Antagonist Pharmacology of Kainate- and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid-Preferring Receptors

TIMOTHY J. WILDING and JAMES E. HUETTNER
Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110
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SUMMARY

Whole-cell recordings were used to study the antagonist pharmacology of two subtypes of non-N-methyl-D-aspartate glutamate receptors: the kainate-preferring subtype expressed by rat dorsal root ganglion (DRG) neurons and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-preferring subtype expressed by neurons from rat cerebral cortex. A series of quinoxaline derivatives were tested for the ability to distinguish between AMPA and kainate receptors, as determined by differential potency. Of the nine compounds studied, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[quinoxaline (NBQX) showed the highest selectivity for AMPA-preferring receptors, whereas 5-chloro-7-trifluoromethyl-2,3-quinoxalinedione (ACEA-1011) showed the highest selectivity for the kainate-preferring subtype. NBQX blocked non-N-methyl-D-aspartate currents in cortical cells with a $K_p$ of 0.3 µM, but in DRG neurons the $K_p$ for NBQX was 3-fold higher (0.9 µM). ACEA-1011 also blocked the currents in DRG cells with a $K_p$ of ~1 µM, but in cortical neurons the $K_p$ for this drug was 10–12 µM. Several additional compounds were tested for selective potency, including 5-nitro-6,7,8,9-tetrahydrobenzo[g]indole-2,3-dione-3-oxime, γ-D-glutamylaminomethylsulphonic acid, and derivatives of kynurenic acid and 1-benzazepine. 5-Nitro-6,7,8,9-tetrahydrobenzo[g]indole-2,3-dione-3-oxime displayed the highest selectivity in this group, blocking kainate receptors with a $K_p$ of 6 µM while inhibiting AMPA receptors with a $K_p$ of >100 µM. The remaining antagonists showed <3-fold selectivity between AMPA and kainate receptor subtypes. Our results suggest that most competitive antagonists block native AMPA and kainate receptors with approximately similar potencies, which is in marked contrast to the substantial differences in potency that have been observed with receptor agonists.

The glutamate-gated ion channels make up three different subfamilies named for the agonists NMDA, AMPA, and kainate. These subtypes were originally defined by pharmacological studies (1), but recent work on cloned GluR subunits has confirmed that NMDA, AMPA, and kainate receptors are distinct molecular entities (2, 3). Comparison of physiological responses evoked at native receptors with those observed with cloned receptor subunits expressed in heterologous cells indicates that AMPA receptors are formed by the subunits GluR1–4 (4, 5), whereas kainate receptors are constructed from GluR5–7 (6–9) in combination with the KA1 and KA2 subunits (10–12). Current understanding of the functional role of these different receptor subtypes is based largely on studies involving selective agonist and antagonist compounds. Both NMDA and AMPA receptors have been shown to mediate rapid excitatory transmission in the CNS, but the role of kainate receptors in neuronal function remains poorly understood.

Early work with kainate demonstrated that cells in hippocampus (13) and the axons of primary sensory neurons (14) are highly sensitive to this compound. Subsequent studies have focused on these cell types to characterize the properties of native kainate receptors. Sensory neurons do not express functional NMDA or AMPA receptors, but a subset of small-diameter DRG neurons produce kainate receptors (15, 16). Molecular studies indicate that the GluR5 and KA2 subunits are expressed at high levels in DRGs (2, 6, 12), and the currents recorded in heterologous cells supplied with these subunits display many of the same physiological properties as native kainate receptor channels in freshly isolated DRG neurons. Sensory neurons do not express functional NMDA or AMPA receptors, but a subset of small-diameter DRG neurons produce kainate receptors (15, 16). Molecular studies indicate that the GluR5 and KA2 subunits are expressed at high levels in DRGs (2, 6, 12), and the currents recorded in heterologous cells supplied with these subunits display many of the same physiological properties as native kainate receptor channels in freshly isolated DRG neurons.
neurons (2, 8, 12). Subunit localization studies in the brain suggest that hippocampal neurons express a number of different kainate receptor mRNAs (6, 7, 9, 10, 12); however, physiological detection of kainate receptors in hippocampal neurons and other CNS cell types has often proved difficult (16, 17). This difficulty arises because CNS neurons both in vivo (17) and in culture (18, 19, 20) seem to express a much higher density of AMPA receptors than kainate receptors. In addition, many kainate receptor agonists, including kainate and domoate, also serve as relatively potent agonists for AMPA receptors (18). Further progress toward understanding the function of neuronal kainate receptors clearly would be aided by the development of better pharmacological agents. Previous work has identified a number of compounds that display some degree of selectivity between AMPA and kainate receptors, including the noncompetitive 2,3-benzodiazepines GYKI 52466 and 53655 (20, 21), as well as competitive drugs such as the oxime NS-102 (22). However, many non-NMDA antagonists have not been directly evaluated for subtype selectivity in physiological assays on native receptors.

In the current study, we determined the relative potency of 13 competitive antagonists at AMPA receptors expressed by cultured rat cortical neurons and at kainate receptors in freshly dissociated rat DRG neurons. Our results show that most competitive antagonists display relatively weak selectivity between AMPA and kainate receptors and are therefore likely to block both subtypes when applied to neurons in situ.

Materials and Methods

Cell preparation

DRG cells were dissociated as described by Wilding and Huettner (21). The cells were maintained overnight at room temperature in Earle’s balanced salt solution (14160, Gibco-BRL; containing 2 mM CaCl₂, 1 mM MgSO₄, 20 mM glucose, and 26 mM NaHCO₃, equilibrated with 5% CO₂/95% O₂). For most experiments, recordings were obtained the day after dissociation. Cortical cells were isolated from newborn Long-Evans rats and maintained in culture as described previously (23). Recordings were obtained from cortical neurons after 6–14 days in culture.

Electrical recording and drug application. Whole-cell recordings of agonist-gated currents were obtained with an Axopatch 200 amplifier (Axon Instruments). Pipette resistance ranged from 1 to 10 MΩ with an internal solution containing 10 mM HEPES, 10 mM EGTA, 5 mM CaCl₂, and 140 mM CsCl, or CsF, titrated to pH 7.40 with CsOH. In most cases, current recorded during agonist applications was compresses by averaging 3 msec of data at 0.1-sec intervals. The recording chamber was continuously perfused with Tyrode’s solution (150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 13 mM HEPES, pH 7.4). The external solution for drug application contained 160 mM NaCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4, 500 μM tetrodotoxin, and 2 μM MK-801. Control, agonist, and antagonist solutions were applied from a bank of microcapillary tubes mounted on a micromanipulator and connected to a series of reservoirs. Solution flow was driven by gravity. To block desensitization of kainate currents in DRG cells (15), concanavalin A was applied at 2 μM for 5–10 min before recording. Concentration-response curves were generated and analyzed as described previously (24). The dissociation constant for each inhibitor was calculated from the displacement it produced in the EC₅₀ for receptor activation by kainate. More extensive Schid analysis was not undertaken due to the low solubility of many of the antagonists and their apparent low selectivity based on analysis of single doses.

Antagonists were prepared as 10–40 mM stock solutions in DMSO, ethanol (dioxiane), or 50% DMSO/50% ethanol (NS-102). These stocks were diluted into control or agonist-containing solutions so that the final concentration of vehicle was ≤0.5%. Pure DMSO, ethanol, or DMSO/ethanol was added to solutions lacking antagonist so that all of the solutions used in an experiment would contain the same levels of vehicle. All drug stock solutions were stored at −20°C. AMPA, GAMS, and CNQX were purchased from Research Biochemicals International. Kainate, L-glutamiciic acid, tetrodotoxin, and concanavalin A were obtained from Sigma Chemical Co. QX and HXQCA were purchased from Aldrich. 6CIQX, 6,7diCIQX, and 5NQX were obtained from Tocris Cookson. The remaining drugs were kindly provided by the following companies: ACEA Pharmaceuticals, a wholly owned subsidiary of CoCenys (ACEA-1011, ACEA-1021, and 8C1DDHb), Marion Merrell Dow (5,7diClKyn), Merck, Sharp, and Dohme (diazocilpine), Novo Nordisk (NBQX), and NeuroSearch (NS-102).

Results

Quinoxaline derivatives. Whole-cell currents activated by kainate were used to study the inhibitory potency of a series of compounds at kainate- and AMPA-prefering receptors. Current through AMPA-prefering receptors was recorded in cultured neocortical neurons, whereas kainate receptors were studied in freshly dissociated DRG neurons. Previous studies (e.g., Ref. 25) have shown that kainate activates large maintained currents through AMPA receptors in CNS neurons. For most experiments, DRG neurons were briefly exposed to concanavalin A at the beginning of the recording session. Treatment with concanavalin A has been shown to eliminate desensitization of kainate receptors without causing any significant change in the agonist concentration required to give half-maximal receptor activation (15, 18). By treating the cells with concanavalin A, we were able to study the steady state inhibition produced by the various antagonists, which would otherwise have been difficult to resolve once receptor were strongly desensitized. The inhibitory action of each antagonist was verified qualitatively in freshly isolated cells that had not been exposed to lectin. We saw no evidence that exposure to concanavalin A had any effect on the relative potency of the antagonists.

Initially, four quinoxalinediones, CNQX, NBQX, ACEA-1011, and ACEA-1021, were tested for their ability to inhibit kainate currents in DRG and cortical neurons. To determine antagonist potency and provide information on the mechanism of inhibition, we examined the effect of a fixed concentration of each antagonist on the concentration-response relation for kainate. Fig. 1 shows the inhibition of whole-cell kainate current by NBQX (26) and by ACEA-1011 (27, 28). In the absence of antagonist, kainate currents in cortical neurons were half-maximal at a concentration of 160 μM, whereas in DRG cells, the half-maximal concentration was 6 μM. In both cell types, the dose-response relation for kainate was shifted toward higher concentrations by the inclusion of NBQX or ACEA-1011. Inhibition by the drugs was overcome with saturating doses of kainate, which is consistent with a competitive mechanism of antagonism.

Comparison among the first four compounds that we tested revealed that all four drugs blocked with similar potency at kainate receptors (Kᵢ ≈ 1 μM), but they displayed a much broader range of potencies against AMPA receptors (0.3 < Kᵢ).
In terms of subtype selectivity, NBQX was ~3-fold more potent against AMPA-preferring receptors, CNQX showed nearly identical potency at both receptors, and ACEA-1021 and ACEA-1011 were ~3-fold and 10–12-fold more potent at kainate-preferring receptors, respectively. Based on these observations, we tested a number of additional quinoxaline derivatives in the hope of finding a compound with micromolar potency against kainate receptors but much lower affinity for the AMPA subtype. As shown in Fig. 2 and summarized in Table 1, all five of these additional compounds (QX, 6ClQX, 6,7diClQX, 5NQX, and HQXCA) were somewhat selective for kainate versus AMPA receptors, but none of the drugs showed >5-fold difference in potency at the two receptor subtypes (see Fig. 5).

**Other antagonists.** In addition to the quinoxaline derivatives, we tested several other compounds that have been shown to exhibit some degree of differential potency between AMPA and kainate. The compound GAMS was a relatively weak antagonist of kainate current in both cell types (Fig. 3 and Table 1). The $K_b$ for GAMS was 360 μM in DRG neurons and 750 μM in cortical cells, yielding a selectivity ratio of 2.1. Because this degree of selectivity was considerably lower than that obtained in several previous studies (29–31), we also tested cortical neurons for the action of GAMS against steady state currents gated by AMPA (Fig. 4D). In all six of the cells tested, GAMS inhibited the current evoked by low doses of AMPA. The $K_b$ value for inhibition of steady state AMPA current was ~400 μM. In two of the cells (e.g., Fig.

### Table 1

**Activation of AMPA and kainate receptors in the presence and absence of antagonists**

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<td>Dose</td>
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<td>Slope factor</td>
<td>Antagonist $K_b$</td>
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**Fig. 1.** Antagonism of whole-cell kainate currents by NBQX and ACEA-1011. A, Currents activated by kainate alone (left), kainate plus 2 μM NBQX (middle), and kainate plus 30 μM ACEA-1011 (right) in three different cortical neurons. Filled bars, duration of agonist exposure. Numbers above each bar, kainate concentration (in μM). A control response to 10 mM kainate alone (10 mM C) is shown at the end of each antagonist series. B, Currents evoked by kainate alone (left), kainate plus 10 μM NBQX (middle), and kainate plus 30 μM ACEA-1011 (right) in three different DRG neurons that had been treated with concanavalin A to block desensitization. Holding potential, −70 mV. Scales left to right: (A) 100 pA, 35 sec; 150 pA, 20 sec; 250 pA, 20 sec; and (B) 200 pA, 25 sec; 50 pA, 40 sec; 50 pA, 25 sec.
of the relations for kainate alone and kainate plus NS-102 were an 18-20-fold gest that has been previously described (32, 33). Taken together, for kainate in DRG neurons, suggesting a for kainate in relation Fig. 2. Effect of quinoxaline derivatives on the concentration-response relation for kainate in DRG (A) and cortical (B) neurons. Data are mean ± standard error of the normalized currents (I/I_control). Smooth curves, best fit of I/I_control = 1/(1 + (EC50/kainate)) to all of the data points for each concentration-response relation. Curve parameters and antagonist concentrations are given in Table 1 (n is the slope factor). Data for cortical cells tested with ACEA-1021 come from Ref. 24, and data for five of the eight cortical cells tested with ACEA-1011 come from Ref. 28.

4D), the response to saturating AMPA (250 μM) was slightly potentiated in the presence of GAMS (1 mM), a phenomenon that has been previously described (32, 33). Taken together, these results indicate very little selectivity by GAMS between AMPA and kainate subtypes.

Significantly greater selectivity was observed for NS-102 (22). As shown in Fig. 3, 10 μM NS-102, which is close to the limit of aqueous solubility, produced a 3-fold shift in the EC50 for kainate in DRG neurons, suggesting a Ks of 5-6 μM for this drug. In cultured cortical neurons, 10 μM NS-102 consistently produced a slight reduction in current gated by 40 μM to 2.5 mM kainate, but the data in Fig. 3 indicate a Ks of ≥100 μM at cortical receptors (see Discussion). These results suggest an 18-20-fold selectivity in favor of the kainate-prefering receptors (Table 1). Because the concentration-response relations for kainate alone and kainate plus NS-102 were collected in different cells, we were concerned that the small shift observed with NS-102 in cortical cells might simply reflect random variability in the responses to kainate and not a direct inhibitory action by the drug. To control for this possibility, several neurons were tested with various concentrations of kainate alone, as well as kainate plus NS-102 (Fig. 4, A and B). In six cortical cells, 10 μM NS-102 produced 6 ± 2% inhibition of currents gated by 160 μM kainate, which is consistent with our estimated dissociation constant of 114 μM. Similar experiments performed with AMPA instead of kainate gave the same result (Fig. 4C); 10 μM NS-102 produced ~6-10% inhibition against concentrations of AMPA near the EC50 (5 μM) but did not inhibit the steady state current evoked by a saturating dose of AMPA (250 μM).

Finally, both the 5,7-dichloro derivative of kynurenic acid and the 8-chloro derivative of dihydro-2,5-dioxo-3-hydroxy-1H-benzazepine produced modest inhibition of kainate current in DRG and cortical neurons (34, 35). Neither drug, however, displayed significant selectivity between the two receptor subtypes (Fig. 3 and Table 1).
have highlighted their differential sensitivity to modulation of AMPA- and kainate-preferring receptor pharmacology. Relative to NBQX ($K_a = 0.3 \mu M$). In DRG cells, NBQX was only slightly less potent ($K_a = 0.9 \mu M$), and the full sequence of antagonist potency covered a narrower range of apparent affinities: NBQX (1) = ACEA 1021 (1) = ACEA 1011 (1.1) > CNQX (1.7) > NS-102 (6.7) > 8CIDDHB (11) > 6,7diClQX (14) > 5,7diClKyn (22) > 6ClQX (42) > QX (60) > 5NQX (119) > HQXCA (142) > GAMS (397). As a result, drugs with lower affinity in cortical neurons, such as ACEA-1011 and NS-102, showed the highest selectivity for receptors expressed by DRG cells (Fig. 5). With the ratio of antagonist dissociation constants in cortical cells versus DRG neurons used as a gauge of relative potency, the following selectivity sequence is derived: NBQX (0.3) > CNQX (0.9) > 8CIDDHB (1) > 5,7diClKyn (1.1) > 6ClQX (1.7) > HQXCA (1.8) > 6,7diClQX (2.0) > GAMS (2.1) > 5NQX (2.7) > ACEA-1021 (2.8) > QX (3.5) > ACEA-1011 (12) > NS-102 (19), where the number in parenthesis is $K_a/k_D$.

NS-102 showed the highest selectivity for kainate-preferring receptors among the 13 antagonists that we tested. This compound displaces kainate from low affinity binding sites in rat cortical membranes (39) and was found to antagonize kainate-preferring receptors on embryonic hippocampal neurons (40). Selective inhibition by NS-102 also has been observed for receptors expressed in 293 cells. Verdoorn et al. (22) found that homomeric channels formed by the kainate receptor subunit GluR6 were significantly more sensitive to block by NS-102 than heteromeric receptors formed by coexpression of the AMPA receptor subunits GluR2 and GluR4. Because NS-102 is poorly soluble in aqueous solutions, we did not test any concentration of $>10 \mu M$. In DRG cells, this dose produced a shift of ~3-fold in the $EC_{50}$ for kainate, whereas in cortical neurons, the effect was much smaller (see Fig. 2). The ratio of apparent dissociation constants for NS-102 favored inhibition at kainate receptors by nearly 20-fold, although in terms of absolute affinity NS-102 was 6-fold less potent at kainate receptors than were several of the quinoxalinediones (Fig. 5).

ACEA-1011 showed ~12-fold selectivity for kainate-preferring versus AMPA-preferring receptors. This compound, along with the related drug ACEA-1021, displays even higher
affinity for the glycine modulatory site on the NMDA receptor than for either of the non-NMDA receptors (24, 28). ACEA-1011 blocks glycine potentiation of NMDA receptors with a $K_i$ of $\sim 0.5 \mu M$ (27, 28), whereas ACEA-1021 is considerably more potent ($K_i = 5 \, \text{nm}$) (24). As expected from previous work (26, 41, 42), NBQX and CNQX were the most potent antagonists against AMPA receptors in rat cortical neurons. Our apparent dissociation constants for these drugs are largely more potent for AMPA and kainate receptors (29, 30). Most recently, a study found that GAMS blocked kainate currents with a virtually no effect on currents evoked by AMPA (31). Our results in cells from rat cortex and DRGs indicate substantial lower selectivity. Using kainate as the agonist, we obtained $K_i$ values of 360 $\mu M$ in DRG neurons and 750 $\mu M$ in cortical cells. Further work will be needed to understand the basis for this apparent difference between native receptors expressed by a specific population of cells and those expressed in oocytes after injection of whole-brain mRNA.

**Comparison with agonists.** In contrast to the current study, in which we found relatively low selectivity between AMPA and kainate receptors (29, 30), most recently, a study of receptors expressed in Xenopus oocytes from whole chick brain mRNA found that GAMS blocked kainate currents with an apparent dissociation constant of $\sim 50 \mu M$ but had virtually no effect on currents evoked by AMPA (31). Our results in cells from rat cortex and DRGs indicate substantial lower selectivity. Using kainate as the agonist, we obtained $K_i$ values of 360 $\mu M$ in DRG neurons and 750 $\mu M$ in cortical cells. Further work will be needed to understand the basis for this apparent difference between native receptors expressed by a specific population of cells and those expressed in oocytes after injection of whole-brain mRNA.

**Functional implications.** Since their initial characterization several years ago (26, 44), quinoxalinediones such as CNQX and NBQX have become the standard antagonists for inhibition of neuronal AMPA receptors. Our results, together with those of other recent studies (7, 22, 37), indicate that these compounds also produce potent inhibition at kainate receptors. Compared with the noncompetitive 2,3-benzodiazepine YGYK 53655, which shows $>200$-fold higher affinity at AMPA than at kainate receptors (20, 21), even NBQX displayed only a 3-fold difference in potency for inhibition of whole-cell currents. Although previous work (26, 44) demonstrated relatively strong selectivity for quinoxalinediones between high affinity AMPA and kainate binding sites, recent analysis of cloned receptor subunits suggests that functional ion channels correspond to subunit combinations with lower affinity for kainate ($K_i = 50-100 \, \text{nm}$), which include the GluR5 or GluR6 subunit (12). High affinity kainate binding sites ($K_i$ for kainate $= 5-10 \, \text{nm}$), formed by expression of the KA1 or KA2 subunits in isolation, do not make functional channels (12).

In the current study, we determined antagonist potency against kainate receptors in DRG neurons that are thought to include the GluR5 and KA2 subunits (2, 6, 8). Recent work on homomeric GluR6 receptors expressed in 293 cells revealed similar potencies for CNQX and NS-102 in that system (22). However, a study of kainate receptors in embryonic hippocampal neurons (40), which are thought to express the GluR6 and, in some cells, GluR5 subunit (45), found NS-102 to be significantly more potent than CNQX. Clearly, further work will be needed to determine whether the various antagonists used in this study show similar potency against kainate receptors resulting from other subunit combinations that may be expressed by other cells in the nervous system. Nevertheless, in the case of DRG cells, our results emphasize the broad overlap in potency of competitive antagonists at kainate receptors and CNS AMPA receptors.

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**References.**


