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Role For Ionotropic and Metabotropic Receptors in Quisqualate-Stimulated Inositol Polyphosphate Accumulation in Rat Cerebral Cortex

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SUMMARY

The actions of the excitatory amino acid quisqualate (QA) on inositol polyphosphate accumulation in cerebral cortex slices have been assessed using both [^{3}H]inositol prelabeling and mass measurements over relatively short incubation periods. QA stimulated accumulation of all the inositol polyphosphates, with similar EC_{50} values (2.8 ± 0.7 μM). High performance liquid chromatography analysis of isomeric forms of inositol polyphosphates and specific mass assays revealed that both phosphorylation and dephosphorylation products of inositol-1,4,5-trisphosphate accumulate. A large component of the QA-stimulated inositol polyphosphate accumulation was inhibited by the ionotropic antagonist 6,7-dinitroquinoxaline-2,3-dione in a competitive manner. This implied that the QA response may be due to entry of Ca^{2+} via voltage-sensitive calcium channels as a consequence of an ionotropic receptor-induced depolarization. In support of this mechanism, the QA-induced response was dependent on the presence of extracellular calcium, whereas the well characterized muscarinic receptor agonist response to carbachol showed only a slight reduction under the same conditions. The concentration-dependent (EC_{50} 8.8 ± 3 μM) response to the selective ionotropic agonist amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) differed from that to QA or carbachol, in that accumulation of only [^{3}H]inositol mono- and bisphosphates was stimulated, with no increase in the [^{3}H]inositol tris- or tetrakisphosphates. Use of the metabotropic agonist (*trans*)-(±)-1-aminocyclopentyl-1,3-dicarboxylate (ACPD), however, produced concentration-dependent increases in all [^{3}H]inositol polyphosphates. Although both AMPA and ACPD responses alone were smaller in magnitude than that to QA, when present together AMPA and ACPD produced additive responses on [^{3}H]inositol mono- and bisphosphate and a marked synergistic increase in [^{3}H]inositol tetrakisphosphate accumulation, resulting in a response similar to that seen for QA. These data suggest that QA-evoked responses in rat cortex slices are the result of a complex interaction mediated through both ionotropic and metabotropic receptors, in which Ca^{2+} entry may stimulate accumulation of inositol mono- and bisphosphate directly and divert the metabolism of inositol-1,4,5-trisphosphate to inositol-1,3,4,5-tetrakisphosphate.

There is now much evidence that the two major fast excitatory amino acid neurotransmitters, glutamate and aspartate, mediate their actions in the central nervous system via ionotropic and metabotropic receptors (1, 2). At least two of these receptors are gated ion channels; these are named according to their selective agonists, *N*-methyl D-aspartate and AMPA, and both allow the entry of Na^+ and K^+ and exhibit different permeabilities for Ca^{2+}. However, the metabotropic receptor stimulated by QA has recently been cloned (3), is linked via a guanine nucleotide-binding protein to phosphoinositidase C, and, upon activation, yields the second messenger Ins(1,4,5)P_3 (4, 5), resulting in the mobilization of intracellular Ca^{2+} (6, 7). Recent evidence suggests that ACPD may be a selective metabotropic agonist, in contrast to QA, which is also a potent agonist at AMPA sites (8, 9).

Although there have been several studies that have revealed stimulatory effects of various excitatory amino acid agonists on phosphoinositide hydrolysis (10, 11), it has not been clear that responses are mediated solely by metabotropic receptors. In particular, the vast majority of studies have assessed total labeled inositol phosphates over long periods and, because it is now established that depolarizing stimuli can induce a phosphoinositide response secondary to Ca^{2+} entry into cells (12–15), the characterization of these excitatory amino acid responses remains particularly unclear.

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$, *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis-, and hexakisphosphate derivatives, respectively, with assignment of phosphate locants where appropriate; KRB, Krebs-Ringer bicarbonate buffer; ACPD, (*trans*)-(±)-1-aminocyclopentyl-1,3-dicarboxylic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; QA, quisqualic acid; [Ca^{2+}]$_i$, intracellular calcium concentration; HPLC, high performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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In the present experiments, we have attempted to address this problem by performing a detailed analysis of inositol polyphosphate accumulations, using HPLC and $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ mass assays, over short periods after excitatory amino acid receptor activation in rat cerebral cortex slices. We reveal clear indications that phosphoinositide responses can be mediated by both ionotropic and metabotropic receptors. These can only be distinguished by analysis of isomeric species of inositol polyphosphates, which reveals that a marked synergistic response is elicited when both receptors are activated.

Experimental Procedures

Materials. QA and carbachol were from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). DNQX, AMPA, and ACPD were from Tocris Neuramin (Buckhurst Hill, Essex, UK). *myo*-[2-^{3}H]Inositol (12–17 Ci/mmol) was purchased and [^{3}H]$Ins(1,4,5)P_3$ (17–20 Ci/mmol) and [^{32}P]$Ins(1,3,4,5)P_4$ (100–200 Ci/mmol) were gifts from New England Nuclear (Du Pont Biotechnology, Stevenage, Herts, UK). Dowex anion exchange resin AG1-X8 (200–400 mesh), formate form, was from Bio-Rad (Watford, Herts, UK). All other chemicals were of analytical grade from Fisons plc (Loughborough, Leicestershire, UK).

Incubation techniques. These have been described previously (12, 14). Briefly, cross-chopped cerebral cortex slices (350 × 350 μm) were washed and incubated in KHB containing either 1.2 mM $CaCl_2$ or no added calcium (where indicated); after 60 min, aliquots of packed slices were transferred to flat-bottomed vials containing *myo*-[2-^{3}H]inositol (2.5 μCi) in KHB (final volume, 300 μl), for an additional 60 min. After this period, agents were added, for 5 min unless otherwise indicated. Lithium was not present in any of the studies. Reactions were terminated by addition of cold perchloric acid (10%; 300 μl), and the [^{3}H]inositol phosphates were extracted and neutralized as described originally by Downes *et al.* (16). The [^{3}H]inositol phosphates were separated on columns containing Dowex resin AG1-X8 (200–400 mesh; formate form) and the radioactivity in each sample was determined by scintillation counting (14). In tissue extracts, ^{3}H-labeled peaks eluted with profiles similar to those of $InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$ standards; however, no radioactivity was eluted as $InsP_5$ or $InsP_6$, using the present short (60 min) prelabeling protocol.

Inositol polyphosphate analysis. HPLC separations of the [^{3}H]inositol phosphates were performed by a modification of the method of Dean and Moyer (17), as described previously (18). Briefly, separation was achieved using a Partisil (10-μm) SAX analytical column (Technicol), equipped with a precolumn packed with Whatman Pellicular anion exchange resin, and elution with gradients comprising water and $(NH_4)H_2PO_4$, adjusted to pH 3.7 with H_3PO_4. Samples were routinely spiked with 50–100 nmol each of adenosine and guanosine mono-, di-, and triphosphates before injection, and nucleotides were detected by continuous UV monitoring of the column eluate at 254 nm. Authentic ^{3}H-labeled standards and/or coelution with internal ^{14}C- or ^{32}P-labeled inositol phosphate standards were used to identify sample [^{3}H]inositol phosphates (18).

Mass measurements of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_3$ were performed exactly as described previously (4, 19).

Data analysis was performed to assess the EC_{50} (concentration of drug producing 50% of maximal stimulation) value by computer-assisted curve-fitting, using ALLFIT (20). Statistical significance was assessed by Student's *t* test, and differences were considered significant when $p < 0.05$.

Results

Characteristics of QA-stimulated phosphoinositide metabolism. QA was able to enhance accumulation of all the [^{3}H]inositol (poly)phosphates, with similar EC_{50} values (2.8 ± 0.7 μM) and maximal responses at 50 μM QA (Fig. 1). Time course studies (see Fig. 4) indicated that at 5 min the response to QA (50 μM) was maximal and, therefore, all studies were performed using this incubation period.

When present at a concentration of 100 μM, the ionotropic receptor antagonist DNQX was able to competitively shift the

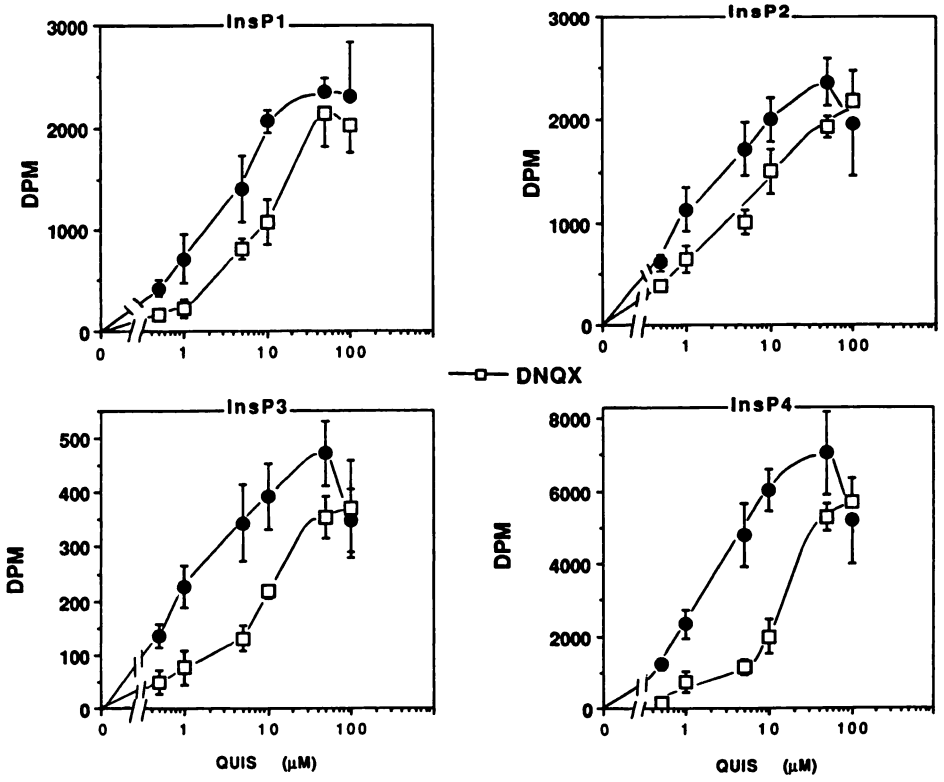

Fig. 1. Concentration-response curves for QA in the absence (●) and presence (□) of the ionotropic receptor antagonist DNQX (100 μM). [^{3}H]Inositol-labeled rat cerebral cortex slices were incubated for 5 min with the concentrations of QA (*QUIS*) shown. DNQX (100 μM) was added 5 min before addition of QA. [^{3}H]Inositol phosphates were extracted and determined as described in Experimental Procedures. Data are the means ± standard errors of four or five separate experiments performed in triplicate. Basal values of 1400 ± 239 for $InsP_1$, 393 ± 54 for $InsP_2$, 148 ± 16 for $InsP_3$, and 122 ± 19 for $InsP_4$ dpm were subtracted.

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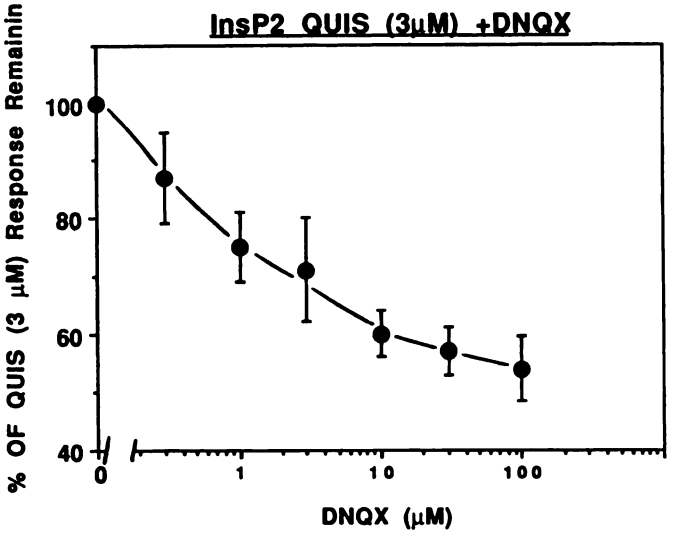

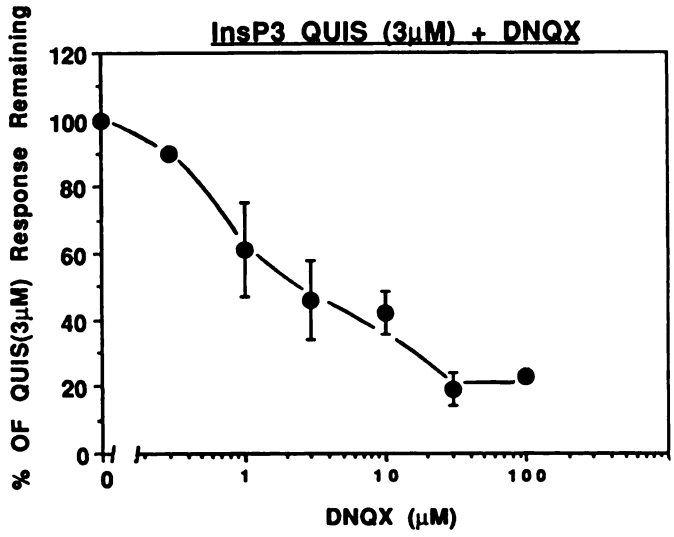

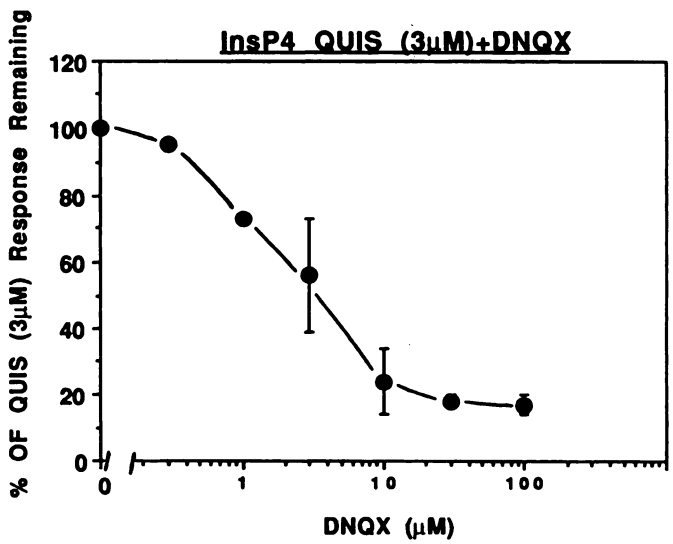

QA concentration-response curves for all the [^{3}H]inositol (poly)phosphates (Fig. 1). However, the shift was greater for $InsP_3$ and $InsP_4$ than for $InsP_1$ and $InsP_2$. The basis of this difference appears to relate to the extent of inhibition observed by DNQX against a single concentration of QA (3 μM) (Fig. 2). Only 50% of the [^{3}H]$InsP_2$ response appears to be sensitive, whereas greater than 80% of the [^{3}H]$InsP_4$ accumulation was suppressed by DNQX, but with similar IC_{50} values (approximately 3 μM).

Further analysis of these responses has been made using specific mass assays for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (4, 19). The data in Fig. 3 show clearly that QA is able to stimulate mass accumulations of these inositol polyphosphates and that DNQX (at 50 μM) can block a substantial part of these responses. The shape of the Ins(1,3,4,5)P_4 curve is very similar to that for $InsP_4$ shown in Fig. 1, whereas that for Ins(1,4,5)P_3 mass differs, in that at lower concentrations of QA the accumulation of Ins(1,4,5)P_3 does not appear to be reduced by DNQX. It should be noted, of course, that the [^{3}H]$InsP_3$ data in Fig. 1 comprise both [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3.

The actions of QA (5 μM) and the effect of DNQX were further studied by HPLC analysis (Table 1). Significant accumulations of several inositol phosphate isomers followed QA (5 μM) stimulation, and these were suppressed by DNQX, in good agreement with the data in Figs. 1 and 3. It should be noted in these experiments that there was not a detectable accumulation of [^{3}H]Ins(1,4,5)P_3 at 5 min after QA. However, the clear and marked accumulation of Ins(1,3,4,5)P_4 and Ins(1,4)P_2 strongly indicates a rapid turnover of Ins(1,4,5)P_3 by both phosphorylation and dephosphorylation routes (see Ref. 18). Data shown in Fig. 4 reveal the time courses of the QA response in cerebral cortex slices and the inhibition by DNQX.

Calcium dependence of QA responses. Because removal of the ionotropic component of the QA response suppressed a large proportion of the [^{3}H]inositol polyphosphate response, the calcium dependency of the QA actions was next examined. Omission of $CaCl_2$ from the KRB solution throughout the washing, labeling, and experimental periods revealed that the QA response was particularly dependent on the presence of extracellular calcium (Fig. 5). In comparison, the muscarinic receptor agonist carbachol was able to produce similar responses in either the presence or absence of extracellular calcium, although in the case of $InsP_1$ a Ca^{2+} dependency was also apparent. These results, therefore, indicate that the activation of receptors by QA is dependent on extracellular calcium and support further the results with DNQX showing that ionotropic QA receptors play a substantial role in stimulating the accumulation of [^{3}H]inositol polyphosphates.

Actions of the ionotropic agonist AMPA and the metabotropic agonist ACPD on phosphoinositide metabolism. The ionotropic receptor agonist AMPA was found to cause stimulation of [^{3}H]inositol phosphate accumulation (Table 2), in a concentration-dependent fashion (Fig. 6). Responses were severely reduced in the absence of extracellular Ca^{2+} (data not shown) and, in contrast to both QA and carbachol, AMPA did not cause significant accumulation of [^{3}H]$InsP_3$ or [^{3}H]

Fig. 2. Concentration-response curves for the ionotropic receptor antagonist DNQX, on [^{3}H]$InsP_{2-4}$ accumulation stimulated by QA (*QUIS*) at 3 μM. DNQX was added 5 min before agonist, and incubation periods were for 5 min. Data are shown as means ± standard errors of three separate experiments performed in triplicate.

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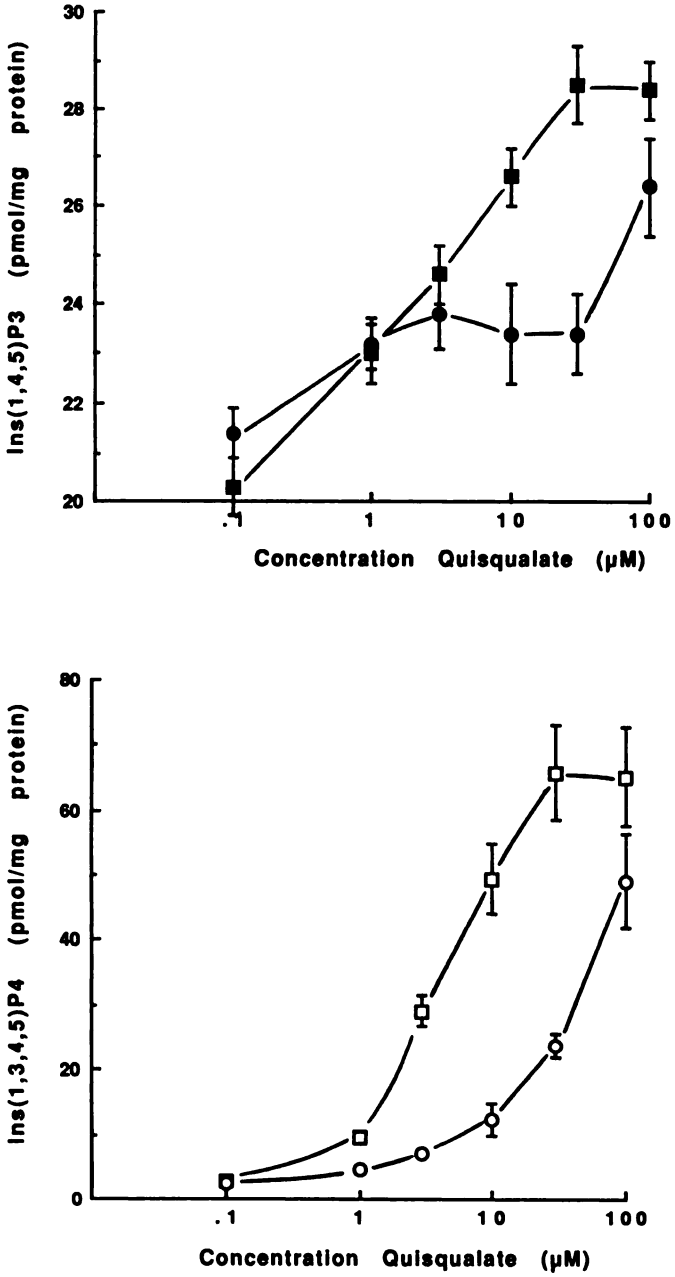

Fig. 3. Concentration-response curves for QA in the absence (■, □) or presence (●, ○) of DNQX (50 μM), on mass accumulations of Ins(1,4,5)P_3 (■, ●) and Ins(1,3,4,5)P_4 (□, ○). DNQX was added 5 min before addition of QA and then incubated for an additional 5 min. Data are shown as means ± standard errors of at least three separate experiments performed in triplicate.

$InsP_4$ but did increase [^{3}H]$InsP_1$ and [^{3}H]$InsP_2$ (the [^{3}H]$InsP_1$ data are not shown in Fig. 6 for the sake of clarity; see Table 2). The ionotropic receptor antagonist DNQX (100 μM) was able to produce a shift to the right in the curve for $InsP_2$, although quantification of this antagonism was not possible because maximal responses to AMPA were not obtained at 100 μM in the presence of antagonist (Fig. 6).

A different pattern of production of [^{3}H]inositol phosphates was obtained when the metabotropic receptor agonist ACPD was used. Concentration-related accumulation of all the [^{3}H] inositol polyphosphates was observed (Table 2; Fig. 7) and was independent of extracellular Ca^{2+} and totally insensitive to 100

TABLE 1

Effects of DNQX on QA-stimulated [^{3}H]inositol polyphosphate isomer accumulation in rat cerebral cortex slices

DNQX (100 μM) was added 5 min before addition of QA (5 μM). Incubations were carried out for 5 min. The data are means ± standard errors of three separate experiments.

	[^{3}H]inositol (poly)phosphate accumulation		
	Control	QA	QA + DNQX
	dpm		
Ins(1)P_1	1043 ± 66	2111 ± 253[a]	1534 ± 19[a]
Ins(4)P_1	669 ± 67	956 ± 97[a]	751 ± 35
Ins(1,3)P_2	172 ± 19	284 ± 46[a]	148 ± 17
Ins(1,4)P_2	417 ± 45	1442 ± 186[a]	1041 ± 99[a]
Ins(1,3,4)P_3	168 ± 22	276 ± 28[a]	195 ± 31
Ins(1,4,5)P_3	243 ± 25	298 ± 13	285 ± 28
Ins(1,3,4,5)P_4	198 ± 13	1331 ± 119[a]	337 ± 33[a]

[a] Statistical significance (Student's *t* test for unpaired observations) for differences from control values are indicated for $p < 0.05$.

μM DNQX (data not shown). Responses to AMPA alone or with ACPD are also shown. Here again, AMPA (100 μM) induced accumulation of [^{3}H]$InsP_1$ and [^{3}H]$InsP_2$ but not [^{3}H] $InsP_3$ or [^{3}H]$InsP_4$. The final bar on the histogram (Fig. 7) shows the effect of ACPD (300 μM) in the presence of AMPA (100 μM) and reveals that, whereas [^{3}H]$InsP_1$ and $InsP_2$ accumulations were additive, those of [^{3}H]$InsP_3$ and $InsP_4$ were synergistic, particularly for the latter inositol polyphosphate. Time course studies confirmed that production of [^{3}H]inositol polyphosphates was maximal at the 5-min time point used for these incubations (data not shown). HPLC analyses were performed to confirm the [^{3}H]inositol polyphosphate isomeric composition of stimulations caused by ACPD (300 μM), AMPA (100 μM), or both together (Table 2). As expected, ACPD (300 μM) increased all [^{3}H]inositol polyphosphates, with the most marked effects being on Ins(4)P_1 (105%), Ins(1,4)P_2 (270%), and Ins(1,3,4,5)P_4 (135%). In contrast, although AMPA (100 μM) could increase Ins(4)P_1 and Ins(1,4)P_2 to a similar degree (117 and 198%, respectively), there was no increase in Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 accumulation or in that of their subsequent hydrolysis products of Ins(1,3,4,5)P_4. When ACPD and AMPA were co-incubated together, all the [^{3}H]inositol polyphosphate isomers were increased, but the most dramatic synergistic change was seen in Ins(1,3,4,5)P_4 accumulation, in agreement with the data in Fig. 7.

Discussion

Evidence for the existence of a metabotropic receptor for excitatory amino acids has come from various experimental approaches. Firstly, many groups have reported effects, predominantly of QA and ibotenate, on phosphoinositide hydrolysis in various brain preparations. These include cultured striatal neurons (21–23), cultured cerebellar granule cells (24), forebrain synaptoneurosomes (25), cultured cortical astrocytes (26), and cerebral cortex (27, 28), and clear support for metabotropic receptors on normal neurons has come from measurement of $[Ca^{2+}]_i$ transients in single hippocampal neurons (7). Secondly, metabotropic receptors can be expressed in *Xenopus* oocytes after injection of rat brain mRNA, and the properties of an oscillating Ca^{2+}-dependent Cl^- current characteristic of Ins(1,4,5)P_3 generation can be pharmacologically distinguished (29). Finally, it has very recently been reported that a cDNA

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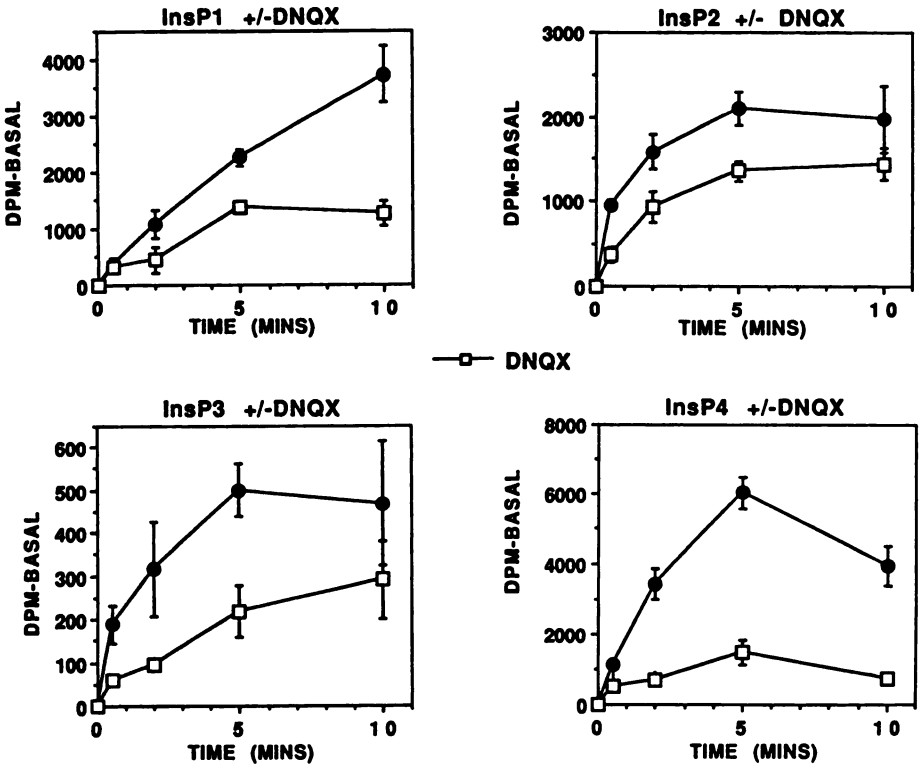

Fig. 4. Time courses of the effects of 100 μM DNQX (□) on the accumulation of [^{3}H]inositol (poly)phosphates stimulated by 50 μM QA (●). DNQX was added 5 min before agonist. Data are means ± standard errors of three separate experiments performed in triplicate. Basal values of 1433 ± 201 for $InsP_1$, 374 ± 34 for $InsP_2$, 156 ± 12 for $InsP_3$, and 98 ± 9 dpm for $InsP_4$ have been subtracted.

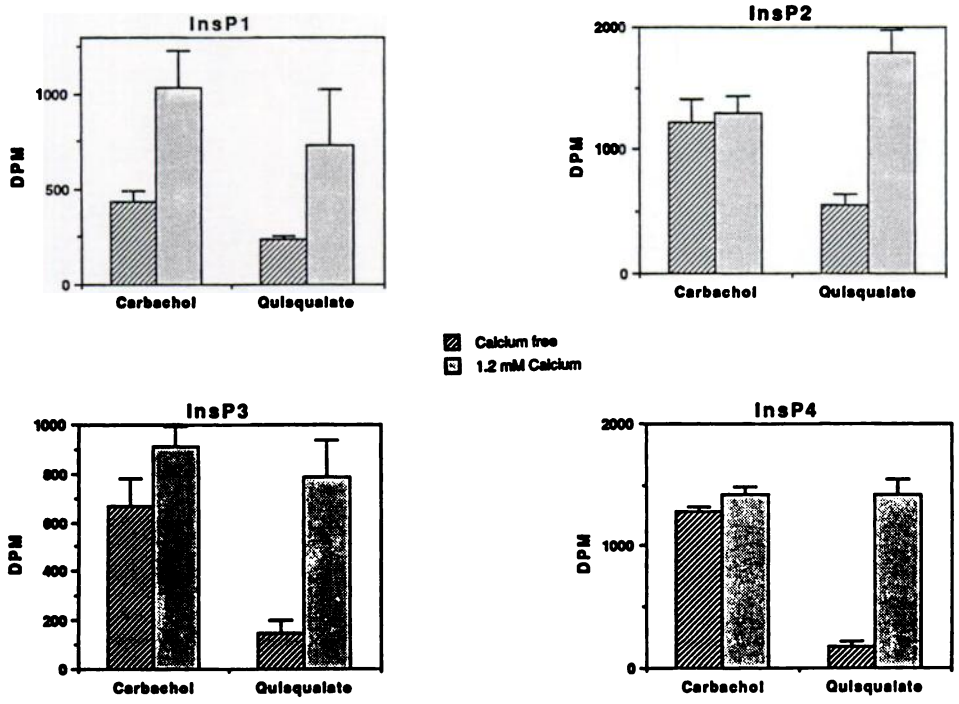

Fig. 5. Effects of either no added Ca^{2+} (■) or 1.2 mM Ca^{2+} (▨) in the KRB incubation medium on [^{3}H]inositol phosphate accumulation stimulated by carbachol (1 mM) or QA (50 μM). Cerebral cortex slices were washed, were incubated with [^{3}H]inositol, and underwent the experimental incubations (5 min) either with or without added Ca^{2+}. Data are means ± standard errors of three to six separate experiments, each performed in triplicate. Subtracted basal values for [^{3}H]$InsP_{1-4}$ were, respectively, 1221 ± 141, 172 ± 25, 80 ± 20, and 56 ± 7 dpm for calcium-free medium and 924 ± 84, 209 ± 42, 111 ± 16, and 99 ± 32 dpm for medium containing 1.2 mM Ca^{2+}.

of a metabotropic glutamate receptor from rat cerebellum has been cloned, characterized, and expressed (3).

However, effects of excitatory amino acids on neuronal phosphoinositide metabolism have been confused by responses to ionotropic agonists such as *N*-methyl-D-aspartate and kainate (see Ref. 11), and the vast majority of studies have examined total [^{3}H]inositol phosphates in the presence of lithium over long stimulation periods. This experimental approach may reveal Ca^{2+}-dependent [^{3}H]IP_1 and [^{3}H]IP_2 formation that is independent of Ins(1,4,5)P_3 formation, particularly in the presence of lithium, which disrupts the resynthesis of the phosphoinositides (see Refs. 12, 14, 15, and 30–33). We have attempted to clarify these issues with a detailed analysis of inositol polyphosphate accumulation, using HPLC separation of isomeric species and mass determination of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, in rat cerebral cortex slices.

The concentration-response and HPLC profiles, together with comparison with the muscarinic receptor agonist car-

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TABLE 2

Effects of ACPD, AMPA, and ACPD plus AMPA on accumulation of [^{3}H]inositol (poly)phosphate isomers in rat cerebral cortex slices

ACPD (300 μM) and AMPA (100 μM) were added to slice preparations for 5 min. Data were obtained by HPLC, as described in Experimental Procedures, and are means ± standard errors for three or four separate experiments.

	[^{3}H]inositol phosphate accumulation			
	Basal	ACPD	AMPA	ACPD + AMPA
	dpm			
Ins(1)P_1	979 ± 30	1617 ± 283[a]	1384 ± 165[a]	2097 ± 248[a]
Ins(4)P_1	474 ± 66	982 ± 60[a]	1028 ± 186[a]	1088 ± 121[a]
Ins(1,3)P_2	183 ± 19	338 ± 41[a]	245 ± 15	603 ± 71[a]
Ins(1,4)P_2	447 ± 55	1656 ± 129[a]	1336 ± 179[a]	2955 ± 477[a]
Ins(1,3,4)P_3	217 ± 21	422 ± 76[a]	188 ± 29	574 ± 102[a]
Ins(1,4,5)P_3	284 ± 32	439 ± 54	334 ± 26	541 ± 39[a]
Ins(1,3,4,5)P_4	197 ± 12	463 ± 82[a]	236 ± 15	2186 ± 273[a]

[a] Statistical significance (Student's *t* test for unpaired observations) for differences from control values are indicated for $p < 0.05$.

bachol, indicated that QA was able to produce marked accumulation of various inositol polyphosphates using both radiolabeling and mass determination protocols. Previously, we had demonstrated that, at a maximal effective concentration, QA (50 μM) was the only agonist tested that was able to produce increases in Ins(1,4,5)P_3 concentration, measured at 10, 60, and 300 sec after agonist challenge, that approach those observed for carbachol in cerebral cortex slices (4). As far as we are aware, these are the only studies to show increased inositol polyphosphate and isomer responses to QA at short time periods in the absence of lithium in mature cerebral cortex. In embryonic striatal neurones, Ambrosini and Meldolesi (5) demonstrated increases, evoked by QA at 15 sec, in total [^{3}H] inositol phosphates and in [^{3}H]Ins(1,4,5)P_3 accumulation. In their system, QA did not stimulate the accumulation of the various inositol phosphates to the same extent as carbachol. Our present studies, showing the effect of QA on labeled inositol polyphosphate isomers, demonstrated only a relatively small increase in [^{3}H]Ins(1,4,5)P_3 accumulation after 5 min of incubation. However, clear accumulation of phosphorylation and dephosphorylation products suggests a continual turnover of this second messenger (see Ref. 18). Some difference would also appear to exist between the measurements of Ins(1,4,5)P_3 using radiolabeling and mass determinations, in that the mass measurements after QA appear to be more comparable to those after carbachol than do those of the labeled species (4). This suggests there may be changes in the specific activity of [^{3}H]Ins(1,4,5)P_3 with different agonists, but this requires more study.

QA is known to act on two receptor subtypes (1), with only the metabotropic QA receptors being coupled directly to phosphoinositidase C activation, with subsequent 4.5-hydrolysis and generation of the intracellular calcium-mobilizing messenger Ins(1,4,5)P_3. QA can also act on ionotropic QA receptors, which allow Na^+ entry, leading to cell depolarization and consequent activation of voltage-sensitive calcium channels (34). The resulting increase in intracellular Ca^{2+} can activate phosphoinositidase C to form inositol phosphates, particularly in excitable cells (12, 14, 15, 32, 35). Thus, there are two mechanisms available (via ionotropic and metabotropic receptors) for QA to stimulate formation of [^{3}H]inositol phosphates, with the potential of substantial synergism between them (12, 36). In this study, we have used a relatively selective ionotropic antagonist (DNQX) and ionotropic and metabotropic agonists (AMPA and ACPD, respectively) in an attempt to dissect the relative contributions made by activation of ionotropic and metabo-

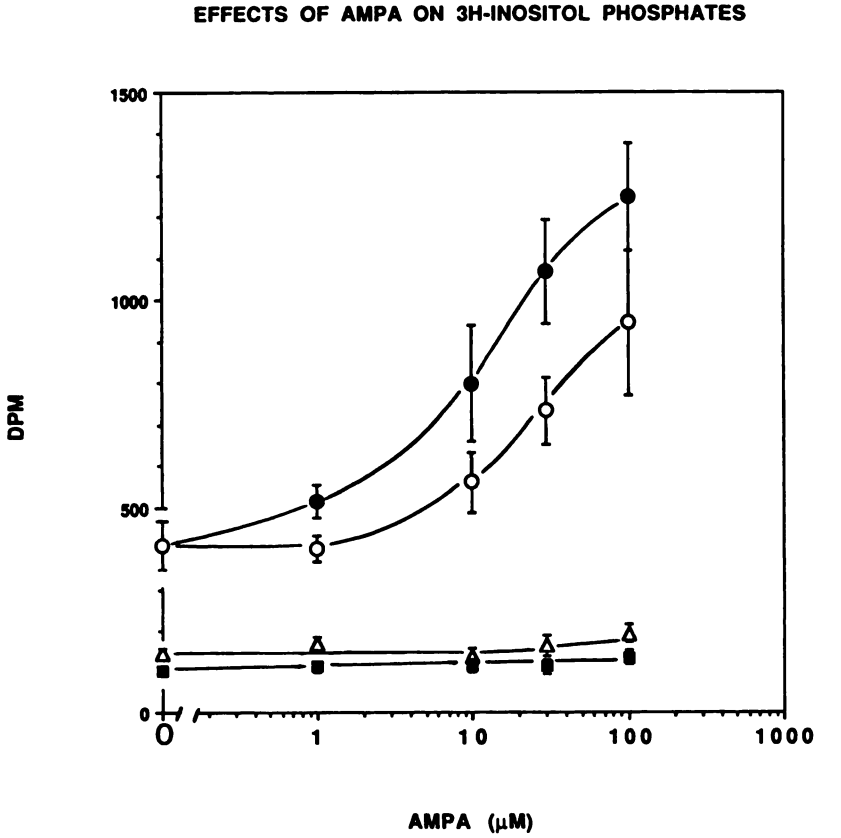

Fig. 6. Effects of the ionotropic receptor agonist AMPA on accumulation of [^{3}H] $InsP_2$ (●), [^{3}H]$InsP_3$ (△), and [^{3}H]$InsP_4$ (■). Cerebral cortex slices were incubated with AMPA for a period of 5 min. Data are means ± standard errors of three to seven experiments performed in triplicate. When present, DNQX (100 μM) was added 5 min before agonist addition (○).

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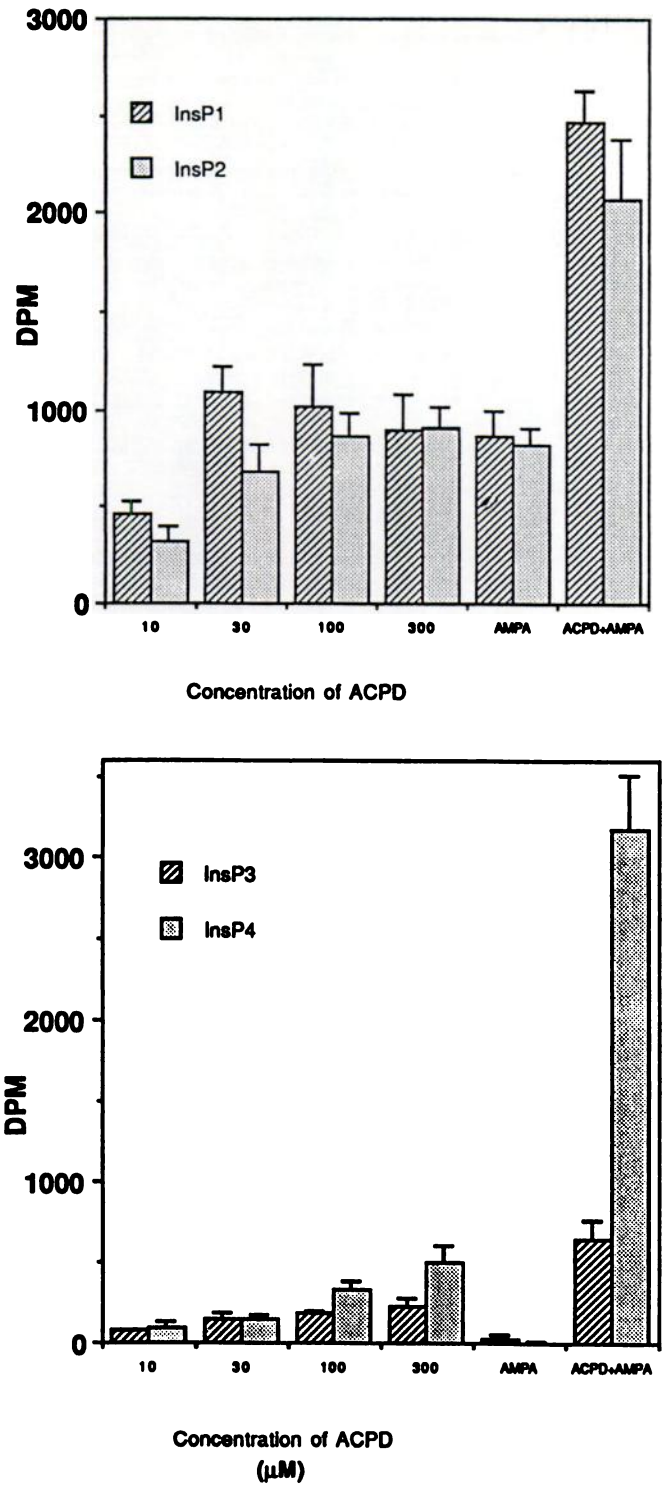

Fig. 7. Effects of the metabotropic receptor agonist ACPD and the ionotropic receptor agonist AMPA on [^{3}H]inositol (poly)phosphate accumulation. *Upper*, [^{3}H]$InsP_1$ and $InsP_2$; *lower*, [^{3}H]$InsP_3$ and $InsP_4$. ACPD at 10–300 μM, AMPA at 100 μM, and ACPD at 300 μM in the presence of AMPA (100 μM) were added to [^{3}H]inositol-prelabeled cerebral cortex slices for a 5-min incubation period. The data are means ± standard errors of three to six separate experiments performed in triplicate. Basal values of 2686 ± 214, 799 ± 79, 344 ± 19, and 312 ± 18 dpm were subtracted for [^{3}H]$InsP_{1-4}$, respectively.

tropic QA receptors in [^{3}H]inositol polyphosphate accumulation. Using DNQX, we were surprised at how much of the QA-stimulated tritiated and mass inositol polyphosphate response was apparently dependent on activation of the ionotropic receptor, with inhibition being observed at the earliest time points. The mass measurements of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ stimulated by QA were also inhibited competitively by DNQX, with parallel shifts of the $Ins(1,3,4,5)P_4$ curve, although the shift for $Ins(1,4,5)P_3$ was more complex, with inhibition only being apparent at higher QA concentrations. This complex antagonism and the inability to completely suppress [^{3}H]$InsP_2$ accumulation with DNQX highlight a mixed ionotropic and metabotropic response with complex precursor-product relationships.

Although other studies have shown that quinoxalines can inhibit the electrophysiological actions of QA in a competitive manner (37, 38), investigations of the effects on inositol phosphates appear to be at variance with our results. Palmer *et al.* (22) and Blackstone *et al.* (39) failed to obtain inhibition of QA-stimulated [^{3}H]inositol phosphate accumulation with DNQX or other related quinoxalines in hippocampal and cerebellar slices, respectively. Indeed, Blackstone *et al.* (39) failed to obtain inhibition of 100 μM QA with 1 mM CNQX. These disparities with our results may relate to regional differences and/or to the long incubation periods used by these authors, obscuring initial events that include ionotropic receptor activation. In addition, the incomplete inhibition by DNQX seen in the present study for certain inositol phosphates may have been obscured when total [^{3}H]inositol phosphates were monitored in the presence of lithium.

Because it is clear that, in cerebral cortex at least, a large component of the QA-stimulated phosphoinositide response is due to ionotropic receptor effects, it was perhaps to be expected that the ability of QA to stimulate accumulation of [^{3}H]inositol phosphates was particularly sensitive to the presence of extracellular calcium. Other investigators have reported variable effects; for example, in frontal cortex Nicoletti *et al.* (40) found no stimulatory effects of QA, but in hippocampal slices the ibotenate-stimulated accumulation of [^{3}H]inositol phosphates over a 60-min period was unaffected by low Ca^{2+} but was abolished by addition of EGTA.

The structural analogue of ibotenate, AMPA, which is considered to be a selective ionotropic agonist (1, 41), was not able to produce significant increases in [^{3}H]$InsP_3$ or [^{3}H]$InsP_4$ but induced accumulation of [^{3}H]$InsP_1$ and [^{3}H]$InsP_2$. This rather unusual pattern of accumulation is identical to previous observations from this laboratory with agents that raise intracellular calcium directly, such as ionophores and toxins (14, 15), and may reflect activation of the 5-phosphatase and/or a breakdown (secondary to elevated Ca^{2+}) of phosphatidylinositol 4-monophosphate (30, 31, 42). Although negligible effects of AMPA on phosphoinositide metabolism in hippocampal slices have been reported (22, 23), this may again relate to regional differences or to the problems of assaying total [^{3}H]inositol phosphates in the presence of lithium over long incubation periods.

Activation of QA metabotropic receptors using the specific agonist ACPD resulted in a more typical response associated with phosphatidylinositol 4,5-bisphosphate hydrolysis. However, when this agent was co-incubated with the ionotropic agonist AMPA, there was additive accumulation of [^{3}H]$InsP_1$ and [^{3}H]$InsP_2$ but synergistic accumulation of [^{3}H]$InsP_3$ and

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particularly [^{3}H]InsP$_4$, resembling responses produced by QA alone. It can be suggested that the increase in $[Ca^{2+}]_i$ (and/or its localization) is greater after AMPA-induced entry than after ACPD-induced mobilization. We have previously reported examples of marked synergistic accumulation of [^{3}H]InsP$_4$ in depolarized cerebral cortex slices incubated with carbachol (12). It was argued that the elevated intracellular Ca^{2+} stimulates Ca^{2+}/calmodulin-dependent InsP$_3$ 3-kinase (43, 44), favoring Ins(1,3,4,5)P$_4$ accumulation. Such a mechanism clearly accommodates the synergy observed in these studies between ACPD and AMPA.

In summary, the phosphoinositide responses obtained when both ionotropic and metabotropic excitatory amino acid receptors are activated by AMPA and ACPD, respectively, resemble those seen when QA is used alone as an agonist. Thus, the ionotropic antagonist DNQX is able to remove a large proportion of the QA response, presumably that which is due to Ca^{2+}-induced synergy. These results have not only demonstrated the actions of QA but also more clearly defined the roles played by ionotropic and metabotropic receptors in the QA-induced phosphoinositide response in cerebral cortex. Our data suggest that ionotropic and metabotropic QA receptors probably coexist on the same cells and they emphasize the integration of these responses in the cerebral cortex.

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References

1. Monaghan, D. T., R. J. Bridges, and C. W. Cotman. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.* **29**:365–402 (1989).
2. Watkins, J. C., P. Krogsgaard-Larsen, and T. Honoré. Structure activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.* **11**:25–33 (1990).
3. Masu, M., Y. Tanabe, K. Tsuchida, R. Shigemoto, and S. Nakanishi. Sequence and expression of a metaobotropic glutamate receptor. *Nature (Lond.)* **349**:760–765 (1991).
4. Challiss, R. A. J., I. H. Batty, and S. R. Nahorski. Mass measurements of Ins(1,4,5)P$_3$ in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem. Biophys. Res. Commun.* **157**:684–691 (1988).
5. Ambrosini, A., and J. Meldolesi. Muscarinic and quisqualate receptor-dependent phosphoinositide hydrolysis in primary cultures of striatal and hippocampal neurons: evidence for differential mechanisms of activation. *J. Neurochem.* **53**:825–833 (1989).
6. Murphy, S. N., and R. J. Miller. A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **85**:8737–8741 (1988).
7. Murphy, S. N., and R. J. Miller. Two distinct quisqualate receptors regulate Ca^{2+} homeostasis in hippocampal neurons *in vitro*. *Mol. Pharmacol.* **35**:671–680 (1989).
8. Palmer, E., D. T. Monaghan, and C. W. Cotman. Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. *Eur. J. Pharmacol.* **166**:585–587 (1989).
9. Manzoni, O., L. Fagni, J. P. Pin, F. Rassendren, F. Poulat, F. Sladeczek, and J. Bockaert. (*trans*)-1-Aminocyclopentyl-1,3-dicarboxylate stimulates quisqualate phosphoinositide-coupled receptors, but not ionotropic glutamate receptors in striatal neurons and *Xenopus* oocytes. *Mol. Pharmacol.* **38**:1–6 (1990).
10. Sladeczek, F., M. Récasens, and J. Bockaert. A new mechanism for glutamate receptor action: phosphoinositide hydrolysis. *Trends Neurosci.* **11**:545–549 (1988).
11. Smart, T. G. Excitatory amino acids: the involvement of second messengers in the signal transduction process. *Cell. Mol. Neurobiol.* **9**:193–206 (1989).
12. Baird, J. G., and S. R. Nahorski. Potassium depolarisation markedly enhances muscarinic receptor stimulated inositol tetrakisphosphate accumulation in rat cerebral cortical slices. *Biochem. Biophys. Res. Commun.* **141**:1130–1137 (1986).
13. Court, J. A., C. J. Fowler, J. M. Candy, P. R. Hoban, and C. J. Smith. Raising the ambient potassium ion concentration enhances carbachol stimulated phosphoinositide hydrolysis in rat brain hippocampal and cerebral cortical miniprisms. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **334**:10–16 (1986).
14. Baird, J. G., and S. R. Nahorski. Increased intracellular calcium stimulates [^{3}H]inositol phosphate accumulation in rat cerebral cortical slices. *J. Neurochem.* **54**:555–561 (1990).
15. Baird, J. G., and S. R. Nahorski. Differences between muscarinic receptor and Ca^{2+} induced inositol polyphosphate isomer accumulation in rat cerebral cortex slices. *Biochem. J.* **267**:835–838 (1990).
16. Downes, C. P., P. T. Hawkins, and R. F. Irvine. Inositol 1,3,4,5-tetrakisphosphate and not phosphatidylinositol 3,4-bisphosphate is the probable precursor of inositol 1,3,4-trisphosphate in agonist stimulated parotid gland. *Biochem. J.* **238**:501–506 (1986).
17. Dean, N. M., and J. D. Moyer. Metabolism of inositol bis-, tris-, tetrakis- and pentakis-phosphates in GH$_3$ cells. *Biochem. J.* **250**:493–500 (1988).
18. Batty, I. H., A. J. Letcher, and S. R. Nahorski. Accumulation of inositol polyphosphate isomers in agonist stimulated cerebral cortex slices. *Biochem. J.* **258**:23–32 (1989).
19. Challiss, R. A. J., and S. R. Nahorski. Neurotransmitter- and depolarization-stimulated accumulation of inositol 1,3,4,5-tetrakisphosphate mass in rat cerebral cortex slices. *J. Neurochem.* **54**:2138–2141 (1990).
20. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
21. Schmidt, B. H., S. Weiss, M. Sebben, D. E. Kemp, J. Bockaert, and F. Sladeczek. Dual action of excitatory amino acids on the metabolism of inositol phosphates in striatal neurons. *Mol. Pharmacol.* **32**:364–368 (1987).
22. Palmer, E., D. T. Monaghan, and C. W. Cotman. Glutamate receptors and phosphoinositide metabolism: stimulation via quisqualate receptors is inhibited by *N*-methyl D-aspartate receptor activation. *Mol. Brain. Res.* **4**:161–165 (1988).
23. Schoepp, D. D., and B. G. Johnson. Excitatory amino acid agonist-antagonist interactions at 2-amino-4-phosphonobutyric acid-sensitive quisqualate receptors coupled to phosphoinositide hydrolysis in slices of rat hippocampus. *J. Neurochem.* **50**:1605–1613 (1988).
24. Nicoletti, F., J. L. Meek, M. J. Iadarla, D. M. Chuang, B. L. Roth, and E. Costa. Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J. Neurochem.* **46**:40–46 (1986).
25. Récasens, M., I. Sassetti, A. Nourigat, F. Sladeczek, and J. Bockaert. Characterization of subtypes of excitatory amino acid receptors involved in the stimulation of inositol phosphate synthesis in rat brain synaptoneurosomes. *Eur. J. Pharmacol.* **141**:87–93 (1987).
26. Pearce, B., J. Albrecht, C. Morrow, and S. Murphy. Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux. *Neurosci Lett.* **72**:335–340 (1986).
27. Godfrey, P. P., C. J. Wilkins, W. Tyler, and S. P. Watson. Stimulatory and inhibitory actions of excitatory amino acids on inositol phospholipid metabolism in rat cerebral cortex. *Br. J. Pharmacol.* **95**:131–138 (1988).
28. Noble, E. P., E. Sincini, D. Bergmann, and G. T. Bruggencate. Excitatory amino acids inhibit stimulated phosphoinositide hydrolysis in rat prefrontal cortex. *Life Sci.* **44**:19–26 (1989).
29. Sugiyama, H., I. Ito, and C. Hirono. A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature (Lond.)* **325**:531–533 (1987).
30. Brammer, M. J., I. Hajimohammadreza, S. Sardiwal, and K. Weaver. Is inositol bisphosphate the product of A23187 and carbachol-mediated polyphosphoinositide breakdown in synaptosomes? *J. Neurochem.* **51**:514–521 (1988).
31. Brammer, M. J., and K. Weaver. Kinetic analysis of A23187-mediated polyphosphoinositide breakdown in rat cortical synaptosomes suggests that inositol bisphosphate does not arise primarily by degradation of inositol trisphosphate. *J. Neurochem.* **53**:399–407 (1989).
32. Eberhard, D. A., and R. W. Holz. Intracellular Ca^{2+} activates phospholipase C. *Trends Neurosci.* **11**:517–520 (1988).
33. Kennedy, E. D., R. A. J. Challiss, and S. R. Nahorski. Lithium reduces the accumulation of inositol polyphosphate second messengers following cholinergic stimulation of cerebral cortex slices. *J. Neurochem.* **53**:1652–1655 (1989).
34. Mayer, M. L., and R. J. Miller. Excitatory amino acid receptors, second messengers and regulation of intracellular Ca^{2+} in mammalian neurons. *Trends Pharmacol. Sci.* **11**:254–260 (1990).
35. Kendall, D. A., and S. R. Nahorski. Dihydropyridine Ca^{2+} channel activators and antagonists influence depolarisation-evoked inositol phospholipid hydrolysis in brain. *Eur. J. Pharmacol.* **115**:31–36 (1985).
36. Eva, C., and E. Costa. Potassium ion facilitation of phosphoinositide turnover activation by muscarinic receptor agonists in rat brain. *J. Neurochem.* **46**:1429–1435 (1986).

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37. Birch, P. J., C. J. Grossman, and A. G. Hayes. Kynurenate and FG9041 have both competitive and non-competitive antagonist actions at excitatory amino acid receptors. *Eur. J. Pharmacol.* **151**:313–315 (1988).
38. Drejer, J., and T. Honoré. New quinoxalinediones show potent antagonism of quisqualate responses in cultured mouse cortical neurones. *Neurosci. Lett.* **87**:104–108 (1988).
39. Blackstone, C. D., S. Supattapone, and S. H. Snyder. Inositol phospholipid-linked glutamate receptors mediate cerebellar parallel fiber-Purkinje cell synaptic transmission. *Proc. Natl. Acad. Sci. USA* **86**:4316–4320 (1989).
40. Nicoletti, F., M. J. Iadarola, J. T. Wroblewski, and E. Costa. Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: developmental change and interaction with *alpha*$_1$-adrenoceptors. *Proc. Natl. Acad. Sci. USA* **83**:1931–1935 (1986).
41. Krogsgaard-Larson, P., T. Honoré, J. J. Hansen, D. R. Curtis, and D. Lodge. New class of glutamate agonist structurally related to ibotenic acid. *Nature (Lond.)* **284**:64–66 (1980).
42. Kolesnick, R. N., and M. C. Gershengorn. Ca^{2+} ionophores affect phosphoinositide metabolism differently than thyrotropin-releasing hormone in GH_3 pituitary cells. *J. Biol. Chem.* **259**:9514–9519 (1984).
43. Ryu, S. H., S. Y. Lee, K. Y. Lee, and S. G. Rhee. Catalytic properties of inositol trisphosphate kinase in activation by Ca^{2+} and calmodulin. *FASEB J.* **1**:388–393 (1987).
44. Takazawa, K., H. Passareiro, J. E. Dumont, and C. Erneux. Ca^{2+}/calmodulin-sensitive inositol 1,4,5-trisphosphate 3-kinase in rat and bovine brain tissues. *Biochem. Biophys. Res. Commun.* **153**:632–641 (1988).

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