Both Enantiomers of 1-Aminocyclopentyl-1,3-dicarboxylate Are Full Agonists of Metabotropic Glutamate Receptors Coupled to Phospholipase C

OLIVIER MANZONI, LAURENT PREZEAU, FRANCOIS A. RASSENDREN, FRITZ SLADECZEK, KEN CURRY, and JOEL BOCKAERT

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, F-34094 Montpellier, Cedex 5, France (O.M., L.P., F.S., J.B.), Centre de Recherches de Biochimie Macromoléculaire CNRS-INSERM, 34033 Montpellier, Cedex 1, France (F.A.R.), and The University of British Columbia, Department of Physiology, Vancouver, Canada V6T 1W5 (K.C.)

Received December 11, 1991; Accepted May 12, 1992

SUMMARY

We tested the effects of two enantiomers of a glutamate analogue, (trans)-1-aminocyclopentyl-1,3-dicarboxylate (t-ACPD), in striatal and cerebellar neurons in primary culture, as well as in Xenopus oocytes injected with cerebellar rat RNA. In the presence of MK-801, to avoid N-methyl-p-aspartate receptor activation, and 3 μ M tetrodotoxin, both enantiomers [(1R,3S)- and (1S,3R)-t-ACPD] stimulated inositol phosphate (InsP) formation both in striatal neurons after 9–11 days in vitro [EC₅₀, 3.7 ± 1.1 μ M, three experiments, and 33 ± 7.5 μ M, three experiments; maximal stimulatory effects, $252 \pm 15\%$, 13 experiments, and $269 \pm 15\%$ of basal InsP formation, 14 experiments, for (1R,3S)and (1S,3R)-t-ACPD, respectively] and in cerebellar granule cells after 9–11 days in vitro [EC₅₀, 50 \pm 18 μ M, four experiments, and 307 \pm 92 μ M, four experiments; maximal stimulatory effects, 401 \pm 71%, eight experiments, and 423 \pm 75% of basal InsP formation, eight experiments, for (1R,3S)- and (1S,3R)-t-ACPD, respectively]. These effects were not additive, indicating that

both enantiomers acted at the same receptor molecule. When we monitored t-ACPD-induced increases in intracellular Ca2+ concentration ([Ca²⁺]_i) with fura-2 ratio-imaging, we found that both enantiomers could elicit similar increase in [Ca2+], in the presence of 1 µM MK-801 and 3 µM tetrodotoxin; these effects were also observed in the absence of external Ca2+. Moreover, in Xenopus oocytes injected with adult rat cerebellar RNA, both drugs elicited oscillatory increases of a Ca2+-dependent chloride conductance, with similar efficacy, with (1R,3S)-t-ACPD being the more potent isomer. These data are in contradiction to previous reports showing that, in "immature" cerebellar neurons and adult hippocampal slices, (1S,3R)-t-ACPD was either the only active enantiomer or a full agonist of metabotropic receptors, with (1R,3S)-t-ACPD being ineffective or a partial agonist. However, performing these experiments in immature (2-3 days in vitro) striatal or cerebellar neurons, we found that only (1S,3R)t-ACPD was active in stimulating $[Ca^{2+}]_{i}$.

Downloaded from molpharm.aspetjournals.org at Bibl Univ Med Odontologie on October 4, 2012

The excitatory amino acids aspartate and GLU are generally considered the major excitatory neurotransmitters in the vertebrate central nervous system. Three types of ionotropic receptors are stimulated by excitatory amino acids, the NMDA, the AMPA, and the kainate receptors (1). In striatal neurons in primary cultures, we have shown that excitatory amino acid receptors are able to stimulate phospholipase C and, therefore, the formation of InsP via a guanine nucleotide-binding proteincoupled QA-preferring receptor called the metabotropic GLU receptor (2-4). Molecular cloning recently revealed a family of metabotropic receptors termed mGluR1 to mGluR4 (5-7). We demonstrated that the metabotropic GLU receptor was able to activate protein kinase C and that its activity was regulated by protein kinase C (8). Only a few specific drugs have been shown to interact with the metabotropic GLU receptor. In striatal neurons, we have recently shown that t-ACPD, a drug proposed as a metabotropic receptor-specific agonist in hippocampal slices (9, 10), activates InsP formation (11). In this system, neither AMPA nor NMDA receptors were activated by concentrations of t-ACPD (0.1-1 mM) that induced a maximal InsP response, showing that t-ACPD was a metabotropic receptorspecific agonist (11). In these experiments, we used a racemic mixture (1:1) of two enantiomers of t-ACPD (1S,3R and 1R,3S).

This work was supported by grants from CNRS (Centre National de la Recherche Scientifique), INSERM (Institut National de la Santé et de la Recherche Médicale), Bayer France/Troponwerke (FRG), DRET (Direction des Recherches Etudes et Techniques) (Contract 91/161), and International Human Frontier of Science Program Organization. K.C. is a Canada MRC Fellow.

ABBREVIATIONS: GLU, glutamate; *t*-ACPD, (*trans*)-1-aminocyclopentyl-1,3-dicarboxylate; InsP, inositol phosphate(s); TTX, tetrodotoxin; NMDA, *N*-methyl-D-aspartate; [Ca²⁺], intracellular calcium concentration; AMPA, α -aminohydroxy-5-methyl-4-isoxazolzpropionate; QA, quisqualate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; fura-2/AM, fura-2/acetoxymethyl ester.

CULAR PHARMAC

It has been reported that, in cerebellar neurons maintained in culture for 2 days, only (1S,3R)-t-ACPD was able to stimulate the metabotropic GLU receptor and induce calcium mobilization (12). Measuring InsP formation in hippocampal slices, Schoepp *et al.* (13) showed that (1R,3S)-t-ACPD was a partial agonist with weak potency in neonatal slices [(1S,3R)-t-ACPD being a full agonist] and was almost ineffective in adult slices.

When we tested the two enantiomers, (1S,3R)- and (1R,3S)t-ACPD, with metabotropic GLU receptors of striatal neurons maintained for 9–11 days *in vitro*, we found that both enantiomers were active. We, therefore, decided to study in more detail the possible origin of this discrepancy, using different models expressing the metabotropic GLU receptor, i.e., striatal neurons, cerebellar granule cells, and *Xenopus* oocytes injected with rat cerebellum RNA.

Experimental Procedures

Materials

Agents used for this study were obtained from the following sources: EGTA from Sigma (L'Isle d'Abeau, France); racemic t-ACPD and CNQX from Tocris Neuramin (Essex, England); MK-801, as a gift, from Merck Sharp and Dome; myo- $[2-^{3}H]$ inositol from C.E.A. (Saclay, France); the 12-well clusters and 35-mm-diameter dishes for tissue cultures from Costar (Cambridge, MA); Dulbecco's minimal essential medium and F-12 nutrient from GIBCO Europe (Paris, France); fura-2/AM from Sigma; and GLU-pyruvate transaminase and pyruvic acid from Boehringer Mannheim (France). All other compounds were of the highest possible grade, from commercial sources.

Methods

Neuronal cultures. Primary cultures of mouse striatal neurons were prepared in serum-free medium, as previously described (14), and mouse cerebellar granule cells were maintained in culture for 3 or 9-10 days before experimentation, as described by Van-Vliet *et al.* (15).

InsP formation. InsP formation in striatal neurons and in cerebellar granule cells was examined as previously described (16). After 11 days in vitro, the culture medium was replaced with HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂, 1.1 mM CaCl₂, 0.1% glucose, 0.1% bovine serum albumin, 20 mM HEPES, pH 7.2), and neurons were incubated for 10 min with 10 mM LiCl, in order to block InsP degradation. In order to avoid any contamination by endogenous GLU, InsP formation in cerebellar granule cells was measured in the presence of GLU-pyruvate transaminase (1 unit/ml) and pyruvic acid (1 mm). Agents were used at the indicated concentrations and at the indicated times. The incubation temperature was 37°, and the reaction was stopped after an additional 30 min, by replacement of the incubation medium with 5% perchloric acid, on ice. The InsP were extracted and separated by ion exchange chromatography. The elution procedure was simplified by application of 24 ml of 1 M ammonium formate/0.1 M formic acid buffer immediately after the 40 mM sodium formate elution, in order to elute all InsP in a single fraction.

Preparation of t-ACPD enantiomers. Both (1R,3S)- and (1S,3R)-t-ACPD were prepared and separated as previously described (18).

 $[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ measurements were performed as we previously described (13). Briefly, after 5–13 days *in vitro*, mouse striatal neurons or cerebellar granule cells were loaded with the fluorescent calcium chelator fura-2/AM, by incubation with 3.3 μ M fura-2/AM for 1 hr at 37°, in HEPES-buffered saline without bovine serum albumin. After the incubation, the cells were washed three times in the same buffer. The coverslips containing the loaded and washed cells were mounted for microscopic analysis.

For $[Ca^{2+}]_i$ measurements by ratio-imaging, the cells were alternatively excited with 340- and 380-nm light, and the emission was measured at 500 nm (18), on a Zeiss Axiovert 10 inverted microscope equipped with a photomultiplier and a MSP 21 microprocessor (responsible for the filter changes). The images were taken by a low light level SIT LH 4036 camera from Lhésa (Paris, France) and digitized in real time by a Quantel Crystal imaging system. During the 2-sec excitation period at each wavelength, we averaged eight videoframes/digitized image. Each image was coded on $512 \times 576 \times 8$ bits. The camera dark noise was subtracted from the recorded crude images. [Ca²⁺]_i was estimated from the images obtained after division, using the procedure described by Grynkiewicz *et al.* (17). The constants necessary for this procedure were obtained with fura-2 free acid standard solutions. The whole-image treatments were done with Turbo-Pascal homemade software. For pharmacological experiments on cell soma, [Ca²⁺]_i was determined on the final image individually for each cell soma, using the Crystal particle analysis package together with homemade software.

Expression of rat brain RNA in *Xenopus* oocytes and electrophysiological detection of metabotropic GLU receptors. Adult female mouse cerebellar RNA was prepared as previously described (19), using a phenol-chloroform extraction procedure. *Xenopus* oocytes were dissociated with a collagenase treatment (2 mg/ml, type 1a) and kept in ND96 (96 mM NaCl, 1 mM KCl, 2 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5 with NaOH), with 50 units/liter gentamicin. RNA injection was performed with a Drummond micropipette; each oocyte was injected with 50–70 nl of brain RNA, at 2 mg/ml. After injection, oocytes were kept in ND96 for 3–4 days at 19°, to allow protein expression. Electrophysiological recordings were carried out with a Dagan Corner Stone double-microelectrode voltage amplifier. Oocytes were placed in a 300- μ l perfusion chamber and continuously bathed with ND96. Pharmacological agents were applied directly to the bath, with the perfusion being stopped.

Data analysis. Estimates of EC₅₀ (potency) and E_{max} (efficacy) were obtained by fitting the equation

$$E = E_{\max} \frac{L}{\text{EC}_{50} + L}$$

to the observed response E, as a function of drug concentration L, using nonlinear regression analysis (Grafit; Erith Software Ltd., UK).

Results

t-ACPD Enantiomer-Induced InsP Formation and Intracellular Ca²⁺ Mobilization in Differentiated Neurons

Cross-reactivity of t-ACPD enantiomers with NMDA receptors has been reported in some systems (4) but not in others (11). To avoid any interactions, we performed all our experiments in the presence of 10 μ M MK-801. Moreover, to avoid activation of voltage-sensitive sodium channels, all experiments were performed in the presence of 3 μ M TTX.

InsP formation. As shown in Fig. 1, both (1R,3S)- and (1S,3R)-t-ACPD were able to stimulate InsP formation in striatal neurons and in cerebellar granule cells after 11 days *in vitro*. In striatal neurons, the EC₅₀ values were $3.7 \pm 1.1 \,\mu$ M (three experiments) and $33 \pm 7.5 \,\mu$ M (three experiments) for (1R,3S)- and (1S,3R)-t-ACPD, respectively (compared with $16.5 \pm 3.5 \,\mu$ M, three experiments, for the racemic commercial mixture). The maximal effects of both enantiomers were not additive (1 mM induced effects were $252 \pm 15\%$ of basal formation, 13 experiments, and $269 \pm 15\%$ of basal formation, 14 experiments, for (1R,3S)- and (1S,3R)-t-ACPD, respectively, compared with 303 ± 41 , five experiments, for a mixture of both enantiomers) and not different from the effects of the commercial racemic mixture.

In cerebellar granule cells after 11 days in vitro, we found EC_{50} values of $50 \pm 18 \,\mu\text{M}$ (four experiments) and $307 \pm 92 \,\mu\text{M}$ (four experiments) for (1R,3S)- and (1S,3R)-t-ACPD, respectively (in this set of experiments, the EC_{50} of the racemic





Fig. 1. A, Dose-response curves for (1*R*,3*S*)- and (1*S*,3*R*)-*t*-ACPD stimulation of InsP formation in striatal neurons after 11 days *in vitro*. Experiments were performed in the presence of 1 μ M MK-801, to avoid NMDA receptor activation. Values represent means ± standard errors of three to five independent experiments, in which each data point was obtained in duplicate. Basal InsP formation was 875 ± 58 dpm. B, Dose-response curves for (1*R*,3*S*)- and (1*S*,3*R*)-*t*-ACPD stimulation of InsP formation in cerebellar granule cells after 11 days *in vitro*. Experiments were performed in the presence of 1 μ M MK-801, to avoid NMDA receptor activation. Values represent means ± standard errors of three or four independent experiments, in which each data point was obtained in duplicate. Basal InsP formation was 1580 ± 123 dpm.

commercial mixture was equal to $114 \pm 40 \mu$ M, four experiments). Maximal effects were at $401 \pm 71\%$ of basal formation (eight experiments) and $423 \pm 75\%$ of basal formation (eight experiments) for (1R,3S)- and (1S,3R)-t-ACPD, respectively.

Intracellular calcium mobilization. As expected from InsP experiments, both enantiomers were able to increase $[Ca^{2+}]_i$ in striatal neurons after 9–11 days in vitro. We examined a total of 380 cells in five different preparations and found that 114 cells responded to 1 mM (1S,3R)-t-ACPD and 118-1 mM (1R,3S)-t-ACPD. Among these 380 cells, 95 responded to both enantiomers. Fig. 2A shows a typical experiment, in which we measured the effects of both enantiomers in 77 cells; 13 responded to 1 mM (1R, 3S)-t-ACPD applied first, with an average maximal increase of $[Ca^{2+}]_i$ equal to 237 ± 36 nM. A subsequent application of 1 mm (1S,3R)-t-ACPD elicited an increase of $[Ca^{2+}]_i$ equal to 221 ± 55 nM in three neurons [that all responded to (1R,3S)-t-ACPD; basal $[Ca^{2+}]_i$ was 44 ± 14 nM]. These effects were still observed in a buffer without added Ca^{2+} , showing that they were not a consequence of extracellular calcium entry into the cell. In a typical experiment, repeated three times, we found, in the absence of external Ca^{2+} , that basal $[Ca^{2+}]_i$ was 111 ± 15 nM (n = 17) and maximal $[Ca^{2+}]_i$ induced by application of 1 mM (1S,3R)-t-ACPD was 184 \pm 7 nM (n = 11); this value reached 193 ± 19 nM (n = 17) in the presence of 1 mm (1R, 3S)-t-ACPD. Under these experimental conditions, NMDA (100 μ M) was not able to increase [Ca²⁺]_i. We tried to perform dose-response curves for both enantiomers; we never detected reliable increases in the $[Ca^{2+}]_i$ with doses



Fig. 2. Effects of maximal doses (1 mM) of (1*R*,3*S*)- and (1*S*,3*R*)-*t*-ACPD in increasing the [Ca²⁺], in differentiated striatal neurons and cerebellar granule cells in primary culture. A, Effects of both enantiomers in striatal neurons after 11 days *in vitro*. Data represent means ± standard errors of [Ca²⁺], determinations performed in three to 13 neurons, in the presence of 1 μ M MK-801 and 3 μ M TTX. B, Same as in A but with cerebellar granule cells after 11 days *in vitro*. Data represent means ± standard errors in the presence of 1 μ M MK-801 and 3 μ M TTX. B, Same as in A but with cerebellar granule cells after 11 days *in vitro*. Data represent means ± standard errors of [Ca²⁺], determinations performed in 15 to 54 neurons, in the presence of 1 μ M MK-801 and 3 μ M TTX. ***, $\rho < 0.001$ as determined with Student's *t* test.

lower than 100 μ M, regardless of the enantiomer used, whereas maximal responses were obtained at 500 μ M for some cells and 1000 μ M for others [basal, 85 ± 8 nM (n = 14); maximal [Ca²⁺]_i, 196 ± 13 nM (n = 14) and 164 ± 16 nM (n = 6), in the presence of 100 μ M (1S,3R)- or (1R,3S)-t-ACPD, respectively].

Similar results were observed in differentiated cerebellar granule cells. Indeed, of 59 cerebellar granular neurons tested after 11-12 days *in vitro*, a total of 54 cells responded to 1 mM (1R,3S)-t-ACPD applied first (mean maximal $[Ca^{2+}]_i$, 242 ± 16 nM) and 15 responded to (1S,3R)-t-ACPD applied second (mean maximal $[Ca^{2+}]_i$, 188 ± 22 nM; basal $[Ca^{2+}]_i$, 89 ± 21 nM) (Fig. 2B). The same pattern of responses was observed in the absence of external Ca^{2+} (not shown).

Rat Cerebellar RNA-Injected Xenopus oocytes

To ensure that the response to both enantiomers was not a feature restricted to cultured neurons, we measured the effects of increasing concentrations of (1R,3S)- or (1S,3R)-t-ACPD on agonist-elicited oscillatory increases of Ca²⁺-dependent chloride conductance in *Xenopus* oocytes injected with adult female rat cerebellar RNA (Fig. 3A). We found that both enantiomers elicited dose-dependent effects, with (1R,3S)-t-ACPD being more potent. Interestingly, the effects of both enantiomers were

Bspet





Fig. 3. A, Dose=response curves for (1R,3S)= and (1S,3R)=t=ACPD stim= ulation of CI⁼ currents in adult cerebellar RNA-injected Xenopus occytes. In order to avoid desensitization of GLU metabotropic receptors, doseresponse experiments were performed as follows. Each occyte was tested for expression of t-ACPD receptors by a short stimulation (10sec) with 1 mm racemic mixture. Then, one agonist concentration was applied to the occyte. For each concentration, six to eight occytes were used; the results are expressed as means ± standard errors of six to eight occytes for each drug concentration. B, Effects of 1 mm (1A,3S)= and (1S,3A)-t-ACPD on Xenopus occytes injected with various adult rat cerebellar ANA concentrations. Occytes from the same female were injected with 30, 50, or 100 nl of RNA (2 mg/ml), yielding the indicated final quantity of RNA. Responses to 1 mm (17,39)- or 1 mm (19,37)-t-ACPD were tested on each occyte of each batch. In order to avoid interpretation of desensitization as a difference in efficacy, each stereoisomer was applied alternately to the same occyte until desensitization was observed. Moreover, from one occyte to another, (1\$,3A)= or (1R,39)=t=ACPD, alternately, was applied first. Data are means ± stand= ard errors of the indicated number of experiments (number in parentheses on top of bars).

not additive when tested at 1 mM (data not shown), supporting the idea that the two agonists were acting at the same receptor.

It has been suggested that (1R,3S)-t-ACPD could be a partial agonist at a receptor fully activated by (1S,3R)-t-ACPD (10). There was a possibility that we did not detect partial agonist properties of (1R,3S)-t-ACPD in *Xenopus* oocytes, due to an artificially high amplification between second messenger production (inositol 1,4,5-trisphosphate) and the final response (increase of Ca²⁺-activated Cl⁼ currents). We, therefore, injected various oocytes with various RNA concentrations (corresponding to various receptor concentrations). Fig. 3B shows that the ratio between (1S,3R)- and (1R,3S)-t-ACPD-elicited maximal responses (obtained with 1 mM) did not vary with the amount of receptors expressed. In addition, it shows that the amount of receptors expressed in the dose-response experiments did not produce saturating curents. Therefore, these experiments suggest that (1R,3S)- and (1S,3R)-t-ACPD are both full agonists of the same receptor.

Intracellular Calcium Measurements in Undifferentiated Neurons

A previous report by Irving *et al.* (12) suggested that only the (1S,3R)-*t*-ACPD enantiomer was able to increase $[Ca^{2+}]_i$ in cerebellar granule cells maintained in culture for 2–3 days. In order to determine whether the difference between this report and our experimental results was due to an age-dependent phenomenon, we decided to measure *t*-ACPD-induced Ca²⁺ mobilization in both cerebellar and striatal neurons after 3 days *in vitro*.

We examined a total of 260 striatal neurons in four different preparations and found that no cells responded to 1 mM (1R,3S)-t-ACPD, whereas 89 cells responded to 1 mM (1S,3R)t-ACPD (Fig. 4A). The mean maximal $[Ca^{2+}]_i$ induced by application of 1 mM (1S,3R)-t-ACPD was 140 ± 10 nM (basal $[Ca^{2+}]_i$, 49 ± 6 nM). Application of 1 mM (1S,3R)-t-ACPD also





test.

elicited an increase in $[Ca^{2+}]_i$ when tested in the absence of added external Ca^{2+} [83 ± 14 nM and 196 ± 21 nM, n = 12, in the absence and the presence of 1 mM (1S,3R)-t-ACPD, respectively]. Here again, we could not detect reliable stimulation of the $[Ca^{2+}]_i$ with doses lower than 100 μ M (data not shown). This demonstrated that (1S,3R)-t-ACPD is the only active enantiomer of t-ACPD in striatal neurons, after 2-3 days in vitro.

We tested 59 immature granule cells (2-3 days in vitro) and found, as previously described (12), that 19 cells responded to 1 mM (1S,3R)-t-ACPD. No significant effects of (1R,3S)-t-ACPD (applied first) [mean maximal $[Ca^{2+}]_i$ induced by 1 mM (1S,3R)-t-ACPD was 365 ± 69 nM (basal level was 86 ± 10 nM, n = 19), compared with 108 ± 23 nM for 1 mM (1R,3S)-t-ACPD] (Fig. 4B) could be detected. Similar effects were observed in the absence of external Ca^{2+} (data not shown).

Discussion

The main findings of this study are that both enantiomers can activate GLU metabotropic receptors and that the pharmacological characteristics of the metabotropic GLU receptor responses in striatal neurons and cerebellar granule cells are modified during their *in vitro* maintenance in primary culture.

As observed by Irving et al. (12), when granule cells were kept in vitro for 2 or 3 days the metabotropic GLU receptorinduced intracellular Ca^{2+} release was sensitive only to (1S,3R)t-ACPD. This response was also observed in striatal neurons, in the absence or the presence of external Ca^{2+} . Note that we were unable to measure any increase in InsP formation after t-ACPD stimulation in neurons after 2-3 days in vitro. This could be due to technical limitations, such as the nonlabeling of the phospholipid pool mobilized by the metabotropic receptor activation. Interestingly, we also were unsuccessful in measuring inositol 1.4.5-trisphosphate production with a radio-receptor assay. These failures could be due to technical reasons or to the metabotropic receptor-induced increase in $[Ca^{2+}]_i$ not being mediated by InsP production at that particular developmental stage. However, this explanation is unlikely, because (1S,3R)-t-ACPD-induced $[Ca^{2+}]_i$ increase could be observed even in the absence of external Ca^{2+} .

After 9-11 days in vitro, both the $[Ca^{2+}]_i$ and the InsP metabotropic GLU responses were triggered by (1S,3R)- and (1R,3S)-t-ACPD. Such a developmental pattern was observed in primary cultured striatal neurons and cerebellar granule cells. After 2-3 days in vitro the metabotropic GLU receptor was sensitive only to (1S,3R)-t-ACPD, whereas after 10-12 days in vitro it was sensitive to both enantiomers. Interestingly, in both types of "differentiated" neurons, (1R,3S)-t-ACPD was more potent than (1S,3R)-t-ACPD in stimulating InsP formation.

The exact reason for such a difference in the pharmacological specificity of the metabotropic GLU receptors during development is unknown, but one possibility is that metabotropic GLU receptor molecules expressed at the beginning of the plating are different from those expressed after 10 days *in vitro*. However, we cannot exclude other, more complex, explanations. Note that, when RNA was prepared from 10-day-old rat cerebellum and injected into *Xenopus* oocytes, the expressed metabotropic GLU receptor was sensitive to both (1S,3R)- and (1R,3S)-t-ACPD. The observation that neither the agonist-induced InsP formation in mouse cultured neurons nor the Cl⁻

currents in rat cerebellar RNA-injected *Xenopus* oocytes obtained with the two enantiomers were additive suggests that the two metabotropic GLU responses [one sensitive to only (1S,3R)-t-ACPD and one sensitive to both enantiomers] are not expressed simultaneously.

We have shown that neuronal differentiation, synapse formation, and neurotransmitter release rapidly increase between 8 and 10 days in vitro in these cultures (14). It is possible that changes in gene expression of receptors occurs during this period. We have already described such developmental changes in the expression of pharmacologically different muscarinic receptors in striatal neurons (20). Modification of the metabotropic GLU responses, after 2-3 days in vitro and 10 days in vitro, to stimulation by (1S,3R)- and (1R,3S)-t-ACPD enantiomers is additional pharmacological evidence for the existence of different metabotropic GLU receptors. However, more data are in favor of such a heterogeneity. Recently, four different metabotropic receptors have been cloned (7). In rat cerebellar cortical slices, the peak of agonist-induced InsP formation is at 11 days for t-ACPD and at 7 days for ibotenate or QA (4). In mature rat hippocampus ibotenate had a greater efficacy than QA, whereas the opposite was true in synaptoneurosomes of rat forebrain. More generally, studies of agonist-induced phosphoinositide hydrolysis have demonstrated regional and developmental differences and, when rat brain RNA is expressed in Xenopus oocytes, one can inhibit t-ACPD but not QA effects with DL-2-amino-3-phosphopropionate (for a review, see Ref. 4). Finally, AP3 and DL-2-amino-4-phosphonobutyrate had a greater efficacy and potency in inhibiting agonist-induced InsP formation in mature than in immature brain structures (21).

Our results are in contradiction to observations made in hippocampal slices (13). In slices from neonatal rat (1R,3S)-t-ACPD is a partial agonist of a receptor that is fully activated by (1S,3R)-t-ACPD, whereas in adult tissue (1S,3R)-t-ACPD is the only active enantiomer [with (1R,3S)-t-ACPD being totally inactive in stimulating InsP formation]. One can propose several explanations for such drastic discrepancies with our own data. Firstly, one might expect the existence of more than two types of metabotropic receptors coupled to phospholipase C, some being expressed in the hippocampus and others in cerebellum and striatum. Secondly, hippocampal slices are a rather complicated model, where complex interactions (between glial cells and neurons) are likely to occur. Indeed differences in uptake mechanisms and interactions with glial metabotropic receptors could interfere with neuronal metabotropic responses.

The fact that we were able to measure, in rat cerebellum RNA-injected Xenopus oocytes, responses to both (1S,3R)- and (1R,3S)-t-ACPD demonstrates that the (1R,3S)-t-ACPD response is not an exclusive feature of mouse cultured neurons after 9–11 days in vitro. Therefore, the conclusion that (1S,3R)-t-ACPD is the only active enantiomer of t-ACPD on metabotropic receptors (12-14) has to be revised.

Nevertheless, molecular cloning of several metabotropic GLU receptors will give the definitive answer to these questions (5-7). It will be interesting to check the responsiveness of the different clones to (1S,3R)- and (1R,3R)-t-ACPD and to determine whether different metabotropic receptor subtypes are expressed during neuronal development.

Bspet

Acknowledgments

The authors would like to thank A. Sahuquet for creating all the fura-2 software, Dr. P. Mangeat for helpful discussions during the set-up of the fura-2 system, Dr. M. Lafon-Cazal for providing us with cultures of cerebellar granule neurons, Dr. C. Waeber for his help with the curve-fitting software, A. Turner-Madeuf for excellent secretarial assistance, and M. Passama for the artwork.

References

- Monaghan, D. T., R. J. Bridges, and C. W. Cotman. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 29:365-402 (1989).
- Sladeczek, F., J. P. Pin, M. Récasens, J. Bockaert, and S. Weiss. Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature (Lond.)* 317:717-719 (1985).
- Schoepp, D. D., J. Bockaert, and F. Sladeczek. Metabotropic excitatory amino acid receptors: pharmacological and functional characteristics. *Trends Phar*macol. Sci. 11:508-515 (1990).
- Masu, M., Y. Tanabe, K. Tsuchida, R. Shigemoto, and S. Nakanishi. Sequence and expression of a metabotropic glutamate receptor. *Nature (Lond.)* 349:760-769 (1991).
- Houamed, K. M., J. L. Kuijper, T. L. Gilbert, B. A. Haldeman, P. J. O'Hara, E. R. Mulvihill, W. Almers, and F. S. Hagen. Cloning, expression and gene structure of a G-protein-coupled glutamate receptor from rat brain. *Science* (Washington D. C.) 252:1318-1321 (1991).
- Tanabe, Y., M. Masu, T. Ishii, R. Shigemoto, and S. Nakanishi. A family of metabotropic glutamate receptors. *Neuron* 8:169-172 (1992).
- Manzoni, O. J. J., F. Finiels-Marlier, I. Sassetti, J. Bockaert, C. Le Peuch, and F. Sladeczek. The glutamate receptor of the Qp-type activates protein kinase C and is regulated by protein kinase C. *Neurosci. Lett.* 109:146-151 (1990).
- Palmer, E., D. T. Monaghan, and C. W. Cotman. Trans-ACPD, a selective agonist of the phosphoinositide coupled excitatory amino acid receptor. Eur. J. Pharmacol. 166:585-588 (1989).
- Desai, M. A., and J. P. Conn. Selective activation of phosphoinositide hydrolysis by a rigid analogue of glutamate. *Neurosci. Lett.* 109:157-161 (1990).
- 11. Manzoni, O. J. J., L. Fagni, J. P. Pin, F. Rassendren, F. Poulat, F. A. J.

Sladeczek, and J. Bockaert. t-ACPD stimulates quisqualate phosphoinositidecoupled receptors but not ionotropic glutamate receptors in striatal neurons and Xenopus oocytes. Mol. Pharmacol. **38:**1-6 (1990).

- Irving, A. J., G. Schofield, J. C. Watkins, D. C. Sunter, and G. L. Collingridge. 1S,3R-ACPD stimulates and L-AP3 blocks calcium mobilization in rat cerebellar neurons. Eur. J. Pharmacol. 186:363-365 (1991).
- Schoepp, D. D., B. G. Johnson, R. A. True, and J. A. Monn. Comparison of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD)- and 1R,3S-ACPD stimulated brain phosphoinositide hydrolysis. *Eur. J. Phar*macol. 207:351-353 (1991).
- Weiss, S., J. P. Pin, M. Sebben, D. E. Kemp, F. Sladeczek, J. Gabrion, and J. Bockaert. Synaptogenesis of cultured striatal neurons in serum-free medium: a morphological and biochemical study. *Proc. Natl. Acad. Sci. USA* 83:2238-2242 (1986).
- Van-Vliet, B. J., M. Sebben, A. Dumuis, J. Gabrion, J. Bockaert, and J. P. Pin. Endogenous amino acid release from cultured cerebellar neuronal cells: effect of tetanus toxin on glutamate release. J. Neurochem. 52:1229-1239 (1989).
- Manzoni, O. J. J., F. Poulat, E. Do, A. Sahuquet, I. Sassetti, J. Bockaert, and F. Sladeczek. Pharmacological characterization of the quisqualate receptor coupled to phospholipase C (Qp) in striatal neurons. *Eur. J. Pharmacol.* 207:231-241 (1991).
- Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:2440-2450 (1985).
- Curry, K., M. J. Peet, D. S. K. Magnuson, and H. McLennan. Synthesis, resolution, and absolute configuration of the isomers of the neuronal excitant 1-amino-1,3-cyclopentadicarboxylic acid. J. Med. Chem. 31:864-867 (1988).
- Rassendren, F., P. Lory, J. P. Pin, J. Bockaert, and J. Nargeot. A specific quisqualate agonist inhibits kainate responses induced in *Xenopus* oocytes injected with rat brain RNA. *Neurosci. Lett.* **99**:333-339 (1989).
- Schmidt, B. H., O. J. J. Manzoni, M. Royer, J. Bockaert, and F. A. J. Sladeczek. Cholinergic inositol phosphate formation in striatal neurons is mediated by distinct mechanisms. *Eur. J. Pharmacol.* 206:87-97 (1991).
- Schoepp, D. D., and B. G. Johnson. Inhibition of excitatory amino acidstimulated phosphoinositide hydrolysis in the neonatal rat hippocampus by 2-amino-3-phosphonopropionate. J. Neurochem. 53:1865-1868 (1989).

Send reprint requests to: Olivier Manzoni, Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, 34094 Montpellier, Cedex 5, France.