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MOLECULAR PHARMACOLOGY, 47:1057–1064 (1995).

Metabotropic Glutamate Receptors Negatively Coupled to Adenylate Cyclase Inhibit *N*-Methyl-D-aspartate Receptor Activity and Prevent Neurotoxicity in Mesencephalic Neurons *In Vitro*

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Received January 23, 1995; Accepted February 2, 1995

SUMMARY

The functional effects of G protein-linked glutamate receptor activation have been studied in mouse mesencephalic neurons *in vitro*. We have been able to identify two receptor classes, one linked to phosphoinositide hydrolysis and another that inhibits adenylate cyclase. The agonist (1*S*,3*R*)-aminocyclopentane-1,3-dicarboxylate (ACPD) affected the two responses with similar potency (EC_{50} = 2 and 7 μM, respectively). In contrast, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine selectively decreased adenylate cyclase activity (EC_{50} = 150 nM), without interfering with the phosphoinositide pathway. Activation of ion channel-linked glutamate receptors in mesencephalic neurons leads to cGMP formation. In this study, we demonstrate that cell pretreatment with ACPD or (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine prevented, in a dose-dependent fashion, *N*-methyl-D-aspartate (NMDA)-induced cGMP formation but not the kainate-stimulated response. The pharmacological profile suggests that receptors that are negatively coupled to adenylate cyclase are responsible for this effect. Coexposure of neurons to ACPD and Ba^{2+}, a K^+ channel blocker, counteracted the ACPD-induced blockade of NMDA receptors, suggesting that activation of K^+ conductances could be involved in the post-transduction events triggered by metabotropic receptors in the mesencephalon. Neuronal treatment with NMDA for 10 min caused a reduction in mitochondrial activity. Direct inhibition of nitric oxide synthase with the inhibitor N^G-nitro-L-arginine or removal of extracellular nitric oxide with reduced hemoglobin did not prevent this metabolic impairment, thus excluding a role for nitric oxide in this test for excitotoxicity. On the contrary, the mitochondrial function was maintained when neurons exposed to NMDA were preincubated with metabotropic receptor agonists. To summarize, our results suggest that metabotropic receptors that are negatively coupled to adenylate cyclase exert modulatory control specifically on NMDA receptor activity. This event could also contribute to the reduction of neurotoxic effects due to NMDA receptor hyperactivity.

The mesencephalic nuclei substantia nigra and ventral tegmental area receive excitatory glutamatergic afferent innervation mainly from the frontal cortex (1, 2) and from the subthalamic (3, 4) and peduncolopontine (5) nuclei. Functional investigations of the role of glutamate in these areas have been carried out both in mesencephalic slices and in primary neuronal cultures (6–11). These studies focused primarily on the effects of ion channel-linked (ionotropic) GluRs of the NMDA and AMPA/kainate subtypes (12) on dopaminergic neurons. In addition, depolarization of substantia nigra dopaminergic cells was observed after stimulation with ACPD, which elicited a slow inward Na^+ current (13). This ligand is selective for other GluRs, which are referred to as mGluRs because of their link to G proteins and intracellular transducers. The recent cloning of mGluR genes revealed a wide heterogeneity of receptor proteins (14), and it is now known that these receptors influence neuronal activity through modulation of a variety of intracellular events (extensively reviewed in Refs. 15 and 16).

Interest concerning the function of mGluRs in the basal ganglia is now growing, because mRNAs for different mGluR types have been detected in distinct neuronal populations, by *in situ* hybridization analysis (17, 18). The substantia nigra pars compacta expresses moderate levels of mGluR1 (at the dopaminergic cell level) and low levels of mGluR4. mGluR3 is present only in neurons and glial cells of the pars reticulata.

ABBREVIATIONS: GluR, glutamate receptor; ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate; CCGI, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)-glycine; NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; PI, phosphoinositide(s); InsP, inositol phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; Quis, quisqualate; AP3, 2-amino-3-phosphonopropionate; NO, nitric oxide; TCA, trichloroacetic acid; RIA, radioimmunoassay.

Both substantia nigra regions were labeled by the probe recognizing mGluR5, whereas no staining was detectable for mGluR2 message (17). RNA for mGluR7 was also detected (18).

Endogenous glutamate, released in the ventral mesencephalon, could simultaneously activate ionotropic GluRs and mGluRs, and the neuronal activity may be the outcome of their mutual modulation through the generation of intracellular signals. We focused our attention on the possible interactions between ion channel- and G protein-linked GluRs in mouse mesencephalic primary cultures enriched in substantia nigra and ventral tegmental area neurons. By measuring second messenger levels, we provide evidence for multiple intracellular pathways triggered by the diverse GluR classes. Notably, we demonstrate that stimulation of mGluRs that inhibit adenylate cyclase causes a negative modulation of NMDA receptor activity.

It has been suggested that persistent and excessive stimulation of NMDA receptors could contribute to the dopaminergic cell damage that occurs with age and in Parkinson's disease (19, 20). A dysfunction in glutamate release could also be responsible for the alteration of the neuronal circuits connecting the ventral tegmental area to the prefrontal cortex that is observed in psychotic patients (20). In this work, we used mesencephalic cell cultures as a model to investigate whether glutamatergic agonists may cause neuronal damage in this brain region. Our results show that NMDA receptor hyperactivity brought about a precocious mitochondrial impairment in mesencephalic neurons and that the neurotoxic action of NMDA could be prevented by mGluR activation.

Experimental Procedures

Materials. Tissue culture media were from Flow Laboratories (Milan, Italy). ^{125}I-cGMP and ^{125}I-cAMP RIA kits and *myo*-[2-^{3}H]-inositol were from Amersham International (Buckinghamshire, UK). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise. Excitatory amino acid ligands were from Tocris (Bristol, UK).

Mesencephalic neuronal cultures. Neurons were obtained from Swiss albino mouse fetuses (embryonic day 13; Charles River, Calco, Italy) by dissection of the anteroventral part of the mesencephalon, according to the method of Prochiantz *et al.* (21). The dissociated cells were plated in 33-mm Falcon culture dishes that had been coated first with 1.5 μg/ml polyornithine and then with 7% (v/v) fetal calf serum in Hanks' salts. The culture medium was composed of a mixture (1:1) of Dulbecco's minimal essential medium and F-12 medium, supplemented with glucose (33 mM), glutamine (2 mM), and HEPES (5 mM). Fetal calf serum was replaced by a mixture of hormones, protein, and salts composed of insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenium salt (30 nM). Under these conditions nonneuronal cell proliferation is greatly reduced and glial cells represent <5% of the total population (22). Immunocytochemistry revealed the presence of tyrosine hydroxylase- and glutamate decarboxylase-positive cells (data not shown).

Determination of cGMP. After 7–9 days *in vitro*, the growth medium was removed and cells were washed twice with a modified Krebs-Ringer medium buffered with HEPES-NaOH, pH 7.4, which contained 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 12 mM glucose. Incubation medium also included 1 mM IBMX, a blocker of cGMP and cAMP phosphodiesterases, and 1 μM tetrodotoxin, to block plasma membrane depolarization and endogenous neurotransmitter release. Experiments were performed at room temperature. After a 10-min preincubation to allow IBMX incorporation and a 5-min treatment with mGluR agonists, neurons were exposed to NMDA and kainate for 1 min, at which time the production of cGMP was maximal (23). NMDA studies were performed in Mg^{2+}-free buffer, preceded by a brief wash of neurons with the same buffer. The reaction was terminated by addition of cold perchloric acid (0.4 N). After at least 15 min in the cold, the preparations were carefully collected, transferred to plastic Eppendorf tubes, and centrifuged at 8000 × *g* for 5 min. The supernatant was transferred to other tubes and neutralized with K_2CO_3, and aliquots were tested for cGMP content by using a commercially available ^{125}I-cGMP RIA kit, as reported previously (23). The pellets from the original centrifugation were dissolved in 0.5 N NaOH and protein content was measured by the method of Lowry *et al.* (24), with bovine serum albumin as the standard.

Determination of cAMP. After preincubation with IBMX for 10 min, neurons were exposed to mGluR agonists for 10 min at room temperature. Experiments were performed either in the presence or in the absence of 10 μM forskolin. Samples were treated as described for cGMP determination. Aliquots of the supernatants were tested for cAMP content by using a commercially available ^{125}I-cAMP RIA kit.

PI hydrolysis. After 7–8 days *in vitro*, neurons were incubated for 24 hr with 2 μCi/ml *myo*-[2-^{3}H]inositol (specific activity, 17 Ci/mmol; Amersham) to label plasma membrane PI. After two washes with the incubation buffer, neurons were stimulated for 10 min with the agonists, in the presence of 5 mM LiCl (to avoid inositol monophosphate degradation) and 1 μM tetrodotoxin. The reaction, conducted at room temperature, was terminated by treatment of the monolayers with 15% ice-cold TCA. After at least 15 min in the cold, the preparations were carefully collected, transferred to plastic Eppendorf tubes, centrifuged at 8000 × *g* for 5 min, and processed as reported previously (22), to separate the pool of labeled InsPs obtained by PI hydrolysis. Briefly, after TCA extraction with diethyl ether, samples were neutralized with sodium tetraborate and the [^{3}H]InsPs were bound to AG1-X8 resin (Bio-Rad) and together eluted with 0.1 M formic acid/1.2 M ammonium formate. The pellets from the initial TCA precipitation were dissolved in 0.5 N NaOH and processed for measurement of the protein content.

NMDA toxicity. Mesencephalic neurons were used for neurotoxicity studies after 7–8 days *in vitro*. After replacement of the growth medium with Mg^{2+}-free buffer, NMDA (plus 1 μM glycine and 1 μM tetrodotoxin) was added to the neuronal cultures for 10 min at room temperature. The NMDA-containing solution was then removed and the original medium was added back to the dishes, which were returned to a 37° incubator, containing 5% carbon dioxide, for various times (30 min, 1 hr, 3 hr, or 24 hr) to allow manifestation of injury. Incubation for 1 hr at 37°, after the 10-min NMDA exposure, was the shortest period in which toxicity was manifested. This incubation time was used in all experiments reported herein. Rinsing of the cells before replacement of their medium or addition of fresh growth medium was found to greatly damage the cultures. Therefore, such manipulations were not routinely performed and the conditioned medium of each dish was set aside during the exposure to NMDA and added back to the dishes. To quantify early cytological damage, mitochondrial activity was assessed by a colorimetric assay with MTT (25).

MTT assay. Yellow MTT is converted to the blue formazan product only by metabolically active mitochondria, and the absorbance is directly proportional to the number of viable cells (25). MTT was added to the cultures at 0.5 mg/ml (final concentration) 1 hr after NMDA treatment, and color was allowed to develop for an additional 1 hr. An equal volume of 0.08 N HCl/isopropanol was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into spectrophotometric cuvettes and read at a test wavelength of 570 nm and a reference wavelength of 630 nm. Data are expressed in arbitrary units, where the absorbance difference (absorbance at 570 nm minus absorbance at 630 nm) in the control samples equals 1 unit.

Statistical analysis. Results are expressed as means ± standard errors. The statistical significance of the differences between means was analyzed by the Student *t* test.

Results

mGluR activation inhibits NMDA receptor-induced cGMP formation in mesencephalic neurons. NMDA-induced cGMP formation was completely blocked when mesencephalic neuronal cultures were pretreated for 5 min with the selective mGluR agonists ACPD (100 μM), CCGI (1 μM) (26), or DHPG (100 μM) (27) before stimulation for 1 min with NMDA (Fig. 1). In contrast, ACPD and CCGI did not affect cGMP formation stimulated by kainate (Fig. 1). Dose-response curves obtained with ACPD and CCGI revealed that these two compounds may affect NMDA receptor activity at very low doses (Fig. 2), with half-maximal effects at approximately 0.5 μM and 10 nM, respectively. The 5-min pretreatment with CCGI had a negligible effect on basal cGMP levels, whereas, in some experiments, ACPD slightly decreased basal values as well (about 10%). Preincubation for 5 min with Quis (10 μM), tested in a single experiment, potently increased cGMP levels, both under control conditions and in the presence of 100 μM NMDA (115% and 169% increases above basal levels, respectively). Quis may also affect AMPA/kainate receptors (12) and, indeed, the activation of this ionotropic GluR class may account for the results obtained. For this reason, the effects of this nonselective agonist on cGMP levels were not further investigated in this study.

To understand which mechanism coupled mGluR with NMDA receptor activity, we exposed neurons to the highest concentration of ACPD tested (100 μM) together with Ba^{2+} at 100 μM, a concentration at which it blocks some K^+ conductances (28–30). Ba^{2+}, by itself, did not modify basal or NMDA-induced cGMP accumulation, but it completely abolished the negative modulation exerted by ACPD on NMDA receptor activity (Fig. 3). General blockers such as tetraethylammonium and 4-aminopyridine (used in the 0.1–10 mM range) or iberiotoxin (1–10 nM), which is selective for the big-conductance, Ca^{2+}-dependent, K^+ channels (31), gave controversial results when added together with mGluR ligands and also greatly affected basal cGMP values (data not shown).

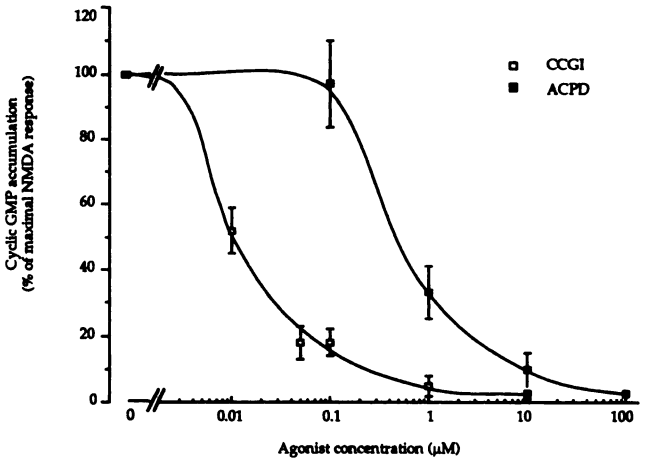

Fig. 2. Dose-response curves for mGluR agonist effects on NMDA-induced cGMP accumulation in mesencephalic neurons. After 5-min pretreatment with the mGluR agonists (at the concentrations indicated), cells were incubated for 1 min with or without NMDA (100 μM). Results are shown as percentages of the maximal NMDA response, which was 60 ± 10% above basal cGMP values (3.20 ± 0.31 pmol/mg of protein), and are the means ± standard errors of at least three experiments performed in triplicate with different cell preparations. EC_{50} values for inhibition were 10 nM for CCGI and 500 nM for ACPD.

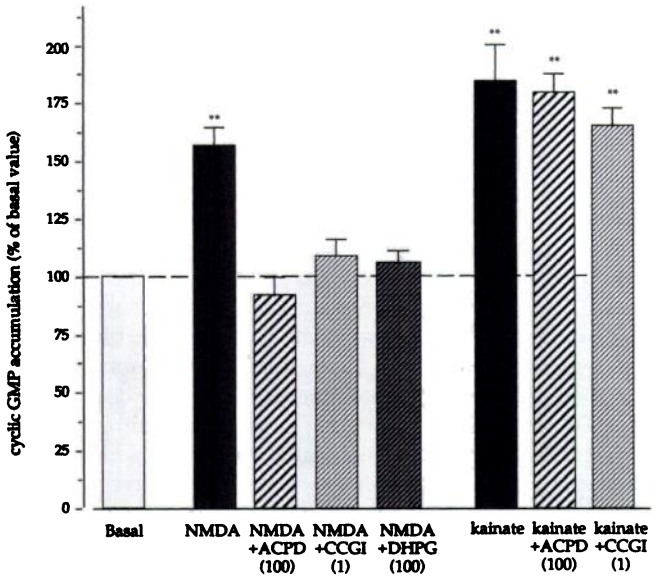

Fig. 1. Effects of mGluR agonists on NMDA- and kainate-induced cGMP accumulation in mesencephalic neurons. After 5-min pretreatment with the mGluR agonists (at the concentrations given in parentheses, in μM), cells were incubated for 1 min with or without NMDA or kainate (both at 100 μM). Results are shown as percentages of basal cGMP values (2.09 ± 0.24 pmol/mg of protein) and are the means ± standard errors of three to five experiments performed in triplicate with different cell preparations. **, $p < 0.01$ in a one-tailed *t* test, compared with controls.

mGluRs modulate second messenger levels in mesencephalic neuronal cultures. To identify which mGluR subtype was responsible for the inhibition of the NMDA-mediated cGMP formation, we investigated the ability of

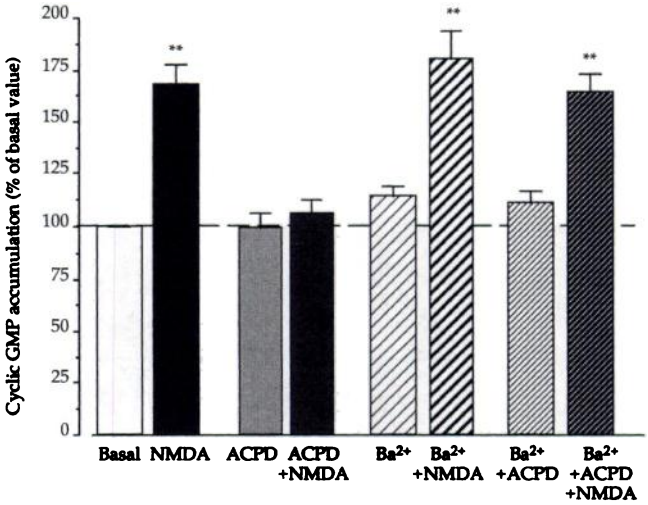

Fig. 3. Ba^{2+} prevention of the ACPD-induced inhibition of NMDA receptor activity. After 5-min pre-exposure to ACPD and/or Ba^{2+} (both at 100 μM), cells were treated for 1 min with or without NMDA (100 μM). Results are shown as percentages of basal cGMP values (2.62 ± 0.28 pmol/mg of protein) and are the means ± standard errors of four experiments performed in triplicate with different cell preparations. **, $p < 0.01$ in a one-tailed *t* test, compared with basal values.

selective mGluR ligands to modulate the intracellular levels of the two second messengers, i.e., InsPs and cAMP (15). As can be seen in Fig. 4, an increase in [^{3}H]InsPs could be induced by ACPD, Quis, and DHPG, with similar potencies. The mGluR agonist CCGI did not affect PI hydrolysis in the concentration range used (Fig. 4). The noncompetitive antagonist AP3 (15) at 10 μM reduced responses to ACPD or Quis by 45–50% (Table 1).

The mGluR agonists were tested for their effects on adenylate cyclase. To compare the influence of mGluRs on this second messenger pathway with their effects on NMDA receptors, we evaluated whether basal cAMP values were modified by the diverse agonists. ACPD and CCGI decreased basal cAMP levels, with E_{max} values for inhibition of 23% at 500 μM ACPD and 36% at 5 μM CCGI (Fig. 5A). DHPG at 100 μM, the concentration used in the studies of NMDA receptor activity shown in Fig. 1, reduced basal cAMP levels by 30 ± 2% (data not shown), whereas the effect of Quis was always stimulatory (Fig. 5A). In Fig. 5B, we report the dose-dependent reduction in cAMP levels observed with mGluR ligands when the enzyme was activated by forskolin (10 μM). The rank order of potency was CCGI ≫ ACPD > DHPG = Quis. The inhibition of forskolin-induced responses was never complete and reached 43% with 5 μM CCGI or 48% with 500 μM ACPD. The maximal inhibition with Quis or DHPG was about 35%. At a higher concentration (500 μM) Quis had a stimulatory effect (data not shown). As mentioned above, however, the latter compound may also affect ionotropic GluRs and, therefore, the results obtained must be carefully interpreted, because more complex intracellular mechanisms may be involved.

mGluR activation protects mesencephalic neurons from NMDA-induced toxicity. L-Glutamate and, more selectively, NMDA and kainate could damage mesencephalic neurons when administered at high concentrations. In particular, in a subpopulation of neurons kainate (100–500 μM) produced acute toxic phenomena, characterized by neuronal swelling that was evident after 5 min of exposure to the neurotoxin (data not shown). NMDA did not cause appreciable morphological changes in 1–3 hr, as determined by visual observation of the cultures. However, its toxicity was detectable by measurement of the ability of neuronal mitochondria to convert the MTT salt into blue formazan (25). Metabolic impairment was already evident in the mesencephalic neurons at 1 hr after their treatment with NMDA for 10 min. This NMDA-related toxicity was dose dependent (Fig. 6A), with maximal effects being seen at 500 μM, and it was completely antagonized by the NMDA channel blocker MK-801 (10 μM) (12), added for 10 min together with NMDA (Fig. 6B).

Treatment with ACPD has been shown to exert neuroprotection from NMDA toxicity in various neuronal cell types (32, 33). We investigated whether the mGluR-mediated modulation of NMDA receptor activity could play a neuroprotective role also in our neuronal system. Indeed, ACPD, CCGI, and DHPG at concentrations that inhibited NMDA-induced cGMP formation (Fig. 1) completely prevented NMDA injury of mesencephalic neurons (Fig. 7).

It has been proposed that excessive release of NO may be neurotoxic in cultured neurons (34). Because the NO-cGMP pathway triggered by NMDA receptors was blocked by mGluR agonists (Fig. 1), we tested whether their mechanism of neuroprotection was dependent on NO synthase inhibition. Preincubation of neurons with the NO synthase blocker N^G-nitro-L-arginine, at doses that completely inhibited cGMP formation (23), did not protect neurons from the toxic effects of NMDA. In addition, 100 μM N^G-nitro-L-arginine was toxic by itself (Fig. 8). Reduced hemoglobin (10 μM), which acts as a chelator of NO released in the extracellular space (33), was also unable to block NMDA-dependent mitochondrial damage and was toxic by itself (Fig. 8).

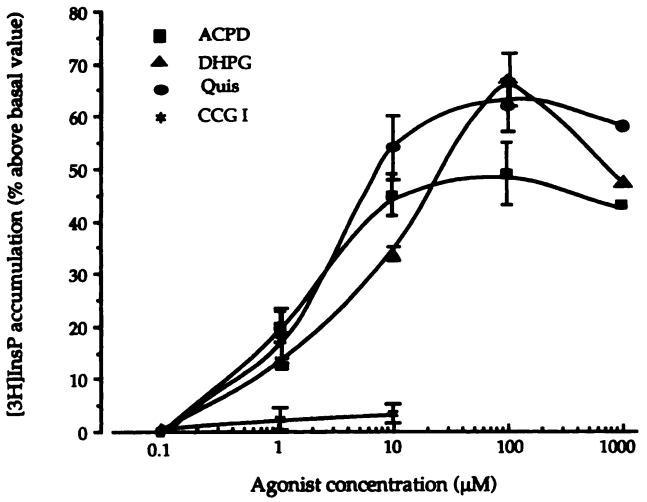

Fig. 4. Dose-response curves for mGluR agonist effects on [^{3}H]InsP formation in mesencephalic neurons. Cells labeled with *myo*-[2-^{3}H]-inositol were stimulated for 10 min with ACPD, CCGI, DHPG, or Quis at the indicated doses. Values, expressed as percentages of basal values (1925 ± 177 cpm/mg of protein), are the means ± standard errors of at least three independent experiments performed in triplicate with different neuronal preparations. Agonists were tested at 1 mM only once. CCGI was ineffective at all concentrations tested; EC_{50} values for the other agonists were 2 μM for ACPD, 2.5 μM for Quis, and 10 μM for DHPG.

TABLE 1

Effects of mGluR ligands on [^{3}H]InsP formation in mesencephalic neurons *in vitro*

Neurons were preincubated for 5 min in the presence or absence of AP3 and were then treated for 10 min with the mGluR agonists ACPD or Quis. Values are expressed as percentages of basal values (2588 ± 280 cpm/mg of protein) and are the averages ± standard errors of at least three independent experiments performed in triplicate.

	[^{3}H]InsP accumulation
	% of basal
Basal	100
ACPD (10 μM)	148 ± 5[a]
Quis (10 μM)	144 ± 4[a]
AP3 (10 μM)	107 ± 8
AP3 + ACPD (both 10 μM)	126 ± 2[b]
AP3 + Quis (both 10 μM)	122 ± 4[b]

[a] $p < 0.01$ in a one-tailed t test, compared with basal levels.
[b] $p < 0.05$.

Discussion

In this study, we provide new evidence for a functional role of mGluRs in mesencephalic neurons *in vitro*. Our main finding is that the stimulation of mGluRs may negatively control the activity of another GluR type, namely the NMDA subtype.

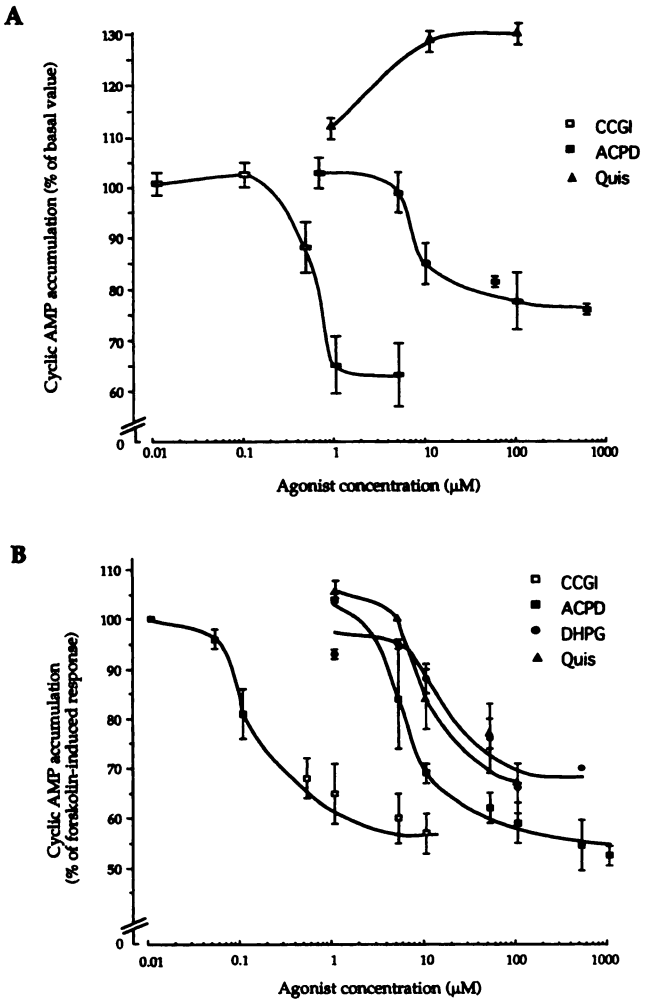

Fig. 5. Concentration-dependent inhibition by mGluR agonists of adenylate cyclase activity in mesencephalic neurons. Neurons were incubated for 10 min under basal conditions or in the presence of forskolin (10 μM), with increasing concentrations of mGluR ligands. A, Effects of ACPD, CCGI, or Quis on basal cAMP levels. Values are expressed as percentages of basal cAMP values (44.48 ± 4 pmol/mg of protein, means ± standard errors of three experiments). B, Effects of ACPD, CCGI, DHPG, or Quis on forskolin-induced cAMP accumulation. Values are expressed as percentages of forskolin-stimulated cAMP levels (494 ± 70 pmol/mg of protein) and are the means ± standard errors of three or four independent experiments performed in triplicate with different neuronal preparations. Under basal conditions (A) Quis was stimulatory, whereas the EC_{50} values of inhibition for the other agonists were 7 μM for ACPD and 500 nM for CCGI. In the presence of forskolin (B) the EC_{50} values were 7 μM for ACPD, 150 nM for CCGI, 12 μM for Quis, and 20 μM for DHPG.

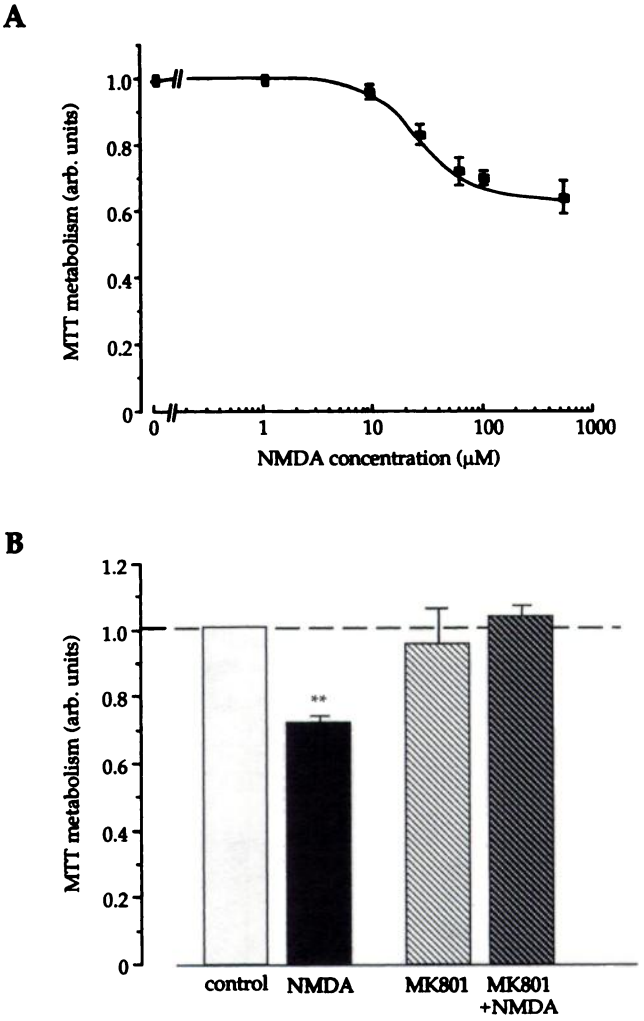

Fig. 6. NMDA-induced toxicity in mesencephalic neuronal cultures. A, Dose-response curves for NMDA effects on MTT conversion, after 10-min incubation under control conditions or in the presence of increasing concentrations of NMDA (1–500 μM). B, Effect of the NMDA antagonist MK-801 (10 μM), added to the cultures during 10-min exposure to 100 μM NMDA. After replacement of NMDA-containing buffer with the original growth medium, dishes were returned to the incubator for 1 hr and then MTT was added for an additional 1 hr, as described in detail in Experimental Procedures. Data (absorbance difference, i.e., absorbance at 570 nm minus absorbance at 630 nm) have been converted to arbitrary units (control values = 1.0) and are the means ± standard errors of at least three independent experiments performed in quadruplicate. **, $p < 0.01$ for 100 μM NMDA in a one-tailed t test, compared with controls.

We have previously shown that, in mesencephalic neurons, ionotropic GluR activation stimulates intracellular generation of cGMP (23). In the present work, we used this biochemical variable as a tool to estimate NMDA or kainate receptor activity after neuronal exposure to selective mGluR agonists. Whereas kainate receptor-induced cGMP formation remained unaffected by ACPD or CCGI, these compounds caused a dose-dependent block of the NMDA response (Figs. 1 and 2).

The recent discovery of selective agonists for the various mGluRs added new information regarding their function in normal and pathological neuronal transmission (reviewed in Ref. 15). In general, slow excitatory events and neurotoxic action appear to be related to PI-linked mGluR activation (35, 36), whereas the inhibitory effects of mGluRs have been considered secondary to the activity of receptors negatively linked to adenylate cyclase (15). An exception is represented by cerebellar neurons, where PI-linked mGluRs reduce cellular excitability (37) and NMDA-related toxicity (33). Our goal was to identify the subtypes functionally expressed in mesencephalic neuronal preparations and the concentration ranges for selective mGluR agonists at which selectivity for a particular subtype was manifested.

Our results clearly demonstrate that mesencephalic neurons *in vitro* express both mGluRs that stimulate PI hydrolysis and mGluRs that inhibit adenylate cyclase (Figs. 4 and 5; Table 1). This is in agreement with the *in situ* hybridiza-

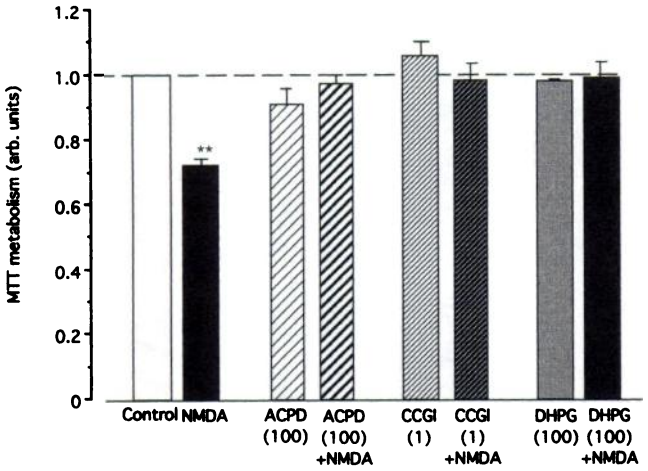

Fig. 7. Effects of mGluR agonists on the NMDA-induced toxicity in mesencephalic neuronal cultures. Neurons were pretreated for 5 min with ACPD (100 μM), CCGI (1 μM), or DHPG (100 μM) and then exposed to NMDA (100 μM) for 10 min. Data (absorbance at 570 nm minus absorbance at 630 nm) have been converted to arbitrary units (control values = 1.0) and are the means ± standard errors of two or three independent experiments performed in quadruplicate. **, $p < 0.01$ in a one-tailed *t* test for NMDA values, compared with controls.

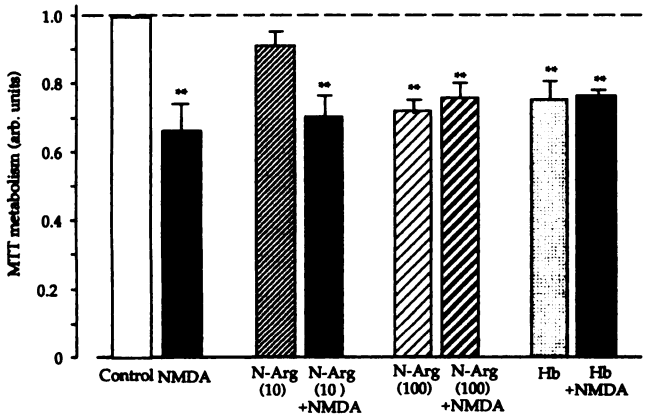

Fig. 8. Effect of NO deprivation on NMDA-induced toxicity in mesencephalic neuronal cultures. Neurons were pretreated for 5 min with N^G-nitro-L-arginine (*N-Arg*) (10 or 100 μM) or reduced hemoglobin (*Hb*) (1 μM) and then exposed to NMDA (100 μM) for 10 min. Data (absorbance at 570 nm minus absorbance at 630 nm) have been converted to arbitrary units (control values = 1.0) and are the means ± standard errors of at least three independent experiments performed in quadruplicate. **, $p < 0.01$ in a one-tailed *t* test, compared with controls.

tion evidence for the expression of multiple mGluR mRNAs in the substantia nigra (17). ACPD and Quis increased intracellular InsPs with similar potencies. Such results differ from those obtained in other cell preparations, where Quis stimulates InsP formation more potently than does ACPD (15). However, Quis may also act on AMPA/kainate receptors, and InsP values measured may be the result of complex intracellular interactions that remain to be investigated. ACPD- and Quis-induced InsP formation was partially reduced by the noncompetitive antagonist AP3 (Table 1). ACPD triggered InsP formation and reduced basal and forskolin-stimulated adenylate cyclase activities with similar EC_{50} values. Another compound, DHPG, has been very recently proposed to be highly selective for the PI-coupled receptors (38). Indeed, it increased InsP levels also in mesencephalic cultures (Fig. 4) but, in our cell preparation, it also affected cAMP levels (Fig. 5B). Therefore, ACPD and DHPG did not discriminate between the two second messenger pathways, and it was not possible to determine, from their actions, which mGluR was responsible for NMDA receptor inhibition. In mesencephalic neurons, PI-linked receptors potentiate NMDA receptor activity, via protein kinase C (23), and a similar effect has been demonstrated in the hippocampus (39). Hence, we expected that activation of PI-linked mGluRs would produce a potentiation and not a block of NMDA-induced cGMP formation. The possibility that such an event occurred in our cell preparation cannot be excluded, but it was probably restricted to a small percentage of neurons and masked by the other prominent effect of negative modulation of NMDA responses.

CCGI was proposed to be selective for mGluR2 and mGluR3 expressed in a non-neuronal cell line, with an EC_{50} that was >1 order of magnitude lower than that for its effects on mGluR1, mGluR4, or mGluR5 (14, 26). In our neuronal preparation, this compound reduced basal and forskolin-stimulated cAMP levels at very low doses (maximal effect at approximately 1–5 μM). We consider it relevant that, at up to 10 μM, CCGI did not stimulate PI turnover but completely prevented NMDA receptor activation. These results strongly suggest that the mGluR responsible for the blockade of NMDA receptors observed herein is a subtype negatively coupled to adenylate cyclase. According to its pharmacology (14, 15), with CCGI being almost 2 orders of magnitude more potent than ACPD, and considering the *in situ* hybridization evidence reported in the literature (17), this receptor is likely the mGluR3 subtype.

The ability of mGluRs to modulate cell excitability through voltage-operated channels has been demonstrated in neurons from several brain areas (16). In mesencephalic neurons, the ACPD modulation of NMDA receptor activity was prevented by Ba^{2+} (Fig. 3). Although this ion does not allow identification of the class of K^+ channels involved, a possible explanation for this result is that ACPD elicited an outward current that hyperpolarized the cells, thus reducing the opening probability of the NMDA receptors, which undergo voltage-dependent Mg^{2+} block (12, 29). This would also be a possible explanation for why kainate receptors, which are voltage insensitive (12), were not influenced by ACPD or CCGI. The application of other K^+ channel blockers did not contribute further to the clarification of the mechanism(s) of such modulation. Indeed, we failed to mimic the action of Ba^{2+} with tetraethylammonium, 4-aminopyridine, or iberiotoxin, because by themselves they caused large increases in cGMP. However, other examples in the literature suggest that some K^+ conductances are particularly susceptible to block by external Ba^{2+} (28–30, 40), compared with other blockers. A contribution of neurotransmitter release appears unlikely, because of the presence of tetrodotoxin in the incubation buffer. In the substantia nigra pars compacta, other neurotransmitter receptors that are negatively linked to adenylate cyclase (dopamine D_2 and γ-aminobutyric acid type B receptors) activate outward K^+ currents and hyperpolarize dopaminergic neurons (28), causing also a decrease in NMDA receptor activity (29). The present study suggests that this could be a general mechanism (41) that is used also by

mGluR3 (or related mGluRs) prominently expressed in the substantia nigra pars reticulata (17).

The action of glutamate in the ventral mesencephalon is the object of increasing interest because of its contribution to human pathological processes, such as parkinsonism or psychoses (26). We used mesencephalic neuronal cultures as a model to study the early neurotoxic events induced by glutamate in this brain area. Here we show that 10-min treatment of mesencephalic neurons with NMDA induced mitochondrial damage that was already appreciable after 1 hr (Fig. 6). Pretreatment of mesencephalic neurons with ACPD (100 μM), CCGI (1 μM), or DHPG (100 μM) completely prevented NMDA-induced mitochondrial metabolic impairment (Fig. 7). It has been proposed that an important component of NMDA toxicity is due to NO release (33). The possibility that mGluR agonists exerted their neuroprotection via inhibition of NMDA-dependent NO formation was investigated. However, formation of this compound did not seem to be relevant for the manifestation of mitochondrial damage, at least under the neurotoxic conditions used in our study. Indeed, neither the NO synthase inhibitor N^G-nitro-L-arginine nor the removal of NO with reduced hemoglobin could prevent NMDA-mediated injury (Fig. 8), and other intracellular events must be considered (42).

To our knowledge, the data shown herein and the results of the electrophysiological study concerning the ACPD-mediated excitation of dopaminergic cells (13) are the only functional indications of mGluR existence in mesencephalic neurons. We think that our results can contribute to the understanding of the regulation of GluR subtypes in this brain region. Moreover, our study strongly encourages the development of compounds that are highly selective for mGluRs negatively linked to adenylate cyclase (as neuroprotective agents), which could have relevance also for human neuropathologies involving dysfunction of mesencephalic nuclei.

Acknowledgments

We thank Dr. P. Calabresi and Dr. N. B. Mercuri for helpful comments.

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