0026-895X/90/010001-06$02.00/0

MOLECULAR PHARMACOLOGY, 38:1-6

ACCELERATED COMMUNICATION

(*trans*)-1-Amino-cyclopentyl-1,3-dicarboxylate Stimulates Quisqualate Phosphoinositide-Coupled Receptors but Not Ionotropic Glutamate Receptors in Striatal Neurons and *Xenopus* Oocytes

OLIVIER MANZONI, LAURENT FAGNI, JEAN-PHILIPPE PIN, FRANÇOIS RASSENDREN, FRANCIS POULAT, FRITZ SLADECZEK, and JOËL BOCKAERT

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, 34094 Montpellier Cedex 5, France (O.M., L.F., J.-P.P., F.P., F.S., J.B.), and Centre de Recherche de Biochimie Macromoleculaire CNRS/INSERM, 34033 Montpellier Cedex 1, France (F.R.)

Received December 27, 1989; Accepted April 9, 1990

SUMMARY

The effects of a novel glutamate analogue, (*trans*)-1-amino-cyclopentyl-1,3-dicarboxylate (ACPD), have been tested in striatal neurons in primary culture and in *Xenopus* oocytes injected with rat brain RNA. Both systems have been previously shown to contain well characterized metabotropic receptors coupled to phospholipase C (Q_p), as well as ionotropic glutamate receptors. In striatal neurons, ACPD stimulated inositol phosphate (InsP) accumulation (EC_{50} = 9.7 ± 2.5 μM; maximal effect, 184.7 ± 11.6% of basal accumulation). This effect of ACPD was likely to be mediated by Q_p receptors, because maximal ACPD and quisqualate-induced InsP formation were not additive. In contrast, the effects of ACPD and norepinephrine on InsP formation were additive. ACPD-induced InsP formation was not antagonised by antagonists of muscarinic and α_1-adrenergic receptors (1 μM atropine and 0.1 μM prazosin, respectively). In *Xenopus* oocytes, ACPD and quisqualate induced an oscillatory increase of a Ca^{2+}-dependent chloride conductance, which is characteristic of the activation of phospholipase C-coupled receptors in this model. The specificity of ACPD on Q_p receptors was demonstrated by testing the effect of this drug on quisqualate/kainate as well as on *N*-methyl-D-aspartate ionotropic receptors. In striatal neurons, the activation of quisqualate/kainate and *N*-methyl-D-aspartate receptors was tested by measurement of [^{3}H]-γ-aminobutyric acid release and by electrophysiological recordings using the patch-clamp technique. At concentrations as high as 1 mM, ACPD was inactive on these ionotropic receptors, either as agonist or as antagonist. In conclusion, ACPD appeared to be a highly specific agonist of Q_p receptors, with no activity on ionotropic glutamate receptors. It will be a useful drug to study the physiological properties of Q_p receptors in vertebrate brains.

Glutamate and aspartate are the two major fast excitatory neurotransmitters in the vertebrate central nervous system. Their actions are mediated through both ionotropic and metabotropic receptors (1, 2). Three of these receptors are gated ionic channels and have been termed, according to their specific agonists, NMDA, KA, and QA receptors (1). Recent findings indicate that QA and KA might activate at least one common receptor, which is termed either QA/KA or the AMPA receptor, because it is specifically activated by the latter drug (3–5). A fourth putative glutamate receptor is sensitive to 2-amino-4-phosphonobutyrate and triggers hyperpolarizing currents (1).

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), and Bayer France and DRET (Direction des Recherches Etudes et Techniques) contract 88/163.

In addition to these receptors, there is now a very well characterized phosphoinositide-coupled receptor stimulated by QA that we have proposed to term Q_p (2). Q_p receptors were first described to stimulate total InsP production in striatal neurons (6). They have been found thereafter in a great variety of systems such as cerebellar granule cells, astrocytes in primary culture, synaptoneurosomes, and brain slices (for reviews see Refs. 1 and 2). In addition, it has been shown that in neuronal cultures these receptors stimulate both inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate production (7, 8), protein kinase C activation (9), and intracellular Ca^{2+} mobilization (10, 11). Q_p receptors are easily expressed in *Xenopus* oocytes after injection of rat brain RNA (5, 12). In this model, Q_p receptors increase Cl^- conductance through the following cascade: production of inositol 1,4,5-trisphosphate, mobiliza-

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; ACPD, (*trans*)-1-amino-cyclopentyl-1,3-dicarboxylate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethylene glycol bis-(β-amino-ethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; GABA, γ-aminobutyric acid; KA, Kainate; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; InsP, inositol phosphate; PLC, phospholipase C; QA, quisqualate; Q_p receptor, quisqualate metabotropic receptor; NE, norepinephrine.

tion of intracellular Ca^{2+} stores, and activation of a Ca^{2+}-dependent Cl^- channel (12).

The pharmacology of the Q_p receptor is now well defined in these different models. The Q_p receptor is stimulated by submicromolar concentrations of QA and glutamate, respectively, but not by KA, NMDA, or AMPA (1, 2, 13). It is not inhibited by NMDA or AMPA receptor antagonists but is blocked by phorbol esters (9). Recently, Palmer *et al.* (14) demonstrated that ACPD, a novel glutamate analogue, was a potent activator of InsP metabolism in hippocampal slices and that this compound does not interact with NMDA, KA, or AMPA binding sites.

It was, therefore, interesting to study the effect of ACPD on models in which responses to excitatory amino acids are mediated through very well characterized QA/KA, NMDA, and Q_p receptors. We show here that ACPD is inactive on NMDA and QA/KA receptors in striatal neurons and *Xenopus* oocytes, their activity being measured by both electrophysiological and biochemical methods. In contrast, ACPD clearly stimulates Q_p receptors in striatal neurons.

Experimental Procedures

Materials

Agents used for this study were obtained from the following sources: QA, NMDA, KA, and EGTA from Sigma (L'Isle d'Abeau, France); AMPA and ACPD from Tocris Neuramin (Essex, England); *myo*-[2-^{3}H]inositol from C.E.A. (Saclay, France); 4-amino-*n*-[2,3-^{3}H]butyric acid from Amersham (Paris, France); the six-well clusters and 35-mm diameter dishes for tissue cultures from Costar (Cambridge, MA); and Dulbecco's minimal essential medium and F-12 nutrient from GIBCO Europe (Paris, France). All other compounds are of the highest possible grade from commercial sources.

Methods

Striatal neuronal cultures. Primary cultures of striatal neurons were prepared in serum-free medium as previously described (15). Briefly, striata were removed from 14–15-day-old mouse embryos (Iffa Credo, Lyon, France). The cells were gently dissociated in culture medium with a fire-narrowed Pasteur pipette and plated (1.8×10^6 cells/dish for biochemical experiments and 1.0×10^6 cells/dish for electrophysiological experiments) in 35-mm diameter Costar culture dishes (six-well clusters, 2 ml/well) that were previously coated with poly-L-ornithine (15 μg/ml; molecular weight 40,000) and medium containing 10% fetal calf serum, successively. Culture medium was composed of a 1:1 mixture of Dulbecco's minimal essential medium and F-12 nutrient, supplemented with glucose (30 mM), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES buffer (5 mM). For electrophysiological experiments, 2% fetal calf serum was also added to the medium 48 hr after plating. Under these conditions, the cultures have been shown to be highly enriched in neurons that form many mature and functional synapses (15). Only 7% of the cells have been identified as astrocytes (representing 5% of the surface area) after 12 days *in vitro*.

[^{3}H]GABA release experiments. The release of [^{3}H]GABA from striatal neurons was assayed after 14 days *in vitro*, as previously described (16), in the presence of amino oxyacetic acid (100 μM) to avoid [^{3}H]GABA degradation. Briefly, neurons were washed and exposed for 30 min to 2 ml of medium (128 mM NaCl, 5 mM KCl, 1.1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4, 10 mM glucose, 20 mM HEPES, pH 7.4) containing 0.1 μCi of [^{3}H]GABA. Medium was then changed every 4 min. After six changes of medium, neurons were stimulated with the indicated concentrations of excitatory amino acid agonists for 4 min. The radioactivity released in the medium and that remaining in the cells was determined as previously described (4). Results were expressed as the percentage of radioactivity released from the cells.

Electrophysiological patch-clamp technique. Recordings were performed at room temperature from 10- to 12-days *in vitro* neurons, using the whole-cell configuration of the patch-clamp technique described by Hamill *et al.* (17). External solution contained 140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 1 μM tetrodotoxin, and 10 mM HEPES-Na, pH 7.4. Patch pipettes (3–5 MΩ) were filled with an internal medium containing 140 mM CsCl, 0.5 mM $CaCl_2$, 5 mM EGTA-Tris, and 10 mM HEPES-Tris, pH 7.2. Drugs were applied using the perfusion technique described by Johnson and Ascher (18), slightly modified.

Expression of rat brain RNA in *Xenopus* oocytes and electrophysiological detection of Q_p and Q_i. Fifteen-day-old rat brain RNA was prepared as previously described (5), using a phenol-chloroform extraction procedure. *Xenopus* oocytes were dissociated with a collagenase treatment (2 mg/ml, type 1A) and kept in a Barth modified solution. RNA injection was performed with a Drumond micropipette, each oocyte being injected with 50–70 nl of brain RNA at 2 mg/ml. After injection, oocytes were kept in Barth modified solution for 3–4 days at 19° to allow protein expression. Electrophysiological recordings were carried out with a home-made double-microelectrode voltage amplifier. Oocytes were placed in a 1.5-ml perfusion chamber and continuously bathed with Barth modified solution. Pharmacological agents were applied directly in the bath, the perfusion being stopped. For intracellular injection of EGTA, 50 nl of 20 mM EGTA, pH 7 were injected in the oocyte using a Drumond micropipette.

InsP accumulation. Insp accumulation in striatal neurons was examined as previously described (6). After 13 days *in vitro*, the culture medium containing 1 μCi/ml ^{3}H-myoinositol was replaced with buffer consisting of 146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 20 mM HEPES, 10% glucose, and 10% bovine serum albumin and neurons were incubated at 37° for 10 min in the presence of 10 mM LiCl. The agonists were then added for a 30-min incubation period. At the end of this time, the reaction was stopped by replacement of the incubation medium with 5% perchloric acid, on ice. The InsP were then extracted and purified as previously described (9).

Results

ACPD stimulated Q_p receptors in cultured striatal neurons. ACPD stimulated basal InsP accumulation in a dose-dependent manner (Fig. 1a). ACPD produced a maximal stimulation of 184.7 ± 11.6% of basal InsP formation, with an EC_{50} of 9.7 ± 2.5 μM. In the same experiments, we tested QA-induced InsP formation; the maximal effect was 248.3 ± 9.3% of basal InsP accumulation, with an EC_{50} of 0.16 ± 0.09 μM (Fig. 1a). In all the experiments, ACPD was less potent and had a lower efficacy than QA in increasing InsP formation. ACPD (100 μM) and QA (1 μM) stimulations of InsP were not additive (Fig. 1b). Note that the effect of QA (1 μM) plus ACPD (300 μM) was close to the effect of ACPD (300 μM) alone but lower than the effect of QA (1 μM). This suggests that ACPD behaves as a partial agonist on Q_p receptors. The responses induced by ACPD (100 μM) and NE (100 μM) a dose inducing a maximal InsP formation; see Ref. 19) were additive (Fig. 1c). Because striatal neurons also possess α_1-adrenergic and muscarinic receptors associated with PLC (19), the effect of prazosin (0.1 μM), an α_1-adrenergic receptor antagonist, and atropine (1 μM), a muscarinic receptor antagonist, were tested on 300 μM ACPD-stimulated InsP formation. No inhibitory effect of these drugs were observed (210.75 ± 20.6; 230 ± 17.3, and 190 ± 23.4% of basal InsP formation for control, prazosin-treated, and atropine-treated, respectively).

ACPD stimulated Q_p but not QA/KA receptors expressed in *Xenopus* oocytes. As previously reported (5, 12),

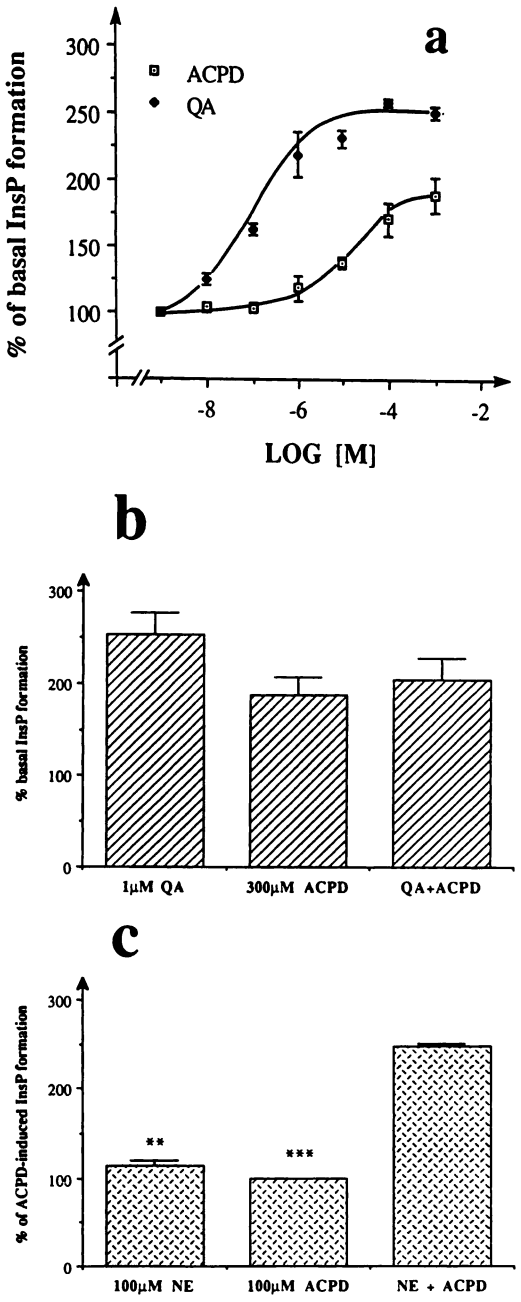

Fig. 1. Stimulation by ACPD and QA of InsP accumulation in cultured striatal neurons. a, Dose-response curve for ACPD stimulation of InsP accumulation. Values represent means ± standard errors of three independent experiments, in which each data point was obtained in duplicate. Basal InsP accumulation was 930 ± 39 dpm. b, Nonadditivity of ACPD and QA effects on InsP accumulation. Basal InsP accumulation was 1370 ± 51 dpm. c, Additivity of ACPD and NE effects on ACPD-induced InsP accumulation. Basal InsP accumulation was 1134 ± 43 dpm. Values of NE, ACPD, and NE plus ACPD were significantly different (**, $p < 0.03$; ***$p < 0.001$), as determined with Student's t test.

QA (100 μM) induced a two-component current in rat brain RNA-injected oocytes. The first component was a smooth inward current with very rapid onset. The second component was a very large delayed oscillatory current, which persisted after removal of the agonist (Fig. 2a). This oscillation was due to activation of Ca^{2+}-dependent chloride channels, because its reversal potential was in the range of −30 mV and because this response could be blocked by intracellular injection of EGTA without affecting the smooth inward current (Fig. 2c). This latter residual current was blocked by 100 μM CNQX (Fig. 2e). Similar results were obtained with AMPA, a specific QA/KA agonist. Therefore, the smooth inward current was due to the stimulation of QA/KA and, as has already been shown (5, 12, 13), the oscillatory current was mediated by the Q_p receptor.

When ACPD (1 mM) was applied on the same oocyte, only an oscillatory current was elicited (Fig. 2b). This current presented the same characteristics as the Q_p receptor-mediated current described above [i.e., it could be blocked by intracellular injection of EGTA (Fig. 2d) and its reversal potential was in the range of −30 mV]. This indicates that ACPD activated a receptor coupled to PLC but not QA/KA receptors. We noticed that the oscillations induced by 1 mM ACPD (a concentration that gives maximal stimulation of InsP formation) were lower in intensity than those induced by QA. This difference was markedly larger than the range of variation of the QA response in the same oocyte. This was not due to a desensitization of the response, because the same results were obtained when ACPD was first applied onto the oocyte. However, no further quantitative comparison between QA and ACPD responses was performed.

ACPD did not activate QA/KA receptors in cultured striatal neurons. Activation of QA/KA receptors by ACPD in striatal neurons was tested either by measurement of [^{3}H] GABA release or by electrophysiological recordings using the patch-clamp technique. When stimulated with QA/KA receptor agonists, striatal neurons were shown to release GABA through the reversal of the GABA uptake system (16). This response appears, therefore, as a relatively direct consequence of the activation of the QA/KA receptors. Treatment of striatal neurons with concanavalin A (100 μg/ml) for 2 hr before the experiment has been shown to strongly potentiate the QA or AMPA response, without modifying the NMDA response or the GABA release mechanism (4). This treatment also decreased the apparent affinity of QA (4). As shown in Fig. 3a, ACPD was without significant effect even at concentrations as high as 1 mM, whereas under these conditions QA strongly stimulated the release of GABA in a dose-dependent manner. ACPD was also tested as an antagonist at QA/KA receptors. The dose-response curve of KA was not modified in the presence of concentrations of ACPD as high as 300 μM (for example, 21.5 ± 3.0 and 21.8 ± 2.8% of GABA release evoked by 100 μM KA, in the absence and presence of 300 μM ACPD, respectively).

QA (Fig. 3b) and KA (not shown) induced inward current responses in striatal neurons clamped at −60 mV, using the whole-cell patch configuration. The responses were dose dependent. Threshold responses were obtained with 1 μM KA and 0.1 to 0.5 μM QA. Maximal QA responses were observed with concentrations of 5 to 10 μM. QA and KA responses reversed between −10 and 10 mV and the current-voltage relation of these responses did not display any significant rectification in the potential range of −80 to 80 mV. This pattern was typical of non-NMDA-type currents described in other preparations (20).

In neurons that showed sensitivity to QA (Fig. 1b) or KA (100 μM; not shown), ACPD, at concentrations up to 1 mM, did not induce any detectable ionotropic response at holding potentials ranging from −100 to 100 mV (Fig. 3b). This absence of effect of ACPD was also observed when the drug application

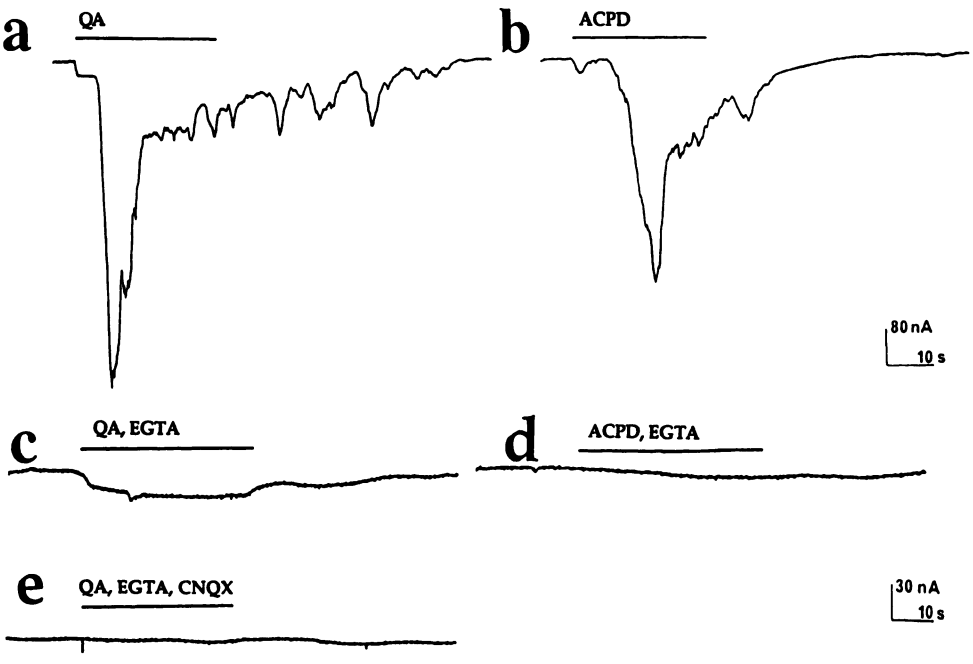

Fig. 2. Stimulation by ACPD of Q_p but not of QA/KA receptors in *Xenopus* oocytes previously injected with rat brain RNA. Oocytes were voltage clamped at −80 mV. Agonists were applied directly in the bath using a 20-μl pipette, with the perfusion being stopped. The time of stimulation is indicated by the *bars* above the *current traces*. The concentrations of pharmacological agents were 100 μM and 1 mM for QA and ACPD, respectively. When indicated, 50 nl of EGTA (20 mM, pH 7) were injected into the oocyte with a Drumond micropipette, leading to an intracellular concentration in the range of 0.5 mM. All experiments were performed on the same oocyte and repeated three times.

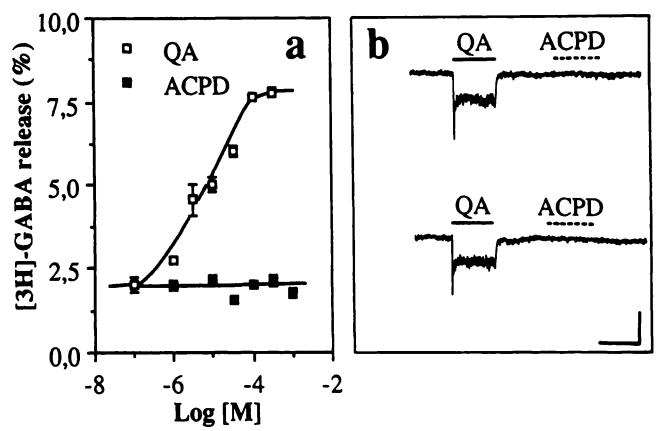

Fig. 3. ACPD did not activate QA/KA receptors in cultured striatal neurons. a, The release of [^{3}H]GABA from concanavalin A-treated neurons was determined as described in Experimental Procedures. The percentage of radioactivity released from the cells was measured after a 4-min stimulation period with increasing concentrations of QA or ACPD. Results are means ± standard errors of triplicate determinations. Similar results were obtained twice. b, The two consecutive whole-cell recordings (digitized at 70 Hz) were obtained from one neuron at a holding potential of −60 mV. ——, 2 μM QA; – – –, 100 μM ACPD applications. *Calibration bars*, 50 pA and 10 sec. Similar results were obtained in 10 other cells.

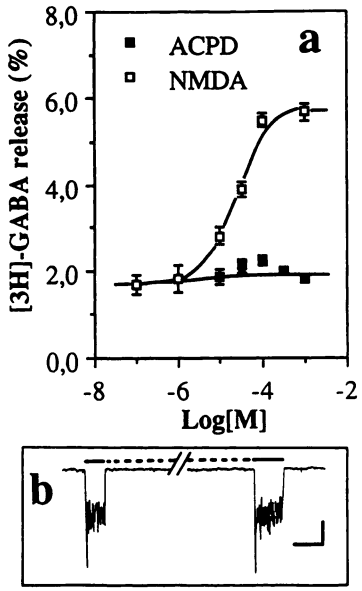

Fig. 4. ACPD did not activate NMDA receptors in cultured striatal neurons. a, The release of [^{3}H]GABA from striatal neurons was determined as described in Experimental Procedures, in the absence of Mg^{2+}. The percentage of radioactivity released from the cells was measured after a 4-min stimulation with increasing concentrations of NMDA or ACPD. Results are means ± standard errors of triplicate determinations. Similar results were obtained twice. b, The whole-cell recording (digitized at 17 Hz) was obtained from one neuron, in the absence of Mg^{2+} and in the presence of 1 μM glycine, at a holding potential of −60 mV. The two NMDA (100 μM) applications (——) were separated by a 5-min application of ACPD (100 μM) (– – –). *Calibrations bars*, 50 pA and 10 sec. Similar results were obtained from five other cells.

lasted for up to 5 min, although QA or KA responses of the cell could still be elicited by subsequent brief applications of the agonists.

ACPD did not activate NMDA receptors in cultured striatal neurons. Activation of NMDA receptors by ACPD in striatal neurons was also tested either by measurement of [^{3}H] GABA release or by electrophysiological recordings using the patch-clamp technique. As previously described (21), the GABA release stimulated by NMDA in the absence of Mg^{2+} clearly reflects the activation of NMDA receptors present in these neurons (4). Under these conditions, NMDA strongly stimulated the release of GABA in a dose-dependent manner, whereas ACPD was without significant effect, even at concentrations as high as 1 mM (Fig. 4a). We also tested the antagonist effect of 30 μM ACPD on NMDA-induced GABA release, and the dose-response curve of NMDA was not affected by ACPD (2.6 ± 0.5 and 2.7 ± 0.3% of GABA release evoked by 100 μM NMDA, in the absence and presence of 300 μM ACPD, respectively).

In whole-cell patch recording, NMDA induced inward currents at negative holding membrane potentials. The current

response reversed around 0 mV. This typical NMDA response was obtained in the absence of Mg^{2+} and in the presence of 1 μM glycine in order to fully activate NMDA receptors (18). Under this condition the current-voltage relationship of the NMDA response was linear between −80 and +40 mV. The threshold response was obtained with concentrations of 1 to 5 μM and the maximal response with 100 μM. In neurons that exhibited NMDA responses, ACPD (1 mM) did not produce any response, whatever the holding potential or the time of application (Fig. 4b).

These results clearly show that ACPD did not activate any of the known ionotropic glutamate receptors in cultured striatal neurons.

Discussion

In cultured striatal neurons and in *Xenopus* oocytes injected with rat brain RNA, the glutamate receptor that triggers InsP formation has been well characterized (2, 5, 12, 13, 22). The receptor we have proposed to name the Q_p receptor (2) is likely to be coupled to a GTP-binding protein in both systems (7, 12). QA is the most potent agonist of the Q_p receptor, whereas AMPA, a potent agonist of QA/KA receptors, is inactive (2, 5, 13). Moreover Q_p-mediated responses are not blocked by any known antagonist of NMDA or QA/KA ionotropic receptors (22, 23)[1] but are inhibited by phorbol esters, which are inactive on QA/KA responses (8, 9).

In hippocampal slices the pharmacology of glutamate-stimulated InsP formation shows different features than in striatal neurons, as suggested by various reports (Ref. 24 and for reviews see Refs. 1 and 2). Different receptor types might be involved in this glutamate response. Because ACPD stimulation of InsP formation has only been reported in hippocampal slices (14), it was, therefore, important to study the ACPD effect on less complex models. It was of particular interest to systematically compare the effect of ACPD on the three major subtypes of glutamate receptors: Q_p, NMDA, and KA/QA. This was the reason we decided to study the ACPD effects on striatal neurons in primary culture and in *Xenopus* oocytes injected with rat brain RNA.

Measuring [^{3}H]GABA release or using patch-clamp electrophysiological techniques, we have clearly demonstrated here that ACPD, even at high concentrations, was unable to stimulate NMDA and QA/KA ionotropic receptors in cultured striatal neurons. In contrast, ACPD was able to stimulate InsP accumulation in these cells, but it was less potent and less efficient than QA in activating Q_p receptors. This result contrasts with those of Palmer *et al.* (14), who found a larger maximal effect with ACPD than with QA. This discrepancy argues in favor of the hypothesis of different Q_p receptors in hippocampal and striatal neurons. The ACPD effect was likely to result from Q_p receptor activation, because it was not additive with the QA response and not antagonized by antagonists of the other PLC-coupled receptors present in these cells [i.e., α_1-adrenergic and muscarinic receptors (19)]. In the same preparation, ACPD and NE stimulations of InsP accumulation were additive. The results obtained with *Xenopus* oocytes previously injected with rat brain RNA lead to the same conclusion. In this model, ACPD induced a response typically resulting from the activation of a PLC-coupled receptor, i.e., an oscillatory Cl^- current that was inhibited by EGTA injection into the oocyte. Because no residual inward current could be seen with ACPD after intracellular injection of EGTA, we can conclude that in this system, as in striatal neurons, the QA/KA receptors are not sensitive to ACPD. Because *Xenopus* oocytes were injected with RNA prepared from whole brains, it seems clear that the specificity of ACPD for the Q_p receptor type is not a particularity of cultured striatal neurons.

In conclusion, these results extend previous data reported by Palmer *et al.* (14) that ACPD does not interact with NMDA and QA/KA receptors. Moreover, we present arguments in favor of its action on the most well characterized glutamate-phosphoinositide-coupled receptors, the Q_p receptor. Because glutamate receptors have been designated so far in terms of their specific agonists, it would be correct, as proposed by Monaghan *et al.* (1), to call the previously named Q_p receptors the ACPD receptors.

One of the most pressing questions remaining to be elucidated is the precise physiological role of these ACPD receptors. Several hypotheses have already been proposed. First, as already discussed (2), ACPD receptors might be implicated in neuronal development (24, 25). Second, ACPD receptors might be involved in synaptic plasticity, such as the long term potentiation phenomenon. This latter hypothesis is supported by the inhibition of both long term potentiation induction (26) and ACPD receptor-mediated responses after pertussis toxin treatment (7, 12, 27) but also by the demonstration that InsP production and kinase C activation may play a role in this phenomenon (for review see Ref. 28). Finally, a neurotoxic effect of QA, not sensitive to CNQX, has been recently described, leading to the proposal that this toxicity could result from ACPD receptor stimulation (29). The present demonstration that ACPD is a specific activator of Q_p receptors should certainly help to elucidate some of these questions.

Acknowledgments

We would like to thank Angie Turner-Madeuf for excellent secretarial assistance.

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