Reassessment of the Ca\textsuperscript{2+} Sensing Property of a Type I Metabotropic Glutamate Receptor by Simultaneous Measurement of Inositol 1,4,5-Trisphosphate and Ca\textsuperscript{2+} in Single Cells*

Mark S. Nash‡, Ruth Saunders§, Kenneth W. Young, R. A. John Challiss, and Stefan R. Nahorski

From the Department of Cell Physiology and Pharmacology, Medical Sciences Building, University of Leicester, P. O. Box 138, University Road, Leicester, LE1 9HN, United Kingdom

Transient transfection of Chinese hamster ovary or baby hamster kidney cells expressing the Group I metabotropic glutamate receptor mGlu\textsubscript{1}\alpha with green fluorescent protein-tagged pleckstrin homology domain of phospholipase C\textsubscript{8i} allows real-time detection of inositol 1,4,5-trisphosphate. Loading with Fura-2 enables simultaneous measurement of intracellular Ca\textsuperscript{2+} within the same cell. Using this technique we have studied the extracellular calcium-sensing property of the mGlu\textsubscript{1}\alpha receptor. Quisqualate, in extracellular medium containing 1.3 mM Ca\textsuperscript{2+}, increased inositol 1,4,5-trisphosphate in all cells. This followed a typical peak and plateau pattern and was paralleled by concurrent increases in intracellular Ca\textsuperscript{2+} concentration. Under nominally Ca\textsuperscript{2+}-free conditions similar initial peaks in inositol 1,4,5-trisphosphate and Ca\textsuperscript{2+} concentration occurred with little change in either agonist potency or efficacy. However, sustained inositol 1,4,5-trisphosphate production was substantially reduced and the plateau in Ca\textsuperscript{2+} concentration absent. Depletion of intracellular Ca\textsuperscript{2+} stores using thapsigargin abolished quisqualate-induced increases in intracellular Ca\textsuperscript{2+} and markedly reduced inositol 1,4,5-trisphosphate production. These data suggest that the mGlu\textsubscript{1}\alpha receptor is not a calcium-sensing receptor because the initial response to agonist is not sensitive to extracellular Ca\textsuperscript{2+} concentration. However, prolonged activation of phospholipase C requires extracellular Ca\textsuperscript{2+}, while the initial burst of activity is highly dependent on Ca\textsuperscript{2+} mobilization from intracellular stores.

The Group I subfamily of metabotropic glutamate receptors, mGlu\textsubscript{1} and mGlu\textsubscript{5}, couple to phospholipase C (PLC) via G\textsubscript{q/11} proteins to stimulate inositol 1,4,5-trisphosphate (IP\textsubscript{3}) production and to mobilize intracellular calcium (Ca\textsuperscript{2+}) stores (1, 2). Recently, the close structural similarity of the amino-terminal domain of mGlu receptors with that of the calcium-sensing receptor led to the suggestion that mGlu receptors may also respond to changes in extracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{o}) (3–5). Thus, Ca\textsuperscript{2+}\textsubscript{o} has been reported to both potentiate the stimulation of inositol phosphate (InsP) accumulation by the mGlu\textsubscript{1}\alpha receptor (4) as well as directly activate both Group I mGlu receptors (5). Since Ca\textsuperscript{2+}\textsubscript{o} also potentiates agonist binding and potency at GABA\textsubscript{B} receptors (6, 7) Ca\textsuperscript{2+}\textsubscript{o} sensing may, in fact, be a general property of family 3 G protein-coupled receptors.

The ability to respond to changes in Ca\textsuperscript{2+}\textsubscript{o} has profound implications for receptor signaling in the central nervous system where local fluctuations in synaptic [Ca\textsuperscript{2+}]\textsubscript{i} can occur as a result of activation of calcium permeant cation channels (8). Moreover, a novel form of intercellular communication has recently been identified where Ca\textsuperscript{2+} extruded from one cell, following agonist-driven Ca\textsuperscript{2+}\textsubscript{i} mobilization, stimulates neighboring cells expressing calcium-sensing receptors (9). Since mGlu receptors are expressed widely in the central nervous system this form of communication may have important consequences for our understanding of neuronal and glial cell interactions. There is, however, indirect evidence against the Ca\textsuperscript{2+}\textsubscript{o} sensing property of Group I mGlu receptors. Several studies have determined the origin of mGlu receptor-mediated Ca\textsuperscript{2+}\textsubscript{i} responses; intracellular store release or extracellular Ca\textsuperscript{2+} entry, by removing Ca\textsuperscript{2+}\textsubscript{o}. From these, mGlu5 receptor-mediated Ca\textsuperscript{2+}\textsubscript{i} release in astrocytes, cortical neurons (10–12), and HEK-293 (13), and initial mGlu1a receptor responses in HEK-293 (14) and A9 cells (15) were reported to be unaffected by removing Ca\textsuperscript{2+}\textsubscript{o}. Moreover, the recently published x-ray crystallography structure of the NH\textsubscript{2} terminus of the mGlu1a receptor identified a high affinity cation-biding site, which suggests that Ca\textsuperscript{2+} is more likely to be a “scaffold factor” rather than a physiological ligand (16). Given the potential importance of Ca\textsuperscript{2+}\textsubscript{o} sensing by these receptors such a fundamental question concerning their activation requires an unambiguous answer.

To address this issue we have made real-time concurrent measurements of IP\textsubscript{3} and Ca\textsuperscript{2+}, in single cells expressing the mGlu\textsubscript{1}\alpha receptor using a recently developed technique that utilizes an enhanced green fluorescent protein-tagged pleckstrin homology domain of phospholipase C\textsubscript{8i} (eGFP-PH\textsubscript{PLC\textsubscript{8i}}) to detect IP\textsubscript{3} in Fura-2 loaded cells (17–19). PH\textsubscript{PLC\textsubscript{8i}} binds selectively to phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) over all other inositol lipids (20) and a fusion construct of this PH domain cells expressing mGlu1a receptors; [3H]InsP\textsubscript{3}, [3H]inositol phosphates; KHB, Krebs-Henseleit buffer; PH, pleckstrin homology.

* This work was supported by Programme Grants 16895 and 062495/z/00, and an Equipment Grant (061050/z/00) from the Wellcome Trust.
‡ To whom correspondence should be addressed. Tel.: 0116-252-3075; Fax: 0116-252-5045; E-mail: msn2@le.ac.uk.
§ Supported by a Medical Research Council of Great Britain Studentship.
\* The abbreviations used are: mGlu1, metabotropic glutamate receptor type 1; mGlu5, metabotropic glutamate receptor type 5; PLC, phospholipase C; G proteins, heterotrimeric GTP-binding regulatory proteins; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; Ca\textsuperscript{2+}, intracellular calcium; [Ca\textsuperscript{2+}]\textsubscript{i}, extracellular calcium concentration; eGFP-PH\textsubscript{PLC\textsubscript{8i}}, enhanced green fluorescent protein-tagged pleckstrin homology domain of phospholipase C\textsubscript{8i}; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; CHO-mGlu1a, Chinese hamster ovary cells expressing mGlu1a receptors; IPTG, isopropyl-\beta-D-thiogalactoside; BHK-mGlu1a, baby hamster kidney cells expressing mGlu1a receptors; [3H]InsP\textsubscript{3}, [3H]inositol phosphates; KHB, Krebs-Henseleit buffer; PH, pleckstrin homology.
domain with eGFP associates with the plasma membrane (18, 19). Hirose et al. (17) recently demonstrated that PHPLCα has higher affinity for the soluble head group of PI(3,4,5)P3, IP3, and that PLC-induced elevations in IP3 cause translocation of the fusion protein to the cytosol. The extent of membrane association of eGFP-PHPLCα can thus be used to evaluate cellular IP3 levels and, by preloading with Fura-2, simultaneous measurements of [Ca2+]i are possible in the same cell. This technique overcomes many of the inherent pitfalls in other assays by allowing simultaneous temporal analysis of two crucial indices of PLC signaling in single cells rather than in cell populations. Our data clearly indicate that the initial response to mGlu1a receptor activation is not sensitive to Ca2+i, demonstrating that it is not a true Ca2+i-sensing receptor. However, for prolonged IP3 production Ca2+i entry is required. Moreover, Ca2+i mobilization from intracellular stores by IP3 is found to be essential for amplification of the initial response to agonist.

EXPERIMENTAL PROCEDURES

Materials—Vector containing the fusion construct between eGFP and the PH domain of PLCα1 was kindly provided by Professor T. Meyer (State University of New York at Stony Brook, NY). Detailed information regarding this construct can be found in Stauffer et al. (18). Standard chemicals and biochemicals were from Sigma (Poole, UK) unless otherwise indicated. Radiochemicals were from Amersham Pharmacia Biotech (Amersham, United Kingdom). Quisqualate and 15,3-ACPD were from Tocris-Cookson (Bristol, UK). Fura-2 AM was from Molecular Probes (Cambridge, UK) and Dowex anion exchange resin AG1-X8 (200–400 mesh, 400–500 mesh) from Bio-Rad (Watford, UK). All materials for cell culture were supplied by Life Technologies, Inc. (Paisley, UK).

Cell Culture—A description of the LacsSwitch inducible expression system (Stratagene) used to express human mGlu1a in Chinese hamster ovary cells (CHO-lac-mGlu1a) is provided elsewhere (21, 22). For these studies on the CHO-lac-mGlu1a cells, maximal levels of receptor expression were used throughout and achieved by preincubation with 100 μM IPTG for greater than 18 h (21). This results in expression levels of ~50,000 receptors per cell (400 fmol/mg protein). To minimize the exposure to glutamate, CHO-lac-mGlu1a cells were grown in Dulbecco’s modified Eagle’s medium with Glutamax™ containing 10% fetal calf serum, proline (44 mg/ml), fungizone (2.5 mg/ml), penicillin (10 units/ml), streptomycin (100 μg/ml), and pyruvate (5 mM) was added to the medium. Agonist-induced cell death was prevented by the addition of 25 μg/ml of heparin. The extent of membrane association of mGlu1a receptors demonstrated sensitivity of these receptors to Ca2+i (4). Similar findings were also obtained for CHO cells expressing mGlu1a (Fig. 1). Importantly, we demonstrate here that varying the [Ca2+]i, in the absence of quisqualate, from 0 to 4 mM had no effect itself on the accumulation of [3H]InsP. This differs from the work of Kubo et al. (5), which suggested that calcium was an agonist at Group I mGlu receptors. The data do, however, support the view that Ca2+i potentiates the response of mGlu1a receptors to agonist. In nominally [Ca2+]i-free medium minimal stimulation of [3H]InsP accumulation occurred in CHO-lac-mGlu1a cells exposed to either the Group I mGlu receptor agonist agonist quisqualate or the partial agonist 1RS-ACPD (Fig. 1A). As the [Ca2+]i was raised, [3H]InsP accumulation increased up to a maximum at 1.3 mM [Ca2+]i. This effect was more marked for the partial agonist 15RS-ACPD (Fig. 1A). These data were determined in a separate series of concentration dependence experiments in 1.3 mM [Ca2+]i, containing KHB and were −6.41 ± 0.08 (log EC50 (M), n = 4, Emax = −19.2-fold) for quisqualate and −4.6 ± 0.11 (log EC50 (M), n = 3, Emax = −16.9-fold) for 15RS-ACPD. The effect was not due to a reduction in initial PIP2 levels because comparable levels for the total membrane phosphoinositides were detected for cells at 1.3 mM [Ca2+]i (data not shown). The cellular levels were unaffected by treatment with 10 μM quisqualate in nominal [Ca2+]i, but reduced by 30% in cells in 1.3 mM [Ca2+]i, which represents InsP formation by hydrolysis of PIP2. The combined data thus suggest that maximal receptor activation of PLC requires the presence of both quisqualate and Ca2+i. This supports the view that the mGlu1a receptor is an extracellular calcium-sensing

mGlu1α receptors do Not Sense Extracellular Ca2+.  

**Initial Response to Agonist Is Not Dependent on Extracellular Calcium**—When the translocation of eGFP-PH<sub>PLC<i></i></sub>B to the cytoplasm (Fig. 2) is graphically represented as the ratio of fluorescence to the basal against time, IP<i><i></i></i><sub>3</sub> production is found to follow a clear peak and plateau pattern (Fig. 3). In the nominal absence of Ca<sup>2+</sup> (determined to be <1 μM free Ca<sup>2+</sup>)), the initial peak is similar, but sustained IP<sub>3</sub> production is markedly lower than in the presence of 1.3 mM Ca<sup>2+</sup>. Analysis of the combined data from 23 cells revealed that while the peak response to 10 μM quisqualate is unchanged the plateau level (determined at 200 s) is decreased by 72 ± 3% (Table I). Similar data were obtained when CHO-lac-mGlu1α cells were challenged with the submaximal quisqualate concentration of 1 μM (Table I) with a decrease in sustained production of 81 ± 3% in the absence of Ca<sup>2+</sup>. The relatively slight decrease in peak response observed at this concentration can be attributed to small amounts of store depletion during the wash phase.

For the dual imaging experiments plotting the changes in cytosolic eGFP-PH<sub>PLC<i></i></sub>B fluorescence and [Ca<sup>2+</sup>]<sub>i</sub>, against time revealed the remarkably similar responses to prolonged quisqualate (10 μM) treatment. An initial peak in IP<sub>3</sub> production followed agonist addition, which fell to a steady state level, and this was mirrored closely by changes in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 4A). In the nominal absence of Ca<sup>2+</sup>, the initial peaks in IP<sub>3</sub> (expressed as a fluorescent ratio relative to basal, 1.28 ± 0.03 versus 1.33 ± 0.04 in 1.3 mM [Ca<sup>2+</sup>]<sub>i</sub>) and [Ca<sup>2+</sup>]<sub>i</sub> (601 ± 35 versus 465 ± 37 nM in 1.3 mM [Ca<sup>2+</sup>]<sub>i</sub>) were unchanged (Fig. 4B). However, the secondary plateau phase (measured at 200 s) of [Ca<sup>2+</sup>]<sub>i</sub>, was absent (7 ± 4 nM versus 214 ± 23 nM in 1.3 mM [Ca<sup>2+</sup>]<sub>i</sub>) and, although the peak in IP<sub>3</sub> production was clearly more prolonged than for [Ca<sup>2+</sup>]<sub>i</sub>, by 200 s it had returned to near basal levels (1.04 ± 0.02 versus 1.12 ± 0.03 in 1.3 mM [Ca<sup>2+</sup>]<sub>i</sub>) (Fig. 4B). Concentration-dependent effects of quisqualate on IP<sub>3</sub> pro-

---

**Fig. 1**. Effect of Ca<sup>2+</sup><sub>i</sub> on stimulation of [H][InsP accumulation and Ca<sup>2+</sup> mobilization by mGlu1α receptors. A, CHO-lac-mGlu1α cells were preincubated in KHB containing the indicated [Ca<sup>2+</sup>]o for 30 min before challenging with 30 μM quisqualate (●), 1 mM ACPD (○), or 30 μM quisqualate (●) for an additional 15 min. LiCl (10 mM) was added 10 min before addition of the agonists. Basal stimulation was addition of KHB alone (●). Data are expressed as mean ± S.E.M. from four separate experiments. B, representative trace for a single CHO-lac-mGlu1α cell challenged for 5 min with 3 μM quisqualate in the presence of the indicated [Ca<sup>2+</sup>]o. Cells were pre-treated with the appropriate concentration for 2 min before and after agonist challenge and perfused with KHB containing 1.3 mM CaCl<sub>2</sub> for 15 min between each treatment. The trace has been edited to show only the period of agonist exposure for clarity.

---

**Differential Sensitivity of Peak and Plateau Quisqualate Ca<sup>2+</sup> Responses to [Ca<sup>2+</sup>]<sub>i</sub>**—To study this phenomenon further, measurements of agonist-induced effects on [Ca<sup>2+</sup>]o, at the mGlu1α receptor were made (Fig. 1). A representative trace from a single CHO-lac-mGlu1α cell shows the result of a sustained challenge with 3 μM quisqualate (Fig. 1B). The characteristic response, represented by the effect in 1.3 mM Ca<sup>2+</sup> containing buffer, is a rapid initial peak due to release of Ca<sup>2+</sup> from intracellular stores followed by a sustained phase of Ca<sup>2+</sup><sub>i</sub> entry. Quisqualate dose dependently stimulated this initial increase in peak [Ca<sup>2+</sup>]o, with a log EC<sub>50</sub> (μM) value of −6.9 ± 0.1. The trace (Fig. 1B) illustrates the typical effect of varying [Ca<sup>2+</sup>]<sub>i</sub>, on both the peak and sustained phase of the response to maximal agonist concentration. Ca<sup>2+</sup> o failed to influence the peak response to agonist challenge but increased [Ca<sup>2+</sup>]o, during the sustained phase. In at least six separate experiments, no effect of modifying [Ca<sup>2+</sup>]o, on peak response of mGlu1α receptors could be observed. However, the plateau [Ca<sup>2+</sup>]o, was consistently augmented by increasing [Ca<sup>2+</sup>]o, Similar findings were obtained when submaximal concentrations of quisqualate (0.7 μM) were used (data not shown). Moreover, variations in [Ca<sup>2+</sup>]o, have similar effects on agonist-induced plateau levels of [Ca<sup>2+</sup>]o, in CHO cells expressing m3 muscarinic receptors. These results are incompatible with the view that mGlu1α receptors are calcium-sensing receptors.

**Single Cell Measurement of IP<sub>3</sub>**—A confocal image through the mid-section of CHO-lac-mGlu1α cells 48 h after transfection confirmed the localization of eGFP-PH<sub>PLC<i></i></sub>B to the plasma membrane (Fig. 2, A and D). The majority of the fluorescent signal was concentrated over the periphery of the cells, with only a small signal detected in the cytosol. In control cells expressing eGFP alone the signal was located over the cytosol (data not shown). This is consistent with previous work (17–19) demonstrating the association of the PH<sub>PLC<i></i></sub>B domain with plasma membrane PIP<sub>2</sub>. Challenge with 10 μM quisqualate in KHB containing 1.3 mM Ca<sup>2+</sup> induced translocation of the fusion protein to the cytosol (Fig. 2B) and this decreased slightly during sustained exposure to the agonist (Fig. 2C). In the nominal absence of Ca<sup>2+</sup>, the initial peak response to 10 μM quisqualate challenge was similar (Fig. 2E), however, the plateau level of cytosolic fluorescence was markedly lower than in the presence of 1.3 mM Ca<sup>2+</sup> (Fig. 2F).

Using an epifluorescence microscope the halo of eGFP fluorescence around the transfected CHO-lac-mGlu1α cells was still evident (Fig. 2J). A pseudo-color representation of the [Ca<sup>2+</sup>]o, determined for these resting cells is shown in Fig. 2G. Upon addition of quisqualate (10 μM) a clear increase in the [Ca<sup>2+</sup>]o, occurred (Fig. 2H), which returned to a lower sustained level in the continued presence of the agonist (Fig. 2I). Concurrent with these increases in [Ca<sup>2+</sup>]o, agonist-induced translocation of the eGFP signal to the cytoplasm was observed (Fig. 2K) and this also decreased during persistent agonist exposure (Fig. 2L).

---

Fig. 2. Imaging of agonist-induced changes in \([\text{Ca}^{2+}]_o\) and eGFP-PH\(_{PLC\alpha}\) localization in individual CHO-lac-mGlu1\(\alpha\) cells. Confocal images (A-F) of CHO-lac-mGlu1 cells transiently transfected with eGFP-PH\(_{PLC\alpha}\) and challenged with 10 \(\mu\)M quisqualate in the presence of 1.3 mM \([\text{Ca}^{2+}]_o\) (A-C) or nominal (D-F) calcium. Basal (A and D), peak (B and E), and sustained (C and F) responses to agonist are shown. G-L shows dual imaging experiments to detect changes in both [Ca\(^{2+}\)\(_o\)] and [IP\(_3\)] in CHO-lac-mGlu1\(\alpha\) cells transfected with eGFP-PH\(_{PLC\alpha}\) and loaded with Fura-2. G-I, pseudocolor images of [Ca\(^{2+}\)\(_o\)] before (G), 10 s after (H), and ~2 min after (I) prolonged perfusion with 10 \(\mu\)M quisqualate. The eGFP fluorescence associated with this same group of CHO-lac-mGlu1\(\alpha\) cells at the same times is shown in J-L.

Fig. 3. Effect of Ca\(^{2+}\)\(_o\) on mGlu1\(\alpha\) receptor-mediated IP\(_3\) production in a single CHO-lac-mGlu1\(\alpha\) cell. Representative traces showing quisqualate (10 \(\mu\)M) (indicated by the dashed line) mediated IP\(_3\) production in a single CHO-lac-mGlu1\(\alpha\) cell in the presence of 1.3 mM (solid line) or nominal (dotted line) calcium containing KHB. After perfusion with KHB containing nominal [Ca\(^{2+}\)\(_o\)] the cell was challenged with quisqualate in KHB containing the same [Ca\(^{2+}\)\(_o\)] for 3 min and then washed free of agonist. After washing in KHB containing 1.3 mM Ca\(^{2+}\) for 15 min the cell was rechallenged with quisqualate using the same protocol in the presence of 1.3 mM Ca\(^{2+}\)\(_o\). Data are shown as the ratio of cytosolic eGFP fluorescence at each time point relative to the basal.

### Table I

*Effect of Ca\(^{2+}\)\(_o\) on agonist-induced eGFP-PH\(_{PLC\alpha}\) translocation in CHO-lac-mGlu1\(\alpha\)*

<table>
<thead>
<tr>
<th>Challenge</th>
<th>eGFP-PH(_{PLC\alpha}) fluorescence relative to basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 (\mu)M Quisqualate:</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>3.09 ± 0.11</td>
</tr>
<tr>
<td>Plateau</td>
<td>2.45 ± 0.08</td>
</tr>
<tr>
<td>1 (\mu)M Quisqualate:</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>2.81 ± 0.11</td>
</tr>
<tr>
<td>Plateau</td>
<td>2.23 ± 0.10</td>
</tr>
</tbody>
</table>

\(*p < 0.05\) compared to levels for 1.3 mM [Ca\(^{2+}\)\(_o\)], using Student’s \(t\) test.

The data in Table I show that the initial peak fluorescence was comparable in 1.3 mM [Ca\(^{2+}\)\(_o\)] and 1.3 mM [Ca\(^{2+}\)\(_o\)] cells, but the peak [Ca\(^{2+}\)\(_o\)] was lower in the nominal Ca\(^{2+}\)\(_o\) condition. The peak fluorescence for the nominal condition was significantly lower than for 1.3 mM [Ca\(^{2+}\)\(_o\)]. The plateau fluorescence was similar in both conditions.

Moreover, the peak fluorescence was significantly higher in the 1.3 mM [Ca\(^{2+}\)\(_o\)] condition, and the plateau fluorescence was similar in both conditions.

### Discussion

The data obtained from analysis of peak heights and plateau levels thus argue very strongly for a failure of extracellular calcium to influence the initial response of PLC to mGlu1\(\alpha\) receptor activation. However, removing Ca\(^{2+}\)\(_o\) reduces the sustained component of IP\(_3\) production.

To eliminate the possibility that Ca\(^{2+}\)\(_o\) altered the dynamics of the response to agonist challenge the times at which the signals peaked were determined. No difference was detected in the point at which IP\(_3\) or Ca\(^{2+}\)\(_o\) concentration started to rise in the presence or absence of Ca\(^{2+}\)\(_o\). Moreover, the time at which the response peaked was unaffected by removing Ca\(^{2+}\)\(_o\) with peak levels of IP\(_3\) observed 14.3 ± 0.9 and 13.9 ± 1.1 s.
after the initial rise in the presence and absence of Ca\(^{2+}\)_o, respectively. Peak Ca\(^{2+}\)_i levels were detected 8.5 ± 0.5 and 9.8 ± 0.9 s after the initiation of the response in 1.3 mM and nominal [Ca\(^{2+}\)]_i, respectively. The values represent mean time (± S.E.M.) from analysis of more than 17 cells exposed to 10 μM quisqualate. Therefore, Ca\(^{2+}\)_o does not regulate the rate at which the receptor is able to activate PLC after agonist binding or affect the speed of release of Ca\(^{2+}\) from intracellular stores. The rates at which the peak IP\(_3\) and Ca\(^{2+}\) responses declined were, however, dependent on Ca\(^{2+}\)_o with an increased rate evident for both parameters. It is noteworthy that the effect of nominally Ca\(^{2+}\) free conditions was more pronounced on the rate at which [Ca\(^{2+}\)]_i declines than on that for IP\(_3\) such that in the absence of Ca\(^{2+}\)_o, the increase in IP\(_3\) is far more prolonged than that of [Ca\(^{2+}\)]_i, (graphically evident in Fig. 4B).

Depletion of Intracellular Stores Attenuates the Initial Response—Since Ca\(^{2+}\)_o, failed to influence the early receptor-induced events it was interesting to determine whether Ca\(^{2+}\) released from the intracellular stores affected mGlu1a receptor signaling. Control cells that were perfused with KHB containing no Ca\(^{2+}\) for 10 min and then challenged with 10 μM quisqualate (Fig. 5A) responded with a transient peak in [Ca\(^{2+}\)]_i (392 ± 42 nM above basal, n = 22) and a relatively more pronounced peak in IP\(_3\) (1.40 ± 0.05, n = 20, fluorescent ratio relative to basal). When cells were first challenged with 2 μM thapsigargin (Fig. 5B) there was an increase in [Ca\(^{2+}\)]_i (249 ± 23 nM above basal, n = 9), which steadily returned to basal as the intracellular Ca\(^{2+}\) stores depleted due to inhibition of the Ca\(^{2+}\)-ATPase. Importantly, no effect on IP\(_3\) synthesis was detected (1.02 ± 0.01, n = 9) indicating that store Ca\(^{2+}\) does not stimulate PLC in the absence of receptor activation. When quisqualate was then perfused over these cells there was no change in [Ca\(^{2+}\)]_i (6 ± 3 nM, n = 22) and only a very small increase in IP\(_3\) production (1.11 ± 0.03, n = 22).

Raising [Ca\(^{2+}\)]_o Differentially Affects IP\(_3\) Production and [Ca\(^{2+}\)]_i Mobilization—Perfusion of CHO-lac-mGlu1a cells with KHB (1.3 mM Ca\(^{2+}\)) followed by a 4-min challenge with quisqualate (10 μM) in KHB containing either 1.3 or 2.6 mM Ca\(^{2+}\) revealed an interesting discrepancy between the effect of Ca\(^{2+}\)_o on IP\(_3\) production and [Ca\(^{2+}\)]_i, (Fig. 6). No difference in the peak height or plateau level of translocation of eGFP-PH\(_{PLC_\delta}\) fluorescence to the cytosol was observed in 1.3 or 2.6 mM [Ca\(^{2+}\)]_o (Fig. 6A). In contrast, an increase in [Ca\(^{2+}\)]_i, for both parameters after quisqualate challenge was detected (Fig. 6B). Increasing Ca\(^{2+}\)_o alone had no effect on the [Ca\(^{2+}\)]_i, (data not shown).

Rat mGlu1a Receptor Responds to Ca\(^{2+}\)_o, in a Similar Manner to Human mGlu1a Receptor—To eliminate the possibility that the Ca\(^{2+}\)_o, sensing property of mGlu1a receptors reflects species differences since CHO-lac-mGlu1a cells express human receptors rather than the rat receptor used previously (4, 5) similar experiments were performed on BHK-mGlu1a cells. Transient transfection with eGFP-PH\(_{PLC_\delta}\) resulted in the enrichment of fluorescence over the plasma membrane (Fig. 7A). Challenge with 1 μM quisqualate induced translocation of the fusion protein to the cytosol and this decreased during sustained agonist exposure (Fig. 7A). In the absence of added Ca\(^{2+}\) the same initial peak response was recorded but sustained

Fig. 4. Comparison of mGlu1a receptor-induced IP\(_3\) production and Ca\(^{2+}\)_i mobilization in nominal and 1.3 mM Ca\(^{2+}\)_o. Representative traces of simultaneous measurements of [Ca\(^{2+}\)]_i, (dotted lines) and cytosolic eGFP-PH\(_{PLC_\delta}\) fluorescence (solid lines) in single CHO-lac-mGlu1a cells exposed to quisqualate (10 μM) (indicated by the dashed lines) in KHB containing 1.3 mM (A) or nominal (B) extracellular calcium. Cells were perfused for 3 min (5 ml/min) with KHB containing no added Ca\(^{2+}\) or 1.3 mM CaCl\(_2\) prior to perfusion with quisqualate in the appropriate KHB solution. C and D, dose-response curves for the changes in [Ca\(^{2+}\)]_i, (dotted lines) and the eGFP-PH\(_{PLC_\delta}\) ratio (solid lines) in 1.3 mM (C) and nominal (D) [Ca\(^{2+}\)]_i. Peak (solid symbols) and plateau (open symbols) levels are shown for the increase in both [Ca\(^{2+}\)]_i above basal (solid lines) (N.B the values for eGFP-PH\(_{PLC_\delta}\) translocation are significantly smaller than those obtained confocally because of interference from different focal planes increases the background fluorescence in these non-confocal experiments.
For the purposes of this study nominal $[\text{Ca}^{2+}]$, was used because this maintains the integrity of the intracellular $\text{Ca}^{2+}$ pool for longer compared with chelation of $\text{Ca}^{2+}$ with EGTA, which can rapidly cause store depletion (25). The failure of $\text{Ca}^{2+}$, to affect the initial events in mGlu1 receptor signaling suggests that the early processes involved in receptor activation, i.e. agonist binding, coupling to the G proteins, PLC$\beta$ activation and IP$_3$ synthesis and release of $\text{Ca}^{2+}$ from intracellular stores, occur independently of $\text{Ca}^{2+}$. Clearly this differs from the activation of the calcium-sensing receptor where $\text{Ca}^{2+}$ binding stimulates IP$_3$ production, rapid $\text{Ca}^{2+}$, transients, and coupling to Go$_{q11}$ (26–28).

In contrast, sustained increases in CHO-lac-mGlu1a for both $[\text{Ca}^{2+}]$, and $[\text{IP}_3]$, were highly dependent upon $\text{Ca}^{2+}$.o. This we believe offers the most probable explanation for the discrepancy between the InsP accumulation assays and the real-time analysis of IP$_3$. Obviously, the contribution of the secondary phase of IP$_3$ production to accumulative measurements made over 15 min is much greater than the initial transient peak IP$_3$ synthesis, which is lost within 2–3 min. Sustained elevations in $\text{Ca}^{2+}$, during prolonged agonist challenge commonly arise from opening of plasma membrane capacitative $\text{Ca}^{2+}$ channels, possibly synonymous with Trp channels (29), as a result of store depletion. In contrast, activation of mGlu1a receptors has been reported to induce a rapid and complete uncoupling from PLC following by opening of a receptor-operated $\text{Ca}^{2+}$ channel (14). The low level of sustained IP$_3$ synthesis in the absence of $\text{Ca}^{2+}$.o, observed using the confocal microscope is in disagreement with this suggestion. However, the predominant role of $\text{Ca}^{2+}$-dependent PLCs (e.g. PLC$\delta$; 30, 31) during this secondary phase is indicated and is similar to M$_3$ muscarinic receptors where IP$_3$ production was also found to continue in the absence of $\text{Ca}^{2+}$.o at a decreased level (32, 33). The data also indicate that the initial peak in $[\text{Ca}^{2+}]$, predominantly represents $\text{Ca}^{2+}$ release from intracellular stores with no involvement of $\text{Ca}^{2+}$.o entry. Two distinct phases are evidently involved in the response to activation of mGlu1a receptors.

We have previously reported a sensitivity of M$_3$ muscarinic and bradykinin receptor-induced IP$_3$ responses in SH-SY5Y cells to store depletion using thapsigargin (32–34) and an important observation described here is the almost complete dependence of IP$_3$ production by the mGlu1a receptor on $\text{Ca}^{2+}$ released from the intracellular stores. This sensitivity of mGlu1a receptors to intracellular store $\text{Ca}^{2+}$ may reflect the selective recruitment of PLC isoenzymes responsive to variations in $[\text{Ca}^{2+}]$.o. Furthermore, the association of the mGlu1a receptor with Homer/Vesl proteins (35, 36) allows for potential cross-linking with IP$_3$ and/or ryosinad receptors (37). Theoretically this could hold mGlu1a receptors in close proximity to the site of $\text{Ca}^{2+}$ release from the endoplasmic reticulum such that high local concentrations of $\text{Ca}^{2+}$ may dramatically potentiate the activity of $\text{Ca}^{2+}$-sensitive PLCs. Irrespective of the origins of this sensitivity it is important to note that any inadvertent depletion of $\text{Ca}^{2+}$, stores prior to assaying mGlu1a receptor activity will give results that could be erroneously interpreted as demonstrating $\text{Ca}^{2+}$.o sensitivity. Numerous authors have reported store depletion during prolonged incubation in $\text{Ca}^{2+}$-free buffers (e.g. Ref. 25).

Kubo et al. (5) identified Ser$^{166}$ as controlling the $\text{Ca}^{2+}$.o, sensing properties of rat mGlu1a receptors. This apparent inconsistency in $\text{Ca}^{2+}$.o, sensing property of mGlu1a receptors does not reflect species differences, since the rat mGlu1a receptor expressed in BHK cells responded in a similar manner to the human receptor. The x-ray crystallography structure of the mGlu1a receptor revealed the presence of a high affinity cat-

**FIG. 5.** Effect of thapsigargin pretreatment on the stimulation of IP$_3$ production and $\text{Ca}^{2+}$ mobilization by quisqualate in nominal $[\text{Ca}^{2+}]$. A, representative trace of a CHO-lac-mGlu1a cell perfused with KHB containing no added CaCl$_2$ for 10 min before perfusion with 10 $\mu$M quisqualate (indicated by the dot-dash-dot line). Simultaneous measurements of $[\text{Ca}^{2+}]$, (dotted line) and eGFP-PH$_{PLC}$ ratio (solid line) were taken throughout this period. B, example trace of an experiment where cells were perfused for 3 min with KHB ($[\text{Ca}^{2+}]$), challenged with 2 $\mu$M thapsigargin in $\text{Ca}^{2+}$-free KHB (indicated by the dashed line), reperfused with KHB ($[\text{Ca}^{2+}]$) and finally perfused with 10 $\mu$M quisqualate (dot-dash-dot line).

**DISCUSSION**

In the present work, the establishment of a real-time assay for the measurement of [IP$_3$], in single cells has revealed that the mGlu1a receptor does not show $\text{Ca}^{2+}$.o, sensing properties. Although previous data from InsP accumulation experiments (Fig. 1; Ref. 4) supported the view that $\text{Ca}^{2+}$, modulates mGlu1a receptor activity, no differences in the early phases of IP$_3$ production or $\text{Ca}^{2+}$ mobilization in the presence or absence of $\text{Ca}^{2+}$.o were observed. Each of the following parameters appeared unchanged by removal of $\text{Ca}^{2+}$, the maximal response to quisqualate, the agonist potency, and the dynamics of the peak response for both IP$_3$ production and $\text{Ca}^{2+}$ mobilization.

Increases in cytosolic fluorescence were dramatically decreased (Fig. 7A). This effect is more clearly observed when the translocation data are plotted against time for the response to quisqualate in the presence of 1.3 mM (solid line) or nominal (dashed line) $[\text{Ca}^{2+}]$. The combined data demonstrate that while peak responses are unchanged plateau levels induced by sustained agonist challenge are significantly decreased (Fig. 7B). Experiments performed using 10 $\mu$M quisqualate (maximal agonist concentration) similarly recorded no difference in the peak response in the presence or absence of added $\text{Ca}^{2+}$.o, but showed dramatically reduced sustained levels (data not shown).

In the present study, the establishment of a real-time assay for the measurement of [IP$_3$], in single cells has revealed that the mGlu1a receptor does not show $\text{Ca}^{2+}$.o, sensing properties. Although previous data from InsP accumulation experiments (Fig. 1; Ref. 4) supported the view that $\text{Ca}^{2+}$, modulates mGlu1a receptor activity, no differences in the early phases of IP$_3$ production or $\text{Ca}^{2+}$ mobilization in the presence or absence of $\text{Ca}^{2+}$.o were observed. Each of the following parameters appeared unchanged by removal of $\text{Ca}^{2+}$, the maximal response to quisqualate, the agonist potency, and the dynamics of the peak response for both IP$_3$ production and $\text{Ca}^{2+}$ mobilization.
Fig. 6. Effect of increasing the extracellular Ca\textsuperscript{2+} concentration on IP\textsubscript{3} production and Ca\textsuperscript{2+} mobilization. CHO-lac-mGlu1\textalpha{} cells were preincubated for 3 min with 1.3 mM Ca\textsuperscript{2+} containing KHB before imaging. After 15 s, 10 \mu{}M quisqualate in KHB containing either 1.3 or 2.6 mM Ca\textsuperscript{2+} was perfused over the cells. Dual measurements of the changes in cytosolic eGFP-PHPLC\textsubscript{i} fluorescence ratio (A) and [Ca\textsuperscript{2+}], above basal (B) with time were made. The peak (hatched bars) and plateau (open bars) levels of each trace were recorded and expressed as the mean \pm{} S.E.M. of at least 12 individual cells from three separate experiments.

Fig. 7. Effect of Ca\textsuperscript{2+}, on IP\textsubscript{3} production in BHK cells expressing the rat mGlu1\textalpha{} receptor. BHK-mGlu1\textalpha{} cells were transiently transfected with eGFP-PHPLC\textsubscript{i} for 48 h and then imaged with an UltraVIEW confocal system. After perfusion with KHB containing nominal [Ca\textsuperscript{2+}] the cells were challenged with 1 \mu{}M quisqualate in buffer with the same [Ca\textsuperscript{2+}] for 3 min and then washed free of agonist. After washing for 10–15 min (1.3 mM Ca\textsuperscript{2+}) cells were re-challenged with quisqualate using the same protocol in the presence of 1.3 mM Ca\textsuperscript{2+}. A, confocal images showing the eGFP fluorescence associated with the basal, peak, and plateau responses induced by 1 \mu{}M quisqualate for experiments performed in 1.3 mM or nominal [Ca\textsuperscript{2+}]. B, representative traces showing the effect of 1 \mu{}M quisqualate (horizontal line) on IP\textsubscript{3} production in a single BHK-mGlu1\textalpha{} cell in the presence of 1.3 mM (open bars) or nominal (solid bars) [Ca\textsuperscript{2+}]. *p < 0.01 when compared with response in 1.3 mM [Ca\textsuperscript{2+}] using Student’s paired t test.

REFERENCES
mGlu1α Receptors do Not Sense Extracellular Ca^{2+}
Reassessment of the Ca$^{2+}$ Sensing Property of a Type I Metabotropic Glutamate Receptor by Simultaneous Measurement of Inositol 1,4,5-Trisphosphate and Ca$^{2+}$ in Single Cells

Mark S. Nash, Ruth Saunders, Kenneth W. Young, R. A. John Challiss and Stefan R. Nahorski

doi: 10.1074/jbc.M007600200 originally published online February 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M007600200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 12 of which can be accessed free at http://www.jbc.org/content/276/22/19286.full.html#ref-list-1