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Pharmacological characterization of the metabotropic glutamate receptor inhibiting D-[^{3}H]-aspartate output in rat striatum

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1 The effects of several agonists of the metabotropic glutamate receptor (mGluR) were studied in adult rat striatal slices by measuring (i) KCl (30 mM)-induced output of previously taken up D-[^{3}H]-aspartate (Asp), (ii) forskolin (30 μM)-induced adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation and (iii) phophoinositide (PI) hydrolysis.

2 K^+-induced efflux of D-[^{3}H]-Asp was inhibited by the following mGluR agonists: (1S,3S,4S)-(carboxycyclopropyl)glycine (L-CCG-I), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) and quisqualic acid (Quis). 2-Amino-4-phosphonobutyrate (L-AP4) was inactive up to 300 μM. The maximal inhibition of D-[^{3}H]-Asp output was 60 ± 8%. The EC_{50}s of mGluR agonists were: 0.5 μM for L-CCG-I, 100 μM for 1S,3R-ACPD and 100 μM for Quis.

3 Forskolin-induced cyclic AMP accumulation was also inhibited by mGluR agonists. The maximal inhibition was 50 ± 4% and was obtained at a concentration of 10 μM for L-CCG-I and 100 μM for 1S,3R-ACPD. The EC_{50}s for this inhibition were: 0.9 μM for L-CCG-I and 20 μM for 1S,3R-ACPD. Quis (300 μM) inhibited cyclic AMP accumulation by approximately 20%. L-AP4 slightly potentiated cyclic AMP accumulation.

4 PI hydrolysis was stimulated by mGluR agonists. The most potent compound was Quis (100 μM), which increased inositol phosphate formation up to 2.2 fold over control values. Its EC_{50} was 15 μM. L-CCG-I and 1S,3R-ACPD increased inositol phosphate formation by approximately 1.8 fold and their EC_{50} values were 30 and 25 μM, respectively. L-AP4 did not affect PI hydrolysis.

5 In conclusion, mGluR agonists that reduce D-[^{3}H]-Asp output have a pharmacological profile similar to that of mGluR agonists inhibiting cyclic AMP accumulation. L-CCG-I appears to be a relatively selective agonist for the mGluR receptor which inhibits D-[^{3}H]-Asp efflux and cyclic AMP accumulation, while Quis appears to act preferentially on the mGluR receptor linked to the metabolism of PIs.

Keywords: Metabotropic glutamate receptors; excitatory amino acid release; (2S,3S,4S)-(carboxycyclopropyl) glycine (L-CCG-I); (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD); quisqualic acid; cyclic AMP; synaptic plasticity, phosphoinositide hydrolysis

Introduction

Molecular cloning has revealed the existence of at least six metabotropic glutamate receptors (mGluRs; mGluR1 to mGluR6) (Houamed *et al.*, 1991; Masu *et al.*, 1991; Tanabe *et al.*, 1992; Nakanishi, 1992). These six subtypes can be classified into three different groups according to their sequence similarities: one group consists of mGluR1 and mGluR5, which are preferentially linked, through G proteins, to phosphoinositide hydrolysis (PI); the second group includes mGluR2 and mGluR3, which are coupled to the inhibition of forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation; the third group includes mGluR4 and mGluR6, is thought to be similarly linked to the inhibition of adenylate cyclase. The pharmacological characterization of these receptors is far from being completed because of the lack of selective agonists and antagonists for each receptor subtype. Experiments using transfected cells appear to be particularly useful for this goal (Aramori & Nakanishi, 1991; Hayashi *et al.*, 1992). To date, most of the literature data on the role of the mGluRs in brain function has been obtained by using (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), the prototype agonist of mGluRs (Aronica *et al.*, 1991; Anwyl, 1991; Baskys & Malenka, 1992; Schoepp & Conn, 1993). Different steroisomers of 1S,3R-ACPD seem to be useful for the classification of mGluRs (Cartmell *et al.*, 1993b).

It has been shown recently that appropriate concentrations of 1S,3R-ACPD, in the presence of arachidonic acid, enhance glutamate exocytosis from cerebrocortical synaptosomes (Herrero *et al.*, 1992). This observation has been considered particularly important for the understanding of the overall function of glutamatergic synapses and the role that mGluRs may play in the mechanisms of synaptic potentiation and neuronal plasticity (Nicholls, 1992). In fact, the importance of mGluR activation for the stimulation of neurotransmitter release and for the induction and maintenance of long term potentiation has been repeatedly stressed (Anwyl, 1991; Bortolotto & Collingridge, 1992; Zheng & Gallagher, 1992). However, using electrophysiological approaches, it has also been shown that 1S,3R-ACPD and quisqualic acid (Quis), a non-selective mGluR agonist, cause a synaptic inhibition that has been ascribed to the decrease of transmitter release from glutamatergic terminals (Lovinger, 1991; Calabresi *et al.*, 1992; Rannie & Shinnick-Gallagher, 1992). These observations were particularly well-defined in the rat striatum, a brain structure enriched in glutamatergic presynaptic terminals which originate primarily in the frontal cortex and are known to modulate the function of several neuronal populations (Wood *et al.*, 1979).

In order to understand the role of mGluRs in brain func-

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tion and to set up simple models for the identification of new mGluR agonists and antagonists, we performed experiments aimed at evaluating how mGluR stimulation could affect excitatory amino acid release from brain slices. We here show that mGluR agonists significantly inhibited the depolarization-induced output of D-[^{3}H]-aspartate (Asp) from rat striatal slices. After evaluating the actions of several proposed mGluR agonists on D-[^{3}H]-Asp efflux, on the inhibition of cyclic AMP accumulation and on PI metabolism, we noticed that the pharmacological properties of the mGluR type controlling transmitter output were different from those of the receptor stimulating PI metabolism but similar to the profile of the mGluR modulating cyclic AMP accumulation. Thus, we propose that glutamate release may be inhibited by presynaptic glutamate receptors modulating cyclic AMP formation in a manner that may be compared to the control of noradrenaline output exerted by α_2-auto-adrenoceptors (Langer, 1980; Starke, 1987).

Methods

Preparation of rat striatal slices

Male Wistar rats (Nossan strain, Milan, Italy), weighing 180–200 g were used. After decapitation, their striata were rapidly removed and placed into ice-cold Krebs-bicarbonate buffer (in mM: NaCl 122, KCl 3.1, $MgSO_4$ 1.2, KH_2PO_4 0.4, $CaCl_2$ 1.3, $NaHCO_3$ 25 and glucose 10). Transverse slices (350 μm thick) were prepared with a McIlwain tissue chopper and then left to stand, dipped into Krebs-bicarbonate solution gassed with 95% O_2/5% CO_2, for 1 h at 37°C in order to allow functional recovery.

Studies on D-[^{3}H]-Asp release

Recovered slices were incubated for 45 min at 37°C in oxygenated Krebs solution containing D-[^{3}H]-Asp (final concentration 50 nM) and subsequently washed for 30 min by changing the incubation medium twice. The labelled slices were transferred (two per chamber) to Perspex chambers (0.3 ml volume) (see Beani *et al.*, 1978; Moroni *et al.*, 1981) and superfused with gassed (95% O_2/5% CO_2) Krebs solution at 37°C. Preliminary experiments showed that after 30 min of superfusion, a quasi steady state efflux of tritium was reached. Different mGluR agonists were added 5 min before slices were challenged for 5 min with a 30 mM KCl-containing buffer (with isomolar reduction of NaCl) (see Figure 1 for experimental details). The superfusion solutions were directly collected in counting vials. Instagel was added and the radioactivity was counted in a Packard (Tri Carb 1500) liquid scintillation analyzer. Tritium output was calculated as the percentage of total radioactivity (c.p.m. tritium in the perfusate/c.p.m. tritium in the slices at the beginning of the respective 5 min superfusion period).

Measurements of cyclic AMP formation

Striatal slices (two per tube) were placed for 30 min in 500 μl of oxygenated (95% O_2/5% CO_2) Krebs solution at 37°C. After this time, 5 μl of either forskolin (final concentration, 30 μM) or vehicle, 3-isobutyl-1-methylxanthine (IBMX, final concentration 1 mM), and 5 μl of a solution containing mGluR agonists were added. Tubes were then placed in a shaking water bath for 15 min. The incubation was terminated by adding 0.75 ml of ice-cold 12 mM disodium EDTA solution to each tube. The samples were immediately homogenized in a Tetronix Tissuemixer and then boiled for 10 min. After centrifugation the supernatants were frozen at − 80°C until cyclic AMP was measured. Cyclic AMP levels were determined with a cyclic AMP radioimmunoassay kit.

Studies on PI hydrolysis

Slices prepared as previously described were incubated for 2 h with [^{3}H]-inositol (20 μCi ml^{-1}). They were then washed in 50 ml of freshly oxygenated buffer and transferred to test-tubes (two slices each) with 500 μl of drug-containing medium and gently stirred in the presence of 10 mM LiCl by bubbling 95% O_2/5% CO_2. After 15 min at 37°C, the reaction was stopped by the addition of 1.88 ml of chloroform/methanol (1:2). The phases were separated by adding 0.65 ml of chloroform and 0.65 ml of water and, after brief sonication, by centrifuging the tubes at 800 *g* for 10 min. The upper phase (2 ml), which contained the water-soluble [^{3}H]-inositol phosphates (inositol monophosphate, IP; inositol 1,4-bisphosphate, IP_2 inositol 1,4,5-trisphosphate, IP_3) was transferred to test-tubes and water (3 ml) was added. The inositol phosphates were then separated on Dowex AG 1-X 8 anion exchange resins (formate form, 100–200 mesh) according to Berridge & Irvine (1984), as previously described (Pellegrini-Giampietro *et al.*, 1988). The radioactivity in portions (8 ml) of these fractions was determined by scintillation counting. Calculations were performed on the sum of IP, IP_2 and IP_3 (d.p.m. mg^{-1} protein).

Protein assay

The protein content of the samples was measured according to Lowry *et al.* (1951).

Materials

(1S,3S,4S)-(Carboxycyclopropyl)glycine (L-CCG-I) was synthesized as described by Pellicciari *et al.* (1986, 1988, 1991). In those papers the active compounds were named cyclopropylglutamate stereoisomers (CGA), to stress the analogy with glutamate; (1S,3S,4S)-CGA corresponded to L-CGA B and is here named L-CCG-I. The nomenclature used by Shinozaki *et al.* (1989) and by Tocris Neuramin (Bristol, U.K.) has now been introduced in order to ensure unambiguous assignment of the molecule. 1S,3R-ACPD, Quis and 2-amino-4-phosphonobutyrate (L-AP4) were purchased from Tocris Neuramin (Bristol, U.K.). D-[^{3}H]-Asp (10–30 Ci mmol^{-1}) and [^{3}H]-cyclic AMP radioimmunoassay kit (25 Ci mmol^{-1}) were from Amersham (Amity PG, Milan, Italy). *myo* 2-[^{3}H]-N-inositol (10–20 Ci mmol^{-1}) was from New England Nuclear (Du Pont de Nemours, Milan, Italy). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), and Dowex AG-1-X 8 anion exchange resin (100–200 mesh) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The scintillation fluid (Instagel) was from Packard (Groningen, The Netherlands). All other reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Statistical analysis

Statistical significance in all experiments was evaluated by performing the analysis of variance. The EC_{50}s were defined as the concentration which caused 50% of the maximal effects. Preliminary experiments showed that the maximal effects obtainable were: inhibition of 50% of cyclic AMP formation; inhibition of 60% of D-[^{3}H]-Asp release; accumulation of inositol phosphates of 2.2 times over controls for Quis and 1.8 times for L-CCG-I and 1S,3R-ACPD (see Figures 3, 4 and 5).

Results

Effects of mGluR agonists on K$^+$-induced D-[^{3}H]-Asp striatal output

In striatal slices prelabelled with D-[^{3}H]-Asp and superfused for a 50 min period, application of buffer containing 30 mM

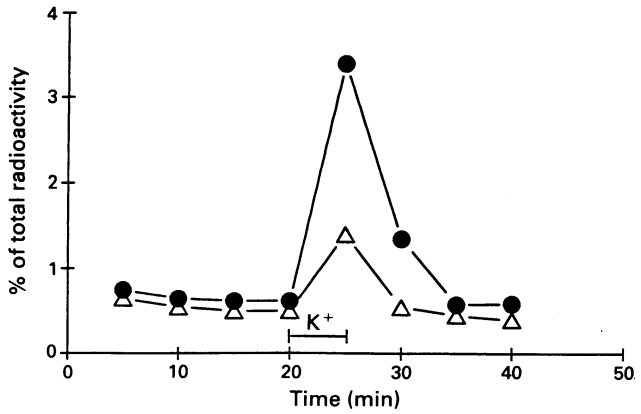

Figure 1 Striatal slices were incubated with D-[^{3}H]-aspartate (D-[^{3}H]-Asp) for 45 min at 37°C and subsequently washed twice prior to being placed in the superfusion chambers. They were then superfused for 30 min before starting the collection of the samples. After the collection of 3 samples the slices were challenged for 5 min with a 30 mM KCl-containing solution (with isomolar reduction of NaCl). The slices contained $150 \pm 20 \times 10^3$ c.p.m. mg^{-1} protein at the beginning of the collection of superfusates. Each point is the percentage of total radioactivity (radioactivity present in the perfusate/radioactivity present in the slices at the beginning of the respective 5 min superfusion period). At least 5 duplicate experiments were performed; s.e.mean were within 15% of the reported values. (●) Normal Krebs; (△) Ca^{2+}-free Krebs.

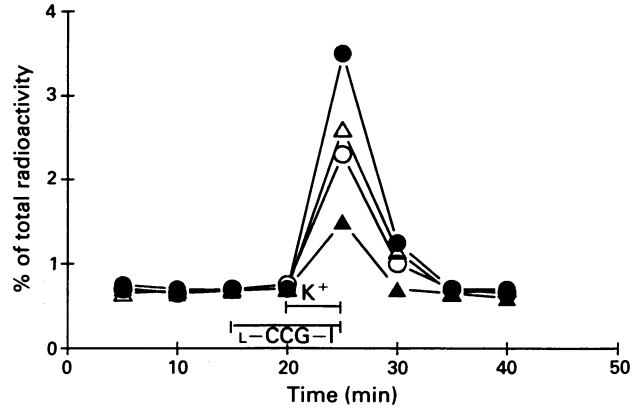

Figure 2 Dose-dependent effects of (1S,3S,4S)-(carboxycyclopropyl) glycine (L-CGG-I) on K^+-induced D-[^{3}H]-aspartate (D-[^{3}H]-Asp) output. Different concentrations of L-CCG-I were added to the superfusion fluid 5 min before KCl (30 mM). Each point represents the mean obtained in at least 5 different experiments conducted in duplicate (see Figure 1 for the experimental protocol). (●) Controls; (△) L-CCG-I 0.1 μM; (○) L-CCG-I 0.3 μM; (▲) L-CCG-I 1 μM.

KCl increased D-[^{3}H]-Asp output by 3.5 ± 0.7 times. This output was reduced by $50 \pm 7\%$ when $CaCl_2$ was omitted from the solution and EDTA (1 mM) was added (Figure 1). When L-CCG-I was present in the superfusion solution 5 min before and during the K^+-induced depolarization, the output of radioactivity was reduced in a dose-dependent manner. The maximum effect ($60 \pm 8\%$ over control values) was obtained at 1 μM L-CCG-I (Figure 2). 1S,3R-ACPD (30–300 μM) and Quis (100–300 μM) had similar effects (Figure 3), while L-AP4 did not affect tritium output. The EC_{50}s for the inhibition of this output were: 0.5 μM for L-CCG-I; 100 μM for 1S,3R-ACPD, and 100 μM for Quis.

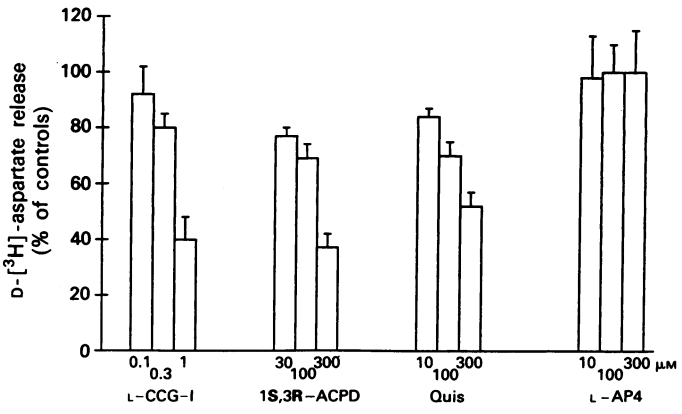

Figure 3 Different concentrations of mGluR agonists (L-CCG-I, 1S-3R-ACPD, Quis, L-AP4) were added 5 min before depolarization of striatal slices with 30 mM of KCl (see Figure 1 for details). Each column represents radioactivity output expressed as percentage of controls and represents the mean ± s.e.mean of at least 5 experiments run in duplicate. For abbreviations, see text.

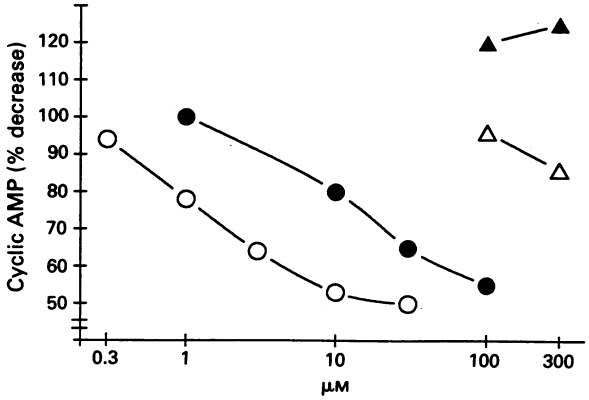

Figure 4 Striatal slices were incubated for 15 min at 37°C with various concentrations of L-CCG-I, 1S,3R-ACPD, Quis and L-AP4 in the presence of 30 μM forskolin and 1 mM IBMX. The content of cyclic AMP of each sample was measured by radioimmunoassay. Data are the means of 5 experiments run in triplicate and are expressed as percentage over the forskolin-stimulated values in each experiment, s.e.mean, were consistently within 10% of the reported values. (○) L-CCG-I; (●) 1S,3R-ACPD; (△) Quis; (▲) L-AP4. For abbreviations, see text.

Effects of mGluR agonists on forskolin-stimulated cyclic AMP accumulation

The addition of forskolin (30 μM) to striatal slices incubated in the presence of 1 mM IBMX increased their content in cyclic AMP from 4.7 ± 0.2 to 102 ± 10 pmol mg^{-1} protein. When agonists of mGluRs were added, the stimulation was significantly reduced. Figure 4 shows that L-CCG-I (10 μM) reduced the effect of forskolin by approximately 50%. This was the maximal inhibition we could achieve. Similar effects were evoked by larger concentrations of 1S,3R-ACPD (100 μM), while Quis was less active. The EC_{50}s for this effect were: 0.9 μM for L-CCG-I, 20 μM for 1S,3R-ACPD, and 300 μM for Quis. Conversely, L-AP4 slightly potentiated forskolin-stimulated cyclic AMP accumulation.

The effects of mGluR agonists on the stimulation of PI hydrolysis

The addition of mGluR agonists to striatal slices increased PI metabolism in a concentration-dependent manner. Figure 5

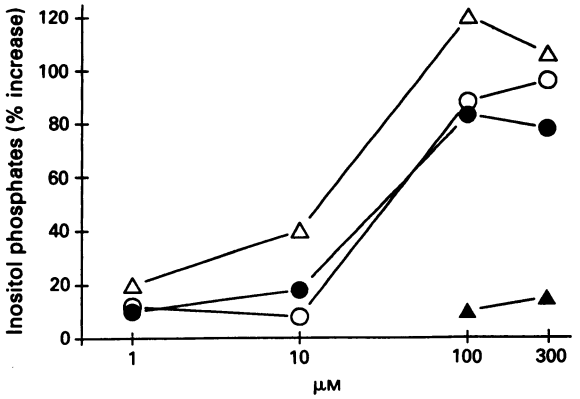

Figure 5 Slices prelabelled with [^{3}H]-inositol were exposed to different concentrations of mGluR agonists (Quis, 1S,3R-ACPD, L-CGG-I and L-AP4) for 15 min in the presence of 10 mM LiCl. Data are the means of at least 3 experiments run in triplicate and are expressed as percentage increases over basal values in each experiment. The basal value was 15910 ± 1267 d.p.m. mg^{-1} protein and represent the sum of radioactivity found in the fractions corresponding to [^{3}H]-IP, [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$. (○) L-CCG-I; (●) 1S,3R-ACPD; (△) Quis; (▲) L-AP4. For abbreviations, see text.

shows that Quis (100 μM) increased 2.2 fold the formation of inositol phosphates. At the same concentration, the stimulation evoked by L-CCG-I and 1S,3R-ACPD was approximately 1.8 fold. L-AP4 was inactive. The EC_{50}s were: 15 μM for Quis, 25 μM for 1S,3R-ACPD, and 30 μM for L-CCG-I.

Discussion

Studies on the characterization of the physiological and pharmacological properties of mGluRs have been impeded by the lack of selective agonists and antagonists. In fact, the commonly used agonsits 1S,3R-ACPD or Quis do not distinguish among the different receptor subtypes and the recently described antagonists derived from phenylglycine seem to have partial agonist properties and are not completely characterized (Ito *et al.*, 1992; Eaton *et al.*, 1993; Birse *et al.*, 1993). Other compounds such as L-Asp-β-hydroxamate seem to have mixed agonist-antagonist activity on the stimulation of PI metabolism (Porter *et al.*, 1992). In a similar manner, 2-amino-3-phosphonopropionate (L-AP3), once considered a selective mGluR antagonist (Schoepp & Johnson 1989), has been demonstrated to have agonist-antagonist activity leading to rapid desensitization of the receptor (Lonart *et al.*, 1992). In mammalian cells expressing the cloned mGluR subtypes, Hayashi *et al.* (1992) noticed that L-CCG-I, another proposed agonist of mGluRs (Nakagawa *et al.*, 1990; Ishida *et al.*, 1990; Kudo *et al.*, 1991), had an EC_{50} of 0.3 μM when studied on mGluR2 mediating the inhibition of forskolin-induced cyclic AMP accumulation and of 50 μM when studied on mGluR1 mediating increase of phosphoinositide metabolism. This could suggest that L-CCG-I is a relatively selective agonist for the subtype of mGluR negatively linked to the cyclase system. This receptor subtype has been described in different brain slice preparations (Schoepp *et al.*, 1992; Cartmell *et al.*, 1992; Manzoni *et al.*, 1992; Casabona *et al.*, 1992). In rat striatal slices L-CCG-I had an EC_{50} of 0.9 μM for the inhibition of cyclic AMP accumulation and of 30 μM for the stimulation of PI metabolism thus confirming, in a different model, data obtained in transfected cells (Hayashi *et al.*, 1992). Furthermore, L-CCG-I significantly inhibited K^+-induced output of D-[^{3}H]-Asp. This output, once considered as a gross index of synaptic transmitter release (Potashner, 1978; Notman *et al.*, 1984; Arqueros *et al.*, 1985) is now regarded with some suspect because D-[^{3}H]-Asp penetrates poorly into the synaptic vescicles (see: McMahon & Nicholls, 1991). The apparent calcium dependency of D-[^{3}H]-Asp release under our experimental conditions and preliminary results obtained by measuring the effects of mGluR agonists on the K^+ (30 mM)-evoked release of endogenous glutamate from striatal slices suggest that we are studying actions on the depolarization-induced output of synpatic transmitter. However, the possibility that mGluR agonists affect the cytoplasmic output of glutamate (with an action on the excitatory amino acid carrier ?) and not the exocytotically released transmitter cannot be ruled out by the present experiments. Since the EC_{50} of L-CCG-I for the inhibition of D-[^{3}H]-Asp efflux and that for the blockade of cyclic AMP accumulation are in the same range and in view of the fact that the orders of potency of mGluR agonists for both actions were comparable, it is reasonable to propose that the receptor involved might be the same. Receptors inhibiting cyclic AMP accumulation and modulating transmitter release have been widely studied in the adrenergic system and selective agonists for these receptors are now useful therapeutic tools (Langer, 1980; Starke, 1987; Schoffelmeer *et al.*, 1988).

Glutamate receptors capable of inhibiting D-[^{3}H]-Asp output have previously been reported (McBean & Roberts, 1981) and a number of studies have demonstrated presynaptic glutamate receptors stimulated by L-AP4 that inhibit synaptic excitation (Koerner & Cotman, 1981; Gannon *et al.*, 1989; Forsythe & Clements, 1990; Trombley & Westbrook, 1992). L-AP4 has recently been proposed as a prototype agonist of mGluR4 (Thomsen *et al.*, 1992), one of the mGluR types negatively linked to adenylate cyclase and inhibition of transmitter release (Nakanishi, 1992). Since L-AP4 binding sites have been described in the striatum (Butcher *et al.*, 1983), we also tested L-AP4 in our experiments. Our results indicate that L-AP4 (100–300 μM) did not inhibit D-[^{3}H]-Asp release and slightly stimulated forskolin-induced cyclic AMP accumulation. Therefore, the receptor subtype involved in the control of transmitter release in the rat striatum does not appear to be mGluR4. This observation is in line with *in situ* hybridization studies suggesting that mGluR4 receptors are neither present in the striatum nor in the cortex where the major glutamatergic input to the striatum originates (Tanabe *et al.*, 1993). However, L-AP4 inhibits the forskolin-induced cyclic AMP accumulation in the guinea-pig cortex (Cartmell *et al.*, 1993a) and it is not clear whether or not this inhibition is associated with a decrease of transmitter output.

1S,3R-ACPD and Quis inhibited D-[^{3}H]-Asp output and forskolin-induced cyclic AMP accumulation at concentrations very similar to those stimulating PI hydrolysis (this paper) and potentiating transmitter release, in the presence of arachidonic acid, in other brain areas (Herrero *et al.*, 1992). It has been demonstrated that arachidonic acid release from striatal neurones is promoted by the concerted action of Quis on both ionotropic and metabotropic receptors (Dumuis *et al.*, 1990), nevertheless in striatal slices Quis diminished D-[^{3}H]-Asp output. The demonstration that similar concentrations of 1S,3R-ACPD, a prototype agonist of the mGluRs, reduced D-[^{3}H]-Asp output in the striatum (this study) and increased glutamate release in the neocortex (Herrero *et al.*, 1992) may offer an explanation for the contradictory results thus far reported on the pharmacological and toxic effects of this mGluR agonist. In fact, 1S,3R-ACPD has been shown both to reduce and increase synaptic transmission and to cause and prevent excitotoxic neuronal damage. Different receptors are probably responsible for the numerous effects of this compound. For instance, Koh *et al.* (1991) reported that 1S,3R-ACPD could protect cortical neuronal cultures from the toxic action of N-methyl-D-aspartate. Similar results have been obtained in the retina (Siliprandi *et al.*, 1992) and in the mouse hippocampus (Chiamulera *et al.*, 1992) *in vivo*. Conversely, Sacaan & Schoepp (1992) described an excitotoxic action for this mGluR agonist in the rat hippocampus. The possible interaction of 1S,3R-ACPD with different

mGluR subtypes and the differential expression of these receptors in different species and brain areas could easily explain these apparently contradictory observations.

The acceptable selectivity of LCCG-I for the mGluR receptor linked to the inhibition of cyclic AMP and the inhibition of transmitter output shows that it is possible to obtain compounds that significantly reduce transmitter release at glutamatergic synapses by acting on a specific mGluR subtype. Experiments along this line are in progress in our laboratory. The availability of selective agonists for specific mGluR subtypes could help in understanding the overall control of the function of glutamatergic synapses and could be useful in pathological situations linked to excitotoxic events such as epilepsy, neurodegeneration and brain ischaemia (see: Guidotti, 1990; Meldrum 1990).

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