Characterization of the metabotropic glutamate receptors mediating phospholipase C activation and calcium release in cerebellar granule cells: calcium-dependence of the phospholipase C response

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Abstract

In this study we have determined the metabotropic glutamate receptors (mGluRs) involved in the glutamate activation of phospholipase C (PLC) and Ca2+ mobilization in cerebellar granule cells at 9 days in vitro; and studied the Ca2+ modulation of the PLC response. Both PLC activation and Ca2+ signalling were found to be mediated exclusively by the mGluR1 subtype, although both group I mGluRs, mGluR1α and mGluR5, could be detected in cell extracts. Exposure of cells to medium devoid of Ca2+ for various times before agonist stimulation reduced the PLC response, which was quickly recovered following the re-exposure of cells to Ca2+-containing medium. The extent of the glutamate response correlated well with changes in the cytosolic Ca2+ concentration. On the other hand, loading of the intracellular Ca2+ stores by a transient depolarization followed by washing in nondepolarizing buffer, allowed glutamate to release stored Ca2+ in the majority of cells and enhanced glutamate activation of PLC. Under such conditions, the absence of extracellular Ca2+ during stimulation and the chelation of cytosolic Ca2+ with BAPTA/AM inhibited both glutamate-elicited Ca2+ response and PLC activation. Overall, these results indicate that the mGluR-mediated activation of PLC depends on the presence of extracellular Ca2+ and can be modulated by moderate changes of cytosolic Ca2+. Furthermore, ryanodine reduced PLC stimulation by glutamate in predepolarized cells but not in control cells, suggesting that ryanodine receptors could play a role in the potentiation of the mGluR-mediated activation of PLC by Ca2+ release in predepolarized cells.

Introduction

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system playing an important role in a wide variety of physiological and pathological processes. Metabotropic glutamate receptors (mGluRs) belong to a distinct family of G-protein-coupled receptors which also includes the GABAB receptor and the Ca2+-sensing receptor (Brown et al., 1993; Kaupmann et al., 1997). Currently, eight mGluRs have been cloned and they are subdivided into three groups. Group I mGluRs (mGluR1 and 5) are coupled to phosphoinositide phospholipase C (PLC), whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8) are either negatively coupled to adenylate cyclase or linked to ion channels (for recent reviews see Conn & Pin, 1997; Schoepf et al., 1999).

Stimulation of PLC leads to the generation of inositol 1,4,5-trisphosphate (InsP3), which mobilizes intracellular Ca2+. In some cell systems, the InsP3-elicited increase of cytosolic Ca2+ concentration ([Ca2+]i) can be further enhanced by Ca2+-induced Ca2+ release through ryanodine receptors (Taylor & Broad, 1998). In neurons, a good quantitative correlation between InsP3 formation and changes in [Ca2+]i, has been reported generally, although recent data indicate this correlation can be dependent on the agonist used (Martin et al., 1999; del Río et al., 1999); and it has been suggested that intracellular Ca2+ mobilization is independent of PLC activation (Chavis et al., 1996). On the other hand, agonist stimulation of PLC has been shown to be Ca2+-dependent in several receptor systems. For example, in neuronal cells, a good correlation between increases in [Ca2+]i, and PLC activation has been demonstrated (Willars & Nahoski, 1995; Masgrau et al., 2000), but we have also recently shown that muscarinic activation of PLC is regulated by the Ca2+ levels of the intracellular stores, rather than by changes of [Ca2+]i, in cerebellar granule cells (CGCs) (Masgrau et al., 2000). Whether this regulatory mechanism also operates in other PLC-linked receptors has not been explored yet.

Cerebellar granule neurons in primary culture have been used widely as a neuronal model system to study GluR-mediated signalling pathways. CGCs contain group I mGluRs, as shown by different studies, but divergent results have been reported regarding their expression during culture development (Aronica et al., 1993; Prézeau et al., 1994; Santi et al., 1994; Copani et al., 1998). On the other hand, the contribution of the two subtypes of group I mGluRs to the activation of PLC and Ca2+ mobilization in CGCs has not yet...
been clearly established, although it has been suggested that the mGluR1 subtype could mediate the activation of PLC in CGCs after 4–6 days in vitro (DIV) (Toms et al., 1995).

In the present study we have first examined the expression of both mGluR1α and mGluR5 in CGCs after different days in vitro and determined the group I mGluR subtype involved in the glutamate-induced PLC activation and Ca2+ mobilization at 9 DIV. We have also studied the relationship between both responses at this stage.

Part of the present observations have been previously reported in an abstract from the Society for Neuroscience Meeting (1999).

Materials and methods

Cell culture

Primary cultures of cerebellar granule cells were prepared from 7–8-day-old rats, as previously described (Masgrau et al., 2000). Briefly, rats were decapitated and cerebella immediately dissected out. After removing meninges and blood vessels, the tissue was minced and incubated for 10 min at 37 °C in Ca2+-free Krebs-Ringer buffer containing 0.0025% trypsin. The tissue was then mechanically triturated through a fine-polished glass pipette and filtered through a 40-μm nylon mesh in the presence of 0.52 mg/mL soybean trypsin inhibitor and 170 IU/mL DNase. After centrifugation at 500 g, cells were plated on poly-L-lysine precoated glass coverslips or plates at a density of 1–1.5 × 106 viable cells per millilitre in Basal Eagle’s medium, supplemented with 10% fetal calf serum, 33 mM glucose, 2 mM glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin and 25 mM KCl. After 24 h, cytoseine arabinoside was added to a final concentration of 10 μM to prevent glial cell proliferation. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2/95% air and, unless indicated, routinely used after 9 DIV.

Immunoblot analysis

Cells were cultured in six-well multidishes. After 3, 6, 9 or 12 DIV, cells were washed for 30 min with Krebs-Henseleit/HEPES (KH) buffer at 37 °C and then placed on ice to solubilize cells with 500 μL of sample buffer at high reducing conditions: 125 mM Tris-HCl, 50 mM dithiothreitol, 4% sodium dodecylsulfate, 20% glycerol and 0.01% bromophenol blue, pH 6.8. Samples were collected and 500 μL of water, which was used to rinse the wells, was added. Western blots were carried out using 5% SDS-PAGE minigels, loading 10 μg of protein per lane. Gels were electroblotted onto nitrocellulose membranes and blots were blocked in 5% nonfat dry milk/TBST buffer (20 mM Tris-HCl, 0.9% NaCl, 0.1% Tween, pH 7.5) overnight and then incubated 2 h with a 1 : 1000 dilution in 5% nonfat dry milk/TBST buffer of antiserum raised against the C-terminus of the mGluR1 receptor or 1 : 3000 dilution of antiserum raised against the C-terminus of the mGluR5 receptor. Blots were washed with three changes of the TBST buffer for 30 min and incubated for 2 h with a 1 : 1000 dilution (in 5% nonfat dry milk/TBST buffer) of a horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody. Immunoreactive proteins were detected with enhanced chemiluminescence reagents. As positive and negative controls of the mGluR1α and mGluR5 immunodetection, an inducible expression system consisting of Chinese hamster ovary (CHO) cells transfected with either hmGluR1α or hmGluR5a (Hermans et al., 1997) was used. In this case, cells were plated in 24-well multidishes and kept in culture medium for 48 h before the experiment. When required, isopropyl-beta-D-thiogalactopyranoside (IPTG; 5, 10 or 100 μM) was added to the medium during the final 20 h to induce mGluR1α and mGluR5a expression. For the

PLC activity

PLC activity was assayed by measuring the accumulation of 3H-labelled inositol mono- and polyphosphates ([H]-InsPs) in the presence of 10 mM LiCl, as previously described (Masgrau et al., 2000). Cells cultured in 24-well plates were prelabelled with 2 μCi/mL [3H]myo-inositol for 4 h. Media were then removed and cells were washed and incubated for 30 min in KH buffer (in mM: NaCl, 113; KCl, 4.7; KH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 25; glucose, 11; and HEPES, 5, with or without CaCl2 1.3 added, pH 7.4 equilibrated with 5% O2/95% CO2 at 37 °C). When CaCl2 was omitted in the KH buffer, the extracellular Ca2+ concentrations were lower than 1 μM as fluorometrically assessed with fura-2. Buffer was then aspirated and cells were incubated for 1 min with 0.5 mL of buffer containing 10 mM LiCl with or without agonist. When appropriate, cells were preincubated with 20 min with antagonists or other compounds before the addition of agonists and these compounds were also present during stimulation. Reactions were terminated with 0.8 mL ice-cold methanol, cells were then scraped and transferred to test tubes with 0.9 mL chloroform and two phases were generated by adding 0.75 mL of 0.17 mM HCl. After 5-min centrifugation at 2000 g, 1 mL aliquots of the aqueous phases were neutralized with 1.5 m NH4OH and applied to a Dowex (AG1-X8) formate column. [H]-InsPs were eluted with 9 mL of 1 M ammonium formate/0.1 M formic and counted by liquid scintillation spectrometry. The organic phases containing [3H]-lipids were washed with 1.55 mL methanol/water (1 : 1, v/v) and 0.2 mL aliquots were also counted for radioactivity. Accumulation of [3H]-InsPs was calculated as the percentage of [H]-lipids.

Intracellular Ca2+ concentration

Experiments were performed with cells grown on glass coverslips. Briefly, culture medium was removed and cells loaded with the Ca2+ indicator fura-2 by bathing them in a KH buffer containing 2 μM fura-2/AM for 60 min at room temperature. Cells were then rinsed and the coverslips mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. When cells were loaded with both fura-2 and BAPTA, BAPTA/AM was added at this moment. From this point, cells were maintained in KH buffer at 37 °C. After excitation at 340 and 380 nm, fluorescent images at wavelengths above 510 nm were collected with an intensified charge-coupled device camera (Photonics Science, Robertsbridge, East Sussex, UK) and the 340 : 380 ratios were converted to approximated Ca2+ concentrations using the Applied Imaging Quantecel 700 System (Applied Imaging International Ltd, Sunderland, UK). A calibration curve was performed according to the method of Grynkiewicz et al. (1985). When [Ca2+]i levels were estimated, 20–25 cells were analysed in each culture, and the mean values from different cultures were averaged. When appropriate, cells were preincubated for 20 min with antagonists or other compounds before the addition of agonists and these compounds were also present during stimulation.

Data analysis

Concentration–response and inhibition curves were analysed by nonlinear regression with the program GRAPHPAD PRISM (GraphPad Software Inc.). The computed parameter estimates (EC50 and Emax for concentration–response curves and IC50 for inhibition curves) for each group of n-replicated experiments were expressed as
mean ± SEM and, for display purposes, they were used to generate a
logistic curve that was drawn through the experimental data.

Statistical significance was evaluated by performing analysis of
variance followed by Dunnett’s test for multiple comparisons, using
the SAS (Cary, NC, USA) statistical package. Significance was taken
at P < 0.05.

Materials

L-Glutamic acid, L-quisqualic acid, CHPG, DHPG, 1S,3R-ACPD,
AIDA, CNQX, CPCCOEt, D-AP5, MCPG, MPEP and SIB-1893
were purchased from Tocris-Cookson (UK). The anti-mGluR1α
antibody was from Chemicon (CA, USA) and the anti-mGluR5
antibody was from Upstate Biotechnology (NY, USA). Fura-2/AM,
trypsin, soybean trypsin inhibitor, DNase I, poly-l-lysine and the
horseradish peroxidase-conjugated goat antirabbit and antimouse
secondary antibodies were from Sigma-Aldrich, (Laeufel ingen, Switzerland). [3H]myo-inositol and ECL enhanced
chemiluminescence reagents were from Amersham Ibérica (Spain).
All cell culture media and reagents were purchased from GIBCO Life
Technologies (UK). Other chemicals used were of analytical grade.

Results

Western blot analysis of the expression of mGluR1α and
mGluR5 in CGCs

As a first step in the identification of the group I mGluRs involved in
glutamate-elicited PLC activation and Ca2+ mobilization in CGCs,
the expression of mGluR1α and mGluR5 proteins was studied using
polyclonal selective antibodies, and hmGluR1α- and hmGluR5α-transfected CHO cells were used as controls to exclude
any cross-reactation. As shown in Fig. 1, both receptors were detected
in CGC cultures at the different DIV studied. mGluR1α migrated
as a unique band whereas mGluR5 showed two different bands. In
transfected CHO cells, both mGluR1α and mGluR5α receptors also
migrated as two different bands in agreement with previous results
(Hermans et al., 1998a), and this has been attributed to a different
glycosylation of the receptor inside the same cell (Mody et al., 1999).
This or other post-translational modifications might also account for
the different pattern of mGluR1α detected in CGCs and transfected
CHO cells.

Determination of the receptor subtypes involved in the mGluR
activation of PLC

As our results show that CGCs express both subtypes of group I
mGluRs at 9 DIV, a time when cultures are considered mature, we
performed the pharmacological characterization of mGluR-induced
PLC activation at this stage. PLC activity was measured as the
accumulation of [3H]-InsPs after 30 min of agonist application and, in
order to eliminate involvement of iGluRs, cells were preincubated for
20 min with 30 μM CNQX and 50 μM AP5 in all the experiments in
which glutamate or quisqualate responses were determined.

Glutamate, the mGluR agonist 1S,3R-ACPD and the group I
mGluR agonists, quisqualate and DHPG, but not the mGluR5-
selective agonist CHPG (Doherty et al., 1997), stimulated PLC
activity in CGCs in a dose-dependent manner (Fig. 2A). Quisqualate and
DHPG were the most potent agonists with EC50 values of
8.2 ± 1.1 μM (n = 4) and 9.1 ± 1.1 μM (n = 3), respectively, fol-
lowed by 1S,3R-ACPD (56.4 ± 6.4 μM, n = 3), and Glu
(119.1 ± 1.2 μM, n = 3). Glu exhibited the highest maximal effect
(1344 ± 47% of basal), followed by quisqualate (725 ± 25), DHPG
(362 ± 8) and 1S,3R-ACPD (296 ± 5). The rank order of agonist
potency fits the pharmacological profile reported for group I mGluR-
coupled activation of PLC (Conn & Pin, 1997; Schoepf et al., 1999)
and the lack of effect of CHPG suggests that mGluR5 are not
involved in the response.

Further characterization of the glutamate-induced [3H]-InsP accu-
mulation was achieved by studying the effects of different group I
mGluR antagonists. Figure 2B shows the effect of both MCPG and
AIDA, which have been reported to show higher potency antagoniz-
ing the glutamate effect on mGluR1 than mGluR5 in transfected cells
(Brabet et al., 1995; Moroni et al., 1997) and the specific mGluR1
antagonist CPCCOEt (Casabona et al., 1997; Hermans et al., 1998b).
All these antagonists inhibited the accumulation of [3H]-InsPs elicited
by 100 μM glutamate with IC50 values of 66.5 ± 12.0 μM,
91.8 ± 15.0 μM and 12.1 ± 1.4 μM, respectively (n = 3).
Recently, SIB-1893 and the structurally related compound, MPEP, have
been described as potent and selective mGluR5 antagonists which inhibit
hmGluR5α-elicited responses in expression systems with IC50 values
of 0.29 μM and 39 nM, respectively, and present IC50 values higher
than 100 μM at hmGluR1β-mediated responses (Gasparini et al.,
2001 Federation of European Neuroscience Societies, European Journal of Neuroscience, 13, 248–256
inhibition was observed at very high concentrations (100 μM) of MPEP, as shown in Fig. 2C. None of the antagonists mentioned above had any significant effect on basal PLC activity. These results clearly support the conclusion that mGluR1 is the receptor subtype mediating the glutamate stimulation of PLC in CGCs after 9 DIV.

Determination of the receptor subtypes involved in the mGluR-elicited Ca²⁺ mobilization

Glutamate-elicited changes in [Ca²⁺], were routinely studied in fura-2-loaded CGCs at 9 DIV after a transient depolarization of 1 min incubation with 40 mM KCl, a treatment that allows Ca²⁺ entry and replenishment of the intracellular Ca²⁺ stores (Murphy & Miller, 1989; Irving et al., 1992; Fohrman et al., 1993; Masgrau et al., 2000), followed by washing in nondepolarizing KH buffer, before the addition of agonists. When glutamate or quisqualate responses were determined, cells were preincubated for 20 min with 30 μM CNQX and 50 μM AP5.

The glutamate-evoked changes in [Ca²⁺], showed heterogeneity, both within cells of the same culture and between independent cultures. Glutamate (1 mM) induced a single-peaked increase of [Ca²⁺], which averaged 1.84 ± 0.08-fold of basal (Fig. 3A) in approximately 60% of the cells in five of the eight imaged cultures and in more than 90% of the cells in the other three. In the absence of extracellular Ca²⁺, glutamate also elicited a [Ca²⁺], increase but the average response was smaller (1.27 ± 0.02-fold of basal, n = 3) than the signal found in the presence of 1.3 mM Ca²⁺ (Fig. 3A).

The effect of other mGluR agonists was also studied. Quisqualate (30 μM), 1S,3R-ACPD (300 μM) and DHPG (100 μM) also induced peaked increases of [Ca²⁺], as shown in Fig. 3B for DHPG, amounting 1.60 ± 0.07, 2.03 ± 0.34 and 1.85 ± 0.23-fold over basal, respectively (n = 3). The percentage of responding cells was low and depended on the agonist (quisqualate: 35–45%, 1S,3R-ACPD: 50–55%, DHPG: 15–20%). The responses to these agonists were totally dependent on the presence of extracellular Ca²⁺, as shown in Fig. 3 and previously reported for 1S,3R-ACPD (Irving et al., 1992). On the other hand, the mGluR5 selective agonist CHPG was unable to induce any [Ca²⁺], increase, even at high concentrations (2 mM) (Fig. 3C). Finally, incubation of the cells with the mGluR1-selective antagonist CPCCOEt (100 μM), resulted in a total inhibition of the glutamate (1 mM)-elicited Ca²⁺ response (Fig. 3D). All these results indicate that the mGluR subtype mediating Ca²⁺ responses, in addition to PLC stimulation, is the mGluR1.

Ca²⁺-dependence of PLC activation by glutamate

Once determined as the receptor responsible for glutamate-induced Ca²⁺ responses and PLC activation, we have studied the Ca²⁺-dependence of mGluR1 activation of PLC in CGCs at 9 DIV measuring the accumulation of [³H]-InsPs in [³H]myo-inositol-labelled cells after 1 min of agonist stimulation in the presence of 10 mM LiCl. The extracellular Ca²⁺-dependence of the response was determined in cells preincubated for 30 min in KH buffer in the presence of the iGluR antagonists CNQX (30 μM) and AP5 (50 μM), and stimulated for 1 min with 1 mM glutamate. When extracellular Ca²⁺ (1.3 mM) was present during both preincubation and stimulation, glutamate evoked a significant stimulation of [³H]-InsPs accumulation (172.8 ± 9.8% of basal, n = 3). Replacement of the medium by a nominally Ca²⁺-free KH for different time intervals before glutamate addition resulted in a rapid decrease of the net agonist response (48.8 ± 9.1, 48.8 ± 5.1, 59.9 ± 11.4 and 66.0 ± 11.1% reduction after 1, 3, 10 and 30 min of Ca²⁺ omission, respectively; n = 3, Fig. 4). On the other hand, after 30 min preincubation in the absence of Ca²⁺, replacing the medium for a
buffer containing 1.3 mM Ca\(^{2+}\) at various time intervals before glutamate stimulation resulted in a complete recovery of the response which, even after only 1 min of Ca\(^{2+}\) addition, was not significantly different from that shown by control cells preincubated and stimulated in the presence of extracellular Ca\(^{2+}\) (Fig. 4).

Since the omission of extracellular Ca\(^{2+}\) also results in a rapid lowering of [Ca\(^{2+}\)], (from 69.7 ± 2.1 nM to 44.7 ± 3.0 nM, n = 3), which returns to control levels in less than 1 min after Ca\(^{2+}\) addition (Masgrau et al., 2000), the rapid effects of the above treatments on the PLC activation by glutamate could reflect the effects of [Ca\(^{2+}\)] changes on the response. We explored this possibility using cells in which the intracellular Ca\(^{2+}\) stores had been replenished by a transient depolarization, a treatment that, as described above, is required to show a glutamate-elicited increase of [Ca\(^{2+}\)], in a majority of cells. Cells were incubated for 1 min with 40 mM K\(^+\) and then washed in a nondepolarizing KH buffer. One minute later, cells were exposed to 1 mM glutamate and 10 mM LiCl also for 1 min in the same medium. As shown in Fig. 5, the previous transient depolarization did not change the basal accumulation of [Ca\(^{2+}\)], but notably decreased both the peak and plateau of [Ca\(^{2+}\)] elevation elicited by 40 mM K\(^+\) and suppressed the glutamate-evoked Ca\(^{2+}\) mobilization (Fig. 6A).

Finally, we studied the involvement of ryanodine receptors in the Ca\(^{2+}\)-dependence of PLC activation by glutamate, since these Ca\(^{2+}\) channels have been shown to play a major role in the mGluR-mediated intracellular Ca\(^{2+}\) release in depolarized CGCs (del Río et al., 1999). As shown in Fig. 7, inhibition of ryanodine receptors with 10 μM ryanodine had no effect in control cells but resulted in a...

Fig. 3. Characterization of the mGluR-mediating Ca\(^{2+}\) signalling in cerebellar granule cells. (A–C) Cells were stimulated with either 1 mM glutamate (Glu), 100 μM DHPG or 2 mM CHPG for 1 min before and after a transient depolarization with 40 mM KCl, as indicated. In the cases of glutamate and DHPG, cells were also exposed to the agonists in the absence of extracellular Ca\(^{2+}\). In the case of glutamate, cells were preincubated for 20 min with the iGluR antagonists CNQX (30 μM) and AP5 (50 μM). (D) Cells were preincubated for 20 min with 100 μM CPCCOEt, 30 μM CNQX and 50 μM AP5, and then stimulated with 1 mM Glu before and after a transient depolarization with 40 mM KCl. The traces are from individual cells representative of 25 different cells analysed. Similar results were obtained in three independent cultures.
significant reduction (42.3 ± 11.2%, n = 3) of the glutamate stimulation of PLC in predepolarized cells, being the resultant response similar to that found in control cells in the absence of ryanodine. These results suggest the involvement of Ca\(^{2+}\) release through ryanodine receptors in the potentiation of the mGluR-mediated PLC activation in predepolarized cells.

**Discussion**

The initial aim of this work was to determine the presence of group I mGluRs in primary cultures of CGCs and their involvement in the glutamate stimulation of PLC and Ca\(^{2+}\) responses at 9 DIV. Using selective polyclonal antibodies, we have detected both mGluR1 and mGluR5 in these cultures and, whereas mGluR1 expression was found constant from 3 to 12 DIV, the level of mGluR5 increased with time in culture. Among the different expression patterns previously reported by other authors (Aronica et al., 1993; Prézeau et al., 1994; Santi et al., 1994; Copani et al., 1998), our results are in partial agreement with a study by Santi et al. (1994) showing that the content of mGluR1 mRNA was relatively constant through culture development, except by a lower expression at 9 DIV, whereas the mGluR5-mRNA level increased gradually with time.

The pharmacological characterization of the mGluR-mediated responses was performed at 9 DIV, when CGCs are considered mature (Thangnipon et al., 1983) and express both subtypes of group I mGluRs. At this stage, mGluR1, but not mGluR5, mediated the activation of PLC. The rank order of agonist potency corresponded to that described for group I mGluR-elicited responses in various nerve tissue preparations (Conn & Pin, 1997; Sacaan et al., 1998; Schoepf et al., 1999; Servitja et al., 1999) and in CGCs at 4–6 DIV for part of these agonists (Toms et al., 1995).
was inhibited by MCPG, AIDA and the selective mGluR1 antagonist CPCCOEt, with IC$_{50}$ values corresponding to the involvement of mGluR1 (Hermans et al., 1998b; Schoepp et al., 1999). On the other hand, the lack of PLC activation by the mGluR5-selective agonist CHPG (Doherty et al., 1997) and the inability of the recently described mGluR5-selective antagonists, SIB-1893 and MPEP (Gasparini et al., 1999; Varney et al., 1999), to inhibit the glutamate response with the expected potency indicated that mGluR5, although expressed, does not participate in the glutamate activation of PLC. A similar conclusion was reached when mGluR-mediated Ca$^{2+}$ mobilization was characterized, since all the above agonists, but not CHPG, were able to increase [Ca$^{2+}$], in depolarized CGCs and CPCCOEt blocked the glutamate-induced Ca$^{2+}$ response. The basis for the lack of involvement of mGluR5 in the glutamate responses in cultured CGCs is unknown, and additional experimental approaches are needed to explore the several possibilities that could be considered, including the occurrence of a low coupling efficiency between the receptors and PLC, targeting of the receptor to a cell compartment different from plasma membrane or desensitization evoked by the glutamate released by CGCs grown in depolarizing conditions.

Discrepant results have been previously reported on the ability of mGluR agonists to mobilize Ca$^{2+}$ in cultured CGCs. Whereas some authors showed mGluR-mediated increase of [Ca$^{2+}$], in resting cells (Courtney & Nicholls, 1990; Pizzi et al., 1996; Simpson et al., 1996), others found the response only after loading the intracellular Ca$^{2+}$ stores (Irving et al., 1992) or in depolarizing conditions (del Río et al., 1999). The above discrepancies, however, could be due to the different stages of culture development studied, being calcium signals recorded in resting cells when measurements were performed at 3–6 DIV, but not, or very scarcely observed, as in our study, at 9 DIV. The number of resting cells responding to mGluR agonists could be related to the number of receptors in the cell membrane, as suggested by results from Hermans et al. (1998a). In CGCs, this would not refer to the global mGluR1 expression but to the actual number of functional receptors, which could be lower after 9 DIV as a consequence of desensitization evoked by endogenous glutamate. On the other hand, our results also show that the percentage of CGCs showing a Ca$^{2+}$ response to mGluR agonists also depends on the loading state of the intracellular Ca$^{2+}$ stores, in agreement with recent results showing that filling the Ca$^{2+}$ stores may enhance the frequency of elementary Ca$^{2+}$ release signals and their coupling to produce global Ca$^{2+}$ responses in PC12 cells and hippocampal neurons (Koizumi et al., 1999).

The second aim of this study was to characterize the Ca$^{2+}$-dependence of the glutamate activation of PLC in CGCs. In previous studies we have described how muscarinic receptor-mediated activation of PLC in these cells is modulated by the filling state of the intracellular Ca$^{2+}$ stores rather than by moderate changes of [Ca$^{2+}$], (Masgrau et al., 2000). In the present work, a similar study was performed to characterize the mGluR1-mediated activation of PLC in CGCs. The results showed that the glutamate response is regulated by small changes in [Ca$^{2+}$]. This was first shown by the rapid modulation of the PLC response by omission and readdition of extracellular Ca$^{2+}$, which was better correlated with the rapid changes of [Ca$^{2+}$], evoked by these treatments (Masgrau et al., 2000) than with the more gradual changes in the intracellular Ca$^{2+}$ stores that become progressively depleted in a Ca$^{2+}$-free medium (Fohrman et al., 1993). Furthermore, the potentiation of the PLC response
achieved by a previous transient depolarization was inhibited by both omission of extracellular Ca\(^{2+}\) during stimulation and cytosolic Ca\(^{2+}\) chelation with BAPTA/AM. Since the glutamate-elicited increase of [Ca\(^{2+}\)]\(i\) in predepolarized cells was also reduced by the absence of extracellular Ca\(^{2+}\) during stimulation and abolished by treatment with BAPTA/AM, it can be concluded that the potentiation of the PLC activation by glutamate observed after filling the stores by a transient depolarization may result from the increased ability of the stores to release Ca\(^{2+}\) and increase [Ca\(^{2+}\)]\(i\) under these conditions. In all these experiments, however, the possibility of a direct modulation of mGluR1 activity by external Ca\(^{2+}\) cannot be ruled out, since recent reports have shown that changes of Ca\(^{2+}\) concentration in the millimolar range result in regulation of glutamate activation of PLC in heterologous expression systems (Kubo et al., 1998; Saunders et al., 1998).

Our results indicate that, in contrast to the muscarinic activation of PLC (Masgrau et al., 2000), mGluR-mediated PLC response is highly sensitive to relatively small changes of [Ca\(^{2+}\)]\(i\), such as those resulting from changes of extracellular Ca\(^{2+}\) concentration or from the intracellular Ca\(^{2+}\) release that takes place in predepolarized CGCs. Thus, different Ca\(^{2+}\)-dependent regulatory mechanisms of the agonist activation of PLC appear to be relevant in cultured CGCs depending on the receptors involved. The basis for this difference is unknown at present. Besides a different localization of both receptors, it can be speculated that different PLC isoenzymes with different Ca\(^{2+}\) sensitivity may be involved in mGluR and muscarinic receptor stimulation, as already suggested by Irving et al. (1992).

Increasing evidence suggests a complex relationship between group I mGluRs, Ca\(^{2+}\) stores and Ca\(^{2+}\) channels in neurons, and a major role of ryanodine receptors in mGluR-mediated Ca\(^{2+}\) signals has been proposed (Chavis et al., 1996; Fagni et al., 2000). These authors showed that mGluR1 agonists facilitate a PLC-independent coupling between ryanodine receptors and 1-type Ca\(^{2+}\) channels in mouse CGCs under depolarizing conditions; and del Río et al. (1999) found a greater ryanodine inhibition of the Ca\(^{2+}\) release elicited by mGluR agonists than by muscarinic agonists in mildly depolarized CGCs. In this context, we have described here that ryanodine also inhibits the potentiation of mGluR1-mediated PLC activation in store-filled cells able to release intracellular Ca\(^{2+}\). All these data indicate that mGluR-evoked Ca\(^{2+}\) release in predepolarized CGCs may occur mostly through ryanodine receptors, and that this response may regulate the extent of PLC activation by the same agonists.

In conclusion, we have shown that PLC activation and Ca\(^{2+}\) mobilization are mediated by mGluR1 in cultured CGCs. In addition, there is a different Ca\(^{2+}\)-dependence of PLC activation by mGluR1 and muscarinic receptors, such that mGluRs are modulated by moderate changes of [Ca\(^{2+}\)]\(i\). Our results support the conclusion that, although mediating the same signalling pathway, different PLC-linked receptors can show different Ca\(^{2+}\)-dependence and regulatory mechanisms.

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Abbreviations

AIDA, 1-aminoindan-1,5-dicarbonylic acid; 1S,3R-ACPD, (1S,3R)-1-amino- cyclopentane-1,3-dicarbonylic acid; AM, acetylomethyl ester; BAPTA/AM, 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (acetoxymethyl ester); [Ca\(^{2+}\)]\(i\), cytosolic Ca\(^{2+}\) concentration; CGCs, cerebellar granule cells; CHO, Chinese hamster ovary; CHPG, (RS)-2-chloro-5-hydroxyphenylglycine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPCCOEt, cyclopro- pan[b]chromen-1a-carboxylic acid ethylester; D-AP5, D(-)-2-amino-5-phosphono- pumpatoic acid; DHPG, (S)-3,5-dihydroxyphenylglycine; InsPs, inositol phosphates; InsP3, inositol 1,4,5-trisphosphate; IPTG, ; KH buffer, Krebs-Henseleit/HEPES buffer; MCPG, (S)-2-methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; MPEP, 2-Methyl-6-(phenylethyl- nyl)-pyridine; PLC, phosphoinositide phospholipase C; SIB-1893, (E)-2- methyl-6-(2-phenylethyl)pyridine; TBST buffer, comprised of 20 mM Tris-HCl, 0.15 m NaCl and 0.1% Tween, pH 7.5.

References


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