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Excitatory Amino Acid Receptors Coupled with Guanylate Cyclase in Primary Cultures of Cerebellar Granule Cells

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Primary cultures of cerebellar granule cells have been used in pharmacologically and functionally characterizing excitatory amino acid recognition sites coupled with guanylate cyclase. When granule cells were incubated in physiological culture conditions (Locke's solution, pH 7.4), only kainate and, to a lesser extent, L-glutamate increased cyclic GMP (cGMP) levels. Under these conditions, L-aspartate, N-methyl-D-aspartate (NMDA), and quisqualate were inactive. When granule cells were incubated in the absence of extracellular Mg^{2+} or in the presence of the depolarizing agent veratrine, L-glutamate, L-aspartate, and NMDA became as effective as kainate in enhancing cGMP formation. The action of kainate was preferentially antagonized by 2,3-*cis*-piperidindicarboxylate, whereas the action of L-glutamate was preferentially antagonized by (±)2-amino-5-phosphonovalerate. These data suggest that 2 different excitatory amino acid recognition sites (activated by kainate or by L-glutamate, L-aspartate, and NMDA, respectively) are coupled with guanylate cyclase in primary cultures of cerebellar granule cells: While the coupling of the recognition site for kainate with guanylate cyclase operates under resting conditions and in the presence of Mg^{2+}, the coupling of the recognition site for L-glutamate, L-aspartate, and NMDA with guanylate cyclase requires depolarizing conditions or the absence of extracellular Mg^{2+}.

The putative dicarboxylic excitatory amino acid transmitters—L-glutamate, L-aspartate, and a number of their structural analogs—excite CNS neurons by activating specific membrane receptors (Foster and Fagg, 1984). Three major classes of dicarboxylic amino acid receptors have been defined by electrophysiological criteria and radioligand binding techniques: (1) the "*N*-methyl-D-aspartate (NMDA)-preferring" receptor, which is selectively antagonized by some α-amino-ω-phosphonocarboxylic acids, including (±)2-amino-5-phosphonovalerate (APV); (2) the "quisqualate-preferring" receptor, at which L-glutamate diethylester (GDEE) acts as a selective but weak antagonist; and (3) the "kainate-preferring" receptor, which is insensitive to inhibition by NMDA-receptor antagonists or GDEE. Broad-spectrum antagonists, like 2,3-*cis*-piperidindicarboxylate (PDA) or γ-glutamylglycine can also antagonize the "kainate-preferring" receptor.

Activation of excitatory amino acid receptors triggers various transmembrane biochemical signals, which include increased levels of cyclic nucleotides (Ferrendelli et al., 1974; Mao et al., 1974; Schmidt et al., 1977; Shimizu and Yamamura, 1977), increased turnover of inositol phospholipids (Nicoletti et al., 1986a, b), and an increase in calcium ion flux (Wroblewski et al., 1985). Hence, excitatory amino acid receptors can be characterized pharmacologically by investigating the activation of specific transduction signals triggered by application of agonists.

An area of the brain that has attracted considerable attention is the cerebellum, where L-glutamate or L-aspartate may function as a neurotransmitter for climbing and parallel fibers (Young et al., 1974; Hudson et al., 1976; Sandoval and Cotman, 1978; Rea et al., 1980; Gallo et al., 1982; Wiklund et al., 1982). *In vivo* stimulation of these fibers elicits a prompt and massive increase in cerebellar cGMP content without changes in cAMP (Biggio and Guidotti, 1976; Biggio et al., 1977a, b); intracerebroventricular injection of L-glutamate can reproduce this effect (Mao et al., 1974). Moreover, kainate, L-glutamate, and L-aspartate induce large increases of cGMP and, in some instances, increase cAMP content in rat cerebellar slices (Ferrendelli et al., 1974; Schmidt et al., 1977; Garthwaite, 1982). Thus, the excitatory amino acid-induced increase in cyclic nucleotides, in general, and cerebellar cGMP, in particular, has been used as a biochemical index to study signal transduction following application of agonist(s) and antagonist(s) (Garthwaite and Wilkin, 1982; Foster and Roberts, 1981; Ferkany et al., 1982; Garthwaite and Gilligan, 1984).

Here we have used primary cultures of cerebellar granule cells to characterize the excitatory amino acid receptors with regard to their ability to increase cellular cGMP content. In these cultures, the major cell type (>92–95% of the entire cell population) is represented by granule cells, which undergo a morphological and biochemical differentiation similar to granule cells "*in vivo*" (Garthwaite and Balazs, 1981). Granule cells in culture are rich in the L-glutamate-synthesizing enzyme glutaminase (Patel and Balazs, 1975), and they release L-glutamate and (to a lesser extent) L-aspartate in response to depolarizing stimuli (Gallo et al., 1982). Granule cells in culture express specific recognition sites for excitatory amino acids (Wroblewski et al., unpublished observations), as they do in intact cerebellum (Greenamyre et al., 1985). Activation of these sites by excitatory amino acids triggers specific transmembrane biochemical processes, including activation of inositol phospholipid hydrolysis (Nicoletti et al., 1986b) and stimulation of $^{45}Ca^{2+}$ uptake (Wroblewski et al., 1985).

Materials and Methods

Cell cultures. Cerebellar granule cell cultures were obtained from 8-d-old rats, as previously described by Gallo et al. (1985). Cells were re-

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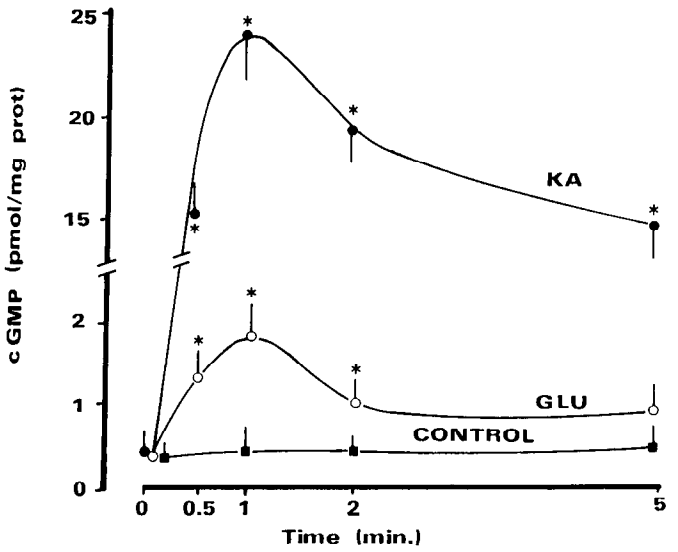

Figure 1. Time-dependent increase in cGMP levels elicited by kainate (*KA*, 100 μM) and L-glutamate (*GLU*, 100 μM) in primary cultures of cerebellar granule cells. Values are means ± SEM of at least 4 determinations. $p < 0.01$ (Student's *t* test) if compared with control values.

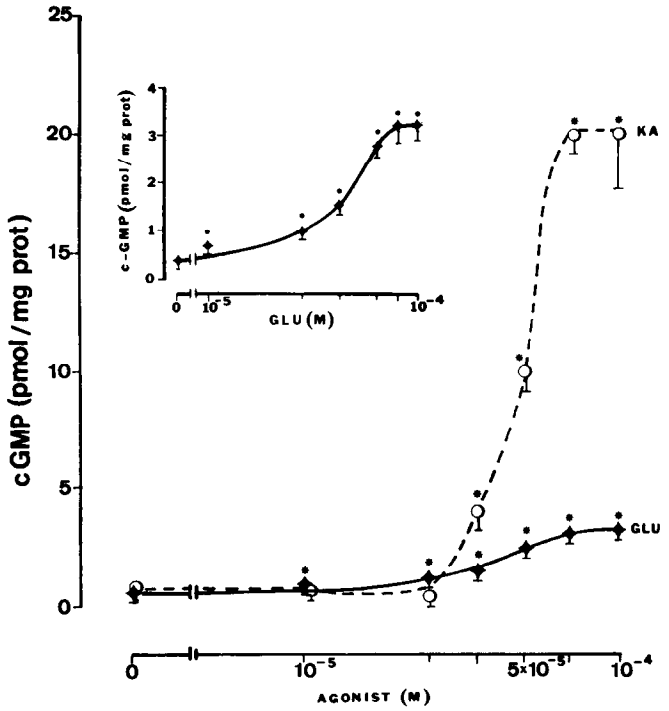

Figure 2. Concentration-dependent increase in cGMP levels elicited by kainate (○) or L-glutamate (◆) in primary cultures of cerebellar granule cells. Values are means ± SEM of at least 4 determinations. $p < 0.01$ (Student's *t* test) if compared with control values. *Insert,* Concentration-dependent stimulation of cGMP induced by L-glutamate plotted on expanded scale.

suspended in the following culture medium: basal Eagle's medium (BME) (Flow Labs), 10% fetal calf serum (FCS) (Gibco), 25 mM KCl, 2 mM gutamine and 100 μg/ml gentamycin (Gibco). The cells were seeded (2.5×10^6 cells/dish) onto 35 mm Falcon dishes coated with poly(L-lysine) (5 μg/ml) and then incubated at 37°C in a humidified atmosphere, 5% CO/95% air. Cytosine arabinofuranoside (Ara-C), 10 μM, was added after 16 hr of culture to inhibit the replication of non-neuronal cells. Cerebellar astrocytic cultures were prepared as previously described (Woodhams et al., 1981) and plated onto 35 mm Falcon plastic dishes. T he culture medium was BME containing 10% FCS, 2 mM glutamine, and 100 μg/ml gentamycin. The KCl concentration in this medium was 4.7 mM and no Ara-C was added to these cultures. Astroglial cultures were incubated in the same conditions as neuronal cultures.

Immunocytochemistry. Immunocytochemistry of neuronal and astrocytic cultures was performed as previously reported (Nicoletti et al., 1986b). After 8 d in culture, the cells were washed with 0.2 M PBS (pH 7.4) and then fixed with 4% formaldehyde plus 0.1% glutaraldehyde in PBS for 10 min at room temperature. The cells were first incubated for 20 min with 1:10 dilution of normal goat antiserum (NGS) and then incubated with specific antisera at 4°C for 24 hr in PBS containing 0.1% Saponin (Sigma, St. Louis) and 0.1% NGS. This incubation was terminated by several washings followed by incubation with secondary antibodies in PBS/saponin/NGS for 30 min. After washing, the cells were coverslipped with buffered glycerol and viewed with a fluorescence microscope (Leitz). Polyclonal rabbit antiserum to glial fibrillary acid protein (GFAP; Dako) was diluted 1:1000; sheep antiserum to glutamic acid decarboxylase (GAD, E.C. 4.1.1.15, a gift from Dr. J. Kopin) was diluted 1:800; monoclonal mouse antisera to phosphorylated and non-phosphorylated neurofilaments (Sternberger-Mayer) were diluted 1:2000. Secondary antibodies were affinity-purified and conjugated to fluorescein isothiocyanate (FITC) goat anti-mouse IgG (1:100, Jackson Immunoresearch).

For double-immunofluorescence staining the cells were incubated with a mixture of both primary antibodies at the same dilutions described above and incubated after washings with 2 appropriate antibodies with different fluorescent labels, so that glial cells were emitting red (RITC) and neuronal cells green (FITC) fluorescence.

Measurement of cGMP and cAMP content. Dishes containing 8-d-old cell cultures were washed twice with 1 ml Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4) prewarmed at 37°C. The cells were incubated with 1 ml Locke's solution immediately prior to addition of drugs dissolved in Locke's solutions. Control cells received an equal volume of Locke's solution. Following incubation for up to 5 min, the incubation medium was removed and 1 ml of 0.4 *N* $HClO_4$ added. Then the cells were harvested and the suspension was centrifuged at 10,000 × *g* for 10 min. The supernatants, after $HClO_4$ precipitation by K_2CO_3, were processed for cyclic nucleotide assay using radioimmunological methods, as previously described (Harper and Brooker, 1975). Protein in the pellet was determined as described by Lowry et al. (1951), using BSA as the standard. The protein content in each sample ranged between 85 and 120 μg.

Measurement of GAD. GAD activity was measured in control cells and in cells pretreated with 50 μM kainate for 18 hr to eliminate GABAergic neurons. Cells were harvested and homogenized in 100 mM sodium phosphate buffer (pH 6.5) containing 20 mM 2-mercaptoethanol and 0.05% (wt/vol) Triton X-100. Incubations were carried out in samples of homogenate after adding final concentrations of 4 mM pyridoxal phosphate and 50 mM L-glutamate for 60 min at 37°C. The reaction rate was found to be linear up to 2 hr. The reaction was stopped by adding a final concentration of 0.2 *N* $HClO_4$. Control samples were added with $HClO_4$ before incubation. The enzyme activity was calculated from the changes in GABA concentration measured by HPLC and was expressed as nmol GABA/mg protein/hr.

Materials. Kainate, L-glutamate, NMDA, quisqualate, (±)APV, veratrine, and forskolin were purchased from Sigma; tetrodotoxin (TTX) from Calbiochem; isobutylmethylxantine (IBMX) from Aldrich; PDA from Cambridge Research Biochemicals.

Results

Increase in cGMP content induced by excitatory amino acids in primary cultures of cerebellar granule cells

Kainate and, to a lesser extent, L-glutamate enhanced cGMP content in 8- to 10-day-old primary cultures of cerebellar granule cells. Addition of 100 μM kainate to the cultures increased cGMP formation by approximately 60-fold, while the addition of L-glutamate (100 μM) increased cGMP content by only 4- to 5-fold (Fig. 1). This increase peaked at 1 min of incubation and was reduced by 40% after 5 min (Fig. 1). Hence, 1 min incubation was routinely used in our study. Kainate and L-glutamate did

Table 1. Increase in cGMP content elicited by kainate in the presence or absence of 3-isobutylmethylxantine (IBMX)

IBMX (100 μM)	cGMP (pmol/mg protein) Control	Kainic acid (50 μM)	Increase
Absent	0.37 ± 0.08	7.4 ± 1.2	20×
Present	2.2 ± 0.5	48 ± 3.5	22×

Cultures were preincubated with IBMX for 20 min prior to the addition of kainate. cGMP content was measured 1 min after kainate additions. Each value represents the mean ± SEM of 5 culture dishes, each assayed in duplicate.

not affect cAMP content in cultures of cerebellar granule cells (cAMP valucs pmol/mg protein: control, 25 ± 2; 100 μM kainate, 32 ± 4 at 1 min and 28 ± 3 at 15 min; 100 μM L-glutamate, 28 ± 3 at 1 min and 24 ± 2 at 15 min; $n = 8$), in contrast to 10^{-4} M forskolin, which elevated cAMP levels more than 200-fold: from 25 ± 4 to 480 ± 72 pmol/mg protein (incubation time, 5 min; $n = 3$). To test whether the phosphodiesterases were involved in the effect of kainate on cGMP, we used the phosphodiesterase inhibitor IBMX. Addition of 100 μM IBMX to the granule cell cultures significantly enhanced cGMP levels. In the presence of IBMX, kainate increased cGMP content by a greater extent; however, when expressed as a percentage of basal values, this increase appeared similar in the presence or absence of IBMX (Table 1). This suggests that the increase in cGMP content induced by kainate results from an activation of guanylate cyclase rather than from an inhibition of phosphodiesterases. The increase in cGMP content induced by kainate and L-glutamate was concentration dependent, with apparent EC_{50} of 50 and 30 μM, respectively (Fig. 2). L-Aspartate, quisqualate, and NMDA failed to increase cGMP content even at the highest concentrations (Table 2).

Tentative identification of the cell type containing L-glutamate or kainate receptors coupled with guanylate cyclase

Double-immunofluorescence studies with monoclonal antiserum to phosphorylated and nonphosphorylated neurofilaments and with polyclonal rabbit antiserum to GFAPs indicated that 97% of the cells in culture were neurons, and less than 3% were glial or endothelial cells. GABAergic neurons constituted less than 5% of the neuronal population, as shown by specific

Table 2. cGMP content in primary cultures of cerebellar granule cells treated with different excitatory amino acids

Amino acid	cGMP (pmol/mg protein)
Control	0.41 ± 0.03
Kainate (100 μM)	28 ± 4.4[a]
L-Glutamate (100 μM)	3.2 0.5[a]
L-Aspartate (100 μM)	0.51 ± 0.05
NMDA (100 μM)	0.44 ± 0.15
Quisqualate (100 μM)	0.42 ± 0.16

Values are means ± SEM of 4–18 determinations.
[a] $p < 0.01$, compared with control.

GAD (E.C. 4.1.1.15) immunostaining (Fig. 3), while the majority of neuronal cells (approximately 92% of the entire cell population) was morphologically identified as granule cells (Fig. 3). To determine the cell specificity of the increase in cGMP content elicited by excitatory amino acids, the action of kainate or L-glutamate was studied in cultures of cerebellar astrocytes or in cultures of cerebellar neurons pretreated with 10 μM kainate for 18 hr. Such treatment caused a substantial loss in GABAergic neurons, as reflected by a more than 90% decrease in GAD activity (control cells, 35 ± 3.6; kainate-treated cells, 2.2 ± 0.25 nmol GABA/mg protein/hr) and by the disappearance of GAD-like immunoreactivity (Fig. 3*B*). Kainate and L-glutamate failed to increase cGMP content in cultures of astrocytes (Table 3), but they were still effective in cultures of cerebellar neurons pretreated with kainate (Table 3).

Conditions affecting the increase in cGMP content induced by excitatory amino acids

Influence of veratrine

Veratrine, a mixture of alkaloids containing the depolarizing agent veratridine, was used as a tool to evaluate the influence of depolarizing conditions on the increase in cGMP elicited by excitatory amino acids. Addition of veratrine to the incubation buffer elicited a concentration-dependent increase in cGMP content with an apparent EC_{50} value of 7.5 μg/ml. A maximal increase (ranging from 30- to 60-fold) was obtained with 15 μg/ml veratrine (Table 4). This increase was comparable to that induced by maximal concentrations (100 μM) of kainate (Table 4). The action of veratrine was completely antagonized by 0.5

Table 3. Kainate and L-glutamate increase cGMP levels in cultures of cerebellar granule cells pretreated with kainate to destroy GABAergic neurons, and fail to induce changes in cGMP content in cultures of cerebellar astrocytes

Culture	cGMP (pmol/mg protein) Control	Kainate (100 μM)	L-Glutamate (100 μM)
Cerebellar neurons	0.41 ± 0.030	28 ± 4.4[b]	3.2 ± 0.50[b]
Cerebellar neurons pretreated with 10 μM kainate[a]	0.37 ± 0.10	23 ± 2.6[b]	2.4 ± 0.80[b]
Astrocytes	0.23 ± 0.022	0.25 ± 0.34	0.19 ± 0.030

Values are means ± SEM of at least 4 determinations.
[a] Six-day-old cultures were pretreated for 18 hr with 10 μM kainate. This treatment destroyed GABAergic neurons (see Fig. 3). Kainate was removed and the cells were maintained for 12 hr in culture medium before being used for the experiment.
[b] $p < 0.01$ if compared with the respective control.

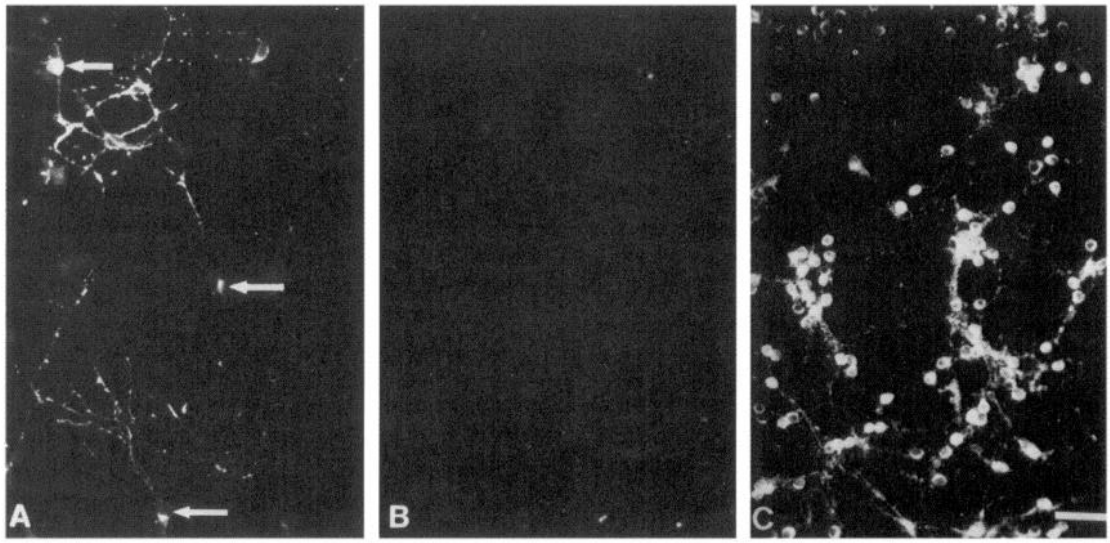

Figure 3. GAD (*A* and *B*) and neurofilament (*C*) immunostaining in primary cultures of cerebellar granule cells: Treatment with kainate selectively destroys GABAergic neurons (*B*). *A*, Control cell cultures showing few GAD-positive cell bodies (*arrows*) and a network of fibers and terminals. *B*, Culture 18 hr after 10 μM kainate treatment showing no cell or fiber staining with GAD antibodies. After kainate treatment cells were maintained for 12 hr in normal culture medium before being used in the experiment. *C*, Double-staining with neurofilament antisera reveals the presence of numerous granule cell-like neurons after kainate treatment. Bar, 30 μm.

μM TTX. This concentration of TTX did not substantially affect the stimulation of cGMP formation elicited by kainate, but it reduced the stimulation of L-glutamate by approximately 40% (Table 4). Veratrine, kainate, and L-glutamate failed to increase cGMP content if the incubation was performed in the absence of extracellular Ca^{2+} or in the presence of high concentrations of Mg^{2+} (see Table 4). In the presence of low concentrations of veratrine (4 μg/ml), the increase of cGMP content induced by L-glutamate was synergistic (Table 5), whereas the increase in cGMP levels induced by kainate appeared to be additive (Table 5). L-Aspartate, and to a lesser extent NMDA, synergistically enhanced cGMP content in the presence of veratrine (Table 5). In the presence of kainate (35 μM), the increase in cGMP content elicited by L-glutamate was only additive (Table 5), whereas L-aspartate and NMDA failed to facilitate the kainate-induced increase in cGMP content (Table 5).

Influence of extracellular Mg^{2+}

When Mg^{2+} ions were omitted from the incubation buffer, basal cGMP levels were enhanced approximately 2-fold (Fig. 4). In the absence of extracellular Mg^{2+}, the actions of L-glutamate (Fig. 4*A*), NMDA (Fig. 4*C*), and L-aspartate (Table 6) were greatly amplified (up to 30-fold increase in cGMP level). Under these conditions, the increase in cGMP content induced by kainate was not substantially modified (Fig. 4*B*). Quisqualate also enhanced cGMP content in the absence of extracellular Mg^{2+} (Fig. 4*D*) but to a smaller extent than L-glutamate or NMDA.

Pharmacological characterization of excitatory amino acid receptors coupled with guanylate cyclase

Among the different dicarboxylic amino acid receptor antagonists that we have tested, PDA effectively antagonized the increase in cGMP content elicited by kainate (Fig. 5, and Table 6). The inhibition by PDA of the half-maximal effective dose of kainate was concentration dependent, with an apparent IC_{50} of approximately 50 μM (Fig. 5*a*). Concentrations of 1 mM PDA also antagonized the increase in cGMP content elicited by a maximal effective dose of kainate (Table 6). (±)APV was less potent than PDA in antagonizing the action of a 50 μM kainate (Fig. 5*a*) and was virtually ineffective in antagonizing the in-

Table 4. Effect of TTX and divalent ions on the increase of cGMP elicited by veratrine, kainate and L-glutamate

	cGMP (pmol/mg protein)			
Stimulating agent	Control	TTX (5×10^{-7} M)	No Ca^{2+} 1 mM EGTA[a]	Mg^{2+} (10 mM)[b]
None	0.4 ± 0.08	0.4 ± 0.02	0.08 ± 0.006[c]	0.2 ± 0.05[c]
Kainate, 10^{-4} M	19 ± 1.2	21 ± 0.9	0.07 ± 0.01[c]	1.3 ± 0.3[c]
Veratrine, 15 μg/ml	24 ± 1.8	0.3 ± 0.03[a]	0.1 ± 0.03[c]	0.9 ± 0.2[c]
L-Glutamate, 10^{-4} M	2.8 ± 0.2	1.7 ± 0.12[c]	0.1 ± 0.02[c]	0.8 ± 0.2[c]

TTX was dissolved in a small volume of glacial acetic acid and then diluted in the incubation buffer. TTX or the control vehicle was added to the cultures together with KA or veratrine. cGMP determinations were carried out 1 min after the addition of the stimulating agents. Each value is the mean ± SEM of 5 determinations.

[a] $CaCl_2$ was omitted and 1 mM EGTA substituted.

[b] Sodium chloride concentration was reduced to give isotonic conditions in the presence of 10 mM $MgCl_2$.

[c] $p < 0.01$ compared with the respective control values.

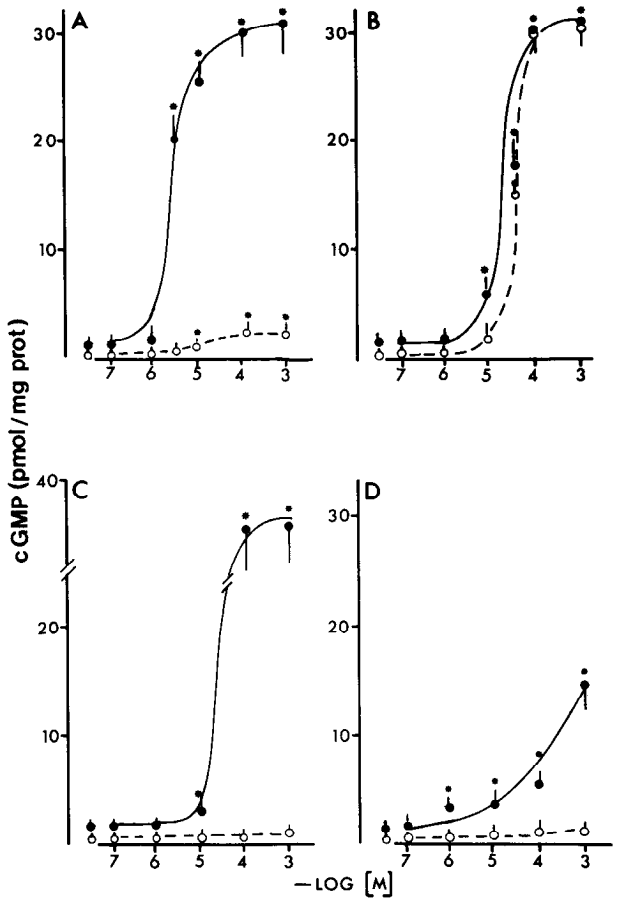

Figure 4. Concentration-dependent stimulation of cGMP formation by L-glutamate (*A*), kainate (*B*), NMDA (*C*), and quisqualate (*D*) in primary cultures of cerebellar granule cells incubated in the presence of 1 mM Mg^{2+} (○) or in the absence (●) of extracellular Mg^{2+}. Values are means ± SEM of at least 4 determinations. $p < 0.01$ (Student's *t* test) compared with values obtained in the respective control group.

crease of cGMP content elicited by 100 μM kainate (Table 6).

In contrast, the stimulation of cGMP formation elicited by a half-maximal effective dose of L-glutamate was potently antagonized by (±)APV (IC_{50} = 10 μM) but was less sensitive to the inhibition by PDA, both in the absence (Fig. 5*b*) and in the presence of Mg^{2+} (data not shown). A maximal effective dose of L-glutamate (100 μM) was antagonized by 1 mM (±)APV but not by 1 mM PDA (Table 6). Concentrations of 1 mM (±)APV and PDA antagonized the increase in cGMP content elicited by NMDA, L-aspartate, and quisqualate, and (±) APV also reduced the basal formation of cGMP in the absence of extracellular Mg^{2+} (Table 6). GDEE partially reduced the increase in cGMP content elicited by L-glutamate and quisqualate but failed to antagonize the action of kainate (Table 6). In addition to excitatory amino acid receptor agonists or antagonists, we have tested compounds (picrotoxin, 1 μM; bicuculline, 1 μM; muscimol, 1 μM; diazepam, 10 μM; ACh, 100 μM, + eserine, 10 μM) known to alter cerebellar cGMP content *in vivo* (Biggio et al., 1977a, b) or in tissue slice preparations (Ferrendelli et al., 1974). These substances failed to change the granule cell cGMP content either in the absence or in the presence of kainate.

Table 5. Increase in cGMP levels induced by dicarboxylic amino acids in primary cultures of cerebellar granule cells: interactions with veratrine and kainate

Amino acid	None	Veratrine (4 μg/ml)	Kainate (35 μM)
Control	0.52 ± 0.12	6.2 ± 0.51	4.9 ± 0.62
L-Glutamate (100 μM)	2.6 ± 0.19[c]	31 ± 0.7[a]	7.5 ± 0.81[a]
L-Aspartate (100 μM)	0.52 ± 0.06	15 ± 1.9[a]	5.4 ± 0.37
NMDA (100 μM)	0.44 ± 0.02	8.3 ± 0.7[b]	5.4 ± 0.37
Quisqualate (100 μM)	0.52 ± 0.09	6.5 ± 0.82	—
Kainate (35 μM)	4.9 ± 0.62[c]	13 ± 1.8[a]	—

Values are means ± SEM of at least 4 determinations.
[a] $p < 0.01$ and [b] $p < 0.05$ when compared with the respective controls (None, Veratrine, and Kainate).

Discussion

Activation of excitatory amino acid receptors enhances cGMP formation in the rat cerebellum *in vivo* (Mao et al., 1974; Biggio and Guidotti, 1976), rat cerebellar slices (Ferrendelli et al., 1974; Schmidt et al., 1977; Ferkany et al., 1982; Garthwaite, 1982; Foster and Roberts, 1984) or dispersed cerebellar cells (Garthwaite and Balazs, 1981). Using these preparations, the heterogeneity of the neuronal cell population and the presence of glial cells, which take up glutamate and aspartate from the synaptic cleft (Balcar and Johnston, 1973), may distort the evaluation of the intrinsic potency of excitatory amino acid receptor agonists and antagonists (Garthwaite, 1985).

Table 6. Increase in cGMP content elicited by dicarboxylic amino acids in Mg^{2+}-free buffer: inhibition by dicarboxylic amino acid receptor antagonists

	cGMP (pmol/mg protein)			
Amino acid	None	(±)APV (1 mM)	PDA (1 mM)	GDEE (1 mM)
Control	1.1 ± 0.17	0.84 ± 0.07[a]	1.2 ± 0.3	1.0 ± 0.08
L-Glutamate (100 μM)	21 ± 3.5	2.2 ± 0.08[a]	21 ± 1.9	14 ± 1.6[a]
Kainate (100 μM)	22 ± 4.2	19 ± 1.9	9.4 ± 1.3[a]	21 ± 3.0
NMDA (100 μM)	20 ± 5.8	1.3 ± 0.1[a]	3.9 ± 0.3[a]	—
Quisqualate (100 μM)	8.0 ± 1.6	0.83 ± 0.05[a]	1.3 ± 0.25[a]	2.8 ± 0.4[a]
L-Aspartate (100 μM)	25 ± 1.3	1.6 ± 0.20[a]	5.8 ± 0.24[a]	—

Values are means ± SEM of at least 4 determinations.
[a] $p < 0.01$ compared with values obtained in the absence of antagonists.

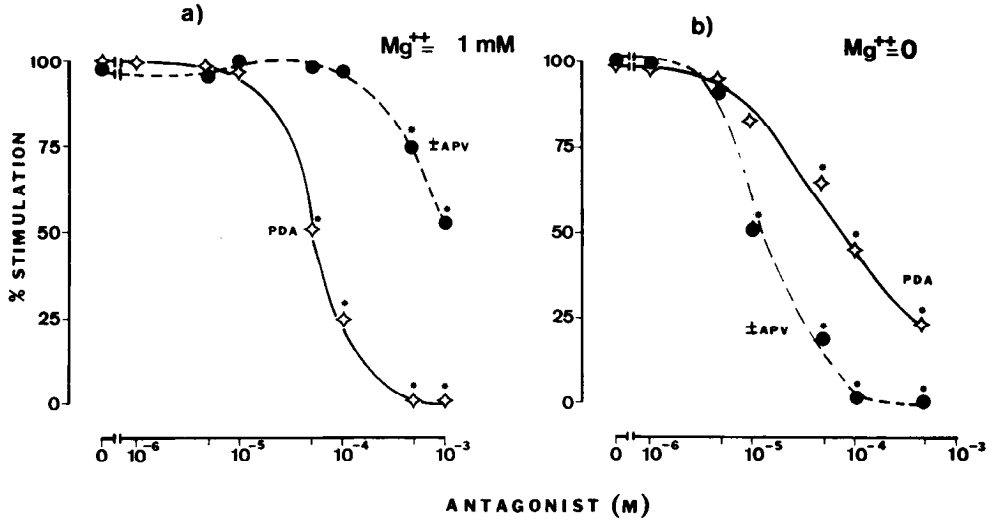

Figure 5. Concentration-dependent inhibition of kainate- and L-glutamate-stimulated cGMP formation by PDA and (±)APV in primary cultures of cerebellar granule cells. The cGMP content (pmol/mg/protein) of untreated cell cultures was 0.43 ± 0.06 in the presence of 1 mM Mg^{2+} and 0.92 ± 0.1 in the absence of Mg^{2+}. a, Addition of kainate (50 μM) to normal Locke's solution increased cGMP values to 15 ± 2.1 pmol/mg protein. b, Addition of L-glutamate (5 μM) to Mg^{2+}-free Locke's solution increased cGMP values to 18 ± 1 pmol/mg protein. Each value is the mean of 8 individual samples from 2 separate experiments (standard mean less than 10%). Values are expressed as a percentage of the increase induced by kainate and L-glutamate above basal cGMP content. $*p < 0.01$ compared with values obtained in the absence of antagonists.

Primary cultures of cerebellar neurons offer an attractive model to study putative second messenger systems associated with excitatory amino acid receptors. In this culture, glial cells are nearly absent and the neuronal population is homogeneous, mostly represented by granule cells (>92–95% of the entire cell population); moreover, primary cultures of cerebellar neurons express excitatory amino acid recognition sites coupled with specific membrane transducing systems (Wroblewski et al., 1985; Nicoletti et al., 1986b). We report here that the addition of excitatory amino acid receptor agonists to cultured cerebellar granule cells induces remarkable changes in cGMP content without modifying cAMP content. The action of kainate on cAMP content of cerebellar granule cells in primary culture is not substantially affected by the absence of extracellular Mg^{2+}, by the presence of the depolarizing agent veratrine, or by the presence of TTX, and is preferentially antagonized by PDA, a ligand of excitatory amino acid receptors that inhibits the electrophysiological action of kainate (Watkins and Evans, 1981). Hence, it appears that cultured cerebellar granule cells express a "kainate-preferring receptor," associated with guanylate cyclase, whose pharmacological profile is similar to that defined by electrophysiological or radioligand binding studies. In addition to a kainate receptor, granule cells in culture express another excitatory amino acid receptor whose activation is transduced into stimulation of guanylate cyclase only in the absence of extracellular Mg^{2+} or in the presence of depolarizing stimuli. This receptor is activated by L-glutamate, L-aspartate, and NMDA and is preferentially antagonized by (±)APV, which has been described as a selective "NMDA" receptor antagonist (Watkins and Evans, 1981; Foster and Fagg, 1984). When granule cells are incubated in standard culture conditions, the stimulation of this receptor by L-glutamate, L-aspartate, and NMDA fails to induce substantial changes in cGMP content. However, in the absence of extracellular Mg^{2+} or in depolarized conditions, the occupancy of this recognition site is transduced into a potent activation of guanylate cyclase. Thus, in primary cultures of cerebellar granule cells, a receptor for kainate and a receptor for L-glutamate, L-aspartate, and NMDA can be differentiated pharmacologically and functionally. While kainate activates guanylate cyclase to the same extent in all the conditions examined, L-glutamate, L-aspartate, and NMDA stimulate guanylate cyclase only in conditions that provide a reduction of extracellular Mg^{2+} concentration or a membrane depolarization. Electrophysiological data indicate that the opening of specific membrane ionic channels triggered by the activation of NMDA receptors is inhibited by Mg^{2+} and that this inhibition can be reversed by either depolarizing the cells or decreasing the extracellular Mg^{2+} concentration (Mayer et al., 1984; Nowak et al., 1984). The activation of guanylate cyclase by excitatory amino acids might result from the opening of specific membrane ionic channels, leading directly or indirectly to an enhanced influx of extracellular Ca^{2+}. A class of ionic channels that transduces the binding of L-glutamate, L-aspartate, and NMDA to their recognition sites into activation of guanylate cyclase might be modulated by Mg^{2+}. Membrane depolarization induced by veratrine might reverse the Mg^{2+} inhibition through allosteric changes of some Mg^{2+}-sensitive proteins that regulate these channels. Hence, we speculate that the following chain of events is responsible for the activation of guanylate cyclase by L-glutamate, L-aspartate, or NMDA: (1) in depolarizing conditions or in the absence of extracellular Mg^{2+}, the occupancy of specific recognition site by L-glutamate, L-aspartate, or NMDA leads to the opening of "receptor-regulated" cationic channels; (2) Ca^{2+} ions enter the granule cells through these channels or through other channels that become permeable because of the depolarization; (3) the consequent increase in intracellular Ca^{2+} activates guanylate cyclase. On the other hand, kainate would activate guanylate cyclase through the opening of a different class of channels that are Mg^{2+}-independent and are unaffected by depolarization. A critical role for Ca^{2+} in the activation of guanylate cyclase is supported by the following observations: (1) kainate enhances $^{45}Ca^{2+}$ uptake in primary cultures of cerebellar granule cells, whereas L-aspartate and NMDA enhance $^{45}Ca^{2+}$ uptake only in the absence of extracellular Mg^{2+} or in the presence of veratrine (Wroblewski et al., 1985); (2) excitatory amino acids fail to enhance cGMP content when Ca^{2+} is omitted from the incubation medium (see Table 4).

In conclusion, 2 different excitatory amino acid recognition sites (activated by kainate or by L-glutamate, L-aspartate, and NMDA, respectively) are located in the membrane of cerebellar granule cells. While the coupling of the kainate recognition site with guanylate cyclase occurs under resting conditions and in

the presence of Mg^{2+}, the coupling of the recognition sites for L-glutamate, L-aspartate, and NMDA with guanylate cyclase requires depolarizing conditions or decreased Mg^{2+} concentrations. Garthwaite (1982), using cerebellar slices prepared from newborn rat brain, reported that the absence of Mg^{2+} potentiated the stimulation of cGMP synthesis elicited by NMDA but failed to induce changes in the actions of L-glutamate or L-aspartate. This difference is not observed in cultures of cerebellar granule cells. Garthwaite (1982) suggested that, in neonatal cerebellar slices, the stimulation of cGMP synthesis by L-glutamate and L-aspartate was mediated by a specific quisqualate recognition site. In primary cultures of cerebellar granule cells, such a receptor is not coupled with guanylate cyclase, at least under the same conditions in which receptors for L-glutamate and L-aspartate are coupled. The relatively modest increase in cGMP formation that we observed with high concentrations of quisqualate in Mg^{2+}-free conditions is partially antagonized by GDEE and completely prevented by (±)APV and PDA, suggesting that, in our model, high concentrations of quisqualate interact with the receptor that is preferentially sensitive to L-glutamate, L-aspartate, and NMDA.

The selective increase in cGMP content induced by kainate in granule cells in the presence of Mg^{2+} under resting conditions can be used to study the conformational constraints that determine the intrinsic activity of kainate-like compounds. Since kainate is not present in mammalian CNS, it can be postulated that an endogenous agonist for kainate recognition sites might exist and play a physiological role. Stimulation of guanylate cyclase in cultures of cerebellar granule cells might be a useful tool for the detection and purification of such an endogenous compound.

We have recently reported that, in primary cultures of cerebellar granule cells, excitatory amino acids activate inositol phospholipid metabolism with the following order of potency: glutamate > quisqualate > kainate > aspartate > NMDA (Nicoletti et al., 1986b). The different pharmacological profile revealed by the capacity of excitatory amino acids to stimulate cGMP formation suggests that stimulation of guanylate cyclase and activation of inositol phospholipid hydrolysis are independent signals generated by specific ligands of excitatory amino acid receptors. The physiological relevance of these second messenger systems in the function of excitatory amino acid receptors is currently under investigation.

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