Functional regulation of metabotropic glutamate receptor type 1c: a role for phosphorylation in the desensitization of the receptor

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Abstract The phosphorylation and desensitization of metabotropic glutamate receptor type 1c in response to agonist and phorbol esters has been studied. Specific immunoprecipitation of mGluR1c from cells treated with agonist or PMA showed a time-dependent increase in the phosphorylation of a membrane protein with the same molecular weight as the dimeric form of the receptor. Measurements of inositol phosphate production showed a rapid functional desensitization of about 90% after agonist treatment, whereas treatment with PMA caused only a 30% loss in the same time. The extent of receptor phosphorylation following the different treatments paralleled the desensitization of the receptor. These results strongly suggest that phosphorylation of the dimeric form of mGluR1c, as a functionally active form, may play a role in its rapid desensitization.

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Key words: mGluR1c; Phosphorylation; Desensitization; Cell surface expression; Metabotropic; Dimer

1. Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian brain and acts at multiple receptor types. These receptors can be divided into two groups namely the ionotropic receptors, which form ion channels, and metabotropic receptors coupled by G-proteins to various second messengers systems [1]. Eight members of the mGluRs family have been identified and categorized into three subgroups on the basis of their sequence homology, agonist selectivity and signal transduction pathway. Group I contains mGluRs 1 and 5, which are coupled to phospholipase C in transfected cells, and quisqualic acid (Quis) is their most potent agonist. Five splice variants of mGluR1 have been described, namely metabotropic glutamate receptor (mGluR)1α, mGluR1β, mGluR1c, mGluR1d and mGluR1e [1,2], all of them differing in the length of the C terminal tail.

G-protein-coupled receptors (GPCRs) undergo homologous [3] and heterologous desensitization [4]. In the former the receptor loses its responsiveness to a subsequent stimulation after activation by its agonist, in the latter responsiveness is lost after activation of a different receptor or cell signalling pathway. Receptor desensitization is a complex process which can involve phosphorylation of the molecule, its uncoupling from G-proteins, its internalization/sequestration, and ultimately its down-regulation. In the well-characterized β_2-adrenergic system, desensitization involves phosphorylation of some serine-threonine residues of the receptor by two different mechanisms. On the basis of these studies, it has been suggested that after a short period of agonist exposure, the receptors uncouple from G proteins due to phosphorylation catalyzed by receptor-specific kinases (e.g. β-adrenergic receptor-associated kinase-1 and -2) and/or kinases regulated by second messengers (e.g. PKA and PKC). Phosphorylation of the receptor by kinases increases the affinity of the receptor for cytosolic factors (arrestins and adapters) that impairs the ability of the receptor to interact with G proteins [5].

In this study we have examined the phosphorylation and desensitization of mGluR1c in response to Quis and PMA treatment, using a permanently transfected CHO-K1 cell line. Phosphorylation was measured using specific monoclonal antibodies against phospho-serine and by $^{32}P_i$ metabolic labelling of CHO-K1 cells, as described previously [6]. The time course desensitization was determined by agonist inositol phosphate generation after preincubation with Quis or PMA. Together, these approaches have allowed us to delineate the kinetics of regulation of mGluR1c permanently expressed in Chinese hamster ovary cells.

2. Materials and methods

2.1. Antibodies

The F1 serum was raised against a histidine tagged fusion protein containing an amino terminal sequence of mGluR1, residues 121–341 [7]. Purified anti-phosphoserine (clone 1C8) monoclonal antibody was purchased from BIOMOL (Hamburg, Germany) and used as described before [6]. Monoclonal anti-β-tubulin clone TUB 2.1 was obtained from Sigma. Fluorescein (FITC)-conjugate affinity purified donkey anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Horseradish-peroxidase(HRP)-conjugate swine anti-rabbit IgG and HRP-conjugate swine anti-mouse IgG were from Dako. Horseradish-peroxidase(HRP)-conjugate rabbit anti-mouse IgM was from Sigma.

2.2. Cell culture, membrane preparation and protein determination

CHO-K1 cells were transfected with an in-house expression vector encoding the rat mGluR1c and the resistance marker aminoglycoside phosphotransferase (neo[r]) under control of the single CMV promoter. The continued presence of Geneticin G-418 in the culture medium was essential to select and to maintain the positive clones. CHO-K1 permanently transfected with mGluR1c were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 7.5% decomplemented dialyzed fetal bovine serum, 1 mM glutamine, 0.3 mM proline, 4.5 g/l glucose and 500 µg/ml Geneticin G-418.

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Before incubation with any drug, cells were grown in glutamate-free medium (ICN) in the absence of both glutamine and glutamic acid for 3 h.

Membranes of cells were prepared by shearing them in 10 mM $NaHCO_3$ as previously described [7] and protein was measured by the bicinchoninic acid method.

2.3. SDS/PAGE, immunoblotting and immunoprecipitation

Cell membranes were treated with SDS-PAGE sample buffer [7], before loading onto the polyacrylamide gels. Electrophoresis was performed using 6% polyacrylamide gels [8]. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore), using a semi-dry transfer system buffer [7] and immunoblotted using anti-mGluR1 affinity purified F1 antibody (F1-Ab; 2–4 μg/ml) [9]. For immunoblotting with anti-phosphoserine antibody clone 1C8 cells were first immunoprecipitated with F1-Ab and then immunoblotted as described previously [6]. Immunoreactive bands were detected with HRP-conjugated anti-rabbit (1:5000), anti-mouse (1:5000) and/or anti-mouse IgM (1:2000) followed by chemiluminescence detection (Pierce).

2.4. Biotinylation of cell surface proteins

Biotinylation was performed as described previously [10] using 50 μg/ml sulfo-NHS-LC-biotin (Pierce) in borate buffer for 5 min at room temperature. Biotinylated cell surface proteins were isolated using Streptavidin-agarose beads and immunoblotted as described above.

2.5. mGluR1c phosphorylation

Whole cell phosphorylation studies were carried out in a manner similar to that described previously [6,11]. Briefly, CHO-K1 cells permanently transfected with mGluR1c were incubated with [^{32}P]orthophosphate (1 mCi/ml) for 4 h at 37°C in 5% CO_2 in phosphate/glutamine-free medium. The cells were then incubated for the indicated times with the medium alone or the medium plus the indicated concentrations of PMA or the agonist Quis, washed three times with ice-cold phosphate-buffered saline (PBS), and scraped in ice-cold lysis buffer for 1 h on ice. The solubilized preparation was then centrifuged at $80\,000 \times g$ for 90 min. The supernatant was processed for immunoprecipitation as described above. Immune complexes were dissociated by heating to 100°C for 5 min and resolved by SDS-polyacrylamide gel electrophoresis in 6% gels. The gels were run, dried and autoradiographed.

2.6. Inositol phosphate (IP) accumulation

CHO-K1 cells permanently transfected with mGluR1c were grown (overnight) in glutamine/inositol-free medium (DMEM supplemented with 7.5% fetal bovine serum, dialyzed against inositol-free DMEM) containing myo-[^{3}H]inositol (5 μCi/ml; Amersham, UK). Washed cells, subsequently incubated with the indicated reagents, were collected, washed exhaustively in pre-warmed (37°C) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered Kreb's solution (20 mM HEPES, pH 7.4; 145 mM NaCl; 5 mM KCl; 1.2 mM $CaCl_2$; 1.3 mM $MgCl_2$; 1.2 mM NaH_2PO_4 and 10 mM glucose) and incubated (10^6/0.2 ml of PBS) containing 10 mM LiCl for 10 min. Phosphoinositide hydrolysis was initiated by the addition of 0.1 ml of pre-warmed HEPES/Kreb's containing the indicated Quis concentration. The total IP pool was measured as described previously [9]. The EC_{50} of agonist induced IP accumulation was determined by non-linear regression as described previously [6].

3. Results

In a solubilized fraction prepared from a CHO-K1 cell line permanently transfected with mGluR1c, the F1 antibody [7] consistently immunoblotted two major immunoreactive proteins of apparent molecular weight of 94 kDa and 190 kDa (Fig. 1, lysate). The lower of these molecular weights corresponds to that expected for the receptor and whilst the higher molecular weight is approximately twice that of the lower, suggesting the existence of an SDS-resistant dimeric species of the receptor. These results are in good agreement with
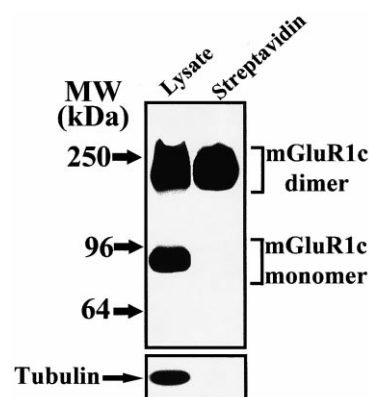

Fig. 1. Cell surface biotinylation of mGluR1c permanently expressed in CHO-K1 cells. CHO-K1 cells permanently transfected with mGluR1c were cell surface biotinylated by incubation with 50 μg/ml of sulfo-NHS-LC-biotin in borate buffer as described in Section 2. The labelled proteins were isolated with streptavidin-agarose beads and complexes were applied to 6% SDS-PAGE gels (streptavidin lane) to be analyzed by immunoblotting using a polyclonal anti-mGluR1 affinity purified F1 antibody (2–4 μg/ml) or a monoclonal anti-β-tubulin (1:200) (inset panel). A portion of solubilized extract was analyzed by SDS-PAGE and immunoblotted under the same conditions (lysate lane). Immunoreactive bands were detected with a swine anti-rabbit (1:5000) or a swine anti-mouse (1:5000) secondary antibody conjugated to horseradish peroxidase followed by chemiluminescence detection.

previously reported studies using transiently transfected HEK cells [12] and with the results obtained with other antibodies to these receptors [7,11]. In order to differentiate which form of the receptor was present in the plasma membrane, we performed cell surface protein biotinylation and subsequent streptavidin isolation (Fig. 1) of the biotinylated cell surface proteins. Under these conditions, the dimeric form of the receptor was the dominant form found in the streptavidin isolated proteins, where often none of the monomeric form could be detected (Fig. 1, streptavidin). No tubulin could be detected in the streptavidin isolated, showing that the biotin ester had not penetrated the cell membrane (Fig. 1, inset panel).

CHO-K1 cells permanently transfected with mGluR1c were metabolically labeled with ^{32}P and phosphorylation was induced by a 5 or 45 min treatment with 100 μM Quis (agonist) or 100 nM PMA (protein kinase C activator). Following the incubation, mGluR1c molecules were immunoprecipitated using the affinity-purified specific anti-mGluR1 F1 antibody. The immunoprecipitate from untreated cells, analyzed by SDS-PAGE and autoradiography, gave rise to a major phosphorylated band of around 190 kDa, similar in size to the dimeric form of the receptor (compare Fig. 1 with Fig. 2A, lane 1). Under similar conditions, Quis induced a rapid time-dependent increase in the phosphorylation of the dimeric form of the receptor together with a small increment in the phosphorylation of a 94 kDa protein, probably corresponding to the monomeric form of the receptor (Fig. 2A). Phosphorylation of mGluR1c was also observed when cells were treated with PMA, showing a smaller but more sustained phosphorylation of the receptor (Fig. 2A). Immunoprecipitates from agonist or PMA treated cells were also analyzed by immunoblotting using a monoclonal antibody against phospho-serine residues (see Section 2). Using this, stimulation of CHO-K1 cells with Quis led to a rapid time-dependent phosphorylation
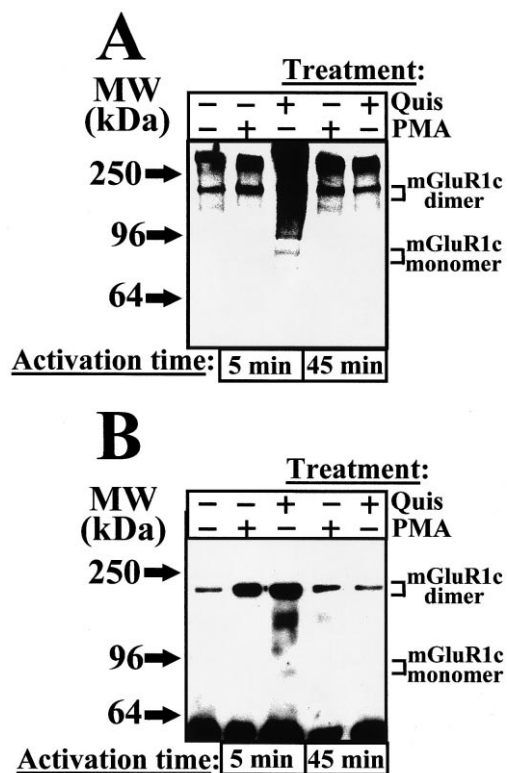
Fig. 2. Phosphorylation of mGluR1c. A: Phosphorylation as determined by ^{32}P metabolic labelling. CHO-K1 cells permanently transfected with mGluR1c were metabolically labelled with [^{32}P]orthophosphate as described in Section 2. After washing, cells were treated with 100 µM Quis or 100 nM PMA for 5, or for 45 min, and the receptor was isolated by immunoprecipitation with anti-mGluR1 affinity purified F1 antibody (5 µg/ml), applied to 6% SDS-PAGE gels and autoradiographed. B: Phosphorylation as determined by immunoblotting using anti-phospho-serine antibodies. CHO-K1 cells permanently transfected with mGluR1c were stimulated with 100 µM Quis or 100 nM PMA for 5, or for 45 min and then processed for immunoprecipitation using the anti-mGluR1 affinity purified F1 antibody (5 µg/ml). The immunocomplexes were separated in a 6% SDS-PAGE gel and then immunoblotted using an anti-phospho-serine antibody clone 1C8 as described in Section 2.

of the receptor again mainly in its dimeric form (Fig. 2B), in agreement with the results previously obtained using ^{32}P$_i$ (Fig. 2A). A similar time course for the phosphorylation of the dimeric form of mGluR1c following stimulation with PMA was also observed using the anti-phosphoserine antibodies (Fig. 2B). It should be noted that re-probing the anti-phosphoserine blots with the mGluR1 F1 antibody gave an immunoreactive band which overlaid that visualized by the former, confirming that indeed the 190 kDa protein contained mGluR1c and was most probably the dimeric form of the receptor.

In order to characterize the protein kinase involved in this rapid and transient phosphorylation of mGluR1c permanently expressed in CHO-K1 cells, we analyzed the phosphorylation level of mGluR1c after 5 min of exposure to stimuli in the presence or absence of the specific protein kinase C inhibitor Ro318220 (Fig. 3). Under these conditions, we found that the degree of basal phosphorylation of the dimeric form of mGluR1c is diminished in the presence of the inhibitor, suggesting a role for PKC in the basal phosphorylation level of mGluR1c. When the cells were stimulated with agonist for

5 min, Ro318220 only partially inhibited the agonist induced phosphorylation. Finally, when cells were stimulated with PMA, the PKC inhibitor totally blocked the receptor phosphorylation (Fig. 3). These results suggest the existence of a protein kinase other than protein kinase C producing the rapid agonist mediated phosphorylation.

Experiments of second messenger production were carried out to analyze the effect of phosphorylation on signalling via mGluR1c. In CHO-K1 cells permanently transfected with mGluR1c, the full agonist Quis increased the level of IP with an EC_{50} of 1.3 ± 0.6 µM (Fig. 4A).

Cells were pretreated with 100 µM Quis or 100 nM PMA for 5 min, extensively washed to eliminate the excess of the reagents and re-stimulated with 100 µM Quis in HBSS/Krebs buffer containing 10 mM LiCl for 5, 15 and 45 min. The responsiveness of treated cells was compared with cells preincubated with buffer alone. Under these conditions, pretreatment of cells with agonist causes a maximal desensitization ($\sim 90\%$) of mGluR1c responsiveness at the earliest time point studied (5 min) followed by a recovery to nearly normal levels after 45 min. When PMA induced desensitization was analyzed, we observed that it also was maximal following a 5 min treatment but caused only a loss of 20% of mGluR1c maximal responsiveness. The time-course of agonist and PMA desensitization correlate well with the time-course of agonist and PMA induced receptor phosphorylation, suggesting a relationship between short term phosphorylation of mGluR1c and short term desensitization of the receptor (Fig. 4B)

Next, we analyzed the effect of Ro318220 on the PMA and agonist short time induced desensitization. In control CHO-K1 cells permanently expressing mGluR1c a 10 min pretreatment of cells with Ro318220 before the agonist stimulation, induced an increment of 50% in the total IP production when compared with non-pretreated cells. However, agonist induced desensitization of $90 \pm 5\%$ was minimally affected by the specific PKC inhibitor remaining at $70 \pm 10\%$. On the oth-
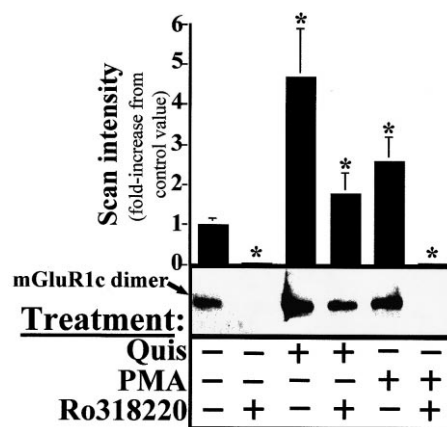

Fig. 3. Characterization of the protein kinase involved in the mGluR1c phosphorylation. CHO-K1 cells permanently transfected with mGluR1c were preincubated in absence or presence of 100 nM Ro318220 during 10 min and then stimulated with 100 µM Quis or 100 nM PMA during 5 min. Cells were processed for immunoprecipitation using the anti-mGluR1 affinity purified F1 antibody (5 µg/ml) and the immunocomplexes were analyzed as described before. The immunoreactive dimer band on the X-ray film was measured by densitometric scanning and plotted on the upper panel. The results are the mean ± S.E.M. of three independent quantifications. *, $P < 0.01$ when compared with unstimulated cells.
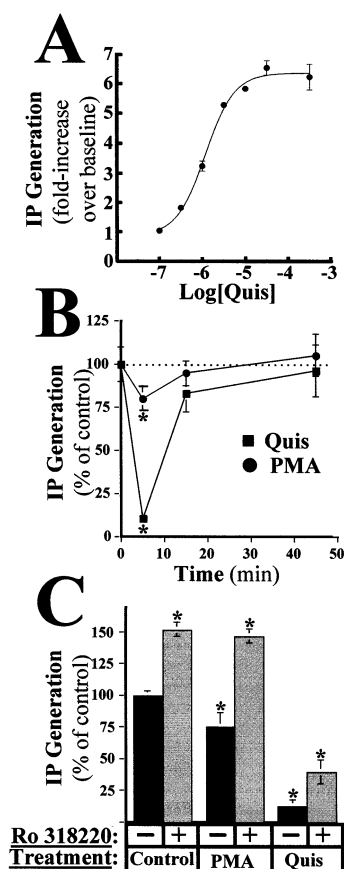
Fig. 4. Functional characterization of mGluR1c permanently expressed in CHO-K1 cells. A: concentration dependence of IP generation in response to Quis stimulation. Cells were stimulated with varying concentrations of Quis during 20 min. IP generations were determined as described in Section 2. Values are expressed as fold-increment in the IP production over the baseline (non-stimulated cells). The EC_{50} was determined by non-linear regression (see Section 2). B: Time-course desensitization of IP generation to Quis. The time course of desensitization after exposure of cells to 100 µM Quis (filled square) or 100 nM PMA (filled circle) is shown. After 5 min pretreatment, the cells were extensively washed to eliminate the excess of agonist or PMA and re-stimulated with 100 µM Quis during 5, 15 and 45 min. C: The effect Ro318220 on short time (5 min) mGluR1c induced desensitization. Cells were preincubated in absence or presence of 100 nM Ro318220 for 10 min before the Quis or PMA stimulation. IPs were isolated as indicated in Section 2. Data are plotted as mean ± S.E.M. values of quintuplicate determinations. *, $P < 0.01$ when compared with unstimulated cells.

er hand, the small desensitization of mGluR1c IP generation induced by PMA alone of 20 ± 8% was entirely blocked by pretreatment of the cells with 100 nM Ro318220 (Fig. 4C). These results show that the PMA induced desensitization of mGluR1c is mediated by PKC, whereas the agonist induced desensitization of mGluR1c is mainly mediated by another protein kinase.

4. Discussion

Recently, evidence has emerged that the seven transmembrane GPCRs may be present as dimers in the plasma membrane. To date several GPCRs have been shown to form dimers when expressed in mammalian cells, namely the β-adrenergic receptor [13], the D2 dopamine receptor [14], the

opioid receptor [15], the adenosine A_1 receptor [16] and mGluR5 [17]. The precise function of GPCR dimerization has not been fully elucidated, for example the β-adrenergic receptor dimerization is important for efficient receptor G-protein coupling [13], whereas for the opioid receptor homo-dimerization seems to have a role in the regulation of receptor internalization [15].

In the case of mGluRs group I, they appear to be disulfide bonded dimers with the N-terminal domain containing the residues responsible for their dimerization [17,18]. Here we show that mGluR1c appears to be preferentially isolated from biotinylated cells as an SDS-resistant dimer. Similar results were also obtained following cell surface biotinylation of the other short C-terminal subtype of mGluR1, mGluR1β [10]. Since the N-terminal domain of mGluR1α alone forms a disulfide bonded dimer [18] it is likely that both mGluR1β and mGluR1c will be similarly bonded and dimeric. The failure of the mGluR1β and mGluR1c dimers to dissociate in SDS-sample buffer even in the presence of reducing agents may be due to their lack of the large C-terminal domain found in mGluR1α. The absence of this domain could permit strong hydrophobic interactions between the transmembrane domains following receptor solubilization resulting in SDS-resistant dimer formation. The small amount of monomeric mGluR1c found in the total cell extracts may reflect the presence inside the cell of non-dimerized receptor.

Agonist stimulated phosphorylation of GPCRs plays a central regulatory role in transmembrane signalling [3,5]. Studies on the phosphorylation of GPCRs have found correlation between functional desensitization and receptor phosphorylation including phospholipase C-coupled receptors (e.g. the m1-muscarinic receptors [19], the adenosine A_1 receptor [6], the neurokinin 2 receptor [20], the platelet activating factor receptor [21] and the α_{1B}-adrenergic receptor [22]). mGluR1c is phosphorylated following agonist and PMA treatment with a time course that was consistent with an involvement in receptor desensitization. The phosphorylation was transient, being rapidly reversed and diminishing to nearly basal levels after treatment with PMA, and to basal levels following exposure to agonist after 45 min. Previously. we have shown that mGluR1α expressed in BHK cells, undergoes a similar rapid and reversible phosphorylation following exposure to Quis [11]. Similar time-courses of phosphorylation following agonist treatment has been described for other G-protein coupled receptors (e.g. the cholecystokinin receptor [23]). There is considerable evidence to show that the group I mGluRs undergo a rapid desensitization following agonist treatment and that protein kinase C may be implicated in this process. Thus, the treatment of synaptosomes, brain slices or cultured cerebellar granule cells with protein kinase C activators results in either the complete or partial abolition of agonist responses [24–27]. The differences reported in these studies between the total or partial inhibition of agonist responses following phorbol ester treatment, may reflect the varying levels of expression of mGluR1 and mGluR5 in the different brain areas used for experimentation. In addition, the presence of multiple receptor subtypes in these preparations makes a comparison of these results with ours, obtained using a single cell type and one receptor subtype, difficult. Nevertheless, we have observed a partial inhibition of agonist responses following phorbol ester treatment of the CHO cells

expressing mGluR1c and rapid and full densensitization following agonist application.

It is interesting to note that two types of protein kinase, differentiated by their sensitivity to the highly specific PKC inhibitor Ro318220, appear to phosphorylate mGluR1c. Thus whilst PKC may have a role in the desensitization of mGluR1c another protein kinase(s), possibly one of the G-protein coupled receptor kinases, whose existence in CHO-K1 cells has been demonstrated [28], may be responsible for the majority of the rapid agonist mediated desensitization of mGluR1c in this cell line. In this context it is interesting to note that in other studies of the agonist response of cells permanently transfected with mGluR1α, pretreatment of the cells with phorbol esters only partially abolished their agonist mediated IP responses as seen for mGluR1c in this study [29,30]. In BHK cells permanently expressing mGluR1α the basal level of receptor phosphorylation was significantly higher than that seen for mGluR1c in this study [11]. Pretreatment of the BHK cells prior to agonist treatment with the specific protein kinase inhibitor, Ro318220, reduced the basal level of mGluR1α phosphorylation but did not prevent it, suggesting that a kinase other than PKC might be involved in its phosphorylation [11]. Whilst bearing in mind the caveat that recombinant receptors expressed in cell lines lack many of the regulatory elements that would normally exist in their normal cell environment these results suggest that kinases other than protein kinase C may be involved in the desensitization of the Group I mGluRs. The relative importance of protein kinase C in their desensitization may vary with the subtype of the receptor and/or the cell type in which it is expressed.

In conclusion, the results presented here suggest that mGluR1c is present at the cell surface of CHO-K1 cells as a dimer, which is the target of agonist mediated phosphorylation. The time course of the phosphorylation of the receptor correlates with its desensitization kinetics and the inhibition of protein kinase C causes only a partial reversal of desensitization indicating that other kinases may be involved in this process.

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