Differential internalisation of mGluR1 splice variants in response to agonist and phorbol esters in permanently transfected BHK cells

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Abstract The internalisation of metabotropic glutamate receptor (mGluR1α) and its splice variant (mGluR1β), in response to agonist and phorbol esters (PMA), has been studied. Both mGluR1α and mGluR1β exhibit a similar rate of internalisation following PMA treatment, with a shift in their distribution from plasma membrane to endosome-enriched membrane fractions. Agonist challenge however caused a rapid loss, within 5–10 min, of mGluR1β but not mGluR1α from the cell surface. These results show that the two forms of mGluR1 show different internalisation responses to agonist and suggest that the C-terminal region of the molecule plays an important role in this phenomenon.

Key words: Metabotropic glutamate receptor; Internalization; Splice variant; Protein kinase C; Immunoblotting

1. Introduction

Metabotropic glutamate receptors (mGluRs) constitute a family of large G-protein coupled receptors which show little sequence homology with the superfamily of smaller G-protein linked receptors [1]. Eight members of the mGluR family have been identified, several of which generate different subtypes by alternative mRNA splicing [2]. These eight mGluR receptors and their subtypes may be categorised into three subgroups on the basis of their sequence homology, agonist selectivity and signal transduction pathway. The first group contains mGluR1 and mGluR5 which share the strongest sequence homology (62% identical at the amino acid level), 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 mGluR1α, mGluR1β, mGluR1c, mGluR1d and mGluR1e [2–4]. In mGluR1β, the insertion of an 85 base sequence in the carboxy-terminal tail introduces a stop codon that results in a 318 amino acid deletion and also changes the reading frame resulting in a different C-terminal amino acid sequence from that of mGluR1α. The functional significance of the different splice variants has not yet been fully explored. It has been suggested that the C-termini of mGluRs, which are intracellular, might play a role in the subcellular targeting of the receptor [5] and elements of the C-terminus close to the inner surface of the plasma membrane have been shown to be important for G-protein coupling [2].

G-protein coupled receptors undergo homologous [6] and heterologous desensitisation [7]. 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 desensitisation is a complex process which can involve phosphorylation of the molecule, its uncoupling from G-proteins, its internalisation/sequestration, and ultimately its down-regulation. However, the role of receptor internalisation in the process of desensitisation is not well defined. Desensitisation can occur independently of internalisation, and consequently other functions have been attributed to receptor internalisation, such as a role in receptor re-sensitisation [8,9].

In this study we have examined the internalisation of mGlur1α and mGlur1β, in response to quisqualic acid and phorbol 12-myristate 13-acetate (PMA) treatment, using permanently transfected BHK cells. Cell surface receptor was monitored using ^{125}I-labelled antibodies raised against N-terminal epitopes of mGluR1. This together with immunoblotting of different membrane fractions following challenge of the cells with PMA and quisqualic acid has allowed us to delineate the kinetics of internalisation of mGluR1α and mGluR1β and to show that these two subtypes of mGluR exhibit different internalisation responses to quisqualic acid.

2. Materials and methods

2.1. Antibodies

Two antisera were used in this study. One, designated R1, was raised against four synthetic peptides based on N-terminal sequences present in mGluR1, namely residues 59–75, 124–140, 139–157 and 367–385. This pseudo-polyclonal antiserum was affinity purified as previously described [10]. All the initial experiments were performed with this antibody and confirmed using the second antibody described below.

The second serum, F1, was raised against a histidine tagged fusion protein containing an amino-terminal sequence of mGluR1, residues 121–341. This was produced by cloning an *Eco*RI-*Hin*dIII fragment of pmGR1 [11] encoding these residues into the bacterial expression vector pET-28a$^+$ (Novagen, Inc.). Induction and purification of the fusion protein was performed as recommended by the manufacturers. Two rabbits were inoculated subcutaneously with 0.5 mg of the fusion protein in Freund's complete adjuvant, and subsequent injections were performed every 4 weeks using Freund's incomplete adjuvant. Blood was taken 10 days after each injection. In order to affinity purify the specific antibodies, the same fragment (amino acid residues 121–341 of rat mGluR1) was subcloned into the pGEX-4T1 bacterial expression vector (Pharmacia Biotech) and the resulting GST fusion protein was purified on glutathione agarose (Sigma). These sera were affinity purified on a cyanogen bromide coupled GST fusion protein column. Affinity purified antibody (F1-Ab) was eluted using low pH as described previously [12] and dialyzed against phosphate buffered saline (PBS).

2.2. Radioiodination of R1-Ab and F1-Ab

The affinity purified antibodies (5 μg) were radiolabelled with ^{125}I (100 μCi) (ICN) using chloramine-T [13]. The reaction product ([^{125}I]R1-Ab) was separated from free ^{125}I by chromatography on a

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PD-10 Sephadex G-25 fine grade column (Pharmacia). Elution was performed with phosphate-buffered saline (PBS), pH 7.4 containing 1% bovine serum albumin (BSA). The specific activity was routinely 2.5–3.35×10^9 dpm/nmol.

2.3. Tissue culture

BHK 570 cells stably expressing the mGluR1α or mGluR1β receptor were grown as previously described [10]. Membranes of cells were prepared by shearing cells in 10 mM $NaHCO_3$ as previously described [14] and protein was measured by the bicinchoninic acid method [15].

2.4. SDS-PAGE and immunoblotting

Cell membranes were treated with SDS-PAGE sample buffer [12], before loading onto the polyacrylamide gels. Electrophoresis was performed using 7.5% polyacrylamide gels [16]. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore), using a semi-dry transfer system [17]. After blocking with 5% (w/v) dry milk in PBS containing 0.05% Tween-20 (PBS-T), PVDF membranes were washed in PBS-T and incubated overnight at 4°C with the specific affinity purified R1 or F1 antibody (2–4 μg/ml) in TBS-T-milk. Immunoreactive bands were detected with swine anti-rabbit antibody conjugated to horseradish peroxidase followed by chemiluminescence detection (Amersham) [12].

2.5. Radioiodinated antibody binding experiments

For internalisation experiments, cells were grown in glutamate free medium (ICN) in the absence of both glutamine and glutamic acid for 3 h before the addition of 100 μM quisqualic acid or 1 μM PMA. After the indicated incubation time, cells were quickly chilled at 4°C, washed in cold PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were washed in PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. After 30 min incubation in buffer A containing 1% BSA (buffer B), cells were incubated with 1 μg/ml of [^{125}I]R1-Ab or [^{125}I]F1-Ab overnight at 4°C. Cells were washed five times with buffer B, disrupted with 1% SDS and the bound radioactivity determined using a Beckmann LS 5000 CE scintillation counter.

2.6. Sucrose gradients

High- and low-density membranes were separated on sucrose step gradients as previously described [18]. Membranes at the 15–38% sucrose interface (low-density fraction) and 38–60% sucrose interface (high-density fraction) were collected, diluted with water and centrifuged at 100 000×*g* for 1 h at 4°C. Pellets were resuspended in 50 μl of 10 mM $NaHCO_3$ and the protein concentration was determined as described above. The receptors were detected by immunoblotting using R1-Ab.

The immunoblots were quantified by densitometric scanning. The band intensities on X-ray film corresponding to mGlur1α and mGlur1β (monomer+dimer) receptors were measured using the volume integration function of the computer program NIH Image. The results are expressed as the percentages of the total receptor (high-density fraction+low-density fraction) in each treatment.

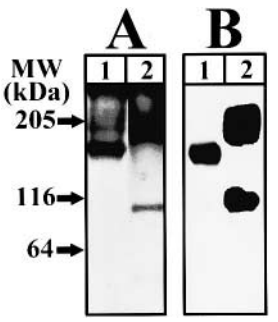

Fig. 1. Immunoblot showing expression of mGluR1α and mGluR1β in permanently transfected BHK cells. Twenty to 40 μg of protein from cell membranes were applied to 7.5% SDS-PAGE gels; the proteins were transferred to PVDF and the blots were incubated with R1 (A) or F1 (B). Lane 1, BHK cells permanently transfected with mGluR1α; lane 2, BHK cells permanently transfected with mGluR1β.

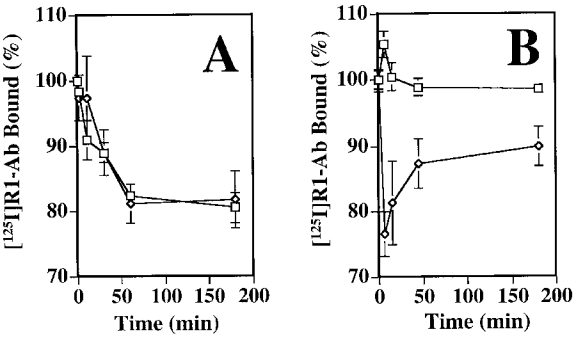

Fig. 2. Binding of [^{125}I]R1-Ab to BHK cells permanently transfected with mGluR1α (□) or mGluR1β (◇) receptor subtypes. Permanently transfected BHK cells were grown in glutamate free medium for 3 h before the addition of 1 μM PMA (A) or 100 μM quisqualic acid (B) for the indicated time. Cells were processed as indicated in Section 2 and then incubated overnight at 4°C with 1 μg/ml of [^{125}I]R1-Ab. The radioactivity that remained bound to the cells after washing was counted. Values are the mean±S.E.M. of quadruplicate determinations. Similar results were obtained in three different experiments.

3. Results

Two antibodies raised against protein sequences in the amino terminus of mGluR1 have been used in this study. One generated using four synthetic peptides contained in this region of the molecule (termed R1) and the other raised against a fusion protein containing the amino acid residues 121–341 of rat mGluR1 (termed F1). Both antibodies immunoblotted a major immunoreactive protein of apparent molecular weight of 150 kDa in membranes prepared from a BHK cell line permanently transfected for mGluR1α (Fig. 1), together with higher molecular weight bands which may represent oligomers of the receptor. In membranes prepared from a BHK cell line containing mGluR1β, the R1 and F1 yielded two immunoreactive bands, with apparent molecular weights of 190 kDa and 94 kDa (Fig. 1), which may correspond to receptor dimer and monomer respectively. These results are in good agreement with previously reported studies using the BHK cell lines and with the results obtained with other antibodies to these receptors [10–19].

The effect of quisqualic acid, a full agonist of mGluR1, and the protein kinase C activator, PMA, on receptor cell surface expression was examined. Following activation for varying times by the different agents the cells were quickly chilled, fixed, and the amount of receptors remaining on the cell surface determined by [^{125}I]R1 binding. BHK mGluR1α and BHK mGluR1β containing cell lines showed a similar time-course of internalisation ($t_{1/2}$ = 18±6 min and 32±8 min, respectively) in response to PMA (Fig. 2A), with maximal internalisation (~20%) of mGluR1α or mGluR1β after a 45 min exposure to 1 μM PMA. When cells where activated with 100 μM quisqualic acid the time-course of surface receptor loss in the two cell lines was completely different. BHK cells containing mGluR1β showed a rapid loss of receptor (up to ~25%), within 5 min, followed by a slow recovery reaching ~90% of the initial receptor density after a 3 h exposure to the agonist. BHK cells containing mGluR1α did not show detectable receptor loss following a short time exposure to agonist (Fig. 2B). Similar results were observed when the other N-terminal antibody [^{125}I]F1 was used (data not shown).

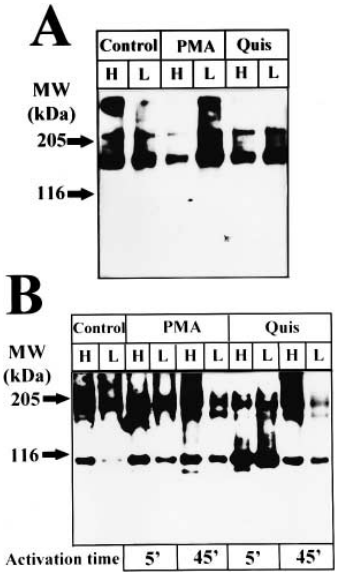

Fig. 3. Receptor internalization of mGluR1α and mGluR1β in BHK cells as detected by immunoblotting. BHK cells permanently transfected with mGluR1α (A) or mGluR1β (B) were grown in glutamate free medium for 3 h before the addition of 1 μM PMA (PMA), 100 μM quisqualic acid (Quis) or vehicle (Control) for 45 min (A) or 5 min and 45 min (B). Cells were quickly chilled at 4°C and high- (H) and low-density (L) membranes were separated on a sucrose gradient (see Section 2). 20 μg of protein from each fraction was applied to 7.5% SDS-PAGE gels and the receptor present was detected by immunoblotting using the R1 antibody. Similar results were obtained in three different experiments.

The different behaviour of the two receptor subtypes in response to quisqualic acid exposure suggests that the region responsible for agonist mediated fast internalisation of mGluR1β is located in the last 22 amino acids of mGluR1β.

In order to confirm that the loss of cell surface receptor described above was due to receptor internalisation we prepared cellular fractions enriched in plasma membranes and endosomal compartments from cells exposed to either PMA or quisqualic acid.

In control BHK mGluR1α cells the percentage of receptor present in the low-density membrane fraction was consistently around 50% of the total receptor (Fig. 3A, Control), as determined by densitometric scanning of the immunoblot of the corresponding fractionated membranes. After activation of PKC for 45 min with 1 μM PMA there was a shift ranging from 20–30% of mGluR1α from the high- to the low-density membrane fraction in three different experiments (Fig. 3A). However, quisqualic acid treatment of the cells caused no significant alteration in the distribution of mGluR1α in membrane fractions (Fig. 3A).

When BHK cells containing mGluR1β cells were similarly examined two activation times of drug intervention were chosen, namely 5 and 45 min, in order to study the fast agonist effect on surface mGluR1β expression described above. In the BHK cells permanently transfected with mGluR1β the amount of receptor present in the low-density membrane fraction was consistently about 32 ± 2% of the total receptor (Fig. 3B). Activation of PKC with 1 μM PMA revealed a time dependent redistribution of receptor from high- to the low-density fraction (Fig. 3B). Densitometry of immunoblots in three different experiments indicated that the amount of total receptor in the low density membrane fraction increased by 10–15% after 5 min and by 15–20% after 45 min of PMA treatment. The appearance of the lower molecular weight bands in the PMA treated lanes, especially noticeable after 45 min may reflect the degradation of the receptor after entry of at least some of it, into the lysosomal compartment. In contrast, when the cells where activated with 100 μM quisqualic acid the amount of receptor in the low density fraction increased markedly (by 18–25%) after 5 min (Fig. 3B) and declined subsequently. It was also notable that the amount of 94 kDa mGluR1β monomer in the low-density fraction appeared to increase following PMA and especially quisqualic acid treatment.

Thus there is evidence that there is a reduction in receptor number following treatment of the cells with PMA, and that mGluR1β rapidly enters a light membrane compartment in response to quisqualic acid whereas mGluR1α does not. The data on the redistribution of the cell surface receptor obtained from the fractionation of the cell membranes correlates well with that obtained by binding of [^{125}I]R1 and [^{125}I]F1, suggesting that the latter was due to receptor internalisation.

4. Discussion

Although the internalisation process has been well characterised for several different G-protein coupled receptors (GPCRs) [20], the internalisation of metabotropic glutamate receptors (mGluRs) has not previously been reported. Here we report two novel findings, namely that mGluR1β, but not mGluR1α undergoes rapid removal from the cell surface following agonist stimulation and that both receptor subtypes undergo a slower internalisation following treatment of cells with PMA. These responses were observed using two different antibodies directed against different N-terminal epitopes and were confirmed by membrane fractionation to determine the plasma membrane receptor content.

The splice variants of mGluR1 have been reported to show little difference in their agonist responses and all of them activate phospholipase C in heterologous expression systems [2]. However, expression in mammalian cells of the long isoform of mGluR1 (mGluR1α) results in an increase in the basal PLC activity with a concomitant doubling of basal inositol phosphate formation [21]. In contrast, when the short isoforms mGluR1β and mGluR1c were similarly expressed in heterologous expression systems basal inositol phosphate production was unaffected [21]. Similar results have been reported for mGluR5 and its splice variants which utilise the same second messenger pathway as mGluR1 in transfected cells [22]. This has led to the suggestion that the long intracellular tail of mGluR1α is responsible either for constitutive coupling of the receptor to intracellular G-proteins or to the maintenance of the receptor in an activated state.

Our results suggest that carboxy-terminal domain in mGluR1 is not only important in the coupling to the G-protein, but may also be an important determinant in regulating agonist induced internalisation. This suggests that intracellular sequences of both splice variants contains sequences that can interact with other cytoplasmic proteins and cause intracellular internalisation in a manner analogous to the interactions of other G-protein coupled receptors with arrestin [20]. However, the mGluR1s do not contain motifs similar to those found in other GPCRs. In particular, the highly conserved

DRYXXV/IXXPL sequence, found in the second intracellular loop of GPCRs [23,24], and the highly conserved motif $NP(X)_{2,3}Y$ found toward the cytoplasmic face of the putative seventh transmembrane domain in these proteins [9,25,26] are not found in the mGluR1 receptors. This suggests that other internalisation sequences remain to be found in this new and different group of GPCRs. Additional studies are needed to elucidate which amino acids in mGluR1β are responsible for its rapid internalisation in response to agonist. The results presented here also suggest that the carboxy-terminal domain of these receptors does not play an important role in the protein kinase C mediated internalisation of mGluR1s splice variants and that consequently the determinants responsible for this internalisation lie elsewhere in the molecule.

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