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Rapid internalization and surface expression of a functional, fluorescently tagged G-protein-coupled glutamate receptor

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L-Glutamate is the principal excitatory neurotransmitter in the vertebrate central nervous system, where it mediates many of its actions via G-protein-coupled metabotropic glutamate (mGlu) receptors. Since little is known about the dynamics of mGlu receptors at the plasma membrane, we have constructed a fusion protein comprising the mGlu receptor subtype 1α ($mGlu_{1\alpha}$) and green fluorescent protein (GFP). Using imaging of Ca^{2+} release from intracellular stores as a functional assay, the agonist pharmacology of this fluorescently tagged receptor was found to be similar to that of the wild-type receptor when expressed in HEK-293 cells. Receptor movement and function were measured simultaneously by combined imaging of Ca^{2+}, using fura-red, and GFP fluorescence in single cells. Exposure to agonist induced a rapid loss of up to 30 % of membrane-associated fluorescence, with a corresponding decrease in the functional response. Following removal of the agonist there was recovery of both the membrane fluorescence and the functional response. These data suggest that the surface expression of G-protein-coupled glutamate receptors might be rapidly regulated in response to agonist activation.

Key words: green fluorescent protein, $mGlu_1$ receptor, metabotropic glutamate receptor, receptor movement.

INTRODUCTION

Following the initial cloning of the metabotropic glutamate (mGlu) receptor $mGlu_{1\alpha}$ [1,2], eight separate mGlu receptors have now been identified (for reviews, see [3,4]). Together with the recently cloned γ-aminobutyric acid $(GABA)_B$ receptor [5] and the Ca^{2+}-sensing receptor [6], they form a distinct superfamily of G-protein-coupled receptors (GPCRs) which, while retaining a seven-transmembrane-domain motif, show little other sequence or structural similarity with the muscarinic/adrenergic receptor superfamily. The mGlu and $GABA_B$ receptors are large in comparison with other GPCRs, and possess an extensive extracellular N-terminal domain, in which the ligand-binding domain is localized, and an intracellular C-terminus.

On the basis of their sequence similarity, pharmacology and signal-transduction mechanisms, mGlu receptors have been classified into three groups [3]. Group I consists of $mGlu_1$ and $mGlu_5$ receptors, which are coupled to phospholipase C [1,7], activate protein kinase C and release Ca^{2+} from intracellular stores. In contrast, in expression systems, Group II ($mGlu_2$ and $mGlu_3$) and Group III ($mGlu_{4,6-8}$) receptors are negatively coupled to adenylate cyclase. Analysis of their regional and cellular localizations, the development of selective pharmacological agents and the generation of mice with specific receptor knock-outs is enabling the function of these receptors to be elucidated. For example, $mGlu_1$ receptors are widely distributed in the brain, and they are enriched in the perisynaptic membrane [8–11]. They can be activated during synaptic transmission [12], and might be important for certain forms of synaptic plasticity involved in learning and memory [13–16] and in neuronal damage [17,18]. Within microsomes, the $mGlu_{1\alpha}$ receptor is oriented with the C-terminal region exposed to the cytoplasm [19], suggesting that

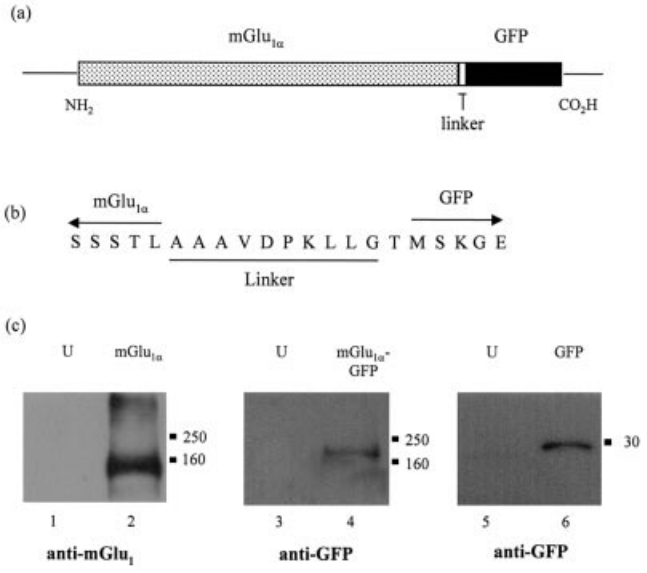

Figure 1 Structure and immunoblot analysis of $mGlu_1$–GFP expression in HEK-293 cells

(**a**) GFP was linked to the C-terminus of the $mGlu_{1\alpha}$ receptor following removal of the stop codon. A linker peptide was inserted to allow the GFP to fold independently. (**b**) Amino acid sequence of the fusion region, showing the C-terminal end of the $mGlu_{1\alpha}$ receptor, the linker region and the N-terminus of the GFP. (**c**) Immunoblot analysis of HEK-293 cells expressing $mGlu_{1\alpha}$ (lane 2), $mGlu_{1\alpha}$–GFP (lane 4) and GFP (lane 6). Protein from control, untransfected cells (U) are in lanes 1, 3 and 5. Immunoblots were probed with anti-$mGlu_1$ (lanes 1 and 2) or anti-GFP (lanes 3–6) antisera. Secondary antibodies were incubated with HRP-conjugated antibodies, and blots were revealed by enhanced chemiluminescence.

Abbreviations used: (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-amino-1,3-cyclopentane dicarboxylic acid; L-AP4, L(+)-2-amino-4-phosphonobutanoic acid; (*RS*)-CHPG, (*RS*)-2-chloro-5-hydroxyphenylglycine; (*S*)-DHPG, (*S*)-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's modified Eagle's medium; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; (*S*)-MCPG, (*S*)-α-methyl-4-carboxyphenylglycine; mGlu, metabotropic glutamate.

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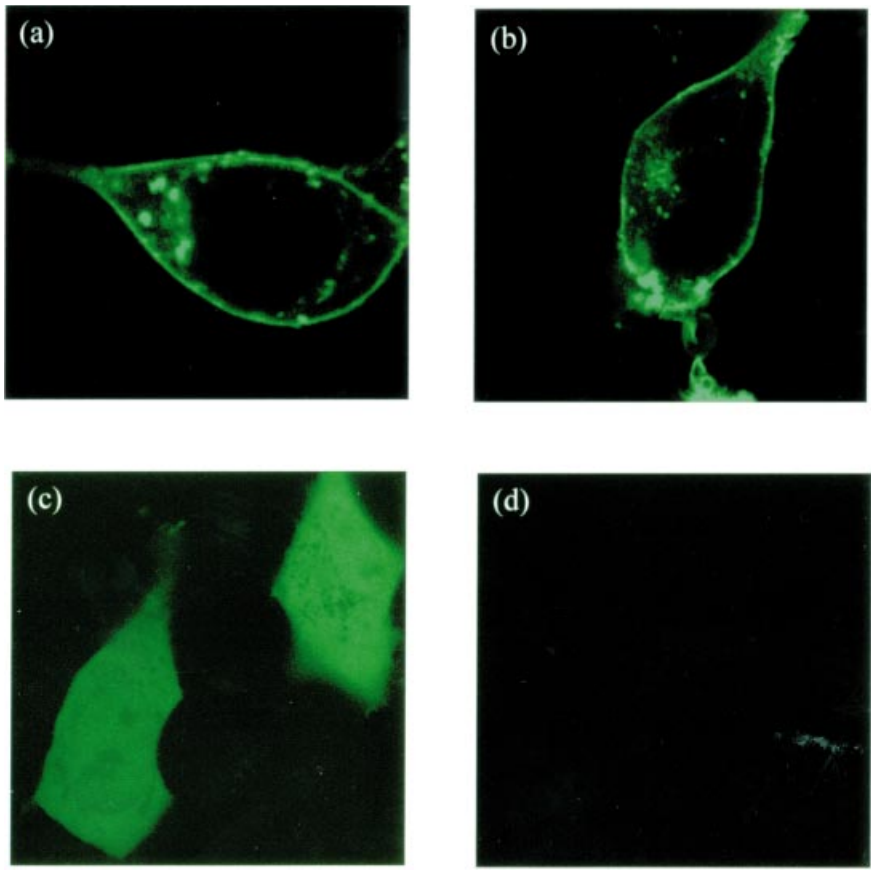

Figure 2 Localization of $mGlu_{1\alpha}$–GFP expressed in HEK-293 cells

Cells were transfected with $mGlu_{1\alpha}$–GFP (**a**, **b**) or GFP (**c**) and viewed under a Bio-Rad MRC1024 confocal microscope with illumination at 488 nm. In cells transfected with $mGlu_{1\alpha}$–GFP, fluorescence was concentrated at the plasma membrane, in intracellular vesicles and in perinuclear membrane compartments. In cells transfected with GFP alone, the fluorescence distribution was even in intensity. No fluorescence was seen in untransfected cells (**d**).

insertion and removal from the plasma membrane is likely to be mediated via C-terminal interactions with other proteins. One recently identified class of proteins that can interact with $mGlu_1$ receptors is the Homer family of small PDZ binding proteins (PDZ is a 90-amino-acid repeat first found in **P**SD-95 **D**lg **Z**O-1) [20–22], which are thought to act as adapter molecules attaching the receptors to proteins in the cytoskeletal matrix. Currently, however, very little is known about the targeting and trafficking of mGlu receptors in cells.

A technique which has been used successfully to investigate the localization and movement of receptors is to image fluorescently tagged receptors in living cells using green fluorescent protein (GFP). A wide range of GFP fusion proteins have now been made and, in many cases, the addition of GFP has had little effect on receptor function or trafficking (e.g. [23–26]). In particular, GFP fusions of the β_2-adrenoceptor and cholecystokinin A receptors have recently been shown to be fully functional in terms of their pharmacology and internalization/recycling behaviour [27–29]. We report here the construction and functional expression of $mGlu_{1\alpha}$–GFP, the first GFP fusion protein of a member of the mGlu receptor superfamily. We demonstrate that this fusion protein is fully functional in a heterologous expression system, and that it can move rapidly to and from the plasma membrane in response to the level of receptor activation.

EXPERIMENTAL

Materials

A red-shifted GFP, cloned into pCMX, was generously given by Dr. J. Pines (Cambridge, U.K.). pcDNA1.amp and the TOPO TA cloning kit were obtained from Invitrogen (Leek, The Netherlands). Anti-$mGlu_1$ antiserum was purchased from UBS (Lake Placid, NY, U.S.A.) and anti-GFP anti-serum was from Clontech (Palo Alto, CA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Paisley, Renfrewshire, U.K.) and SuperFect was from Qiagen (Crawley, West Sussex, U.K.). All other cell culture reagents were from Sigma–Aldrich (Poole, Dorset, U.K.). (*S*)-3,5-Dihydroxyphenylglycine [(*S*)-DHPG], (1*S*,3*R*)-1-amino-1,3-cyclopentane dicarboxylic acid [(1*S*,3*R*)-ACPD] and L(+)-2-amino-4-phosphonobutanoic acid (L-AP4) were obtained from Tocris Cookson Ltd. (Bristol, U.K.). (*RS*)-2-Chloro-5-hydroxyphenylglycine [(*RS*)-CHPG] and LY317206 were generously given by Dr. D. E. Jane, School of Medical Sciences, Bristol, and Eli Lilly and Co., Indianapolis, IN, U.S.A., respectively. All chemicals used were of analytical grade.

$mGlu_{1\alpha}$–GFP construction

The $mGlu_{1\alpha}$-coding sequence (GenBank accession number X57569) was cloned into the cytomegalovirus-driven mammalian

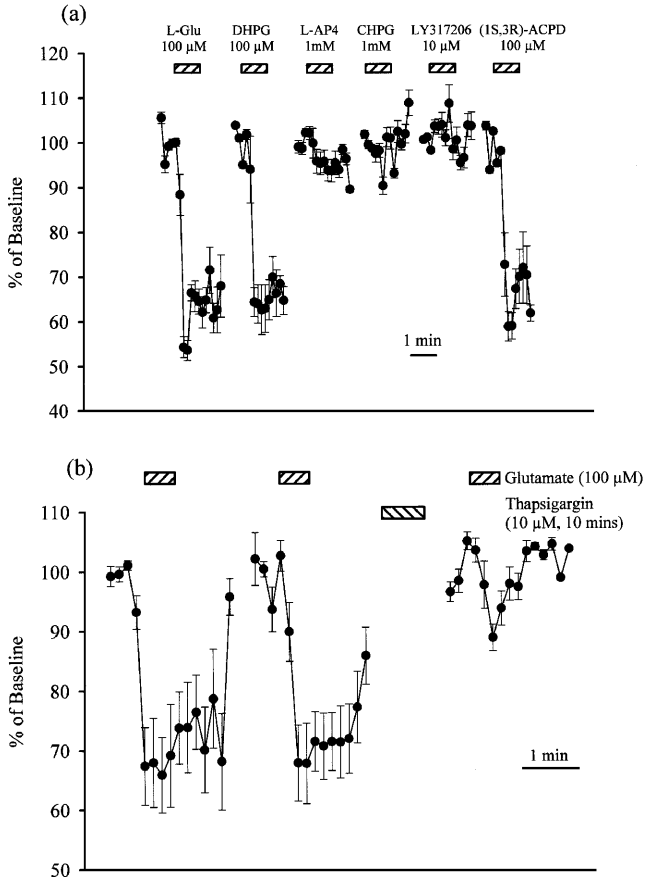

Figure 3 Functional analysis of $mGlu_{1\alpha}$–GFP expressed in HEK-293 cells

(**a**) HEK-293 cells were transfected with $mGlu_{1\alpha}$–GFP and incubated with various agonists at the indicated concentrations for the durations shown by the bars. Ca^{2+} mobilization, recorded as a decrease in fura-red fluorescence, was elicited only by those agonists that activate the $mGlu_1$ receptor. (**b**) Functional responses are antagonized by the prior incubation of the cells with 10 μM thapsigargin, which depletes intracellular Ca^{2+} stores by preventing their refilling. Following thapsigargin treatment, responses were greatly decreased within two applications of agonist (second application shown). In parallel controls, at least four reproducible responses to 100 μM L-glutamate were observed. Data are presented as means ± S.E.M. from six individual cells taken from at least two different transfections.

expression vector pcDNA1.amp. A 297-bp region from the unique *Cla*I restriction site to the 3′ end of the coding sequence was then amplified using primers (+)5′-GGG GAG GAC ATC GAT GAT GAC-3′ and (−)5′-CGC GAC TCT CTC GAG GTA ACC TGC GGC CGC CGC CAG GGT GGA AGA GCT-3′ to remove the TAG stop codon and insert a downstream *Not*I restriction site. The amplified fragment was cloned into pCR2.1 TA vector using the TOPO TA cloning kit, and sequenced to ensure fidelity of amplification. The sequence-verified mutated region was then ligated back into the $mGlu_{1\alpha}$-receptor-coding sequence to make a full-length $mGlu_{1\alpha}$ receptor minus the stop codon ($mGlu_{1\alpha}$-STOP). A red-shifted GFP incorporating a $Ser^{65} \rightarrow Thr$ mutation was then amplified using primers (+)5′-GCA TTC GTA GCG GCC GCG GTC GAC CCC AAG CTT CTA GGT ACC-3′ and (−)5′-TGG CCT CGA GCC TCT AGA TAC CTA-3′, adding an upstream *Not*I restriction site and downstream universal stop sequence. The amplified GFP fragment was digested with *Not*I/*Xho*I, and cloned into $mGlu_{1\alpha}$-STOP to create $mGlu_{1\alpha}$–GFP. A schematic representation of the construct is shown in Figure 1(a), and the amino acid sequence of the fusion region is shown in Figure 1(b).

Cell culture and transfection

HEK-293 cells were cultured in DMEM, containing 1 $g \cdot l^{-1}$ D-glucose and supplemented with 2 mM L-glutamine and 10 % (v/v) dialysed horse serum. Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. For transfection experiments, cells were grown on glass coverslips (22-mm diameter, grade 1 thickness, coated with 25 μM poly-D-lysine), and transfected using SuperFect according to the manufacturer's instructions. Briefly, 0.3–1.0 μg of each DNA was mixed with 15 μl of DMEM and 3 μl of SuperFect reagent per coverslip, and incubated at room temperature for 10–15 min. Aliquots of complete medium (100 μl) per coverslip were then added to the DNA/SuperFect mixture. Cells were washed twice in PBS, and incubated in 100 μl of transfection solution at 37 °C for 2–3 h. Cells were washed a further two times with PBS, and then cultured in complete medium. For transfections into HEK-293 cells, medium was additionally supplemented with 250 μM (*S*)-α-methyl-4-carboxyphenylglycine (MCPG) to lessen any potentially excitotoxic effects of the $mGlu_{1\alpha}$–GFP fusion on this cell line. Transfected cells were used within 48 h.

Confocal imaging

For imaging of GFP fluorescence alone, cells transfected with either GFP or $mGlu_{1\alpha}$–GFP were viewed under a Bio-Rad (Hemel Hempstead, Herts., U.K.) MRC1024 laser-scanning confocal microscope equipped with an argon-ion laser using 488 nm excitation/515 nm long-pass emission filters. For combined GFP and Ca^{2+} imaging, cells were loaded with the membrane-permeable dye fura red-AM (10 μM, 60 min, 37 °C) and viewed under either a Bio-Rad MRC 600 or an MRC 1024 laser-scanning confocal microscope equipped with argon-ion lasers using a 488 nm excitation filter and 525/20 nm and 660DF50 nm (Omega) band-pass emission filters to image GFP and fura-red signals respectively. In all experiments, cells transfected with the $mGlu_{1\alpha}$–GFP receptor, but not loaded with fura-red, were used in parallel to ensure no 'bleed-through' was evident from the green to red channels. Cells were continuously perfused with Krebs balanced salt solution (124 mM NaCl/2.9 mM KCl/1.4 mM NaH_2PO_4,H_2O/25 mM $NaHCO_3$/1 mM $MgSO_4$,$7H_2O$/2 mM $CaCl_2$/25 mM D-glucose), continuously gassed with O_2/CO_2 (19:1) at a rate of approx. 2 $ml \cdot min^{-1}$. Kalman integrations of five individual images were obtained every 10–120 s. Agonists were added to the perfusate as 2 ml aliquots. The fluorescence of individual cells was measured on a Macintosh computer using the NIH Image program. Membrane-associated fluorescence density was measured by drawing a line along the cell-perimeter fluorescence on each individual frame of an image series. A histogram of the fluorescence density along the line was displayed using Lasersharp 3.0 software (Bio-Rad), and the mean fluorescence density was used as a measure of $mGlu_{1\alpha}$-GFP fusion density.

SDS/PAGE and Western blotting

For immunoblot experiments, cells transfected with the appropriate DNA were solubilized with 100 μl of 10 % (v/v) SDS and the protein was precipitated using methanol and chloroform, as described in [30]. Discontinuous SDS/PAGE [31] was performed using a Bio-Rad Mini Protean II upright electrophoresis system and either 6 % or 7.5 % resolving polyacrylamide gels, as

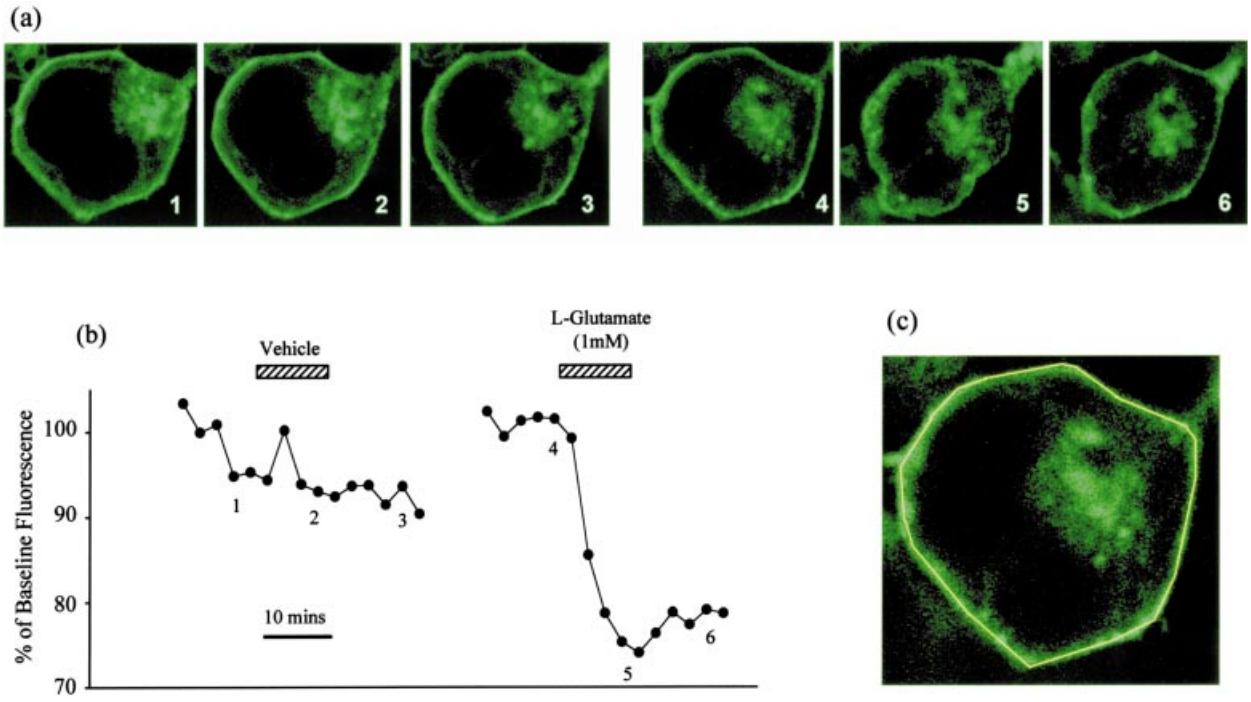

Figure 4 Effect of L-glutamate exposure on the membrane-associated fluorescence in a representative cell expressing $mGlu_{1\alpha}$–GFP

(**a**) Confocal images, obtained every 2 min, of a cell exposed to vehicle or L-glutamate (1 mM) for 10 min. Note the transient alteration in morphology during exposure to L-glutamate (panel 5). (**b**) Graphical representation of the membrane-associated fluorescence density measured along a line drawn around the perimeter of the cell (**c**). The numbers correspond to the images shown in (**a**). A separate line was drawn for each frame of the image series to compensate for shape changes. Movies (in both Quicktime and AVI format) of the actions of buffer vehicle and 1 mM L-glutamate on this cell can be seen at http://www.BiochemJ.org/bj/341/bj3410415add.htm

described previously [19]. Following separation, proteins were transferred to nitrocellulose (Immobilon P) under wet-blot conditions (100 V, 1 h), blocked with 5% (w/v) milk overnight, and then probed with antisera raised against the $mGlu_{1\alpha}$ receptor C-terminus (obtained from UBS; at a dilution of 1:2000, 2 h at room temperature). Immunoreactivity was detected using horseradish-peroxidase-conjugated secondary antibody and chemiluminescent detection (Boehringer Mannheim, Mannheim, Germany). In some experiments, the blots were then stripped and re-probed with an antibody raised against GFP (Clontech; at a dilution of 1:1000), as described above.

Data analysis

Dose–response profiles were analysed by non-linear least-squares analysis, and EC_{50} values were derived using SigmaPlot (Jandel Scientific, Erkrath, Germany). The K_i value for the antagonist was calculated using the equation $K_i = L/r\text{-}1$ where L is the antagonist concentration and r is the ratio of EC_{50} values obtained in the presence and absence of antagonist. Unless stated otherwise, results are given as means ± S.E.M. Statistical analysis was achieved by Student's paired t test.

RESULTS AND DISCUSSION

Expression of $mGlu_{1\alpha}$–GFP in mammalian cell lines

HEK-293 cells, transfected with GFP, $mGlu_{1\alpha}$ receptor or the $mGlu_{1\alpha}$–GFP fusion protein, were analysed by Western blotting (Figure 1c). In good agreement with the predicted size of the recombinant receptor [1], anti-($mGlu_1$ receptor) antisera, which recognize the extreme C-terminus of the receptor, yielded a band of approx. 150 kDa in cells transfected with the $mGlu_{1\alpha}$ receptor. In cells expressing the $mGlu_{1\alpha}$–GFP receptor fusion protein, this band was absent, but a fainter band of approx. 180 kDa (the predicted size of the fusion protein) was detected. An anti-GFP antiserum also recognized a 180 kDa band in $mGlu_{1\alpha}$–GFP receptor-expressing cells, in addition to a band at 30 kDa in cells transfected with GFP alone. None of these bands was detectable in untransfected cells.

Representative confocal microscope images of HEK-293 cells transfected with the $mGlu_{1\alpha}$–GFP fusion protein are shown in Figure 2. A highly localized, ring-like distribution of fluorescence was detected which was co-incident with the edge of the cell (Figures 2a and 2b), consistent with an association of the $mGlu_{1\alpha}$–GFP receptor with the plasma membrane. Plasma-membrane-associated fluorescence was generally even in terms of the intensity, although some cells did show a more punctate fluorescence. In addition, spots of intense fluorescence were present in the cytosolic compartment. No fluorescence was detected in the nucleus. In contrast, the fluorescence distribution in cells transfected with GFP alone was diffuse (Figure 2c), and present throughout the cytosol and nucleus, as has been reported previously (e.g. [32]), consistent with GFP itself not being localized to any particular intracellular compartment. No fluorescence was detected in untransfected cells that were observed under the same experimental conditions (Figure 2d).

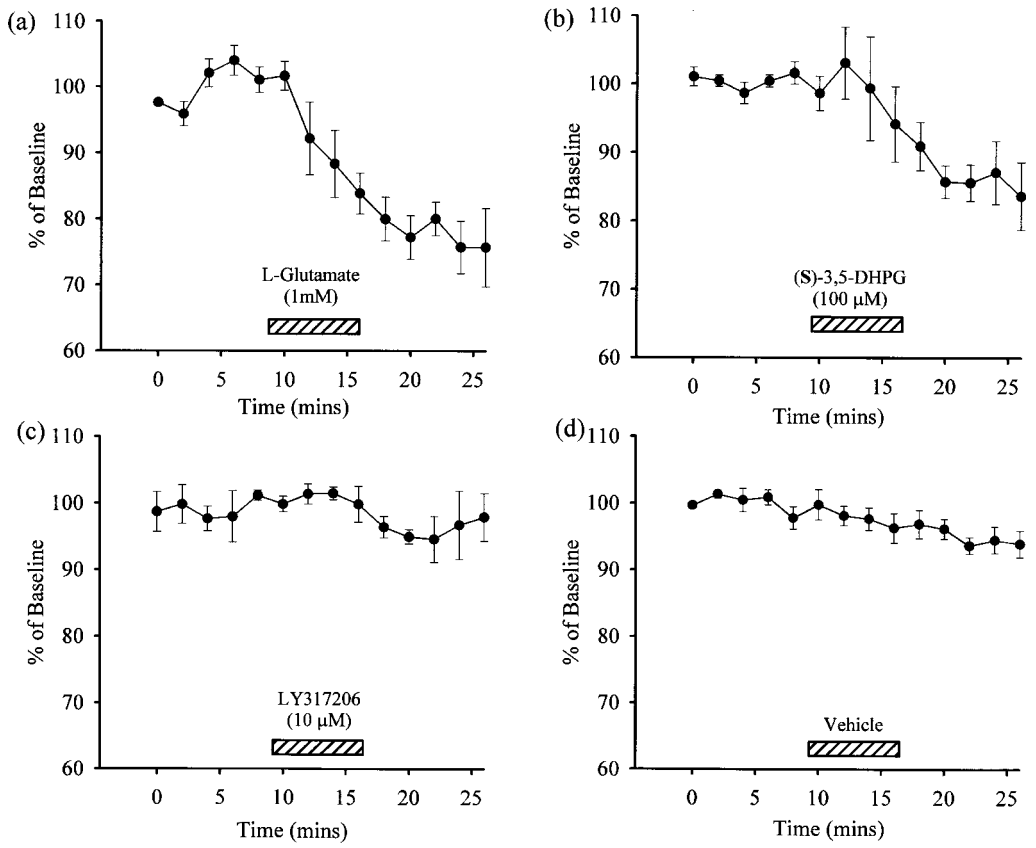

Figure 5 Quantification of the effects of mGlu receptor agonists on membrane-associated fluorescence in HEK-293 cells expressing $mGlu_{1\alpha}$–GFP

Cells were exposed to 1 mM L-glutamate (**a**; $n = 5$), 100 μM (*S*)-DHPG (**b**; $n = 5$), 10 μM LY317206 (**c**; $n = 3$) or vehicle (**d**; $n = 5$). Data are means $\pm$ S.E.M. for cells taken from at least three separate transfections.

Functional characterization of $mGlu_{1\alpha}$–GFP in HEK-293 cells

To determine whether the $mGlu_{1\alpha}$–GFP fusion protein formed a functional receptor, Ca^{2+} release from intracellular stores was investigated in individual cells using the indicator fura-red, which decreases its fluorescence on binding Ca^{2+} (Figure 3a). Responses were obtained with the endogenous ligand, L-glutamate (100 μM), the prototypical, broad-spectrum mGlu receptor agonist (1*S*,3*R*)-ACPD (100 μM) and the group I-specific mGlu receptor agonist (*S*)-DHPG (100 μM). In contrast, the group II and III mGlu-receptor-selective agonists LY317206 (10 μM) and L-AP4 (1 mM) respectively had no effect. No specific agonists for $mGlu_1$ receptors exist, but an $mGlu_5$-selective agonist, (*RS*)-CHPG (1 mM), did not elicit Ca^{2+} release. Dose–response curves for L-glutamate were constructed in $mGlu_{1\alpha}$ and $mGlu_{1\alpha}$–GFP-receptor-expressing cells, and yielded similar EC_{50} values [1.1 ± 0.26 μM and 0.94 ± 0.20 μM respectively ($n = 3$), and results not shown]. Co-application of 1 mM (*S*)-MCPG, a commonly used group I/II mGlu receptor antagonist, induced a parallel shift in the dose–response profile, indicating competitive antagonism. The K_i obtained was determined as 153 ± 19 μM [mean $\pm$ S.E.M. ($n = 3$), and results not shown]. Thus the $mGlu_{1\alpha}$–GFP fusion protein forms a functional receptor, which retains the pharmacology of the wild-type receptor. Fluorescence changes were greatly decreased by treatment with thapsigargin (10 μM; Figure 3b), verifying that the responses were due to Ca^{2+} release from intracellular stores. These results demonstrate that the $mGlu_{1\alpha}$–GFP receptor is able to couple to an effector mechanism in a manner similar to that of wild-type receptors.

Agonist-induced changes in mGlu receptor surface expression and function

Members of other classes of GPCRs internalize on prolonged exposure to agonist (e.g. [33–36]). L-Glutamate (1 mM) over a 10 min period caused a rapid decrease in plasma-membrane-associated fluorescence in $mGlu_{1\alpha}$–GFP receptor-expressing cells (Figure 4). The effect of L-glutamate was associated with a transient change in the shape of the cells, which involved increased crenellation of the cell surface (Figure 4a, panel 5; a movie in both Quicktime and AVI format can be seen at http://www.BiochemJ.org/bj/341/bj3410415add.htm). This morphological change was reversed within 2 min of agonist removal, whereas the membrane-associated fluorescence signal took longer to recover (Figure 4b). Pooled data from five individual cells are shown in Figure 5. L-Glutamate induced a $30 \pm 2\%$ decrease in the plasma-membrane-associated fluorescence, with a half-time ($t_{\frac{1}{2}}$) of 6.7 ± 0.9 min (Figure 5a). The group I mGlu-receptor-specific agonist, (*S*)-DHPG, produced a similar effect (Figure 5b). In contrast, neither the group II-specific agonist LY317206 (Figure 5c) nor buffer vehicle (Figure 5d) had any significant effect. We investigated further the effects of L-glutamate, the endogenous ligand for this receptor. The loss of membrane-associated fluorescence induced by L-glutamate was dose-depe-

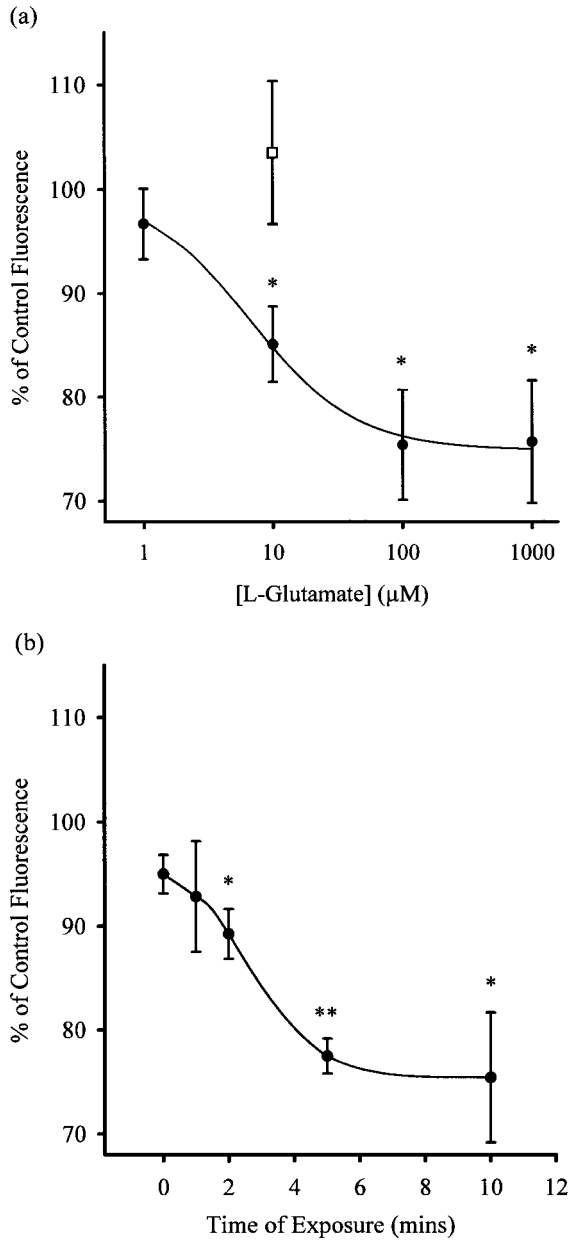

Figure 6 Effect of L-glutamate on membrane-associated fluorescence in HEK-293 cells expressing $mGlu_{1\alpha}$–GFP

(**a**) Concentration response of membrane-associated fluorescence loss. Cells were exposed to increasing concentrations of L-glutamate for 10 min in the absence (●) or presence (□) of 1 mM (*S*)-MCPG. (**b**) Time required to induce receptor internalization was assessed following increasing periods of incubation with 1 mM L-glutamate. The intensity of the membrane fluorescence was measured 10 min after the initial L-glutamate incubation. Loss of fluorescence at zero time thus represents a slow run-down in fluorescence (see Figure 5d). Data shown are means ± S.E.M. ($n = 5$), with cells taken from at least three transfections. $^{*}P < 0.05$; $^{**}P < 0.01$.

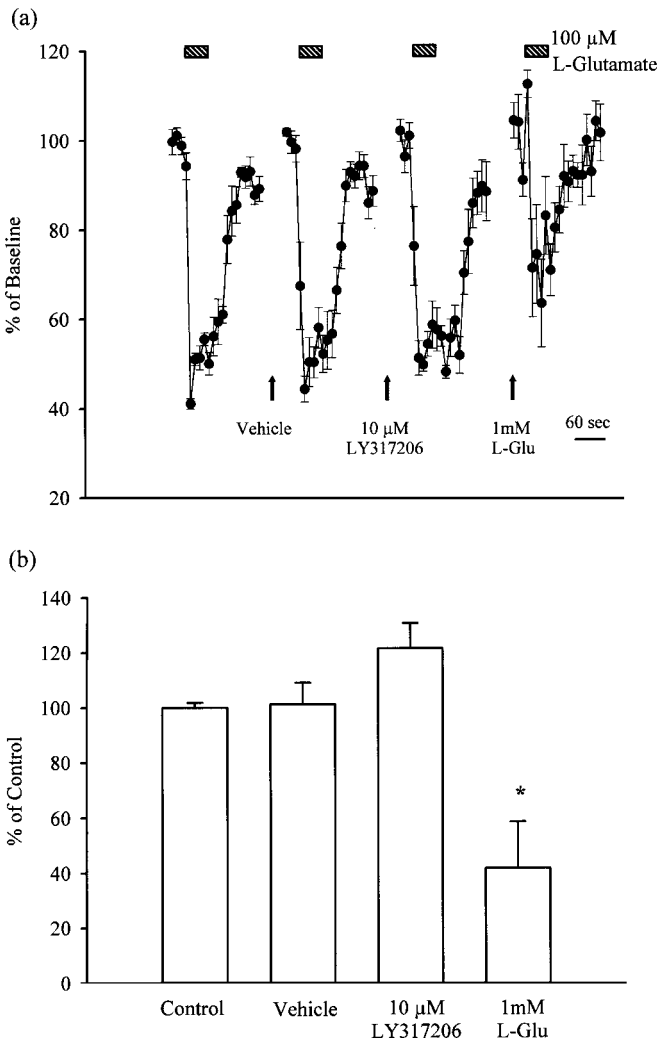

Figure 7 Quantification of the effects of mGlu receptor agonists on functional responses in HEK-293 cells expressing $mGlu_{1\alpha}$–GFP

Ca^{2+} mobilization in response to 100 μM L-glutamate was determined 2 min after 10-min incubations (indicated by filled arrows) with buffer vehicle, LY317206 (10 μM) or L-glutamate (1 mM). (**a**) Pooled responses of five individual cells (means ± S.E.M.) from two transfections. (**b**) Integrated response for the same cells as in (**a**). $^{*}P < 0.05$.

ndent (Figure 6a), with an EC_{50} of 6.7 ± 1.3 μM ($n = 5$). In addition, the internalization induced by 10 μM L-glutamate was completely blocked by co-application of 1 mM (*S*)-MCPG. We also determined the minimum exposure time to 1 mM L-glutamate required to detect a decrease in plasma-membrane-associated fluorescence (Figure 6b). An exposure of 2 min was required to produce a significant decrease (11 ± 2%; $P < 0.05$, $n = 5$), whereas a 5-min exposure resulted in a near-maximal 23 ± 2% ($P < 0.01$, $n = 5$) decrease in membrane-associated fluorescence.

The magnitude and time course of the decrease in plasma-membrane-associated $mGlu_{1\alpha}$–GFP receptor fluorescence is similar to the agonist-induced decrease in cell-surface expression of the alternative splice variant $mGlu_{1\beta}$ receptor immunoreactivity [37] when expressed in BHK cells. To determine whether the agonist-induced decrease in fluorescence observed in the present study was due to the removal of functional receptors from the membrane, we performed further Ca^{2+}-imaging experiments in $mGlu_{1\alpha}$–GFP-expressing cells. Exposure to either vehicle or LY317206 for 10 min did not affect the subsequent ability of L-glutamate to mobilize Ca^{2+} from intracellular stores. In contrast, exposure to L-glutamate caused a decrease in the subsequent Ca^{2+}-mobilizing response to L-glutamate (100 μM; $n = 5$, $P < 0.05$) (Figure 7). In terms of the integral of the Ca^{2+} mobilization, this treatment led to a ≈ 50% decrease in functional response.

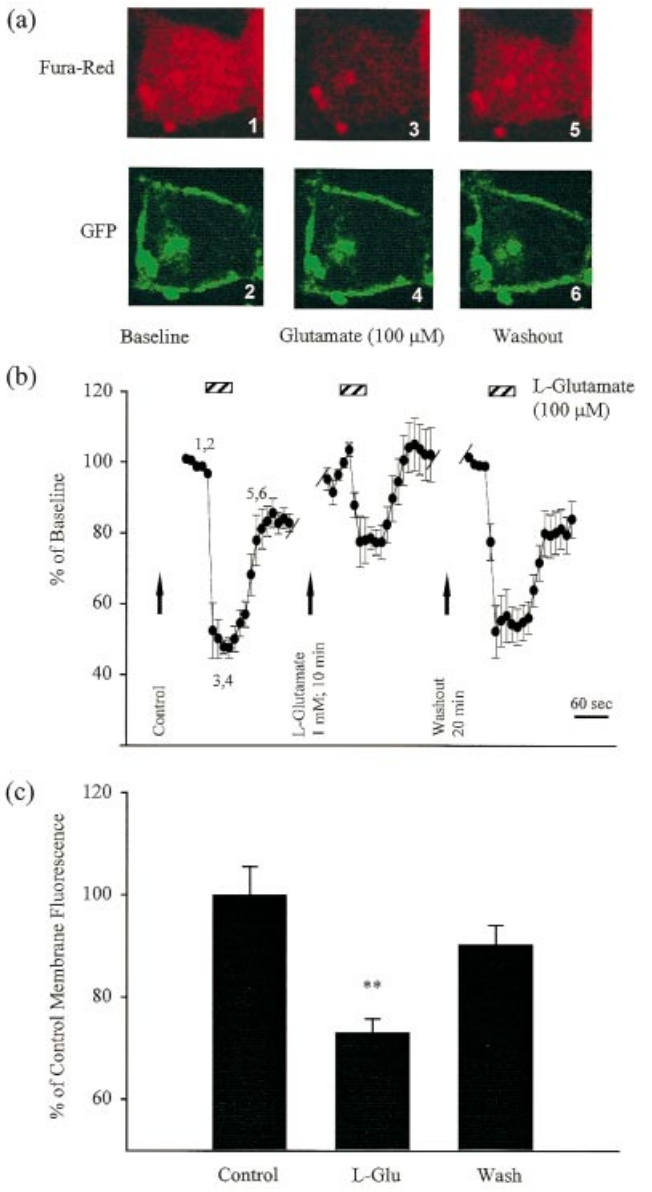

Figure 8 Simultaneous imaging of localization and functional responses of the $mGlu_{1\alpha}$–GFP receptor expressed in HEK-293 cells

(**a**) Images of the Ca^{2+} response measured by fura-red (red) and $mGlu_1$–GFP fluorescence (green) are shown for a cell 20 s before (panels 1,2), 20 s after (panels 3,4) and 100 s after (images 5,6) application of L-glutamate (100 μM). Note that the decrease in fluorescence from fura-red can be measured simultaneously, but independently, of the fluorescence from GFP, which remains unchanged during the functional response. Images are taken at the points indicated during the control response in (**b**). (**b**) Pooled functional response of six cells from three separate transfections (means $\pm$ S.E.M.) imaged as in (**a**) following a 10-min exposure to L-glutamate (1 mM) and following a 20-min period of wash-out. (**c**) Membrane-associated fluorescence for the same cells. $^{**}P < 0.01$.

Simultaneous measurements of receptor movement and function

The relationship between the intensity of plasma-membrane-associated fluorescence and the functional response in individual cells was investigated further by simultaneous measurement of the Ca^{2+} response and $mGlu_{1\alpha}$–GFP fluorescence. The use of fura-red, which has an emission profile that can be separated from GFP, allowed us to undertake these experiments, but did necessitate a slight decrease in image quality. In agreement with the experiments performed separately (Figures 4–7), L-glutamate (1 mM, 10 min) caused a decrease in both membrane fluorescence and the Ca^{2+}-mobilizing response to a subsequent challenge with L-glutamate (Figure 8). In these experiments we also examined whether there was any recovery in either the membrane fluorescence or the functional response following wash-out of L-glutamate; after 20 min of wash-out, there was recovery of both parameters.

Concluding remarks

Our data show that mGlu receptors are rapidly internalized following exposure to agonist, with a time course similar to that of other classes of GPCRs [34–37] and GPCR–GFP fusion constructs [28–30]. This suggests that, despite their structural differences, similar mechanisms might exist to regulate the surface expression of both superfamilies of receptors. The loss of surface $mGlu_{1\alpha}$–GFP also follows a similar time course and magnitude to that of $mGlu_{1\beta}$, as determined by using an N-terminal-directed antibody [37]. Surprisingly, in that study surface expression of $mGlu_{1\alpha}$ was unaffected by agonist exposure. The discrepancy between that study and the data reported here might be due to the different cell lines used (BHK and HEK-293 respectively), since internalization of GPCRs coupled to phospholipase C has previously been shown to be dependent on cell type [36].

The reasons for mGlu receptor internalization in response to agonist application are currently unclear, but one possibility is that rapid internalization could be related to a neuroprotective mechanism. For example, transient ischaemic episodes lead to an accumulation of extracellular L-glutamate in the brain [38], and it is known that blockade of $mGlu_1$ receptor activation can confer protection against post-traumatic neuronal death [17]. Thus the loss of $mGlu_1$ receptors from the plasma membrane might be part of a neuroprotective response to overexcitation of neurones. The ability of mGlu receptors to return to the cell surface following removal of agonist would allow the recovery of physiological function once an insult had passed. A second, non-exclusive possibility is that internalized receptor could serve an as-yet-unidentified signalling function. During synaptic transmission the extracellular concentration of L-glutamate in the vicinity of mGlu receptors probably exceeds 1 mM [39]. However, whether the L-glutamate concentration remains elevated for a sufficiently lengthy period of time under certain physiological conditions, e.g. during repetitive synaptic activation, for significant mGlu receptor internalization to occur has yet to be determined.

This work was supported by the MRC and BIOTEC (grant no. BIO4 CT96 0049). V.C. is an MRC Co-operative Awards in Science and Engineering Ph.D. student in collaboration with Pfizer U.K. Ltd.

REFERENCES

1 Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. and Nakanishi, S. (1991) Nature (London) **349**, 760–765
2 Houamed, K. M., Kuijper, J. L., Gilbert, T. L., Haldeman, B. A., Ohara, P. J., Mulvihill, E. R., Almers, W. and Hagen, F. S. (1991) Science **252**, 1318–1321
3 Pin, J.-P. and Duvoisin, R. (1995) Neuropharmacology **34**, 1–26
4 Conn, P. J. and Pin, J.-P. (1997) Annu. Rev. Pharmacol. Toxicol. **37**, 205–237
5 Kaupmann, K., Huggel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W. and Bettler, B. (1997) Nature (London) **386**, 239–246
6 Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J. and Hebert, S. C. (1993) Nature (London) **366**, 575–580
7 Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1992) J. Biol. Chem. **267**, 13361–13368

8 Baude, A., Nusser, Z., Roberts, J. D. B., Mulvihill, E., McIlhinney, R. A. J. and Somogyi, P. (1993) Neuron **11**, 771–787
9 Luján, R., Nusser, Z., Roberts, J. D. B., Shigemoto, R. and Somogyi, P. (1996) Eur. J. Neurosci. **8**, 1488–1500
10 Petralia, R. S., Wang, Y. X., Singh, S., Wu, C., Shi, L. R., Wei, J. and Wenthold, R. J. (1997) J. Chem. Neuroanat. **13**, 77–93
11 Rubio, M. E. and Wenthold, R. J. (1997) Neuron **18**, 939–950
12 Batchelor, A. M., Knopfel, T., Gasparini, F. and Garthwaite, J. (1997) Neuropharmacology **36**, 401–403
13 Aiba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., Zwingman, T. A. and Tonegawa, S. (1994) Cell **79**, 377–388
14 Aiba, A., Chen, C., Herrup, K., Rosenmund, C., Stevens, C. F. and Tonegawa, S. (1994) Cell **79**, 365–375
15 Conquet, F., Bashir, Z. I., Davies, C. H., Daniel, H., Ferraguti, F., Bordi, F., Franz-Bacon, K., Reggiani, A., Matarese, V., Conde, F., Collingridge, G. L. and Crepel, F. (1994) Nature (London) **372**, 237–243
16 Bordi, F., Reggiani, A. and Conquet, F. (1997) Brain Res. **761**, 121–126
17 Mukhin, A. G., Ivonava, A. and Faden, A. I. (1997) Neuroreport **8**, 2561–2566
18 Heinrich-Noack, P., Hatton, C. D. and Reymann, K. G. (1998) Neuroreport **9**, 985–988
19 Seal, A. J., Collingridge, G. L. and Henley, J. M. (1994) Neuropharmacology **33**, 1065–1070
20 Brakeman, P. R., Lanahan, A. A., O'Brien, R., Roche, K., Barnes, C. A., Huganir, R. L. and Worley, P. F. (1997) Nature (London) **386**, 284–288
21 Xiao, B., Tu, J. C., Petralia, R. S., Yuan, J. P., Doan, A., Breder, C. D., Ruggiero, A., Lanahan, A. A., Wenthold, R. J. and Worley, P. F. (1998) Neuron **21**, 707–716
22 Tu, J. C., Xiao, B., Yuan, J. P., Lanahan, A. A., Leoffert, K., Li, M., Linden, D. J. and Worley, P. F. (1998) Neuron **21**, 717–726
23 Georget, V., Lobaccaro, J. M., Terouanne, B., Mangeat, P., Nicolas, J. C. and Sultan, C. (1997) Mol. Cell. Endocrinol. **129**, 17–26
24 Sakai, N., Sasaki, K., Ikegaki, N., Shirai, Y., Ono, Y. and Saito, N. (1997) J. Cell Biol. **139**, 1465–1476
25 Barak, L. S., Ferguson, S. S., Zhang, J. and Caron, M. G. (1997) J. Biol. Chem. **272**, 27497–27500
26 Feng, X., Zhang, J., Barak, L. S., Meyer, T., Caron, M. C. and Hannun, Y. A. (1998) J. Biol. Chem. **273**, 10755–10762
27 Barak, L. S., Ferguson, S. S. G., Zhang, J., Martenson, C., Meyer, T. and Caron, M. G. (1997) Mol. Pharmacol. **51**, 177–184
28 Kallal, L., Gagnon, A. W., Penn, R. B. and Benovic, J. L. (1998) J. Biol. Chem. **273**, 322–328
29 Tarasova, N. I., Stauber, R. H., Choi, J. K., Hudson, E. A., Czerwinski, G., Miller, J. L., Pavlakis, G. N., Michejda, C. J. and Wank, S. A. (1997) J. Biol. Chem. **272**, 14817–14824
30 Wessel, D. and Flugge, U. I. (1984) Anal. Biochem. **138**, 141–143
31 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
32 John, S. A., Monck, J. R., Weiss, J. N. and Ribalet, B. (1998) J. Physiol. **510**, 333–345
33 Koenig, J. A. and Edwardson, J. M. (1994) J. Biol. Chem. **269**, 17174–17182
34 Barak, L. S., Menard, L., Ferguson, S. S. G., Colapietro, A. and Caron, M. G. (1995) Biochemistry **34**, 15407–15414
35 von Kastrow, M. and Kobilka, B. M. (1994) J. Biol. Chem. **269**, 18448–18452
36 Koenig, J. A. and Edwardson, J. M. (1996) Mol. Pharmacol. **49**, 351–359
37 Ciruela, F. and McIlhinney, R. A. J. (1997) FEBS Lett. **418**, 83–86
38 Arvin, B., Moncada, C., LePeillet, E., Chapman, A. and Meldrum, B. S. (1992) NeuroReport **3**, 235–238
39 Clements, J. D., Lester, R. A. J., Tong, G., Jahr, C. E. and Westbrook, G. L. (1992) Science **258**, 1498–1501

Received 11 January 1998/30 March 1999; accepted 30 April 1999