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ACCELERATED COMMUNICATION

Pharmacological Discrimination of GluR5 and GluR6 Kainate Receptor Subtypes by (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl]decahydroisoquinoline-3 carboxylic-acid

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SUMMARY

The pharmacological tools available for the discrimination of kainate receptor subtypes are limited. We examined the effects of (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid (LY293558) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline (NBQX) on inward currents associated with activation of non-*N*-methyl-D-aspartate (NMDA) receptors in acutely isolated rat cerebellar Purkinje neurons, rat dorsal root ganglion neurons, and human embryonic kidney 293 cells transfected with human glutamate receptors (GluR) 5 and 6. LY293558 and NBQX inhibited kainate-induced currents in cerebellar Purkinje cells, DRG neurons, and human GluR5-transfected cells. In contrast, human embryonic kidney 293 cells expressing GluR6 receptors, although blocked by NBQX, were unaffected by LY293558 at concentrations of ≤100 μM. The selective antagonism by LY293558 of GluR5 receptors should allow the determination of the functional role of GluR5 and GluR6 in more complex systems.

Glutamate is the major excitatory neurotransmitter in the brain and can act on three major types of ligand-gated ion channels that are defined by the activity of the subtype-selective agonists NMDA, kainate, and AMPA (1). Non-NMDA ion/channel-linked glutamate receptor proteins that have been cloned include kainate- and AMPA-preferring subtypes (2). AMPA receptors, although activated by kainate, are distinct from kainate receptors in several respects. Molecular biological studies have determined that they are composed of subunits (GluR1–4) that can assemble to form functional channels. AMPA evokes desensitizing currents from these receptor channels, whereas the responses to kainate are nondesensitizing (3). Desensitization of AMPA responses is removed by cyclothiazide (4), and AMPA receptors are selectively inhibited by the noncompetitive AMPA antagonist GYKI53655 (5). Five kainate receptors have been cloned and classified as either high affinity (KA1 and KA2) or low affinity (GluR5, GluR6, and GluR7) kainate receptors. GluR5 and GluR6 but not GluR7, KA1, and KA2 receptors form functional ion channels when expressed in homomeric configurations (6–13). Desensitization of kainate-induced responses at both GluR5 and GluR6 is removed by the plant lectin concanavalin A (6, 14, 15). Human GluR5, when expressed in its homomeric configuration, is activated by kainate and only weakly activated by AMPA (14), whereas GluR6 forms a homomeric receptor channel that can be selectively activated by kainate but not by AMPA (13). GluR5 is widely distributed throughout the central nervous system (2) and most tissues express kainate receptors along with AMPA receptors. However, DRG neurons have been shown to express GluRs that are preferentially activated by kainate (15), and Northern blot analysis for probes of individual AMPA and kainate receptor subunits demonstrates that GluR5 mRNA predominates in DRG cell bodies (6). This observation coupled with

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate: DRG, dorsal root ganglion; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; HEK, human embryonic kidney; MEM, minimum essential medium; BSA, bovine serum albumin; LY293558, (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GluR, glutamate receptor; KA, kainate receptor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

the selective modulation of kainate responses in DRG neurons by concanavalin A and not the AMPA receptor modulator cyclothiazide suggests that GluR5 receptors mediate the observed kainate-induced currents in DRG neurons.

Agents that antagonize AMPA receptors (but also kainate receptors) include the quinoxaline compounds, most notably NBQX, which is active when administered systemically and thus has been very useful in defining the potential of this class of compounds (16). LY293558 is also a competitive antagonist of AMPA receptors (17). In the present study, we compared the effects of LY293558 with those of NBQX on AMPA receptor-mediated responses in rat cerebellar Purkinje cells. It has been reported that both AMPA- and kainate-preferring receptors are present in cerebellar Purkinje cells; these include GluR1, GluR2, and GluR3 AMPA-preferring subunits (18–20) and GluR5 and KA1 ionotropic glutamate receptors (8, 21). The presence of functional AMPA-preferring receptors is established in cerebellar Purkinje cells, and the presence of functional kainate-preferring conductances has been reported in rat cerebellar Purkinje cells maintained in slice culture (22). We used acutely isolated cerebellar Purkinje cells under experimental conditions that are likely to preclude the involvement of the kainate-preferring class of receptors. In addition, we compared the actions of NBQX and LY293558 on kainate responses in acutely isolated rat DRG neurons and on both human GluR5 and GluR6 receptors transfected into HEK 293 cells (13, 14). The results of our study reveal a novel selectivity profile for block of AMPA and kainate receptors; in addition to its ability to block AMPA receptors, LY293558 inhibits GluR5 but not GluR6 kainate receptors.

Materials and Methods

Cerebellar Purkinje neurons were isolated via modification of the method of Mintz *et al.* (23). The cerebellar vermes of 6–11-day-old rat pups (Sprague-Dawley, Harlan) were removed and transferred to buffer containing 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 1 mM HEPES, 1 mM glucose, and 0.1% phenol red, pH 7. Tissue was cleaned, chopped, and digested with buffer containing 1 mg/ml Protease XXIII (Sigma Chemical Co.) at 37° for 6 min. After digestion and being washed, the tissue was transferred to buffer supplemented with 1 mg/ml BSA (Sigma) and 1 mg/ml trypsin inhibitor. Cells were dissociated by trituration and plated onto poly-L-lysine-coated (Sigma) (50 μg/ml) glass coverslips. Purkinje cells were identified morphologically from the surrounding granule cells based on their large cell bodies (15–30 μM). DRG neurons were isolated via modification of the method of Moises *et al.* (24). DRGs were dissected from the lumbar and thoracic regions of 4–7-day-old rats and treated with collagenase (type II, 3 mg/ml) for 50 min at 37° in a solution consisting of 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 1 mM HEPES, and 1 mM glucose, pH 7.4. After centrifugation and removal of the enzyme solution, a solution of MEM (GIBCO Laboratories) with BSA (20 mg/ml) was added, and the tissue was resuspended and centrifuged. This procedure was repeated, and the MEM/BSA solution was replaced with an MEM solution supplemented with 1.5 mg/ml $NaHCO_3$, 300 ng/ml nerve growth factor, 10 μg/ml penicillin/streptomycin, and 10 mM glucose. Cells were mechanically dispersed with the use of a fine-tipped plastic Pasteur pipette and plated onto poly-L-lysine-coated (50 μg/ml) glass coverslips. All recordings were performed within 36 hr of plating.

Stable cell lines of HEK 293 cells transfected with cDNA coding for the human GluR5(Q) and GluR6(Q) receptors were established as previously reported (13, 14). For electrophysiological recordings, cells were dissociated by trituration and plated onto poly-L-lysine-coated (10 μg/ml) coverslips. Whole-cell voltage-clamp recordings were made from cells with use of the tight-seal whole-cell configuration of the patch-clamp technique (25). Glass fragments of coverslips

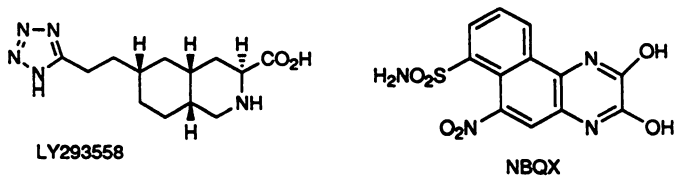

Fig. 1. Structures of LY293558 and NBQX.

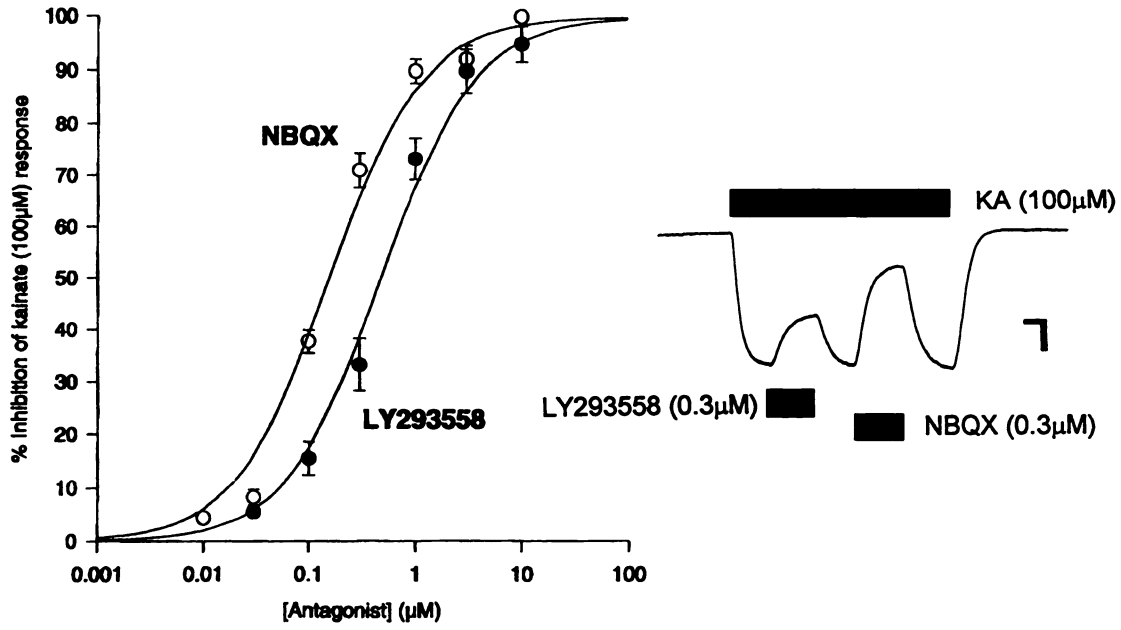

Fig. 2. Inhibitions produced by NBQX and LY293558 of inward currents evoked by kainate (100 μM) in cerebellar Purkinje neurons (V_h = −70 mV). Current traces show the inhibition of kainate-induced (100 μM) current by LY293558 (0.3 μM) and NBQX (0.3 μM). *Vertical scale bars*, 200 pA; *horizontal scale bars*, 5 sec.

with adherent cells were placed in a perfusion recording chamber and rinsed thoroughly with recording buffer containing 138 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES, and 10 mM glucose, pH 7.4 with NaOH (osmolality, 315 mOsm/kg). Pipette solutions contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 0.1 mM EGTA, pH 7.2 with KOH (osmolality, 285 mOsm/kg). For DRG neurons, intracellular solutions were composed of 125 mM Cs methanesulfonate, 15 mM CsCl, 5 mM Cs BAPTA, 10 mM HEPES, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$, and 2 mM MgATP, pH to 7.2. Currents were recorded on either an Axopatch ID or a List EPC-7 amplifier. Pipette of resistance 1.5–2.5 MΩ were used. The holding potential for all experiments described was −70 mV.

Drug application for Purkinje cells and GluR6-expressing HEK 293 cells occurred via bath perfusion, and exchange of solutions occurred within ~15 sec. For DRG neurons and GluR5(Q)-expressing HEK 293 cells, drug application occurred via a series of multibarrel perfusion lines (Biologic Inc.). Solution exchange was ~100 msec for these two systems. Experiments in HEK 293 cells expressing human GluR6, GluR5, and DRG neurons were performed after preincubation in buffer containing concanavalin A (250 μg/ml, Type IV, Sigma) to prevent agonist-induced receptor desensitization.

Drugs used in the study were NBQX (Tocris Cookson, Bristol, UK), LY293558 (Lilly Research Laboratories, Indianapolis, IN), and concanavalin A (Sigma). NBQX was dissolved in dimethylsulfoxide with stock concentrations that limited final exposure of tissue to dimethylsulfoxide to <0.2%. LY293558 was dissolved as stock concentration in equimolar NaOH. Curve fitting was based on the following equation: percent inhibition (y) = $100(D^n/D^n + EC_{50}{}^n)$, where the slope of the lines, n, is fixed to a value of 1, and D is the antagonist concentration. Estimates of dissociation constants (pK_B) for antagonists were made through analysis of competitive agonist/antagonist interactions by nonlinear regression as described by Lew and Angus (26).

Results and Discussion

AMPA receptor-mediated responses in cerebellar Purkinje cells are inhibited by LY293558 and NBQX. The structures of NBQX and LY293558 are shown in Fig. 1. In the present study, we used kainate to activate AMPA receptors. Under our experimental conditions in the absence of the allosteric modulator cyclothiazide, kainate produces nondesensitizing inward currents. Kainate-induced currents in cerebellar Purkinje neurons were unaffected by concanavalin A. In the presence of concanavalin A (250 μg/ml), kainate (100 μM) responses were 92 ± 4% (four cells) of kainate responses in the absence of concanavalin A, whereas both AMPA (1 μM) and kainate (30 μM) responses were potentiated by cyclothiazide (30 μM) by 620 ± 90% (three cells) and 58 ± 14% (four cells), respectively. Our experimental paradigm, which uses slow application of agonist in the absence of concanavalin A, is therefore likely to preclude involvement of rapidly desensitizing responses to kainate acting at kainate-preferring receptors. Both NBQX and LY293558 inhibited kainate-activated (100 μM) inward currents in acutely isolated cerebellar Purkinje neurons. Fig. 2 shows current traces of typical inhibitions produced by LY293558 and NBQX and a plot of the concentration-dependent inhibition curves for both compounds. Estimated IC_{50} values of 0.16 ± 0.02 μM (five cells) and 0.47 ± 0.05 μM (five cells) were obtained for NBQX and LY293558, respectively.

LY293558 and NBQX inhibit kainate-induced currents in DRG neurons and human GluR5. As previously reported (6, 15, 27), application of kainate to acutely isolated DRG neurons evoked inward currents that were potentiated by concanavalin A but not by cyclothiazide (not shown). To remove receptor desensitization, we routinely preincubated DRG neurons for ≥10 min in concanavalin A (250 μg/ml). Of 108 cells examined, kainate induced currents in 57% of cells, with an EC_{50} value of 12.1 ± 0.6 μM (eight cells). The effects of NBQX and LY293558 on kainate-induced (30 μM) currents in DRG neurons are shown in Fig. 3A. In recordings from eight cells, NBQX and LY293558 produced reversible and concentration-dependent inhibitions of kainate-induced currents in DRG neurons. In contrast to cerebellar Purkinje neurons, NBQX was marginally less potent than LY293558 in DRG neurons, with IC_{50} values of 2.9 ± 0.2 and 1.0 ± 0.1 μM, respectively. The inhibition of kainate-induced currents by NBQX and LY293558 was competitive and could be overcome by saturating concentrations of kainate. Estimates of antagonist dissociation constants (pK_B values) for these compounds based on nonlinear regression analysis resulted in pK_Bvalues of 5.97 ± 0.14 and 6.24 ± 0.07 for NBQX and LY293558, respectively. Studies were also performed on human GluR5 receptors stably transfected into HEK 293 cells (14). This channel, expressed in its homomeric form, is activated by kainate and domoate, with approximate EC_{50} values of 16.1 ± 1.0 μM (six cells) and 0.3 ± 0.1 μM (11 cells), respectively. In a similar manner to that observed with DRG

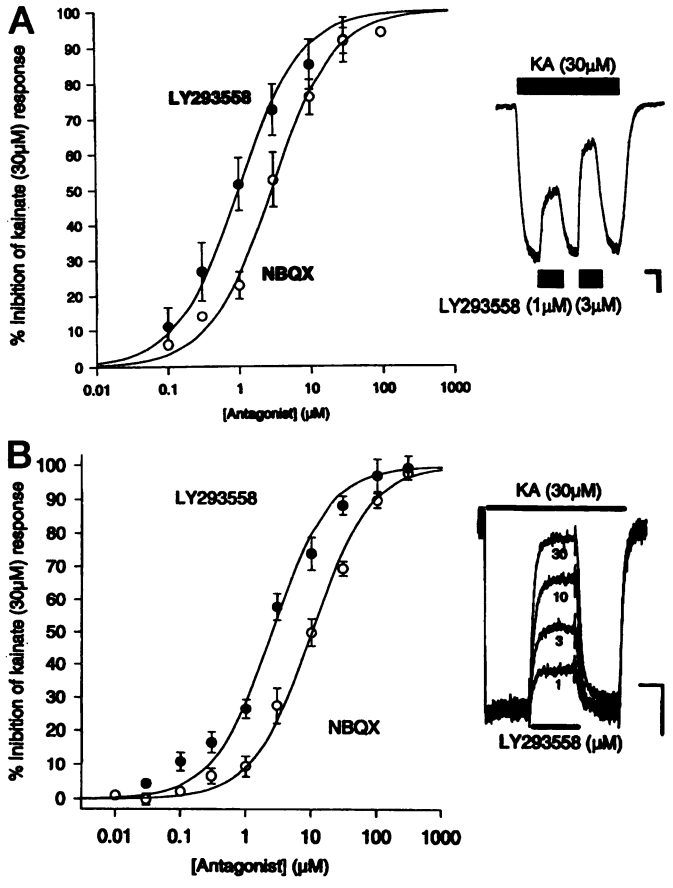

Fig. 3. A, Inhibition of kainate (30 μM) inward currents by NBQX and LY293558 in acutely isolated DRG neurons. *Inset*, current trace illustrating the concentration-dependent inhibition of a kainate current by LY293558. *Vertical scale bars*, 100 pA; *horizontal scale bars*, 2 sec. B, Inhibition of kainate (30 μM) inward currents by NBQX and LY293558 in HEK 293 cells stably expressing GluR5 kainate receptors. *Inset*, current trace illustrating the concentration-dependent inhibition of a kainate current by LY293558. *Vertical scale bars*, 50 pA; *horizontal scale bars*, 2 sec.

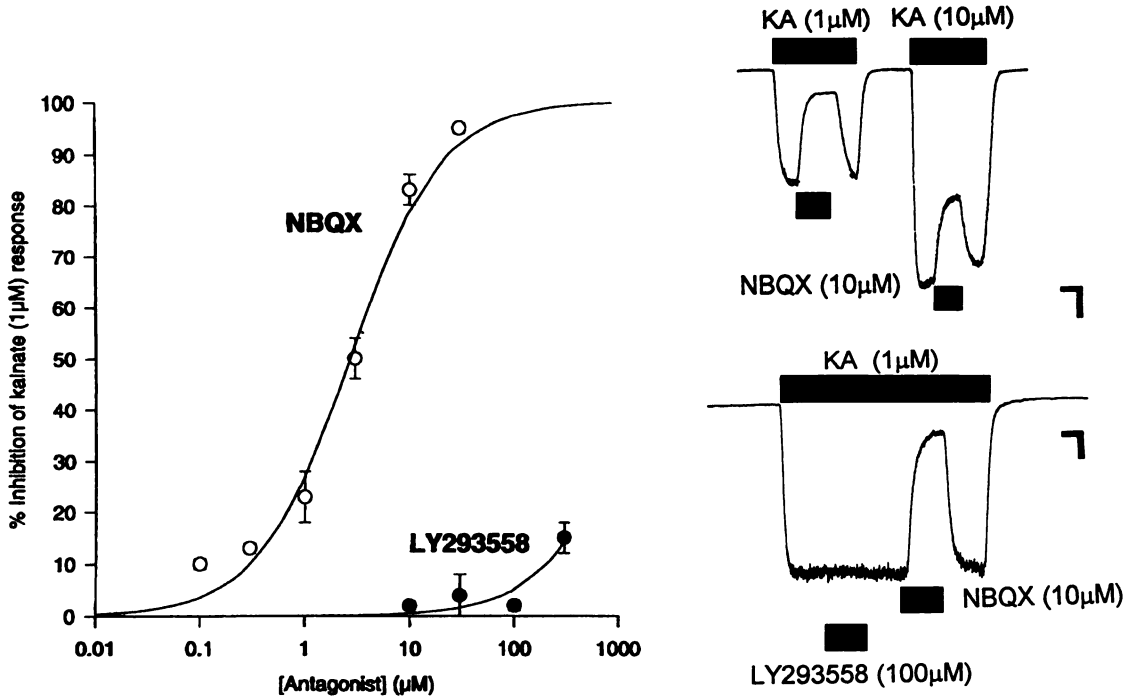

Fig. 4. Inhibition of inward currents evoked by kainate (1 μM) in HEK 293 cells expressing the GluR6 receptor by NBQX (four cells) but not by LY293558 (four cells). Current traces show the effect of NBQX (10 μM) at two different kainate concentrations (1 and 10 μM). *Vertical and horizontal scale bars: top inset*, 200 pA and 30 sec; *bottom inset*, 100 pA and 5 sec, respectively.

neurons, both NBQX and LY293558 inhibited kainate-evoked (30 μM) currents (Fig. 3B). Calculated IC_{50} values of 11.6 ± 3.8 μM (three cells) and 2.5 ± 0.3 μM (six cells) were obtained for NBQX and LY293558, respectively. The inhibition of kainate-induced currents by NBQX and LY293558 was competitive and could be overcome with saturating concentrations of kainate, and calculated pK_B values of 5.35 ± 0.01 and 6.24 ± 0.06 were obtained.

LY293558 discriminates between GluR5 and GluR6 kainate receptors. The effects of NBQX and LY293558 were also examined on human GluR6 glutamate receptors stably transfected into HEK 293 cells. As with GluR5 ionotropic glutamate receptors and kainate-activated currents in DRG neurons, agonist-induced desensitization of rat GluR6 receptors has been shown to be prevented by the plant lectin concanavalin A (250 μg/ml) (13). Steady state removal of desensitization of human GluR6 was achieved within 10 min of concanavalin A treatment. The effect of concanavalin A was maintained for ≥2 hr after its washout (data not shown). Currents activated by kainate (1 μM) in these cells were reversibly blocked by NBQX, with an estimated IC_{50} value of 2.76 ± 0.35 μM (four cells). However, LY293558 failed to produce a significant inhibition of kainate-induced currents at concentration of 100 μM and only small effects at concentrations as high as 300 μM (Fig. 4).

The compounds LY293558 and NBQX (a tetrazole-substituted decahydroisoquinoline and quinoxalinedione, respectively) are structurally distinct from each other. Nevertheless, both have been shown to be competitive antagonists of AMPA receptors in *in situ* preparations. For example, LY293558 (as racemic compound LY215490) prevents both AMPA- and kainate-induced depolarizations in rat neocortical slices (17), with ~5-fold selectivity toward blocking AMPA. NBQX will also antagonize depolarizations to both AMPA and kainate. In the rat neocortical slice, NBQX is ~30-fold more potent against AMPA-evoked depolarizations than kainate-evoked depolarization (16). However, in the rat spinal cord, both LY293558 and NBQX seem to be less selective between AMPA- and kainate-induced increases in firing rate. These responses are enhanced by cyclothiazide and therefore likely to be mediated by GluR1–4 receptors. Because kainate can activate both AMPA- and kainate-preferring subtypes, the selectivity of both of these compounds between kainate-preferring glutamate receptors (GluR5 and GluR6) remained to be investigated. Previous work has shown that the quinoxalines such as CNQX and NBQX lack the ability to discriminate between AMPA- and kainate-preferring receptors (12, 15, 28, 29). Such a lack of selectivity was confirmed in the present experiments. The observed concentration-dependent inhibition of inward currents activated by kainate in cerebellar Purkinje cells by both LY293558 and NBQX is in good agreement with the previously reported activity of these compounds at AMPA receptors in cortical slices and spinal cord preparations. To date, the ability to discriminate between AMPA- and kainate-preferring receptors has resided in the use of compounds that allosterically modulate desensitization of receptors, cyclothiazide for AMPA receptors and concanavalin A for kainate receptors (6). The present results suggest that LY293558 is able to block not only responses mediated by AMPA receptors but also those mediated by either homomeric GluR5 and native GluR5 in DRG neurons. However, unlike NBQX, LY293558 does not affect GluR6 receptor-mediated responses. LY293558 is also inactive at heteromeric human GluR6 and KA2 kainate receptors,[1] although studies remain to be conducted on other heteromeric combi-

[1] D. Bleakman, B. Ballyk, and R. K. Kamboj, unpublished observations.

nations of kainate receptors. Previous studies have demonstrated that selective antagonism of AMPA responses may be achieved with the noncompetitive AMPA receptor antagonist GYKI53655 (5).[2] The activity profile of LY293558 should prove useful for unmasking responses mediated by GluR6 receptors. For example, AMPA-mediated responses can be selectively removed with GYKI53655, and the combined use of GYKI53655 and LY293558 should leave intact GluR6-mediated responses. Such pharmacological tools should allow a greater understanding of the role of kainate receptors in synaptic function.

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