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Glutamate-stimulated production of inositol phosphates is mediated by Ca^{2+} influx in oligodendrocyte progenitors

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Abstract

The effect of glutamate on the accumulation of [^{3}H]inositol phosphates was examined in oligodendrocyte progenitor cultures prepared from rat brains. Glutamate, and the analogues α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate, caused a concentration- and time-dependent increase in [^{3}H]inositol trisphosphate (IP_3) formation and the effect was blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a competitive AMPA and kainate receptor antagonist. Similarly, the more selective, noncompetitive antagonist of AMPA receptors, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466), significantly reduced the effect of both AMPA and kainate. In contrast, antagonists of *N*-methyl-D-aspartate (NMDA) receptor, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine (MK-801) and *R*(−)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), and antagonists of metabotropic receptors, L(+)-2-amino-3-phosphono-propanoic acid (L-AP3) and α-methyl-4-carboxyphenylglycine (MCPG), were ineffective. These results suggest that the effect of glutamate on [^{3}H]IP_3 accumulation is mediated through ionotropic AMPA receptors. Cyclothiazide, an inhibitor of AMPA receptor desensitization, strongly potentiated the AMPA and kainate-stimulated [^{3}H]IP_3 formation as well as the uptake of $^{45}Ca^{2+}$ in line with the previous findings. $^{45}Ca^{2+}$ uptake evoked by AMPA or kainate, in combination with cyclothiazide, was also prevented by both CNQX and GYKI 52466. Glutamate-stimulated [^{3}H]IP_3 accumulation was prevented by EGTA, suggesting a requirement for extracellular calcium. Pre-incubation with the voltage-gated Ca^{2+} channel blockers, diltiazem, nifedipine and $CdCl_2$, partially prevented the glutamate-induced [^{3}H]IP_3 accumulation as well as $^{45}Ca^{2+}$ uptake. Similarly, the Na^+/Ca^{2+} exchanger blockers benzamil and 3,4-dichlorobenzamil reduced significantly kainate-stimulated $^{45}Ca^{2+}$ uptake. These data indicate that glutamate-induced [^{3}H]IP_3 accumulation is triggered by calcium influx via AMPA receptors, voltage-gated calcium channels and the Na^+/Ca^{2+} exchanger operating in reverse mode. © 1997 Elsevier Science B.V.

Keywords: AMPA receptor; Extracellular calcium; Glutamate; Inositol phosphate; Oligodendrocyte

1. Introduction

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, binds to two groups of receptors classified into ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels which further subdivide into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors according to their agonist selectivity. Activation of ionotropic receptors opens ion channels permeable to Na^+, K^+ and Ca^{2+}. Glutamate also activates metabotropic receptors coupled through G-proteins to second messenger pathways. Their activation increases phospholipase C activity which hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 mobilizes intracellular Ca^{2+} ($[Ca^{2+}]_i$) while diacylglycerol activates protein kinase C. Recent cloning and expression studies have identified several genes encoding glutamate receptor subunits that are expressed extensively in central nervous system neurons and glia (for review, see Hollmann and Heinemann, 1994; Gallo et al., 1995).

It is well established that cells of the oligodendrocyte lineage express glutamate-gated ion channels both in culture and in situ (for review, see Gallo and Russell, 1995). Glutamate and related agonists depolarize oligodendrocytes and their progenitors through ionotropic non-NMDA (AMPA/kainate) receptors (Barres et al., 1990; Wyllie et al., 1991; Borges et al., 1994; Gallo et al., 1994b;

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Holzwarth et al., 1994). The analysis of glutamate receptor currents in single cells revealed different desensitization kinetics and affinity for agonists, suggesting the co-expression of functional AMPA- and kainate-preferring receptors (Gallo et al., 1994b; Patneau et al., 1994). Furthermore, molecular analysis of glutamate receptors in oligodendrocyte progenitors has demonstrated the expression of AMPA- and kainate-preferring subunit mRNAs and proteins (Jensen and Chiu, 1993; Gallo et al., 1994a,b; Holzwarth et al., 1994; Patneau et al., 1994; Yoshioka et al., 1995; Garcia-Barcina and Matute, 1996). Ca^{2+}-imaging experiments, in antigenically identified cultured oligodendrocyte progenitors, further supported the electrophysiological studies showing that activation of AMPA or kainate receptors produced large and transient increases in $[Ca^{2+}]_i$ (Holzwarth et al., 1994; Holtzclaw et al., 1995; Pende et al., 1994; Meucci et al., 1996). More recent studies have also provided evidence for the presence of metabotropic (Holtzclaw et al., 1995) and NMDA (Wang et al., 1996) receptors in oligodendrocyte progenitors obtained from cortical and neurohypophysial cultures, respectively.

The potential functions of glutamate receptors in oligodendrocyte progenitors are beginning to be elucidated. Thus, NMDA receptors in neurohypophysial explant cultures play a critical role in oligodendrocyte progenitor migration and regulate polysialic acid-neural cell adhesion molecule expression (Wang et al., 1996). On the other hand, prolonged activation of kainate-responsive non-NMDA receptors mediates excitotoxicity in cells of the oligodendroglial lineage when exposed to millimolar concentrations of the agonist (Yoshioka et al., 1995). We and others have recently shown that glutamate, at concentrations which are not toxic to the cells, inhibited proliferation through activation of AMPA or kainate receptors, suggesting a role in cellular development (Liu and Almazan, 1995; Gallo et al., 1996). In addition, activation of AMPA or kainate receptors increased the expression of the immediate early genes NGFI-A (Gallo et al., 1994b; Pende et al., 1994) and c-fos (Pende et al., 1994; Liu and Almazan, 1995). Since glutamate-induced c-fos expression was dependent both on extracellular calcium influx and downstream protein kinase C activation (Liu and Almazan, 1995), it was of interest to further elucidate the molecular events linking receptor activation to gene expression. The objective of the present study was to determine whether phospholipase C participates in the signaling cascade initiated by glutamate in oligodendrocyte progenitors and the role of extracellular calcium.

We have found that stimulation of AMPA receptors causes activation of phospholipase C through a mechanism that requires influx of calcium from the extracellular milieu. Cyclothiazide, an agent which blocks desensitization of the AMPA receptors, in combination with AMPA or kainate produced a large increase in $^{45}Ca^{2+}$ uptake and potentiated the effects of the agonists on PI hydrolysis.

2. Materials and methods

2.1. Materials

L-glutamic acid, (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), kainic acid, *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD), *N*-methyl-D-aspartic acid (NMDA), quisqualate, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclo-hepten-5,10-imine (MK-801), 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), *R*(−)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), L(+)-2-amino-3-phosphono-propanoic acid (L-AP3), GYKI 52466, α-methyl-4-carboxyphenylglycine (MCPG), cyclothiazide, benzamil, nifedipine and diltiazem were purchased from Research Biochemicals International (Natick, MA, USA); *N*-methyl-D-glucamine from ICN (Montreal, QC); *myo*-[^{3}H]Inositol (12.3 Ci/mmol) from DuPont (Mississauga, ON, Canada); $^{45}Ca^{2+}$ (21 mCi/mg) from Amersham (Oakville, ON, Canada); Dowex AG1-X8 resin 100–200 mesh formate form from Bio-Rad (Mississauga, ON, USA); ammonium formate and formic acid from VWR (Mont-Royal, QC, Canada). Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium and inositol-free DMEM as well as phosphate buffered saline, 7.5% bovine serum albumin fraction V, fetal calf serum, penicillin/streptomycin were from Gibco (Burlington, ON, Canada); PDGF AA, bFGF from UBI (Lake Placid, NY, USA); poly-D-lysine, poly-D-ornithine, Triton-X-100 from Sigma (St Louis, MO, USA). All other reagents were obtained from standard suppliers.

2.2. Cell culture

Primary cultures of oligodendrocyte progenitors were prepared from the brains of newborn Sprague–Dawley rats as described by Almazan et al. (1993) as modified from McCarthy and de Vellis (1980). The meninges and blood vessels were removed from the cerebral hemispheres in Ham's F12 medium. The tissue suspension was passed through a 230 μm nylon mesh and collected by filtering through a 150 μm nylon mesh. The resulting suspension was centrifuged for 7 min at 1000 rpm and then resuspended in DMEM supplemented with 10% heat-inactivated fetal calf serum. Cells were plated on poly-L-ornithine-precoated 80 cm^2 flasks and incubated at 37°C with 5% CO_2 in air. Culture medium was changed after 3 d and every two days thereafter. The initial mixed glial cultures, grown for 9 to 11 d, were placed on a rotary shaker at 225 rpm at 37°C for 3 h to remove loosely attached macrophages. Oligodendrocyte progenitors were detached following shaking for 18 h at 260 rpm. The cells were filtered through a 30 μm nylon mesh and plated on bacterial grade Petri dishes for 3 h. Under these conditions, astrocytes and microglia attached to the plastic surface and oligodendrocyte progenitors remained in suspension. The

final cell suspension was plated on 6-well dishes (Falcon) pre-coated with poly-D-lysine. Cultures were maintained in serum-free medium containing 2.5 ng/ml PDGF AA and 2.5 ng/ml bFGF to promote self-renewal and prevent differentiation (Bogler et al., 1990) and medium was changed every two days. Serum-free medium consisted of a DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1% bovine serum albumin, 25 μg/ml human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 μg/ml insulin, 16 μg/ml putrescine, 30 nM selenium, 50 units/ml penicillin and 50 μg/ml streptomycin as previously reported (Almazan et al., 1993).

The cultures were characterized immunocytochemically using antibodies against cell type-specific markers (Cohen and Almazan, 1994; Radhakrishna and Almazan, 1994). More than 95% of the cells reacted positively with mouse monoclonal antibody A2B5, a marker for oligodendrocyte progenitors in culture while less than 5% were galactocerebroside-positive oligodendrocytes, glial fibrillary acidic protein-positive astrocytes or complement type-3-positive microglia (Cohen et al., 1996). These oligodendrocyte progenitor-enriched cell cultures were used in the present study.

2.3. Measurement of [^{3}H]inositol phosphates accumulation

Formation of [^{3}H]inositol phosphates (IPs) was measured as described by Berridge et al. (1983). The cells were pre-labelled with 1 μCi/ml *myo*-[^{3}H]inositol in inositol-free DMEM containing all components in the serum-free medium including PDGF AA and bFGF overnight. LiCl, at a final concentration of 10 mM, was added for 15 min to inhibit inositol-1-phosphatase activity. Labelled cells were then challenged with various glutamate agents at 37°C for 15 min. Antagonists, when used, were added 10 min prior to the agonist stimulation. Reactions were stopped by replacement of the incubation medium with 1 ml of ice-cold methanol. Cells were harvested and the suspension was mixed with 2 ml of ice-cold chloroform/water (1:1; v/v). The extracts were mixed vigorously and centrifuged to facilitate phase separation. The upper aqueous phase, containing IPs, was applied to the Dowex AG1-X8 column (anion-exchange resin, formate form). Elution of the different components was as follows: free inositol, with water; glycerophosphoinositol, with 60 mM sodium formate/5 mM disodium tetraborate; inositol monophosphate (IP_1), with 0.2 M ammonium formate in 0.1 M formic acid; inositol bisphosphate (IP_2), with 0.4 M ammonium formate in 0.1 M formic acid; inositol trisphosphate (IP_3), with 0.8 M ammonium formate in 0.1 M formic acid; and inositol tetrakisphosphate (IP_4), with 1.2 M ammonium formate in 0.1 M formic acid. Radioactivity was determined by liquid scintillation counting. Elution of the IPs from columns were confirmed by using a mixture of IP_1, IP_2, IP_3 and IP_4 as standards from DuPont (Mississauga, ON). Mass measurements of inositol-1,4,5-trisphosphate or inositol-1,3,4-trisphosphate were not performed. Therefore, the fraction collected as [^{3}H]IP_3 should represent a mixture of the two isomers as reported by others (Baird and Nahorski, 1990).

2.4. Measurement of $^{45}Ca^{2+}$ influx

Oligodendrocyte progenitors were grown for 4–5 days in serum-free medium supplemented with bFGF and PDGF (2.5 ng/ml each), and $^{45}Ca^{2+}$ influx was determined according to the protocol described by Hack and Balász (1995). Cells were preincubated for 30 min in a Mg^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, 5 mM Hepes, pH 7.4) at 37°C. Antagonists or blockers were added 5 min prior to stimulation with AMPA or kainate receptor agonists. Stimulation was achieved by replacing incubation solution with 0.5 ml of Locke's solution containing 0.5 μCi $^{45}Ca^{2+}$ and the various pharmacological agents tested. After 5 min, the cells were washed three times with ice-cold buffer (154 mM choline chloride, 2 mM EGTA, and 10 mM Hepes, pH 7.4) then solubilized in 0.1 M NaOH/0.1% Triton X-100. Radioactivity was determined by liquid scintillation counting.

2.5. Data analysis

One way analysis of variance followed by the Tukey–Kramer test was used to determine statistical significance; P values less than 0.05 were considered significant.

3. Results

3.1. Effects of glutamate agonists on [^{3}H]IPs accumulation: Concentration and time-dependency

Exposure of progenitors to glutamate at a range of concentrations from 1 to 1000 μM for 15 min caused a concentration-dependent increase in the accumulation of [^{3}H]IP_3 (Fig. 1) as well as other inositol phosphate metabolites including [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_4 (data not shown). Glutamate was the most potent activator of phosphoinositide hydrolysis (EC_{50} = 27 μM), causing a maximal increase in [^{3}H]IP_3 formation at 1 mM (315% of basal level); it was followed by AMPA (EC_{50} = 34 μM) which achieved its maximal effect at 500 μM (379% of basal level) and showed similar efficacy to glutamate. Kainate exhibited the lowest potency, with an EC_{50} of 81 μM, but the greatest efficacy, with a maximal increase in [^{3}H]IP_3 at 500 μM (685% of basal level).

To determine the time-course of glutamate-stimulated

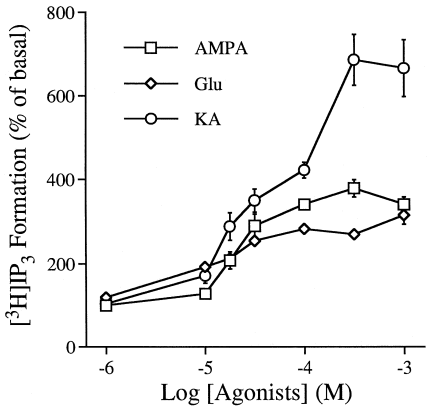

Fig. 1. Concentration-dependence of glutamate agonists-induced [^{3}H]IP_3 accumulation in oligodendrocyte progenitors. Cells were incubated with 1 μCi/ml of *myo*-[^{3}H]inositol overnight at 37°C. Progenitors were stimulated with increasing concentrations of glutamate (Glu), AMPA and kainate (KA) (1–1000 μM) for 15 min in the presence of 10 mM LiCl. [^{3}H]IP_3 was eluted from the columns as described in Section 2. Data are expressed as mean ± SEM (bars) of three independent experiments performed in triplicate. Differences with basal values: 50 μM ($P < 0.05$), 100 μM ($P < 0.001$), 500 μM ($P < 0.01$) and 1000 μM Glu ($P < 0.001$); 50, 100, 500 and 1000 μM AMPA ($P < 0.001$); 25, 50, 100, 500 and 1000 μM KA ($P < 0.001$).

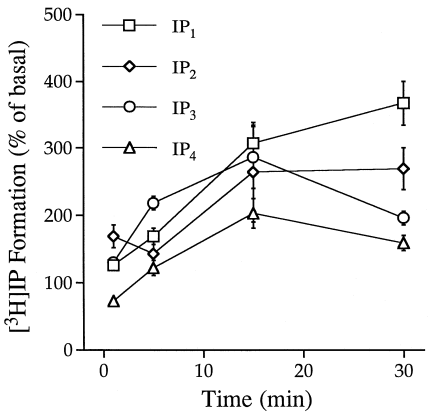

Fig. 2. Time-dependence of glutamate-stimulated [^{3}H]IPs formation in oligodendrocyte progenitors. Progenitors were incubated with glutamate (100 μM) for the indicated time periods. [^{3}H]IPs were collected as described in Section 2. Data are mean ± SEM of two separate experiments performed in triplicate.

phosphoinositide breakdown, 100 μM glutamate was added for 15 s to 30 min in the presence of LiCl. A slight increase in [^{3}H]IP_3 accumulation was detected after 15 s of stimulation (112% of basal level, Table 1), reached its maximum (286% of basal level) at 15 min and decreased to lower levels by 30 min (Fig. 2). [^{3}H]IP_4 increased in parallel with [^{3}H]IP_3 but to a lesser extent (Table 1). [^{3}H]IP_1 and [^{3}H]IP_2 levels significantly increased by 1 ($P < 0.05$) and 5 ($P < 0.01$) min, respectively, and kept increasing at 30 min. Table 1 shows that the absolute values (dpm/culture dish) for the accumulation of [^{3}H]IP_1 were higher than for the other [^{3}H]IPs.

Table 1
Time-dependence of glutamate-induced [^{3}H]IPs accumulation in oligodendrocyte progenitors

Time	Glutamate-induced [^{3}H]IPs accumulation (dpm)			
	[^{3}H]IP_1	[^{3}H]IP_2	[^{3}H]IP_3	[^{3}H]IP_4
Basal	482 ± 36	154 ± 27	109 ± 10	37 ± 2
15 s	374 ± 24	152 ± 21	122 ± 9	36 ± 4
45 s	499 ± 79	92 ± 7	147 ± 8	52 ± 8
1 min	605 ± 40	261 ± 26	142 ± 7	27 ± 2
5 min	815 ± 57	220 ± 28	238 ± 11[b]	45 ± 4
15 min	1482 ± 130[a]	407 ± 114[c]	312 ± 50[a]	75 ± 8[b]
30 min	1767 ± 158[a]	414 ± 48[c]	214 ± 11[c]	59 ± 4

Oligodendrocyte progenitors were incubated overnight with 1 μCi/ml of *myo*-[^{3}H]inositol and then exposed to 100 μM glutamate in the presence of 10 mM LiCl for the indicated time periods. [^{3}H]IPs were eluted from the columns as described in Section 2. Data are mean ± SEM of two separate experiments performed in triplicate. Values indicated differ significantly from basal values: [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$.

3.2. Effect of glutamate antagonists on agonists-stimulated [^{3}H]IP_3 accumulation

To determine the specific receptor subtype(s) mediating glutamate-stimulated [^{3}H]IP_3 formation, we tested selective antagonists in combination with glutamate. CNQX, a competitive AMPA/kainate receptor antagonist, blocked glutamate-induced [^{3}H]IP_3 accumulation in a concentration-dependent manner (Fig. 4). Complete blockade was achieved with 50 μM CNQX (Fig. 3). In contrast, stimulation by glutamate was not affected by the non-competitive NMDA receptor antagonist MK-801 (10 μM) or the competitive antagonist CPP (100 μM) (Fig. 3). Furthermore, no significant increase in the accumulation of [^{3}H]IP_3

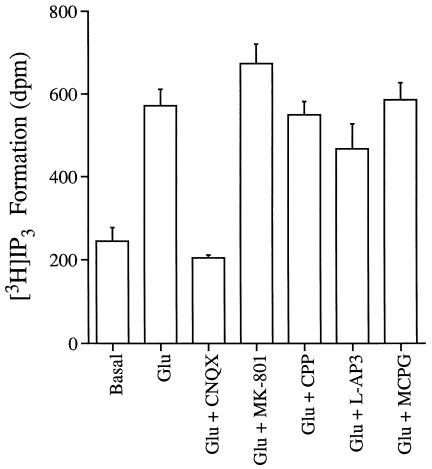

Fig. 3. Effect of glutamate antagonists on glutamate (Glu)-stimulated [^{3}H]IP_3 accumulation in oligodendrocyte progenitors. [^{3}H]inositol-labelled progenitors were preincubated with antagonists, CNQX (50 μM), MK-801 (10 μM), CPP (100 μM), L-AP3 (100 μM), MCPG (250 μM) for 10 min prior to glutamate (100 μM) stimulation. Data are mean ± SEM of four independent experiments performed in triplicate. Values differ significantly from Glu stimulation alone: Glu + CNQX ($P < 0.001$).

Table 2
Effects of glutamate agonists on $[^3H]IP_3$ accumulation in astrocytes and oligodendrocyte progenitors

Treatment	$[^3H]IP_3$ accumulation (dpm)	
	astrocytes	progenitors
Basal	217 ± 11	245 ± 23
Glu	307 ± 29	572 ± 40[a]
Quisqualate	616 ± 42[a]	—
trans-(1*S*,3*R*)-ACPD	417 ± 22[b]	228 ± 39
NMDA	—	203 ± 47

Astroglial cells and oligodendrocyte progenitors were stimulated with indicated agonists (100 μM for astrocytes and 300 μM for oligodendrocyte progenitors) for 15 min. $[^3H]IP_3$ formation was measured as described in Section 2. Data represent mean ± SEM values from two separate experiments performed in triplicate. Statistical significance differed from basal values: [a]$P < 0.001$, [b]$P < 0.01$.

induced by 300 μM NMDA was observed (Table 2). These results suggest that glutamate-stimulated $[^3H]IP_3$ accumulation in oligodendrocyte progenitors was mediated by ionotropic AMPA/kainate receptor activation, but not through NMDA receptor activation.

Since metabotropic receptors are G-protein-coupled and have the potential to activate phospholipase C, we next examined whether $[^3H]IP_3$ formation was mediated by their activation with glutamate. The metabotropic receptor antagonists L-AP3 (100 μM) and MCPG (250 μM) did not prevent glutamate-stimulated $[^3H]IP_3$ formation (Fig. 3), and the agonist, *trans*-(1*S*,3*R*)-ACPD, at 300 μM did not increase $[^3H]IP_3$ accumulation above basal levels (Table 2). These results indicate that metabotropic glutamate receptors are not involved in phospholipase C activation in oligodendrocyte progenitors in culture.

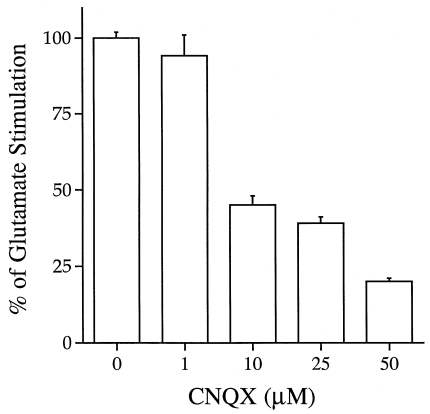

Fig. 4. Effect of CNQX on glutamate-induced $[^3H]IP_3$ accumulation in oligodendrocyte progenitors. Cultures were preincubated with CNQX from 1 μM to 50 μM for 10 min prior to glutamate (100 μM) stimulation. Data represent mean ± SEM of two separate experiments performed in triplicate. Statistical differences observed with glutamate stimulation: 10 μM and 25 μM CNQX ($P < 0.01$) and 50 μM CNQX ($P < 0.001$).

Table 3
Role of extracellular Ca^{2+} in glutamate (Glu)-stimulated $[^3H]IP_3$ formation in oligodendrocyte progenitors

Treatment	$[^3H]IP_3$ formation (dpm)	% of basal level
Basal	328 ± 14	100
Glu	1117 ± 74[a]	341
Glu + EGTA 2 mM	561 ± 19[b]	171
Glu + EGTA 4 mM	476 ± 26[c]	145
A23187 5 μM	636 ± 41	194
Glu + A23187	2575 ± 281[a]	785

EGTA was added 5 min before glutamate (100 μM) and the Ca^{2+} ionophore, A23187, was added for 15 min. Data represent mean ± SEM from three independent experiments performed in triplicate. Statistical differences are indicated: [a]$P < 0.001$ versus basal values, [b]$P < 0.01$, [c]$P < 0.001$ versus Glu.

3.3. Effects of glutamate agonists on $[^3H]IP_3$ accumulation in astrocytes

To exclude the possibility that the effect of glutamate in $[^3H]IP_3$ formation in our cultures could be partly due by the presence of contaminating astrocytes, we stimulated astrocyte cultures under similar conditions. A small increase in $[^3H]IP_3$ formation (141% of basal level) was effected by 100 μM glutamate, whereas, 100 μM quisqualate or *trans*-(1*S*,3*R*)-ACPD, two known agonists of metabotropic receptors, stimulated $[^3H]IP_3$ accumulation by 284% and 192% of basal levels, respectively (Table 2). Milani et al. (1989) showed that the effects of glutamate and quisqualate were not blocked by the AMPA/kainate receptor antagonist DNQX in astrocyte cultures. Our results are consistent with previous work (Milani et al., 1989; Pearce et al., 1990) showing that quisqualate is the most potent agonist to stimulate PI hydrolysis in astrocytes and its effect is mediated through metabotropic receptor activation. Thus, the pharmacology of glutamate-induced $[^3H]IP_3$ formation in oligodendrocyte progenitors differs from that observed in astrocytes. From these results it is concluded that the increases in PI hydrolysis observed in our cultures is not due to metabotropic receptor activation in the small number (less than 5%) of astrocytes contaminating the preparations.

3.4. Glutamate-stimulated $[^3H]IP_3$ accumulation is dependent on extracellular Ca^{2+}

Since activation of AMPA/kainate receptors causes a rapid and transient elevation of $[Ca^{2+}]_i$ (Kastritsis and McCarthy, 1993; Borges et al., 1994; Holzwarth et al., 1994; Pende et al., 1994; Holtzclaw et al., 1995; Takeda et al., 1995), we next studied the role of extracellular Ca^{2+} in glutamate-stimulated $[^3H]IP_3$ formation. To chelate extracellular Ca^{2+}, EGTA was added to the culture medium before glutamate application. Glutamate-stimulated $[^3H]IP_3$ accumulation was significantly reduced (70%) in the presence of 2 mM EGTA and further declined (82%) with 4

Table 4
Effect of Ca^{2+}-channel blockers on glutamate (Glu)-stimulated [^{3}H]IP_3 accumulation in oligodendrocyte progenitors

Treatment	[^{3}H]IP_3 formation (dpm)	% of Glu stimulation
Basal	274 ± 16	—
Glu	718 ± 6[a]	100
Glu + diltiazem	483 ± 20[b]	47
Glu + nifedipine	471 ± 45[c]	44
Basal	180 ± 11	—
Glu	628 ± 60[a]	100
Glu + $CdCl_2$	360 ± 14[b]	40
Basal	198 ± 9	—
Glu	494 ± 33[a]	100
K^+	256 ± 12[d]	20
K^+ + $CdCl_2$	168 ± 12	—

Ca^{2+}-channel blockers were given to [^{3}H]inositol-labelled cells for 10 min followed by glutamate or K^+ stimulation for 15 min. The concentrations used were as follows: Glu, 100 μM; Diltiazem, 50 μM; Nifedipine, 50 μM; $CdCl_2$, 1 mM and K^+, 50 mM. Data are mean ± SEM of three independent experiments performed in triplicate. Statistical differences observed with Glu are: [b]$P < 0.001$; [c]$P < 0.01$; differences with basal are: [a]$P < 0.001$; [d]$P < 0.01$.

Table 5
Effects of cyclothiazide on AMPA- and kainate (KA)-induced $^{45}Ca^{2+}$ uptake in oligodendrocyte progenitors

Treatment	$^{45}Ca^{2+}$ uptake (nmol/min per mg protein)	% of basal uptake
Basal	6.4 ± 0.6	100
AMPA (100 μM)	9.7 ± 0.7	152
KA (100 μM)	14.7 ± 0.9[a]	230
Cyclo (50 μM)	6.0 ± 0.5	94
AMPA + cyclo	52.0 ± 2.6[b]	813
KA + cyclo	61.0 ± 3.7[b]	953
KA + cyclo + CNQX	7.6 ± 6.6[c]	119
KA + cyclo + GYKI	9.6 ± 0.7[c]	150
AMPA + cyclo + CNQX	8.9 ± 0.8[d]	139
AMPA + cyclo + GYKI	9.4 ± 0.5[d]	147
Basal	7.1 ± 0.3	100
NMDA	5.9 ± 0.1	83
trans-(1*S*, 3*R*)-ACPD	6.8 ± 0.1	95

$^{45}Ca^{2+}$ uptake in oligodendrocyte progenitor cultures was determined as described in Section 2. The concentrations used were as follows: CNQX, 10 μM; GYKI 52466, 50 μM; NMDA, 300 μM; *trans*-(1*S*,3*R*)-ACPD, 300 μM. Values are mean ± SEM of one out of 5 independent experiments performed in quadruplicate. Statistical differences observed with basal: [a]$P < 0.05$, [b]$P < 0.001$; with KA + cyclo: [c]$P < 0.001$; with AMPA + cyclo: [d]$P < 0.001$.

mM EGTA (Table 3). Moreover, the Ca^{2+} ionophore, A23187, (5 μM) caused a slight increase in [^{3}H]IP_3 formation (194% of basal level), while coincubation with glutamate produced an additive response (785% of basal level) suggesting that they were acting through similar mechanisms (Table 3).

To further explore the route of extracellular Ca^{2+} influx in glutamate-induced [^{3}H]IP_3 formation, several Ca^{2+} channel blockers were tested. The inorganic Ca^{2+} channel blocker, $CdCl_2$, at 1 mM partially prevented glutamate-stimulated [^{3}H]IP_3 accumulation (40% of glutamate stimulation) (Table 4). Furthermore, two L-type voltage-dependent Ca^{2+} channel blockers, diltiazem and nifedipine, both at 50 μM significantly reduced glutamate-induced [^{3}H]IP_3 formation by 53% and 56%, respectively. In addition, depolarization induced by high extracellular K^+ (50 mM) resulted in an increased production of [^{3}H]IP_3 (Table 4). The effect of elevated extracellular K^+ was completely inhibited by 1 mM $CdCl_2$. These results strongly suggest that extracellular Ca^{2+} plays an important role in phospholipase C activation by glutamate, and that L-type voltage-dependent Ca^{2+} channels participate in transmembrane Ca^{2+} influx.

3.5. Effect of cyclothiazide on AMPA or kainate-stimulated PI hydrolysis

An important characteristic of AMPA receptors is their rapid desensitization in the presence of agonists. Desensitization can be blocked with the allosteric modulator cyclothiazide, which has been shown to potentiate the pharmacological responses produced by activation of AMPA receptors (Patneau et al., 1994; Yamada and Tang, 1993). Thus, oligodendrocyte progenitors were pretreated with cyclothiazide for 5 min prior to stimulation with AMPA or kainate. Cyclothiazide alone had no effect on [^{3}H]IP_3 production (data not shown), but strongly potentiated the effects of AMPA and kainate. Cyclothiazide, at 50 μM, caused a 3.1-fold and a 2.8-fold increase in AMPA-in-

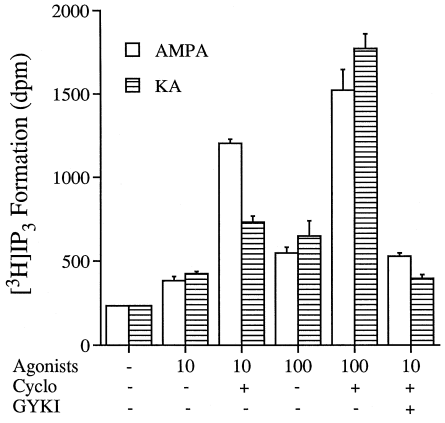

Fig. 5. Effects of cyclothiazide (Cyclo) on AMPA- and kainate (KA)-stimulated [^{3}H]IP_3 formation in oligodendrocyte progenitors. Cells were exposed to AMPA or kainate, either alone or in the presence of cyclothiazide (50 μM), or cyclothiazide (50 μM) + GYKI 52466 (50 μM). Data are mean ± SEM of two independent experiments performed in triplicate. Statistical significances are indicated: 100 μM AMPA, 100 μM kainate ($P < 0.05$); 10 μM AMPA + cyclo, 100 μM AMPA + cyclo, 10 μM kainate + cyclo, 100 μM kainate + cyclo ($P < 0.001$) versus basal; 10 μM AMPA + cyclo + GYKI versus 10 μM AMPA + cyclo ($P < 0.001$); 10 μM kainate + cyclo + GYKI versus 10 μM kainate + cyclo ($P < 0.05$).

duced [^{3}H]IP_3 formation at AMPA concentrations of 10 and 100 μM, respectively (Fig. 5). Similarly, the effects of kainate at 10 and 100 μM were potentiated by 1.7-fold and 2.7-fold, respectively. Both AMPA- and kainate-stimulated [^{3}H]IP_3 increases obtained in the presence of cyclothiazide were strongly inhibited by the AMPA receptor antagonist, GYKI 52466, at 50 μM suggesting that AMPA- and kainate-induced [^{3}H]IP_3 formation in oligodendrocyte progenitors is mediated through AMPA receptors.

3.6. Effects of cyclothiazide on AMPA- and kainate-induced $^{45}Ca^{2+}$ uptake

The substantial increase in [^{3}H]IP_3 accumulation brought about by blockade of AMPA receptor desensitization with cyclothiazide also led to a pronounced increase in AMPA- and kainate-induced $^{45}Ca^{2+}$ uptake (Table 5). Under our experimental conditions, 5 min of stimulation with kainate (100 μM) alone produced a statistically significant increase in $^{45}Ca^{2+}$ uptake (230% of basal level), while the increase produced by 100 μM AMPA was smaller (152% of basal level). Cyclothiazide (50 μM) potentiated the effect of AMPA (100 μM) by 13.7-fold and the effect of kainate (100 μM) by 6.6-fold. Both CNQX (10 μM) and GYKI 52466 (50 μM) antagonized the combined action of the agonists plus cyclothiazide on $^{45}Ca^{2+}$ uptake. The possible involvement of NMDA and metabotropic receptors-mediated $^{45}Ca^{2+}$ uptake was excluded since both NMDA and *trans*-(1*S*,3*R*)-ACPD at 300 μM did not stimulate $^{45}Ca^{2+}$ uptake into the cells (Table 5). These results indicate that $^{45}Ca^{2+}$ uptake evoked by both AMPA and kainate is mediated through AMPA receptors in oligodendrocyte progenitors.

Table 6
Effects of calcium channel blockers on kainate and cyclothiazide-induced $^{45}Ca^{2+}$-uptake in oligodendrocyte progenitors

Treatment	$^{45}Ca^{2+}$ uptake (nmol/min per mg protein)	% of maximal KA + cyclo uptake
Basal	5.2 ± 0.3	—
Diltiazem (100 μM)	4.9 ± 0.4	—
Nifedipine (100 μM)	4.5 ± 0.3	—
KA + cyclo	71.9 ± 6.0	100
KA + cyclo + dil (10 μM)	63.1 ± 5.0[a]	87
KA + cyclo + dil (100 μM)	46.0 ± 2.5[b]	61
KA + cyclo + nif (10 μM)	46.3 ± 2.5[b]	61
KA + cyclo + nif (100 μM)	28.0 ± 2.2[b]	34
Basal	7.0 ± 0.6	—
$CdCl_2$ (100 μM)	9.6 ± 0.9	—
KA + cyclo	31.6 ± 1.7	100
KA + cyclo + $CdCl_2$	21.3 ± 0.8[a]	67

Progenitors were preincubated with diltiazem (dil), nifedipine (nif), $CdCl_2$, followed by KA (100 μM) plus cyclothiazide (50 μM) stimulation. Values are mean ± SEM of one out of 4 independent experiments performed in quadruplicate. Statistical differences observed with KA + cyclo alone: [a]$P < 0.05$, [b]$P < 0.001$.

Table 7
Effects of extracellular Na^+ removal on kainate and cyclothiazide-stimulated $^{45}Ca^{2+}$-uptake in oligodendrocyte progenitors

Treatment	$^{45}Ca^{2+}$ uptake (nmol/min per mg protein)	% of basal uptake
Basal	7.0 ± 0.6	100
Benzamil (50 μM)	10.9 ± 0.7	156
KA + cyclo	31.6 ± 1.7	451
KA + cyclo + benzamil	13.3 ± 0.4[a]	190
Basal (Na^+-free)	9.4 ± 0.4	134
KA + cyclo (Na^+-free)	48.7 ± 2.2[b]	696
Basal	7.0 ± 0.2	100
DCB (25 μM)	7.1 ± 0.8	101
KA + cyclo	40.3 ± 1.4	576
KA + cyclo + DCB	19.7 ± 0.5[a]	281

Cells were pretreated with benzamil and 3,4-dichlorobenzamil (DCB) followed by KA (100 μM) and cyclothiazide (25 μM) stimulation. Values are mean ± SEM of one out of 2 independent experiments performed in quadruplicate. Statistical differences observed with KA + cyclo: [a]$P < 0.001$; with basal (Na^+-free medium): [b]$P < 0.001$.

3.7. Role of voltage-dependent Ca^{2+} channels and the Na^+/Ca^{2+} exchanger in kainate-induced $^{45}Ca^{2+}$ uptake

To gain further insight into the mechanism of Ca^{2+} influx, the potential involvement of voltage-dependent Ca^{2+} channels and the Na^+/Ca^{2+} exchanger was investigated. Oligodendrocyte progenitors were preincubated with 10 μM or 100 μM of the L-type voltage-dependent Ca^{2+} channel inhibitors, diltiazem and nifedipine, prior to stimulation with kainate (100 μM) and cyclothiazide (50 μM). At equimolar concentrations (100 μM), nifedipine was more potent, inhibiting $^{45}Ca^{2+}$ uptake induced by kainate and cyclothiazide by 65% compared to the 46% reduction obtained with diltiazem (Table 6). Under similar conditions, pretreatment with a low concentration of $CdCl_2$ (100 μM) produced a significant reduction (40%) in $^{45}Ca^{2+}$ uptake (Table 6). Since a reversal of the Na^+/Ca^{2+} exchanger has been shown to mediate influx of extracellular Ca^{2+} in astroglial cells as intracellular Na^+ levels increased after depolarization (Goldman et al., 1994; Takuma et al., 1994; Holgado and Beauge, 1995), activation of AMPA receptors could subsequently lead to activation of the Na^+/Ca^{2+} exchanger. This possibility was tested by pretreating oligodendrocyte progenitors with benzamil, an inhibitor of the Na^+/Ca^{2+} exchanger, prior to stimulation with kainate plus cyclothiazide. Benzamil (50 μM) alone had an insignificant effect on the basal level, but reduced the effect of kainate and cyclothiazide by more than 50% (Table 7). Similar reduction of kainate plus cyclothiazide-induced $^{45}Ca^{2+}$ uptake was achieved by a potent Na^+/Ca^{2+} exchanger inhibitor, 3,4-dichlorobenzamil (Table 7). Another strategy to demonstrate a contribution by the Na^+/Ca^{2+} exchanger to kainate-induced $^{45}Ca^{2+}$ uptake is to replace extracellular Na^+ with isosmotic *N*-methyl-D-glucamine, a condition favoring rever-

sal of the Na^+/Ca^{2+} exchanger. Although basal $^{45}Ca^{2+}$ uptake was not significantly altered, the kainate- and cyclothiazide-evoked increase in $^{45}Ca^{2+}$ uptake was potentiated in the absence of extracellular Na^+ (Table 7). Our results suggest that oligodendrocyte progenitors possess a Na^+/Ca^{2+} exchanger which may contribute to kainate-stimulated $^{45}Ca^{2+}$ uptake, although further experiments are required to confirm these findings.

4. Discussion

In this paper we report that (1) activation of AMPA receptors in cultured oligodendrocyte progenitors leads to the formation of [^{3}H]inositol phosphates (IPs) in a time- and concentration-dependent manner, (2) the increases in [^{3}H]IPs are dependent on extracellular Ca^{2+} influx, (3) cyclothiazide strongly potentiates the AMPA- and kainate-stimulated formation of [^{3}H]IPs as well as $^{45}Ca^{2+}$ uptake and (4) both voltage-dependent Ca^{2+} channels and the Na^+/Ca^{2+} exchanger are implicated in AMPA receptor-mediated $^{45}Ca^{2+}$ influx and the subsequent increase in [^{3}H]IPs formation.

Several lines of evidence have shown that in cells of the oligodendrocyte lineage glutamate and selective agonists elicit a variety of responses, including membrane depolarization (Wyllie et al., 1991), an increase in $[Ca^{2+}]_i$ (Borges et al., 1994; Holzwarth et al., 1994; Pende et al., 1994; Holtzclaw et al., 1995; Meucci et al., 1996), transient blockade of a delayed rectifying K^+ current (Borges et al., 1994; Berger, 1995; Borges and Kettenmann, 1995), decrease in cellular proliferation (Liu and Almazan, 1995; Gallo et al., 1996) as well as changes in gene expression (Gallo et al., 1994b; Pende et al., 1994; Liu and Almazan, 1995). These biological responses result mainly from the activation of glutamate receptors of the AMPA/kainate subtypes. We have previously reported that induction of c-*fos* by AMPA/kainate receptors was dependent on extracellular Ca^{2+} and downstream activation of protein kinase C which could be activated by the diacylglycerol generated by phospholipase C activation (Liu and Almazan, 1995). In the present study, we show that receptor-mediated hydrolysis of phosphoinositides and subsequent generation of second messengers can provide a signalling pathway leading to increased gene expression. Glutamate and the agonists AMPA and kainate stimulated a rapid breakdown of phosphoinositides in a concentration-dependent manner. Kainate was the most efficacious activator of phosphoinositide hydrolysis. This finding is in agreement with that of Holtzclaw et al. (1995) showing that kainate is the most effective agonist for $[Ca^{2+}]_i$ responses. Since oligodendrocyte progenitors have been shown to express AMPA- and kainate-preferring receptors (Patneau et al., 1994) and two recent reports have also presented evidence for the presence of NMDA and metabotropic receptors mediating $[Ca^{2+}]_i$ increases in restricted populations of oligodendrocyte progenitors (Wang et al., 1996; Holtzclaw et al., 1995), we attempted to determine the receptor subtype implicated in phospholipase C activation. The accumulation of [^{3}H]IP_3 induced by glutamate was blocked by the AMPA/kainate receptor antagonist CNQX as well as by GYKI 52466. Although the benzodiazepine, GYKI 52466, is not an absolutely selective antagonist for AMPA receptors, in our experiments the concentration needed to completely prevent both AMPA- and kainate-mediated responses has been shown to selectively block depolarization of AMPA but not kainate receptors (Paternain et al., 1995). Moreover, in our experiments, the contribution of G-protein coupled metabotropic receptors was excluded since the agonist *trans*-(1*S*,3*R*)-ACPD did not induce [^{3}H]IP_3 formation and the antagonists L-AP3 and MCPG did not block the effect of glutamate. Similarly, the NMDA receptor antagonists MK-801 and CPP were ineffective in blocking the effect of glutamate. Taken together, our data suggest that glutamate-stimulated [^{3}H]IP_3 accumulation is mediated through ionotropic AMPA receptors, while NMDA, metabotropic and kainate receptors are not involved. Consistent with our observations, glutamate-stimulated Ca^{2+} transients are also mediated by AMPA receptors (Holtzclaw et al., 1995; Meucci et al., 1996) which are abundantly expressed in cells of the oligodendrocyte lineage (Jensen and Chiu, 1993; Gallo et al., 1994a,b; Holzwarth et al., 1994; Patneau et al., 1994; Yoshioka et al., 1995; Garcia-Barcina and Matute, 1996). In contrast, metabotropic receptors may only be expressed in a small population of oligodendrocyte progenitors from rat cortical cultures and varied according to their developmental stage or their culture conditions (Holtzclaw et al., 1995). Similarly, the expression of NMDA receptors appears to be restricted to oligodendrocyte progenitors from the neurohypophysis (Wang et al., 1996) and previous studies on cells derived from brain or optic nerve have also failed to detect functional responses (Barres et al., 1990; Wyllie et al., 1991; Fulton et al., 1992; Gallo et al., 1994a,b).

The biochemical mechanisms mediating phosphoinositide responses to glutamate agonists appear to be dependent on extracellular Ca^{2+}. Indeed, when extracellular Ca^{2+} was chelated by EGTA the glutamate response was abolished. Moreover, depolarizing concentrations of extracellular K^+ or the Ca^{2+} ionophore, A23187, alone were capable of eliciting [^{3}H]IP_3 accumulation, and the combination of ionophore and glutamate produced additive responses. These findings suggest that activation of AMPA receptors stimulates phosphoinositide hydrolysis in a Ca^{2+}-dependent manner. Since none of the known ionotropic glutamate receptors is directly linked to phospholipase C and phosphoinositide breakdown, this increase in [^{3}H]IP_3 formation must result from the Ca^{2+}-mediated activation of phospholipase C. Several authors have reported that activation of AMPA receptors causes a rapid and transient increase in $[Ca^{2+}]_i$ (Holzwarth et al., 1994;

Borges et al., 1994; Holtzclaw et al., 1995; Meucci et al., 1996). Thus, it appears likely that Ca^{2+} influx is the primary trigger for phospholipase C activation, as has been reported in other systems (Eberhard and Holz, 1988; Baird and Nahorski, 1990; Chandler and Crews, 1990). Since the three classes of mammalian phospholipase Cs (β, γ, δ) require calcium as a cofactor for enzymatic activity (for review, see Exton, 1996), the subtype implicated in glutamate-stimulated PI hydrolysis can not be directly inferred. Phospholipase Cβ and phospholipase Cγ are activated by G-protein-coupled or tyrosine kinase receptors and are respectively regulated by G-proteins or tyrosine phosphorylation. We have observed that the tyrosine kinase inhibitor genistein does not modify the response of phospholipase C to glutamate (unpublished results) suggesting that phospholipase Cγ is not involved. Phospholipase Cδ appears as a good candidate, however neither the receptors nor the transducers that are coupled to this isoform are known.

A characteristic feature of AMPA receptors is their remarkably rapid desensitization. Recent findings indicate that AMPA-induced receptor desensitization can be effectively blocked by cyclothiazide (Yamada and Tang, 1993; Patneau et al., 1994). This allosteric modulator has been shown to block the desensitizing currents evoked by AMPA and to potentiate the steady-state currents produced by kainate in glial cells of the oligodendrocyte lineage (Patneau et al., 1994; Berger, 1995). Similarly, the Ca^{2+} transients evoked by AMPA and kainate have been shown to be greatly potentiated by cyclothiazide in cortical oligodendrocyte progenitors and the CG-4 cell line (Pende et al., 1994; Holtzclaw et al., 1995). In the present study, cyclothiazide not only potentiated AMPA and kainate-stimulated phosphoinositide hydrolysis, but also enhanced the action of these agonists on $^{45}Ca^{2+}$ uptake. The fact that AMPA and kainate are capable of inducing $^{45}Ca^{2+}$ influx into progenitors in the presence of cyclothiazide further strengthens our hypothesis that subsequent phospholipase C activation is primarily mediated by Ca^{2+} influx. The effects of the agonists themselves, as well as the cyclothiazide-induced potentiation of agonist responses, were all blocked by the AMPA receptor antagonist, GYKI 52466, indicating that $^{45}Ca^{2+}$ uptake and subsequent $[^3H]IP_3$ accumulation were specifically mediated through AMPA receptors. The levels of $[^3H]IP_3$ formation and $^{45}Ca^{2+}$ uptake induced by agonists plus cyclothiazide were consistently higher than those effected by agonists alone. Thus, there appears to be a correlation between the magnitude of $^{45}Ca^{2+}$ influx and level of $[^3H]IP_3$ accumulation induced by AMPA receptor activation, further supporting the notion that Ca^{2+} influx is responsible for the phospholipase C activation that leads to $[^3H]IP_3$ accumulation.

Apart from receptor-gated channels, oligodendrocyte progenitors also have voltage-dependent Ca^{2+} channels (Borges et al., 1994; Kirischuk et al., 1995; Verkhratsky and Kettenmann, 1996), including the L-type (Williamson et al., 1997), which can be activated subsequently via glutamate-mediated depolarization (Borges et al., 1994). Potassium-induced depolarization activates voltage-dependent Ca^{2+} channels causing increases in intracellular Ca^{2+} (Kirchhoff and Kettenmann, 1992; Pende et al., 1994) and NGFI-A gene expression (Pende et al., 1994) in oligodendrocyte progenitors. Our results show that under similar experimental conditions, high concentrations of extracellular K^+ is sufficient to stimulate $[^3H]IP_3$ formation. Furthermore, diltiazem and nifedipine, inhibitors of L-type voltage-dependent Ca^{2+} channels, and the inorganic Ca^{2+} channel blocker $CaCl_2$ caused a significant reduction of the glutamate-mediated $[^3H]IP_3$ formation and $^{45}Ca^{2+}$ uptake. Our data are in agreement with that of Pende et al. (1994) showing that another voltage-dependent Ca^{2+} channel blocker, nimodipine, only partially inhibited kainate or glutamate-induced $[Ca^{2+}]_i$ increases. Since these drugs did not fully block phospholipase C activation and $^{45}Ca^{2+}$ uptake, even at higher concentrations, our results imply that at least a fraction of the Ca^{2+} must have passed though the receptor-gated channels. This view is supported by the observation that kainate-induced $[Ca^{2+}]_i$ increase was blocked by Joro spider toxin, which selectively blocks AMPA receptors that lack the GluR2 subunit and, therefore, exhibit a high Ca^{2+} permeability both in the CG-4 cell line and in oligodendrocyte progenitor preparations (Meucci et al., 1996). Interestingly, the sensitivity of AMPA receptors to this toxin was found to decrease as progenitors differentiated into oligodendrocytes while the proportion of the GluR2 subunit increased (Meucci et al., 1996). The conclusion that oligodendrocyte progenitors express Ca^{2+}-permeable AMPA receptors was also reached by Holzwarth et al. (1994), as kainate-mediated $[Ca^{2+}]_i$ increase was maintained when all external Na^+ was replaced with the nonpermeable cation, *N*-methyl-D-glucamine, an experimental condition which prevents depolarization. It should be noted, however, that reduction of the Na^+ electrochemical gradient by lowering the extracellular Na^+ concentration may alter the operation of the Na^+/Ca^{2+} exchanger and therefore cause an increase in $[Ca^{2+}]_i$ (Courtney et al., 1995). Kiedrowski et al. (1994) have reported that intracellular Na^+ increases occurring during and after glutamate application is an important factor which impairs the extrusion of Ca^{2+} by the Na^+/Ca^{2+} exchanger. In this manner, reversal of the Na^+/Ca^{2+} exchanger can be driven by increasing intracellular Na^+ as a result of kainate-evoked depolarization (Courtney et al., 1995) or by lowering extracellular Na^+ as has been reported in astrocytes (Goldman et al., 1994; Takuma et al., 1994; Holgado and Beauge, 1995; Golovina et al., 1996). To test the possibility that a component of kainate evoked $^{45}Ca^{2+}$ uptake and subsequent phospholipase C activation is dependent on Na^+/Ca^{2+} exchanger, we examined whether these responses are sensitive to benzamil and 3,4-dichlorobenzamil, relatively selective in-

hibitors of the Na^+/Ca^{2+} exchanger (Slaughter et al., 1988). The significant reduction afforded by benzamil and 3,4-dichlorobenzamil on $^{45}Ca^{2+}$ uptake evoked by kainate plus cyclothiazide might reflect Ca^{2+} import due to reversal of the Na^+/Ca^{2+} exchanger. Further evidence implicating the Na^+/Ca^{2+} exchanger was provided by experiments performed under extracellular Na^+-free conditions, which drive operation of the exchanger in the reverse mode. Thus, replacing Na^+ with isosmotic *N*-methyl-D-glucamine resulted in a large increase in kainate-induced $^{45}Ca^{2+}$ uptake while having no effect on unstimulated cells. Although the absolute contribution of the Na^+/Ca^{2+} exchanger must await more specific inhibitors, our findings suggest that one of the mechanisms implicated in AMPA receptors-induced Ca^{2+} uptake is the Na^+/Ca^{2+} exchanger operating in reverse mode as a consequence of simultaneous membrane depolarization and rise in intracellular Na^+. However, we cannot exclude the possibility that IP_3-induced release of Ca^{2+} from intracellular stores further contributes to sustaining phospholipase C activity by positive feedback.

In conclusion, AMPA receptor-mediated phospholipase C activation is a direct consequence of Ca^{2+} influx through receptor-gated channels, voltage-dependent Ca^{2+} channels and reversal of the Na^+/Ca^{2+} exchanger. Phospholipase C activation leads to generation of IP_3 and diacylglycerol, which in turn respectively mobilize Ca^{2+} from intracellular stores and activate protein kinase C. The latter two second messengers have been implicated in the downstream activation of mitogen-activated protein kinases (Pende et al., 1997; Almazan and Larocca, 1997; Liu et al., data not shown) and induction of the immediate early gene, c-*fos* (Liu and Almazan, 1995). Thus, phospholipase C activation and the resultant increase in IP_3 serves as an important link between AMPA receptor activation and gene expression. However, it remains to be determined whether this signal transduction pathway mediates the decrease in oligodendrocyte progenitor proliferation or has other biological effects.

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