0026-895X/94/040570-08$03.00/0

MOLECULAR PHARMACOLOGY, 45:570–577

Pharmacological Characterization of Metabotropic Glutamate Receptors in Several Types of Brain Cells in Primary Cultures

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Received July 9, 1993; Accepted December 20, 1993

SUMMARY

Several cDNAs coding for metabotropic glutamate receptors (mGluR1–7) have now been isolated. mGluR1 and -5 are positively coupled to phospholipase C, whereas mGluR2, -3, -4, -6, and -7 are negatively coupled to adenylyl cyclase (AC) when they are expressed in Chinese hamster ovary or baby hamster kidney cells. However, the exact transduction mechanisms of these receptors in their natural environment remain to be determined. In a previous work, we demonstrated that striatal neurons in primary culture expressed a mGluR that is negatively coupled to AC and that has a pharmacology different from that of mGluR2. In the present study, the pharmacology of mGluRs negatively coupled to AC in several neuronal types and in glial cells was compared with the pharmacology of mGluR2, -3, and -4. Like striatal neurons, cerebral cortical neurons express a mGluR that is able to inhibit AC both in intact cells and in membrane preparations, via a pertussis toxin-sensitive G protein. This mGluR has a pharmacological profile similar to that of mGluR3, because quisqualate is active at relatively low concentrations ($EC_{50} < 100$ μM). Similar experiments revealed that cerebellar granule cells expressed mGluR2-like and mGluR4-like receptors. Striatal glial cells also expressed a mGluR negatively coupled to AC via a pertussis toxin-sensitive G protein. However, only glutamate and aspartate, and not quisqualate, 2-(carboxycyclopropyl)glycine, *trans*-1-aminocyclopentane-1,3-dicarboxylate, or L-2-amino-4-phosphonobutyrate, were agonists for this glial mGluR. This pharmacology is different from that of any cloned mGluR. Reverse transcription associated with polymerase chain reaction revealed that mGluR2 and mGluR3 mRNAs are present in striatal, cortical, and cerebellar neurons but not in striatal glial cells. Interestingly, mGluR4 mRNA was found at a high level in cerebellar granule cells and at a lower level in cortical neurons and glial cells. However, the mGluR4-specific agonist L-2-amino-4-phosphonobutyrate was found to inhibit AC very slightly in granule cells only. In conclusion, our data show that mGluR2- and mGluR3-like receptors can directly inhibit AC in neurons, and they raise the question of whether mGluR4 is really negatively coupled to AC in its normal environment. We also present evidence for a new mGluR subtype expressed in glial cells.

Glutamate is the major excitatory neurotransmitter in the central nervous system and activates both ionotropic receptors and mGluRs. The ionotropic glutamate receptors are ligand-gated channels classified into three main subtypes named according to their specific agonists, i.e., NMDA, AMPA, and KAI (1). The mGluRs are coupled to intracellular effector proteins (phospholipase C, AC, or cGMP phosphodiesterase) or to ionic channels (K^+ or Ca^{2+} channels) via G proteins. These receptors were first described as being glutamate receptors coupled to phospholipase C in neurons (2–5). The existence of such mGluRs was confirmed by the cloning of several mGluRs (mGluR1 and mGluR5) that are indeed coupled to this second messenger pathway (6–9). More recently, several mGluR clones have been isolated that are homologous to mGluR1 but inhibit cAMP production when expressed in cell lines (10–12). This second group of mGluRs consists of glutamate-preferring receptors (mGluR2 and mGluR3) and L-AP4-preferring receptors (mGluR4 and mGluR6) (11–13). The transduction mechanisms of these receptors in their natural environment remain, however, to be elucidated.

We previously demonstrated that several EAAs, i.e., glutamate, *t*-ACPD, and QA, inhibit FK-induced cAMP production in cultured striatal neurons via a PTX-sensitive G protein (14,

This work was supported by grants from CNRS, INSERM, Bayer France/Troponwerke (Germany), and International Human Frontier Science Program Organization (Strasbourg, France), by Direction des Recherches Etudes et Techniques Contract 91161 and by Rhône-Poulenc Rorer, Recherche et Developpement. K.C. is a Medical Research Council of Canada Fellow.

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino,3-hydroxy-5-methyl-4-isoxazolepropionic acid; KAI, kainate; L-AP4, L-2-amino-4-phosphonobutyrate; L-AP3, L-2-amino-3-phosphonopropionate; ACPD, 1-aminocyclopentane-1,3-dicarboxylate; AC, adenylyl cyclase; QA, quisqualate; CCGI, 2-(carboxycyclopropyl)glycine I; CCGII, 2-(carboxycyclopropyl)glycine II; FK, forskolin; EAA, excitatory amino acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PTX, pertussis toxin; PCR, polymerase chain reaction; RT, reverse transcription; HEPES, 4-(2-hydroxyethyl)-1-piperazine-*N*′-[2- ethanesulfonic acid]; IBMX, 3-isobutyl-1-methylxanthine; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; SDS, sodium dodecyl sulfate.

15). These results were obtained both in intact cells and with membrane preparations, demonstrating the direct coupling of these receptors to inhibitory G proteins. Their pharmacology is significantly different from that reported for mGluR2 (10) and mGluR4 (11, 12) but is close to that of mGluR3 (11).

The aim of the present study was, first, to establish the existence of mGluRs directly inhibiting AC in several types of brain cells and, second, to compare their pharmacology with that of the cloned mGluRs. For this purpose, we used well characterized primary neuronal cultures prepared from mouse cerebral cortex, striatum, or cerebellum and primary cultures of striatal astrocytes. We chose these cellular types because, in adult rat brain, mGluR2 mRNAs are highly expressed in cerebral cortex and are weakly expressed in the striatum and the cerebellar cortex (except in Golgi cells) (10), mGluR3 mRNAs are found mainly in the cerebral cortex and the striatum and in glial cells and to a lesser extent in cerebellar granule cells (11), and mGluR4 mRNAs are highly expressed in cerebellar granules cells and weakly expressed in striatum and cerebellar cortex (11).

Experimental Procedures

Materials. Agents used for this study were obtained from the following sources. *t*-ACPD, QA, AMPA, and CNQX were from Tocris Neuramin (Buckhurst Hill, Essex, England). MK-801 was a gift from Merck Sharp and Dohme (Darmstadt, Germany). [2-^{3}H]Adenine was from Amersham (Little Chalfort, Buckinghamshire, UK), [α-^{32}P]ATP from NEN (les Ulis, France), the 12-well clusters for tissue cultures from Costar (Broadway, Cambridge, MA) or Falcon (Becton Dickinson, Grenoble, France), and Dulbecco's minimal essential medium and F-12 nutrient from GIBCO Europe (Paris, France). IBMX, NMDA, KAI, and FK were from Sigma (L'Isle d'Abeau, France). PTX was from List Biological Laboratories (Campbell, CA). All other compounds were of the highest available grade from commercial sources. L-CCGI was prepared from L-methionine in 12 steps, according to a procedure described by Shinozaki and co-workers (16, 17), and (1*S*,3*S*)-ACPD was synthesized as described previously (18).

Cerebral cortical neuronal cultures. Primary cultures of cortical neurons were prepared as described for striatal neurons (14), in serum-free medium. Briefly, pregnant mice (Iffa Credo, Lyon, France) were killed by decapitation and 16-day-old embryos were removed and decapitated. Cortical structures were dissected, and cells were gently dissociated in culture medium with a fire-narrowed Pasteur pipette and were plated (10^6 cells/well, 1 ml/well), in medium containing 10% fetal calf serum, in 12-well cluster plates that had been previously coated with poly-L-ornithine (15 μg/ml; M_r 40,000). Culture medium was composed of a 1:1 mixture of Dulbecco's minimal essential medium and F-12 nutrient supplemented with 30 mM glucose, 2 mM glutamine, 3 mM sodium bicarbonate, and 5 mM HEPES buffer. Under these conditions, cultures are highly enriched in neurons that form many mature and functional synapses (19). Only 7% of the cells have been identified as astrocytes after 12 days *in vitro*.

Cerebellar granule cell cultures. One-week-old mice were killed by decapitation. The cerebellum was removed sterilely. Before experimentation, the cells were maintained in culture for 10 days, as described (20), in 12-well culture dishes (10^6 cells/dish) that had been previously coated with poly-L-ornithine (15 μg/ml; M_r 40,000). Culture medium was composed of a 1:1 mixture of Dulbecco's minimum essential medium and F-12 nutrient, supplemented with 30 mM glucose, 2 mM glutamine, 3 mM sodium bicarbonate, 25 mM KCl, 6.6% fetal calf serum, 3.4% horse decomplemented serum, and 5 mM HEPES. Cytosine arabinoside was added 48 hr after plating to prevent proliferation of non-neuronal cells.

Striatal glial cell cultures. The striatal structures were obtained as described previously (14). The culture medium used always contained 10% fetal calf serum. The medium was changed after 1 week and subsequently twice each week until the cells reached confluence, at approximately 3 weeks (21, 22).

Measurement of cAMP formation in intact cells. The cellular cAMP content was measured using the prelabeling technique, as described previously (23). After 6 days, cells were washed and incubated at 37° (5% CO_2/95% air mixture) with 2 μCi/ml [^{3}H]adenine (24 Ci/mol). After 2 hr, cultures were washed and incubated for 10 min at 37° in 1 ml of HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% glucose, 20 mM HEPES, pH 7.2) containing 0.75 mM IBMX, tested agents, and 10 μM FK. Ca^{2+} was omitted from the incubation buffer to avoid Ca^{2+} influx, which may influence AC activity as well as the release of neurotransmitters. Antagonists were preincubated for 5 min before the beginning of the reaction. The reaction was stopped by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid. Cells were scraped with a rubber policeman and 100 μl of 5 mM ATP and 5 mM cAMP were added to the mixture. Cellular proteins were removed by centrifugation at 5000 × *g* and [^{3}H]ATP and [^{3}H]cAMP were separated by sequential chromatography on Dowex and alumina columns. cAMP formation is expressed as percentage conversion, i.e., % conversion of [^{3}H]ATP to [^{3}H]cAMP = ([^{3}H]cAMP × 100)/([^{3}H]cAMP + [^{3}H]ATP).

Measurement of AC activity in membranes. After removal of the culture medium, cells were washed three times with 5 ml of a homogenate medium (10% sucrose, 1 mM EGTA, 5 mM EDTA, 20 mM Tris·HCl, pH 7.4). Cells were loosened with a rubber scraper and centrifuged for 10 min at 39,000 × *g* at 4°. Pellets were resuspended in 2 ml of the same medium. AC activity was assayed at 30° in a 100-μl incubation medium containing 80 mM Tris·HCl, pH 7.4, 2 mM $MgCl_2$, 50 μM ATP, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 1 mM IBMX, 1 mM cAMP, 50 μM GTP, 1 μCi of [α-^{32}P]ATP, and 2 nCi of [^{3}H]cAMP. Membranes were incubated for 10 min and the reaction was stopped by addition of 900 μl of a mixture containing 5.5 mM Tris·HCl, pH 7.4, 0.4 mM ATP, 0.6 mM cAMP, 0.1 N HCl, and 10 mM $CaCl_2$. The cAMP was isolated as previously described by Salomon *et al.* (24).

PTX treatment and ADP-ribosylation experiments. For PTX treatment, cells were incubated with the toxin at the indicated final concentration. Cells were then washed and incubated with [^{3}H]adenine for cAMP determination, as described above. For the back-ADP-ribosylation experiment, cells were loosened with a rubber scraper and membranes were pelleted for 15 min at 4° in an Eppendorf centrifuge and were resuspended in 20 mM Tris·HCl buffer, pH 8.0, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.05% Lubrol PX. Samples were then incubated, unless otherwise indicated, for 60 min at 30° in 70 mM Tris·HCl buffer, pH 8.0, containing 0.5 μM NAD, 1.5 μCi of [α-^{32}P]NAD (800 Ci/mmol), 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 1 mM EDTA, 0.1 mM $MgCl_2$, 2 mg/ml L-myristylphosphatidylcholine, 10 mM nicotinamide, and 25 mM dithiothreitol, in a 60-μl final assay volume. The procedure was then followed as described previously (25).

mRNA detection by RT-PCR. This procedure was carried out as previously described by Kawasaki *et al.* (26). Briefly, 1 μg of total RNA from cultured cells, extracted as described (27), was transcribed into cDNA in a final volume of 20 μl of RT-PCR buffer containing 1 mM levels of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 20 units of RNAsin, 100 pmol of random nonamers, and 200 units of Moloney murine leukemia virus reverse transcriptase (Stratagene). After 10 min at room temperature and 60 min of incubation at 42°, the reaction was stopped by heating at 95° for 10 min. For the PCR amplification, the following reagents were added to 1 μl of the RT reaction (adapted from Ref. 26): 0.2 mM deoxynucleoside triphosphates, 50 pmol of primers, 2 mM $MgCl_2$, 5 μl of 10× enzyme buffer containing 500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1% Triton X-100, and 2.5 units of *Thermus aquaticus* polymerase (Promega). The 40 cycles were as follows: 94° denaturation for 30 sec, annealing of primers at a specific temperature for each set of primers for 30 sec, and extension at 72° for 30 sec. The amplification products were analyzed on agarose gels. Sequences of the oligonucleotides used as primers (Bioprobe,

Montreuil-Sous-Bois, France) were based on the published rat mGluR sequences (6, 7, 10). The mGluR1-specific primers were as follows: sense primer 1a, 5′-AAATCTACAGCAATGCTGGCGA-3′ (bases 779–800); antisense primer 1b, 5′-CTTCGATGACTTCATCTCTGTC-3′ (bases 964–985); the product generated by amplification is 206 bases long. The mGluR2-specific primers were as follows: sense primer 2a, 5′-CCCACTCTCTGCGGGCCGTGCC-3′ (bases 1091–1112); antisense primer 2b, 5′-CCACTGCCTGCCCGCAGATAGGT-3′ (bases 1369–1391); the product generated by amplification is 300 bases long. The mGluR3-specific primers were as follows: sense primer 3a, 5′-GCTCCAACATCCGCAAGTCCTA-3′ (bases 746–767); antisense primer 3b, 5′-TGTCAATGGCCAGGTGCTTGTC-3′ (bases 1120–1141); the product generated by amplification is 395 bases long. The mGluR4-specific primers were as follows: sense primers 4a, 5′-TGAGCTACGTGCTGCTGGCG-3′ (bases 1874–1893); antisense primer 4b, 5′-TGTCGGCTGACTGTGAGGTG-3′ (bases 2221–2240); the product generated by amplification is 566 bases long. The mGluR5-specific primers were as follows: sense primer 5a, 5′-GTCTCCTGATGTCAAGTGGTT-3′ (bases 987–1007); antisense primer 5b, 5′-GGACCACACTTCGTCATCATC-3′ (bases 1480–1500); the product generated by amplification is 514 bases long. The specificity of each set of primers was examined using rat mGluR1, -2, -3, -4, and -5 plasmid DNA as template.

Blotting and hybridization of the amplification products with mGluR probes. The amplification products were blotted on nylon membranes and hybridized with probes specific for each mGluR. These probes were prepared from isolated rat mGluR cDNAs and labeled by random priming. Prehybridization of membranes was performed at 65° for 2 hr in a buffer containing 5× SSPE (99 M NaCl, 0.05 M NaH_2PO_4, 5 mM EDTA, pH 7.4), 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml herring sperm DNA. Hybridization was performed at 65° in 5× SSPE, 1× Denhardt's solution, 0.5% SDS, 100 μg/ml herring sperm DNA, and the membranes were then washed at high stringency (0.1× standard saline citrate (15 mM NaCl, 1.5 mM Na_3 citrate, pH 7), 0.5% SDS) for 1 hr at 65°.

Results

Direct coupling of mGluRs to AC in cortical and cerebellar granule cells. Glutamate activates both mGluRs and ionotropic receptors. Therefore, to avoid any effect of ionotropic receptors, the experiments were performed in the presence of 10 μM MK-801 (a noncompetitive NMDA receptor antagonist) and 30 μM CNQX (a specific competitive antagonist of KAI/AMPA receptors). We used Ca^{2+}-free medium and 3 μM tetrodotoxin (a Na^+ channel blocker) to avoid any agonist-induced intracellular Ca^{2+} increase due to cell depolarization and release of neurotransmitters.

Glutamate inhibited FK-induced cAMP production in cortical neurons (EC_{50} = 53.00 ± 4.55 μM and E_{max} = 53.2 ± 2.0% of inhibition), as well as in cerebellar granule cells (EC_{50} = 32.5 ± 4.2 μM and E_{max} = 42.0 ± 4.2% of inhibition), in a concentration-dependent manner (Fig. 1A). This inhibition was likely direct, because an inhibition of AC activity was also observed with membranes prepared from both types of neuronal cultures (EC_{50} = 30 μM and 35 μM and E_{max} = 20 and 15%, respectively) (Fig. 1B).

Evidence that the glutamate-induced inhibition of cAMP production stimulated by FK is mediated by a PTX-sensitive G protein. To demonstrate that the glutamate-induced inhibition of AC activity was mediated via a G protein, the effect of PTX on entire cells was analyzed. PTX is known to inhibit the G_i subtypes of G proteins, which are always involved in the inhibition of AC by receptors. Preincubation of both cell types with increasing concentrations of PTX

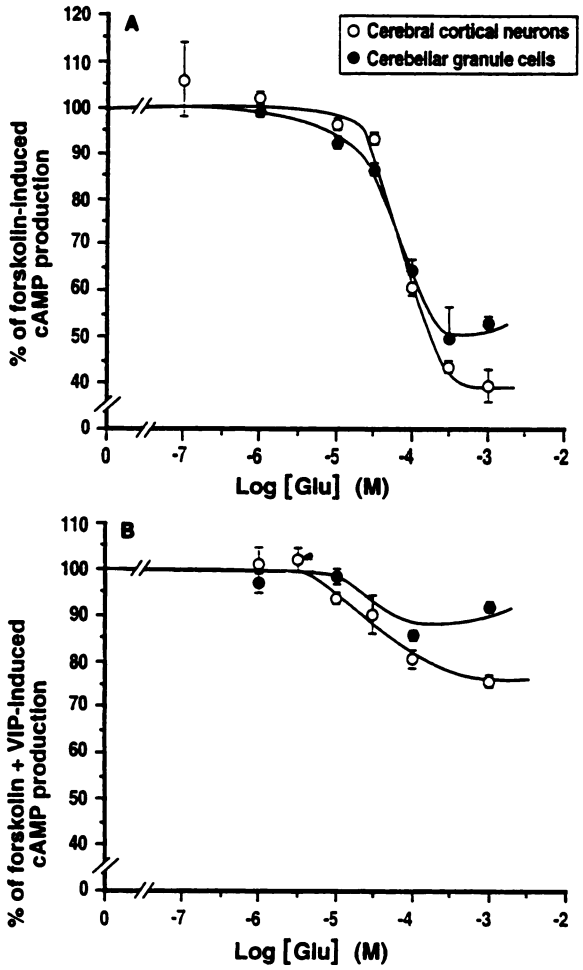

Fig. 1. Concentration-dependent inhibition by glutamate of FK-stimulated cAMP accumulation and AC activity in cortical and cerebellar granule neurons. A, Glutamate-induced inhibition was assayed in entire cells by measuring the intracellular level of cAMP in the presence of 10 μM FK. In cortical neurons, the conversion of [^{3}H]ATP to [^{3}H]cAMP was 3.000 ± 0.450% and 0.243 ± 0.055% in the presence and absence of FK, respectively. These values were 13.800 ± 1.300% and 0.496 ± 0.048% in granule cells of cerebellum. B, In membrane preparations, the effect of glutamate on AC activity was assayed for 5 min in the presence of 1 μM FK and 0.1 μM vasoactive intestinal peptide (*VIP*). Under these conditions, formation of cAMP from ATP was 35.4 ± 6 and 6.4 ± 0.7 pmol/min/mg of protein in the presence and absence of stimulating agents, respectively. These results are representative of a total of three separate experiments. Each value is the mean ± standard error of triplicates.

led to a reduction, but not a total suppression, of glutamate-induced inhibition of cAMP production stimulated by FK (Fig. 2A). To determine whether all PTX-sensitive G proteins present in these neuronal cultures were blocked by this PTX pretreatment, we performed ADP-ribosylation of membranes from cells that had been preincubated with PTX. This experiment indicated that not all PTX-sensitive G proteins were ADP-ribosylated even after the long preincubation period used (16 hr) and with the highest concentration of PTX that could reasonably be used (1000 ng/ml) (Fig. 2B).

Effect of other EAAs on FK-stimulated AC of cerebral cortical neurons and cerebellar granule cells. Although glutamate had the same potency and efficacy in inhibiting AC activity in cortical and cerebellar neurons, the rank orders of potencies for the other agonists were clearly different. Gluta-

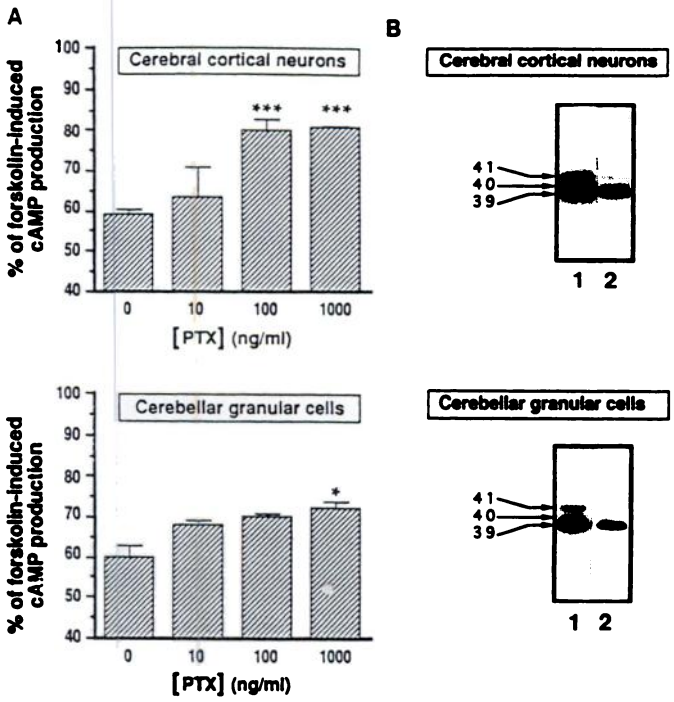

Fig. 2. Effect of PTX on glutamate-induced inhibition of cAMP formation in cortical neurons and cerebellar granule cells. A, Concentration-dependent reduction of glutamate-induced effect by PTX. Neurons were pretreated with PTX, as described in Experimental Procedures, before being incubated with glutamate (300 μM). In the presence of FK, conversion of [^{3}H]ATP to [^{3}H]cAMP was 2.4 ± 0.2% in cortical neurons and 12.4 ± 1.3% in cerebellar granule cells. Each value is the mean ± standard error of triplicates and is representative of three experiments. B, PTX-catalyzed ADP-ribosylation of cortical and cerebellar granule neuron membranes. *Lane 1*, untreated membranes; *lane 2*, membranes prepared from neurons treated overnight with PTX (1000 ng/ml). *Arrows*, apparent molecular mass (in kDa). Only the relevant part of the autoradiogram is shown. This experiment was performed two times. ***, $p<0.001$ estimated with student's *t* test; *, $p<0.05$.

mate was much more efficacious in inhibiting cAMP production than was *t*-ACPD in cerebral cortex and cerebellar granule cells (EC_{50} = 60 ± 20 μM and 177 ± 64 μM and E_{max} = 38 ± 4% and 27 ± 3% inhibition, respectively) (Fig. 3A). In contrast, QA was far more potent in cortical neurons (EC_{50} = 42 ± 11 μM), compared with cerebellar granule cells (EC_{50} > 300 μM) (Fig. 3B).

Recently, several new glutamate receptor agonists have been described [L-CCGI, L-CCGII, and (1*S*,3*S*)-ACPD] (13, 28, 29). The agonist L-CCGI is more potent with mGluR2 than with mGluR1 or mGluR4. In both cerebellar granule and cortical neurons L-CCGI inhibited cAMP formation at low concentrations (EC_{50} = 2.1 ± 1.1 μM and 1.5 ± 0.6 μM, respectively) but had a weak efficacy (E_{max} = 24 ± 4% and 22.0 ± 1, 5%, respectively) (Fig. 3C). Note that L-CCGI-induced inhibition was reversed at concentrations higher than 10 μM in cerebellar granule cells but not in cortical neurons (Fig. 3C). As expected, L-CCGII was less potent than L-CCGI in both cell types (data not shown). (1*S*,3*S*)-ACPD seemed to stimulate more than one receptor, because its concentration-dependent curve sloped only slightly for cortical neurons (EC_{50} and E_{max} values of 14.2 ± 9.0 μM and 30.7 ± 5.4%, respectively) and was biphasic for cerebellar granule cells (EC_{50} = 2.5 ± 0.5 μM and 188.6 ± 11.0 μM and E_{max} = 12.5 ± 2.5% and 15 ± 5%) (Fig. 3D).

AP3, which has been reported to be a putative mGluR antagonist with receptors expressed in cerebral cortical neurons and cerebellar granule cells (data not shown).

L-AP4 effect on FK-stimulated cAMP production in cerebral cortical neurons and cerebellar granule cells. L-AP4 inhibited cAMP production in cerebral cortex with a very low potency. Significant inhibition was observed only for concentrations higher than 100 μM (Fig. 4A). In contrast, L-AP4 inhibited cAMP production in cerebellar granule cells at low concentrations (approximately 3 μM). The efficacy was very weak (E_{max} = 12 ± 1.5%) (Fig. 4B) but was statistically significant ($p \leq 0.003$; paired *t* test; four experiments). At concentrations higher than 100 μM an additional inhibition was observed (Fig. 4B). The inhibitory effect of L-AP4 on AC could be masked by a stimulatory effect of this compound at concentrations higher than 10 μM (note the positive slope of the dose-response curves for concentrations between 10 and 100 μM).

Glutamate-induced inhibition of AC in cultured striatal glial cells. Glutamate inhibited FK-induced cAMP formation in a dose-dependent-manner (EC_{50} = 17.3 ± 6.7 μM and E_{max} = 49.1 ± 3.5% inhibition) in striatal glial cell cultures. Surprisingly, *t*-ACPD (Fig. 5), L-CCGI, and L-AP4 (data not shown) were inactive. QA was a weak agonist (Fig. 5). Some endogenous EAAs, such as aspartate, were good agonists (data not shown). AP3, known to be a putative antagonist of mGluRs, was an agonist at concentrations higher than 500 μM (data not shown).

The inhibition of FK-induced cAMP production by glutamate was partly PTX sensitive (Fig. 6A). Surprisingly, treatment of the cells with 100 ng/ml PTX led to complete ADP-ribosylation of the PTX-sensitive G proteins and to incomplete suppression of the glutamate effect on cAMP production (Fig. 6B).

Characterization of mRNAs coding for mGluRs in cultured brain cells. The presence of mRNAs coding for mGluRs in the neuronal and glial cultures used here and in a previous study (14) was examined by PCR after RT of total RNAs, using specific primers derived from rat mGluR sequences. The amplification products had the expected molecular weight and hybridized with probes specific for each mGluR subtype (Fig. 7). We examined not only the presence of mRNA coding for receptors negatively coupled to AC but also mRNA coding for the phospholipase C-coupled mGluR1 and -5. In good agreement with the *in situ* hybridization experiments in adult rat brain, mRNAs coding for mGluR1 were detected in cerebellar granule cells and not in striatal neurons (Fig. 7). mGluR5 mRNAs were detected in striatal and cortical neurons, as well as in striatal glial cells, but not in cerebellar granule cells (Fig. 7). mGluR2 and mGluR3 mRNAs were found in striatal, cortical, and cerebellar neurons but not in striatal glial cells (Fig. 7). mGluR4 mRNAs were detected in every cell type studied in this work, with a higher apparent level in cerebellar granule cells (Fig. 7).

Discussion

In the three different cell types studied in this report, i.e., cerebral cortical neurons, cerebellar granule cells, and striatal glial cells, glutamate inhibited FK-stimulated cAMP formation. This inhibition was partly PTX sensitive and was also observed in membranes prepared from both neuronal cell types. How-

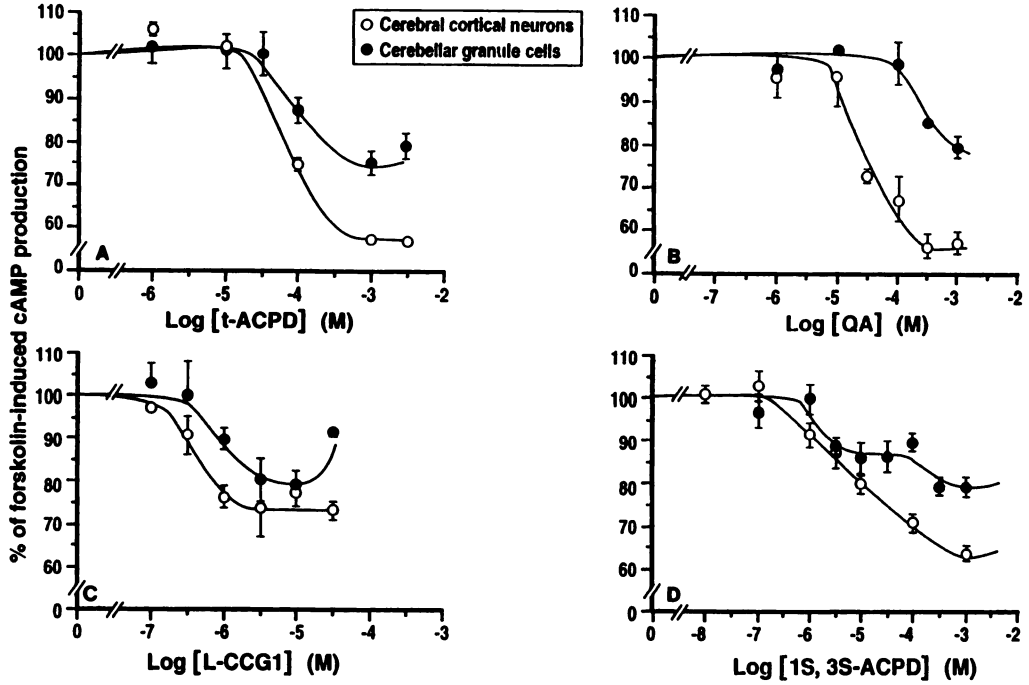

Fig. 3. Effect of several mGluR agonists on FK-induced cAMP production in cortical neurons and in cerebellar granule cells. Neurons were exposed for 10 min to the indicated concentrations of *t*-ACPD (A), QA (B), L-CCGI (C), or (1*S*,3*S*)-ACPD (D), in the presence of 10 μM FK. In cortical neurons the conversion of [^{3}H]ATP to [^{3}H]cAMP was 2.91 ± 0.23% and 0.261 ± 0.022% in the presence and absence of FK, respectively. These values were 13.40 ± 0.72% and 0.50 ± 0.07%, respectively, in cerebellar granule cells. These results are representative of a total of four separate experiments. Each value is the mean ± standard error of triplicates.

ever, we were unable to completely ADP-ribosylate the PTX-sensitive G protein in intact neuronal cells, even with high concentrations of the toxin and very long incubation periods. This is likely due to a difference in the access of PTX to the cytoplasmic compartment of these cells. Nevertheless, these results indicate that mGluRs directly coupled to the G_i subtype of G proteins, and therefore inhibiting AC, do exist in these cells.

The potency of glutamate to inhibit AC is similar in every cell type tested (Table 1). However, the pharmacological profiles established with different agonists clearly indicate that these different cells may express different mGluR subtypes negatively coupled to AC. Based on our experiments, at least three main pharmacological profiles can be distinguished.

The first one is found in cerebral cortical neurons and striatal neurons (Table 1). In these cells, L-CCGI is the most potent agonist, followed by glutamate and QA, which have similar potencies, and then by *t*-ACPD. This pharmacological profile is very similar to that reported for mGluR3 (11, 30), although QA, which is slightly less potent than *t*-ACPD at mGluR3, is slightly more potent than *t*-ACPD at mGluRs expressed in these neurons. The presence of mGluR3-like receptors inhibiting AC in striatal and cerebral cortical neurons is in agreement with the presence of mGluR3 mRNA in the adult rat cortex and striatum (11) and in these cultured cells.

The second pharmacological profile for the inhibition of AC is found in cerebellar granule cells. In these cells also L-CCGI has a high potency; however, QA is far less potent, compared with striatal or cortical neurons. This pharmacological profile resembles that of mGluR2 expressed in Chinese hamster ovary cells (10). The presence of mGluR2-like receptors inhibiting AC in cerebellar neurons in culture is in agreement with the presence of mGluR2 mRNA in these cells.

In striatal glial cells, the pharmacological profile of EAA-induced inhibition of AC is different from that observed in cultured neurons or with any cloned mGluRs and therefore defines our third group. Indeed, among the classical mGluR agonists, only glutamate is able to inhibit AC in these cells, whereas *t*-ACPD, QA, and L-CCGI have no effect or a very small effect. At the RNA level we detected only mGluR4 mRNA and not mRNA coding for mGluR2 or -3. This is surprising, because very recently (31) primary cultures of glial cells prepared from rat cerebral cortex have been shown to express a mGluR3-like receptor. This indicates that, depending on their origin (striatum versus cortex) or their *in vivo* maturation (cells were isolated from either embryos or newborn animals), glial cells may not express the same mGluR subtypes. This is in agreement with what has been reported for different brain regions, in which glial cells do not express the same patterns of receptors (22, 32).

We have certainly simplified the interpretation of our experimental data. These neurons clearly express more than one type of mGluRs negatively coupled to AC. Indeed, mRNAs coding for several mGluRs were detected in each type of cells. This is likely the reason why the E_{max} obtained with glutamate was always higher than those obtained with *t*-ACPD, QA, or L-CCGI (Table 1) and why the dose-response curves for (1*S*,3*S*)-ACPD were shallow (in cerebral cortical neurons) or biphasic (in cerebellar granule cells). This may also explain the apparent slight difference between the pharmacological profiles characterized here and those reported for cloned mGluRs.

It is noteworthy that striatal and cortical neurons, on one hand, and cerebellar granule cells, on the other hand, contain both mGluR2 and mGluR3 mRNAs but the inhibition of AC is mediated mainly by mGluR3-like receptors in striatal and

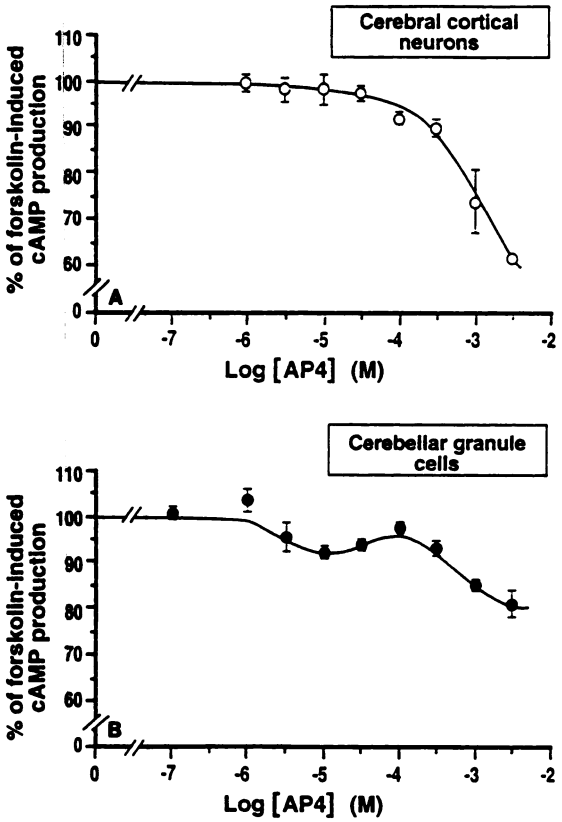

Fig. 4. Concentration-dependent inhibition of FK-stimulated cAMP accumulation by L-AP4 in cortical and cerebellar granule neurons. In cortical neurons, the conversion of [^{3}H]ATP to [^{3}H]cAMP was 2.800 ± 0.200% and 0.335 ± 0.044% in the presence and absence of FK, respectively. These values were 11.0 ± 1.5% and 0.350 ± 0.070% in cerebellar granule cells. These results are representative of a total of four separate experiments. Each value is the mean ± standard error of triplicates.

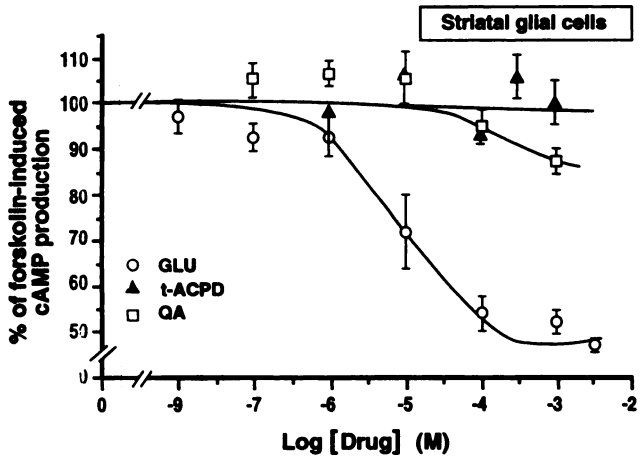

Fig. 5. EAA concentration-dependent inhibition of FK-induced cAMP production in striatal glial cells. Cells were exposed for 10 min to the indicated concentrations of the agonists glutamate, *t*-ACPD, and QA, in the presence of 10 μM FK. The conversion of [^{3}H]ATP to [^{3}H]cAMP was 7.56 ± 1.40% and 0.157 ± 0.016% in the presence and absence of FK, respectively. These results are representative of a total of four separate experiments. Each value is the mean ± standard errror of triplicates.

cortical neurons and mGluR2-like receptors in cerebellar granule cells. The procedure we used to detect specific mRNA does not allow us to estimate the relative amounts of mGluR2 and mGluR3 mRNA, so that, even though both RNAs are present,

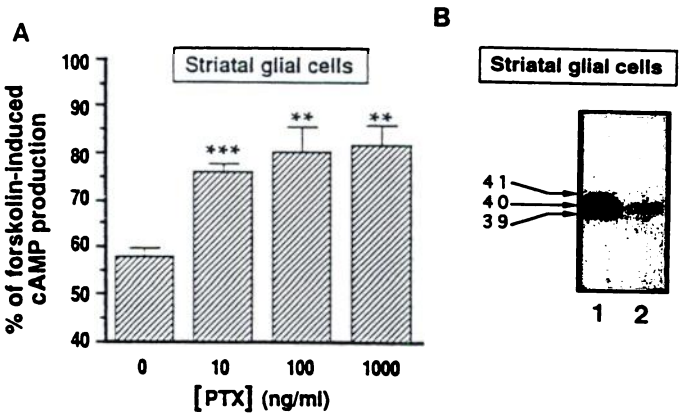

Fig. 6. Effect of PTX on glutamate-induced inhibition of cAMP formation in striatal glial cells. A, Concentration-dependent reduction of glutamate-induced effect by PTX. Glial cells were pretreated overnight with PTX as described in Experimental Procedures before being incubated with glutamate (300 μM). Each value is the mean ± standard error of triplicates and is representative of three experiments. B, PTX-catalyzed ADP-ribosylation of striatal glial cell membrane preparations. *Lane 1*, untreated membranes; *lane 2*, membranes prepared from cells that had been treated overnight with PTX (100 ng/ml). *Arrows*, apparent molecular mass (in kDa). Only the relevant part of the autoradiogram is shown. This experiment is representative of two experiments. ***, $p<0.001$ estimated with Student's *t* test; **, $p<0.03$.

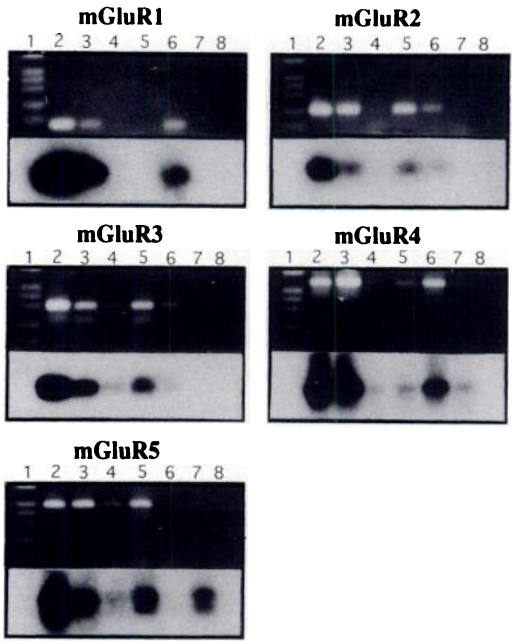

Fig. 7. mGluR mRNA expression determined by RT-PCR analysis and hybridization with mGluR subtype-specific probes, in the different cultured cell types. Total RNAs from adult rat brain, adult mouse brain, cortical neurons, striatal neurons, cerebellar granule cells, and striatal glial cells were transcribed in cDNAs by RT. The presence of the cDNAs for mGluR1, -2, -3, -4, and -5 was determined by PCR amplification using specific oligonucleotide primers, and products were visualized on ethidium bromide-stained agarose gels (*top*). The amplified products were blotted and hybridized with mGluR-specific probes (*bottom*). *Lane 1*, molecular weight markers (from *top* to *bottom*, 653, 517, 453, 394, 298, and 234 base pairs); *lane 2*, adult rat brain; *lane 3*, adult mouse brain; *lane 4*, striatal neurons; *lane 5*, cortical neurons; *lane 6*, cerebellar granule cells; *lane 7*, striatal glial cells; *lane 8*, control without RNA. These results are representative of two distinct experiments performed with two different cultures and mRNA preparations.

TABLE 1

EC_{50} and E_{max} values of mGluR agonists in striatal, cortical, and cerebellar granule neurons and in striatal glial cells, and comparison with the pharmacological profile determined for the different mGluR subtypes

	Glutamate		t-ACPD		QA		L-CCGI		AP4		Comparison with cloned receptors
	EC_{50}	E_{max}[a]	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	
	μM	%	μM	%	μM	%	μM	%	μM	%	
Striatal neurons[b]	53 ± 19	48 ± 4	156 ± 38	40 ± 5	27 ± 10	30 ± 4	8 ± 2	16 ± 3	Inactive up to 100 μM		mGluR3-like
Cerebral cortical neurons	53 ± 4	53 ± 2	60 ± 20	38 ± 4	42 ± 11	48 ± 1	1.5 ± 0.6	22 ± 2	Inactive up to 100 μM		mGluR3-like
Cerebellar granule cells	32 ± 4	42 ± 4	177 ± 64	27 ± 3	>300		2 ± 1	24 ± 4	12% at 10 μM		mGluR2-like + mGluR4-like
Striatal glial cells	17 ± 6	49 ± 3	Inactive		143 ± 25	24 ± 3	Inactive		Inactive		?

[a] E_{max} values are percentage inhibition of FK-stimulated cAMP formation.
[b] Data from Prézeau *et al.* (14).

it is possible that one mRNA type is expressed at a higher level than the other. Moreover, the relative amount of mRNA is not necessarily related to the relative amounts of the proteins encoded by these RNAs.

It is also interesting to note that, although mRNA coding for mGluR4 is present in striatal and cortical neurons and striatal glial cells, the specific mGluR4 agonist L-AP4 does not inhibit cAMP formation at low concentrations. Only in cerebellar granule cell cultures, where a large amount of mGluR4 mRNA is found, is the inhibition of cAMP production by L-AP4 significant with a range of concentrations known to activate mGluR4 receptors (11, 12). However, this effect remains very small. The reason for the weak efficacy of of L-AP4 is unknown. Several possibilities exist, i.e., 1) the efficiency of coupling to AC of mGluR4 is weaker than that of mGluR2/3, as already suggested by expression of these receptors in Chinese hamster ovary cells (11) or baby hamster kidney cells (12), 2) the expression of functional receptors is not in direct relationship to the expression of mRNA, or 3) the inhibition of AC by mGluR4 is in part masked by a stimulatory effect of L-AP4 on this enzyme, as observed in cerebellar granule cells (Fig. 4). This latter effect would be surprising, because L-AP4 has never been shown to stimulate AC. Although our present data do not exclude this possibility, more work is necessary to characterize the effect of this drug on AC activity.

In conclusion, cultured neurons clearly express mGluRs that are negatively coupled to AC and have pharmacological profiles similar to those reported for cloned mGluRs. Cerebral cortical neurons and striatal neurons express mainly (but certainly not exclusively) mGluR3-like receptors, whereas cerebellar granule cells express mGluR2-like receptors and to a lesser extent mGluR4-like receptors. Striatal glial cells in culture express mGluRs, negatively coupled to AC, that have not yet been cloned.

Acknowledgments

We are grateful to Angie Turner-Madeuf, Mireille Passama, and Laurent Charvet for their skillful assistance in the preparation of this manuscript. We wish to thank Dr. Nakanishi for the generous gift of the rat mGluR4 cDNA.

Note Added in Proof

After this report was submitted, Okamoto *et al.* reported the cloning and pharmacological characterization of mGluR7, which is specifically activated by L-AP4, with a low affinity. It is therefore possible that the AC inhibition we observed in cortical and cerebellar granule neurons with high concentration of L-AP4 is due to mGluR7. (Okamoto, N., S. Hori, C. Akazawa, Y. Hayashi, R. Shigemoto, N. Mizuno, and S. Nakamishi. Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. *J. Biol. Chem.* **269**:1231–1236 (1994).)

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