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Inhibition by inorganic ions of a sustained calcium signal evoked by activation of mGlu5 receptors in rat cortical neurons and glia

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1 The effect of mGlu receptor agonists on intracellular calcium (Ca^{2+}) in rat cortical neurons and glial cells was studied. The responses evoked consisted of two phases; an initial transient response followed by a sustained plateau. In both cell types the order of potency of group I mGlu receptor agonists was DHPG > 1S,3R ACPD > 3-HPG.

2 The selective mGlu5 agonist CHPG elicited responses in both cell types as did S4C3-HPG which is thought to be an mGlu5 agonist at high concentrations. S4-CPG had no effect on intracellular Ca^{2+} levels nor did it inhibit the action of 1S,3R ACPD. These results suggest that the responses in both cell types are mediated by mGlu5 receptors.

3 In the absence of extracellular Ca^{2+} ions, 1S,3R ACPD (100 μM) induced only a transient Ca^{2+} response which decayed to baseline with a time constant of approximately 20 s in both cell types. Subsequent readdition of Ca^{2+} (2 mM) to the external solution in the continued presence of 1S,3R ACPD induced a sustained Ca^{2+} plateau.

4 The sustained Ca^{2+} plateau could be blocked by a number of inorganic cations, with an order of potency of $Zn^{2+} \geqslant La^{3+} > Cd^{2+} \geqslant Co^{2+} > Ni^{2+} > Mg^{2+}$. Similar concentrations of Zn^{2+} had little effect on Ca^{2+}-influx evoked by 25 mM K^+.

5 It is concluded that the Ca^{2+}-entry pathway activated by mGlu5 receptors resembles store-operated Ca^{2+}-entry pathways that have been described in other cell types.

Keywords: Metabotropic glutamate receptor; calcium; store-operated calcium entry; zinc; lanthanum; mGlu5; CHPG

Introduction

Metabotropic glutamate (mGlu) receptors are a family of G protein coupled receptors that share sequence homology with $GABA_B$ receptors and Ca^{2+} sensing proteins (Conn & Pin, 1997; Brown *et al*., 1993; Kaupmann *et al*., 1997). They have much less homology with other classes of G protein coupled receptors (see Pin & Duvoisin, 1995). They are involved in many aspects of neuronal function within the CNS including development and synaptic plasticity (e.g. Bashir *et al*., 1993; Lu *et al*., 1997). Eight different mGlu receptors have been cloned (mGlu1–8) and these have been classified into three groups according to their sequence similarities, the signal transduction pathways to which they can couple and the selectivity of certain receptor agonists (Nakanishi & Masu, 1994; Pin & Duvoisin, 1995). Group I receptors (mGlu 1 and 5) activate phospholipase C while activation of Group II (mGlu 2 and 3) and Group III (mGlu 4, 6, 7 and 8) receptors inhibits adenylyl cyclase activity (Pin & Duvoisin, 1995; Conn & Pin, 1997).

In this study, we have investigated the role of mGlu receptors in stimulating an increase in intracellular calcium concentration in neurons and glial cells isolated from rat cerebral cortex. Firstly, we have characterized the subtype of mGlu receptor underlying the response with the aid of a number of selective pharmacological agents. The rise in calcium following mGlu receptor activation consists of two phases – an initial rapid transient rise in calcium followed by a secondary sustained rise which is dependent on the presence of extracellular calcium (see Mathie & Richards, 1997). Such a response profile has been seen following activation of a number of other G protein coupled receptors in other cell types (e.g. Grudt *et al*., 1996). For other receptors, the initial transient response has been attributed to mobilization of calcium from intracellular stores, while the sustained phase may be caused by activation of a store-operated calcium entry pathway (SOC) (Berridge, 1995; Clapham, 1995). Secondly, we have characterized some of the properties of this second phase of the response, in particular its sensitivity to block by inorganic cations, since this property has been used previously to aid the characterization of such SOC pathways in other cell types (e.g. Aussel *et al*., 1996; Hoth & Penner, 1993; Grudt *et al*., 1996; Wayman *et al*., 1996). Preliminary accounts of some of these results have been published (Mathie *et al*., 1996; Prothero *et al*., 1998).

Methods

Cell culture

Neonatal Sprague-Dawley rats (2–5 days old) were anaesthetized with isoflurane and then decapitated. The neocortex was aseptically dissected and placed in HEPES buffered minimum essential medium (M.E.M.). The tissue was mechanically dissociated by passing the tissue through fire-polished pasteur pipettes of decreasing bore. The cell debris was allowed to settle and the supernatant containing disaggregated cells was then spun at $150 \times g$ for 3 min to separate the intact cells. The supernatant was removed and the pellet resuspended in HEPES buffered MEM. The cells were then plated at a density of $0.3-1 \times 10^6$ cells per 50 mm culture dishes containing 5 ml MEM supplemented with 2.5% Foetal Bovine Serum. Each dish contained coverslips that had been coated with poly-L-lysine (0.1 mg ml^{-1}). The cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C for

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5–7 days before use. Occasionally cultures of 10–14 days were used but no differences in the response to agonists were evident. Neurons were identified by their size and morphology (phase bright cell bodies possessing long neurites with few recurrent branches) and astrocytes were identified by their flattened phase-dark appearance (see Figure 1).

Intracellular Ca^{2+} measurements

Ca^{2+} measurements were made with Fura-2 (Molecular Probes, Eugene, OR, U.S.A.) which was loaded into the cells as its AM-ester. Measurements were made using dual excitation (340 and 380 nm) with emission above 420 nm. All experiments were conducted at room temperature (20–27°C). For fluorescence imaging, light was collected *via* a cooled CCD camera (supplied by Digital Pixel, Brighton, U.K.) and analysed using software supplied by Kinetic Imaging (Liverpool, U.K.). To improve the signal-to-noise ratio, the collection times were adjusted to increase the total number of photons accumulated at the lowest intensity of emission. Thus the collection period for 340 nm excitation was three times that for 380 nm excitation (the collection period was generally 600 ms compared to 200 ms). The ratio values in the Figures are not corrected for these different collection times.

In each experiment, ratio values were calculated after subtraction of the background emission; autofluorescence was less than the background signal. Background emission was determined at the end of every experiment by treating the cells with 0.1% Triton. For determination of the local Ca^{2+} from the ratio images, specific areas of interest was chosen. The average ratio value for the designated areas was then calculated and plotted as a function of time. For each experimental procedure, the '*n*' values refer to the number of cells. In each case these cells were taken from a minimum of three coverslips from at least two separate cortical culture preparations. Throughout the text values are given as mean ± s.e.mean.

Calibration of the ratios in the terms of $[Ca^{2+}]$ was carried out *in situ* in several experiments using the ionophore 4Br-A23187. Cells were exposed to a 'zero' calcium solution containing 20 mM EGTA and 0 mM Ca^{2+} to obtain a minimum ratio and a 'high' calcium solution containing 2 mM Ca^{2+} to obtain a maximum ratio, both in the presence of 5 μM 4Br-A23187. Cells were exposed to these zero and high calcium solutions alternately until consistent ratio values were obtained. The average values from these calibrations has been used to quantify the changes in intracellular Ca^{2+} where this was considered appropriate using the following equation (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}] = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{S_{f2}}{S_{b2}}\right)$$

where R is the measured ratio of interest, R_{min} and R_{max} the ratios recorded with zero and high extracellular calcium, S_{f2} and S_{b2} the signals at 380 nm in zero and high calcium and K_D is the apparent dissociation constant for Fura-2.

For 28 cells in six separate calibration experiments the resting calcium concentration was calculated to be 53 ± 14 nM.

Solutions

Cells were bathed in Locke's solution buffered with 16 or 30 mM HEPES. The unbuffered Locke's solution had the following composition (mM) NaCl 140, KCl 3 or 5, $MgCl_2$ 1.8 or 1, $CaCl_2$ 2 and glucose 5.5. The pH was adjusted to 7.3 using 1 M NaOH. The mGlu receptor agonists were diluted from concentrated stocks into the appropriate bathing solution immediately before use. Agonists and inorganic ions were added to the bath for the durations indicated by the bars in the Figures.

Routine chemicals and tissue culture materials were supplied by Sigma and the specific mGlu receptor agonists and antagonists described below were all obtained from Tocris Cookson: the non-selective mGlu receptor agonist, 1S,3R ACPD ((1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid); two Group I mGlu receptor agonists, 3-HPG ((S)-3-hydroxyphenylglycine) and DHPG (3,5-dihydroxyphenylglycine); the mGlu5 receptor agonist, CHPG ((RS)-2 chloro-5-hydroxyphenylglycine); the putative mGlu5 receptor agonist, TADA (trans-azetidine-2,4-dicarboxylic acid); the mGlu1 receptor antagonist/mGlu2 receptor agonist, S4-CPG ((S)-4-carboxyphenylglycine), the mGlu1 receptor antagonist/mGlu5 receptor agonist; S4C3-HPG ((S)-4-carboxy-3-hydroxyphenylglycine) and the group III mGlu receptor agonist, L-AP4 (L(+)-2-amino-4-phosphonobutyric acid).

Results

1S,3R ACPD induced calcium responses in cortical neurons and glial cells

The mGlu receptor agonist, 1S,3R ACPD induced a rise in intracellular calcium concentration in cortical neurons and glial cells. This is illustrated in Figure 1 for both cell types. Typical responses in both cells consisted of an initial transient increase in intracellular calcium followed by a maintained plateau response. The sustained plateau response was maintained for as long as the cells were exposed to the agonist (see Figure 1, neuron 3) and, indeed, could often outlast application of the agonist by several minutes. In some cells, particularly at intermediate agonist concentrations (10–25 μM), the plateau responses were oscillatory. This is illustrated in Figure 1 by neuron number 2. Cells could be divided, broadly, into three groups: those that gave no response, those that gave a sustained plateau response and those that gave an oscillatory response. However, 1S,3R ACPD at 100 μM produced plateau responses in the large majority (around 80%) of either cell type.

A series of experiments were carried out to determine the concentration-response relationships for 1S,3R ACPD (over the concentration range 0.25–250 μM) for both cell types. Data from these experiments are shown in Table 1. It can be seen that the amplitude of the evoked responses increases with agonist concentration for both cell types. 1S,3R ACPD was more potent in eliciting a response in glial cells compared to neurons. 1S,3R ACPD had an EC_{50} of approximately 5 μM for glial cells and 20 μM for neurons. 100 μM 1S,3R ACPD was found to be a maximally effective concentration of 1S,3R ACPD in both cell types, capable of evoking peak calcium ratio signals of up to seven (see Figures 1 and 8) corresponding to a peak calcium concentration of around 1.6 μM. For the particular series of experiments shown in Table 1 all cells are included regardless of whether they produced a detectable response. 1S,3R ACPD (100 μM) induced responses in 80% of neurons and 83% of glial cells respectively; the mean peak calcium signal was 2.37 ± 0.11 (corresponding to a calcium concentration of 241 nM) in glial cells and 2.09 ± 0.19 (183 nM) in neurons. All responses are from cells exposed to the agonist for the first time to avoid problems caused by receptor desensitization at high concentrations or priming at low concentrations.

Pharmacology of mGlu receptor-mediated responses

Although, 1S,3R ACPD is not a selective agonist at any one group of mGlu receptors, the response to 1S,3R ACPD could be mimicked by the Group I selective agonists DHPG (1–300 μM) and 3-HPG (10 μM–1 mM) (Hayashi *et al*., 1994; Brabet *et al*., 1995) as shown in Figures 2 and 3 and Table 1. At maximally effective concentrations, both agonists evoked calcium responses with the same profile as those evoked by 1S,3R ACPD and which consisted of an initial transient phase followed by a sustained plateau. DHPG was much more potent than 3-HPG (compare Figures 2 and 3) and, indeed,

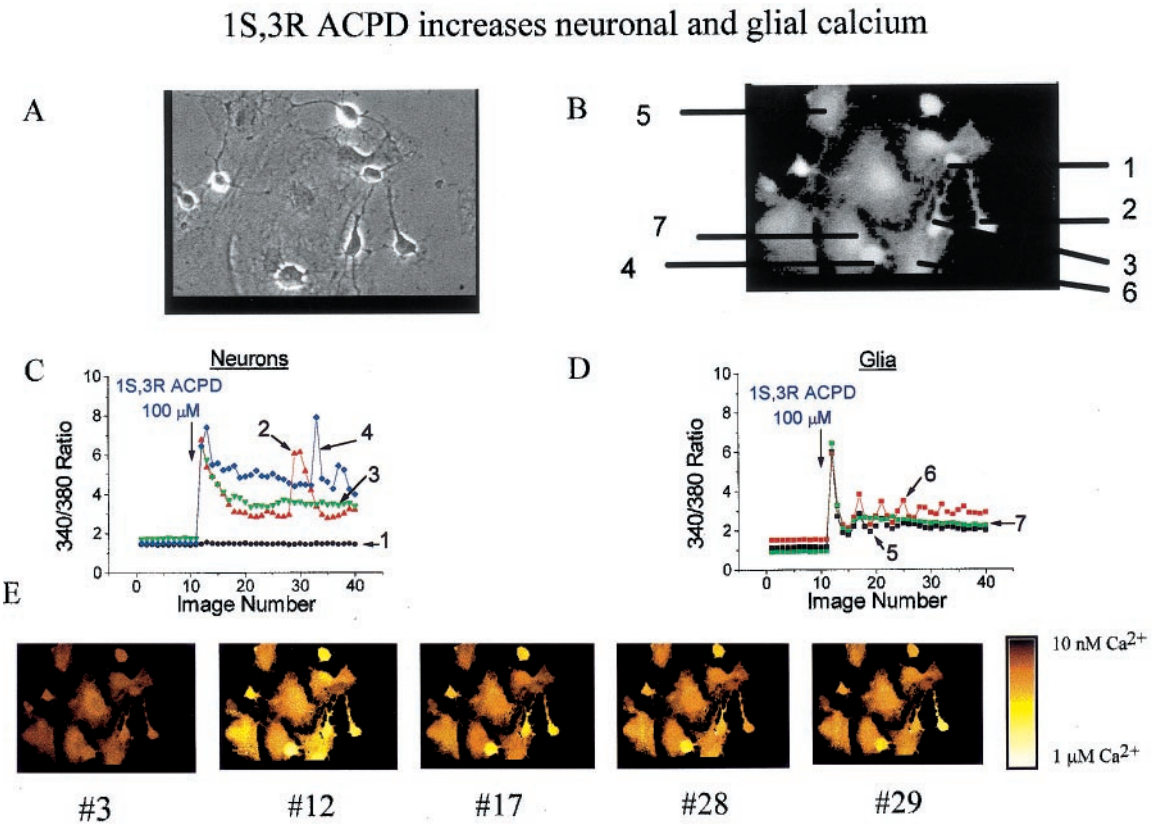

Figure 1 1S,3R ACPD (100 μM) increases the intracellular calcium concentration of cortical neurons and glial cells. (A) Shows a phase-contrast image of a field of cells while (B) shows a fluorescent image following excitation at 380 nm after loading of the cells with Fura-2 showing the neurons and glial cells more clearly. The time course of the effect of 1S,3R ACPD on the 340/380 ratio of the identified neurons and glial cells is shown in (C) and (D) respectively. 1S,3R ACPD was applied at the point indicated by the arrows and was then present for the remainder of the experiment. Images were taken 3.3 s apart. (E) Shows a series of pseudocolour images with changes in Fura-2 ratios for neurons and glial cells at the image numbers indicated. A rise in the Fura-2 ratio is indicated by an increased brightness as shown by the calibration wedge on the far right. Note the typical sustained increase in intracellular calcium concentration following addition of 1S,3R ACPD in some cells exemplified by neuron 3 and the oscillatory behaviour of others shown by neuron 2.

Table 1 Concentration-response relationships for 1S,3R ACPD, 3-HPG and DHPG in neurons and glial cells

		Neurons		*Glia*	
Agonist	*Concentration* (μM)	(n)	*Mean peak calcium ratio*	(n)	*Mean peak calcium ratio*
1S,3R ACPD	0.25	(9)	no responses	(12)	no responses
	1	(22)	1.02±0.06	(8)	0.93±0.09
	2.5	(21)	0.85±0.04	(11)	0.85±0.05
	10	(50)	0.95±0.03	(22)	1.18±0.08
	25	(19)	1.13±0.07	(10)	2.14±0.72
	100	(20)	2.09±0.19	(12)	2.37±0.11
	250	(22)	1.56±0.10	(9)	2.19±0.31
3-HPG	10	(33)	0.88±0.06	(12)	1.56±0.33
	50	(49)	1.14±0.07	(19)	1.39±0.11
	100	(30)	0.97±0.09	(14)	1.79±0.29
	500	(57)	1.21±0.09	(39)	1.83±0.10
	1000	(38)	1.78±0.15	(19)	2.36±0.25
DHPG	1	(8)	1.34±0.07	(17)	1.94±0.32
	3	(23)	2.83±0.59	(22)	2.49±0.57
	30	(30)	3.11±0.41	(22)	3.61±0.65
	100	(16)	3.28±0.49	(12)	3.37±0.60
	300	(25)	2.67±0.43	(15)	4.13±0.82

more potent than 1S,3R ACPD, with 30 μM DHPG appearing to be close to a maximally effective concentration in both neurons and glial cells (Figure 2, Table 1). This concentration evoked a mean peak response of 3.11 ± 0.41 (416 nM) in neurons and 3.61 ± 0.65 (555 nM) in glial cells with 70 and 86% respectively, of either cell type responding. In contrast, 1 mM 3-HPG, (the maximum concentration tested due to the compound's solubility) was only just at the top of the

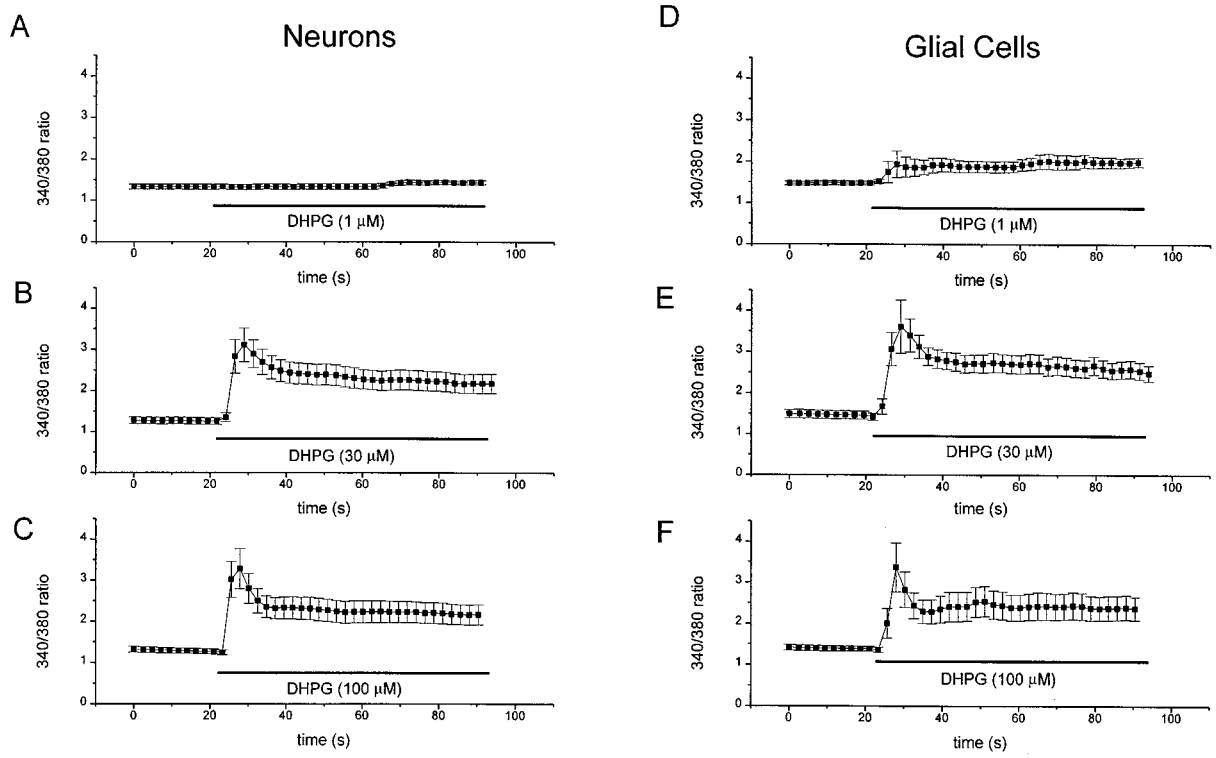

Figure 2 DHPG increases the intracellular calcium concentration of cortical neurons and glial cells. (A–C) The effect of 1, 30 and 100 μM DHPG on cortical neurons. (D–F) The effect of 1, 30 and 100 μM DHPG on cortical glial cells.

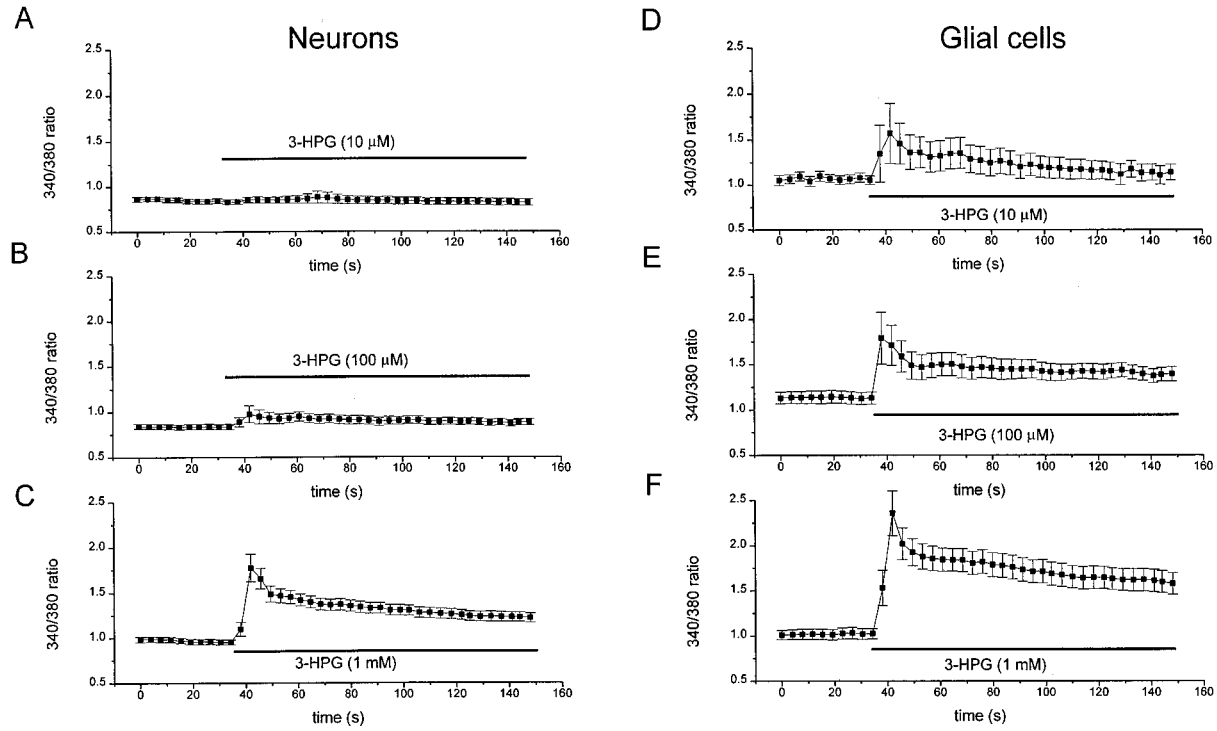

Figure 3 3-HPG increases the intracellular calcium concentration of cortical neurons and glial cells. (A–C) The effect of 10, 100 μM and 1 mM 3-HPG on cortical neurons. (D–F) The effect of 10, 100 μM and 1 mM 3-HPG on cortical glial cells.

concentration-response relationship for neurons inducing mean peak calcium signals of 1.78 ± 0.15 (123 nM) in 82% of neurons (Figure 3, Table 1). At the same concentration, 3-HPG evoked a calcium signal of 2.36 ± 0.25 (239 nM) in glial cells with 89% of cells tested giving a response. For both cell types, the relative effectiveness of these three agonists was DHPG > 1S,3R ACPD > 3-HPG. The Group III agonist L-AP4 (100 μM) had little effect on intracellular calcium (data not shown).

Recently, a number of compounds have been developed which may distinguish between effects mediated by mGlu1 and mGlu5 receptors. In order to determine the nature of the receptor underlying these responses further, four additional pharmacological agents were tested. The first of these was S4-CPG, which has been reported to be a competitive antagonist at mGlu1 receptors but an agonist at mGlu2 receptors. It has no action on mGlu5 receptors (Brabet *et al*., 1995). The second was S4C3-HPG which is reported to be antagonist at mGlu1 receptors but an agonist at mGlu5 receptors at high concentrations (Brabet *et al*., 1995 but see Kingston *et al*., 1995). Thirdly, we used TADA a putative agonist at mGlu5 receptors (Favaron *et al*., 1993; Opitz *et al*., 1995) and fourthly CHPG, a recently developed selective agonist at mGlu5 receptors with no action at mGlu1 receptors (Doherty *et al*., 1997).

Figures 4 and 5 summarize the results obtained with these four compounds. Figure 4A and B shows that the mGlu1 receptor antagonist/mGlu2 receptor agonist, S4-CPG (500 μM) does not evoke any change in calcium in either neurons or glia and does not inhibit the response of the cells to subsequent application of an intermediate concentration of 1S,3R ACPD (25 μM). This would suggest that neither mGlu1 nor mGlu2 receptors underlie the responses seen. Figure 4C and D shows that S4C3-HPG (500 μM) evokes a calcium response in both cell types which would suggest that an mGlu5 receptor underlies the responses. The recently developed, selective, mGlu5 receptor agonist CHPG (100 μM – 1 mM) is also effective at inducing changes in intracellular calcium in both cell types. Figure 5A and B shows that 1 mM CHPG evokes the characteristic transient increase followed by sustained elevation in $[Ca^{2+}]$ in both cell types, with mean peak calcium signals of 3.55 ± 0.19 (537 nM) in 16 out of 28 neurons (57%) and 2.90 ± 0.18 (363 nM) in 18 out of 20 glial cells (90%). Both S4C3-HPG and CHPG evoked responses with the same profile as that evoked by 1S,3R ACPD, 3-HPG and DHPG, namely an initial transient calcium increase followed by a sustained plateau. The putative mGlu5 receptor agonist TADA (500 μM) was unable to evoke changes in intracellular calcium in neurons and only induced a slow rise in intracellular calcium in glial cells. Furthermore, this effect was observed only after a considerable delay following agonist application when compared to the responses seen for the five other effective agonists described above (Figure 5C and D). Thus our data suggest that the rise in calcium following activation of mGlu receptors in both cortical neurons and glial cells is brought about through activation of the mGlu5 receptor subtype (Abe *et al*., 1992).

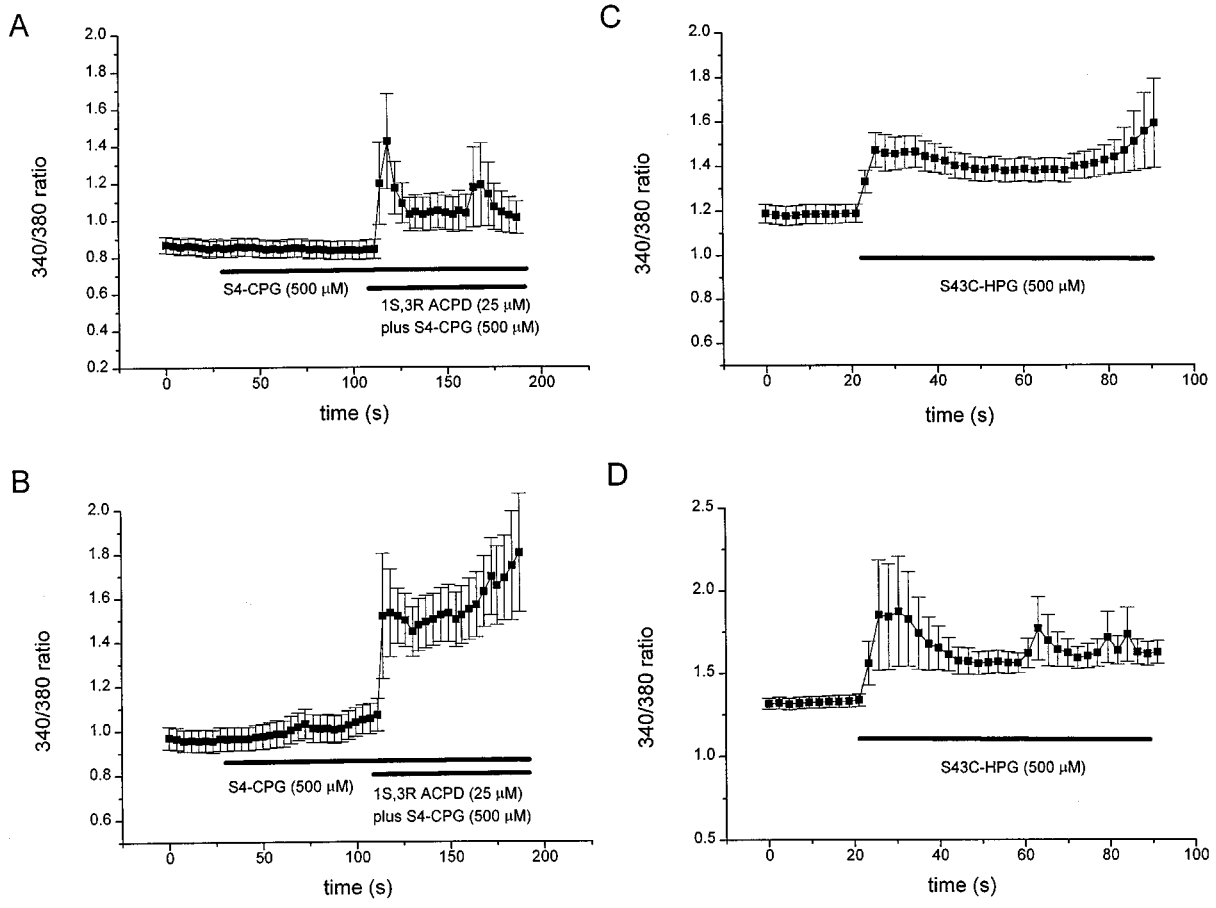

Figure 4 (A and B) The effect of S4-CPG (500 μM) on intracellular calcium levels in cortical neurons (A; $n = 35$) and glial cells (B; $n = 24$) before and after the addition of 1S,3R ACPD (25 μM). (C and D) The effect of S4C3-HPG (500 μM) on intracellular calcium levels in cortical neurons (C; $n = 17$) and glial cells (D; $n = 24$).

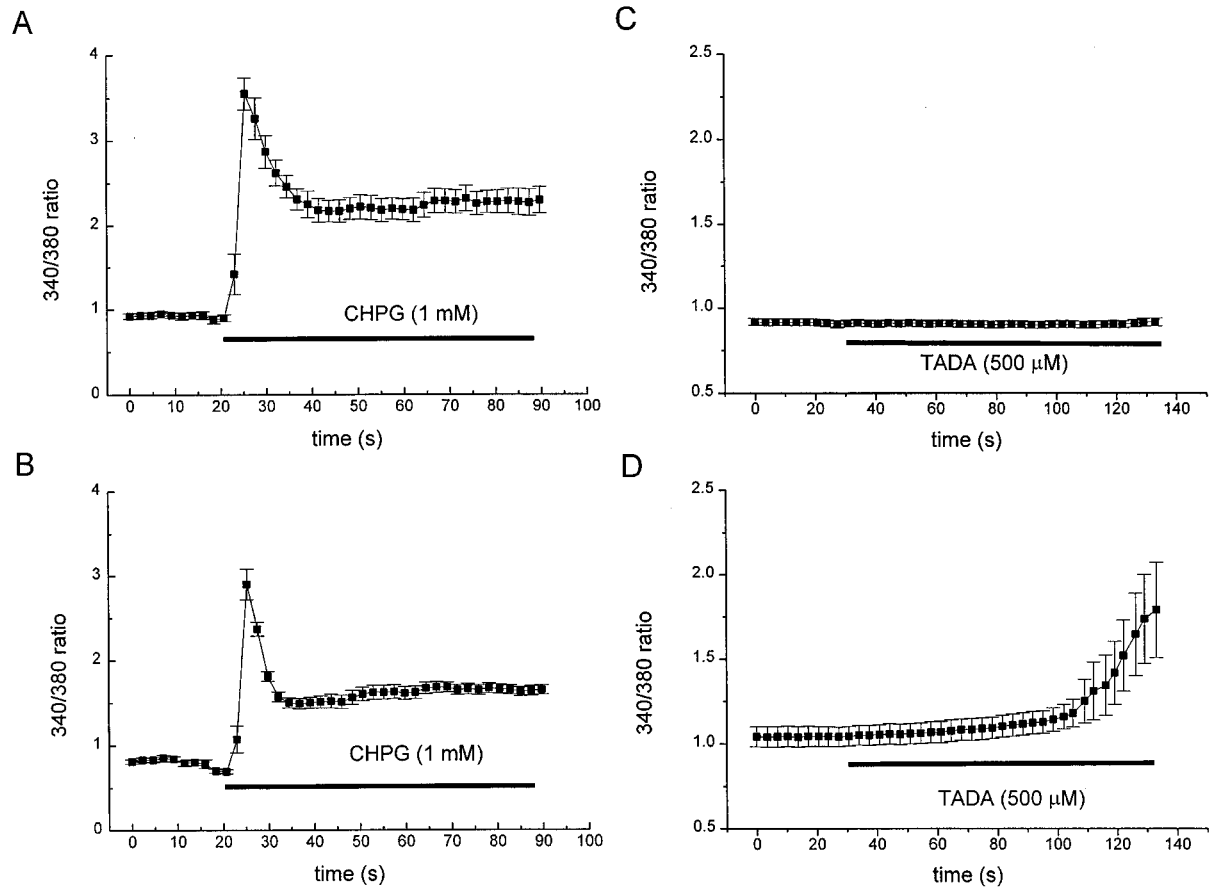

Figure 5 (A and B) The effect of CHPG (1 mM) on intracellular calcium levels in cortical neurons (A; $n=10$) and glial cells (B; $n=11$). (C and D) The effect of TADA (500 μM) on intracellular calcium levels in cortical neurons (C; $n=29$) and glial cells (D; $n=12$).

Dependence of the sustained calcium entry on extracellular calcium

The second part of this study considers the nature of the response evoked by activation of mGlu receptors in cortical neurons and glial cells. 1S,3R ACPD at a maximally effective concentration of 100 μM, evoked responses which, in the majority of cells, consisted of a transient response followed by a plateau phase (see, for example, Figures 1, 7 and 8). When extracellular calcium was removed, 1S,3R ACPD still evoked an increase in intracellular calcium which consisted only of the initial transient phase, the response decaying exponentially to baseline in both cell types with a time constant of 20 s in neurons and 19 s in glial cells (Figure 6). The sustained calcium response could be induced by readdition of calcium (2 mM) to the external solution in the continued presence of 1S,3R ACPD (Figure 6). Thus the initial calcium response depends on the mobilization of calcium from internal stores, while the sustained phase depends on extracellular calcium.

Block of sustained calcium entry by inorganic cations

The activation of this sustained calcium entry resembles the activation of store-operated calcium entry pathways (SOCs) which have been described in a number of other cell types, (see Discussion). The magnitude of the sustained calcium signal evoked by 1S,3R ACPD (100 μM) was 2.15 ± 0.02 ($n=64$) in neurons and 2.22 ± 0.03 ($n=44$) in glial cells in this series of experiments corresponding to a sustained intracellular calcium concentration of 195 and 210 nM, respectively. The peak calcium signals in these cells were 4.44 ± 0.26 ($n=64$) for neurons and 4.90 ± 0.36 ($n=44$) for glial cells. One feature of SOCs is their susceptibility to block by a number of inorganic cations. Figure 7 and Table 2 shows the effect of Zn^{2+} (1–100 μM) on the sustained calcium response to 1S,3R ACPD in neurons. At 100 μM, Zn^{2+} virtually abolished the sustained response in both cell types. Zn^{2+} was more effective in neurons than in glial cells with an estimated IC_{50} of 6.5 μM in neurons but 15 μM in glial cells (see Figure 7 and Table 2). Zinc applied before the addition of 1S,3R ACPD blocked the sustained plateau response but did not affect the transient increase induced by the agonist (data not shown).

A number of other inorganic ions were examined on the sustained calcium influx evoked by 1S,3R ACPD and their relative effectiveness at 100 μM is shown in Figure 8 (see also Table 2). Zn^{2+} and La^{3+} were the most effective ions tested, at this concentration producing $101\pm6\%$ and $78\pm7\%$ block, respectively, in neurons followed by Cd^{2+} ($52\pm7\%$), Co^{2+} ($47\pm8\%$) and Ni^{2+} ($20\pm3\%$). Ni^{2+} when given at a concentration of 400 μM, produced $60\pm9\%$ inhibition of the calcium entry pathway in neurons and $36\pm5\%$ in glial cells, however, a 4 fold increase in concentration to 1.6 mM produced no further increase in the degree of inhibition in either cell type. Mg^{2+}, even at concentration of 10 mM, only produced a partial inhibition of this calcium entry pathway ($47\pm11\%$). For all ions, block was more effective in neurons compared with glial cells (see Table 2) however the relative potency sequence was the same in either cell type and was $Zn^{2+}\geqslant La^{3+}>Cd^{2+}\geqslant Co^{2+}>Ni^{2+}>Mg^{2+}$.

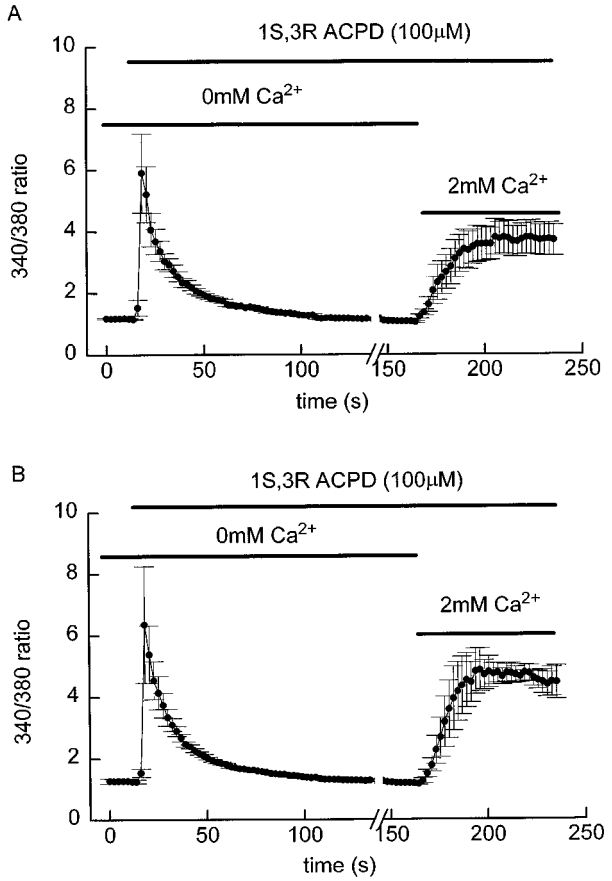

Figure 6 The response to 1S,3R ACPD (100 μM) in the absence of extracellular calcium in neurons (A; $n=7$) and glial cells (B; $n=4$). The response decays exponentially back to baseline in both cell types. Readdition of extracellular calcium (2 mM) induces a sustained rise in calcium in both cell types in the continued presence of 1S,3R ACPD.

In contrast, Zn^{2+} (100 μM) had little detectable effect on calcium entry evoked by depolarization of the neurons with 25 mM K^+ which evokes calcium entry through voltage-gated calcium channels (Figure 9). This K^+-induced calcium entry could, however, be inhibited by both 100 μM Cd^{2+} and 10 μM La^{3+} (Figure 9).

Discussion

Activation of group I mGlu receptors of rat cortical neurons and glial cells induced a rise in the intracellular calcium concentration which consisted of two phases. The first, initial transient phase reflects release of calcium from intracellular stores while the second sustained phase is due to calcium influx across the plasma membrane, possibly *via* a store-operated calcium entry pathway (Berridge, 1995). We have previously shown that responses to 1S,3R ACPD could be inhibited by pretreatment of the cells with the Ca-ATPase inhibitor and store depletor, thapsigargin (0.1–1 μM; Mathie & Richards, 1997). Similar calcium response profiles have now been observed following activation of a number of different G protein coupled receptors in many cell types (see for example, Irving *et al*., 1992; Grudt *et al*., 1996).

We have characterized the nature of the mGlu receptors which underlie these responses using a number of different mGlu receptor agonists. The most convincing evidence was obtained using the recently developed selective mGlu5 receptor agonist, CHPG (Doherty *et al*., 1997). This compound produced clear responses in both cell types at concentrations ranging from 100 μM to 1 mM. The profile of the responses were very similar to those evoked by 1S,3R ACPD. This agonist is the best compound currently available to distinguish between the two types of Group I mGlu receptor as it selectively activates recombinant mGlu5 receptors but not mGlu1 receptors even at concentrations as high as 4 mM (Doherty *et al*., 1997). Our data with two other phenylglycine derivatives (S4-CPG and S4C3-HPG) are also consistent with the idea that mGlu5 receptors underlie the calcium responses (see Brabet *et al*., 1995): S4-CPG has no agonist action nor does it antagonize the effect of 1S,3R ACPD while S4C3-HPG acts as an agonist.

Human recombinant mGlu5 receptors, however, appear to have a pharmacological profile that differs from that of rat as S4-CPG and S4C-HPG act as antagonists at this receptor (Kingston *et al*., 1995). This suggests that there are major species differences between these receptors. It is however, possible that these two compounds are not as reliable as CHPG as indicators of the subtype of mGlu receptors underlying the responses. TADA is the least well characterized of the four compounds tested and it has been suggested to be selective for mGlu5 receptors on the basis of some rather indirect evidence from experiments on native receptors (Favaron *et al*., 1993; Opitz *et al*., 1995) together with a lack of effect on expressed mGlu1 receptors (Favaron *et al*., 1993). We found that this compound had little action on neurons, although it did produce a delayed slow rise in intracellular calcium in glial cells. While this response is very different in profile to that evoked by the other effective agonists, it could lead to a net rise in intracellular calcium of a similar magnitude to that induced by the other agonists.

Immunocytochemical studies with a specific mGlu5 receptor antibody suggests that this receptor is widely expressed throughout the brain but that the cortex is among the areas of the brain with the highest expression levels (Romano *et al*., 1995). Levels of mGlu5 receptor are thought to be at their highest in the neonatal rat brain with around a 2 fold reduction during development (Romano *et al*., 1996). Recent RT–PCR experiments have suggested that cortical glial cells only express the mGlu5 subtype of Group I receptors (e.g. Ciccarelli *et al*., 1997).

Studies with recombinant Group I mGlu receptors have suggested that the profile of the calcium response seen following receptor activation, differs between mGlu1 and mGlu5 receptors (Kawabata *et al*., 1996). Activation of mGlu5 receptors gives rise to oscillatory calcium signals, while activation of mGlu1 leads to a quite different response profile comprising a single peaked calcium response with no maintained plateau. This difference has been attributed to a single amino acid residue (threonine at position 840) present in mGlu5 receptors which can be phosphorylated by protein kinase C (Kawabata *et al*., 1996). The oscillatory responses following mGlu5 receptor activation resemble the responses seen in a proportion of the cells in this study at intermediate agonist concentrations. However, unlike the sustained responses seen in this study at high agonist concentrations, calcium oscillations were seen even in the absence of extracellular calcium. Similar oscillatory responses were observed for native mGlu5 receptors in cortical astrocytes but only when the expression of these receptors was selectively upregulated by basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) (Nakahara *et al*., 1997; see also Miller *et al*., 1995; Balazs *et al*., 1997). Responses seen in untreated cells resembled those seen in this study at high agonist concentra-

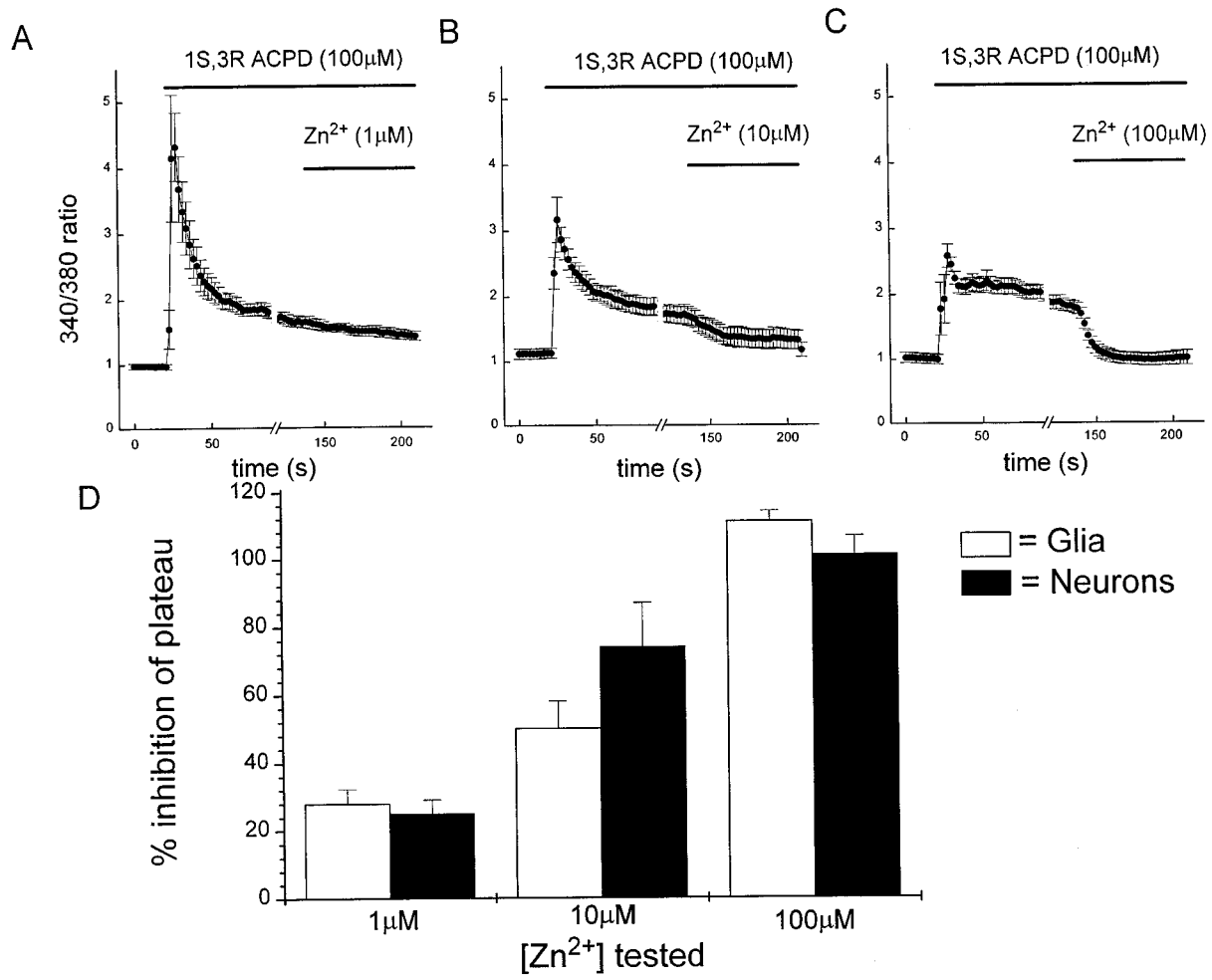

Figure 7 The effect of increasing concentrations of zinc on the sustained plateau evoked by 1S,3R ACPD (100 μM) in cortical neurons. (A–C) Show the effects of 1, 10 and 100 μM zinc in neurons. (D) Shows the percentage inhibition produced by these concentrations of zinc in both cell types measured 37 s after the addition of the ion.

Table 2 Concentration-response relationships for block by inorganic cations of the sustained calcium response following activation of mGluR5 receptors by 1S,3R ACPD (100 μM)

Ion species	*Concentration* (μM)	*Neurons* (n)	*Neurons* *% inhibition of plateau phase ± s.e.mean*§	*Glia* (n)	*Glia* *% inhibition of plateau phase ± s.e.mean*§
Zn^{2+}	1	(11)	25±4	(14)	28±4
	10	(8)	74±13	(5)	50±8
	30	(9)	96±7	(7)	47±7
	50	(17)	75±5	(12)	59±7
	100	(14)	101±6	(6)	111±3
La^{3+}	10	(8)	94±4	(11)	71±8
	25	(11)	102±7	(12)	61±8
	100	(12)	78±7	(12)	59±6
Ni^{2+}	50	(11)	34±7	(7)	29±7
	100	(17)	20±3	(10)	17±3
	200	(7)	71±16	(4)	38±13
	400	(8)	60±9	(13)	36±5
	800	(10)	47±8	(18)	32±4
	1600	(14)	54±9	(19)	36±3
Co^{2+}	55	(8)	39±6	(4)	17±5
	100	(8)	47±4	(4)	23±7
	222	(4)	69±7	(7)	40±4
	444	(10)	66±7	(7)	35±7
	800	(12)	81±4	(12)	57±4
Cd^{2+}	100	(7)	52±7	(4)	36±6
	800	(8)	82±5	(8)	78±14
Mg^{2+}	1000	(6)	47±11	(8)	41±6

§As determined 37 s after ion application.

tions comprising of an initial transient response followed by a sustained plateau (Nakahara *et al*., 1997). Thus the profile of the calcium response seen following mGlu5 receptor activation may depend on the agonist concentration, the density of the receptors and their phosphorylation state.

The sustained plateau response seen in this study could be abolished by the removal of extracellular calcium. This response resembles SOCs that have been described in a number of other cell types which are activated following depletion of intracellular calcium stores (Berridge, 1995). The link between store depletion and SOC channel activation remains to be determined (Clapham, 1995), the two most likely mechanisms being either a diffusible second messenger (Randriamampita & Tsien, 1993) or a direct protein/protein interaction between the SOC channel and the IP_3 receptor (see Putney, 1997). The molecular identity of the channels

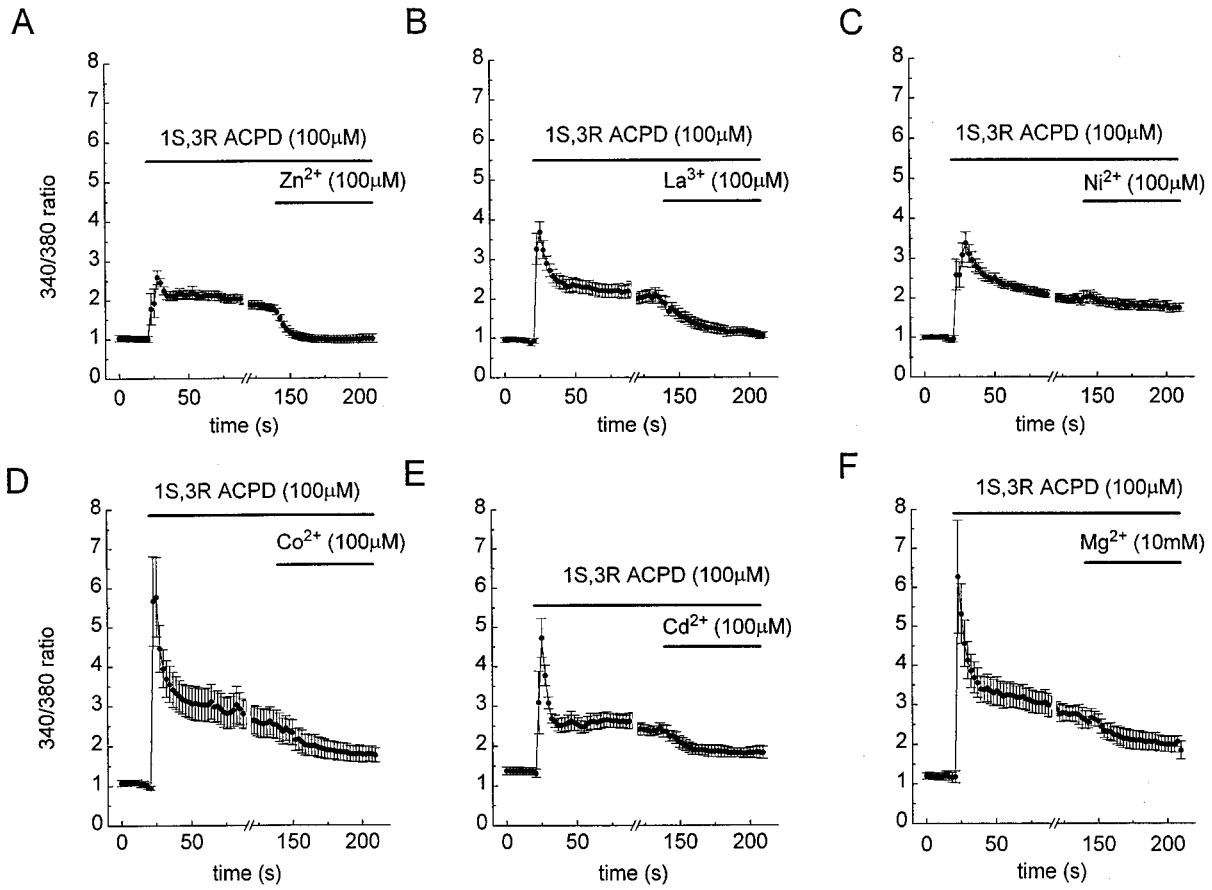

Figure 8 The effect of various inorganic cations on the sustained plateau evoked by 1S,3R ACPD (100 μM) in cortical neurons (A) Zn^{2+} (100 μM); (B) La^{3+} (100 μM); (C) Ni^{2+} (100 μM); (D) Co^{2+} (100 μM); (E) Cd^{2+} (100 μM); (F) Mg^{2+} (10 mM). Inhibition produced by these ions was measured 37 s after the addition of the ion.

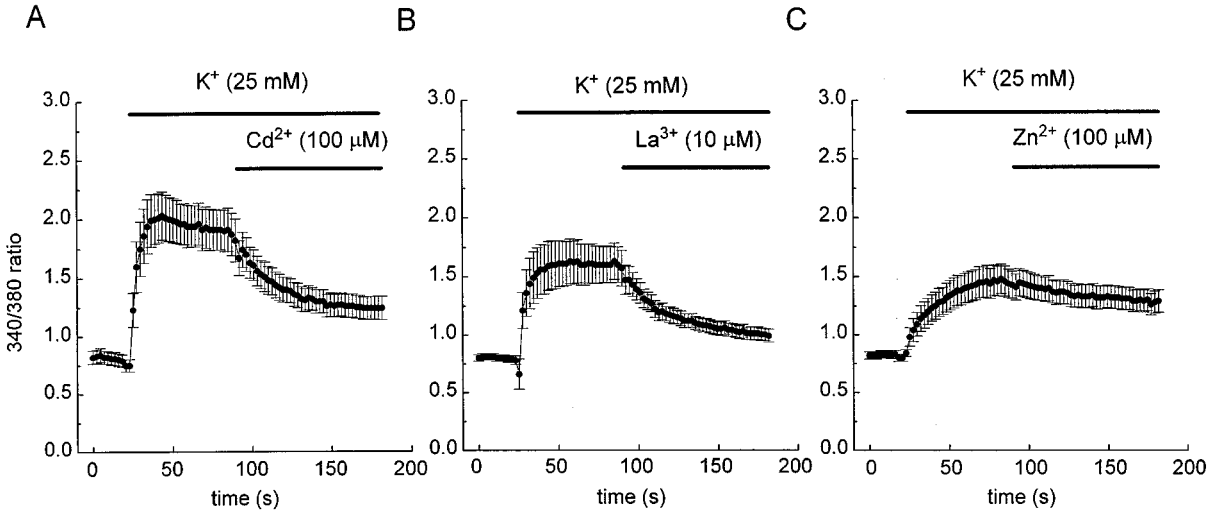

Figure 9 The effect of (A) Cd^{2+} (100 μM; $n=14$); (B) La^{3+} (10 μM; $n=9$) and (C) Zn^{2+} (100 μM; $n=14$) on the calcium response evoked by depolarizing cortical neurons with 25 mM K^+.

underlying SOCs also remains to be established. It has been suggested that the *Drosophila* protein, trp, might be the functional analogue of mammalian SOCs and a number of homologous mammalian genes have recently been cloned (see Clapham, 1996).

The sustained calcium influx evoked by activation of mGlu5 receptors in this study could be blocked by a number of inorganic cations of which Zn^{2+} and La^{3+} were the most effective of the ions tested. This is in contrast to Ca^{2+} influx evoked by depolarization of the cells with 25 mM K^+ in neurons which was blocked by 100 μM La^{3+} and Cd^{2+} but virtually unaffected by the same concentration of Zn^{2+}, consistent with Ca^{2+} entry through voltage-gated Ca^{2+} channels (Wakamori *et al*., 1998). Glial cells gave little or no response to high K^+ treatment in contrast to their responsiveness to mGlu receptor agonists suggesting that depolarization of the cells does not lead to significant glutamate release. The most comprehensive study on the effects of inorganic ions on calcium entry pathways is that of Hoth & Penner (1993) studying I_{crac} currents in mast cells. These authors studied a range of inorganic ions and found a potency series similar but not identical to what we have found in cortical neurons and glial cells. For I_{crac} in mast cells, the order of potency was: $Zn^{2+} \geqslant La^{3+} > Cd^{2+} > Be^{2+} \geqslant Co^{2+} > Mn^{2+} > Ni^{2+} > Sr^{2+} > Ba^{2+}$ (Hoth & Penner, 1993), the only difference from this study being a detectably larger inhibitory effect of Cd^{2+} compared to Co^{2+} for I_{crac} in mast cells. Additionally, however, all of the ions seemed to be about 10 fold less potent at inhibiting I_{crac} than they were at inhibiting the plateau phase of the response to mGlu receptor agonists. Other studies have found quite variable effects of certain inorganic ions on Ca^{2+} influx pathways whether the pathways have been activated by store depletion following G protein stimulation of IP_3 production or whether the stores have been depleted directly with thapsigargin or cyclopiazonic acid. For example, La^{3+} has been shown to block Ca^{2+} entry through the SOC channel in cells treated with thapsigargin at concentrations of 1 mM in salivary cells (Foskett & Wong, 1994), 200 nM in C6-2B glioma cells (Chiono *et al*., 1995), around 20 nM Jurkat T cells (Aussel *et al*., 1996) and 75 nM in SH-SY5Y neuroblastoma cells (Grudt *et al*., 1996). In C6-glioma cells, Wu *et al*. (1997) have found that depletion of intracellular stores with thapsigargin leads to activation of a Ca^{2+} influx pathway which was markedly inhibited by La^{3+} and Ni^{2+}. However, in mouse smooth muscle cells La^{3+} had no effect on Ca^{2+} influx through the SOC pathway at concentrations up to 400 μM (Wayman *et al*., 1996). This suggests that a number of different channels may underlie SOC Ca^{2+} entry in different tissues each with rather different pharmacological characteristics.

Although the response of the glial cells and neurons were broadly similar in all aspects of this study, there were some obvious potency differences. For example, all the mGlu receptor agonists were more potent on glial cells than on neurons producing, at submaximal concentrations, both larger Ca^{2+} signals and a larger proportion of responsive cells. In contrast, the blocking action of divalent cations on SOC Ca^{2+} entry was invariably more pronounced in neurons than in glial cells. The larger agonist-evoked signals in glial cells may reflect subtle differences in the nature of the proteins involved in Ca^{2+} mobilization in the two cell types but more likely, it reflects differences in receptor number or a greater signal amplification at some step in the transduction pathway leading to a greater mobilization of Ca^{2+} in glial cells for a given agonist concentration. In view of the heterologous nature of SOC pathways in different cells (see above), differences in the blocking potency of the different ions may truly reflect different proteins in neurons compared with glial cells underlying Ca^{2+} entry.

The sustained rises in intracellular calcium following mGlu5 receptor activation in rat cortical neurons and glial cells are accompanied by sustained changes in intracellular pH with the neurons acidifying and the glial cells alkalizing (Amos & Chesler, 1998; Amos *et al*., 1998). These changes will have profound consequences for cell function both physiologically, and in pathological situations such as cerebral ischaemia where there is a prolonged rise in extracellular glutamate. The sustained rises in intracellular calcium will stimulate calcium-dependent enzyme activity and Ca^{2+} dependent gene expression (see, for example, Clapham, 1995) which may underlie the plasticity changes seen following stimulation of mGlu receptors (e.g. Bashir *et al*., 1993) and which are impaired in mice lacking mGlu5 receptors (Lu *et al*., 1997). Following cerebral trauma, there is a rise in extracellular glutamate and astrocytes proliferate as part of the repair process. Stimulation of astrocyte mGlu5 receptors enhances glial cell proliferation (Ciccarelli *et al*., 1997) and it is probable that either the sustained rise in Ca^{2+} and/or the subsequent Ca^{2+}-dependent glial cell alkalinization (Amos *et al*., 1998) may serve as the signal for astrocytes to proliferate.

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