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Selective Antagonism of AMPA Receptors Unmasks Kainate Receptor–Mediated Responses in Hippocampal Neurons

Ana V. Paternain,* Miguel Morales,* and Juan Lerma
Departamento de Plasticidad Neural
Instituto Cajal
Consejo Superior de Investigaciones Científicas
Av. Doctor Arce 37
28002 Madrid
Spain

Summary

Although both protein and mRNAs for kainate receptor subunits are abundant in several brain regions, the responsiveness of AMPA receptors to kainate has made it difficult to demonstrate the presence of functional kainate-type receptors in native cells. Recently, however, we have shown that many hippocampal neurons in culture express glutamate receptors of the kainate type. The large nondesensitizing response that kainate induces at AMPA receptors precludes detection and analysis of smaller, rapidly desensitizing currents induced by kainate at kainate receptors. Consequently, the functional significance of these strongly desensitizing glutamate receptors remains enigmatic. We report here that the family of new noncompetitive antagonists of AMPA receptors (GYKI 52466 and 53655) minimally affects kainate-induced responses at kainate receptors while completely blocking AMPA receptor–mediated currents, making it possible to separate the responses mediated by each receptor. These compounds will allow determination of the role played by kainate receptors in synaptic transmission and plasticity in the mammalian brain, as well as evaluation of their involvement in neurotoxicity.

Introduction

Glutamate receptors mediate transmission at the majority of fast excitatory synapses in the vertebrate central nervous system, acting at both N-methyl-D-aspartate (NMDA) and non-NMDA receptors (Mayer and Westbrook, 1987; Collingridge and Lester, 1989). The non-NMDA family of glutamate receptors is composed of at least nine structurally related subunits. Although it is well established that GluR1–R4 are the constituents of the major α-amino-3-hydroxy-5-methyl-4-ixosazole propionic acid (AMPA) native receptor subtypes, there is not a model for the construction of high affinity kainate receptors in native brain cells. GluR5–R7 and KA-1 and KA-2 may constitute the so-called kainate-preferring or kainate-selective receptor in native membranes (for reviews, see Sommer and Seeburg, 1992; Westbrook, 1994). Expression in oocytes and mammalian cell lines of cDNAs coding for the kainate receptor subunits has demonstrated that they generate glutamate receptors with unique properties. Thus, all these subunits could form native kainate receptors when assambled in still undetermined combinations. The recent demonstration in hippocampal neurons of a new class of glutamate receptors activated by kainate and not by AMPA (Lerma et al. 1993) opens the possibility that other receptors, in addition to the AMPA type, could also be involved in fast neurotransmission and/or plasticity, as they also show fast activation and desensitization kinetics. These experiments suggest that AMPA and kainate receptors are coexpressed in young neurons (Lerma et al., 1993), which is consistent with the coexpression of proteins corresponding to AMPA and kainate glutamate receptor subunits in single neurons (Huntley et al., 1993; Craig et al., 1993; Wisden and Seeburg, 1993; Mackler and Eberwine, 1993). However, the lack of compounds selectively affecting one or other receptor hampered separation and analysis of kainate-selective glutamate receptors. Perhaps for that reason, the responses to kainate with properties similar to recombinantly expressed kainate receptors have remained elusive for a long time, and current knowledge on the functional role of kainate receptors is very limited. Recently, an allosteric modulatory site on AMPA receptors has been identified (Ito et al., 1990; Donevan and Rogawski, 1993; Zorumski et al., 1993), and a class of noncompetitive antagonists of AMPA receptor–mediated responses has been developed (Tarnawa et al., 1989; Donevan and Rogawski, 1993). We have found that these antagonists specifically affect AMPA receptor–mediated responses, unmasking the smaller kainate receptor–mediated currents in most of the well developed neurons studied.

Results and Discussion

Rapid perfusion of AMPA in voltage-clamped neurons induces an inward current that rapidly declines to a steady level. In these cells, rapid application of kainate produces a large nondesensitizing response. Both glutamate agonists are acting on the same receptor complex, as has been demonstrated previously using molecular biology techniques (Lambolez et al., 1990) and can be illustrated by cross-desensitization of kainate responses by AMPA (Patneau and Mayer, 1991). In a subset of hippocampal neurons apparently without AMPA receptors, kainate still induced an inward current, but with totally different properties. In these cells, kainate-induced currents developed rapidly and desensitized completely with a rapid time course (see Figure 2B) (Lerma et al., 1993). These responses were not cross-desensitized by a high concentration of AMPA, indicating that kainate activates a totally different, AMPA-insensitive, receptor (Lerma et al., 1993). Transient responses activated by kainate in native membranes are reminiscent of responses obtained in transfected mammalian cells with cDNAs encoding the high affinity kainate receptor subunits (Herb et al., 1992; Sommer et al., 1992). In young cultures (e.g., 1–2 days in vitro), about 30% of the cells show responses exclusively of the kainate type, but the great majority (57%) express both types of non-

*These authors contributed equally to this work.

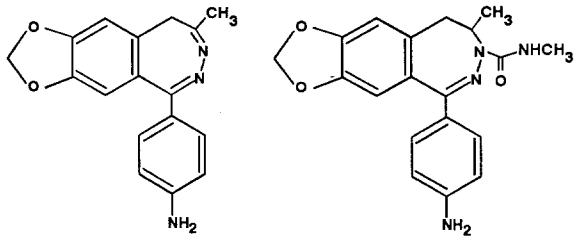

Figure 1. Chemical Structure of 2,3-Benzodiazepines GYKI 52466 and 53655

GYKI 53655, also called LY 300168, is the racemic mixture of (+) and (−) isomers of the (N-methyl)carbamate derivative of GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3- benzodiazepine).

NMDA glutamate receptors. Selective desensitization of AMPA receptors prior to the application of kainate, as described by Lerma et al. (1993), revealed functional kainate receptors in such cells. If kainate receptors are expressed at low levels in these cells, the desensitizing response to kainate is masked by the larger and nondesensitizing response to kainate acting on AMPA receptors. This may well be the situation in more developed neurons, in which responses to kainate are mostly of the AMPA type (i.e., nondesensitizing). The lack of a specific agonist for kainate receptors precludes functional study in more mature systems (e.g., slices or older cultures) in which their participation in synaptic transmission or plasticity could be addressed.

To isolate currents due to the activation of kainate receptors from those resulting from the opening of AMPA receptor channels, we tried the new noncompetitive antagonists of the AMPA receptors, GYKI 52466 and GYKI 53655 (Figure 1). These compounds are antagonists at an allosteric modulatory site present at AMPA receptors (Donevan and Rogawski, 1993; Zorumski et al., 1993) but apparently absent in kainate receptors (Lerma et al., 1993; Patneau et al., 1994). GYKI 52466 potently inhibited the response to AMPA as well as the steady response induced by kainate at AMPA receptors (IC_{50} = 9.8 ± 1.0 μM). In cells lacking AMPA receptors, transient currents induced by kainate were also, but more weakly, reduced in a dose-dependent manner. Estimated half-maximal inhibition was about 450 μM (Figure 2), indicating that this compound is approxi-

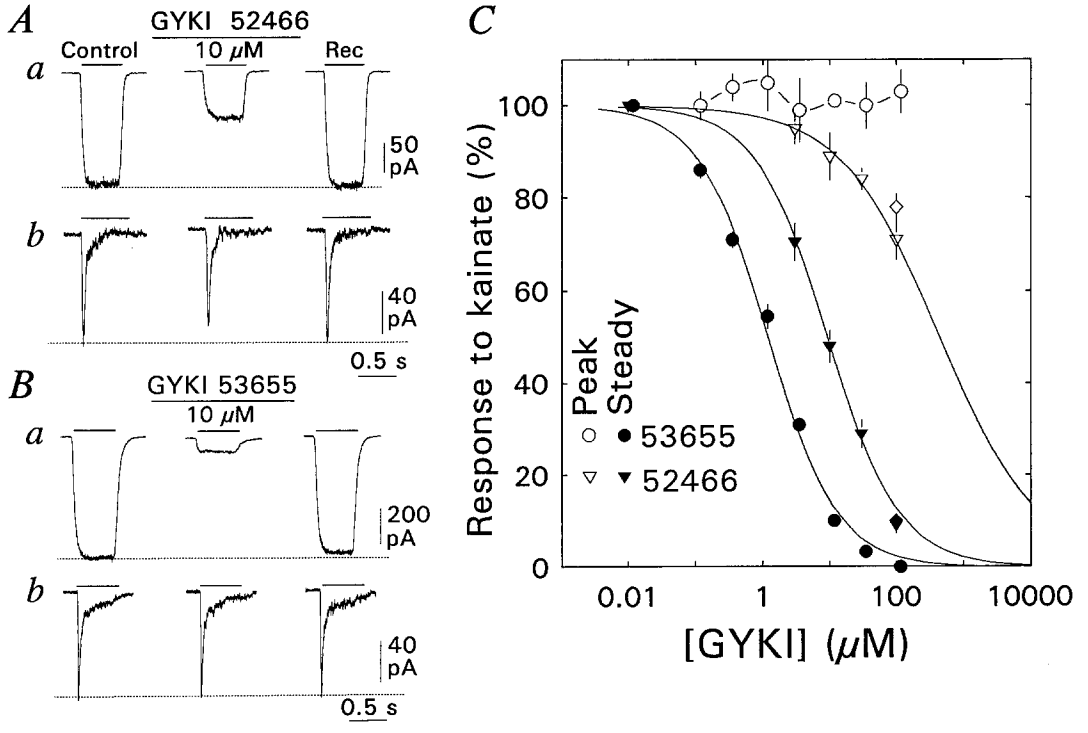

Figure 2. Antagonism of AMPA Receptors by GYKI 52466 and 53655 in Hippocampal Neurons

(A and B) Whole-cell responses induced by rapid application of 100 μM kainate were of the nondesensitizing (a) or rapidly desensitizing (b) type. Steady kainate-induced currents are due to the activation of AMPA receptors, whereas transient responses to kainate are due to the activation of kainate-selective receptors. Responses are shown before application (control), during the concomitant application of 10 μM GYKI 52466 (A) or GYKI 53655 (B), and after GYKI washout (Rec).

(C) Dose–inhibition curves for kainate-induced responses at AMPA (closed symbols) or kainate (open symbols) receptors. Circles, inhibition by GYKI 53655; triangles, the effect of GYKI 52466; diamond, degree of inhibition by GYKI 52466 of responses evoked by 300 μM kainate instead of 100 μM. Points are the mean ± SEM of values obtained from 3–13 cells. IC_{50} values for GYKI 52466 were 9.8 μM and 450 μM for AMPA and kainate receptors, respectively. GYKI 53655 inhibited AMPA receptor–mediated responses with an IC_{50} of 0.9 μM, whereas no effect was observed on kainate receptors (up to 100 μM).

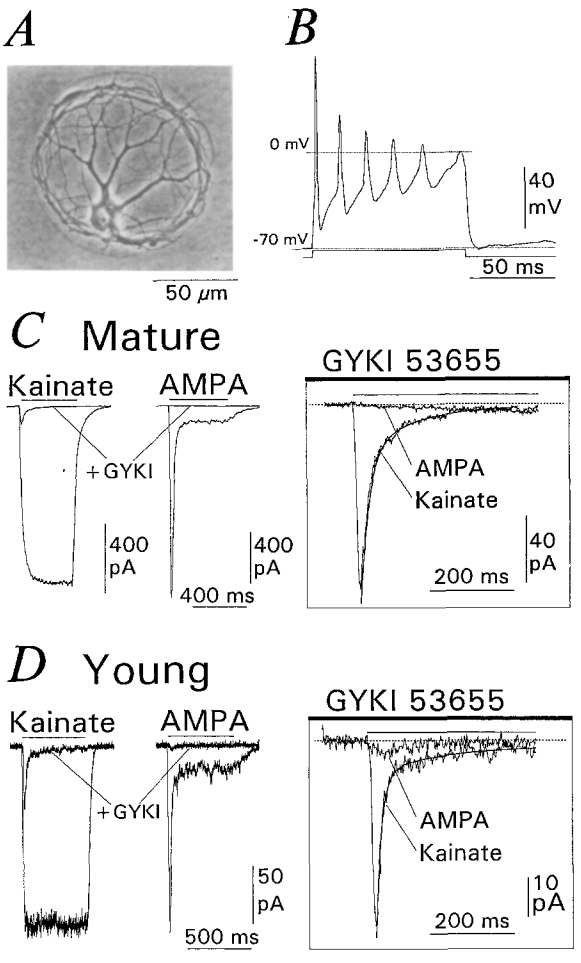

Figure 3. Disclosure of Kainate-Selective, Receptor-Mediated Responses by Specific Antagonism of AMPA Receptors

(A) Photomicrograph showing a typical hippocampal cell in microculture, growing in an island of permissive substrate.

(B) In current-clamp conditions, action potentials were generated in response to a depolarizing pulse.

(C) In this type of cell, kainate induced a very large nondesensitizing response of the type induced by activation of AMPA receptors. GYKI 53655 completely blocked activation of AMPA receptors, leaving a small kainate-activated transient current. This indicates the existence of a population of receptors that totally desensitized upon activation by kainate. Responses in the presence of GYKI are shown at a larger scale in the boxed record. The superimposed solid line represents the fit to the desensitizing process of the sum of two exponentials: τ_{fast} = 19 ms (73%) and τ_{slow} = 111 ms (27%). For this experiment, 500 μM S-AMPA and 300 μM kainate were used, while GYKI 53655 was included at 300 μM to prevent AMPA receptor activation completely.

(D) Similar experiment to that in (C) but carried out on a hippocampal neuron after 3 days in culture. The responses to S-AMPA (200 μM) and kainate (300 μM) in the presence of 100 μM GYKI 53655 are also shown at a larger scale in the inset. The solid line superimposed on the onset of desensitization corresponds to the sum of two exponentials of τ_{fast} = 14 ms (74%) and τ_{slow} = 176 ms (26%).

mately 45 times less potent on kainate than on AMPA receptors. The methyl-carbamoyl analog of GYKI 52466, GYKI 53655 (also LY 300168), however, was more potent in blocking the steady kainate-induced response (IC_{50} = 0.9 ± 0.08 μM) but was totally inactive on peak responses at 100 μM, a concentration that completely blocked steady responses (Figure 2).

Having observed that these noncompetitive antagonists of AMPA receptors were inactive on kainate-selective responses, we examined whether they would be useful tools to unmask kainate receptors in a more mature system, i.e., in hippocampal neurons in microcultures (Bekkers and Stevens, 1991) (Figure 3A). These well developed cells were characterized by having resting potentials of about −50 mV and firing single action potentials spontaneously or upon injection of a short pulse of depolarizing current. Longer pulses induced repetitive firing with some accomodation (Figure 3B). The rapid kainate perfusion induced extremely large nondesensitizing responses (>1 nA; Figure 3C). Administration of kainate together with GYKI 53655 in these well developed hippocampal neurons, apparently lacking kainate receptors, revealed the development of a transient current upon kainate perfusion of much lower magnitude than the steady current induced by kainate in the absence of drug (Figures 3C and 3D). Properties of kainate-induced currents in the presence of GYKI matched those responses obtained in young cultures, but these transient kainate-induced currents were about an order of magnitude smaller than steady kainate-induced responses (120 ± 22 pA versus 1052 ± 132 pA for 300 μM kainate in 100 μM GYKI 53655; n = 15). Clearly, such a large AMPA receptor–mediated current in mature cells masks the presence of the smaller transient response elicited by activation of kainate receptors, making its functional study impossible.

We have reported previously that young neurons expressing only transient currents are not responsive to AMPA. Our data lead us to postulate that rapidly desensitizing currents in hippocampal cells were mediated by receptors containing GluR6 subunits but not KA subunits (Lerma et al., 1993). However, we could not exclude the possibility that heteromeric receptors are expressed later in development. If this were so, nondesensitizing, AMPA-induced responses would be expected in the presence of GYKI 53655, since it is known that heteromeric GluR6/KA-2 recombinant kainate receptors are also activated by AMPA (Herb et al., 1992). To see whether kainate receptors in more mature neurons become sensitive to AMPA, we applied a high concentration of S-AMPA (500 μM) in the presence of a high concentration of GYKI 53655 (300 μM) to microcultured cells showing rapidly desensitizing responses to kainate. In these experiments, and despite the considerably large transient current induced by kainate when AMPA receptors were antagonized (73 ± 10 pA; n = 27), AMPA failed to evoke significant currents (7.7 ± 1.8 pA; n = 25). Figure 3 shows that GYKI 53655 completely blocked activation of AMPA receptors by both kainate and AMPA in young as well as mature cultured neurons. This result indicates that coexpression and/or assembly of KA subunits with GluR6 subunits, hypothetically responsible for these kainate-induced responses, is negligible and that no clear differences exist between the young and more mature microcultured cells.

Interestingly, the frequency of well developed neurons having responses of the kainate type, as revealed by selective AMPA receptor antagonism, was similar in well devel-

oped microcultures and young cultures (Lerma et al., 1993) (81 out of 102 microcultured neurons revealed kainate-selective mediated responses [79%]). From these data we infer that kainate receptors are not specifically regulated during development in culture. In 23 cells of those expressing kainate-selective mediated responses, autaptic currents could be elicited using a voltage-clamp protocol as previously described (Bekkers and Stevens, 1991). Autaptic responses were suppressed completely in the presence of GYKI 53655 (100–300 μM; data not shown), likely indicating that, at least in these cultures, kainate-selective glutamate receptors were not targeted to the synaptic contacts.

In summary, native glutamate receptors of the kainate type are affected poorly or not at all by a new family of compounds that have been proven to be very effective in blocking responses induced at AMPA receptors. This differential antagonism most probably arises from the absence of the allosteric modulatory site for benzothiadiazides in kainate receptors, known to be present in AMPA receptors. Our results demonstrate selective pharmacological properties of kainate and AMPA receptors and provide the manner to separate both responses pharmacologically in intact cells. Since rapid desensitization of AMPA receptors by kainate has been described recently (Patneau et al., 1993), one could still argue that GYKIs favor the desensitizing over the steady response induced by kainate on AMPA receptors. This seems unlikely, since the partial desensitization of AMPA receptors by kainate is seen only in outside-out patches, owing to its rapid nature (1–2 ms of time constant; see Patneau et al., 1993). In addition, not all of the cells expressing AMPA receptors showed these transient responses in the presence of GYKI, which would be expected if GYKIs favored such a desensitizing response.

Previous studies have demonstrated that these compounds do not act on NMDA, metabotropic glutamate, and γ-aminobutyric acid type A receptors (Ouarduoz and Durand, 1991). Moreover, unlike 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX) or 6,7-dinitroquinoxaline-2,3-dione (DNQX; Honoré et al., 1988), the blocking action of the GYKIs could not be surmounted by increasing the agonist concentration (Donevan and Rogawski, 1993; Zorumski et al., 1993) (Figure 2C). Thus, their use in a variety of preparations will allow the role played, if any, by kainate receptors in synaptic transmission and synaptic plasticity to be determined. The cAMP-dependent phosphorylation of some kainate receptor subunits has been shown to increase channel activity (Raymond et al., 1993; Wang et al., 1993), a phenomenon that may be involved in plasticity mechanisms. However, of the three major glutamate receptor channels, the kainate receptor has been studied the least. These receptors are usually invoked to explain the high toxic susceptibility to kainate observed in several brain areas (Coyle, 1983), but there is no conclusive evidence that kainate-induced toxicity does not reflect action of kainate on the very abundant AMPA receptors rather than on specific kainate receptors. Nevertheless, kainate receptor subunits appear to be involved in excitability disorders like kindling (Hikiji et al., 1993). Furthermore, transfection with GluR6 induces neuronal death in specific fields of the hippocampus (Bergold et al., 1993). Pharmacological isolation of responses mediated by kainate receptors will allow us to address these questions. Similarly, these compounds should permit us not only to establish the existence of functional diversity in kainate receptors but also to determine the subunit composition of functional glutamate receptors with high affinity for kainate in different brain areas.

Experimental Procedures

Cells were dissociated mechanically from hippocampi of E17–E18 rat embryos after treatment with trypsin (0.12 mg/ml; 15 min at 37°C) and seeded onto 35 mm petri dishes previously coated with poly-D-lysine (5 mg/ml) and laminin (4 μg/ml). To grow hippocampal neurons in microculture conditions, dissociated cells were plated onto petri dishes coated with 0.2% agarose and sprayed with a solution of poly-D-lysine (5 mg/ml) and laminin (8 μg/ml) as previously described (Bekkers and Stevens, 1991). Cells were incubated in Dulbecco's minimal essential medium supplemented with transferrin (0.1 mg/ml), insulin (5 μg/ml), putrescine (100 μM), progesterone (20 nM), SeO_2 (30 nM), ovalbumin (0.1%), glucose (3.3 mM), sodium pyruvate (1 mM), glutamine (4 mM), and antibiotics in a humidified incubator at 37°C and 5% CO_2. Electrophysiological experiments were carried out 1–3 days after plating conventional cultures. Microcultured neurons were used 10–14 days after plating. Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) using a List EPC-7 amplifier. Cells were rapidly perfused by a fast perfusion system (Lerma, 1992). Currents were filtered at 1 kHz (2 pole Butterworth filter) and acquired at a sampling rate of 1–2 kHz into a personal computer for analysis and display purposes. The external solution was 165 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM glucose, 10 mM HEPES (pH 7.5). Pipettes were filled with 130 mM cesium methanesulfonate, 20 mM CsCl, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 4 mM Mg–ATP, 10 mM EGTA, 10 mM HEPES, buffered to pH 7.3. To record microcultured cells, K^+ substituted for Cs^+ in the internal solution.

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