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Metabotropic and Ionotropic Transducers of Glutamate Signal Inversely Control Cytoplasmic Ca^{2+} Concentration and Excitotoxicity in Cultured Cerebellar Granule Cells: Pivotal Role of Protein Kinase C

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SUMMARY

We investigated the functional role of metabotropic glutamate receptors (mGluRs) in modulating glutamate-affected neuronal intracellular calcium concentration ($[Ca^{2+}]_i$) and cell viability in rat cerebellar granule cells. The mGluR agonist *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (tACPD) induced a transient increase in $[Ca^{2+}]_i$, which seemed to be developmentally regulated and maximal at 4 days *in vitro*. In addition, tACPD significantly prevented the $[Ca^{2+}]_i$ rise produced by glutamate or by *N*-methyl-D-aspartate. The mGluR antagonists L-2-amino-3-phosphonopropionic and (+)-α-methyl-4-carboxyphenylglycine blocked the effects of tACPD, but intrinsically, they magnified the glutamate-mediated $[Ca^{2+}]_i$ elevation. The tACPD-mediated decrease in $[Ca^{2+}]_i$ rise occurred under experimental conditions superimposable on those producing neuroprotection in glutamate-exposed cultures. tACPD affected neither $[Ca^{2+}]_i$ elevation due to KCl nor that evoked by the calcium ionophore A 23187. The inhibitory effect of tACPD was also unaffected by K^+ channel blockade produced by tetraethylammonium. The tACPD effects were fully mimicked by quisqualate and (*RS*)-3,5-dihydroxyphenylglycine, whereas they were only partially reproduced by (2*S*,1′*S*,2′*S*)-2-carboxycyclopropyl-glycine. L-2-Amino-4-phosphonobutyrate was inactive in preventing glutamate-mediated $[Ca^{2+}]_i$ rise and neurotoxicity. The tACPD inhibitory responses seemed to be highly sensitive to protein kinase C blockade by bisindolylmaleimide or staurosporine, whereas they were weakly affected by the cAMP analogue dibutyryl cAMP. The protein kinase C activator 4β-phorbol-12,13-dibutyrate reproduced mGluR-mediated inhibition of both glutamate-induced $[Ca^{2+}]_i$ rise and neurotoxicity. In summary, these data suggest that activation of mGluR1–5 subtypes reduce glutamate-mediated $[Ca^{2+}]_i$ rise through a mechanism involving protein kinase C activation. Such an effect results in neuroprotection.

Glutamate is the major fast excitatory neurotransmitter in the mammalian central nervous system. The interest in glutamate neurotransmission has grown since glutamate was found to control a number of cell functions, including neuronal development (1), neuronal plasticity (2), and neuronal cell death (3). In particular, glutamate has been shown to induce neuron degeneration when it overaccumulates in the intersynaptic space. This may occur *in vivo* under specific conditions, such as hypoxia, ischemia, trauma, and epilepsy (4, 5). Excitotoxicity is thus more generally considered a final common pathway for neuronal injury, even secondary to diseases with different causes (6). The responses mediated by glutamate may result from the coordinated activation of structurally, pharmacologically, and functionally distinct classes of receptors, i.e., iGluRs, which include the NMDA, the AMPA, and the kainate receptor subtypes and the mGluRs. mGluRs are a family (at least eight receptor subtypes) of G protein-associated receptors (7, 8) coupled to multiple signal transduction systems. Although mGluR1 and mGluR5 are linked to polyphosphoinositide hydrolysis, the other subtypes are negatively coupled with adenylate cyclase (8). It is generally accepted that glutamate-mediated neuronal injury is related, at least in part, to a disruption in intracellular calcium homeostasis (10, 11). Excessive stimulation of postsynaptic iGluRs and the concurrent increase in

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ABBREVIATIONS: iGluR, ionotropic glutamate receptors; mGluR, metabotropic glutamate receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; tACPD, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid; NMDA, *N*-methyl-D-aspartate; L-AP3, L-2-amino-3-phosphonopropionic acid; MCPG, (+)-α-methyl-4-carboxyphenyl-glycine; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; L-CCG-I, 2*S*,1′*S*,2′*S*)-2-carboxycyclopropyl-glycine; PDBu, 4β-phorbol-12,13-dibutyrate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; DIV, days *in vitro*; KRS, Krebs-Ringer solution; TEA, tetraethylammonium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl.

$[Ca^{2+}]_i$ for a prolonged period of time have been shown to lead to neurodegeneration in primary cultures of hippocampal (12), cerebral cortical (10, 13), cerebellar (14), spinal (15), and retinal cells (16). Both glutamate-induced neurotoxicity and $[Ca^{2+}]_i$ rise are mediated by ionotropic NMDA and non-NMDA receptors, as revealed by the use of selective antagonists (10, 17, 18). On the contrary, stimulation of mGluRs has been found to attenuate excitotoxicity in different experimental models (19–21). This finding led to the hypothesis of possible "cross-talking" between the different glutamate receptor subtypes, suggesting that mGluRs may act as inhibitory modulators of the iGluR activity. Up to now, the mGluR subtypes involved in hindering neurotoxicity as well as the intracellular mechanisms responsible for mGluR modulatory effects have not been completely clarified.

We previously showed (22) that in primary cultures of rat cerebellar granule cells, neuronal death induced by excitatory amino acids can be significantly prevented by mGluR activation through quisqualate and tACPD. These compounds have been found to mobilize intracellular calcium stores in rat cerebellar granule cells (23) as well as in other neuronal cell populations (24, 25). Because both tACPD-mediated inositol phosphate production and $[Ca^{2+}]_i$ rise occur at concentrations counteracting glutamate toxicity (22), in the current study we investigated the role of mGluR stimulation in glutamate-mediated disturbance of neuronal $[Ca^{2+}]_i$ homeostasis. Moreover, we tentatively characterized the pharmacological profile of mGluRs involved in the control of glutamate transmission and defined the second messenger pathway activated during the neuroprotective process.

Materials and Methods

Cell culture. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rat pups as described previously (22). Cells were plated onto poly-L-lysine-coated dishes and cultured in basal Eagle's medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 μg/ml gentamicin, and 25 mM KCl at a density of 2.5×10^5 cells/cm^2. Cytosine arabinoside (10 μM) was added to the cultures 18 hr after seeding to prevent non-neuronal cell proliferation. Experiments were carried out after culturing the neurons for different days as indicated.

Measurement of $[Ca^{2+}]_i$. Cytosolic free calcium concentration was investigated with the use of microfluorimetry in single cells as previously described (26) with minor modifications. Cells were plated onto poly-L-lysine-coated glass coverslips (100 μg/ml poly-L-lysine) and cultured as described above. At different DIV, cells were loaded with the calcium-sensitive fluorescent dye Fura 2-AM through a 60-min incubation at 37° (4 μM in KRS consisting of 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM HEPES-NaOH, 6 mM glucose, and 1 mg/ml bovine serum albumin, pH 7.4).

Fura 2-AM emission was monitored with an inverted fluorescence microscope (Nikon Diaphot) associated with an intensified charged-coupled device camera (MIRA-100 TE) that recorded the 510 nM fluorescence emission in neurons excited through narrow band-pass filters (340 and 380 nM). The background was subtracted, and the amount of free calcium within the cells was calculated from the ratio of 340/380 nm obtained every 3–4 sec. Calibration was carried out according to external standards of calcium and Fura 2-AM (27). Fluorescence image acquisition and analysis were performed with the use of the Multiple Image Ratioing and Analysis with Calibration system (MIRAcal, Applied Imaging).

Cells were exposed to glutamate for 1.5 min (S1) in the chamber containing Mg^{2+}-free KRS. The cells were then washed with a calcium-free solution and returned to the Mg^{2+}-free KRS. A second glutamate pulse, applied 10 min later, produced an identical or slightly smaller response (S2). Plateau value of $[Ca^{2+}]_i$ rise was calculated based on the mean of four determinations taken at 20, 40, 60, and 80 sec after application of stimulating agents. To study mGluR modulation of glutamate-mediated $[Ca^{2+}]_i$ rise, we added test drugs to the chamber before the second glutamate pulse. Ratios between plateau values of second and first glutamate pulse (S2/S1) were compared to quantify the effect of test drugs.

Measurement of glutamate excitotoxicity. Unless otherwise indicated, cultures were exposed for 15 min to 50 μM glutamate in a Mg^{2+}-free Locke's solution. When present, mGluR agonists were added 5 min before glutamate treatment as described previously (22). Dishes were then returned to cultured conditioned medium at 37° in 95% air/5% CO_2, and cell viability was measured 18–24 hr later. To confirm the correlation between glutamate effect on $[Ca^{2+}]_i$ levels and excitotoxicity, sister cultures were treated with or without tACPD in a Mg^{2+}-free KRS, and after 1 min they were exposed to glutamate for 1.5 min. Afterward, cells were washed with fresh KRS and returned to culture-conditioned medium. Cell viability was evaluated 18 hr later by intravital staining with fluorescein diaceate and propidium iodide mixture, as described previously (22).

Drugs. NMDA, glutamate, PDBu, staurosporine, tetraethylammonium, and dibutyryl cAMP were purchased from Sigma; tACPD, MCPG, L-AP4, L-CCG-I, L-AP3, quisqualate and DHPG were purchased from Tocris; and bisindolylmaleimide was purchased from Calbiochem.

Results

Effect of tACPD on $[Ca^{2+}]_i$ in rat cerebellar granule cells. Rat cerebellar granule cells for $[Ca^{2+}]_i$ recording were cultured for different periods of time from 1–15 DIV. During this period, cells developed a maturation process, as shown by neurite outgrowth, differentiation, and increased vulnerability to glutamate excitotoxicity. To concentrate our study primarily on the events occurring in the cell body, a small recording field (70 μm^2) was used in our experiments.

$[Ca^{2+}]_i$ of resting granule cells at 6 DIV was found to be 42 ± 13 nM (80 cells) (Fig. 1A). tACPD, added for 2 min at 100 μM, induced a transient rise in $[Ca^{2+}]_i$ in 90% of investigated cells (80 cells). Basal level of $[Ca^{2+}]_i$ increased by ~250 nM during the early 15 sec of tACPD application and then rapidly slowed. tACPD effect was also investigated in neurons at different DIV (1–15). As shown in Fig. 1B, tACPD response followed a bell-shaped curve. Maximal responsiveness to tACPD was observed in 4-day-old neurons (40 cells), being almost absent in cells at the first DIV (38 cells) and still present but much lower in 13-day-old cells (53 cells). Resting $[Ca^{2+}]_i$ levels did not modify during the time (15 days) in culture (data not shown).

mGluR activation reduces glutamate-mediated $[Ca^{2+}]_i$ rise. Glutamate administered in a Mg^{2+}-free solution at 50 μM evoked a rapid $[Ca^{2+}]_i$ rise followed by a sustained plateau (S1, 561 ± 52 nM over basal value) in almost all of the neurons investigated (130 cells) at 10–15 DIV (Fig. 2A). A second, nearly identical response to glutamate was seen by repeating the treatment 10 min after washing the cells (S2, 533 ± 59 nM; S2/S1, 0.95 ± 0.05). When the Mg^{2+}-free KRS was replaced by the Mg^{2+}-containing medium, the effect of glutamate seemed markedly depressed (data not shown), suggesting that it mainly involves NMDA receptor activation. Indeed, glutamate-mediated $[Ca^{2+}]_i$ rise was prevented by 1 μM MK 801 (Fig. 2A), a selective antag-

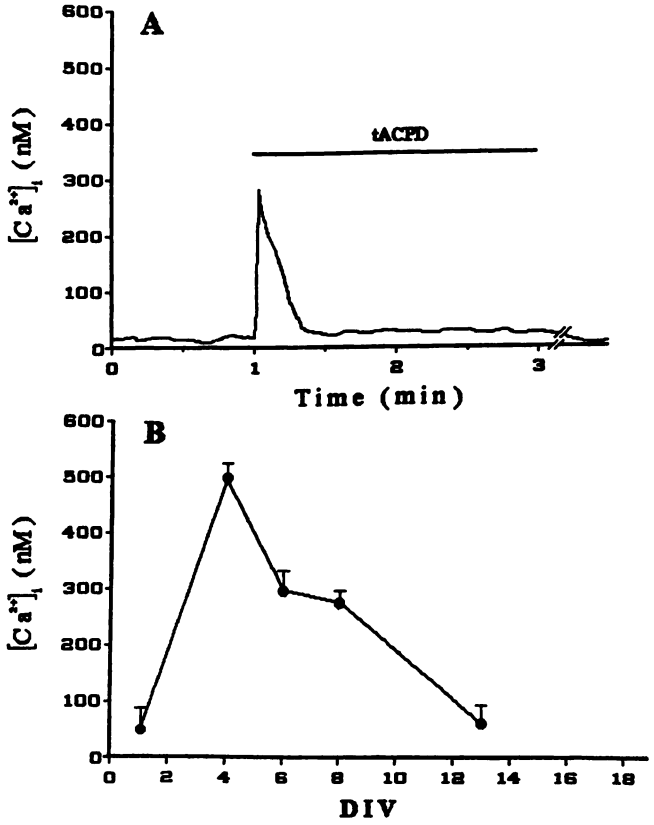

Fig. 1. Effect of bath application of 100 μM tACPD on $[Ca^{2+}]_i$ in single rat cerebellar granule cells. A, Representative of tACPD response in neurons (72 of 80) at 6 DIV. B, Developmental profile of tACPD response. Resting $[Ca^{2+}]_i$ level (40 ± 10 nM) did not change during the time in culture. Maximal neuron responsiveness was found in neurons at 4–8 DIV (40 cells). Lower values were observed at 1 DIV (38 cells) and 13 DIV (53 cells). Values are expressed as mean ± standard error of ≥38 determinations.

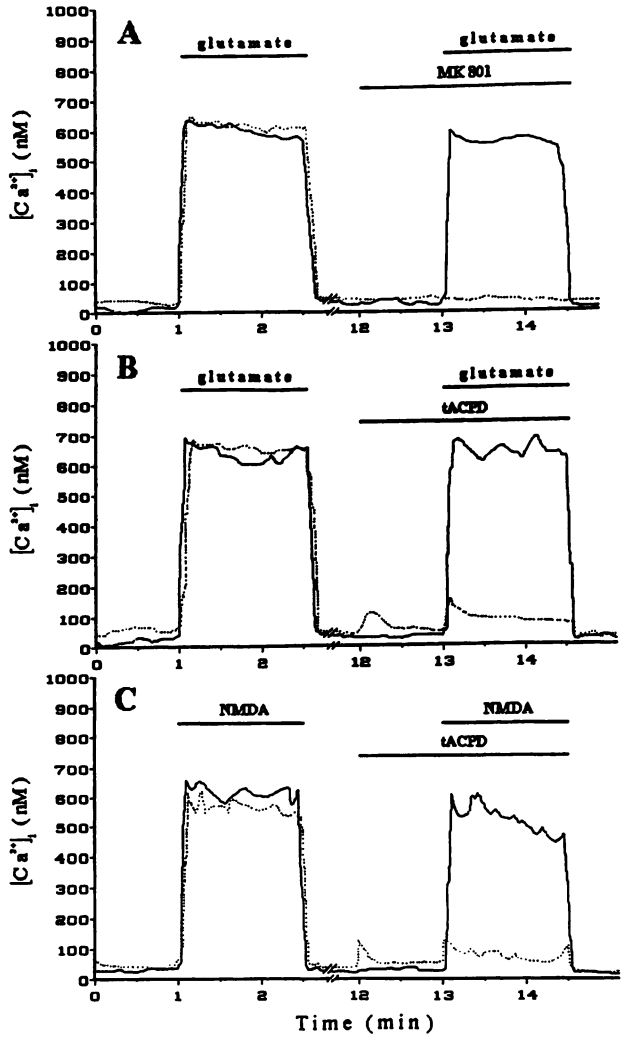

Fig. 2. Reduction in glutamate-evoked $[Ca^{2+}]_i$ increase by tACPD. A, Glutamate (50 μM), in the absence of Mg^{2+}, produced a sustained increase in $[Ca^{2+}]_i$ (S1, first stimulation) in almost all of the neurons investigated (125 of 130 cells). A second, nearly identical response (S2/S1, 0.95 ± 0.05) was produced by additional glutamate at 10 min after washing of the cells (*thick line*). In a parallel experiment, the addition of MK 801 (1 μM) at 1 min before the second glutamate pulse completely abolished glutamate response (*dotted line*). B, Glutamate response observed in control neurons (*thick line*) was reduced by tACPD (100 μM) added to the buffer 1 min before the second glutamate pulse (*dotted line*); tACPD induced a transient increase in resting $[Ca^{2+}]_i$ level (60 ± 20 nM over basal value; 60 cells) and markedly reduced cell (100 of 120) responsiveness to additional glutamate (S2/S1, 0.19 ± 0.02). C, NMDA (100 μM) in a Mg^{2+}-free medium reproduced the glutamate effect (*thick line*; S2/S1, 0.98 ± 0.1). In the presence of tACPD (100 μM), cell responsiveness to NMDA was deeply depressed (*dotted line*; S2/S1, 0.21 ± 0.02). Values are from representative cell recordings.

onist for NMDA receptor, and was reproduced by direct cell application of 100 μM NMDA (Fig. 2C).

Because cell responsiveness to glutamate showed considerable variability between different groups of neurons, test drugs were added before the second glutamate pulse to evaluate the effect of modulators of glutamate receptor function. The size of the second glutamate response was compared with the initial response from the same group of cells. As shown in Fig. 2B, the addition of tACPD 1 min before the second glutamate pulse induced a transient increase (62 ± 18 nM over basal value; 120 cells) in resting $[Ca^{2+}]_i$ level in 70% of 10–15-day-old cells. However, in the presence of tACPD, neuron responsiveness to a further glutamate addition seemed markedly reduced (S2, 89 ± 12 nM). A similar effect was elicited by tACPD on $[Ca^{2+}]_i$ elevation induced by 100 μM NMDA (Fig. 2C). The capability of tACPD to abolish glutamate-mediated $[Ca^{2+}]_i$ rise followed a concentration-dependent course. The calculated IC_{50} value for tACPD was 25 μM, whereas maximal effective concentration was 100 μM (Fig. 3). This range of concentrations seemed to be superimposable on that eliciting neuroprotection in sister cultures exposed to 50 μM glutamate for 1.5 min (Fig. 3).

The transient rise in $[Ca^{2+}]_i$ level and the inhibition of glutamate-mediated $[Ca^{2+}]_i$ increase produced by 100 μM tACPD were highly sensitive to mGluR blockade. As shown in Fig. 4, both tACPD responses (Fig. 4A) were prevented by either the noncompetitive mGluR antagonist L-AP3 (500 μM; Fig. 4C) or the competitive antagonist MCPG (500 μM; Fig. 4B).

These results suggest that glutamate-mediated alteration of calcium homeostasis results from opposite effects elicited by mGluR and iGluR activation. To corroborate this hypothesis, we evaluated the response of glutamate in the presence of L-AP3 or MCPG. As shown in Fig. 5, two pulses of glutamate at 25 μM produced similar increases in $[Ca^{2+}]_i$ (S1, 197 ± 38 nM; S1/S2, 0.92 ± 0.08; 45 cells) in control cerebellar

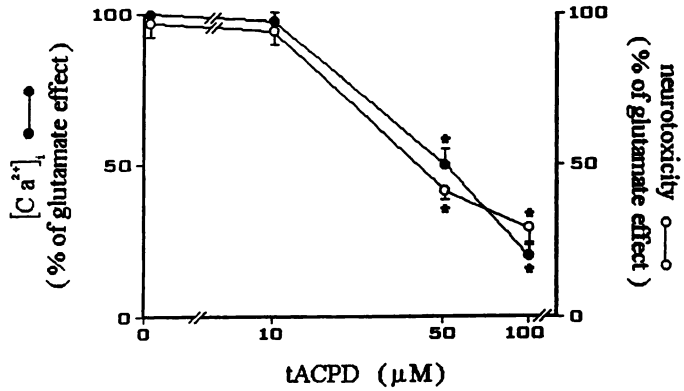

Fig. 3. Concentration-dependent effects of tACPD on glutamate-evoked $[Ca^{2+}]_i$ rise (●) and neurotoxicity (○). Measurement of tACPD effects on glutamate (50 μM)-increased $[Ca^{2+}]_i$ was carried out by evaluating the percentage of S2/S1 values as described in the legend for Fig. 2. In neurotoxicity experiments, cells maintained in Mg^{2+}-free KRS solution were treated with different concentrations or without tACPD at 1 min before exposure (1.5 min) to 50 μM glutamate. Then, cells were returned to culture-conditioned medium at 37° in an atmosphere of 95% air/5% CO_2. Untreated cultures (control) showed 98% of cell viability. Exposure to glutamate reduced cell rescue by 35%. For $[Ca^{2+}]_i$ levels, each point represents the mean ± standard error of S2/S1 values calculated form ≥150 neurons from two separate preparations. For neurotoxicity, data represent mean ± standard error values of three experiments run in triplicate. Wilcoxon's rank sum test was used for the statistical analysis of values. *, $p < 0.01$ versus glutamate alone.

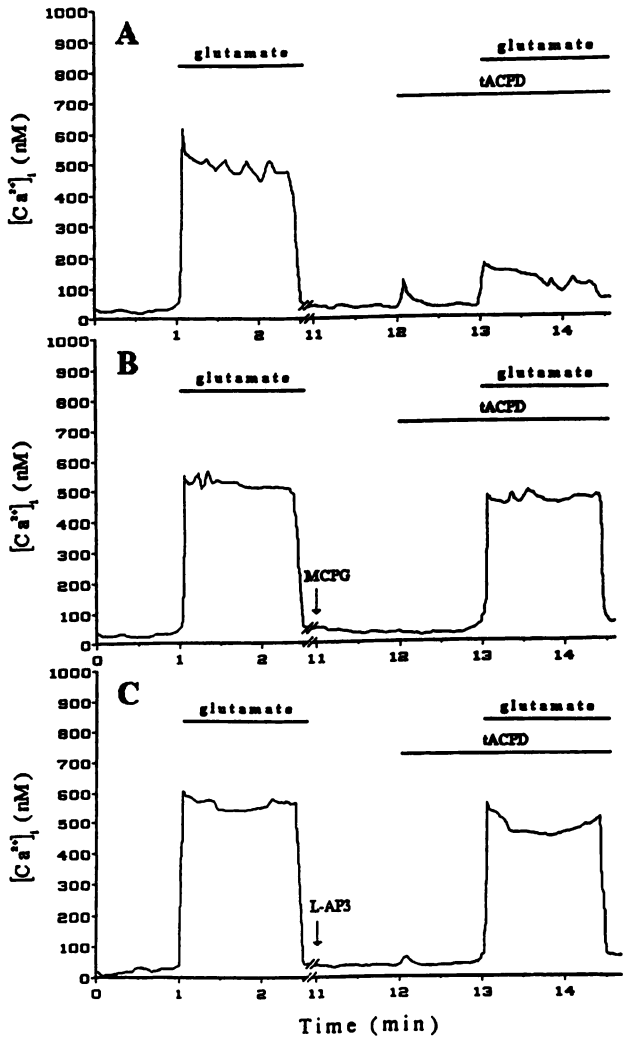

Fig. 4. Blockade of mGluRs prevents tACPD-mediated effects. A, tACPD (100 μM) induced a transient increase in $[Ca^{2+}]_i$ level and inhibited $[Ca^{2+}]_i$ rise mediated by glutamate. Both tACPD-mediated responses were prevented by 500 μM MCPG (B) or by 500 μM L-AP3 (C) applied 1 min before tACPD (2 min before the second glutamate pulse). Values are from representative cell recordings.

neurons. Cell treatment with 500 μM L-AP3 1 min before the second glutamate pulse greatly potentiated cell responsiveness to glutamate (S1/S2, 2.05 ± 0.10; 48 cells). Analogous potentiation of glutamate-induced $[Ca^{2+}]_i$ rise was produced by MCPG at 500 μM. The S1/S2 values were 0.98 ± 0.10 (41 cells) in control cells and 2.13 ± 0.28 (47 cells) in cells administered MCPG at 1 min before S2. The extent of $[Ca^{2+}]_i$ rises produced by 25 μM glutamate in the presence of L-AP3 or MCPG was similar to that elicited by a higher concentration (50 μM) of glutamate alone or by 100 μM NMDA, suggesting that the inhibitory modulation of iGluRs function by mGluRs activation is highly operative during glutamate exposure and ultimately reduces neuron sensitivity to the excitatory stimulus.

Pharmacological characterization of mGluR counteracting glutamate-mediated both $[Ca^{2+}]_i$ rise and neurotoxicity. Because multiple mGluR entities have been found (7, 8), we examined the effect of selective agonists on glutamate-mediated responses in rat cerebellar granule cells as a first step to identify which of the mGluR subtypes mediates the above-mentioned response.

We found that the application of 100 μM quisqualate, which potently, but not selectively, activates mGluR1–5 (8), or of 100 μM DHPG, which preferentially stimulates mGluR1–5 subtypes (9), produced a transient $[Ca^{2+}]_i$ rise (42 ± 9 nM and 60 ± 12 nM over basal values, respectively) in 70% of the examined cells (70 cells for quisqualate, 62 cells for DHPG). In addition, they drastically inhibited the glutamate-mediated response, as shown by the S2/S1 values reported in Fig. 6. On the contrary, L-CCG-I at 1 μM, a concentration selectively stimulating mGluR2 and mGluR3 subtypes (28), did not affect basal neuronal $[Ca^{2+}]_i$ and only partially prevented the glutamate-mediated effect (S2/S1, 0.49 ± 0.05) in ~50% of tested cells (86 cells). L-AP4, which potently activates mGluR4, mGluR6, mGluR7, and mGluR8 (8), modified neither resting nor glutamate-increased $[Ca^{2+}]_i$ levels. It is noteworthy that a close relationship appeared between the effectiveness of compounds to reduce glutamate-mediated $[Ca^{2+}]_i$ rise and their ability to counteract the glutamate-related excitotoxicity, as shown in Fig. 6. Cell viability after glutamate exposure, measured in separate dishes from the same culture preparation, was significantly increased by tACPD, quisqualate, and DHPG; it was only slightly improved by L-CCG-I; and it was not affected by L-AP4.

tACPD does not affect $[Ca^{2+}]_i$ rise due to depolarizing concentrations of KCl or to calcium ionophore. The possible effect of mGluR stimulation on voltage-dependent calcium entry was investigated by exposing neurons to a depolarizing concentration of KCl in the presence or absence of tACPD. As represented in Fig. 7, the addition of 25 mM KCl to the incubation medium resulted in a rapid $[Ca^{2+}]_i$ increase. Initial peak (550 ± 52 nM, 80 cells) was followed by a rapid decline, usually reaching a plateau at levels that were

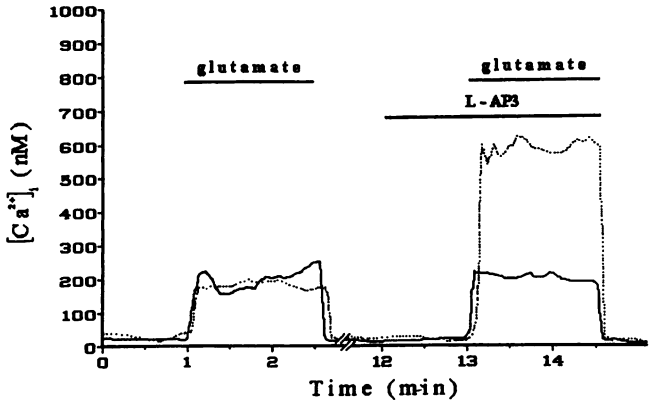

Fig. 5. Blockade of mGluRs by L-AP3 increases granule cell sensitivity to glutamate excitatory transmission. Exposure to 25 μM glutamate for 1.5 min increased $[Ca^{2+}]_i$ by 200 ± 40 nM. An almost identical response was obtained by repeating glutamate treatment 10 min later (S2/S1, 92 ± 8%; 45 cells) (*thick line*). In parallel experiments, L-AP3 (500 μM) was added 1 min before the second glutamate pulse (*dotted line*). L-AP3 markedly increased the intensity of glutamate-evoked $[Ca^{2+}]_i$ rise (S2/S1, 205 ± 10%; 48 cells). Values are from representative cell recordings.

40–50% of the peak. After 10 min, the $[Ca^{2+}]_i$ response to a second KCl application was qualitatively similar to the former but smaller (S2/S1, 0.58 ± 0.11). Application of tACPD between the two pulses did not significantly modify the ratio S2/S1 (0.45 ± 0.08). No cell degeneration was observed after 10 min from KCl application (data not shown).

We investigated the capability of tACPD to modulate either $[Ca^{2+}]_i$ rise or neurotoxicity induced by the calcium ionophore A 23187 (Table 1). The addition of A 23187 at 10 μM produced a sustained increase in $[Ca^{2+}]_i$ in almost all of the cells investigated (253 ± 48 nM; 63 cells). The application of tACPD did not prevent $[Ca^{2+}]_i$ rise induced by A 23187.

The ionophore produced a concentration-dependent cell death. Maximal cell degeneration was found at 100 μM. Pretreatment of the cells with the mGluR agonist tACPD did not prevent neuronal death caused by A 23187.

tACPD inhibition of calcium entry was not mediated by K^+ channel activation. It has been reported that stimulation of glutamate phosphoinositide-coupled receptors activates a large conductance calcium-dependent K^+ channel (29) sensitive to TEA blockade. We investigated the effect of TEA on tACPD-mediated inhibition of glutamate-evoked calcium entry. We observed that the cell treatment with 1 mM TEA did not significantly affect basal $[Ca^{2+}]_i$ values but increased glutamate effectiveness in elevating $[Ca^{2+}]_i$ by 25% (Fig. 8). The K^+ channel blocker did not modify the ability of tACPD to reduce the glutamate-increased $[Ca^{2+}]_i$ levels.

Intracellular pathways involved in mGluR-mediated modulation of both glutamate-elicited $[Ca^{2+}]_i$ rise and cell death. As reported above, maximal inhibition of glutamate-mediated $[Ca^{2+}]_i$ rise and neurotoxicity was produced by agents such as tACPD and quisqualate that preferentially stimulate mGluR1 and mGluR5. Only partial activity was shown by the selective agonist for mGluR2 and mGluR3 subtypes, L-CCG-I, and no activity was shown by L-AP4, the selective agonist for mGluR4, mGluR6, mGluR7, and mGluR8 subtypes.

Because mGluR1 and mGluR5 are positively coupled to phospholipase C, leading to inositol phosphate production and protein kinase C activation (7, 8), in the first set of experiments we evaluated the possible involvement of protein kinase C in mediating the intracellular pathway activated by tACPD and promoting the inhibition of glutamate-induced $[Ca^{2+}]_i$ rise. The selective inhibitor of protein kinase C, bisindolylmaleimide (30), was added to the neurons at 1 μM just after the first glutamate pulse and was maintained during both tACPD and glutamate exposures. As shown in Fig. 9 (*top*), the second pulse of glutamate, which seemed to be dramatically reduced by tACPD in control cultures (S2/S1, 0.15 ± 0.15; 50 cells), was significantly restored in cells pretreated with bisindolylmaleimide (S2/S1, 0.75 ± 0.05; 53 cells). It is noteworthy that the protein kinase C blocker did not affect tACPD-mediated increase of $[Ca^{2+}]_i$ and, in the absence of tACPD, only slightly reduced cell sensitivity to glutamate stimulation (S2/S1, 0.82 ± 0.05) (data not shown). The tACPD inhibition of glutamate-mediated $[Ca^{2+}]_i$ rise was also prevented by the unselective protein kinase C blocker staurosporine (100 nM) (30), which was added to the cultures during loading of the cells with Fura 2-AM and maintained for the entire experiment (Table 2). To confirm these results suggesting a role of protein kinase C in tACPD-mediated inhibitory effects, we tested the capability of the protein kinase C activator PDBu to mimic mGluR-mediated responses. As shown in Fig. 10, PDBu added 3 min before the second glutamate pulse concentration-dependently depressed the glutamate-evoked $[Ca^{2+}]_i$ rise.

In the second set of experiments, we investigated the role of cAMP pathway in tACPD-mediated inhibition of glutamate responses. Cells were treated with the cAMP analogue dibutyryl cAMP at 1 mM. This condition minimized cellular responses mediated by inhibition of adenylate cyclase activity. As shown in Fig. 9 (*top*), dibutyryl cAMP did not modify neuron response to glutamate, whereas it reduced the percentage of tACPD-sensitive cells by 30% (47 cells). In particular, the tACPD response was only partially restored (S2/S1, 0.40 ± 0.10) in cultures treated with dibutyryl cAMP compared with untreated cultures (S2/S1, 0.15 ± 0.05).

In separate experiments, we examined the viability of cells exposed to the above-described treatments (Fig. 9, *bottom*). We found that neuron survival produced by tACPD in glutamate-exposed cells was completely abolished by bisindolylmaleimide, whereas it was not modified by dibutyryl cAMP. Significant survival was also found in cells pretreated with 600 nM PDBu and exposed to excitotoxic pulse of glutamate (Table 3).

Discussion

The present findings demonstrate that the capability of agonists for mGluRs to prevent excitotoxic injury correlates with their ability to reduce glutamate-mediated $[Ca^{2+}]_i$ rise in primary cultures of rat cerebellar granule cells. These effects are mainly mediated by mGluRs positively coupled to polyphosphoinositide turnover, i.e., mGluR1 and mGluR5, and involve protein kinase C activation. The data suggest a novel function for protein kinase C in neuronal signaling, i.e., the negative modulatory effect on NMDA-mediated glutamate excitatory transmission.

mGluR modulation of resting and NMDA-increased $[Ca^{2+}]_i$. In line with previous evidence (23–25), tACPD acti-

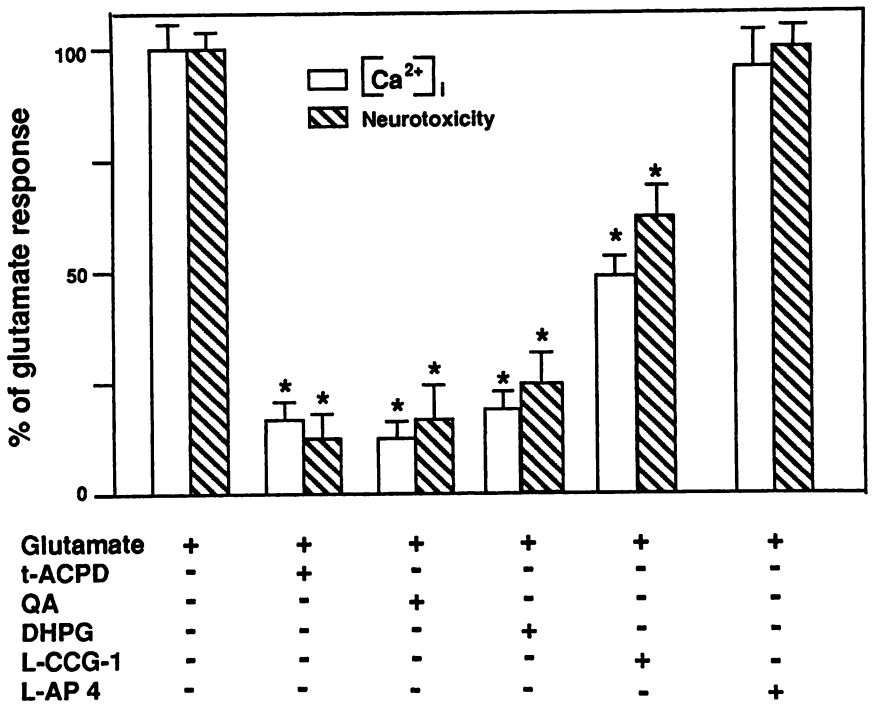

Fig. 6. Pharmacological characterization of mGluRs counteracting both glutamate-mediated $[Ca^{2+}]_i$ increase and neurotoxicity. $[Ca^{2+}]_i$ modifications were evaluated as S2/S1 values obtained by twice stimulating the cells with glutamate 50 μM (see legend for Fig. 2). Drugs tested were tACPD (100 μM), quisqualate (QA, 100 μM), DHPG (100 μM), L-CCG-I (1 μM), and L-AP4 (500 μM). Neurotoxicity experiments were carried out in sister cultures exposed to 50 μM glutamate in the presence or absence of test drugs (see Materials and Methods). Control cells showed 98% viability. The exposure to glutamate reduced cell viability to 30%. Columns represent the mean ± standard error values taken from at least three experiments run in duplicate. For measurement of $[Ca^{2+}]_i$, columns represent the mean ± standard error of percentage of S2/S1 values obtained in cells (see text) from two experiments. Wilcoxon's rank-sum test was used for the statistical analysis of values. *, $p < 0.01$ versus glutamate alone.

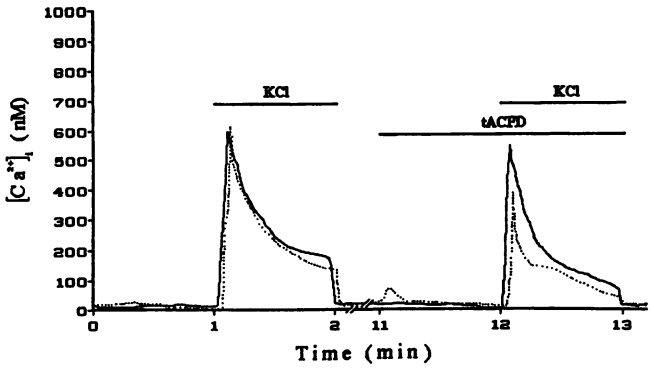

Fig. 7. Inefficacy of tACPD in affecting $[Ca^{2+}]_i$ rise due to depolarizing concentrations of KCl. Cells were exposed for 1 min to 25 mM KCl in KRS (S1). After a 10-min washing interval, cells were exposed to an additional 25 mM KCl pulse. The $[Ca^{2+}]_i$ response to a second application of KCl was qualitatively similar in shape but smaller (S2/S1, 60 ± 10%). The application of 100 μM tACPD between the two pulses did not significantly modify the KCl response (S2/S1, 40 ± 10).

vated a transient increase in $[Ca^{2+}]_i$ that has been reported to be most likely mediated via inositol-1,4,5-trisphosphate-induced calcium release (23). The tACPD-mediated $[Ca^{2+}]_i$ rise was found to follow the developmental profile of mGluR associated with polyphosphoinositide turnover in cultured cerebellar granule cells (31). Indeed, the tACPD-induced increase in $[Ca^{2+}]_i$ was low in magnitude, although consistently present, in cells at 2 DIV. It reached maximal expression in cells at 4 DIV and then progressively decreased in intensity, so that at 13 DIV the estimates were similar to those detectable in cells at 2 DIV.

TABLE 1

Inefficacy of tACPD in preventing $[Ca^{2+}]_i$ rise and neurotoxicity mediated by the calcium ionophore A 23187

For $[Ca^{2+}]_i$ measurement, neurons were exposed to A 23187 for 1.5 min. When present, tACPD was added 1 min before A 23187. Values are mean ± standard error of recordings from 63 neurons. For neurotoxicity experiments, cultures were exposed to A 23187 in Locke's solution for 15 min with or without tACPD. Then, they were returned to culture-conditioned medium at 37° in 95% air/5% CO_2. Cell viability was measured 18 hr later. Data are from three experiments run in triplicate.

Treatment	$[Ca^{2+}]_i$	Cell survival
	nM	%
Solvent	30 ± 8	95 ± 4
A 23187 (10 μM)	253 ± 48	20 ± 5
A 23187 + tACPD (100 μM)	274 ± 47	18 ± 3

The following experiments were performed in cells cultured for 10–15 days. At this age, cerebellar granule cells showed a series of differentiation markers: they seemed to be morphologically stable and, most important, they were sensitive to both NMDA receptor-mediated neurotoxicity and mGluR-mediated neuroprotection.

Changes in glutamate-induced rise in $[Ca^{2+}]_i$ were measured with use of the two-pulses experimental protocol. This method of investigation avoided intercell variations by considering the response of the first pulse as the control value. Glutamate effect was likely to be mediated by stimulation of iGluR NMDA subtype because (i) it was detected in Mg^{2+}-free condition, (ii) it was blocked by MK-801, and (iii) it was fully reproduced by NMDA. tACPD completely prevented glutamate- (as well as NMDA-) induced $[Ca^{2+}]_i$ rise by activating mGluRs sensitive to L-AP3- or MCPG-elicited block-

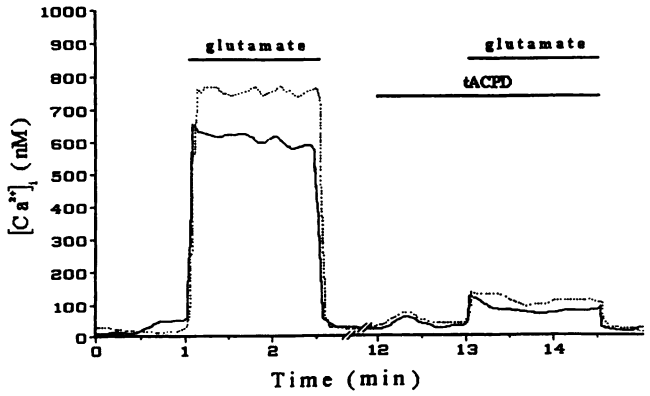

Fig. 8. tACPD inhibition of $[Ca^{2+}]_i$ rise is not mediated by K^+ channel activation. In control cells (53 cells), tACPD (100 μM) reduced glutamate (50 μM)-elicited $[Ca^{2+}]_i$ rise (*thick line*). In treated cultures, TEA at 1 mM concentration was added at the beginning and maintained throughout the experiment (*dotted line*). TEA increased by 25% the responsiveness of cerebellar granule neurons (45 of 50) to 50 μM glutamate but did not modify the inhibitory response to tACPD (50 cells).

ade. Interestingly, the addition of either L-AP3 or MCPG intrinsically magnified the glutamate response, producing higher rises in neuronal $[Ca^{2+}]_i$. This indicates that during glutamate pulse, mGluRs are highly activated and operate a negative modulation of the excitatory stimulus. In particular, it can be assumed that either physiological or pathological modifications in the relative expression of different receptor subtypes might define the threshold of neuron sensitivity to glutamate. It is noteworthy that the preservation of $[Ca^{2+}]_i$ homeostasis by tACPD occurred at concentrations superimposable on those producing neuroprotection.

Specificity of ion channel. We tested the possibility that the prevention of glutamate-induced $[Ca^{2+}]_i$ rise by tACPD was mediated by mGluR modulation of voltage-dependent ion channel. The response elicited by mGluR activation was shown to be specific for NMDA receptor-mediated calcium flux as tACPD poorly modified the $[Ca^{2+}]_i$ increases elicited by depolarizing concentrations of KCl or the ionophore A 23187. These findings apparently contrast with a report (32) describing the inhibitory modulation of L-type calcium channels by the mGluR2/mGluR3 agonists tACPD and L-CCG-I in mouse cerebellar granule cells. However, the shorter exposure of the cells to tACPD in our conditions might account for that discrepancy. The mGluR-mediated prevention of glutamate transmission may be a triggering event that occurs earlier than complete inhibition of L-type calcium current. In this regard, we found that tACPD modified neither the calcium entry nor the neuronal death induced by the calcium ionophore A 23187.

Pharmacological characterization. A main point, often underestimated, is the actual lack of "pure selective" agonists and antagonists for the various mGluR subtypes. Taking this into consideration, we performed a series of pharmacological experiments while aware that their results might give strong suggestions that needed to be supported by data obtained through other approaches.

At least eight different mGluR subtypes have been identified (7, 8). These fall into three groups based on pharmacological criteria and second messenger couplings. Group 1, which includes mGluR1 and mGluR5 receptors, is sensitive to tACPD, quisqualate, and DHPG and is associated with

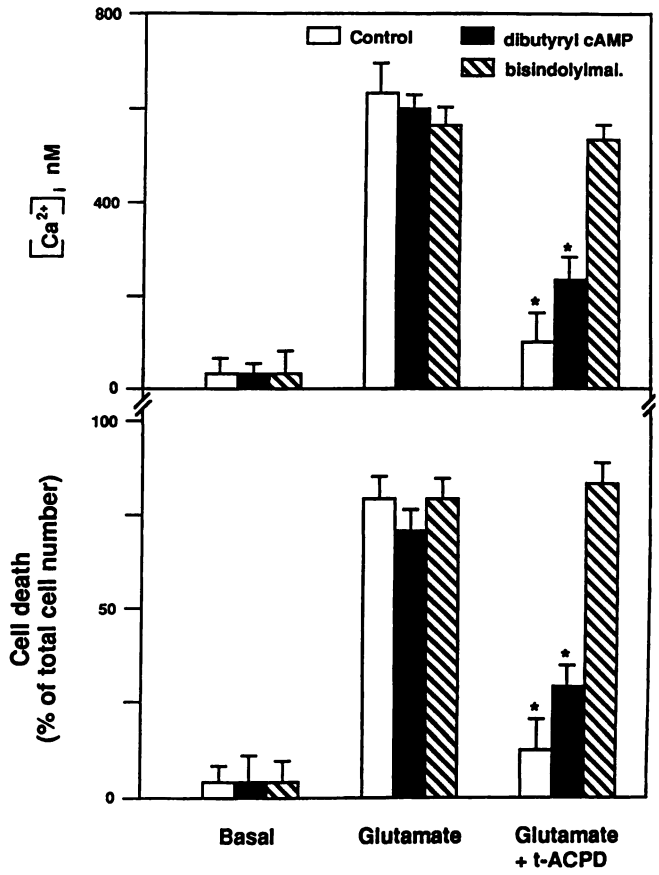

Fig. 9. Second messengers involved in tACPD-mediated effects. *Top*, $[Ca^{2+}]_i$ measured in neurons at resting (*Basal*), after the second glutamate (50 μM) pulse (*Glutamate*), and after the second glutamate pulse in the presence of tACPD (100 μM) (*Glutamate + tACPD*). Experiments were carried out in control, dibutyryl cAMP-treated (1 mM), or bisindolylmaleimide-treated (1 μM) granule cells. Dibutyryl cAMP did not modify S2/S1 value as well as transient elevation of $[Ca^{2+}]_i$ elicited by tACPD (data not shown). The cAMP analogue only partially reduced inhibitory response by mGluR agonist. Bisindolylmaleimide weakly reduced cell responsiveness to glutamate (20% reduction in S2/S1 ratio). Moreover, it did not alter tACPD-mediated transient increase in $[Ca^{2+}]_i$ (data not shown) but completely prevented tACPD-inhibition of glutamate-evoked $[Ca^{2+}]_i$ rise. *Bottom*, cell death produced by glutamate with or without tACPD in control cultures and in cultures treated with dibutyryl cAMP or bisindolylmaleimide. Glutamate incubation caused 75% of cell loss (see Materials and Methods). tACPD with or without dibutyryl cAMP/bisindolylmaleimide was added 5 min before glutamate exposure. Neuroprotection produced by tACPD was poorly modified by dibutyryl cAMP and completely reversed by bisindolylmaleimide.

phosphoinositide hydrolysis. Group 2, which includes mGluR2 and mGluR3 subtypes, is strongly activated by L-CCG-I, tACPD, and, to a lesser extent, by quisqualate and is linked to the inhibition of adenylate cyclase activity. Group 3, which includes mGluR4, mGluR6, mGluR7, and mGluR8 receptors, is activated by L-AP4 and L-serine-*O*-phosphate and is also associated with inhibition of adenylate cyclase. To identify which receptor subtype is mainly involved in the modulation of glutamate excitatory stimuli, we examined the effects of various mGluR agonists on glutamate-mediated functional responses. We found that maximal prevention of both $[Ca^{2+}]_i$ rise and neurotoxicity was produced by tACPD, by quisqualate, and by DHPG; L-CCG-I only partially reduced glutamate effects, whereas L-AP4 was almost inactive on both. These results suggest that mGluRs involved in in-

TABLE 2

Staurosporine prevents tACPD-mediated inhibition of glutamate-evoked $[Ca^{2+}]_i$ rise

tACPD was added 1 min before the second glutamate pulse. Staurosporine, when present, was added during the cell loading with Fura 2-AM and maintained for the entire experiment. Data represent the mean ± standard error of S2 and S2/S1 values obtained in ≥50 neurons from two experiments. Wilcoxon's rank sum test was used for the statistical analysis of values.

Treatment	$[Ca^{2+}]_i$	S2/S1
	nM	
Glutamate (50 μM)	583 ± 62	0.95 ± 0.10
Glutamate + tACPD (100 μM)	178 ± 26[a]	0.20 ± 0.18[a]
Glutamate + staurosporine (100 nM) + tACPD	504 ± 72	0.89 ± 0.15

[a] $p < 0.01$ versus glutamate alone.

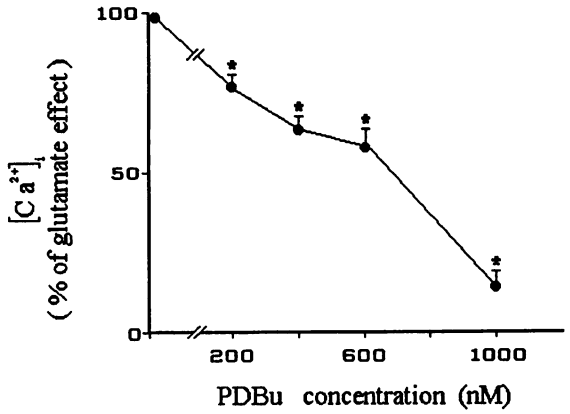

Fig. 10. Concentration-dependent effect of PDBu on glutamate-evoked $[Ca^{2+}]_i$ rise. PDBu was added, at the indicated concentrations, 3 min before the second glutamate pulse. Measurement of PDBu effect on glutamate (50 μM)-increased $[Ca^{2+}]_i$ was carried out by evaluating percentage of S2/S1 values as described in legend for Fig. 2. *Points*, mean ± standard error of S2/S1 values calculated in ≥52 neurons from two preparations.

TABLE 3

Activation of protein kinase C by PDBu protects cultured cerebellar granule cells against excitotoxic degeneration

Cultures were exposed to glutamate for 15 min in a Mg^{2+}-free Locke's solution. When present, PDBu was added 5 min before glutamate treatment. Cultures were then returned to culture-conditioned medium at 37° in 95% air/5% CO_2, and cell viability was measured 18–24 hr later. Values represent mean ± standard error of three experiments run in triplicate. Wilcoxon's rank sum test was used for the statistical analysis of values.

Treatment	Cell survival
	%
Solvent	92 ± 8
Glutamate (50 μM)	41 ± 6[a]
Glutamate + PDBu (600 nM)	79 ± 11

[a] $p < 0.01$ versus control.

hibiting the glutamate responses have the pharmacological profile of mGluR1 and mGluR5 subtypes. The slight effect of L-CCG-I suggests that mGluR2 and mGluR3 are only partially involved in the tACPD-mediated prevention of glutamate-induced $[Ca^{2+}]_i$ rise and neurotoxicity, possibly via partial inhibition of L-type calcium channels (32, 33) and/or slight activation of phospholipase C-associated receptors.

Second messengers: role of protein kinase C. To support the above-mentioned results, we evaluated the involvement of different second messenger-generation systems in the tACPD-mediated inhibitory response. Activation of the cAMP intracellular pathways by the cAMP analogue dibutyryl cAMP to minimize the effects of adenylate cyclase inhibition only partially prevented tACPD reduction in both glutamate-induced $[Ca^{2+}]_i$ rise and neurotoxicity. These data do not rule out control by mGluR2 and mGluR3 of iGluRs as only a restricted number of cells might be able to express these receptor subtypes. Blockade of protein kinase C by the selective inhibitor bisindolylmaleimide (30) or by staurosporine completely counteracted the tACPD reduction in glutamate-evoked responses, i.e., $[Ca^{2+}]_i$ elevation and cell death. Thus, stimulation of protein kinase C could be one of the crucial steps activated by mGluR leading to inhibition of NMDA receptor-mediated calcium entry, which is in agreement with previous data reported by Courtney and Nicholls (34). Indeed, tACPD effects were reproduced by direct activation of protein kinase C; application of PDBu to the cells significantly prevented glutamate-induced $[Ca^{2+}]_i$ rise and neurotoxicity.

Substrate of protein kinase C in this system is still unknown. Several possibilities have been considered. In mouse cerebellar granule cells (29), the stimulation of mGluRs through activation of a large-conductance calcium-dependent K^+ channel has been proposed to reduce ionotropic glutamate receptor-mediated cell excitation. However, in our system, the inhibitory effect of tACPD on glutamate-evoked $[Ca^{2+}]_i$ rise proved to be insensitive to K^+ channel blockade by TEA, suggesting that this mechanism is poorly involved. tACPD was also reported to reduce L-type (32, 33) as well as N-type (35) calcium channels. The former was particularly sensitive to agonists for mGluRs coupled to adenylate cyclase inhibition (mGluR2/mGluR3), whereas the latter was resistant to protein kinase C blockade. It can therefore be argued that different mGluRs and alternative transduction mechanisms are probably controlling glutamate elevation of $[Ca^{2+}]_i$ in cerebellar granule cells. An additional possible substrate of protein kinase C is the NMDA receptor channel. Indeed, NMDA receptor subunits possess consensus phosphorylation sites for protein kinase C (36). Such an hypothesis is intriguing as up to now, protein kinase C has been reported to increase NMDA receptor transmission by reducing Mg^{2+} block of NMDA receptor channel (37). However, the heterogeneity of both NMDA receptor complex (36) and protein kinase C molecules (38) makes necessary further investigations.

Conclusions. We found that the neuroprotective effect of agents acting on mGluRs in cultured cerebellar granule cells (i) is triggered by stimulation of the mGluR1 or mGluR5 subtype, (ii) is mainly mediated by the activation of protein kinase C, and (iii) correlates with a marked reduction in the glutamate-induced $[Ca^{2+}]_i$ rise.

Pathologically increased activity of protein kinase C has been reported to cause neuronal degeneration (39) and to mediate glutamate excitotoxicity in cultured neurons (40). Our data suggest that its physiological activation, as results from mGluR1–5 stimulation, prevents glutamate excitatory response. The functional interaction between iGluRs and mGluRs may represent an elaborated mechanism by which cells, via qualitative and quantitative expression of the different GluR subtypes, define their level of sensitivity to glutamate. This concept may open new visas in the neurobiology of synapse, suggesting a general feature by which postsynaptic signals from a given neurotransmitter integrate to limit the extent of the response of target neuron. Finally, the

identification of drugs acting at specific subtypes of mGluRs, inhibiting iGluR function, may be of pharmacological relevance for their potential application in neurological disorders associated to excitotoxicity.

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