British Journal of Pharmacology (1996) 117, 189–195

Metabotropic glutamate receptors, transmitter output and fatty acids: studies in rat brain slices

Grazia Lombardi, Patrizia Leonardi & [1]Flavio Moroni

Dipartimento di Farmacologia Preclinica e Clinica della Università di Firenze, Viale Morgagni 65; 50134 Firenze, Italy

1 The effects of (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3**R**-ACPD), a non-selective agonist of the metabotropic glutamate receptors (mGluRs), have been studied in rat cortical and striatal slices by measuring the depolarization-induced output of D-[^{3}H]-aspartate (D-[^{3}H]-Asp) and of [^{3}H]-glutamate ([^{3}H]-Glu), neosynthesized from [^{3}H]-glutamine.

2 In cortical slices, 1S,3**R**-ACPD potentiated the depolarization-induced (KCl, 30 mM) output of both D-[^{3}H]-Asp and [^{3}H]-Glu. The potentiation, obtained at 300 μM 1S,3**R**-ACPD was 65±6% for D-[^{3}H]-Asp and 56±10% for [^{3}H]-Glu. Conversely, in striatal slices, 1S,3**R**-ACPD reduced the depolarization-induced transmitter output. The reduction, obtained at 300 μM of the agonist, was 60±8% for D-[^{3}H]-Asp and 50±5% for neosynthesized [^{3}H]-Glu.

3 Bovine serum albumin (BSA, 15 μM), which is able to bind locally produced fatty acids, completely eliminated the potentiating effect 1S,3**R**-ACPD had on D-[^{3}H]-Asp output from cortical slices. Low concentrations of arachidonic acid (1–10 μM) or of oleic acid (1–10 μM) added to BSA-containing perfusion medium, restored this potentiating effect. BSA, however, had no effect on the inhibitory action of 1S,3**R**-ACPD in striatal slices.

4 Bromophenacyl bromide (100 μM), an inhibitor of phospholipase A_2, and RG80267 (100 μM), an inhibitor of diacylglycerol lipase, have been shown to inhibit fatty acid production. These compounds prevented the potentiating effect of 1S,3**R**-ACPD on D-[^{3}H]-Asp-output in cortical slices.

5 Indomethacin (100 μM), an inhibitor of cyclo-oxygenases, plus nordihydroguaiaretic acid (100 μM), an inhibitor of lipoxygenases, increased D-[^{3}H]-Asp output in cortical slices perfused with BSA-containing medium.

6 These experiments suggest that the mGluR-mediated potentiation of transmitter output requires the availability of unsaturated fatty acids, such as arachidonic or oleic acids, in cortical slices. In contrast, the mGluR-induced inhibition of transmitter output is not dependent upon fatty acid availability in striatal slices. The requirement of both unsaturated fatty acids and 1S,3**R**-ACPD in the facilitation of transmitter exocytosis may play an important role in the regulation of synaptic plasticity.

Keywords: (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3**R**-ACPD); excitatory amino acid release; metabotropic glutamate receptors (mGluRs); bovine serum albumin (BSA); arachidonic acid; fatty acids; synaptic plasticity

Introduction

Metabotropic glutamate receptor (mGluR) agonists cause significant changes in the function of presynaptic neuronal terminals, resulting in an increase or a reduction in the depolarization-induced transmitter release (Anwyl, 1991; Lovinger, 1991). We previously observed that (1S,3**R**)-1-amino cyclopentane-1,3-dicarboxylic acid (1S,3**R**-ACPD), a non-selective mGluR agonist (Schoepp et al., 1990; Schoepp & Conn, 1993), reduced the depolarization-induced D-[^{3}H]-aspartate (Asp) output in striatal slices (Lombardi *et al.*, 1993), while in cortical slices, similar concentrations of this compound significantly increased transmitter release (Lombardi *et al.*, 1994). A pharmacological characterization of these effects suggested that the potentiation of transmitter output, in the cortex, was most probably the consequence of 1S,3**R**-ACPD-induced stimulation of mGluRs which are linked to phospholipase C (group 1; namely mGluR1 or mGluR5, see: Nakanishi, 1992 for a review), whereas the inhibition of transmitter output in the striatum was associated with the stimulation of mGluRs which are negatively linked to adenylyl cyclase (group2; namely mGluR2 and/or mGluR3; Lombardi *et al.*, 1994). In the above mentioned studies, we measured the output of D-[^{3}H]-Asp as an index of transmitter release. Since this approach has been considered unsatisfactory due to the poor penetration of isolated synaptic vesicles by D-[^{3}H]-Asp (McMahon & Nicholls, 1991), we decided to also use, under the same experimental settings, the output of [^{3}H]-glutamate ([^{3}H]-Glu), neosynthesized after preincubation of slices with [^{3}H]-glutamine. The use of [^{3}H]-glutamine as a precursor of the transmitter pool of glutamate has been proposed in the past (Cotman & Hamberger, 1979) and studies on the actions of pharmacological agents on the release of both D-[^{3}H]-Asp and [^{3}H]-Glu, neosynthesized from [^{3}H]-glutamine, could uncover possible differences between the pools in which labelled D-[^{3}H]-Asp and [^{3}H]-Glu are stored.

Independent studies have shown that 1S,3**R**-ACPD significantly potentiates the depolarization-induced glutamate exocytosis from cortical synaptosomes. In order for the potentiation to occur, however, the availability of free arachidonic acid or of other unsaturated fatty acids, such as oleic acid, is required (Herrero et al., 1992). This requirement has been explained by assuming that in order to phosphorylate a protein (possibly a K^+ channel), which is responsible for the facilitation of transmitter exocytosis, the presence of both diacylglycerol, formed as a consequence of 1S,3**R**-ACPD-induced activation of phospholipase C, and of an unsaturated fatty acid are necessary (Coffey *et al.*, 1994). In brain slices, mGluR agonists increase the output of the excitatory transmitter without the addition of fatty acids to the superfusion medium (Lombardi *et al.*, 1994). However, in this preparation, fatty acids may be endogenously formed, as a consequence of spontaneous or transmitter-induced activation of membrane phospholipases (Dumuis *et al.*, 1993; Stella *et al.*, 1994). Since

[1] Author for correspondence.

fatty acids are considered particularly important as retrograde messengers during events leading to synaptic plasticity (Williams *et al.*, 1989; Nicholls, 1992), we thought it interesting to test if the presence of a fatty acid is required both for the facilitatory and the inhibitory events induced by mGluR agonists on transmitter output. One of the approaches used to significantly reduce the availability of fatty acids in perfused tissues is the addition to the perfusion fluid of micromolar concentrations of bovine serum albumin (BSA), which possesses active sites able to bind fatty acids (Reed *et al.*, 1975; Rhoads *et al.*, 1983). Moreover, since fatty acid formation in *in vitro* tissues is mainly due to the action of the enzymes phospholipase A_2 and/or diacylglycerol lipase (see: Axelrod *et al.*, 1988 for a review), we also used inhibitors of these enzymes for a better understanding of the role fatty acids play in the mGluR-modulation of transmitter output.

Methods

Preparation of rat brain slices

Male Wistar rats (Nossan strain, Milan) weighing 180–200 g were used. After decapitation, brains were rapidly removed and the parietal cortex or the striatum were taken and placed into ice-cold Krebs-bicarbonate buffer (composition 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 by use of a McIlwain tissue chopper. The slices were then left to stand dipped into Krebs-bicarbonate solution gassed with 95% O_2/5% CO_2 for 1 h at 32°C in order to allow functional recovery.

Release experiments

Recovered slices were incubated either for 45 min in oxygenated Krebs-bicarbonate buffer containing D-[^{3}H]-Asp (final concentration: 50 nM) or for 90 min in a medium containing L-[^{3}H]-glutamine (final concentration: 35 nM) at 32°C. The slices were then washed for 30 min by changing the incubation medium twice. Labelled slices were then transferred to Perspex perfusion chambers (0.8 ml volume) (Beani et al., 1978; Moroni *et al.*, 1981) and perfused with gassed (95% O_2/5% CO_2) Krebs solution at 32°C. Preliminary experiments showed that after 30 min of perfusion, a quasi steady state efflux of tritium was reached. 1S,3R-ACPD was added 5 min before the slices were challenged with the depolarizing buffer solution containing 30 mM KCl (with isomolar reduction of NaCl), according to a previously published protocol (Lombardi *et al.*, 1993). When BSA, fatty acids or the enzyme inhibitors were used, they were added to the perfusion fluid at the beginning of the perfusate collection. The perfusates were collected into sample vials and then analysed for their content of radioactivity. The output of D-[^{3}H]-Asp was counted in a Packard (Tri Carb 1500) liquid scintillation analyser by directly adding 10 ml of Instagel scintillation fluid to the collected solution. [^{3}H]-Glu, newly synthesized from [^{3}H]-glutamine, was measured after purification of the collected perfusate through an anion-exchange chromatography resin. Briefly, the buffer was made alkaline and loaded onto small chromatography columns containing 1 ml of Dowex AG 1-X 8 anion exchange resin (formiate-form, 100–200 mesh). These columns separate basic and neutral amino acids (such as glutamine and γ-aminobutyric acid (GABA)) from the acidic ones (such as aspartate or glutamate). The neutral amino acids are eluted in the void volume and in 2–3 ml of distilled water sequentially added to the columns, while aspartate and glutamate are retained in the resin and then eluted with 2 ml of formic acid 5 M (Gaitonde, 1973; Corradetti *et al.*, 1983). Under our experimental conditions, 85±7% of the radioactivity present in this eluate could be ascribed to authentic [^{3}H]-Glu as identified by using an h.p.l.c. approach previously described (Lombardi & Moroni, 1992). The radioactivity present in the slices was evaluated after tissue solubilisation with 1 ml of solvable tissue solution. D-[^{3}H]-Asp content was directly measured in this solution, while the [^{3}H]-Glu content was measured after purification as described above for the collected samples.

Protein assay

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

Materials

1S,3R-ACPD was purchased from Tocris Neuramin (Bristol, U.K.). D-[^{3}H]-Asp (10–30 Ci mmol^{-1}) and L-[^{3}H]-glutamine (45 Ci mmol^{-1}) were from Amersham (Amity PG, Milan, Italy). Dowex AG-1-X8 anion exchange resin (100–200 mesh), tetrodotoxin, BSA, arachidonic acid, oleic acid, stearic acid, nordihydroguaiaretic acid, indomethacin, bromophenacylbromide were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). RG-80267 (1,6-di[-0-(carbamoyl)cyclohexanone oxime] hexane) was kindly supplied by Rhone-Poulenc Rorer (Collegeville, PA, U.S.A.). Solvable tissue solution (NEF 910G) was from DuPont-NEN (Bad Homburg, Germany). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Arachidonic acid, oleic acid and bromophenacylbromide were dissolved in ethanol (final concentration 0.1% ethanol); RG-80267 was dissolved in dimethyl sulphoxide (final concentration 1%). At these concentrations, both dimethyl sulphoxide and ethanol had no effects. All other compounds were directly dissolved in Krebs-bicarbonate buffer.

Statistical analysis

Statistical significance in all experiments was evaluated by performing the analysis of variance (ANOVA), followed by Tukey's W test for multiple comparison.

Results

Characterization of D-[^{3}H]-Asp and [^{3}H]-Glu output

In cortical or striatal slices prelabelled with D-[^{3}H]-Asp and perfused for a 30 min period, the application of buffer solutions containing depolarizing concentrations of KCl for 5 min significantly increased the output of radioactivity. In striatal slices, this increase was 3.5±0.7 and 10±2 fold when the challenging solutions contained 30 or 50 mM KCl respectively. Similarly, in cortical slices, the increase of radioactivity output was 2.8±0.2 and 8.9±1.1 fold (Figures 1 and 2).

Comparable results were obtained when the experiments were carried out in slices preincubated with [^{3}H]-glutamine and [^{3}H]-Glu output was evaluated. Under these conditions, a depolarizing solution containing 30 mM KCl increased [^{3}H]-Glu output by 2.6±0.1 fold in cortical slices and by 3±0.3 fold in striatal slices. A solution containing 50 mM K increased [^{3}H]-Glu output by 8±0.4 fold in striatal slices and by 7±0.5 in the cortical ones (data not shown).

When depolarization was performed in a nominally Ca^{2+} free medium, the output of both D-[^{3}H]-Asp and that of [^{3}H]-Glu were significantly reduced. Furthermore the depolarization-induced (KCl, 30 mM) D-[^{3}H]-Asp output was reduced by 55±5% in the cortex and by 50±6% in the striatum in the presence of tetrodotoxin (0.5 μM) (Figure 1).

Effects of (1S,3R)-ACPD on D-[^{3}H]-Asp or [^{3}H]-Glu output

1S,3R-ACPD added to the perfusion solution 5 min before and during KCl (30 mM)-induced depolarization, significantly reduced the output of radioactivity from striatal slices, while

increasing the output of radioactivity from cortical slices (Figures 2 and 3). The inhibition obtained with 1S,3R-ACPD (300 μM) was 60±8% when striatal slices were prelabelled with D-[^{3}H]-Asp and 50±5% when [^{3}H]-Glu output was evaluated.

The potentiation obtained with 1S,3R-ACPD (300 μM) was 65±6% when cortical slices were prelabelled with D-[^{3}H]-Asp and 56±10% when [^{3}H]-Glu output was evaluated (Figures 2 and 3).

It is interesting to note that 1S,3R-ACPD (300 μM) did not change the radioactivity output of both D-[^{3}H]-Asp (Figure 2) and [^{3}H]-Glu when the depolarizing solution contained 50 mM KCl. Similarly, 1S,3R-ACPD did not significantly potentiate the depolarization-induced (KCl, 30 mM) D-[^{3}H]-Asp output in cortical slices when $CaCl_2$ was omitted from the perfusion medium (data not shown).

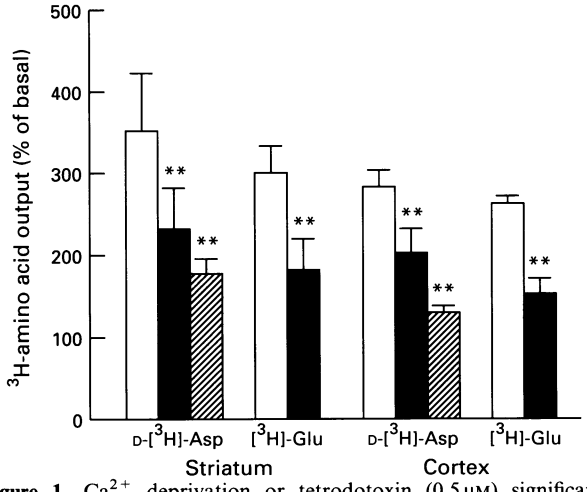

Figure 1 Ca^{2+} deprivation or tetrodotoxin (0.5 μM) significantly reduced the KCl (30 mM)-induced output of D-[^{3}H]-Asp from cortical or striatal slices. The slices were preincubated with D-[^{3}H]-Asp for 45 min or [^{3}H]-glutamine for 90 min at 32°C, washed twice, placed in the perfusion apparatus and perfused with oxygenated Krebs-bicarbonate buffer for 30 min before starting the experiments. At the end of the perfusion period, they contained approximately 5×10^5 c.p.m. mg^{-1} protein when incubated with D-[^{3}H]-Asp and 9×10^4 c.p.m. mg^{-1} protein of [^{3}H]-Glu when preincubated with [^{3}H]-glutamine. Each column represents the mean % increase of basal output (mean±s.e.mean of at least 5 duplicate experiments) of labelled amino acids in the 5 min fraction of perfusion fluid containing the depolarizing concentration of potassium. The basal output (100%) was the average of labelled amino acids present in the two 5 min perfusion samples before the challenge and was: 5200±500 c.p.m. mg^{-1} protein when D-[^{3}H]-Asp was evaluated and 500±20 c.p.m. mg^{-1} protein when [^{3}H]-Glu was evaluated (mean± s.e.mean of at least 80 experiments). Open columns: $CaCl_2$ 1.3 mM; solid columns: $CaCl_2$ 0 mM; hatched columns: $CaCl_2$ 1.3 mM and tetrodotoxin 0.5 μM. **$P<0.01$ vs. Ca^{2+}-containing perfusion buffer.

Effects of BSA and free fatty acids on 1S,3R-ACPD modulation of D-[^{3}H]-Asp output

When BSA (15 μM) was added to the buffer solution, at the beginning of the perfusion period, the effects of 1S,3R-ACPD on the increase of D-[^{3}H]-Asp output from cortical slices were completely prevented (Figure 4). The presence of BSA in the superfusion fluid, however, did not influence 1S,3R-ACPD-induced decrease of D-[^{3}H]-Asp efflux from striatal slices. BSA is able to bind fatty acids in limited amounts (Spector, 1975; Rhoads et al., 1983) and in order to test the role of these fatty acids in the mGluR agonist-induced modulation of transmitter release, low concentrations of a few of them were added to BSA containing superfusion solution. As shown in Figure 5, arachidonic acid (1–10 μM) or oleic acid (1–10 μM), added together with 1S,3R-ACPD to BSA containing buffer solutions, restored the potentiating effect the mGluR agonist had in control experiments. In contrast, stearic acid, a saturated fatty acid which is abundant in brain tissue, did not antagonize BSA activity.

Effects of inhibitors of fatty acid formation on 1S,3R-ACPD modulation of D-[^{3}H]-Asp output

The second approach we utilised to reduce the concentration of fatty acids in brain slices and in the perfusates was to use inhibitors of phospholipase A_2 and diacylglycerol lipase. As shown in Figure 6, when bromophenacyl bromide (100 μM), an inhibitor of phospholipase A_2, was added to the superfusion

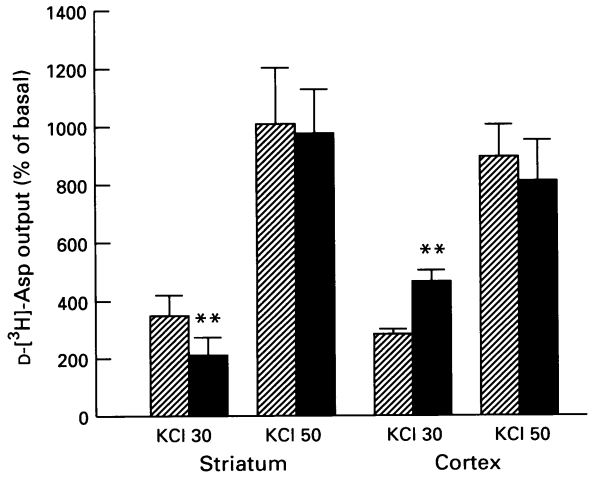

Figure 2 Effects of 1S,3R-ACPD (300 μM) on D-[^{3}H]-Asp output from cortical or striatal slices challenged with depolarizing solutions containing 30 or 50 mM KCl. The mGluR agonist was added to the perfusion solution 5 min before and during the depolarization. Each column represents the % increase of the basal D-[^{3}H]-Asp output (see legend to Figure 1) expressed as means±s.e.mean of at least 5 experiments done in duplicate. Hatched columns: controls; solid columns: 1S,3R-ACPD (300 μM). **$P<0.01$ vs. respective controls.

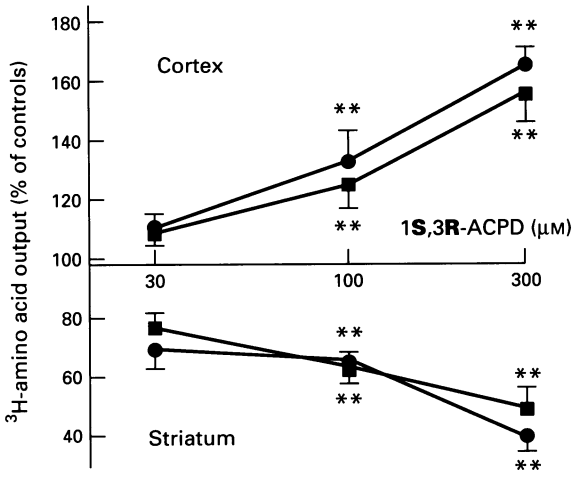

Figure 3 Effects of different concentrations of 1S,3R-ACPD on the depolarization KCl (30 mM)-induced output of D-[^{3}H]-Asp or [^{3}H]-Glu from cortical or striatal slices. Different concentrations of 1S,3R-ACPD were added to the superfusion medium 5 min before and maintained during the depolarization obtained with KCl (30 mM, 5 min). Each point represents the mean change (mean±s.e.mean) of labelled amino acid output expressed as % of the relative controls (^{3}H-amino acid output in slices not exposed to 1S,3R-ACPD). (●) D-[^{3}H]-Asp; (■) [^{3}H]-Glu. **$P<0.01$ vs. controls.

solutions, the 1S,3R-ACPD-induced potentiating effects in cortical slices were reduced. Similar results were obtained with the use of RG-80267 (100 μM), an inhibitor of diacylglycerol lipase. When both inhibitors were simultaneously added, the potentiating effect of 1S,3R-ACPD was prevented (Figure 6).

In the presence of both inhibitors, exogenously added arachidonic acid restored the potentiating effect of 1S,3R-ACPD in cortical slices (Figure 6) and larger concentrations significantly increased the labelled amino acid output. Arachidonic acid (10 μM), however, did not modify the inhibitory action of 1S,3R-ACPD in striatal slices (data not shown).

Effect of inhibitors of arachidonic acid metabolism on 1S,3R-ACPD modulation of D-[^{3}H]-Asp output

In order to clarify if active eicosanoids were involved in the above described effects of arachidonic acid, we used large concentrations of indomethacin (100 μM), an inhibitor of cyclo-oxygenases, and nordihydroguaiaretic acid (100 μM), an inhibitor of lipoxygenases. This concentration of nordihydroguaiaretic acid significantly increased the basal output of D-[^{3}H]-Asp in the absence of BSA, but it had no action on either the basal or the stimulated D-[^{3}H]-Asp output when BSA was present (data not shown). When both inhibitors were present, a low concentration of arachidonic acid (3 μM) induced 1S,3R-ACPD potentiation of transmitter output in cortical slices perfused with BSA containing buffer (Figure 7). Larger concentrations of this fatty acid slightly potentiated the effects of the mGluR agonist. Indomethacin and nordihydroguaiaretic

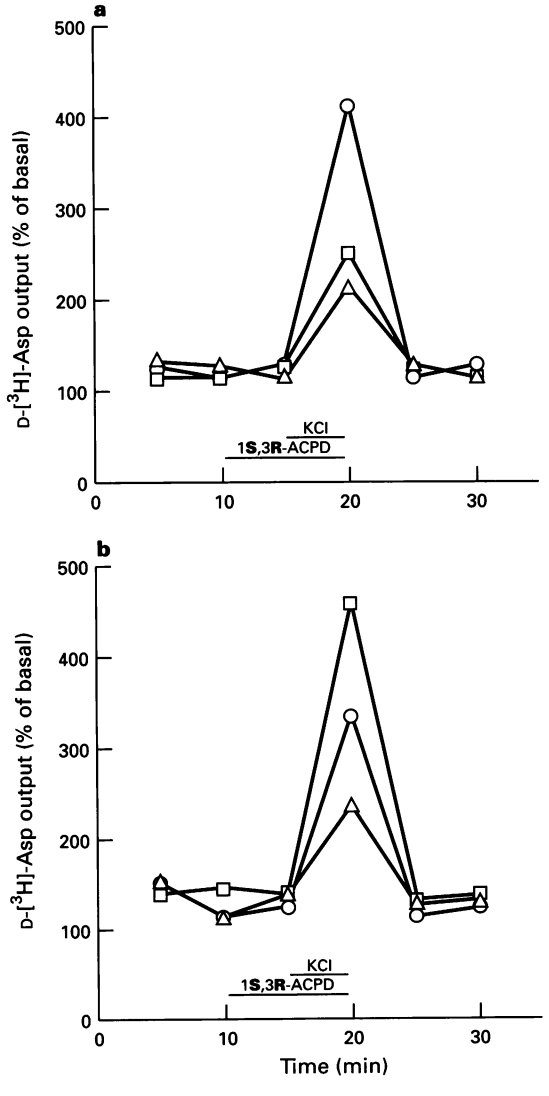

Figure 4 Effects of BSA on the depolarization (KCl, 30 mM)-induced output of D-[^{3}H]-Asp from (a) striatal and (b) cortical slices. BSA (15 μM) was added to the superfusion fluid at the beginning of the collection of perfusates, while 1S,3R-ACPD (300 μM) was added 5 min before and maintained during the depolarization. The points represent the % changes of D-[^{3}H]-Asp output in a typical experiment. (○) Controls; (□) 1S,3R-ACPD (300 μM); (△) 1S,3R-ACPD (300 μM) + BSA (15 μM).

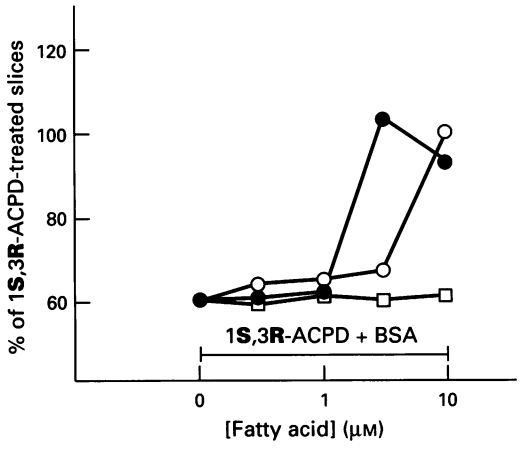

Figure 5 Effects of low concentrations of fatty acids on D-[^{3}H]-Asp output. Cortical slices were perfused with BSA (15 μM) and challenged with a depolarizing solution (KCl, 30 mM) in the presence of 1S,3R-ACPD (300 μM), according to the protocol described in Figure 4. Data are expressed as % of the radioactivity output in control experiments (in which 1S,3R-ACPD was present without BSA or fatty acids). Each point is the mean of 5 duplicate experiments; s.e.mean were within 10% of the values shown. (□) Stearic acid; (●) oleic acid; (○) arachidonic acid.

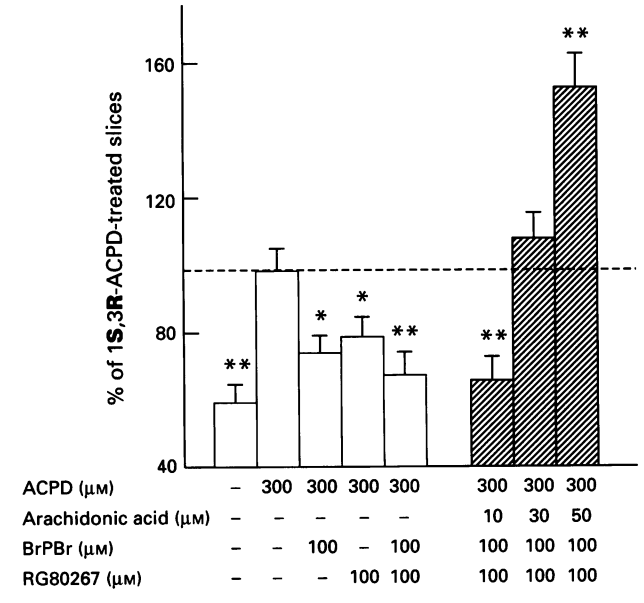

Figure 6 Effects of inhibitors of fatty acid formation on the depolarization (KCl, 30 mM)-induced output of D-[^{3}H]-Asp from cortical slices. Bromophenacyl bromide (BrPBr 100 μM), RG80267 (100 μM) or a combination (100 + 100 μM) of both substances was added to the perfusion fluid at the beginning of the collection of perfusates. Arachidonic acid was added together with 1S,3R-ACPD (300 μM) 5 min before and during the challenge. Each column represents the radioactivity output present in the challenged samples, expressed as % change over control experiments (in which 1S,3R-ACPD was present without enzyme inhibitors). Vertical bars are s.e.mean of at least 4 experiments conducted in duplicate. *$P<0.05$; **$P<0.01$, vs. 1S,3R-ACPD-treated slices.

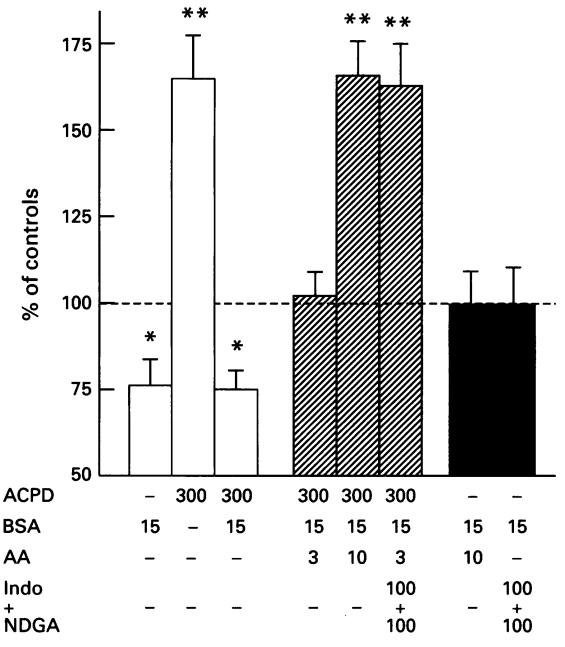

Figure 7 Effects of inhibitors of arachidonic acid (AA) metabolism on the depolarization (KCl, 30 mM)-induced output of D-[^{3}H]-Asp from cortical slices. Indomethacin plus nordihydroguaiaretic acid (Indo + NDGA 100 + 100 μM) were added to BSA containing perfusion fluid together with 1S,3R-ACPD (300 μM) 5 min before the challenge. Each column represents the radioactivity output present in the challenged samples, expressed as % of controls (^{3}H-amino acid output in slices not exposed to 1S,3R-ACPD). Vertical bars are s.e.mean of at least 4 experiments conducted in duplicate. *$P<0.05$; **$P<0.01$ vs. controls.

acid, at concentrations used here, significantly increased the depolarization-induced transmitter output in the presence of BSA. Finally, arachidonic acid (10 μM) did not modify the basal or KCl-evoked outflow of transmitters in the absence of added 1S,3R-ACPD.

Discussion

The present studies show that, in brain slices, the depolarization (KCl, 30 mM)-induced output of both D-[^{3}H]-Asp and [^{3}H]-Glu (neosynthesized after preincubation with [^{3}H]-glutamine) are modulated in a qualitatively and quantitatively similar manner by 1S,3R-ACPD (Figure 3). In particular, in striatal slices, 1S,3R-ACPD (300 μM) inhibits the output of either D-[^{3}H]-Asp or [^{3}H]-Glu by approximately 50%, while in cortical slices, the mGluR agonist potentiates the output of both D-[^{3}H]-Asp and [^{3}H]-Glu in a similar dose-dependent manner. Furthermore, the depolarization-induced output of D-[^{3}H]-Asp or [^{3}H]-Glu has a similar degree of dependence upon the presence of extracellular Ca^{2+} (Figure 1). Our results, showing a good correlation between D-[^{3}H]-Asp output and [^{3}H]-Glu release, suggest that D-[^{3}H]-Asp output is an acceptable index of excitatory transmitter release in brain slices, thus confirming a series of previous observations (Potashner, 1978; Notman *et al.*, 1984; Arqueros *et al.*, 1985; Gallo *et al.*, 1992; Simonato *et al.*, 1994).

Our data also indicate that 1S,3R-ACPD differentially modulates the depolarization-induced (KCl, 30 mM) transmitter exocytosis in the cortex and in the striatum. While the inhibition of transmitter output caused by the mGluR agonist in the striatum does not require the availability of fatty acids, the potentiation of the depolarization-induced transmitter release in cortical slices requires the simultaneous presence of 1S,3R-ACPD and of fatty acids. In fact, BSA, which is able to bind fatty acids (Spector, 1975; Rhoads *et al.*, 1983) or inhibitors of fatty acid formation such as bromophenacyl bromide, an inhibitor of phospholipase A_2 (Hofmann *et al.*, 1982), and RG-80267, an inhibitor of diacylglycerol lipase (Schimmel, 1988), completely prevented the mGluR-induced potentiation of transmitter output. Similar results have been obtained in studies on 4-aminopyridine-induced exocytosis of glutamate from cortical synaptosomes (Herrero *et al.*, 1992; Vazquez *et al.*, 1994; Coffey *et al.*, 1994). Detailed biochemical studies in the above-mentioned model have shown that the simultaneous presence of arachidonic acid and diacylglycerol, formed as a consequence of mGluR stimulation, is necessary to activate synergistically protein kinase C, which is able to phosphorylate several proteins including synapsin 1, MARCKS and a 'delayed rectifier' K^+ channel. The phosphorylation of these proteins probably results in a decreased potassium permeability, which is ultimately responsible for an increase in the number of action potentials and of Ca^{2+} entry in the presynaptic terminal. In the model we used, the potentiation of D-[^{3}H]-Asp release requires the presence of added $CaCl_2$ in the perfusion medium and is dependent on the possibility of obtaining conducted action potentials (see Figure 1 and the results section). Therefore, it is reasonable to propose that the potentiation of transmitter output, we observed in cortical slices, is based on the same biochemical mechanism previously described in synaptosomes (Herrero *et al.*, 1992). In this context it is interesting to note that low concentrations of arachidonate and other unsaturated free fatty acids are able to activate protein kinase C both in brain and in peripheral tissues (McPhail *et al.*, 1984; Seifert *et al.*, 1988). In our model the modulation of transmitter output induced by 1S,3R-ACPD occurred when the depolarizing solution contained 30 mM KCl, while neither the inhibitory nor the excitatory effects of 1S,3R-ACPD were observed when the depolarizing stimulus was a solution containing 50 mM KCl (Figure 2). A precise explanation of this finding requires further experiments, but conducted action potentials are probably still evocable in the presence of 30 mM KCl, as suggested by the experiments with tetrodotoxin, while when the concentration of KCl reaches 50 mM, the neurones are permanently depolarized.

We previously showed that the mGluR involved in the potentiation of transmitter release has pharmacological properties compatible with the so-called group 1 mGluRs (Nakanishi, 1992), which are associated to phospholipase C (Lombardi *et al.*, 1994). Recently, however, we noticed that the pharmacological properties of the mGluR associated to the potentiation of transmitter exocytosis are also compatible with a group of mGluRs associated to phospholipase D (Pellegrini-Giampietro *et al.*, 1994), so further pharmacological studies are necessary to clarify the source of diacylglycerol generated as a result of mGluR stimulation (Vasquez *et al.*, 1994). In contrast, in striatal slices, 1S,3R-ACPD-induced inhibition of transmitter release is due to the activation of a presynaptic mGluR of the 2nd group (namely mGluR2 or mGluR3), negatively associated to adenylyl cyclase (Lombardi *et al.*, 1993; 1994). In order to allow a potentiation of transmitter release, the requirement of arachidonic acid has been regarded with interest because it could be an important step in the formation of synaptic plasticity and long-term potentiation (Nicholls, 1992). In cortical slices, arachidonic acid could be continuously formed due to spontaneous synaptic activity or the joint stimulation of ionotropic and metabotropic glutamate receptors (Dumuis *et al.*, 1988; 1993; Stella *et al.*, 1994). Our experiments show that arachidonic acid could be substituted by other unsaturated fatty acids, such as oleic acid, while stearic acid, a saturated fatty acid which is abundant in brain preparations, is not active. It has been suggested that arachidonic acid and its metabolites, possibly together with highly reactive free radicals which are formed in the course of arachidonic acid metabolism, reduce Glu uptake processes and therefore are able to increase the output of amino acid from brain slices, synaptosomes and glial or neuronal cell cultures (Barbour *et al.*, 1989; Volterra *et al.*, 1992; Simonato *et al.*, 1994). However, the inhibition of uptake processes occurs when the concentration

of arachidonic acid is approximately 100 μM. In the experiments presented here, the concentrations of arachidonic acid were significantly lower (1–10 μM) in a range in which arachidonic acid does not reduce D-[^{3}H]-Asp uptake in brain slices (personal observations). Furthermore, since the additions of large concentrations of inhibitors of arachidonic acid metabolism such as indomethacin and nordihydroguaiaretic acid do not reduce, but actually slightly potentiate arachidonate effects, our experiments strongly support the hypothesis that arachidonic acid metabolites and free radicals formed during its metabolism are not involved in the process of potentiation of transmitter exocytosis.

In conclusion, mGluR agonists may potentiate or inhibit the depolarization-induced transmitter output. In the striatum, the inhibition is mediated by presynaptic mGluRs (namely $mGluR_2$ or $mGluR_3$) negatively associated to adenylyl cyclase (Lombardi *et al.*,1993). In the cortex, the potentiation of transmitter release occurs when 1S,3R-ACPD and unsaturated fatty acids are simultaneously present. The functional role of this biochemical observation in the mechanisms leading to long-term potentiation and to other forms of synaptic plasticity requires further experiments with more selective agonists and antagonists of mGluRs and also studies on cortical preparations obtained from animals lacking in specific mGluR subtypes (Aiba *et al.*, 1994; Conquet *et al.*, 1994).

This study was supported by the National Research Council (C.N.R.), the University of Florence and the European Community Biomed project BMH1-CT 93-1033. The technical assistance of Dr A. Cozzi is gratefully acknowledged.

References

AIBA, A., CHEN, C., HERRUP, K., ROSENMUND, C., STEVENS, C.F. & TONEGAWA, S. (1994). Reduced hippocampal long-term potentiation and context-specific deficit in associative learning in mGluR1 mutant mice. *Cell.*, **79,** 365–375.

ANWYL, R. (1991). The role of the metabotropic receptor in synaptic plasticity. *Trends Pharmacol. Sci.*, **12,** 324–326.

ARQUEROS, L., ABARCA, J. & BUSTOS, G. (1985). Release of D-[^{3}H]-aspartic acid from the rat striatum. *Biochem. Pharmacol.*, **34,** 1217–1224.

AXELROD, J., BURCH, R.M. & JELSEMA, C.L. (1988). Receptor-mediated activation of phospholipase A2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends Neurosci.*, **11,** 117–123.

BARBOUR, B., SZATKOWSKI, M., INGLEDEW, N. & ATTWELL, D. (1989). Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. *Nature*, **342,** 21–28.

BEANI, L., BIANCHI, C., GIACOMELLI, A. & TAMBERI, F. (1978). Noradrenaline inhibition of acetylcholine release from guinea-pig brain. *Eur. J. Pharmacol.*, **48,** 179–193.

COFFEY, E.T., HERRERO, I., SIHRA, T.S., SANCHEZ-PRIETO, J. & NICHOLLS, D.G. (1994). Glutamate exocytosis and MARCKS phosphorylation are enhanced by a metabotropic glutamate receptor coupled to a protein kinase C synergistically activated by diacylglycerol and arachidonic acid. *J. Neurochem.*, **63,** 1303–1310.

CONQUET, F., BASHIR, Z.I., DAVIES, C.H., DANIEL, H., FERRAGUTI, F., BORDI, F., FRANZ-BACON K., REGGIANI, A., MATARESE, V., CONDE', F., COLLINGRIDGE, G.L. & CREPEL, F. (1994). Motor deficit and impairment of synaptic plasticity in mice lacking in mGluR1. *Nature*, **372,** 237–243.

CORRADETTI, R., MONETI, G., MORONI, F., PEPEU, G. & WIERASZKO, A. (1983). Electrical stimulation of the stratum radiatum increases the release and neosynthesis of aspartate, glutamate, and γ-aminobutyric acid in rat hippocampal slices. *J. Neurochem.*, **41,** 1518–1525.

COTMAN, C.W. & HAMBERGER, A. (1979). Glutamate as a cns transmitter: properties of release, inactivation and biosynthesis. In *Amino Acids as Chemical Transmitters. Nato Advanced Study Institute Series* ed. Fonnum, F. pp. 379–412. New York: Plenum Press.

DUMUIS, A., SEBBEN, M., FAGNI, L., PREZEU, L., MANZONI, O., CRAGOE, E.J., Jr. & BOCKAERT, J. (1993). Stimulation by glutamate receptors of arachidonic acid release depends on Na^+/Ca^{2+} exchanger in neonatal cells. *Mol. Pharmacol.*, **43,** 976–981.

DUMUIS, A., SEBBEN, M., HAYNES, L., PIN, J.P. & BOCKAERT, J. (1988). NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature*, **336,** 68–70.

GAITONDE, M.F. (1973). Methods for the isolation and determination of glutamate, glutamine, aspartate and γ-aminobutyrate in brain. In *Research Methods in Neurochemistry*, ed. Marks, N. & Rodnight, R. Vol. 2, pp. 321–359. New York: Plenum Press.

GALLO, V., GIOVANNINI, C. & LEVI, G. (1992). Depression by sodium ions of calcium uptake mediated by non-N-methyl-D-aspartate receptors in cultured cerebellar neurons and correlation with evoked D-[^{3}H]-aspartate release. *J. Neurochem.*, **58,** 406–415.

HERRERO, I., MIRAS-PORTUGAL, M.T. & SÁNCHEZ-PRIETO, J. (1992). Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. *Nature*, **360,** 163–166.

HOFMANN, S.L., PRESCOTT, S.M. & MAJERUS, P.W. (1982). The effects of mepacrine and bromophenacyl bromide on arachidonic acid release in human platelets. *Arch. Biochem. Biophys.*, **215,** 237–244.

LOMBARDI, G., ALESIANI, M., LEONARDI, P., CHERICI, G., PELLICCIARI, R. & MORONI, F. (1993).Pharmacological characterization of the metabotropic glutamate receptor inhibiting D-[^{3}H]-aspartate output in rat striatum. *Br. J. Pharmacol.*, **110,** 1407–1412.

LOMBARDI, G. & MORONI, F. (1992). GM_1 ganglioside reduces ischemia-induced excitatory amino acid output: a microdialysis study in the gerbil hippocampus. *Neurosci. Lett.*, **134,** 171–174.

LOMBARDI, G., PELLEGRINI-GIAMPIETRO, D.E., LEONARDI, P., CHERICI, G., PELLICCIARI, R. & MORONI, F. (1994). The depolarisation-induced outflow of D-[^{3}H]Asp from rat brain slices is modulated by metabotropic glutamate receptors. *Neurochem. Int.*, **24,** 525–532.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 256–275.

LOVINGER, D.M. (1991). Trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) decreases synaptic excitation in rat striatal slices through a presynaptic action. *Neurosci. Lett.*, **129,** 17–21.

MCMAHON, H.T. & NICHOLLS, D.G. (1991). The bioenergetics of neurotransmitter release. *Biochim. Biophys. Acta.*, **1059,** 243–264.

MCPHAIL, L.C., CLAYTON, C.C. & SNYDERMAN, R. (1984). A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. *Science*, **224,** 622–624.

MORONI, F., BIANCHI, C., TANGANELLI, S., MONETI, G. & BEANI, L. (1981). The release of γ-aminobutyric acid, glutamate and acetylcholine from striatal slices: a mass-fragmentographic study. *J. Neurochem.*, **36,** 1691–1697.

NAKANISHI, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science*, **258,** 597–603.

NICHOLLS, D.G. (1992). A retrograde step forward. *Nature*, **360,** 106–107.

NOTMAN, H., WHITNEY, R. & JHAMANDAS, K. (1984). Kainic acid evoked release of D-[^{3}H]aspartate from rat striatum *in vitro:* characterization and pharmacological modulation. *Can. J. Physiol. Pharmacol.*, **62,** 1070–1077.

PELLEGRINI-GIAMPIETRO, D.E., ALBANI, S. & MORONO, F. (1994). Pharmacological characterization of metabotropic glutamate receptors coupled to phospholipase D. *Soc. Neurosci. Abstr.*, **20,** 487.

POTASHNER, S.J. (1987). Effects of tetrodotoxin, calcium and magnesium on the release of amino acids from slices of guinea pig cerebral cortex. *J. Neurochem.*, **31,** 187–195.

REED, R.G., FELDHOFF, R.C., CLUTE, O.L. & PETERS, T. (1975). Fragments of bovine serum albumin produced by limited proteolysis. Conformation and ligand binding. *Biochemistry*, **14,** 4578–4583.

RHOADS, D.E., OSBURN, L.D., PETERSON, N.A. & RAGHUPATHY, E. (1983). Release of neurotransmitter amino acids from synaptosomes: enhancement of calcium-independent efflux by oleic and arachidonic acids. *J. Neurochem.*, **41,** 531–537.

SCHIMMEL, R.J. (1988). The α-adrenergic transduction system in hamster brown adipocytes. *Biochem. J.*, **253,** 93–102.

SCHOEPP, D.D., BOCKAERT, J. & SLADECZEK, F. (1990). Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. *Trends Pharmacol. Sci.*, **11,** 508–515.

SCHOEPP, D.D. & CONN, P.J. (1993). Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol. Sci.*, **14,** 13–20.

SEIFERT, R., SCHACHTELE, C., ROSENTHAL, W. & SCHULTZ, G. (1988). Activation of protein kinase C by *cis*- and *trans*-fatty acids and its potentiation by diacylglycerol. *Biochem. Biophys. Res. Commun.*, **154,** 20–26.

SIMONATO, M., BREGOLA, G., BIANCHI, C. & BEANI, L. (1994). Effect of arachidonic acid on [^{3}H]D-aspartate outflow in the rat hippocampus. *Neurochem. Res.*, **13,** 195–200.

SPECTOR, A.A. (1975). Fatty acid binding to plasma albumin. *J. Lipid Res.*, **16,** 165–179.

STELLA, N., TENCE, M., GLOWINSKI, J. & PREMONT, J. (1994). Glutamate evoked release of arachidonic acid from mouse brain astrocytes. *J. Neurosci.*, **14,** 568–575.

VAZQUEZ, E., HERRERO, I., MIRAS-PORTUGAL, M.T. & SANCHEZ-PRIETO, J. (1994). Role of arachidonic acid in the facilitation of glutamate release from rat cerebrocortical synaptosomes independent of metabotropic glutamate receptor responses. *Neurosci. Lett.*, **174,** 9–13.

VOLTERRA, A., TROTTI, D., CASSUTTI, P., TROMBA, C., SALVAGGIO, A., MELCANGI, R.C. & RACAGNI, G. (1992). High sensitivity of glutamate uptake to extracellular free arachidonic acid levels in rat cortical synaptosomes and astrocytes. *J. Neurochem.*, **59,** 600–606.

WILLIAMS, J.H., ERRINGTON, M.L., LYNCH, M.A. & BLISS, T.V.P. (1989). Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus. *Nature*, **341,** 739–742.

(Received May 10, 1995
Revised July 28, 1995
Accepted August 22, 1995)