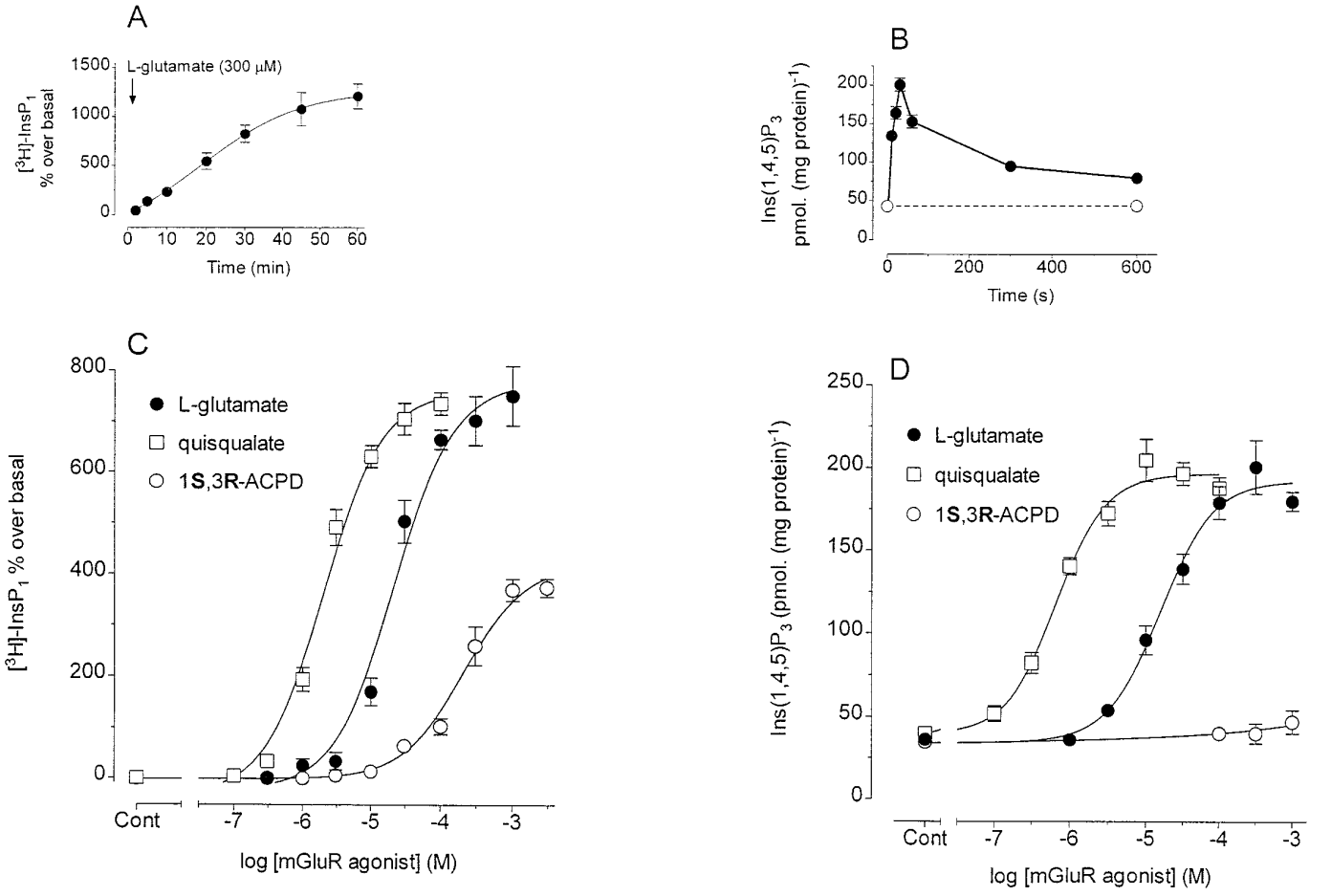

Fig. 1. Time courses and concentration-dependencies of agonist-stimulated [^{3}H]InsP$_1$ and Ins(1,4,5)P$_3$ responses in BHK-mGluR1α cells. For subsequent measurement of [^{3}H]InsP$_1$ (A and C), BHK-mGluR1α cells were cultured in the presence of [^{3}H]inositol (1 μCi/ml) for 48 hr, washed with KHB, and incubated in KHB plus 10 mM LiCl for 30 min before addition of the indicated concentrations of L-glutamate, quisqualate, or 1*S*,3*R*-ACPD for the times indicated (A) or 30 min (C). For [^{3}H]InsP$_1$, data are expressed as increases over basal labeling (644 ± 50 dpm/well; 16 experiments). For Ins(1,4,5)P$_3$ mass determination (B and D), cells were washed with KHB and incubated for 30 min (no LiCl) before the addition of the indicated agonist concentrations for the times indicated (B) or 30 sec (D). All data are shown as mean ± standard error for at least four separate experiments performed in duplicate.

TABLE 1

EC$_{50}$ values for L-glutamate-, quisqualate-, and 1*S*,3*R*-ACPD-stimulated [^{3}H]InsP$_1$ and Ins(1,4,5)P$_3$ mass accumulations in BHK-mGluR1α cells

Concentrations of L-glutamate, quisqualate, and 1*S*,3*R*-ACPD that stimulated half-maximal increases in [^{3}H]InsP$_1$ accumulation (assessed at 30 min in the presence of 10 mM LiCl) or Ins(1,4,5)P$_3$ mass accumulation (assessed at 30 sec) are presented as −log EC$_{50}$ ± standard error values from analyses of at least three concentration-response curves, each performed in duplicate.

Agonist	−log EC$_{50}$	
	[^{3}H]InsP$_1$	Ins(1,4,5)P$_3$
	M	
L-Glutamate	4.67 ± 0.08	4.80 ± 0.09
Quisqualate	5.70 ± 0.05	6.18 ± 0.07
1*S*,3*R*-ACPD	3.67 ± 0.07	N.D.

N.D., An EC$_{50}$ for this response could not be calculated.

by L-glutamate or quisqualate. L-Glutamate and quisqualate caused similar maximal 4–5-fold increases in Ins(1,4,5)P$_3$ mass, with quisqualate again more potent that L-glutamate (Table 1). In contrast, 1*S*,3*R*-ACPD failed to increase significantly the steady state concentration of Ins(1,4,5)P$_3$ above basal levels, even at 1 mM.

Agonist-independent activity of mGluR1α. In agreement with the results obtained by Prézeau *et al.* (21) in LLC-PK1 and human embryonic kidney 293 cells transiently transfected to express mGluR1α, direct comparison between the stably transfected BHK-mGluR1α cell line and vector-transfected BHK cells (labeled to equilibrium with *myo*-[^{3}H]inositol) revealed that basal [^{3}H]InsP$_1$ accumulation was increased in the mGluR1α-expressing cells (BHK-570, 16,536 ± 245; BHK-mGluR1α, 26,088 ± 1,030 dpm/mg of protein; four experiments; $p < 0.001$). This increase was not attributable to receptor activation by endogenous L-glutamate because pretreatment of cells with GPT and pyruvate had no effect on the different basal [^{3}H]InsP$_1$ accumulations seen in vector- and mGluR1α-transfected cells (data not shown).

Effects of PTX. The treatment of BHK-mGluR1α cells for 22–24 hr with 1, 10, or 100 ng/ml PTX had dramatic dose-related effects on basal and agonist-stimulated phosphoinositide turnover that were most marked at the highest concentration of PTX used (Fig. 2). Basal [^{3}H]InsP$_1$ accumulation was dramatically elevated (12.8 ± 0.9-fold) after PTX pretreatment. Remarkably, this increase was greater than that seen in control cells stimulated with a

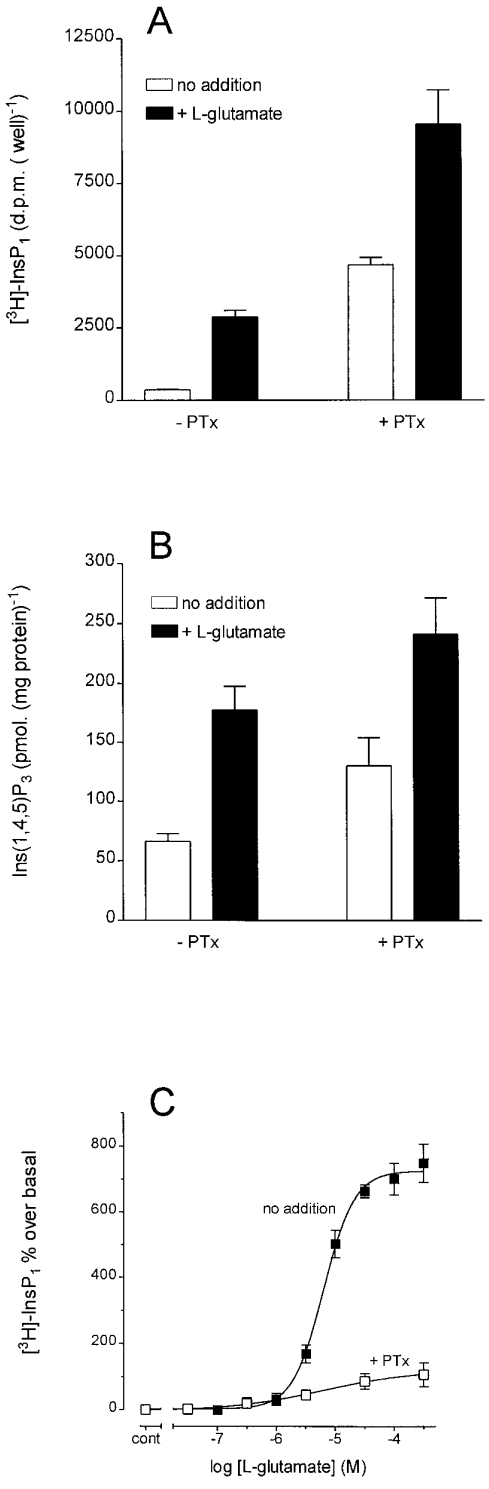

Fig. 2. Effects of PTX treatment on L-glutamate-stimulated [^{3}H]$InsP_1$ and $Ins(1,4,5)P_3$ responses in BHK-mGluR1α cells. Cells were cultured in the absence (for $Ins(1,4,5)P_3$ measurements) or presence (for [^{3}H]$InsP_1$ measurements) of [^{3}H]inositol (1 μCi/ml) for 48 hr. Where indicated, PTX (100 ng/ml) was present for the final 24-hr period. Monolayers were washed with KHB and (A) incubated in KHB plus 10 mM LiCl for 30 min before the addition of vehicle or 300 μM L-glutamate for 30 min. B, Cells were incubated for 30 min (no LiCl) before the addition of vehicle or 300 μM L-glutamate for 30 sec. C, Concentration-effect curves for L-glutamate-stimulated [^{3}H]$InsP_1$ accumulations in control and PTX-treated cells expressed as fold-increases over respective basal values. All data are shown as mean ± standard error for at least four separate experiments performed in duplicate.

maximally effective concentration of L-glutamate (Fig. 2A). However, in PTX-treated cells, L-glutamate still elicited a significant ($p < 0.01$) additional stimulatory effect, which was markedly diminished when expressed in relative terms (see Fig. 2C).

Despite the marked differences in [^{3}H]$InsP_1$ accumulations, [^{3}H]PtdIns, [^{3}H]PtdIns 4-phosphate, and [^{3}H]PtdIns-4,5-bisphosphate levels were similar in control and PTX-treated cells (data not shown), suggesting that equilibrium-labeled BHK cells retain a large cellular [^{3}H]inositol pool that can maintain the labeling and, hence, the specific activity of the [^{3}H]inositol phospholipids for prolonged periods, even under conditions of maximal agonist-stimulated phosphoinositide turnover.

The increase in basal phosphoinositide turnover caused by PTX pretreatment was also observed at the level of $Ins(1,4,5)P_3$ mass, with PTX-pretreated cells with ~100% higher levels of this second messenger (Fig. 2B). L-Glutamate caused significant increases in $Ins(1,4,5)P_3$ accumulation in both control and PTX-treated cells, although as with [^{3}H]$InsP_1$ accumulation the relative increase was less than that after toxin treatment.

In contrast to the marked basal effects of PTX treatment on BHK-mGluR1α cells, toxin treatment had no significant effect on basal [^{3}H]$InsP_1$ accumulation in BHK-mGluR1β cells (Fig. 3). However, after PTX pretreatment, the [^{3}H]$InsP_1$ response evoked by L-glutamate was significantly increased in BHK-mGluR1β cells. PTX treatment of vector-transfected BHK cells had no effect on basal [^{3}H]$InsP_1$ levels, and neither control nor PTX-treated BHK-570 cells exhibited any response to 1–1000 μM L-glutamate (data not shown).

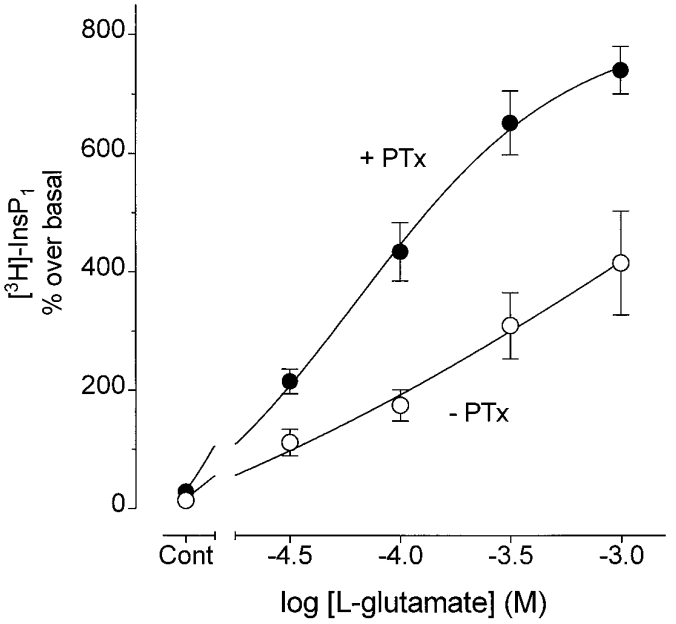

Fig. 3. Effects of PTX treatment on L-glutamate-stimulated [^{3}H]$InsP_1$ accumulation in BHK-mGluR1β cells. BHK-mGluR1β cells were cultured as described in Experimental Procedures. Cells were labeled with [^{3}H]inositol (1 μCi/ml) for 48 hr, and where indicated, PTX (100 ng/ml) was present for the final 24-hr period. [^{3}H]$InsP_1$ accumulations were assessed after incubation in the presence of the indicated concentrations of L-glutamate plus 10 mM LiCl for 30 min. Basal [^{3}H]$InsP_1$ accumulations were control (*Cont*), 389 ± 34; +PTX, 449 ± 41 dpm/well (eight measurements). All data are shown as mean ± standard error for at least four separate experiments performed in duplicate.

Measurement of L-glutamate concentration in the extracellular medium of BHK-570, BHK-mGluR1α, or BHK-mGluR1β cells (taken at a time point coincident with that at which assays to determine [^{3}H]InsP$_1$ accumulations were terminated) revealed in each case similar very low levels of L-glutamate ($\leq$1 μM) for medium taken from either control or PTX-treated cell monolayers.

Effects of PTX and enzymic removal of L-glutamate on agonist-stimulated phosphoinositide responses. Despite the lack of evidence that PTX may result in a change in L-glutamate "handling" in BHK cells, a number of studies have highlighted the problem of glutamate transport and the expression and signaling in cells transfected with cDNA for mGluRs (22, 23). Therefore, we examined the effects of incubation of control and PTX-treated BHK-mGluR1α cell monolayers with high activities of GPT and pyruvate to remove residual extracellular L-glutamate. Although this manipulation had no effect on basal [^{3}H]InsP$_1$ accumulation in control cells, GPT/pyruvate (but not 5 mM pyruvate alone) caused a substantial decrease in PTX-treated cells, such that in the presence of 3 units/ml GPT plus pyruvate, there was a reduction in [^{3}H]InsP$_1$ of >95% (Fig. 4).

These data strongly suggest that the PTX-induced increase in basal [^{3}H]InsP$_1$ is attributable to receptor activation by endogenous L-glutamate. Considering that no difference was found in L-glutamate concentration in incubation medium from control and PTX-treated BHK-mGluR1α cell monolayers, PTX treatment must bring about a radical adaptation by which mGluR1α becomes sensitive to low concentrations ($\leq$1 μM) of L-glutamate. The next series of experiments established that this was the case. As shown in Fig. 5, the concentration dependencies of quisqualate- and 1*S*,3*R*-ACPD-stimulated [^{3}H]InsP$_1$ accumulations differ considerably between control and PTX-treated cells incubated in the presence of 3 units/ml GPT plus 5 mM pyruvate. Thus, for quisqualate, PTX pretreatment significantly increases the maximal response by 75 $\pm$ 9% and dramatically decreases the EC$_{50}$ for quisqualate-stimulated [^{3}H]InsP$_1$ accumulation ~65-fold [$-$log EC$_{50}$: $-$PTX, 5.45 $\pm$ 0.07 (3600 nM); +PTX, 7.26 $\pm$ 0.23 (55 nM) (four experiments)]. Similarly, the maximal [^{3}H]InsP$_1$ response to the partial mGluR1α agonist 1*S*,3*R*-ACPD was increased almost 3-fold and the EC$_{50}$ value decreased almost 10-fold [$-$log EC$_{50}$: $-$PTX, 3.82 $\pm$ 0.42 (152 μM); +PTX, 4.73 $\pm$ 0.27 (19 μM) (four experiments)] in PTX-treated cells.

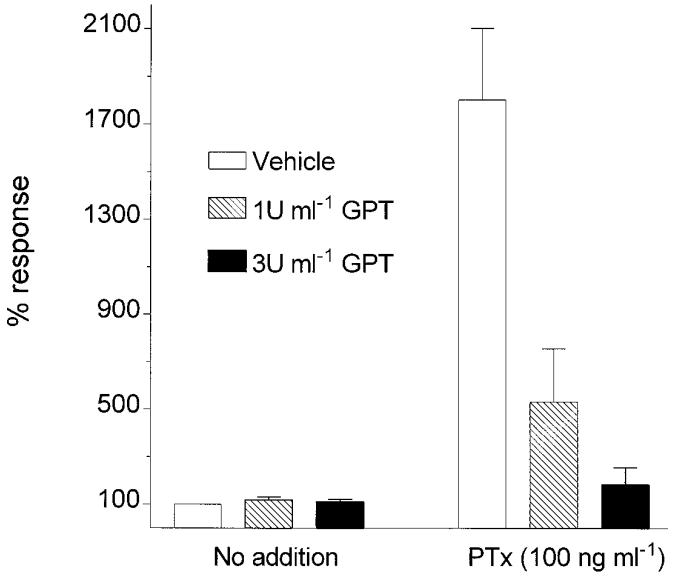

Fig. 4. Effects of enzymic removal of extracellular L-glutamate on basal [^{3}H]InsP$_1$ accumulations in control and PTX-treated BHK-mGluR1α cells. Cells were cultured in the presence of [^{3}H]inositol (1 μCi/ml) for 48 hr, and where indicated, PTX (100 ng/ml) was present for the final 24-hr period. Monolayers were washed with KHB and incubated in KHB in the absence or presence of GPT plus 5 mM pyruvate for 30 min. Then, 10 mM LiCl was then added for an additional 30-min period before acid termination. Data are expressed relative to [^{3}H]InsP$_1$ accumulation in control cells in the absence of GPT/pyruvate and presented as mean $\pm$ standard error for four separate experiments performed in duplicate.

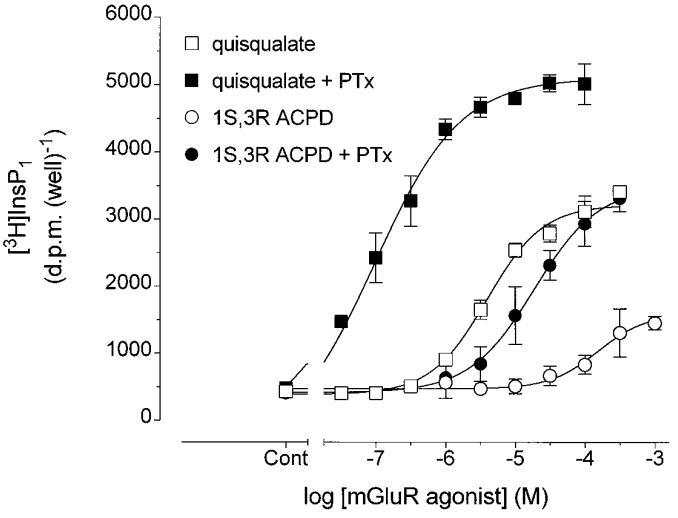

Fig. 5. Concentration-dependent effects of quisqualate and 1*S*,3*R*-ACPD on [^{3}H]InsP$_1$ accumulations in control and PTX-treated BHK-mGluR1α cells in the presence of GPT/pyruvate. Cells were cultured in the presence of [^{3}H]inositol (1 μCi/ml) for 48 hr, and where indicated, PTX (100 ng/ml) was present for the final 24-hr period. Cell monolayers were washed with KHB and incubated in KHB containing GPT (3 units/ml) plus 5 mM pyruvate for 30 min with 10 mM LiCl present for the last 15 min of this preincubation period. The indicated concentrations of quisqualate and 1*S*,3*R*-ACPD were then added to control or PTX-treated cells, and incubations were continued for an additional 30-min period before acid termination. All data are shown as mean $\pm$ standard error for at least four separate experiments performed in duplicate.

Because no endogenous G protein-coupled receptors could be demonstrated in these cells, the effect of PTX pretreatment on receptor-independent mechanisms of stimulating phosphoinositide turnover was also assessed. The nonselective G protein activator AlF_4^- stimulated a modest 2–3-fold increase in [^{3}H]InsP$_1$ accumulation in BHK-mGluR1α cells, and this was significantly enhanced by PTX (Table 2). Similarly, the Ca^{2+}-ionophore ionomycin stimulated a modest increase in [^{3}H]InsP$_1$ accumulation, which was also enhanced after PTX treatment, although in this case the effect did not reach statistical significance (Table 2).

mGluR1α expression levels in control and PTX-treated BHK cells. Western blotting revealed that PTX pretreatment of BHK-mGluR1α cells had no discernible effect on levels of receptor expression (Fig. 6). Thus, the anti-mGluR1α antiserum identified similar levels of a protein of ~150 kDa in both control and PTX-treated BHK cells. In agreement with a previous report (24), additional immunoreactive material ran toward the top of the gel (Fig. 6). The identity of this band is unknown, although it has been pro-

TABLE 2

Effects of PTX pretreatment on [^{3}H]InsP$_1$ accumulations stimulated by quisqualate, AlF_4^-, or ionomycin in BHK-mGluR1α cells

BHK-mGluR1α cell monolayers were labeled with *myo*-[^{3}H]inositol (1 μCi/ml) for 48 hr and exposed to PTX (100 ng/ml) or vehicle for the last 24 hr of the labeling period. Incubations were performed in the presence of GPT (3 units/ml) plus pyruvate (5 mM) as described in Experimental Procedures. After stimulation with NaF (50 mM) plus $AlCl_3$ (10 μM), ionomycin (5 μM), quisqualate (30 μM), or an appropriate vehicle for 30-min incubations was terminated, and [^{3}H]InsP$_1$ was extracted and separated as described in Experimental Procedures. Values are presented as mean ± standard error for three separate experiments performed in duplicate.

Stimulus	Control cells	PTX-treated cells
	dpm/well	
No addition	821 ± 142	942 ± 78
NaF/$AlCl_3$	1932 ± 71	2495 ± 174[a]
No addition	612 ± 34	800 ± 151
Ionomycin	1846 ± 110	2706 ± 436
Quisqualate	3797 ± 333	7567 ± 764[b]

[a] $p < 0.05$.
[b] $p < 0.01$.

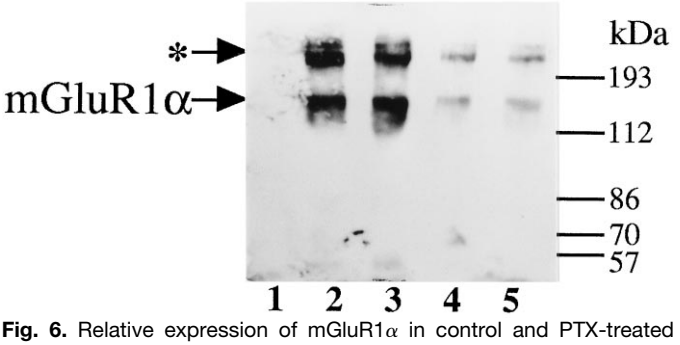

Fig. 6. Relative expression of mGluR1α in control and PTX-treated BHK cells. Cells were cultured in the absence or presence of PTX (100 ng/ml) for 24 hr before the preparation of membranes as described in Experimental Procedures. Representative immunoblot for cell membranes prepared from BHK-mGluR1α (*lanes 2–5*) or untransfected (*BHK570*; *lane 1*) cells using an anti-mGluR1α antiserum. *Lanes 3* and *5*, from BHK-mGluR1α membranes prepared from cells exposed to PTX. *Lanes 1, 2,* and *4*, from vehicle-treated control cells. Membranes were loaded with 10 μg (*lanes 1–3*) or 3 μg (*lanes 4* and *5*) of membrane protein/ml.

posed to represent an aggregated form of the mGluR1α receptor (24).

Lack of effect of PTX on antagonist action at mGluR1α. The inhibitory effects of 4C3HPG (300 μM) on quisqualate-stimulated [^{3}H]InsP$_1$ accumulations in control and PTX-treated BHK-mGluR1α cells incubated in the presence of GPT/pyruvate are shown in Fig. 7. The presence of the mGluR antagonist caused similar apparently parallel rightward shifts in the concentration-effect curves for quisqualate in both control cells [−log EC_{50}: control, 5.44 ± 0.07; +4C3HPG, 4.46 ± 0.09; dose-ratio, 9.6 ± 1.0 (three experiments)] and PTX-treated cells [−log EC_{50}: +PTX, 6.81 ± 0.16; +PTX + 4C3HPG, 5.73 ± 0.13; dose-ratio, 12.5 ± 1.9 (four experiments)] (Fig. 7). Using a rearrangement of the Gaddum equation (K_d = A/(DR − 1), where K_d is the antagonist equilibrium dissociation constant, A is the concentration of antagonist used, and DR is the dose-ratio), we calculated K_d values for 4C3HPG of 35 and 26 μM in control and PTX-treated BHK-mGluR1α cells, respectively.

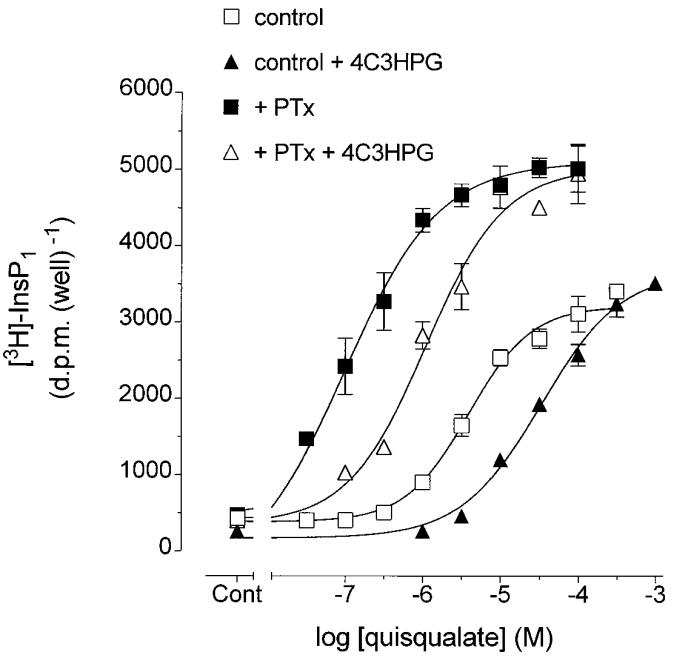

Fig. 7. Antagonism of quisqualate-stimulated [^{3}H]InsP$_1$ accumulations in control and PTX-treated BHK-mGluR1α cells by 4C3HPG. Cells were cultured in the presence of [^{3}H]inositol (1 μCi/ml) for 48 hr, and where indicated, PTX (100 ng/ml) was present for the final 24-hr period. Monolayers were washed with KHB and incubated in KHB containing GPT (3 units/ml) plus 5 mM pyruvate and 10 mM LiCl for 30 min; where indicated, 4C3HPG (300 μM) was present for the last 15 min of this preincubation and throughout the period of stimulation. The indicated concentrations of quisqualate were then added to control or PTX-treated cells, and incubations were continued for an additional 30-min period before acid termination. Data are shown as mean ± standard error for at three (*control*) or four (+*PTX*) separate experiments performed in duplicate.

Discussion

In agreement with reports in BHK (15, 16) and other (13, 22) cell types transfected to express mGluR1α, we have shown that PIC activity is markedly stimulated by the addition of L-glutamate, quisqualate, and, to a lesser extent, 1*S*,3*R*-ACPD. Our data, which assess [^{3}H]InsP$_1$ accumulation in the presence of Li^+ as an index of PIC activation, also confirm the agonist potency ranking order of quisqualate > L-glutamate > 1*S*,3*R*-ACPD (with the latter agent a partial agonist with respect to this response) reported by others. Assessment of concentration-effect curves for agonist-stimulated increases in Ins(1,4,5)P_3 mass yielded EC_{50} values for quisqualate- and L-glutamate-stimulated responses (assessed at 30 sec) that were similar to those obtained for [^{3}H]InsP$_1$ accumulation (assessed at 30 min); however, the partial agonist 1*S*,3*R*-ACPD (even at 1 mM) failed to significantly increase Ins(1,4,5)P_3 accumulation. It should be emphasized that unlike [^{3}H]InsP$_1$ accumulation in the presence of Li^+, Ins(1,4,5)P_3 mass accumulation is a more dynamic measurement that reflects the relative rates of synthesis and breakdown of this messenger; presumably the reduced ability of 1*S*,3*R*-ACPD to stimulate PIC activity via mGluR1α is insufficient to result in a detectable Ins(1,4,5)P_3 accumulation, despite an increased flux through this intermediate (25).

A recent report demonstrated apparent agonist-independent ("constitutive") activity of mGluR1α transiently expressed in either LLC-PK1 or HEK 293 cells (21). In agreement with these data, we have shown here that basal

[^{3}H]InsP$_1$ accumulation is increased (by ~50%) in BHK-mGluR1α cells compared with vector-transfected cells, and basal phosphoinositide hydrolysis rates are not significantly suppressed by the addition of an enzyme/cosubstrate (GPT/pyruvate) system for removing extracellular endogenous glutamate or an mGluR antagonist. To further explore the properties of this apparent agonist-independent activity, we choose to selectively manipulate the G protein populations present in BHK cells by using PTX.

The mGluR family members differ in a number of important respects from other members of the G protein-coupled receptor superfamily. Thus, the ligand binding domain (26, 27) and the intracellular domains of the receptor that control the specificity of linkage to G proteins (28, 29) of mGluRs have little in common with other G protein-coupled receptors. Similarly, in contrast to many receptors that link to β-isozymes of PIC, the type 1 and 5 mGluRs and their splice variants exhibit varying susceptibilities to inhibition of effector coupling by PTX, suggesting that PIC activation is mediated by both $G_{q/11}$- and $G_{i/o}$-type G proteins (13–18). Thus, mGluR1α-mediated activation of phosphoinositide hydrolysis has been reported to be inhibited by 40–80% after PTX pretreatment (13, 15, 16), suggesting that ADP-ribosylation and inactivation of $G_{i/o}$ severely compromise the effector coupling of this splice variant. In the current study, we provided a radically contrasting picture of PTX effects on mGluR1α-mediated phosphoinositide signaling by presenting evidence demonstrating that there is a marked increase in the ability of mGluR agonists to activate PIC after $G_{i/o}$ inactivation in BHK-mGluR1α cells. One possible reason for the disparity between our data and those reported previously (13, 15, 16) may relate to the fact that the earlier studies reported only the effects of agonists as a "fold-over-basal" response, and therefore the major action of PTX on "basal" values may have been overlooked in these studies.

Initial experiments demonstrated that 24-hr pretreatment with 1–100 ng/ml PTX caused a dramatic dose-related increase in [^{3}H]InsP$_1$ accumulation in the absence of added agonist and at a maximally effective dose of toxin was 12–16-fold greater than in control cells. The facts that similar, very low levels of glutamate ($\leq$1 μM) were observed in incubation medium taken from control and PTX-treated cell monolayers and that exogenous L-glutamate stimulated a further increase in [^{3}H]InsP$_1$ accumulation initially suggested that PTX was causing a dramatic unmasking of agonist-independent (constitutive) mGluR1α activity. Despite such evidence, previous work by other groups (22), including studies performed in BHK cells (23), has highlighted the problems that can be associated with systems in which endogenous L-glutamate can contribute to, or account for, receptor activation. Thus, Desai *et al.* (22) reported that mGluR agonists stimulate a much more dramatic increase in phosphoinositide hydrolysis in AV12 cells transfected to express the human mGluR1α in the presence compared with the absence of the cotransfected Na^+-dependent glutamate transporter GLAST (30). Under the assay conditions described by Desai *et al.* (22), extracellular L-glutamate concentration was much greater ($\geq$30 μM) if cells were not engineered to express GLAST, leading to tonic receptor activation and desensitization. However, in the current study, much lower levels of L-glutamate ($\leq$1 μM) were detected, suggesting that BHK cells may differ from AV12 cells in their handling of transplasmalemmal glutamate movements or may already possess a glutamate transporter favoring accumulation from the extracellular medium (31). However, more subtle effects can also occur. Thomsen *et al.* (23) reported that agents that do not directly interact with mGluR1α can, nevertheless, activate phosphoinositide hydrolysis by stimulating heteroexchange mediated by the endogenous glutamate transporter and having the net effect of causing L-glutamate release into the extracellular space sufficient to activate mGluR1α.

In view of this evidence, we carried out further experiments using the enzyme/cosubstrate addition (GPT + 5 mM pyruvate) used by others to reduce extracellular glutamate concentrations (32). Although GPT/pyruvate had no effect on [^{3}H]InsP$_1$ responses in control cells, the elevated basal phosphoinositide hydrolysis in PTX-treated cells was dramatically and fully attenuated under these conditions. In addition, further analysis of the concentration-dependencies of quisqualate- and 1*S*,3*R*-ACPD-stimulated [^{3}H]InsP$_1$ accumulation revealed that in the presence of GPT/pyruvate, PTX pretreatment increased the maximal response elicited by the full agonist quisqualate and dramatically reduced the EC_{50} value (~65-fold) compared with control cells. The partial agonist 1*S*,3*R*-ACPD stimulated a [^{3}H]InsP$_1$ response that was similarly affected with a 2–3-fold increase in the maximal response and a 10-fold leftward shift in EC_{50} value. These dramatic changes in the stimulation of phosphoinositide responses seem to occur without any detectable PTX-induced changes in BHK cell mGluR1α expression assessed by immunoblotting.

The sensitization and increased responsiveness of the phosphoinositide response to mGluR agonists after PTX treatment fully account for the original observations of dramatic elevations in basal [^{3}H]InsP$_1$ accumulation. Thus, although a medium L-glutamate concentration of ~1 μM may have no significant stimulatory effect in control cells, it is sufficient to substantially stimulate phosphoinositide turnover after "sensitization" by PTX treatment. The effect of PTX seems to be much more dramatic in BHK cells expressing the mGluR1α splice variant; although the maximal agonist-stimulated response seen in mGluR1β-expressing BHK cells is increased, a sensitization of the signaling pathway to agonist is not evident. Although further studies are required, these data implicate the carboxyl terminus of mGluR1, in which differential splicing of this receptor occurs (3), as an important domain in the interaction with PTX-sensitive G protein or proteins. Our attempts to examine the effects of PTX on receptor-independent activations of PIC revealed a small enhancement of enzyme activity by AlF_4^--mediated G protein activation in PTX-treated cells, which would be consistent with a dual regulation of this effector by $G_{q/11}$ and $G_{i/o}$ proteins. However, the small responses obtained with AlF_4^- and the lack of knowledge of the relative activation of the different G proteins by this agent preclude confident interpretation.

Our observations strongly suggest that the ability of activated mGluR1α to link to PIC activation (probably via $G_{q/11}$) is negatively modulated by $G_{i/o}$ proteins and that ADP-ribosylation by PTX neutralizes this inhibitory influence (Fig. 8). Consistent with this model is very recent work from this laboratory (33) that provides evidence that glutamate-stimulated [^{35}S]GTPγS binding in BHK-mGluR1α cell mem-

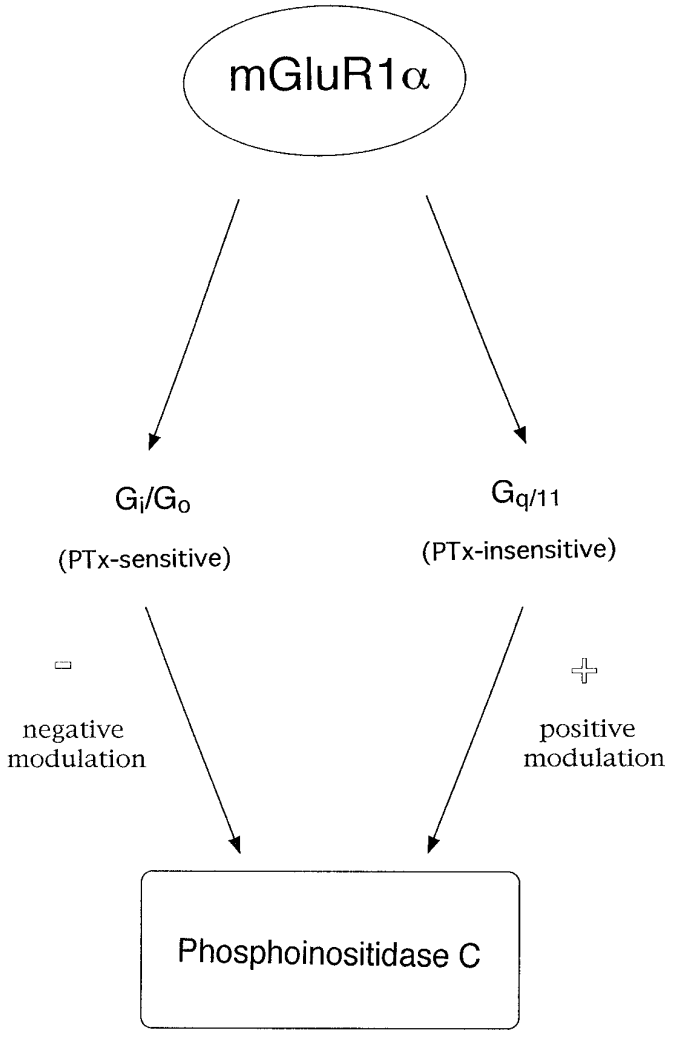

Fig. 8. Possible G protein-mediated dual regulation of PIC activity by mGluR1α in BHK cells and the effect of PTX treatment.

branes involves both PTX-sensitive and -insensitive G proteins. Furthermore, it is interesting to note that the maximum extent of mGluR1α-mediated G protein activation by glutamate is modest (300–400 fmol/mg of protein), suggesting that cell-surface mGluR1α capable of interacting with G proteins are not dramatically overexpressed in this model cell system (33).

Inhibitory effects of PTX-sensitive G proteins on PIC activity have been suspected for some time (34), although whether such modulations occur via direct inhibition of PIC, through activation of a distinct signal transduction pathway, or at a noneffector site in the receptor-effector coupling pathway has often been difficult to establish (34–36). However, a number of studies have reported direct inhibitory $G_{i/o}$ effects on PIC activity in intact cells (37, 38) and permeabilized cell and membrane preparations (37, 39). Of particular interest with respect to the current study is the finding of Watkins *et al.* (40) that $G_{i2\alpha}$ expression can negatively modulate agonist-stimulated PIC activity. They reported that in mouse F9 tetracarcinoma and rat osteosarcoma cells, down-regulation of $G_{i2\alpha}$ by transfection with RNA antisense to this protein or overexpression of a constitutively active Q205L/$G_{i2\alpha}$ caused increases and decreases, respectively, in basal phosphoinositide turnover and potentiated or abolished the Ins(1,4,5)P_3 response to thrombin and an α_1-adrenoceptor agonist (40). Contrary to initial indications, our data provide no evidence for an enhancement of basal phosphoinositide hydrolysis by PTX *per se*, but the current data are consistent with those of Watkins *et al.* (40) with respect to the effect of PTX to enhance agonist-stimulated phosphoinositide responses.

In conclusion ,the present data provide strong evidence that ADP-ribosylation and inactivation of $G_{i/o}$ proteins by PTX result in a dramatic enhancement of mGluR1α-PIC signaling via $G_{q/11}$. It will be important to establish whether this phenomenon occurs for mGluR1α in other cell types, whether it depends on receptor expression levels, and whether it is specific to this subtype. Overall, however, these observations are consistent with a dual regulation of receptor-mediated PIC activity that could be fundamental in controlling the output of phosphoinositide-derived messengers.

Acknowledgments

We thank Dr. D. W. Gray for generating the mGluR1α antiserum used in this study and Dr. J. J. Mackrill for expert assistance with the resolution and identification of mGluR1α using the antiserum.

References

1. Tanabe, Y., M. Masu, T. Ishii, R. Shigemoto, and S. Nakanishi. A family of metabotropic glutamate receptors. *Neuron* **8:**169–179 (1992).
2. Nakanishi, S. Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. *Neuron* **13:**1031–1037 (1994).
3. Pin, J.-P., and R. Duvoisin. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34:**1–26 (1995).
4. Birse, E. F., S. A. Eaton, D. E. Jane, P. L. St. J. Jones, R. H. P. Porter, P. C.-K. Pook, D. C. Sunter, P. M. Udvarhelyi, B. Wharton, P. J. Roberts, T. E. Salt, and J. C. Watkins. Phenylglycine derivatives as new pharmacological tools for investigating the role of metabotropic glutamate receptors in the central nervous system. *Neuroscience* **52:**481–488 (1993).
5. Thomsen, C., E. Boel, and P. D. Suzdak. Actions of phenylglycine analogs at subtypes of the metabotropic glutamate receptor family. *Eur. J. Pharmacol.* **267:**77–84 (1994).
6. Hayashi, Y., N. Sekiyama, S. Nakanishi, D. E. Jane, D. C. Sunter, E. F. Birse, P. M. Udvarhelyi, and J. C. Watkins. Analysis of agonist and antagonist activities of phenylglycine derivatives for different cloned metabotropic glutamate receptor subtypes. *J. Neurosci.* **14:**3370–3377 (1994).
7. Roberts, P. J. Pharmacological tools for the investigation of metabotropic glutamate receptors: phenylglycine derivatives and other selective antagonists: an update. *Neuropharmacology* **34:**813–819 (1995).
8. Bashir, Z. I., Z. A. Bortolotto, C. H. Davies, N. Berretta, A. J. Irving, A. J. Seal, J. M. Henley, D. E. Jane, J. C. Watkins, and G. L. Collingridge. Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature (Lond.)* **363:**347–350 (1993).
9. Chinestra, P., L. Aniksztejn, D. Diabira, and Y. Ben-Ari. (*RS*)-α-Methyl-4-carboxyphenylglycine neither prevents induction nor antagonizes metabotropic glutamate receptors in CA1 hippocampal neurons. *J. Neurophysiol.* **70:**2684–2689 (1993).
10. Manzoni, O. J., M. G. Weisskopf, and R. A. Nicoll. MCPG antagonizes metabotropic glutamate receptors but not long-term potentiation in the hippocampus. *Eur. J. Neurosci.* **6:**1050–1054 (1994).
11. Thomsen, C., H. Klitgaard, M. Sheardown, H. C. Jackson, K. Eskesen, P. Jacobsen, S. Treppendahl, and P. D. Suzdak. (*S*)-4-Carboxy-3-hydroxyphenylglycine, an antagonist of metabotropic glutamate receptor (mGluR) 1α and an agonist of mGluR2, protects against audiogenic seizures in DBA/2 mice. *J. Neurochem.* **62:**2492–2495 (1994).
12. Riedel, G., and K. G. Reymann. Metabotropic glutamate receptors in hippocampal long-term potentiation and learning and memory. *Acta Physiol. Scand.* **157:**1–19 (1996).
13. Aramori, I., and S. Nakanishi. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* **8:**757–765 (1992).
14. Abe, T., H. Sugihara, H. Nawa, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca^{2+} signal transduction. *J. Biol. Chem.* **267:**13361–13368 (1992).
15. Pickering, D. S., C Thomsen, P. D. Suzdak, E. J. Fletcher, R. Robitaille, M. W. Salter, J. F. MacDonald, X.-P. Huang, and D. R. Hampson. A comparison of two alternatively spliced forms of a metabotropic glutamate

receptor coupled to phosphoinositide turnover. *J. Neurochem.* **61:**85–92 (1993).
16. Thomsen, C., E. R. Mulvihill, B. Haldeman, Pickering, D. S., D. R. Hampson, and P. D. Suzdak. A pharmacological characterization of the mGluR1α subtype of the metabotropic glutamate receptor expressed in a cloned baby hamster kidney cell line. *Brain Res.* **619:**22–28 (1993).
17. Joly, C., J. Gomeza, I. Brabet, K. Curry, J. Bockaert, and J.-P. Pin. Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. *J. Neurosci.* **15:**3970–3981 (1995).
18. Kasahara, J., and H. Sugiyama. Inositol phospholipid metabolism in *Xenopus* oocytes mediated by endogenous G_o and G_i proteins. *FEBS Lett.* **355:**41–44 (1994).
19. Challiss, R. A. J., S. Jenkinson, R. Mistry, I. H. Batty, and S. R. Nahorski. Assessment of neuronal phosphoinositide turnover and its disruption by lithium. *Neuroprotocols* **3:**135–144 (1993).
20. Martin, L. J., C. D. Blackstone, R. L. Huganir, and D. L. Price. Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron* **9:**259–270 (1992).
21. Prézeau, L., J. Gomeza, S. Ahern, S. Mary, T. Galvez, J. Bockaert, and J.-P. Pin. Changes in the carboxyl-terminal domain of metabotropic glutamate receptor 1 by alternative splicing generate receptors with differing agonist-independent activity. *Mol. Pharmacol.* **49:**422–429 (1996).
22. Desai, M., J. P. Burnett, N. G. Mayne, and D. D. Schoepp. Cloning and expression of a human metabotropic glutamate receptor 1α: enhanced coupling on cotransfection with a glutamate transporter. *Mol. Pharmacol.* **48:**648–657 (1995).
23. Thomsen, C., L. Hansen, and P. D. Suzdak. L-Glutamate uptake inhibitors may stimulate phosphoinositide hydrolysis in baby hamster kidney cells expressing mGluR1α via heteroexchange with L-glutamate without direct activation of mGluR1α. *J. Neurochem.* **63:**2038–2047 (1994).
24. Alaluf, S., E. R. Mulvihill, and R. A. J. McIlhinney. Rapid agonist mediated phosphorylation of the metabotropic glutamate receptor 1α by protein kinase C in permanently transfected BHK cells. *FEBS Lett.* **367:**301–305 (1995).
25. Mistry, R., and R. A. J. Challiss. Differences in agonist and antagonist activities for two indices of metabotropic glutamate receptor-stimulated phosphoinositide turnover. *Br. J. Pharmacol.* **117:**1735–1743 (1996).
26. O'Hara, P. J., P. O. Sheppard, H. Thøgersen, D. Venezia, B. A. Haldeman, V. McGrane, K. M. Houamed, C. Thomsen, T. L. Gilbert, and E. R. Mulvihill. The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11:**41–52 (1993).
27. Takahashi, K., K. Tsuchida, Y. Tanabe, M. Masu, and S. Nakanishi. Role of the large extracellular domain of metabotropic glutamate receptors in agonist selectivity determination. *J. Biol. Chem.* **268:**19341–19345 (1993).
28. Pin, J.-P., C. Joly, S. F. Heinemann, and J. Bockaert. Domains involved in the specificity of G protein activation in phospholipase C-coupled metabotropic glutamate receptors. *EMBO J.* **13:**342–348 (1994).
29. Gomeza, J., C. Joly, R. Kuhn, T. Knöpfel, J. Bockaert, and J.-P. Pin. The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G proteins. *J. Biol. Chem.* **271:**2199–2205 (1996).
30. Storck, T., S. Schulte, K. Hofmann, and W. Stoffel. Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. *Proc. Natl. Acad. Sci. USA* **89:**10955–10959 (1992).
31. Scott, D. M., and J. A. Pateman. The acidic amino acid transport system of the baby hamster kidney cell line BHK21–C13. *Biochim. Biophys. Acta* **508:**379–388 (1978).
32. Aronica, E., P. Dell'albani, D. F. Condorelli, F. Nicoletti, N. Hack, and R. Balázs. Mechanisms underlying developmental changes in the expression of metabotropic glutamate receptors in cultured cerebellar granule cells: homologous desensitization and interactive effects involving *N*-methyl-D-aspartate receptors. *Mol. Pharmacol.* **44:**981–989 (1993).
33. Akam, E. C., A. M. Carruthers, S. R. Nahorski, and R. A. J. Challiss. Pharmacological characterization of type 1α metabotropic glutamate receptor-stimulated [^{35}S]-GTPγS binding. *Br. J. Pharmacol.* **121:**1203–1209 (1997).
34. Linden, J., and T. M. Delahunty. Receptors that inhibit phosphoinositide breakdown. *Trends Pharmacol. Sci.* **10:**114–120 (1989).
35. Enjalbert, A., G. Guillon, B. Mouillac, V. Audinot, R. Rasolonjanahary, C. Kordon, and J. Bockaert. Dual mechanisms of inhibition by dopamine of basal and thyrotropin-releasing hormone-stimulated inositol phosphate production in anterior pituitary cells: evidence for an inhibition not mediated by voltage-dependent Ca^{2+}-channels. *J. Biol. Chem.* **265:**18816–18822 (1990).
36. Martin, M., J. M. Sanz, M. Ros, and A. Cubero. Metabotropic glutamate receptor analogues inhibit p[NH]ppG-stimulated phospholipase C activity in bovine brain coated vesicles: involvement of a pertussis toxin-sensitive G-protein. *Biochem. J.* **307:**851–857 (1995).
37. Bizzarri, C., M. Di Girolamo, M. C. D'Orazio, and D. Corda. Evidence that a guanine nucleotide-binding protein linked to a muscarinic receptor inhibits directly phospholipase C. *Proc. Natl. Acad. Sci. USA* **87:**4889–4893 (1990).
38. Nakamura, K., T. Nukada, T. Haga, and H. Sugiyama. G protein-mediated inhibition of phosphoinositide metabolism evoked by metabotropic glutamate receptors in frog oocytes. *J. Physiol. (Lond.)* **474:**35–41 (1994).
39. Litosch, I., I. Sulkholutskaya, and C. Weng. G protein-mediated inhibition of phospholipase C activity in a solubilized membrane preparation. *J. Biol. Chem.* **268:**8692–8697 (1993).
40. Watkins, D. C., C. M. Moxham, A. J. Morris, and C. C. Malbon. Suppression of $G_{i\alpha 2}$ enhances phospholipase C signalling. *Biochem. J.* **299:**593–596 (1994).

Send reprint requests to: Dr. R. A. J. Challiss, Department of Cell Physiology & Pharmacology, University of Leicester, P.O. Box 138, Maurice Shock Medical Sciences Building, University Road, Leicester LE1 9HN, United Kingdom. E-mail: jc36@le.ac.uk