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Characterization of the Binding Site for a Novel Class of Noncompetitive α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor Antagonists

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ABSTRACT

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is an ionotropic glutamate receptor that mediates fast excitatory synaptic transmission throughout the central nervous system. In addition to the glutamate binding site, allosteric modulatory sites on the receptor are inferred from the ability of synthetic compounds to affect channel function without interaction with the glutamate binding site. We have identified a novel class of potent, noncompetitive AMPA receptor antagonists typified by CP-465,022 and CP-526,427. The latter compound was radiolabeled and used to elucidate the pharmacology of one allosteric modulatory site. [^{3}H]CP-526,427 labels a single binding site in rat forebrain membranes with a K_d value of 3.3 nM and a B_{max} of 7.0 pmol/mg of protein. The [^{3}H]CP-526,427 binding site does not seem to interact directly with the glutamate binding site but overlaps with that for another class of AMPA receptor antagonists, the 2,3-benzodiazepines. This binding site is distinct from that for the antagonist Evans blue and for several classes of compounds that modulate AMPA receptor desensitization. These results indicate the existence of at least two physically distinct allosteric sites on the AMPA receptor through which channel activity or desensitization is modulated.

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system. Glutamatergic synaptic transmission is mediated by three major subtypes of ionotropic receptors: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (Dingledine et al., 1999). The AMPA receptor mediates fast glutamatergic synaptic transmission. Furthermore, increases in the efficacy of AMPA receptor-mediated synaptic responses underlie at least some forms of long-term potentiation, a process thought to be involved in memory and learning (Bear and Malenka, 1994). This has engendered the hypothesis that compounds that increase AMPA receptor activity may be used to treat cognitive impairment (Staubli et al., 1994). On the other hand, aberrant overactivation of AMPA receptors may play a role in epileptogenesis (Rogawski, 1993; Yamaguchi et al., 1993) and glutamate-induced neuronal death (Buchan et al., 1993; Madsen et al., 1994). Significant advances in understanding the role of AMPA receptors in these pathological processes have come from studies with the glutamate binding site-competitive AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline (NBQX; Sheardown et al., 1990). NBQX, and more recently other quinoxalinedione analogs (Namba et al., 1994; Ohmori et al., 1994), have been found to be broadly effective at blocking seizures in a number of animal models (Namba et al., 1994; Shimizu-Sasamata et al., 1996). These compounds also significantly reduce neuronal loss under experimental conditions, including middle cerebral artery occlusion, brief global cerebral ischemia, and traumatic brain and spinal cord injury (Buchan et al., 1991; Gill et al., 1992;

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline; GYKI-52,466, 4-(8-methyl-9H-1,3-dioxa-6,7-diaza-cyclohepta[*f*]inden-5-yl)-phenylamine; BCP-1, 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine; CX-516, 1-(6-quinoxalinylcarbonyl)-piperidine; Co 102,659, 7-(1-ethyl-propyl)-5-phenyl-7,9-dihydro-1,3-dioxa-6,7-diaza-cyclohepta[*f*]inden-8-one; Co 102,685, 7-cyclohexyl-5-phenyl-7,9-dihydro-1,3-dioxa-6,7-diaza-cyclohepta[*f*]inden-8-one; CP-465,022, 3-(2-Chloro-phenyl)-2-[2-(6-diethylaminomethyl-pyridin-2-yl)-vinyl]-6-fluoro-3H-quinazolin-4-one; CP-526,427, 2-{2-[3-(2-Chloro-phenyl)-6-fluoro-4-oxo-3,4-dihydro-quinazolin-2-yl]-vinyl}-nicotinonitrile; Co 102,581, 5-phenyl-7,9-dihydro-1,3-dioxa-6,7-diaza-cyclohepta[f]inden-8-one; YM-90K, 6-(1*H*-imidazol-1-yl)-7-nitro-2,3(1*h*,4*h*)-quinoxalinedione hydrochloride; BSS, balanced salt solution; RFUs, relative fluorescent units; CP-471,236, 6-Fluoro-3-(2-methyl-pyridin-3-yl)-2-[2-(2-methyl-thiazol-4-yl)-vinyl]-3H-quinazolin-4-one; MK-801, (+)-5-methyl-10,11-dihydro-5-*H*-dibenzo[*a*,*d*]cycloheptan-5,10-imine.

Sheardown et al., 1993; Shimizu-Sasamata et al., 1996; Teng Dong and Wrathall, 1996). NBQX was initially believed to be specific for AMPA receptors based on radioligand binding (Sheardown et al., 1990). However, it is now appreciated to have significant functional inhibitory activity at both kainate and AMPA receptors (Wilding and Huettner, 1996), qualifying conclusions regarding the relative role of AMPA receptors in glutamate-induced neuron loss based on studies with this and related compounds. Nonetheless, the therapeutic potential of compounds such as NBQX has spurred efforts to discover novel compounds that affect AMPA receptor activity.

The search for compounds interacting with the AMPA receptor has resulted in the identification of several classes of allosteric modulators. GYKI-52,466 was identified as the first AMPA receptor antagonist not competitive at the glutamate binding site (Tarnawa et al., 1990, 1992). Structurally similar compounds, the 2,3-benzodiazepines, have now been shown to have a high degree of specificity for AMPA over kainate receptors (Paternain et al., 1995; Wilding and Huettner, 1995). The dye Evans blue has also been shown to be a noncompetitive AMPA receptor antagonist with specificity for receptors containing the GluR1 or 2 subunit (Keller et al., 1993). Several classes of compounds have been identified that potentiate AMPA receptor-mediated responses by inhibiting AMPA receptor desensitization. These include the benzothiadiazides, typified by cyclothiazide (Partin et al., 1993; Yamada and Tang, 1993), and the benzoylpiperidines, typified by 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine (BCP-1) and 1-(6-quinoxalinylcarbonyl)-piperidine (CX-516) (Arai et al., 1994, 1996; Lynch et al., 1997). Recently, Konkoy et al. described two novel 2,3-benzodiazepine derivatives (Co 102,659 and Co 102,685) that potentiate rather than inhibit the activity of AMPA receptors (Konkoy et al., 1998). The multiple effects of these different chemical classes on AMPA receptor activity suggests that the AMPA receptor contains at least one and possibly multiple allosteric binding sites through which activity can be modulated. Furthermore, the 2,3-benzodiazepine binding site may accommodate both agonists and inverse agonists, analogous to the benzodiazepine binding site on the γ-aminobutyric acid$_A$ receptor. Determining the physical and functional relationship between these binding sites, and the mechanisms whereby these compounds effect AMPA receptor activity, remains an important research question.

To identify new compounds that inhibit AMPA receptor function, we screened a large chemical library for inhibitors of AMPA receptor mediated $^{45}Ca^{2+}$ uptake in primary cultures of rat cerebellar granule neurons (assay described below). We discovered that the known anticonvulsant piraquilone (Koe et al., 1986; Fig. 1) effectively blocked this response in a manner not competitive with glutamate-site agonists (Chenard et al., 2000). A medicinal chemistry effort to increase the potency and specificity of this lead for AMPA receptor inhibition led to the identification of a series of quinazolinones typified by CP-465,022 (Welch et al., 1998; and W. M. Welch, F. E. Ewing, J. Huang, F. S. Menniti, M. J. Pagnozzi, K. Kelly, P. A. Seymour, V. Guanowski, S. Guhan, M. R. Guinn, D. Critchett, J. Lazzaro, A. H. Ganong, and B. L. Chenard, submitted). CP-465,022 inhibits AMPA receptor-mediated whole cell currents in rat cortical neurons in primary culture with an IC_{50} value of 30 nM but only weakly inhibits NMDA, γ-aminobutyric acid, or kainate receptor-mediated currents (Lazzaro and Ganong, 1998). The compound does not discriminate among AMPA receptors composed of different homomeric or heteromeric subunit combinations. Furthermore, CP-465,022 inhibits AMPA receptor activity in a noncompetitive manner that is neither voltage- nor use-dependent. When administered to rodents systemically, CP-465,022 inhibits AMPA receptor-mediated synaptic transmission and chemically induced seizures (Seymour et al., 1998). To study further the pharmacology of these AMPA receptor antagonists, a compound in this chemical series, CP-526,427, was radiolabeled and used to characterize the binding site for the class. We show here that inhibition of AMPA receptor activity by CP-465,022 and structural analogs is mediated through an interaction with the binding site labeled by [^{3}H]CP-526,427. This inhibitory binding site overlaps with that for GYKI-52,466 but not for Evans blue. Furthermore, this site is apparently independent of the agonist-binding site and the site(s) involved in inhibiting AMPA receptor desensitization. A preliminary report on characterization of the [^{3}H]CP-526,427 binding site has been published as an abstract (Menniti et al., 1998). The functional activity and specificity of the quinazolinones for AMPA receptor inhibition in vitro and in vivo is described in detail in a manuscript in preparation (Menniti et al.).

Materials and Methods

Chemicals. The chemical synthesis of CP-465,022 and CP-471,236 (Fig. 1) was accomplished by a route outlined in abstract form (Welch et al., 1998) and in detail in a separate publication (W. M. Welch, F. E. Ewing, J. Huang, F. S. Menniti, M. J. Pagnozzi, K.

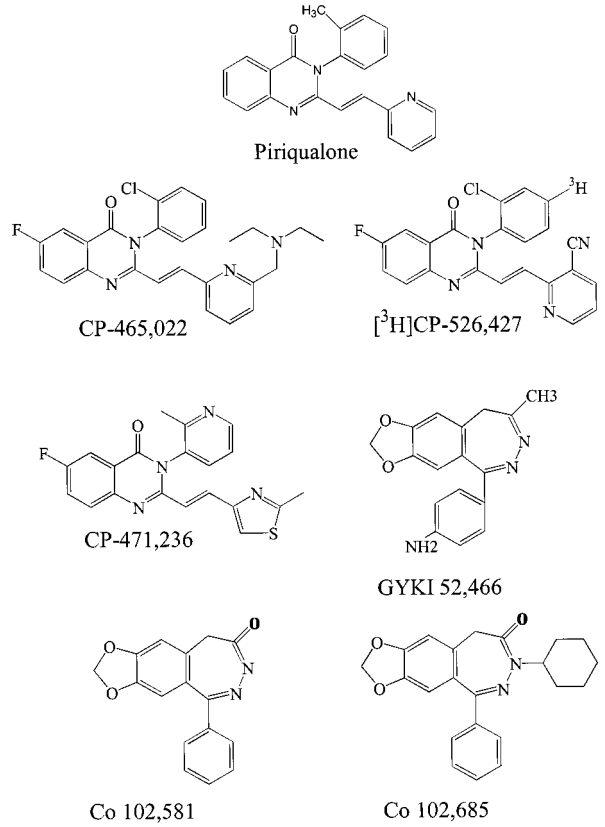

Fig. 1. Structures of piraquilone, CP-465,022, [^{3}H]CP-526,427, CP-471,236, GYKI-52,466, Co 102,581, and Co 102,685.

Kelly, P. A. Seymour, V. Guanowski, S. Guhan, M. R. Guinn, D. Critchett, J. Lazzaro, A. H. Ganong, and B. L. Chenard, submitted). Both CP-465,022 and CP-471,236 are atropisomers that were separated on a preparative scale by HPLC on a Chiracel OD column using 90:10 hexane/ethanol +0.1% diethylamine as eluent. The physiochemical properties and stability of these compounds are described by Newell et al. (2000). [^{3}H]CP-526,427 (Fig. 1) was prepared as follows. Racemic 2-{2-[3-(2-chloro-4-iodo-phenyl)-6-fluoro-4-oxo-3,4-dihydro-quinazolin-2-yl]-vinyl}-nicotinonitrile was prepared by the condensation of 3-(2-chloro-4-iodo-phenyl)-6-fluoro-2-methyl-3H-quinazolin-4-one with 3-cyanopyridin-2-carboxaldehyde in tetrahydrofuran and trifluoroacetic anhydride catalyzed by anhydrous zinc chloride. The racemic compound was separated into its component atropisomers by chromatography on a chiral-phase HPLC column using a mixture of ethanol, acetone, and heptane with 0.1% diethylamine as eluent. Tritiated (*S*)-2-{2-[3-(2-chloro-phenyl)-6-fluoro-4-oxo-3,4-dihydro-quinazolin-2-yl]-vinyl}-nicotinonitrile was prepared by the reductive hydrogenolysis of (*S*)-2-{2-[3-(2-chloro-4-iodo-phenyl)-6-fluoro-4-oxo-3,4-dihydro-quinazolin-2-yl]-vinyl}-nicotinonitrile in ethyl acetate with tritium gas using palladium on carbon as the catalyst. GYKI-52,466 (Chenard et al., 1993), Co 102,685, Co 102, 659, and Co 102,581 (Xia et al., 1997), and YM-90K (Ohmori et al., 1994) were synthesized as described previously. All other compounds were obtained from commercial sources.

Primary Cultures of Rat Cerebellar Granule Neurons. Primary cultures of rat cerebellar granule neurons were prepared as described previously (Parks et al., 1991). Cerebella were removed from 7- to 8-day-old Sprague-Dawley rats, minced into 1-mm pieces, and incubated for 15 min at 37°C in Ca^{2+}-Mg^{2+} free Tyrode's solution containing 0.1% trypsin. The tissue was then triturated using a fine-bore Pasteur pipette. The cell suspension was plated onto poly-D-lysine coated 96-well tissue culture plates at 10^5 cells per well. Medium consisted of minimal essential medium with Earle's salts, 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 25 mM KCl. After 24 h, the medium was replaced with fresh medium containing 10 μM cytosine arabinoside to inhibit cell division. Cultures were used at 5 to 8 days in vitro.

$^{45}Ca^{2+}$ Uptake. Neurons in poly-D-lysine-coated 96-well plates were preincubated for 30 min with different concentrations of compounds in balanced salt solution (BSS; 115 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 11 mM *d*-glucose, and 25 mM HEPES, pH 7.3). They were then exposed at room temperature to 100 μM kainate or NMDA in BSS containing 10 μM glycine, 0.5 mM dithiothreitol, and 0.5 μCi $^{45}Ca^{2+}$ (final specific activity, 2.78 μCi/μmol) in a volume of 100 μl/well. After 10 min, the neurons were then rapidly washed five times with 200 μl/well of ice-cold BSS containing 5 mM EGTA. Neurons were then lysed in 30 μl/well of 0.6% Triton X-100 and radioactivity in aliquots of the lysate were measured with a TopCount microtiter scintillation counter (Packard Instrument Co., Downers Grove, IL).

Measurement of $[Ca^{2+}]_i$. Neurons in 96-well, black/clear, poly-D-lysine-coated tissue culture plates were rinsed once with BSS then incubated for 1 h in BSS containing 4 μM Fluo-4/AM (Molecular Probes, Inc., Eugene, OR). Fluo-4/AM was prepared immediately before use as a 1 mM stock solution in dimethyl sulfoxide with 10% (w/v) pluronic acid. Cells were then washed three times and held in BSS at room temperature and used within 1 h. A fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) was used for simultaneous imaging and fluid addition. Cells were preincubated with test compounds for approximately 6 min, then stimulated with 32 μM AMPA. Changes in fluorescent intensity were measured at a frequency of 1 sample/2 s after AMPA addition. Raw data are expressed in relative fluorescent units (RFUs) after the background fluorescence was subtracted.

[^{3}H]CP-526,427 and [^{3}H]AMPA Binding. The binding of [^{3}H]CP-526,427 was characterized in rat forebrain membranes. Forebrains of adult male Sprague-Dawley rats were homogenized in 0.32 M sucrose at 4°C. The crude nuclear pellet was removed by centrifugation at 1,000*g* for 10 min, and the supernatant centrifuged at 17,000*g* for 25 min. The resulting pellet was resuspended in 5 mM Tris acetate, pH 7.4, at 4°C for 10 min to lyse cellular particles and again centrifuged at 17,000*g*. The resulting pellet was washed twice in Tris acetate, resuspended at 10 mg of protein/ml and stored at −20°C until use. Immediately before binding assays, membranes were thawed, homogenized, and diluted to 0.5 mg of protein/ml with 50 mM Tris·HCl, pH 7.4. Scatchard analyses were performed by incubating different concentrations of radioligand with membranes. For competition assays, compounds were added at various concentrations followed by 3 nM [^{3}H]CP-526,427 (specific activity, 24.36 Ci/mmol). After incubation for 20 min at 30°C in a shaking water bath, samples were filtered onto Whatman GFB glass fiber filters using a MB-48R Cell Harvester (Brandel Research and Development Laboratories, Gaithersburg MD). Filters were washed for 10 s with ice-cold Tris·HCl buffer and the radioactivity trapped on the filter quantified by liquid scintillation counting. Nonspecific binding for [^{3}H]CP-526,427 was determined in parallel incubations containing 10 μM unlabeled CP-526,427 or CP-465,022. Specific binding was defined as total binding minus nonspecific binding.

[^{3}H]AMPA binding was assessed under conditions identical with those described above. Competition experiments were performed in the presence of 10 nM [^{3}H]AMPA (specific activity, 52.6 Ci/mmol; NEN Life Science Products, Boston, MA) and nonspecific binding was determined in parallel incubations containing 10 mM glutamate.

Results

A medicinal chemistry effort to increase the potency and specificity of piraquilone for AMPA receptor inhibition resulted in the identification of a series of novel quinazolinone AMPA receptor antagonists (Fig. 1). To elucidate the pharmacology of these compounds, one of the most potent analogs, CP-526,427, was radiolabeled and a series of ligand binding studies was undertaken. Scatchard analysis of [^{3}H]CP-526,427 binding was performed in triplicate on four preparations of rat forebrain membranes. An example of results from one of these experiments is depicted in Fig. 2. [^{3}H]CP-526,427 binds in a saturable manner to rat forebrain membranes with a K_d value of 3.3 ± 0.3 nM and a B_{max} value of 7.0 ± 3.5 pmol/mg of protein (means ± S.E.M. from the four membrane preparations). These data are best fit by a single binding site model; however, the possibility of a second low-affinity binding site cannot be ruled out. In these experiments, the nonspecific binding was 30 to 40% at ligand concentrations similar to the K_d value. This relatively high value, in light of the apparent high affinity for a specific binding site, most likely results from the lipophilicity of CP-526,427 (calculated LogP = 3.7) and the propensity for nonspecific protein binding exhibited by this chemical series (B. L. Chenard, F. S. Menniti, and W. M. Welch, unpublished observations).

The specific binding of [^{3}H]CP-526,427 is potently displaced by structural analogs including CP-465,022 and CP-471,236 (Fig. 3, top). Both CP-465,022 and CP-471,236 are *S*-configuration atropisomers; the corresponding *R*-atropisomers (CP-465,021 and CP-471,237, respectively) have 100-fold lower potencies for displacement of [^{3}H]CP-526,427 binding (Fig. 3, top; Table 1).

The potency of CP-465,022 and CP-471,236 and the respective *R*-atropisomers was also determined for inhibition of a functional AMPA receptor mediated response; namely, kain-

ate-induced $^{45}Ca^{2+}$ uptake in primary cultures of rat cerebellar granule neurons. Rat cerebellar granule neurons in primary culture elaborate a dense network of processes and form glutamatergic synapses. As has been described by others (Hack and Balazs, 1995), activation of AMPA receptors on these neurons causes depolarization and subsequent activation of NMDA receptors. That the kainate-induced $^{45}Ca^{2+}$ uptake studied here is mediated by AMPA receptor activation is indicated by the following: 1) the EC_{50} value for kainate is approximately 100 μM, consistent with kainate acting as a low-affinity AMPA receptor agonist; 2) the response to kainate is competitively inhibited by AMPA, consistent with AMPA acting as a partial agonist because of desensitization; 3) the response to kainate is inhibited by GYKI-52,466 (see below); and 4) cyclothiazide potentiates the kainate-induced response (M. B. Collins, M. F. Ducat, F. S. Menniti, and M. J. Pagnozzi, unpublished observations). Nevertheless, the bulk of the kainate-induced $^{45}Ca^{2+}$ uptake occurs through the secondary NMDA receptor activation, because 1 μM MK-801 inhibits approximately 70% of the kainate-induced $^{45}Ca^{2+}$ uptake (Fig. 4). CP-465,022 at 100 nM inhibits greater than 90% of the kainate-induced $^{45}Ca^{2+}$ uptake in the rat cerebellar granule neuron cultures (Fig. 4). However, the compound at this concentration is much less effective for inhibition of $^{45}Ca^{2+}$ uptake induced by 100 μM NMDA (approximately 15% inhibition), whereas 1 μM MK-801 completely inhibits the NMDA-induced response (Fig. 4).

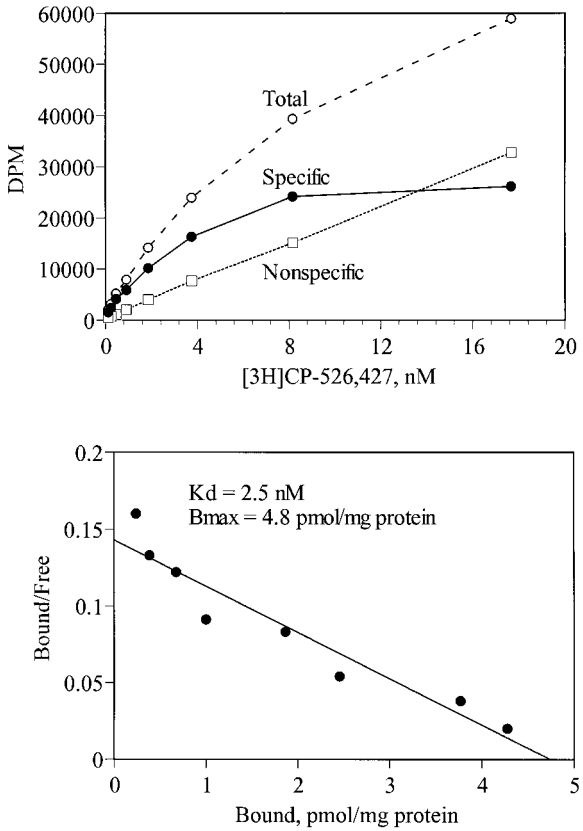

Fig. 2. Scatchard analysis of [^{3}H]CP-526,427 binding to rat forebrain membranes. Top, indicated concentrations of [^{3}H]CP-526,427 were incubated with rat forebrain membranes and bound radioactivity was determined as described under *Materials and Methods* (total binding). Nonspecific binding was determined in the presence of 10 μM CP-465,022 and specific binding was calculated as the difference between total and nonspecific binding at each concentration. Bottom, data are replotted in Scatchard format where the X intercept equals the number of binding sites. Data shown are from a single experiment where each point was determined in triplicate.

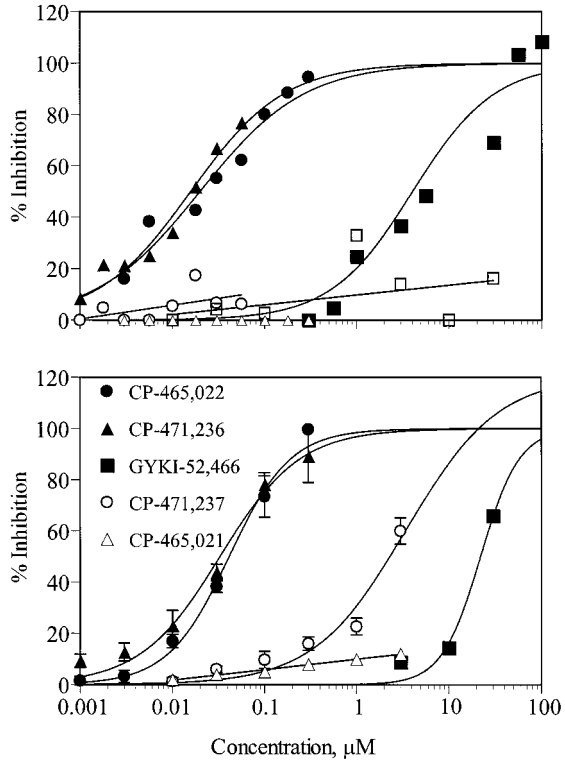

Fig. 3. Inhibition of [^{3}H]CP-526,427 binding to rat forebrain membranes and AMPA receptor mediated $^{45}Ca^{2+}$ uptake in rat cerebellar granule neurons. Top, compounds were incubated at the indicated concentrations with rat forebrain membranes and 3 nM [^{3}H]CP-526,427 and bound radioactivity determined. Nonspecific binding was determined for each point in parallel incubations containing 10 μM CP-465,022 and specific binding was calculated as total minus nonspecifically bound radioactivity. Percent inhibition of [^{3}H]CP-526,427 binding was then calculated as: 100 − [(Specific bound − nonspecific bound)/specific bound when no added drug) × 100]. Bottom, rat cerebellar granule neurons were incubated with compounds at the indicated concentrations and then stimulated with100 μM kainate in the presence of $^{45}Ca^{2+}$. At the end of 10 min, the reaction was stopped and the amount of radioactivity accumulated was determined as described under *Materials and Methods*. Percentage inhibition of $^{45}Ca^{2+}$ uptake was then calculated as: 100 − [($^{45}Ca^{2+}$ uptake − uptake in the absence of kainate)/uptake when no added drug) × 100]. Displacement of [^{3}H]CP-526,427 by Evans blue is shown at the top (□); other symbols are as indicated in the figure. Each point is a triplicate determination from a single representative experiment. Similar results were observed in two to seven additional experiments for each compound.

TABLE 1

Comparison of affinity for the [^{3}H]CP-526,427 binding site and potency for inhibition of a functional AMPA receptor mediated response. IC_{50} values were determined for inhibition of binding of 3 nM [^{3}H]CP-526,427 binding to rat forebrain membranes (Binding IC_{50}) and for inhibition of $^{45}Ca^{2+}$ uptake in primary cultures of rat cerebellar granule neurons induced by 100 μM kainate (Functional IC_{50}) as described under *Materials and Methods*.

Compound	Binding IC_{50} (μM)	*n*	Functional IC_{50} (μM)	*n*
CP-526,427	0.003[a]	4	0.005 ± 0.001	8
CP-465,022	0.025 ± 0.014	6	0.039 ± 0.003	3
CP-465,021	>10	3	>3	3
CP-471,236	0.057 ± 0.08	3	0.028 ± 0.003	6
CP-471,237	>10	4	2.7 ± 0.16	3
GYKI-52,466	12.6 ± 1.2	4	22	1

n, number of experiments.
[a] K_d.

These data indicate that the effect of CP-465,022 on kainate-induced $^{45}Ca^{2+}$ uptake is the result of AMPA, not NMDA, receptor inhibition, consistent with the specificity of this compound for AMPA receptors as determined in other functional assays (Lazzaro and Ganong et al., 1998). Similar results were obtained for a number of other quinazolinone AMPA antagonists in this series (data not shown).

CP-465,022 and CP-471,236 inhibit kainate-induced $^{45}Ca^{2+}$ uptake in the cerebellar granule neuron cultures in a concentration dependent manner (Fig. 3A). In contrast, the corresponding *R*-atropisomers (CP-465,021 and CP-471,237, respectively) have 100-fold lower potencies. In fact, the IC_{50} values for inhibition of the AMPA receptor-mediated functional response corresponds closely with potency for displacement of [^{3}H]CP-526,427 binding (Table 1).

The specific binding of [^{3}H]CP-526,427 was displaced by less than 10% by high concentrations of AMPA receptor agonists glutamate, AMPA, or kainate, or the AMPA receptor competitive antagonist YM-90K (Table 2). The number of [^{3}H]CP-526,427 binding sites was similar in Scatchard analyses conducted in the presence or absence of 1 mM kainate. Furthermore, CP-465,022, CP-471,236, CP-526,427 and related compounds at concentration of 10 μM inhibit [^{3}H]AMPA receptor binding by less than 50%. These data indicate that the [^{3}H]CP-526,427 binding site is distinct from that for glutamate on the AMPA receptor.

A number of compounds structurally unrelated to CP-526,427 were examined for the ability to displace [^{3}H]CP-526,427 specific binding to rat forebrain membranes. Evans blue (Fig. 3, top), cyclothiazide, BCP-1, and CX-516 (Table 2) did not significantly displace [^{3}H]CP-526,427 specific binding at concentrations reported by others to significantly affect AMPA receptor activity. [^{3}H]CP-526,427 specific binding, however, was inhibited by the noncompetitive AMPA receptor antagonist GYKI-52,466 (Fig. 3, top; Table 1). The potency of GYKI-52,466 in the binding assay is similar to that for inhibition of AMPA receptor mediated $^{45}Ca^{2+}$ uptake in rat cerebellar granule neurons (Fig. 3, bottom; Table 1).

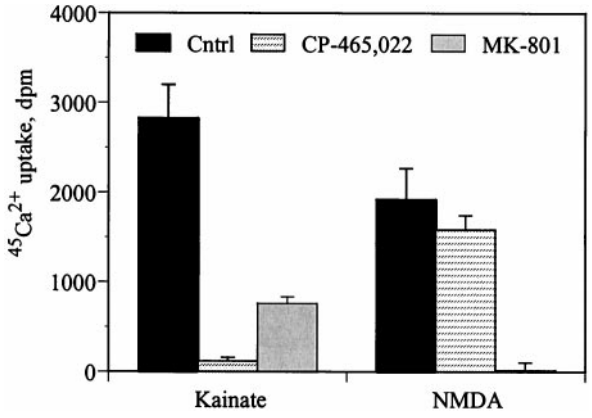

Fig. 4. Effects of CP-465,022 and MK-801 on NMDA- and kainate-induced $^{45}Ca^{2+}$ uptake in rat cerebellar granule neurons. Rat cerebellar granule neurons were incubated with no drug (Cntrl), 100 nM CP-465,022, or 1 μM MK-801 and then stimulated with 100 μM NMDA or kainate in the presence of $^{45}Ca^{2+}$. At the end of 10 min, the reaction was stopped and the amount of radioactivity accumulated was determined as described under *Materials and Methods*. Background $^{45}Ca^{2+}$ uptake was determined in the absence of added NMDA or kainate and subtracted from the stimulated values. Each bar represents the mean (± S.E.M) $^{45}Ca^{2+}$ uptake in DPM/well from three experiments. In each experiment, conditions were replicated in triplicate.

We also examined the activity of structurally related 2,3-benzodiazepine derivatives, some of which potentiate instead of inhibit AMPA receptor activity. Co 102,581 inhibited in a concentration dependent manner the increase in $[Ca^{2+}]_i$ induced by 32 μM AMPA in rat cerebellar granule neurons (Fig. 5, top) with an IC_{50} value of 30.0 μM. This compound is approximately 3-fold less potent than GYKI 52,466 (IC_{50} = 9.2 μM) and 400-fold less potent than CP-465,022 (IC_{50} = 0.074 μM) in this assay (Fig. 5, top). In contrast, Co 102,685 (Fig. 5, top, and Fig. 6) and Co 102,659 (Fig. 5, top) significantly potentiated the increase in $[Ca^{2+}]_i$ induced by 32 μM AMPA in rat cerebellar granule neurons. Co 102,685 was both more potent and efficacious than Co 102,659; however, IC_{50} values could not be calculated in this assay because poor solubility at concentrations higher than 32 μM precluded determination of concentrations yielding maximal effect. Nonetheless, the magnitude of the potentiation of AMPA receptor responses over the concentration range tested is similar to that reported by Konkoy et al. for potentiation of AMPA-stimulated whole-cell currents in rat cortical neurons in primary culture (Konkoy et al., 1998). Co 102,581, GYKI 52,466, and CP-465,022 also inhibited [^{3}H]CP-526,427 binding in a concentration-dependent manner (Fig. 5, bottom). IC_{50} values were similar to those for inhibition of the functional AMPA receptor mediated response (Co 102,581, 64.1 μM; GYKI 52,466, 14.1 μM; and CP-465,022, 0.026 μM). However, Co 102,685 and Co 102,659 failed to displace greater than 50% of [^{3}H]CP-526,427 specific binding at concentrations up to 100 μM (Fig. 5, bottom).

Discussion

CP-526,427 and CP-465,022 typify a recently discovered class of quinazolinone noncompetitive AMPA receptor antagonists. These are among the most potent compounds yet identified that interact with the AMPA receptor. CP-526,427 inhibited an AMPA receptor-mediated functional response in rat cerebellar granule neurons with an IC_{50} value of 5 nM. In comparison, NBQX inhibits [^{3}H]AMPA binding to the glutamate site on the AMPA receptor with a K_d value of 150 nM

TABLE 2

Glutamate site ligands and desensitization modulators weakly displace [^{3}H]CP-526,427 binding to rat forebrain membranes

The percentage displacement of 3 nM [^{3}H]CP-526,427 binding to rat forebrain membranes (% inhibition) was determined for each compound at the indicated concentration. The AMPA receptor affinity for the agonist binding site compounds is the IC_{50} for displacement of [^{3}H]AMPA binding from rat brain membranes and that for the desensitization modulators is EC_{50} for potentiation of AMPA receptor-mediated responses as reported in the cited literature references.

Compound	% Inhibition	*n*	AMPA Receptor Affinity
Agonist binding site			
Glutamate, 1 mM	7.5 ± 10.0	3	1.3 μM[a]
AMPA, 1 mM	14.9 ± 7.8	3	0.3 μM[a]
Kainate, 1 mM	14.3 ± 12.2	3	6 μM[a]
YM-90K, 100 μM	15.5 ± 14.8	3	0.084 μM[b]
Desensitization modulators			
Cyclothiazide, 300 μM	−10.0 ± 10.8	3	12–14 μM[c]
BCP-1, 3 mM	0.3 ± 17.0	3	0.48 mM[d]
CX-516, 3 mM	11.0 ± 8.8	3	0.17 μM[e]

n, number of experiments.
[a] Honore et al., 1982.
[b] Ohmori et al., 1994.
[c] Yamada and Tang, 1993.
[d] Arai et al., 1994.
[e] Arai et al., 1996.

(Sheardown et al., 1990). GYKI-52,466, the prototype of the 2,3-benzodiazepine AMPA receptor antagonists, inhibited AMPA receptor activation-induced $^{45}Ca^{2+}$ uptake in rat cerebellar granule neurons with an IC_{50} of 14 μM, similar to the potency reported by others in functional assays (Donevan and Rogawski, 1993). All of the compounds identified to date that inhibit AMPA receptor desensitization also require micromolar concentrations for efficacy. The potency of CP-526,427 enabled development of a radioligand and binding assay to elucidate the pharmacology of an allosteric modulatory site on the AMPA receptor that is discussed below.

[^{3}H]CP-526,427 binds with high affinity to a presumed single site on rat forebrain membranes. Scatchard analysis indicates a K_d value of 3.3 nM. The K_d value agrees closely with the IC_{50} value for CP-526,427 inhibition of AMPA receptor activation-induced $^{45}Ca^{2+}$ uptake in rat cerebellar granule neurons (present study) and AMPA receptor mediated whole cell currents in cortical neurons (Lazzaro and Ganong, 1998). Furthermore, potency for displacement of [^{3}H]CP-526,427 binding by quinazolinone analogs correlates closely with potency for inhibition of functional AMPA receptor mediated synaptic responses. This indicates that the inhibitory activity of the quinazolinones is mediated through an interaction with the [^{3}H]CP-526,427 binding site.

The [^{3}H]CP-526,427 binding site seems to be distinct from the glutamate binding site on the AMPA receptor. Glutamate and glutamate-binding site AMPA receptor agonists do not displace [^{3}H]CP-526,427 binding; conversely, compounds interacting with the [^{3}H]CP-526,427 site fail to inhibit [^{3}H]AMPA binding in the same membrane preparation. Furthermore, the glutamate site agonist kainate does not affect the affinity or number of [^{3}H]CP-526,427 binding sites. These data are consistent with the fact that compounds interacting with the [^{3}H]CP-526,427 binding site inhibit functional AMPA receptor responses in a manner not competitive with agonist concentration.

[^{3}H]AMPA labels two binding sites in rat forebrain membranes: a high-affinity, low-abundance site and a low-affinity, high-abundance site (Honoré et al., 1982). The ability to detect the low affinity [^{3}H]AMPA binding site is increased when incubations are performed at low temperature and in the presence site of the chaotropic agent thiocyanate (Honoré and Drejer, 1988), although this latter reagent was not employed by Honoré et al. In the present study, the number of [^{3}H]CP-526,427 binding sites observed, 7.0 pmol/mg of protein, is similar to that for the low-affinity [^{3}H]AMPA binding sites reported previously under similar assay conditions [i.e., in the absence of thiocyanate, 6.5 pmol/mg of protein (Honoré et al., 1982)]. We have also observed a similar density of low-affinity [^{3}H]AMPA binding sites under this assay condition (14.3 pmol/mg of protein; K. A. Kelly and F. S. Menniti, unpublished observation). The number of [^{3}H]CP-526,427 binding sites is also similar to that reported for the low-affinity binding site for the AMPA receptor specific radioligand (*S*)-[^{3}H]5-fluorowillardine (in the absence of thiocyanate, 10.8 pmol/mg of protein, Hawkins et al., 1995). Although qualitative, these comparisons suggest that the [^{3}H]CP-526,427 binding site is expressed on AMPA receptors that also contain the low-affinity [^{3}H]AMPA binding site.

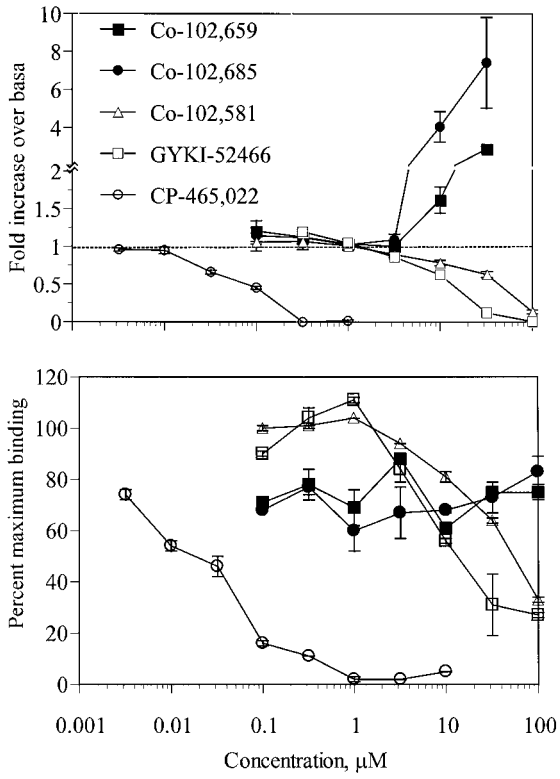

Fig. 5. Effects on AMPA receptor mediated increase in $[Ca^{2+}]_i$ in rat cerebellar granule neurons and inhibition of [^{3}H]CP-526,427 binding to rat forebrain membranes. Top, rat cerebellar granule neurons were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4, incubated with compounds at the indicated concentrations, and then stimulated with 32 μM AMPA. The peak change in $[Ca^{2+}]_i$ in each well, which typically occurred within 20 s of AMPA addition, was used for further calculations. Increases or decreases in $[Ca^{2+}]_i$ are expressed as the fold change relative to the $[Ca^{2+}]_i$ in neurons stimulated with AMPA only. Each point is the mean (± S.E.M) from two (GYKI-52,466), four (CP-465,022), or six (Co-102,659, Co-102,685, and Co-102,581) experiments in which each condition was replicated in four or eight wells. Bottom, compounds were incubated at the indicated concentrations with rat forebrain membranes and 3 nM [^{3}H]CP-526,427 and bound radioactivity determined. Nonspecific binding was determined for each point in parallel incubations containing 10 μM CP-465,022 and specific binding was calculated as total minus nonspecifically bound radioactivity. Percent of maximum binding of [^{3}H]CP-526,427 was then calculated as: (Specific bound − nonspecific bound)/specific bound when no added drug) × 100. Each point is the mean (± S.E.M) from two or three experiments where each condition was determined in triplicate.

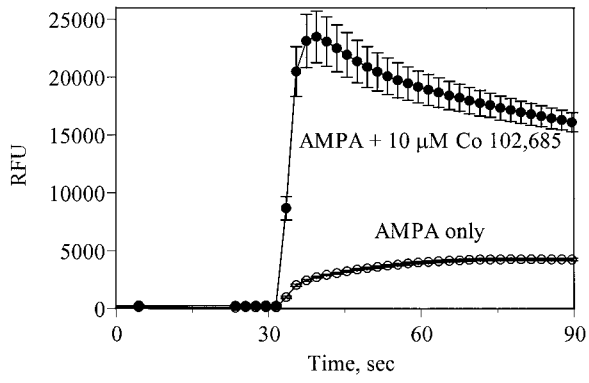

Fig. 6. Effect of Co 102,685 on AMPA-induced increase in $[Ca^{2+}]_i$ in rat cerebellar granule neurons. Rat cerebellar granule neurons loaded with the calcium-sensitive fluorescent dye fluo-4 were preincubated in the presence or absence of 10 μM Co 102,685. Thirty seconds after initiation of data collection, neurons were stimulated with 32 μM AMPA and data collected for an additional 30 s. $[Ca^{2+}]_i$ is represented as RFUs as described under *Materials and Methods*. Each point is the mean (± S.E.M.) RFUs from eight similarly treated wells from a single experiment.

Standley et al. suggest that the low-affinity [^{3}H]AMPA binding site represents the mature, functional pool of AMPA receptors, based on an analysis of [^{3}H]AMPA receptor binding in rat brain slices using autoradiography (Standley et al., 1998). That [^{3}H]CP-526,427 labels such a functional pool of AMPA receptors is consistent with the ability of compounds that bind to this site to completely inhibit AMPA receptor activity in native neuronal preparations. It will be interesting to more thoroughly explore the relationship between the [^{3}H]AMPA and [^{3}H]CP-526,427 binding sites.

There are now several chemical classes of compounds that interact with the AMPA receptor at allosteric modulatory sites. Two classes of antagonists, the 2,3-benzodiazepines, typified by GYKI-52,466 and Co 102,581, and the dye Evans blue, have been identified in addition to CP-526,427 and analogs. The finding that GYKI-52,466 and Co 102,581 displace [^{3}H]CP-526,427 binding with potency similar to that for inhibition of functional AMPA receptor-mediated responses indicates that the 2,3-benzodiazepines and the quinazolinones interact with overlapping sites on the receptor. Furthermore, Weiser et al. have suggested that the 2,3-benzodiazepines and Evans blue interact at different sites on the AMPA receptor, based on differences in the kinetics of block and the failure of GYKI-52,466 to occlude block by Evans blue (Weiser et al., 1996). The finding of the present study, that GYKI-52,466 but not Evans blue displaces [^{3}H]CP-526,427 binding, is consistent with this conclusion. Thus, there seem to be at least two sites through which AMPA receptor activity can be allosterically inhibited: the quinazolinone/2,3-benzodiazepine site and the Evans blue site.

Several classes of synthetic compounds have also been identified that allosterically modulate AMPA receptor desensitization. In the present study, the benzothiadiazide cyclothiazide failed to displace [^{3}H]CP-526,427 binding. It was originally hypothesized that the inhibitory effect of GYKI-52,466 resulted from increased desensitization through an interaction with the site also modulated by cyclothiazide (Zorumski et al., 1993). However, analyses of the functional effects of the 2,3 benzodiazepines on channel gating kinetics indicated that the compounds do not directly effect desensitization (Donevan and Rogawski, 1993; Partin and Mayer, 1996). Furthermore, Partin and Mayer found that a point mutation of GluR1 (S750Q) abolishes the effect of cyclothiazide to block AMPA receptor desensitization but has no effect on the ability of GYKI-52,466 to inhibit receptor activity. The binding data presented here corroborate this earlier functional data confirming that the quinazolinone/2,3-benzodiazepine binding site is distinct from the site of action of the benzothiadiazides. The benzoylpiperidines BCP-1 and CX-516 also inhibit AMPA receptor desensitization; however, differential effects on flip and flop splice variants of the AMPA receptor and on agonist affinity suggest that this class interacts with a site on the AMPA receptor that is different from that of the benzothiadiazides (Kessler et al., 1998). In the present study, the benzopyridines also failed to displace [^{3}H]CP-526,427 binding, suggesting that these compounds interact with yet another unique site on the AMPA receptor.

Recently, Konkoy et al. demonstrated that the 2,3-benzodiazepine derivatives, Co 102,659 and Co 102,685, potentiate AMPA receptor activity (Konkoy et al., 1998). This observation raised the possibility that the 2,3-benzodiazepine binding site might accept agonists (Co 102,659 and Co 102,685) as well as inverse agonists (CP-526,427 and GYKI-52,466). In the present study, Co 102,659 and Co 102,685 were found to potentiate the AMPA receptor activation-dependent increase in $[Ca^{2+}]_i$ in rat cerebellar granule neurons. The potency and efficacy was similar to that reported for potentiation of whole-cell currents in rat cortical neurons. However, these compounds failed to displace [^{3}H]CP-526,427 binding from rat forebrain membranes at similar concentrations. Thus, despite the structural similarities, the ability of Co 102,659 and Co 102,685 to potentiate AMPA receptor responses may be mediated by an interaction with a site distinct from that through which the antagonist 2,3-benzodiazepines inhibit AMPA receptor responses.

In summary, [^{3}H]CP-526,427 binds to an allosteric site on the AMPA receptor. Both the quinazolinones disclosed here and the 2,3 benzodiazepines inhibit AMPA receptor activity through an interaction with this site. This site seems to be distinct from the glutamate binding site as well as the one or more sites on the receptor through which synthetic compounds modulate AMPA receptor desensitization. The localization of the [^{3}H]CP-526,427 binding within the three-dimensional structure of the AMPA receptor, and how interaction of molecules with this site inhibits receptor activity, remains to be determined. The three dimensional structure of the ionotropic glutamate receptors is beginning to be understood (Paas, 1998). The flip/flop module within the large extracellular loop between the second and third membrane-spanning domains is a native allosteric regulator of AMPA receptor kinetics and desensitization (Sommer et al., 1990). Partin et al. (1995) have determined that a single amino acid within the flip/flop module is critical for the effect of cyclothiazide on AMPA receptor desensitization. This suggests that the cyclothiazide-binding site is part of or interacts closely with the flip/flop module and raises the possibility that other desensitization modulators act in this region. It is also noteworthy that allosteric inhibitors of NMDA receptors, such as ifenprodil and CP-101,606, seem to reside in the distal N terminus of the NR2B subunit (Chenard and Menniti, 1999), suggesting that this region of the NMDA receptor can be involved in modulation of channel activity. Similarly, the analogous region of the AMPA receptor may be a site of action for allosteric inhibitors, given the similar structural organization of AMPA and NMDA receptors. Tools such as [^{3}H]CP-526,427 will be valuable in further understanding the structure/function relationships involved in regulating AMPA receptor activity.

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