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Excitatory amino acid receptor-stimulated phosphoinositide turnover in primary cerebrocortical cultures

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1 Characterization of excitatory amino acid-induced accumulation of [^{3}H]-phosphoinositides was carried out in primary cerebrocortical cultures isolated from foetal rats.

2 All of the excitatory amino acid receptor agonists examined caused concentration-dependent enhancement of phosphoinositide (PI) formation. The most potent excitatory amino acid receptor agonists were quisqualate, (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3**R**)-ACPD), ibotenate and glutamate with mean EC_{50} values of $0.9 \pm 0.4\,\mu$M, $15 \pm 5\,\mu$M, $15 \pm 3\,\mu$M and $41 \pm 8\,\mu$M respectively.

3 The selective ionotropic receptor antagonists kynurenic acid (1 mM), 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX, 10 μM) and ($\pm$)-4-(3-phosphonopropyl)-2 piperazinecarboxylic acid (CPP, 100 μM), failed to block responses to quisqualate, (1S,3**R**)-ACPD or glutamate. D,L-2-Amino-3-phosphonopropionate (D,L-AP3) did not block 1S,3**R**-ACPD or quisqualate-induced PI turnover, but had an additive effect with quisqualate or (1S,3**R**)-ACPD.

4 Exposure of cultures to agonists in the absence of added extracellular calcium reduced the maximal quisqualate response by approximately 45%, revealing a two-component concentration-response curve. Concentration-response curves to ibotenate and glutamate became flattened by omission of extracellular calcium, whereas (1S,3**R**)-ACPD-stimulated PI turnover was unaffected.

5 Pretreatment of cultures with pertussis toxin markedly inhibited PI responses evoked by (1S,3**R**)-ACPD.

6 These results suggest that excitatory amino acid-stimulated PI turnover in cerebrocortical cultures is independent of ionotropic receptor activation and is mediated via specific G-protein-linked metabotropic receptors. The partial dependence of the responses to quisqualate, ibotenate and glutamate on the presence of extracellular calcium suggests that the effects of these agonists may be mediated by more than one receptor subtype.

Keywords: Glutamate metabotropic receptors; (1S,3**R**)-ACPD; quisqualate; ibotenate

Introduction

The amino acid, glutamate, is a major excitatory neurotransmitter in the mammalian central nervous system. Receptors for glutamate have been divided into four major classes including N-methyl-D-aspartate (NMDA), kainate, α-amino-hydroxy-5-methyl -4-isoxazolepropionic acid (AMPA), and metabotropic or (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3**R**)-ACPD) receptors (Monaghan *et al.*, 1989; Watkins *et al.*, 1990). Stimulation of NMDA, AMPA and kainate receptors directly activates cation channels allowing the entry of Na^+ or Ca^{2+}, whereas excitatory amino acids acting via metabotropic receptors stimulate phosphoinositide turnover (see Schoepp *et al.*, 1990a).

Evidence from a large number of studies supports the involvement of excitatory amino acids and their receptors in a range of important processes including the neuronal cell injury associated with a variety of neurological disorders (see Meldrum & Garthwaite, 1990; Farooqui & Horrocks, 1991). Thus, characterization of the pharmacology of these receptors is essential for developing a greater understanding of the mechanisms mediating the effects of excitatory amino acids within the CNS.

Primary cerebrocortical cultures have been used extensively to study the excitotoxicity of excitatory amino acids and as a model system for hypoxia and hypoglycemia-induced cell death (see Choi & Rothman, 1990). Cerebrocortical cultures also provide a useful preparation for the study of the responses of isolated mammalian CNS neurones *in vitro*.

Although quisqualate, ibotenate and glutamate are potent agonists at the metabotropic receptor, complex interactions between ionotropic and metabotropic receptor-mediated responses may exist (Baudry *et al.*, 1986; Nicoletti *et al.*, 1986a; Baird & Nahorski, 1991; Morari *et al.*, 1991). Recently (1S,3**R**)-ACPD has been identified as a selective agonist for the metabotropic glutamate receptor (Palmer *et al.*, 1989). Characterization of excitatory amino acid stimulated phosphoinositide turnover in primary cerebrocortical cultures was examined using (1S,3**R**)-ACPD as a selective metabotropic receptor agonist, and 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F) quinoxaline (NBQX), ($\pm$)-4-(3-phosphonopropyl)-2-piperazinecarboxylic acid (CPP) and kynurenate as selective antagonists for glutamate ionotropic receptors. Dependency of the response on extracellular calcium and the possible involvement of a G-protein were also studied.

Methods

Cerebrocortical cultures

Primary cultures were prepared from foetal rat cerebral cortex as previously described (Marcoux *et al.*, 1990). Briefly, cortical hemispheres were sectioned from foetal rat brain and dissociated in Hank's Balanced salt solution (HBSS) containing 0.1% trypsin. The cells were then triturated and suspended in 50% Dulbecco's Modified Eagle's Medium (DME) and 50% Ham's Nutrient Mixture F-12 (F-12) supplemented with 10% horse serum and 6% foetal calf serum, and were plated out on 24 well plates at an approximate density of

200,000 cells cm^{-2}. After 4 days incubation additions of 5-fluoro-deoxyuridine (15 $\mu g\,ml^{-1}$) and uridine (35 $\mu g\,ml^{-1}$) were made to inhibit further division of non-neuronal cells (Ransom *et al.*, 1977). Feeding were performed as necessary with DME/F-12 with 10% horse serum.

Purified glial cultures were made by removing media from a plated well and exposing mixed culture preparations to air for 10 s. This procedure produced cultures which stained for glial fibrillary acidic protein with no residual neuronal cells (Marcoux *et al.*, 1989).

Comparisons between conditions within experiments were made by use of sister cultures from a single plating, thus maintaining matched cell densities and proportions of neuronal to non-neuronal cell types.

Measurement of [³H]-inositol phosphate accumulation

After 15 days *in vitro* the medium was aspirated and cultures were labelled by adding 0.5 ml DME/F-12 with 10% horse serum containing 1 $\mu Ci\,ml^{-1}$ [³H]-myo-inositol to each well. After 48 h, the medium was aspirated, and the cells washed twice with HBSS containing 10 mM LiCl (HBSS/LiCl). Cultures were then incubated at 37°C for 15 min in HBSS/LiCl to block inositol phosphate degradation (Allison *et al.*, 1976; Berridge *et al.*, 1982). Agonist exposures of 20 min duration were performed at 37°C, and were terminated by replacing the incubation medium with 0.5 ml ice-cold 5% trichloracetic acid (TCA). Each TCA extract was then applied to mini-columns containing Dowex-formate anion exchange resin. To remove free residual [³H]-inositol, columns were washed four times with 3 ml 5 mM inositol. Total [³H]-inositol phosphates were then eluted with two times 2 ml of 1 M ammonium formate containing 0.1 M formic acid. All fractions were collected, added to 15 ml of scintillation cocktail (Beckman Ready Gel), and counted for tritium on a scintillation spectrometer (Packard).

Some of the experiments in the present study were carried out in the absence of added calcium. In this case, cultures were washed twice with calcium-free HBSS/LiCl, and incubated for 15 min in this medium prior to agonist exposure. The same protocol was used for experiments carried out in the absence of added magnesium.

In those experiments involving pertussis toxin, cultures were incubated for 12 h before agonist exposure in media containing 1 $\mu g\,ml^{-1}$ of the toxin.

Data analysis

Agonist-stimulated accumulation of [³H]-phosphoinositides was expressed as a percentage of basal control as determined from cultures exposed to vehicle alone. Mean values are expressed ± s.e.mean. Statistical analysis of differences between means was carried out with Student's *t*-test (two-tailed) and the null hypothesis rejected at $P < 0.05$. EC_{50} and maximum response values for agonists were determined from logistic sigmoid plots generated by a curve fitting programme (Sigmaplot). Sigmoid plots were fitted to mean concentration-effect curves using the equation $f = (a-d)/[1 + (x/c)\ b]$, where a represents the maximum response, b the slope, c the EC_{50} and d the minimum response.

Materials

Myo-[2-³H]-inositol: water/ethanol (9:1) solution was obtained from Amersham, Dowex AG1-X8 Anion exchange resin 100–200 mesh formate form was supplied by Biorad, and (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylate ((1S,3**R**)-ACPD) was from Tocris Neuramin Ltd. (2,3-Dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX) and (±)-4-(3-phosphonopropyl)-2 piperazinecarboxylic acid (CPP) were obtained from Parke-Davis. Glutamic acid, quisqualic acid, kainic acid, ibotenic acid, N-methyl-D-aspartic acid (NMDA), α-amino-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) and kynurenic acid were obtained from Sigma. Pertussis toxin (*Bordetella pertussis*) was purchased from Calbiochem. Drugs were dissolved in HBSS except NBQX which was dissolved in dimethyl sulphoxide (DMSO) and diluted in HBSS to produce a final concentration of <0.1% DMSO.

Results

Quisqualate, ibotenate, (1S,3R)-ACPD and glutamate caused concentration-dependent enhancement of [³H]-phosphoinositide accumulation with EC_{50} values of 0.9 ± 0.4 μM, 15 ± 3 μM, 15 ± 5 μM and 41 ± 8 μM respectively. Results are summarized in Figure 1. Concentration-response curves to (1S, 3**R**)-ACPD, ibotenate and glutamate had similar mean maximal values. The mean maximal response to quisqualate was higher than that of the other agonists. Concentration-response curves for quisqualate were often bell-shaped with concentrations above 3–10 μM producing progressively smaller responses. Only modest increases in phosphoinositide formation were evoked by NMDA (109 ± 3% of basal at 1 mM, $n = 9$), AMPA (131 ± 4% of basal at 1 mM, $n = 6$) or kainate (128 ± 5% of basal at 1 mM, $n = 6$), and estimated EC_{50} values were >1 mM.

In experiments with purified glial cell cultures, [³H]-phosphoinositide accumulation from exposure to quisqualate ($n = 6$) or (1S,3**R**)-ACPD ($n = 6$), was increased significantly ($P < 0.05$, Student's *t* test) above control levels. However, stimulated levels of [³H]-phosphoinositide in glial cultures did not exceed those obtained for basal turnover in mixed neuronal sister cultures derived from the same plating.

Effects of excitatory amino acid receptor antagonists

The selective ionotropic receptor antagonists CPP at 100 μM, NBQX at 10 μM, or kynurenate at 1 mM, concentrations sufficient to block maximal NMDA or AMPA receptor-mediated effects in neuronal preparations *in vitro* (Bertolino *et al.*, 1989; Lodge *et al.*, 1990; Benveniste & Mayer, 1991), had no effect on the concentration-response curve for (1S, 3**R**)-ACPD. Concentration-response curves for quisqualate or

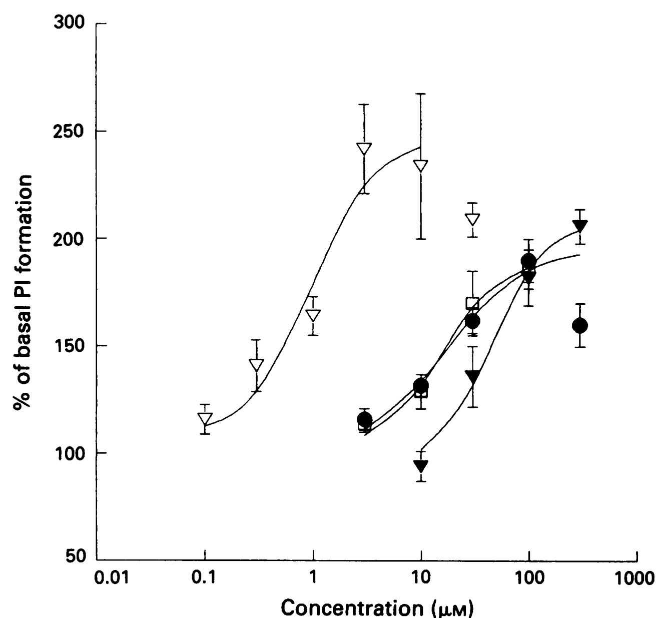


Figure 1 Concentration-dependent phosphoinositide (PI) formation in cerebrocortical cultures in response to quisqualate (▽, $n = 21$), (1S,3**R**)-ACPD (●, $n = 30$), ibotenate (□, $n = 9$), and glutamate (▼, $n = 6$). Values represent means ± s.e.mean of n determinations for each data point taken from between 2 and 10 separate experiments. Basal phosphoinositide accumulation was 738 ± 71 c.p.m.

Table 1 EC_{50} values for quisqualate, (1S,3R)-ACPD and glutamate in the absence and presence of various antagonists

Drugs	EC_{50} (μM)	n
Quisqualate	0.08 ± 0.02	9
Quisqualate + 100 μM CPP	0.18 ± 0.05	9
Quisqualate	0.05 ± 0.04	15
Quisqualate + 10 μM NBQX	0.1 ± 0.01	15
(1S,3R)-ACPD	11 ± 1.6	9
(1S,3R)-ACPD + 100 μM CPP	6 ± 0.8	9
(1S,3R)-ACPD	19 ± 3	6
(1S,3R)-ACPD + 10 μM NBQX	20 ± 0.4	6
(1S,3R)-ACPD	18 ± 8	6
(1S,3R)-ACPD + 1 mM kynurenate	12 ± 2	6
Glutamate	32 ± 3	9
Glutamate + 100 μM CPP	19 ± 1	9
Glutamate	25 ± 2	6
Glutamate + 10 μM NBQX	26 ± 1	6

Values represent the mean ± s.e.mean of n determinations for each data point taken from between 2 and 5 separate experiments. Comparisons between EC_{50} values were made between sister cultures in each experiment. For abbreviations, see text.

glutamate were not significantly altered by CPP (100 μM) or NBQX (10–100 μM). Results are summarized in Table 1. D,L-AP3, the reported glutamate metabotropic receptor antagonist, failed to inhibit (1S,3R)-ACPD or quisqualate-induced phosphoinositide turnover at concentrations up to 1 mM. AP3 alone at a concentration of 1 mM displayed agonist activity and had an additive effect with responses to (1S,3R)-ACPD and quisqualate (see Figure 2).

Calcium-dependence of phosphoinositide response

As illustrated in Figure 3, omission of calcium from the HBSS during agonist exposure caused a flattening of the concentration-response curves for ibotenate and glutamate, but had no effect on responses to (1S,3R)-ACPD. In approximately half of our experiments the quisqualate concentration-response curve appeared biphasic in nature. In these experiments the lower portion of the curve was unaffected by the absence of extracellular calcium, but the upper portion was abolished in medium lacking calcium. The remaining lower section of the curve had a mean maximum value comparable with that for (1S,3R)-ACPD.

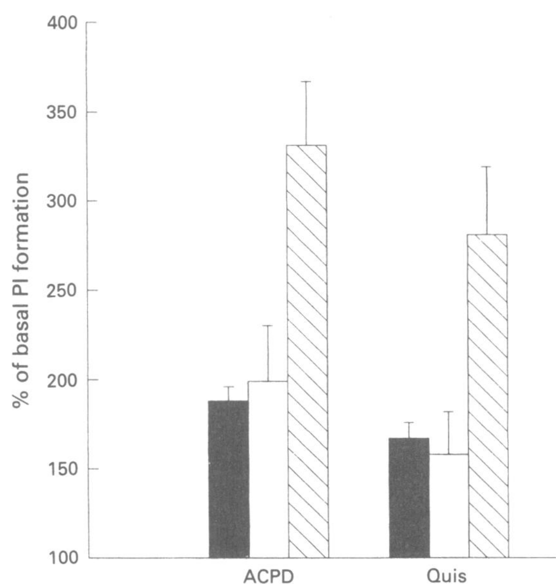


Figure 2 Phosphoinositide formation in response to (1S,3R)-ACPD (100 μM), quisqualate (Quis, 3 μM), or D,L-AP3 (1 mM). Columns represent either (1S,3R)-ACPD or quisqualate alone (solid columns), AP3 alone (open columns), or AP3 in combination with (1S,3R)-ACPD or quisqualate (hatched columns). Values are mean ± s.e.mean from 2 separate experiments with $n = 6$. Comparisons between conditions were made in sister cultures. Basal phosphoinositide formation was 640 ± 15 c.p.m. For abbreviations, see text.

Table 2 EC_{50} values for quisqualate, (1S,3R)-ACPD and glutamate in the presence and absence of magnesium in the exposure medium

Agonist	EC_{50} (μM) *HBSS*	*Mg^{2+}-free*	n
Quisqualate	1.4 ± 0.9	0.5 ± 0.05	21
1S,3R-ACPD	15 ± 5	18 ± 4	30
Glutamate	41 ± 8	52 ± 14	6

Values represent the mean ± s.e.mean of n determinations for each data point taken from between 2 and 10 separate experiments. Comparisons between EC_{50} values were made between sister cultures in each experiment.

Omission of magnesium from the extracellular medium, thus removing the voltage-dependent Mg^{2+} blockade of the NMDA receptor associated ion channel, resulted in an enhancement of the phosphoinositide accumulation evoked by NMDA. However, omission of calcium as well as magnesium from the exposure medium abolished this response (see Figure 4). Responses to glutamate, quisqualate or (1S, 3R)-ACPD were not affected by magnesium-free medium. EC_{50} values obtained in the presence and absence of magnesium are shown in Table 2.

Effect of ionotropic receptor activation on the metabotropic response

The effects of selective activation of NMDA or AMPA receptors on (1S,3R)-ACPD-induced phosphoinositide accumulation were examined to determine whether any interaction occurs between ionotropic receptor-mediated and metabotropic receptor-stimulated phosphoinositide formation. As summarized in Figure 5, when NMDA or AMPA, at concentrations which alone evoked increases in phosphoinositide accumulation, were combined with a maximally effective concentration of (1S,3R)-ACPD, the response was no different from that caused by (1S,3R)-ACPD alone.

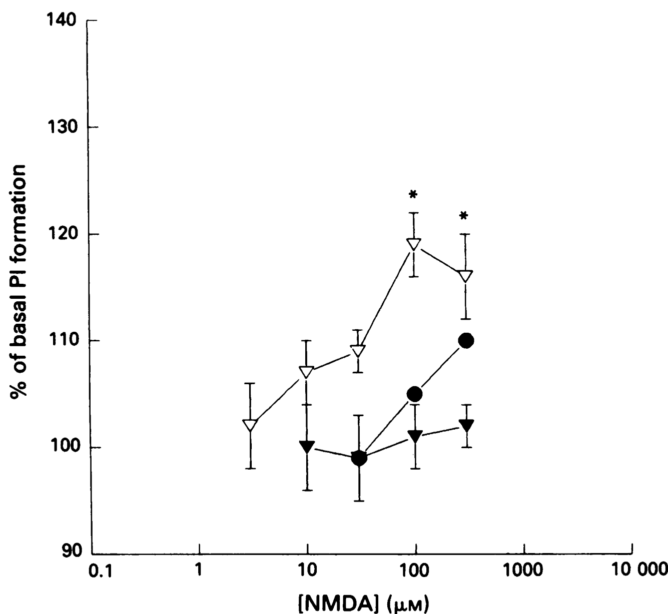
Sensitivity of phosphoinositide response to pertussis toxin pretreatment

Pre-incubation of cultures in pertussis toxin for 12 h before agonist exposure significantly reduced (1S,3R)-ACPD-stimulated [^{3}H]-phosphoinositide accumulation (Figure 6). Pertussis toxin pretreatment had no effect on basal phosphoinositide formation.

Discussion

The following rank-order of potency for excitatory amino acid receptor agonist-stimulated accumulation of [^{3}H]-phosphoinositides was obtained: quisqualate > (1S,3R)-ACPD = ibotenate > glutamate >> AMPA = kainate > NMDA. Although absolute potencies vary across neuronal preparations, this rank-order corresponds well with that described previously by others (Schoepp & Johnson, 1989; Schoepp *et al.*, 1990b; Watkins *et al.*, 1990; Masu *et al.*, 1991). Agonist-stimulated inositol phosphate formation was not inhibited by the ionotropic antagonists kynurenate, CPP or NBQX, suggesting that these responses are mediated by distinct glutamate metabotropic receptors, and are independent of ionotropic receptor activation. Similar findings have been

Figure 3 Phosphoinositide formation in cerebrocortical cultures in the presence (●) and absence (▽) of added extracellular calcium in response to (1S,3R)-ACPD ($n = 12$), quisqualate (Quis, $n = 12$), ibotenate (Ibo, $n = 6$) and glutamate (Glu, $n = 6$). Values represent the mean ± s.e.mean from n determinations for each data point taken from between 2 and 4 separate experiments. Values for nominally calcium-free media significantly less than those obtained with standard HBSS are shown by * when $P < 0.05$ (Student's t test). Basal phosphoinositide accumulation was 735 ± 16 c.p.m. and 561 ± 110 c.p.m. in the presence and absence of added extracellular calcium respectively.



reported for metabotropic quisqualate or (1S,3R)-ACPD responses in other neuronal preparations (Nicoletti *et al.*, 1986b; Schoepp & Johnson, 1988; Palmer *et al.*, 1989; Furuya *et al.*, 1989). Stimulated phosphoinositide accumulation in purified glial cultures was lower than that for basal turnover in mixed cultures suggesting that the responses described in this study are associated primarily with neuronal cell populations.

In the absence of added calcium, a decrease in basal and (1S,3R)-ACPD- stimulated phosphoinositide accumulation was observed. However, the percentage increase above basal phosphoinositide turnover caused by (1S,3R)-ACPD was unaltered. In striatal slices (Doble & Perrier, 1989), synap-

Figure 4 Effects of extracellular magnesium and calcium on phosphoinositide formation in response to N-methyl-D-aspartate (NMDA). Concentration-response curves are shown for cultures exposed to normal medium (●), medium lacking magnesium (▽), or medium lacking magnesium and calcium (▼). Values are mean ± s.e.mean from 3 separate experiments with $n = 9$ for each data point. Values significantly greater than basal phosphoinositide formation are shown by * when $P < 0.05$ (Student's t test). Basal phosphoinositide accumulation was 727 ± 60 c.p.m. for normal HBSS and 721 ± 41 c.p.m. for magnesium-free medium.

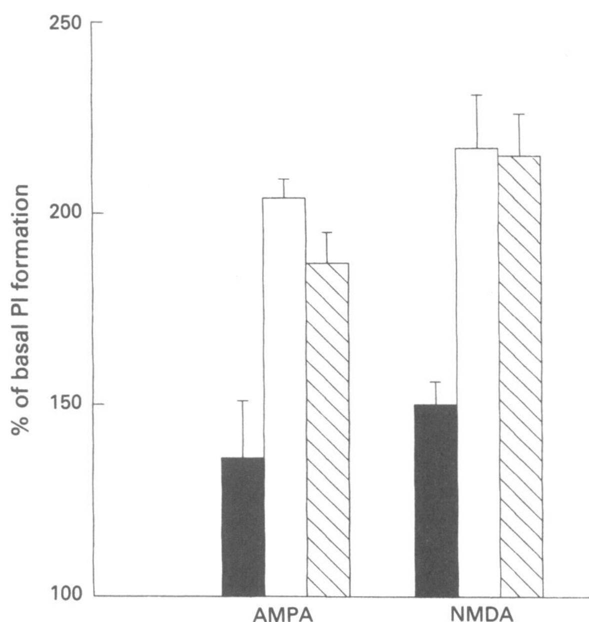


Figure 5 Effect of AMPA (300 μM) or NMDA (100 μM) on phosphoinositide (PI) formation stimulated by 100 μM (1S,3R)-ACPD. Columns represent the effects of AMPA or NMDA alone (solid columns), (1S,3R)-ACPD alone (open columns), or a combined exposure to (1S,3R)-ACPD with AMPA or NMDA (hatched columns). Exposures were performed using magnesium-free HBSS. Values are mean ± s.e.mean from 2 separate experiments with $n = 6$. Basal phosphoinositide accumulation was 819 ± 42 c.p.m. For abbreviations, see text.

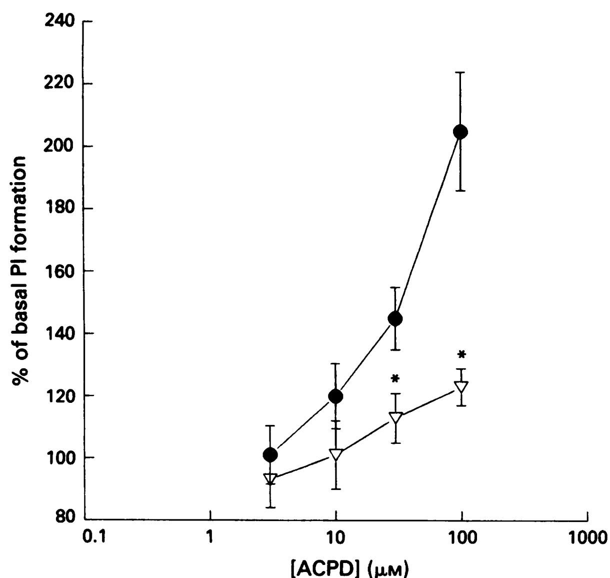


Figure 6 Effect of pertussis toxin pretreatment on phosphoinositide formation in response to (1S,3R)-ACPD. Concentration-response curves are shown for untreated cultures (●) and those exposed to pertussis toxin (PTX) (∇) for 12 h before agonist exposure. Values are mean ± s.e.mean from 3 separate experiments with $n = 9$ for each data point. Values for (1S,3R)-ACPD-stimulated phosphoinositide formation in PTX pretreated cultures significantly less than those obtained in control cultures are shown by * when $P < 0.05$ (Student's t test). Basal phosphoinositide accumulation was 583 ± 62 c.p.m. for control cultures and 631 ± 57 c.p.m. for pertussis pretreated cultures.

toneurosomes (Recasens *et al.*, 1987), and cerebellar granule cells (Nicoletti *et al.*, 1986c) similar results were obtained for quisqualate, ibotenate or glutamate. Reduced phosphoinositide accumulation probably reflects the calcium-dependency of phospholipase C activation, but the extent of stimulation by agonists is relatively calcium-independent (Bennet & Crooke, 1987; Berridge, 1987; Katan & Parker, 1987; Renard *et al.*, 1987). Our results suggest that (1S,3R)-ACPD-stimulated phosphoinositide metabolism is not dependent on influx of extracellular calcium and is mediated by a direct action rather than through calcium-dependent release of other neuroactive substances. However, the partial calcium-dependence of quisqualate, ibotenate and glutamate responses suggests these agonists may activate phosphoinositide metabolism via more than one mechanism.

Variations in the potency of quisqualate at the metabotropic receptor have been reported in different preparations, and may result from the presence of multiple metabotropic receptor subtypes. Evidence for multiple subtypes of metabotropic glutamate receptors also comes from cloning and expression of a family of metabotropic receptors (Tanabe *et al.*, 1992). In cortical neurones (Patel *et al.*, 1990), synaptoneurosomes (Recasens *et al.*, 1987; 1988; Guiramand *et al.*, 1989; Nakagawa *et al.*, 1990), striatal slices (Doble & Perrier, 1989) or retinal slices (Osborne, 1990), EC_{50} values for quisqualate range from 0.03–0.26 μM. However, in slices from hippocampus (Palmer *et al.*, 1988; Schoepp & Johnson, 1988; 1989), cerebellum (Blackstone *et al.*, 1989) or cerebral cortex (Godfrey *et al.*, 1988; Godfrey & Taghavi, 1990; Schoepp *et al.*, 1990b; Schoepp & Hillman, 1990), EC_{50} values range from 2–50 μM. Such variations in EC_{50} values are not reported for (1S,3R)-ACPD, suggesting that this agonist may act more selectively at a particular metabotropic receptor.

In the present study, EC_{50} values for quisqualate varied between experiments depending on the presence or absence of the calcium-sensitive portion of the concentration-response curve. Quisqualate was approximately ten fold less potent with regard to the calcium-sensitive response. This may correspond to the higher EC_{50} values seen in some of the preparations described above. The inconsistent nature and bell-shaped appearance of the upper section of the concentration-response curve suggests that this response may be susceptible to desensitization, responses becoming progressively smaller with increasing concentrations. Alternatively, quisqualate may be acting via a separate mechanism to inhibit calcium-sensitive phosphoinositide formation at these higher concentrations. Since combined activation of metabotropic and ionotropic receptors using (1S,3R)-ACPD with AMPA or NMDA did not produce an additive effect on total phosphoinositide formation, we propose that the upper portion of the quisqualate response is not mediated by ionotropic receptors. Although the effects of CPP or NBQX on the calcium-sensitive upper portion of the quisqualate response could not be determined in this study, further evidence against the involvement of ionotropic receptors comes from the finding that responses to glutamate or ibotenate, while partially sensitive to extracellular calcium, were not inhibited by these antagonists. Thus, the biphasic nature of the concentration-response curve for quisqualate supports the idea that multiple excitatory amino acid receptors, linked to the stimulation of phosphoinositide formation, are associated with cortical neurones in culture. These receptor subtypes may be associated with the same neurones or with different neuronal populations.

Responses to NMDA were enhanced by the omission of magnesium from the exposure medium suggesting that these effects were mediated via activation of NMDA receptors. Removal of calcium from magnesium-free medium abolished responses to NMDA confirming that ionotropic receptor-mediated calcium influx is required for NMDA-stimulated phosphoinositide formation in this preparation. In other systems NMDA produces weak or no effect on phosphoinositide metabolism (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986b; 1987; Recasens *et al.*, 1987; 1988; Godfrey *et al.*, 1988; Schoepp & Johnson, 1988; Gonzales & Moerschbaecher, 1989), and its stimulatory effects can be blocked by NMDA receptor antagonists, supporting the involvement of ionotropic NMDA receptors in this response. Contrary to a number of previous studies showing an inhibitory effect of

NMDA on basal or stimulated phosphoinositide formation (Baudry *et al.*, 1986; Nicoletti *et al.*, 1986a; Schmidt *et al.*, 1987; Godfrey *et al.*, 1988; Gonzales & Moerschbaecher, 1989; Noble *et al.*, 1989; Morrisett *et al.*, 1990; Baird & Nahorski, 1991) in the present study NMDA had no inhibitory effect on basal or (1S,3**R**)-ACPD-stimulated phosphoinositide formation.

D,L-AP3 has been described as an antagonist of metabotropic receptors (Nicoletti *et al.*, 1986b; Schoepp & Johnson, 1989; Hwang *et al.*, 1990), although the high concentrations of AP3 required for blockade have cast some doubt on its selectivity (Baskys, 1992). In the present study D,L-AP3 failed to block (1S,3**R**)-ACPD responses at concentrations up to 1 mM, but at this concentration displayed agonist activity. In striatal neurones AP3 has been described as a partial agonist, inhibiting responses to quisqualate (Olivier *et al.*, 1991). Here, the drug had an additive effect with (1S,3**R**)-ACPD and quisqualate responses at 1 mM, with no evidence of inhibiting their effects, suggesting that AP3 is stimulating phosphoinositide formation by an alternative mechanism.

Exposure of cerebrocortical culture to pertussis toxin before agonist exposure in the present study greatly reduced phosphoinositide accumulation evoked by (1S,3**R**)-ACPD. In *Xenopus* oocytes injected with rat brain mRNA (Sugiyama *et al.*, 1987), in cerebellar granule cells (Nicoletti *et al.*, 1988), and in striatal or hippocampal neurones (Ambrosini & Meldolesi, 1989) pertussis toxin pretreatment also blocked glutamate metabotropic receptor-mediated events. These results suggest that glutamate metabotropic receptors in these preparations and in cerebrocortical neurones in culture are coupled with a pertussis toxin-sensitive G-protein. However, as a point of caution, pertussis toxin acts at several G-proteins (Gilman, 1987), and may be acting indirectly to modulate the phosphoinositide response associated with glutamate metabotropic receptor activation.

In conclusion, the present study has demonstrated that the stimulatory effects of glutamate on phosphoinositide metabolism in primary cerebrocortical cultures are mediated primarily by G-protein-linked metabotropic receptors distinct from ionotropic AMPA or NMDA receptors, and that these metabotropic receptors are associated with neuronal populations present in these cultures. Based on the inconsistent and sometimes biphasic nature of the quisqualate concentration-response curve and the partial sensitivity of the quisqualate response to removal of extracellular calcium, these effects are mediated by more than one receptor subtype. D,L-AP3 was not an effective antagonist of glutamate receptor-stimulated phosphoinositide formation, but had an additive stimulatory effect with quisqualate or (1S,3**R**)-ACPD. Thus, it is essential that more selective antagonists for glutamate metabotropic receptors be developed for further progress to be made in this important area of excitatory amino acid research.

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