The Activation of Inositol Phospholipid Metabolism as a Signal-Transducing System for Excitatory Amino Acids in Primary Cultures of Cerebellar Granule Cells

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L-Glutamic, L-aspartic acids and a number of their structural analogs, including quisqualic, kainic, ibotenic, quinolinic, and *N*-methyl-D-aspartic (NMDA) acids, increase inositol phospholipid hydrolysis when added to primary cultures of cerebellar granule cells, as is reflected by an enhanced formation of ^3H -inositolmonophosphate (^3H -IP₁) in the presence of Li⁺. L-Glutamic acid also enhances the formation of the initial products of inositol phospholipid hydrolysis, ^3H -inositoldi- (^3H -IP₂) and triphosphate (^3H -IP₃). In the absence of extracellular Ca²⁺, L-glutamic acid fails to enhance ^3H -IP₁ formation, but still increases ^3H -IP₂ and ^3H -IP₃ formation.

The stimulation of ^3H -IP₁ formation elicited by L-glutamic acid is reduced by DL-2-amino-5-phosphonovaleric acid (APV) and γ -glutamylglycine and, to a lesser extent, by 2,3-*cis*-piperidindicarboxylic acid (PDA). The stimulation of ^3H -IP₁ formation by kainic acid is antagonized by PDA and γ -glutamylglycine, but it is almost unaffected by APV. The increase in ^3H -IP₁ formation elicited by quisqualic acid is not reduced by any of the dicarboxylic amino acid receptor antagonists that we have tested.

We conclude that different subtypes of excitatory amino acid recognition sites are associated with inositol phospholipid metabolism in primary cultures of cerebellar granule cells.

Although the dicarboxylic amino acid recognition sites located in synaptic membranes have been extensively studied (for a review, see Foster and Fagg, 1984), little is known about the molecular process operative in the transduction of these chemical signals into a specific neuronal response. In rat hippocampal slices, the activation of specific dicarboxylic amino acid recognition sites by ibotenic acid enhances the hydrolysis rate of membrane inositol phospholipids (Nicoletti et al., 1986). Glutamic acid has been found to increase phosphatidylinositol turnover rate in the insect flight muscle (for a review, see Michell, 1975), but it has failed to induce important changes in phosphatidylinositol metabolism in slices from mammalian brain (Brown et al., 1984). However, an accurate assessment of the action of glutamic and aspartic acids on phosphatidylinositol turnover in brain slices is impaired by neuronal and glial uptake and metabolism of these amino acids.

To investigate whether dicarboxylic amino acids change membrane inositol phospholipid hydrolysis, we have used glial-free primary cultures of cerebellar granule cells. These cells have been considered reliable models for the investigation of excitatory amino acid receptor function because they use glutamic acid as neurotransmitter (Sandoval and Cotman, 1978) and they

respond to the application of glutamic, aspartic, and kainic acids with an activation of guanylate cyclase (Novelli et al., unpublished observations).

Materials and Methods

Cell cultures

Cerebellar granule cells were obtained from 8-d-old rats, as described by Gallo et al. (1982). In brief, cells were resuspended and seeded in the following culture medium: Basal Eagle's Medium (BME; Flow Labs), 10% fetal calf serum (FCS; Gibco), 25 mM KCl, 2 mM glutamine, and 100 $\mu\text{g}/\text{ml}$ gentamycin (Gibco), successively plated (2.5×10^6 cells/dish) onto 35 mm Falcon dishes coated with poly-L-lysine, 5 $\mu\text{g}/\text{ml}$, and incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cytosine arabinofuranoside (Ara-C; 10 μM) was added after 16 hr of culture to inhibit the replication of nonneuronal cells. Cerebellar astrocytic cultures were prepared as described by Woodhams et al. (1981). The culture medium was BME containing 10% FCS, 2 mM glutamine, and 100 mg/ml gentamycin. The KCl concentration in this medium was 4.7 mM and no Ara-C was added to these cultures. Neuronal and astrocytic cultures have been characterized by immunocytochemistry.

Immunocytochemistry

After 8 d in culture, the cells were washed with 0.1 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 a 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 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, EC 4.1.1.15; a gift from Dr. J. Kopin) was diluted 1:800; monoclonal mouse antisera to phosphorylated and nonphosphorylated neurofilaments (Sternberger-Mayer) were diluted 1:2000. Secondary antibodies were affinity-purified and conjugated to fluorescein isothiocyanate (FITC) goat anti-mouse IgG (1:100; Cappel) and rhodamine isothiocyanate (RITC) goat anti-rabbit IgG (1:100; Jackson Immunoresearch).

For double immunofluorescence staining, the cells were incubated with a mixture of both primary antibodies at the same dilutions as 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. [For specificity, culture dishes were processed in the full immunohistochemical sequence with one (or both) primary antiserum deleted or replaced with normal rat serum. No detectable staining was observed in these conditions.]

Measurement of inositol phospholipid hydrolysis

The rate of inositol phospholipid hydrolysis was estimated by measuring the formation of ^3H -inositolmonophosphate (^3H -IP₁) in the presence of Li⁺, which blocks the conversion of IP₁ into free inositol (Allison et al., 1976; Berridge et al., 1982). Culture dishes with 8–10-d-old cells received 1 ml fresh medium containing 0.3 μM myo-2- ^3H -inositol (New England Nuclear; specific activity 16.5 Ci/mmol) to label membrane

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Table 1. Stimulation of $^3\text{H-IP}_1$, $^3\text{H-IP}_2$, and $^3\text{H-IP}_3$ formation elicited by L-glutamic acid in primary cultures of cerebellar granule cells in the presence or absence of extracellular Ca^{2+}

	$^3\text{H-IP}_1$		$^3\text{H-IP}_2$ (cpm/100 μg prot.)		$^3\text{H-IP}_3$	
	Control	Glu	Control	Glu	Control	Glu
1.3 mM CaCl_2	560 $\pm$ 54	1250 $\pm$ 180*	910 $\pm$ 150	1970 $\pm$ 60*	110 $\pm$ 28	206 $\pm$ 12*
Omission of CaCl_2 + 1 mM EGTA	290 $\pm$ 32	320 $\pm$ 16	312 $\pm$ 35	740 $\pm$ 46*	99 $\pm$ 9	260 $\pm$ 16*

Cerebellar granule cells were incubated for 2 min in the presence or absence of 50 μM L-glutamic acid (Glu).

* $p < 0.01$, compared with control values.

inositol phospholipids. Twenty-four hours later, the dishes were washed 3 times with 900 μl Krebs/Hensleit buffer (NaCl, 118 mM; KCl, 4.7 mM; CaCl_2 , 1.3 mM; KH_2PO_4 , 1.2 mM; MgSO_4 , 1.2 mM; NaHCO_3 , 25 mM; glucose, 11.7 mM) containing 7 mM LiCl, equilibrated with 95% O_2 /5% CO_2 to a final pH of 7.4, and prewarmed at 37°C. The cells were then incubated with 900 μl of buffer at 37°C for 15 min prior to drug addition and then for an additional 20 min in the presence of dicarboxylic amino acid receptor agonists and antagonists. At the end of the incubation, the buffer was removed by aspiration, 0.3 ml ice-cold water was added, and the reaction (release of $^3\text{H-IP}_1$ from membrane phospholipids) was stopped by freezing the dishes on dry ice. The cells were harvested and the suspension was added to 0.9 ml chloroform/methanol (1:2). After further addition of 0.3 ml water and 0.3 ml chloroform, the suspension was centrifuged at $500 \times g$ for 2 min to facilitate phase separation. The amount of $^3\text{H-IP}_1$ present in the aqueous phase was estimated as described by Berridge et al. (1982). Eight hundred microliters of aqueous phase (diluted 1:3 with water) were added to columns containing 1 ml of Dowex 1 $\times$ 8 resin (100–200 mesh, formate form; Bio Rad) and the phosphate esters were eluted by stepwise addition of formate solutions of increasing strength. Glycerophosphoinositol and inositol 1:2 cyclic phosphate were eluted with 5 mM sodium tetraborate + 60 mM sodium formate; IP_1 was eluted with 0.2 M ammonium formate + 0.1 M formic acid (see Note); the more polar inositol di- (IP_2) and inositol tri- (IP_3) phosphates were eluted with 0.4 M ammonium formate + 0.1 M formic acid and 1 M ammonium formate + 0.1 M formic acid, respectively. The fractions were collected and the radioactivity measured by liquid scintillation spectrometry. Proteins were measured according to Lowry et al. (1951). [Note: The elution profile of inositol monophosphate from the anion exchange column was monitored using inositol-2-monophosphate as an external standard. Twenty microliters of the effluent from the Dowex column were injected into a flow-injection analyzer system consisting of a pump, loop valve, alkaline phosphatase-loaded column, mixing tee, reagent pump, and spectrophotometer. The enzyme-loaded column (3 cm $\times$ 4 mm) contained 100 Sigma units of calf intestinal alkaline phosphatase adsorbed to phenoxylacetylcellulose. The column effluent was mixed with a stream containing 0.4% ammonium molybdate in 1 M nitric acid. The formation of unreduced phosphonomolybdate complex was monitored at 360 nm.]

Table 2. Stimulation of $^3\text{H-IP}_1$ accumulation elicited by some agonists of dicarboxylic amino acid receptors

Dicarboxylic amino acid (10^{-4} M)	$^3\text{H-IP}_1$ accumulation (cpm/100 μg prot.)
None	460 $\pm$ 11 (12)
L-Glutamic acid	2100 $\pm$ 83* (11)
Quisqualic acid	1400 $\pm$ 40* (10)
Kainic acid	1100 $\pm$ 84* (6)
L-Aspartic acid	920 $\pm$ 43* (6)
Ibotenic acid	760 $\pm$ 51* (6)
NMDA	580 $\pm$ 27* (6)
Quinolinic acid	590 $\pm$ 28* (4)

Values are mean $\pm$ SEM of 4–12 determinations (N in parentheses).

* $p < 0.01$, significantly different from basal values when compared to Dunnett's t test.

Measurement of glutamic acid release

Glutamic acid was measured by HPLC essentially as described by Einarsson et al. (1983). Aliquots of the incubation medium containing the released glutamic acid were derived with 9-fluorenylmethyl chloroformate (Sigma), extracted twice with pentane, and the water phase was injected. A 150 $\times$ 4.6 mm I.D. 5 μm Altex Ultrasphere ODS column was used. The eluent contained 12% acetonitrile and 30% methanol in 50 mM Na-acetate buffer, pH 4.0. Between injections, the columns were washed with 50% acetonitrile in Na-acetate buffer. The fluorometric detector (Farrand) was equipped with a 254 nm interference excitation filter and a 300 nm high-pass emission filter.

Chemicals

L-Glutamic, L-aspartic, quisqualic, kainic, ibotenic, *N*-methyl-D-aspartic (NMDA), quinolinic, DL-aminophosphonovaleric (APV), and DL-aminophosphonobutyric (APB) acids, -glutamatediethyl ester (GDEE), were purchased from Sigma. 2,3-Cis-piperidin dicarboxylic acid (PDA) and -glutamylglycine were purchased from Cambridge Research Biochemicals.

Results

We established that neuronal cultures contain more than 97% neurons and only a few (less than 3%) glial cells (Fig. 1). Approximately 92% of the neuronal cells have been morphologically identified as granule cells. Accordingly, these cultured cells have a high density of GABA, benzodiazepines, and *t*-butyl-bicyclophosphate recognition sites (Gallo et al., 1985), which are present also in granule cells *in vivo*. GABAergic neurons (mostly basket and Golgi cells), identified by specific GAD immunostaining, are also present but do not exceed 5% of the cell population (Fig. 2).

Initial experiments were performed to determine whether the stimulatory action of excitatory amino acids on inositol phospholipid hydrolysis was dependent on incubation time. When granule cells were incubated in the presence of 10^{-5} M L-glutamic or kainic acids, $^3\text{H-IP}_1$ formation increased linearly during the first 5 min. $^3\text{H-IP}_1$ accumulation peaked after 20 min and appeared to decrease slightly after 40 min of incubation (Fig. 3). Hence, an incubation time of 20 min was used routinely to study the stimulation of $^3\text{H-IP}_1$ formation. L-Glutamic acid also stimulated the formation of $^3\text{H-IP}_2$ and $^3\text{H-IP}_3$ after 2 min of incubation (Table 1). No changes in $^3\text{H-IP}_2$ or $^3\text{H-IP}_3$ were detectable when cells were incubated for 20 min in the presence of L-glutamic acid (data not shown). The absence of extracellular Ca^{2+} (achieved by the omission of Ca^{2+} from the incubation buffer + addition of 1 mM EGTA) abolished, as expected, the increase in $^3\text{H-IP}_1$ formation elicited by L-glutamic acid (Table 1). However, the stimulation of $^3\text{H-IP}_2$ and $^3\text{H-IP}_3$ by L-glutamic acid was substantially preserved in the absence of extracellular Ca^{2+} (Table 1).

Pharmacology of the increase in $^3\text{H-IP}_1$ formation elicited by excitatory amino acids in primary cultures of cerebellar granule cells

All the excitatory amino acids that we have tested enhanced $^3\text{H-IP}_1$ formation in Li^+ -treated primary cultures of cerebellar

Figure 1. Glial fibrillary acidic protein (*A*) and neurofilament (*B*) immunoreactivity in cultures of cerebellar granule cells. In *A*, only 2 glial cells are seen; *B* shows the neurofilament immunostaining in the same field, with several granule cells (*big arrows*) and 1 small neuron morphologically different from granule cells (*small arrow*). *C*, The same field with phase contrast showing the granule cells (*big arrows*), 1 different neuron (*small arrow*), and 2 glial cells (*arrowheads*). Scale bar, 20 μ m.

Figure 2. Micrograph from cerebellar cultured cells showing GAD immunostaining. No staining was associated with granule cells, whereas there are 2 positive GAD interneurons (arrows) and GAD-positive axons and terminals. Bar = 10 μ m.

granule cells (Table 2, Fig. 4). The apparent EC_{50} values estimated from the concentration-response curves presented in Figure 2 range from 10 to 20 μ M for L-glutamic, L-aspartic, and kainic acids, are approximately 50 μ M for quisqualic acid, and 200 μ M for NMDA. Based on the increase in 3H -IP₁ formation elicited by various concentrations of excitatory amino acids, their rank order of potency is L-glutamic > quisqualic > kainic > L-aspartic > NMDA.

The stimulation of 3H -IP₁ formation by L-glutamic acid is not affected by the concurrent presence of atropine and phentolamine, which antagonize muscarinic cholinergic and α_1 -adrenergic receptors, respectively (data not shown). This evidence suggests that the stimulation of 3H -IP₁ formation elicited by L-glutamic acid is not mediated indirectly through muscarinic or α_1 -adrenergic receptors which, in the CNS, are coupled with inositol phospholipid metabolism (for a review, see Michell, 1975). The increase in 3H -IP₁ formation elicited by L-glutamic acid is significantly reduced by APV, γ -glutamylglycine, or PDA, which are reported to act as antagonists at excitatory amino acid recognition sites (see Foster and Fagg, 1984). Specifically, APV is much more potent than PDA in antagonizing the stimulatory action of L-glutamic acid on inositol phospholipid hydrolysis (Table 3, Fig. 5). APV also antagonizes the stimulation of 3H -IP₁ formation by L-aspartic acid (Table 3). The stimulatory action of low concentrations (10^{-5} M) of kainic acid on 3H -IP₁ formation is antagonized by PDA and γ -glutamylglycine, but is

almost insensitive to the APV inhibition (Table 3, Fig. 4). This evidence suggests that kainic acid acts through a different excitatory amino acid receptor, at which PDA acts as a preferential antagonist. However, an accurate estimation of the excitatory action of kainic acid is difficult to achieve, since kainic acid markedly stimulates glutamate release in cerebellar granule cells (Fig. 6). An enhanced release of glutamic acid might contribute to the accumulation of 3H -IP₁ elicited by concentrations of kainic acid higher than 5×10^{-5} M. Consistent with this hypothesis is that APV fails to antagonize the action of 10^{-5} M kainic acid but is as effective as PDA in reducing the increase in 3H -IP₁ formation elicited by 10^{-4} M kainic acid (Table 3).

The accumulation of 3H -IP₁ elicited by quisqualic acid is not reduced by any of the dicarboxylic amino acid receptor antagonists that we have tested (Table 3). In addition, the action of quisqualic acid fails to be reduced by the GABA receptor blocker bicuculline (10^{-5} M) (data not shown). This evidence suggests that the action of quisqualic acid is not mediated by its decarboxylation into the GABA-mimetic drug quisqualamine.

APB is ineffective in antagonizing the accumulation of 3H -IP₁ elicited by the addition of excitatory amino acid receptor agonists to primary cultures of cerebellar granule cells (Table 3). GDEE, which is reported to act as an excitatory amino acid receptor antagonist (for a review, see Foster and Fagg, 1984), not only fails to antagonize, but even potentiates the increase in 3H -IP₁ formation elicited by L-glutamic and quisqualic acids

Table 3. 3H -IP₁ accumulation elicited by dicarboxylic amino acids in the presence of various transmitter receptor antagonists

3H -IP₁ formation (cpm/100 μ g proteins)

Stimulus	Dicarboxylic amino acid receptor antagonist					
	None	DL-APV (10^{-3} M)	PDA (10^{-3} M)	γ -Glutamyl- glucine (5×10^{-4} M)	GDEE (10^{-3} M)	DL-APB (10^{-3} M)
None	460 $\pm$ 13 (12)	430 $\pm$ 15 (5)	530 $\pm$ 70 (4)	500 $\pm$ 28 (3)	520 $\pm$ 26 (4)	460 $\pm$ 66 (4)
L-Glutamate (5×10^{-6} M)	1200 $\pm$ 59 (6)	470 $\pm$ 23* (6)	700 $\pm$ 18* (6)	440 $\pm$ 24* (4)	1500 $\pm$ 65* (5)	1400 $\pm$ 102 (4)
Kainate (10^{-5} M)	830 $\pm$ 27 (16)	700 $\pm$ 48* (5)	490 $\pm$ 75* (8)	460 $\pm$ 19* (4)	900 $\pm$ 67 (6)	800 $\pm$ 97 (4)
Kainate (10^{-4} M)	1300 $\pm$ 42 (5)	940 $\pm$ 15* (6)	900 $\pm$ 61* (5)	—	—	—
Quisqualate (10^{-5} M)	1400 $\pm$ 219 (7)	1300 $\pm$ 211 (5)	1400 $\pm$ 65 (6)	1500 $\pm$ 130 (4)	1900 $\pm$ 14* (4)	1400 $\pm$ 138 (4)
L-Aspartate (10^{-4} M)	940 $\pm$ 31 (8)	460 $\pm$ 35* (5)	420 $\pm$ 43* (3)	—	—	980 $\pm$ 59 (3)

Antagonists were added to the incubation buffer 5 min prior to agonists. Values are mean $\pm$ SEM of 3–16 determinations (*N* in parentheses).

* $p < 0.01$, significantly different from basal values when compared with Dunnett's *t* test.

Figure 3. Time-dependent accumulation of ³H-IP₁ (expressed as percent stimulation above basal) elicited by L-glutamate and kainic acids in primary cultures of cerebellar granule cells. Control values do not change as a function of the incubation time.

(Table 3). This paradoxical action of GDEE might be due to its enzymatic conversion into glutamic acid during the 20 min incubation time.

Cell specificity of the increase in ³H-IP₁ formation elicited by excitatory amino acids in primary cultures of cerebellar granule cells

To study the cell specificity of the increase in ³H-IP₁ formation elicited by dicarboxylic amino acids, we have investigated the action of glutamic acid in cultures of either cerebellar astrocytes or cerebellar neurons pretreated with 50 μM kainic acid for 18 hr. Such treatment causes a substantial loss in GABAergic interneurons, as is reflected by a 90% decrease in glutamic acid decarboxylase (GAD, EC 4.1.1.15) activity and by the disappearance of GAD-like immunoreactivity (data not shown). L-Glutamic acid (10⁻⁴ M) fails to stimulate inositol phospholipid hydrolysis in cultures of astrocytes (Table 4), but still substantially increases ³H-IP₁ formation in cultures of cerebellar neu-

rons pretreated with kainic acid (Table 4). This treatment changes the stimulatory action of L-glutamic acid on inositol phospholipid hydrolysis by less than 15%.

Discussion

Recent evidence indicates that the hydrolysis of membrane inositol phospholipids is an important signal-transducing system for several neurotransmitters (for a review, see Michell, 1975). Presumably, a receptor-mediated activation of phospholipase C hydrolyzes the polyphosphoinositides phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-diphosphate into inositol phosphates and diacylglycerol. These compounds trigger a cascade of events that leads to mobilization of Ca²⁺ from intracellular stores, release of arachidonic acid from membrane phospholipids or from diacylglycerol, and stimulation of protein phosphorylation by activation of protein kinase C (Berridge, 1984; Michell, 1975; Nishizuka, 1984). The products IP₂ and IP₃ are rapidly dephosphorylated into IP₁, which is recycled back

Figure 4. Dose-dependent accumulation of ³H-IP₁ (expressed as percent stimulation above basal) elicited by various excitatory amino acid receptor agonists in primary cultures of cerebellar granule cells. Values are means ± SEM of 4–12 determinations.

Figure 5. Dose-dependent inhibition of the stimulatory action of 5×10^{-6} M L-glutamic acid (A) and 10^{-5} M kainic acid (B) on $^3\text{H-IP}_1$ formation in primary cultures of cerebellar granule cells. Δ , PDA; $\bullet$, D,L-APV.

to inositol for resynthesis of phosphatidylinositol. Li^+ blocks the conversion of IP_1 into inositol by inhibiting the enzyme inositol-1-phosphatase (3.1.3.25; Allison et al., 1976). In the presence of Li^+ , the rate of accumulation of IP_1 reflects the rate of hydrolysis of membrane inositol phospholipids (Berridge et al., 1982).

Excitatory amino acids activate inositol phospholipid metabolism in primary cultures of cerebellar granule cells. This action seems to be independent of an influx of extracellular Ca^{2+} , since L-glutamic acid also stimulates the formation of the initial products IP_2 and IP_3 in the absence of extracellular Ca^{2+} . In addition, quisqualic, aspartic, and N-methyl-D-aspartic acids enhance inositol phospholipid hydrolysis, but fail to increase substantially $^{45}\text{Ca}^{2+}$ uptake in primary cultures of cerebellar granule cells (Wroblewski et al., 1985). The lack of stimulation of $^3\text{H-IP}_1$ formation by excitatory amino acids in Ca^{2+} -free conditions is in agreement with previous reports (Kendall and Nahorski, 1984). It is possible that the conversion of IP_3 and IP_2 into IP_1 or the direct formation of IP_1 from phosphatidylinositol is dependent on an enhanced influx of extracellular Ca^{2+} .

The stimulatory action of L-glutamic acid on inositol phospholipid hydrolysis is antagonized by APV, which is a potent and selective antagonist of the "NMDA" excitatory amino acid receptor subtype (for a review, see Foster and Fagg, 1984). However, the site whereby L-glutamic acid acts to enhance inositol phospholipid hydrolysis cannot be identified with the "NMDA" receptor, as defined by electrophysiological studies (Watkins and Evans, 1981). In fact, NMDA and the "NMDA" receptor agonists ibotenic and quinolinic acids are weak stimulants of $^3\text{H-IP}_1$ formation (Table 2, Fig. 2). This apparent discrepancy suggests

Table 4. Glutamate-induced $^3\text{H-IP}_1$ accumulation in primary cultures of cerebellar astrocytes or granule cells

	$^3\text{H-IP}_1$ formation (cpm/100 μg prot.)	
	Granule cells	Astrocytes
Controls	460 ± 11 (12)	390 ± 7 (6)
L-Glutamic acid (10^{-4} M)	2100 ± 83 (11)	460 ± 12 (6)

Values are mean $\pm$ SEM of 6–12 individual determinations.

Figure 6. Dose-dependent stimulation of L-glutamic acid release by kainic acid in primary cultures of cerebellar granule cells. Values are means $\pm$ SEM of 4–14 determinations.

that a receptor classification based upon electrophysiological criteria is inadequate for classifying the signal transduction stimulated by excitatory amino acids.

It appears that cerebellar granule cells in culture express a specific recognition site for excitatory amino acids that (1) is coupled with inositol phospholipid metabolism, (2) is preferentially activated by L-glutamic acid, (3) is sensitive to the inhibitory action of APV or γ -glutamylglycine, but (4) does not fall into any of the 3 major excitatory amino acid receptor subtypes defined by electrophysiological or ligand-binding data (Foster and Fagg, 1984).

The stimulatory action of low concentrations of kainic acid is preferentially antagonized by PDA and γ -glutamylglycine. This is consistent with the evidence that, among various excitatory amino acid receptor antagonists, only PDA and γ -glutamylglycine reduce the increase in cGMP content elicited by kainic acid in primary cultures of cerebellar granule cells (Novelli et al., unpublished observations). Hence, in addition to a L-glutamic acid-sensitive site, cerebellar granule cells express a kainic acid-sensitive site associated with inositol phospholipid metabolism. The stimulation of this site by kainic acid enhances glutamic acid release in cerebellar granule cells.

The stimulation of $^3\text{H-IP}_1$ formation by quisqualic acid is insensitive to the antagonism of APV, PDA, and γ -glutamylglycine, which suggests that quisqualic acid activates inositol phospholipid metabolism through a site different from the ones that are preferentially activated by L-glutamic and kainic acids. However, the molecular mechanism whereby quisqualic acid

Table 5. Stimulation of $^3\text{H-IP}_1$ accumulation elicited by L-glutamic acid in primary cultures of cerebellar neurons pretreated with kainic acid (50 μM) for 18 hr to selectively destroy GABAergic neurons

	$^3\text{H-IP}_1$ formation (cpm/100 μg prot.)	
	No pretreatment	Kainic acid pretreatment
Control	480 ± 25 (3)	480 ± 43 (4)
L-Glutamic acid (10^{-4} M)	1900 ± 26 (3) (+298%)	1700 ± 68 (4) (+262%)

Values are the mean $\pm$ SEM of (n) determinations.

enhances inositol phospholipid hydrolysis is still unclear, since the action of quisqualic acid is resistant to all the antagonists we have tested, including GDEE, which is reported as a selective inhibitor of the "quisqualate" preferring receptor subtype (see Foster and Fagg, 1984).

In conclusion, we have shown that, in primary cultures of cerebellar granule cells, at least two different excitatory amino acid receptor subtypes, characterized by a differential sensitivity to the inhibitory action of APV and PDA, are associated with inositol phospholipid metabolism. L-Glutamic acid may act as a potent physiological agonist at the site preferentially inhibited by APV, whereas an endogenous compound, mimicking the action of kainic acid, might act as a physiological agonist at the site preferentially inhibited by PDA. Although the developing cerebellum has been shown to have unique properties with respect to the intracellular biochemical response to excitatory amino acid receptor activation, the stimulation of inositol phospholipid hydrolysis that we have shown is not a peculiar feature of cerebellar granule cells. We have recently observed that the activation of phosphatidylinositol metabolism by excitatory amino acids is a general response throughout the CNS during development (Nicoletti et al., 1986). The role of this signal transduction in the synaptic response to excitatory amino acids is currently under investigation.

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