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Inositolphospholipid-linked glutamate receptors mediate cerebellar parallel-fiber–Purkinje-cell synaptic transmission

(quisqualate/protein kinase C/long-term depression/inositol 1,4,5-trisphosphate/phospholipase C)

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ABSTRACT **In slices of adult rat cerebellum inositolphospholipid turnover is stimulated markedly by glutamate and its rigid analogues quisqualate and ibotenate. The drug and amino acid specificity of the response reflects a quisqualate-preferring excitatory amino acid receptor. The absence of glutamate-enhanced inositolphospholipid turnover in mice with Purkinje-cell degeneration indicates that the inositolphospholipid-linked quisqualate receptor mediates parallel fiber–Purkinje cell synaptic transmission. The quantitative prominence of this synapse accounts for the massive enrichment of elements of the inositolphospholipid system in cerebellar Purkinje cells.**

The inositolphospholipid ("phosphoinositide") (PI) cycle signal-transduction mechanism involves phospholipase C-catalyzed hydrolysis of phosphatidylinosito! 4,5-bisphosphate, forming inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol. $InsP_3$ mobilizes calcium from intracellular stores, whereas diacylglycerol activates protein kinase C (1). The components of this cycle are extraordinarily enriched in the Purkinje cells of the cerebellum. [^{3}H]$InsP_3$ autoradiography reveals densities of $InsP_3$ receptors hundreds of times greater than in peripheral tissues (2, 3). Protein kinase C (2) as well as certain phospholipase C isozymes (4) and their mRNAs (5) are similarly enriched in Purkinje cells. Cellular systems related to calcium, which may be associated with $InsP_3$ actions, are prominent in Purkinje cells, including at least two types of calcium-dependent dendritic membrane potentials (6), a Ca^{2+}-ATPase (7), and three types of calcium-binding proteins—calbindin-D_{28K}, parvalbumin, and calmodulin (8).

Reasons for the concentration of PI cycle elements in Purkinje cells have not been apparent. They may mediate the synaptic activity of inputs to the Purkinje cells. The principal inhibitory synaptic inputs to Purkinje cells are from basket and stellate cells, whose release of γ-aminobutyric acid (GABA) opens chloride channels. The two excitatory inputs are the climbing fibers, emanating from the inferior olives of the brainstem, and the parallel fibers, which derive from cerebellar granule cells. The putative transmitter of the climbing fibers, L-aspartate, enhances calcium permeability via ionotropic receptors (8). L-Glutamate appears to be the transmitter of the parallel fibers (9).

To ascertain which synaptic system accounts for the enrichment of PI-cycle elements within Purkinje cell dendrites, we examined the influence of exogenously applied neurotransmitters and neuromodulators upon PI metabolism in rodent cerebellar slices *in vitro*. We present evidence that parallel fiber–Purkinje cell synaptic transmission involves a quisqualate-preferring receptor coupled to PI hydrolysis.

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MATERIALS AND METHODS

Estimation of Inositolphospholipid Metabolism. Male Sprague–Dawley rats (150–200 g) were sacrificed by decapitation; their brains were removed and dissected over ice. Cross-chopped cerebral cortical or cerebellar slices (350 × 350 μm) were prepared with a McIlwain tissue chopper and dispersed in 50 ml of prewarmed Krebs-bicarbonate buffer (118 mM NaCl/4.7 mM KCl/1.3 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/25 mM $NaHCO_3$/11.7 mM glucose) equilibrated to pH 7.4 with 5% CO_2/95% O_2 (vol/vol). The slices were preincubated at 37°C in a shaking waterbath for 1 hr, during which the buffer was changed twice.

Olivocerebellar projections of some male Sprague–Dawley rats (150–175 g) were lesioned by i.p. injection of 3-acetylpyridine (Sigma) (10). Age-matched controls, injected with saline, were maintained under identical conditions. Rats were sacrificed 14 days after injection, the cerebella were removed, and slices were prepared as described above.

Male Purkinje cell degeneration mice (*pcd/pcd*, C57BR/6J strain) and phenotypically normal littermates (*pcd/+*) used as controls were purchased from The Jackson Laboratory. The mice were sacrificed by cervical dislocation 35–40 days after birth. Cerebella were removed, and slices were prepared as described.

PI turnover was estimated by measuring the accumulation of [^{3}H]inositol phosphates ([^{3}H]$InsP_x$) in the presence of Li^+, which inhibits the enzymatic conversion of inositol-1-phosphate to *myo*-inositol (11). After the preincubation period, 60 μl of gravity-packed tissue slices were transferred to minivials containing 0.3 μM *myo*-[2-^{3}H]inositol (NEN/DuPont; specific activity = 17 Ci/mmol; 1 Ci = 37 GBq) and 5 mM LiCl in 250 μl of oxygenated Krebs-bicarbonate buffer. The vials were gassed with 5% CO_2/95% O_2, capped, and agitated in a shaking waterbath at 37°C for 30 min. Agonists were then added in 10-μl aliquots, the vials were again gassed, and the incubation was continued for another 45 min. Antagonists were added 10 min before the agonists. Similar results were obtained in preliminary experiments when the tissue slices were washed free of [^{3}H]inositol before adding agonists. The reaction was terminated by adding 940 μl of ice-cold $CHCl_3$/methanol (1:2, vol/vol), and the suspension was mixed thoroughly. After further addition of 0.3 ml of $CHCl_3$ and 0.3 ml of H_2O 20 min later, the samples were centrifuged at 1000 × *g* for 5 min (Sorvall model RC3) to facilitate phase separation. The aqueous phase was removed for assay of [^{3}H]$InsP_x$, and 0.2-ml aliquots of the lower organic phase were removed to monitor the incorporation of [^{3}H]inositol into the phospholipids.

Abbreviations: LTD, long-term depression; PI, inositolphospholipid ("phosphoinositide"); $InsP_x$, inositol phosphates; $InsP_3$, inositol 1,4,5-trisphosphate; NMDA, *N*-methyl-D-aspartate.

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[^{3}H]InsP_x was assayed essentially as described by Berridge *et al.* (11). Aliquots of the aqueous phase (0.75 ml) were diluted to 3 ml with H_2O and applied to columns containing 0.25 g of Dowex AG1-X8 resin (200–400 mesh) in the formate form. After four washes with 3 ml of 5 mM *myo*-inositol/0.1 M formic acid, the bound [^{3}H]InsP_x was "batch" eluted with 3 ml of 1.0 M ammonium formate/0.1 M formic acid. Radioactivity was determined by liquid scintillation spectrometry after the addition of 16 ml of the aqueous counting mixture Formula-963 (NEN/DuPont) to the collected fractions. Proteins were measured with bovine serum albumin as the standard (12).

Chemicals. Quisqualate (synthetic), 3-hydroxy-2-quinoxalic acid, kainate, 6,7-dichloro-3-hydroxy-2-quinoxalic acid, and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were obtained from Cambridge Research Biochemicals (Valley Stream, NY). Cerebellin, angiotensin II, corticotropin-releasing factor, and sulfated cholecystokinin octapeptide were purchased from Peninsula Laboratories. Neurotoxin NSTX-3 was from the Peptide Institute (Osaka, Japan), and *N*-acetylaspartylglutamate was provided by Joseph Coyle (Johns Hopkins University School of Medicine, Baltimore). All other chemicals were purchased from Sigma.

RESULTS

Agonist-Stimulated PI Metabolism. Cerebellar PI turnover was estimated by measuring [^{3}H]InsP_x accumulation in [^{3}H]inositol-labeled, Li^+-treated rat cerebellar slices after treatment with putative neuromodulators (Fig. 1, Table 1). The greatest enhancement of [^{3}H]InsP_x accumulation is elicited by excitatory amino acids in a dose-dependent and saturable manner. Glutamate produces a maximal 150% augmentation of [^{3}H]InsP_x formation, with an apparent EC_{50} of 300 μM, whereas aspartate is active but less potent. Quisqualate is the most potent agent examined (EC_{50} = 10 μM) with a maximal stimulation of [^{3}H]InsP_x formation similar to that elicited by glutamate. Ibotenate is similar in potency and efficacy to glutamate, whereas kainate is less potent and substantially less efficacious, increasing [^{3}H]-InsP_x accumulation <35%, even at a concentration of 1 mM. Whether kainate acts directly or by releasing endogenous glutamate (13) is not clear. *N*-Methyl-D-aspartate (NMDA) is inactive at concentrations up to 1 mM, as is *N*-acetylaspartylglutamate, a dipeptide thought to mediate excitatory transmission at certain synapses (14). The rank order of potencies is quisqualate > ibotenate, glutamate > aspartate > kainate > NMDA.

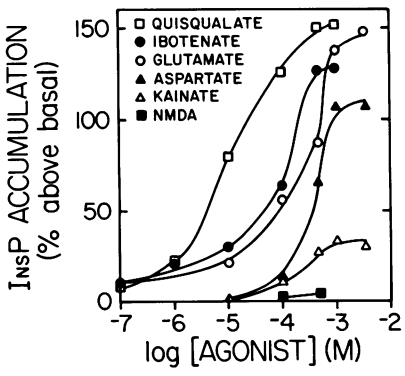

FIG. 1. Dose–response curves of [^{3}H]InsP_x accumulation in rat cerebellar slices stimulated by quisqualate (□), ibotenate (●), glutamate (○), aspartate (▲), kainate (△), and NMDA (■). Results are means of three independent experiments each done in triplicate and are expressed as percentage stimulation above basal values. SEM is <10% of the mean for each data point.

Table 1. Effects of nonexcitatory amino acid receptor agonists and other agents at stimulating inositolphospholipid hydrolysis in rat cerebellar slices

Agent	InsP_x stimulation, % above basal
Receptor agonists	
Angiotensin II (10 μM)	<20
Apomorphine (300 μM)	<20
Baclofen (300 μM)	<20
β-Alanine (3 mM)	<20
Carbamoylcholine (1 mM)	45 ± 7
Cerebellin (10 μM)	<20
2-Chloroadenosine (500 μM)	<20
Cholecystokinin (3 μM)	<20
Corticotropin-releasing factor (2 μM)	<20
Diazepam (100 μM)	<20
α-Aminobutyric acid (3 mM)	<20
Glycine (3 mM)	<20
Histamine (1 mM)	<20
Isoproterenol (3 mM)	<20
Norepinephrine (100 μM)	37 ± 7
Serotonin (1 mM)	63 ± 16
Taurine (1 mM)	<20
Thromboxane B_2 (100 μM)	<20
Other agents	
A23187	<20
Monensin (1 μM)	51 ± 19
Veratridine (30 μM)	43 ± 8

In cases where [^{3}H]InsP_x stimulation was <20%, cerebellar slices were exposed to at least four different concentrations of the agents, with the maximal concentration examined indicated in parentheses. The usual reponses to quisqualate were observed in all experiments. Other values are means ± SEM of at least three determinations.

Of numerous other neurotransmitters and related agents examined, only a few stimulate [^{3}H]InsP_x accumulation in cerebellar slices (Table 1). Norepinephrine (100 μM) produces a maximal 37% augmentation of [^{3}H]InsP_x formation. This may reflect the noradrenergic projection to the cerebellum from the locus ceruleus, which is also thought to activate adenylate cyclase (9). Serotonin (1 mM) produces a 63% increase, which may stem from raphe-cell input to the cerebellum. Carbamoylcholine enhances [^{3}H]InsP_x accumulation 45%, which is much smaller than its effect in the cerebral cortex (Table 2).

The depolarizing drug veratridine (30 μM) stimulates PI hydrolysis 43%, an effect antagonized by the sodium channel blocker tetrodotoxin (2 μM). Tetrodotoxin (2 μM) itself has no effect on either basal or quisqualate-stimulated [^{3}H]InsP_x accumulation. The calcium ionophore A23187 (10 μM) elicits <20% increase in PI metabolism. Taken together, these data suggest that the robust quisqualate-induced increase in PI

Table 2. Comparison of agonist-stimulated inositolphospholipid hydrolysis in tissue slices from rat cerebral cortex and cerebellum

Agonist	[^{3}H]InsP_x, % above basal	
	Cerebral cortex	Cerebellum
Carbamoylcholine	314 ± 26 (10)	45 ± 7 (4)
NMDA	12 ± 2 (2)	2 ± 6 (4)
Kainate	13 ± 4 (2)	34 ± 10 (3)
L-Aspartate	2 ± 5 (2)	66 ± 3 (3)
L-Glutamate	13 ± 3 (2)	87 ± 12 (3)
Ibotenate	52 ± 2 (3)	127 ± 19 (3)
Quisqualate	33 ± 2 (2)	151 ± 11 (3)

All agonists were used at a concentration of 500 μM except for carbamoylcholine (1 mM). Values are means ± SEM of (n) determinations each done in triplicate. Basal formation of [^{3}H]InsP_x was 654 ± 44 cpm/mg of protein for cortical slices and 750 ± 34 cpm/mg of protein for cerebellar slices.

turnover is not secondary to the release of other neurotransmitters, calcium influx through voltage-gated channels activated by depolarization, or calcium influx due to the reverse operation of a Na^+–Ca^{2+} membrane transport exchanger.

We sought to determine which of the four characterized subtypes of excitatory amino acid receptors mediates glutamate effects on cerebellar PI turnover. The NMDA receptor is characterized by high conductance, voltage-dependent Mg^{2+} blockage, and permeability to both calcium and sodium ions (15). Both kainate and quisqualate receptors involve lower conductance and permeability to sodium ions (16, 17). A fourth subtype of excitatory amino acid receptor, which prefers quisqualate, has recently been associated with enhanced PI hydrolysis (18). The failure of NMDA to influence cerebellar PI metabolism argues against a role for the NMDA subtype. Additionally, removal of Mg^{2+} (1.2 mM) from the incubation medium has no effect on the PI hydrolysis elicited by 100 μM quisqualate or 1 mM NMDA (data not shown); nor does the NMDA antagonist D-α-aminoadipate (3 mM), which blocks the stimulation of cGMP by NMDA in mouse cerebellar slices (13), have any effect. We also examined antagonists of the ionotropic non-NMDA receptors. These agents, including glutamate diethyl ester (3 mM), kynurenate (3 mM), neurotoxin NSTX-3 (50 μM), 3-hydroxy-2-quinoxalate (1 mM), 6,7-dinitroquinoxaline-2,3-dione (1 mM), and 6,7-dichloro-3-hydroxy-2-quinoxalate (1 mM), all fail to block the enhancement of PI turnover elicited by 100 μM quisqualate by >10%. Thus the quisqualate effects we have observed do not involve the ionotropic quisqualate receptor. 2-Amino-4-phosphonobutyric acid and *O*-phospho-L-serine antagonize PI hydrolysis elicited by excitatory amino acids, particularly ibotenate, in some preparations (19–21). These agents, even at 1 mM, fail to inhibit the augmentation elicited by 100 μM quisqualate (data not shown). Two distinct PI-linked excitatory amino acid receptors have been proposed, respectively preferring quisqualate and ibotenate (18). We evaluated combinations of ibotenate (0.5 mM) and quisqualate (0.5 mM) but fail to find any additive effects. We also see no additive effects with 0.5 mM aspartate and glutamate (data not shown). Thus, our data suggest that quisqualate and ibotenate enhance PI metabolism through a single metabotropic cerebellar receptor subtype.

Neurotransmitter stimulation of PI turnover usually requires extracellular calcium (22). Omission of calcium decreases the maximal quisqualate-induced enhancement of cerebellar PI hydrolysis ≈55% (Fig. 2). The inclusion of 0.5 mM EGTA along with the omission of calcium from the medium abolishes quisqualate stimulation of PI hydrolysis. It is unlikely that Ca^{2+} is directly activating phospholipase C (23), because both NMDA, which increases membrane permeability to Ca^{2+} ions, and the calcium ionophore A23187 do not enhance PI turnover. Also, depolarization by veratridine, which opens voltage-dependent calcium channels, only modestly increases PI hydrolysis.

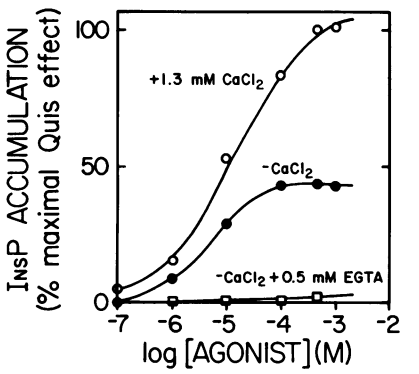

FIG. 2. Calcium dependence of quisqualate-stimulated inositol-phospholipid hydrolysis. Rat cerebellar slices were exposed to various concentrations of quisqualate in Krebs' buffer with $CaCl_2$ omitted (●) or in Krebs' buffer with $CaCl_2$ omitted plus 0.5 mM EGTA (□). The dose–response curve for cerebellar slices exposed to quisqualate in standard Krebs' buffer, which includes 1.3 mM $CaCl_2$, is included for comparison (○). Values are means of triplicate determinations. The experiments were repeated twice with similar results. SD is <12% of the mean for each data point.

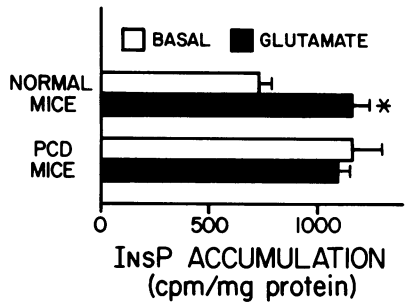

FIG. 3. Comparison between L-glutamate-stimulated inositol-phospholipid hydrolysis in Purkinje-cell-degenerate (PCD) and phenotypically normal C57BR/6J mice. Experimental values represent means ± SD for quadruplicate determinations. Incorporation of [^{3}H]inositol into phospholipids (means ± SD) was 10,840 ± 2400 cpm/mg of protein for cerebellar slices from normal mice and 26,400 ± 2852 cpm/mg of protein for slices from Purkinje-cell-degeneration mice. *, $P < 0.01$, compared with basal.

Regional Differences in Agonist Effects on PI Metabolism. We compared cerebral cortex and cerebellar PI responses to several substances (Table 2). Quisqualate enhances PI hydrolysis five times more in cerebellum than cerebral cortex. As in the cerebellum, PI metabolism in the cerebral cortex is increased more by ibotenate and quisqualate than by aspartate and glutamate. By contrast, carbamoylcholine increases PI hydrolysis 7-fold more in the cerebral cortex than in the cerebellum.

Agonist-Stimulated PI Metabolism in Cerebella from Mutant Mice and Lesioned Rats. To ascertain the cellular locus of cerebellar glutamate augmentation of PI turnover, we examined Purkinje-cell-degeneration mice (Fig. 3). At the age studied (35–40 days), >99% of the Purkinje cells of these mice have degenerated (24). No augmentation of PI metabolism is observed with glutamate (1 mM), though in littermate controls glutamate produces a significant increase. The somewhat greater basal PI turnover in Purkinje-cell-deficient animals appears to reflect their greater incorporation of [^{3}H]inositol into phospholipids.

Treatment with 3-acetylpyridine, which destroys cerebellar climbing fibers (10), fails to alter PI turnover in response to aspartate (1 mM), glutamate (1 mM), kainate (1 mM), and quisqualate (0.5 mM) (data not shown).

DISCUSSION

This study demonstrates a quisqualate-preferring excitatory amino acid receptor linked to PI hydrolysis, which is localized to Purkinje cells. Of the two excitatory inputs to Purkinje cells, climbing fibers and parallel fibers, the latter appears to be the physiologic presynaptic element for this receptor, as climbing fibers influence Purkinje cells by means of an ionotropic aspartate-preferring receptor (25). The quisqualate-preferring PI-linked response has been characterized in various tissue preparations, including *Xenopus* oocytes (26), primary cultures of rat cerebellar granule cells (27), mouse striatal neurons (28), and rat brain slices (19–21, 29). Costa and associates (19) reported stimulation of PI metabolism by ibotenate and quisqualate in hippocampal brain slices. Quisqualate-specific enhancement of PI turnover mediates intracellular Ca^{2+} release in hippocampal neurons (30).

The drug and amino acid specificity in these studies (19–21, 26–30) resembles responses we have observed here. Drug effects on neurophysiologic responses of Purkinje cells to parallel fiber stimulation also support a quisqualate-type receptor at this synapse (31). The decline of quisqualate-sensitive [^{3}H]glutamate binding sites in cerebellar cells after loss of Purkinje cells (32) suggests that the binding sites involve the quisqualate-responsive PI receptors.

Mediation of parallel-fiber input to Purkinje cells by the PI cycle accounts for the massive concentration of PI cycle elements within Purkinje cells, such as the high levels of InsP_3 receptors, protein kinase C, and phospholipase C. The selective localization of G_0 protein in Purkinje cells may reflect a role for G_0 in activating phospholipase C in these cells (33). Cerebellar granule cells are the most numerous neuronal cells in the brain, numbering 10^8 in the rat (34). Each parallel fiber innervates several hundred thousand Purkinje cells, and each Purkinje cell receives converging input from $\approx 10^5$ parallel fibers. Thus, the parallel fiber–Purkinje cell synapse is the most abundant synapse in the brain. Several phospholipase C isozymes have been mapped in the brain by immunohistochemistry (4), and their mRNA has been localized by *in situ* hybridization (5). The isozyme designated PI-PLC-I (35), also designated PLC-A (5) and PLCα (36), is selectively localized to Purkinje cells (5). Thus, the parallel-fiber input to the Purkinje cells presumably stimulates selectively the phospholipase C A isozyme. Protein kinase C isozymes I and II are uniquely associated with Purkinje cells and may represent the subtypes responsive to parallel-fiber input (37).

Purkinje cells are also enriched in several calcium-dependent systems. Dendrites of Purkinje cells possess a calcium-dependent action potential initiated in the tertiary branches of the dendrites, where parallel fibers synapse, in contrast to more proximal portions of the dendrites, where climbing fibers synapse (38). This suggests a preferential association with the parallel-fiber input. Purkinje cells are also enriched in the calcium-binding proteins calmodulin, parvalbumin, and calbindin-D_{28K} (8), of which cerebellar calbindin-D_{28K} occurs only within Purkinje cells (39, 40). These proteins may sequester calcium entering Purkinje cells from the extracellular fluid in response to climbing-fiber firing or after calcium release from intracellular stores by InsP_3.

The Ca^{2+}-ATPase of Purkinje cells is the subtype selectively associated with the endoplasmic reticulum (7). This Ca^{2+} pump may replenish the InsP_3-releasable pool of Ca^{2+}, which occurs in endoplasmic reticulum (1). The relationship of these elements to the Ca^{2+} requirement for quisqualate effects on PI turnover is unclear. This Ca^{2+} requirement may reflect, in part, the role of Ca^{2+} in facilitating ligand binding to quisqualate receptors (41).

When the two excitatory inputs to the Purkinje cells, the climbing fibers and parallel fibers, are conjunctively activated, parallel fiber–Purkinje cell synaptic transmission undergoes long-term depression (LTD), a form of motor learning (8, 9). Applying quisqualate while stimulating climbing fibers also elicits LTD (42), implicating quisqualate-preferring receptors in parallel fiber–Purkinje cell transmission and LTD. Because activation of protein kinase C induces LTD of glutamate-induced responses (43), the PI-linked receptor appears responsible for LTD. The PI system is also involved in hippocampal long-term potentiation, another learning and memory model (44). Identifying the quisqualate-responsive PI receptor as the mediator of parallel fiber–Purkinje cell synaptic transmission may help clarify mechanisms of LTD and related models of learning and memory.

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