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Effects of 5′-Alkyl-Benzothiadiazides on (*R*,*S*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptor Biophysics and Synaptic Responses

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ABSTRACT

Alkyl-substituted benzothiadiazides (BTDs) were tested for their effects on (*R*,*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. In excised patches, the 5′-ethyl derivative "D1" blocked the desensitization of AMPA receptor currents during prolonged application of glutamate (EC_{50}, 36 μM), and it slowed deactivation of responses elicited by 1-ms glutamate pulses greater than 10-fold. [^{3}H]Fluorowillardiine binding to rat synaptic membranes was increased by D1 by a factor of 3.6 (EC_{50}, 17 μM) with a Hill coefficient near 2. In hippocampal slices, the compound reversibly increased excitatory postsynaptic currents and field excitatory postsynaptic potentials (EPSPs) with thresholds around 10 μM. The size of the alkyl substituent influenced both the potency and nature of the drug effect on synaptic currents: 5′-methyl compounds had a 2-fold greater effect on response amplitude than on response duration, whereas 5′-ethyl compounds like D1 caused greater increases in duration than amplitude. In tests with recombinantly expressed AMPA receptor subunits, D1 preferred the glutamate receptor (GluR) subunit GluR4 flip (0.64 μM) over GluR4 flop (5.3 μM); similar affinities but with smaller flip-flop differences were obtained for GluR1 through 3. These results show that D1 and congeners are significantly more potent than the parent compound IDRA-21 and that they differ in two fundamental aspects from cyclothiazide, the most widely studied BTD: 1) D1 markedly increases the agonist affinity of AMPA receptors and 2) it has immediate and large effects on field EPSPs. The large gain in potency conferred by alkyl substitution suggests that the 5′ substituent is in intimate contact with the receptor, with the size of the substituent determining the way in which receptor kinetics is changed.

AMPA-type glutamate receptors are abundant throughout the brain and account for much of the transmission occurring at excitatory synapses. Ito et al. (1990) made the seminal observation that the current through these receptors can be enhanced by the nootropic compound aniracetam. The drug did not influence other types of glutamate receptors, and it had no evident effect on AMPA receptors in the absence of glutamate. Other compounds were subsequently discovered that "up-modulate" or "potentiate" AMPA receptor function in a similar manner, including diazoxide (Yamada and Rothman, 1992), cyclothiazide (Yamada and Tang, 1993), IDRA-21 (Bertolino et al., 1993), and PEPA (Sekiguchi et al., 1997). Using the structural leads given by these compounds, several laboratories have developed entire families of potent AMPA receptor modulators beginning with the ampakines (Arai et al., 1994, 1996b,c, 2000; Staubli et al., 1994a,b) and including the pyridothiadiazines (Pirotte et al., 1998) and the biarylpropylsulfonamides (Ornstein et al., 2000).

The interest in these compounds has been fostered in part by the possibility that some neurological disorders, such as age-related memory impairment, schizophrenia, and perhaps depression, may be associated with lower than normal excitatory transmission in some brain regions (Masliah et al., 1993; Tamminga, 1998). If so, up-modulation of AMPA receptors could potentially be of ther-

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ABBREVIATIONS: AMPA, (*R*,*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; FW, fluorowillardiine; GYKI, GYKI 52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride]; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; SCN^-, thiocyanate; DMSO, dimethyl sulfoxide; BTD, benzothiadiazide; EPSP, excitatory postsynaptic potential; EPSC, excitatory postsynaptic current; HEK, human embryonic kidney; IDRA-21, 7-chloro-3-methyl-3–4-dihydro-2*H*-1,2,4 benzothiadiazine (*S*,*S*)-dioxide; PEPA, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide; ACSF, artificial cerebrospinal fluid; GluR, glutamate receptor; KSCN, potassium thiocyanate; S18986, (*S*)-2,3-dihydro-[3,4]cyclopentano-1,2,4-benzothiadiazine-1,1-dioxide.

apeutic value. Experimental evidence to support this has been obtained in behavioral studies with ampakines (e.g., Granger et al., 1993, 1996; Staubli et al., 1994b; Larson et al., 1996) and recently also with other modulators (Zivkovic et al., 1995; Lebrun et al., 2000; Li et al., 2001). Several of these compounds have been shown to cross the blood-brain barrier to increase excitatory responses in vivo (Staubli et al., 1994a; Vandergriff et al., 2001), and their ability to facilitate long-term synaptic potentiation (Arai and Lynch, 1992, 1996a; Staubli et al., 1994a) has been suggested to account for the observed improvements in various memory tests. The drugs may have similar actions in humans according to preliminary clinical tests (Lynch et al., 1996; Goff et al., 2001).

Most current AMPA receptor modulators belong to one of two structural families, referred to as benzamides (aniracetam and ampakines) and benzothiadiazides (BTDs). PEPA and the more recent biarylpropylsulfonamides fall outside these two categories but share elements with the latter. Behavioral tests have largely involved the benzamides because the first modulators in the BTD family (diazoxide and cyclothiazide) have clinically important peripheral effects and only weakly affect field EPSPs in hippocampal slices, even at concentrations far above their affinities for the AMPA receptor (Larson et al., 1994; Arai and Lynch, 1998; Hjelmstad et al., 1999). Some modulators have substantial effects on extracellular synaptic responses that align well with their affinities for AMPA receptors, whereas others do not; this is thought to be related to how the compounds affect receptor kinetics. Diverse experiments indicate that cyclothiazide acts mainly on desensitization (Johansen et al., 1995; Partin et al., 1996), whereas ampakines also have a strong, even primary, influence on channel gating (Arai and Lynch, 1998). This would explain the observed differences in the effects of the modulators if, as has been argued, the latter process plays a larger role than the former in shaping the size and waveform of undisturbed synaptic responses.

IDRA-21, which does not have the peripheral side effects associated with diazoxide and cyclothiazide and unlike the latter rapidly increases synaptic potentials (Arai et al., 1996a), was the first benzothiadiazide examined in behavioral tests (Zivkovic et al., 1995). However, IDRA-21 has modest potency, with concentrations above 200 μM typically needed to enhance excitatory transmission in hippocampal slices (Arai et al., 1996a). Our efforts as well as those of others (Desos et al., 1996; Pirotte et al., 1998) have therefore been directed at finding analogs that have higher potency yet maintain effect profiles that are suitable for behavioral applications. In the present project, we systematically modified substituents at various locations around the benzothiadiazide core of IDRA-21 and measured the effects on various aspects of AMPA receptor operation. Some of the modifications resulted in compounds that have much greater potency than IDRA-21 and have effects on AMPA receptor kinetics that differ radically from those of cyclothiazide. A comprehensive description of the compounds that were synthesized and examined in this study is given elsewhere (Phillips et al., 2002), and some data have been presented in abstract form (Arai et al., 1999).

Materials and Methods

AMPA Receptor Currents in Excised Patches. Patch-clamp studies were carried out with outside-out patches excised from pyramidal neurons in field CA1 of organotypic hippocampal slices (Arai et al., 1996c, 2000). The slice cultures were prepared from 13 to 14-day-old Sprague-Dawley rats and grown for 2 weeks on cellulose membrane inserts (Millipore CM; Millipore Corp., Bedford, MA). Patches were excised in a medium containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 25 mM D-glucose, and 20 mM HEPES, pH 7.3, and relocated to a chamber perfused with recording medium containing 130 mM NaCl, 3.5 mM KCl, 20 mM HEPES, 0.01 mM dizocilpine maleate, and 0.05 mM D-2-amino-5-phosphonopentanoic acid. Patch pipettes had a resistance of 3 to 8 MΩ and were filled with a solution of 65 mM CsF, 65 mM CsCl, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP disodium salt, and 10 mM HEPES, pH 7.3. A piezo device was employed to switch solutions applied to the patch within a fraction of a millisecond (Arai et al., 1996b). In brief, background medium and agonist containing medium were flowing continuously through two lines of a double pipette that was moved by a piezo device across a distance of 50 μm in 0.4 ms (Arai et al., 1996b,c). Data were acquired with a patch amplifier (AxoPatch-1D; Axon Instruments, Inc., Foster City, CA) at a filter frequency of 5 kHz and digitized at 10 kHz with PClamp/Digidata 1200 (Axon Instruments, Inc.). The holding potential was −50 mV. The drugs were applied at the same concentration in both background and glutamate lines, and background flow lines were switched at least 15 s before applying the first glutamate pulse. Typically, five responses were collected and averaged for each condition. Measurement with each patch was alternated repeatedly between control (A: glutamate alone) and test conditions (B: glutamate + drug). For data analysis, response B was compared with the average of the responses A taken before and after response B, and peak and steady-state currents recorded in the presence of drug were normalized to those without drug. Deactivation rates were determined by fitting the decay phase of the response to a 1-ms glutamate pulse (10 mM) with a single or double exponential function. Drug solutions were prepared from 1000-fold stock solutions in dimethyl sulfoxide (DMSO); the same final concentrations of DMSO (maximum, 0.1%) were included in all drug and control solutions.

Whole-Cell Recording from Pyramidal Cells in Field CA1 of Hippocampal Slices. Male Sprague-Dawley rats of postnatal day 15 to 21 (Harlan, Indianapolis, IN) were decapitated under anesthesia following National Institutes of Health guidelines and an institutionally approved protocol. Transverse hippocampal slices (400 μm) were prepared using a vibratome (Leica Microsystems, Deerfield, IL). The slices were submerged in oxygenated artificial cerebrospinal fluid (ACSF) infused at 0.5 ml/min. The experiments were carried out at ambient temperature. The ACSF contained 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 10 mM glucose, and 10 mM HEPES, pH 7.4. The intrapipette solution contained 130 mM CsF, 10 mM EGTA/K, 2 mM ATP disodium salt, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. Pyramidal cells were visualized with an infrared microscope (BXI50; Olympus, Tokyo, Japan) with differential interference contrast configuration. Synaptic responses were recorded using borosilicate glass electrodes (2–5 MΩ) in response to activation of Schaffer-commissural fibers stimulated by a bipolar nichrome electrode in stratum radiatum. After establishing a stable baseline, the perfusion line was switched to one containing the drug; solution exchange in the recording chamber was complete within 3 min. EPSCs were recorded with AxoPatch 200B and digitized at 10 kHz with Digidata1200/PClamp 7. The holding potential was −70 mV, and the signals were filtered at 5 kHz. Recordings were discarded if the input resistance varied by greater than 10% over the course of the experiment.

Whole-Cell Recordings from HEK 293 Cells. Patch-clamp recordings were carried out in whole-cell configuration from human embryonic kidney (HEK) 293 cells that stably express homomeric

AMPA receptors consisting of GluR3 flop subunits (see Hennegriff et al., 1997). Recordings were made at room temperature in serum-free minimal essential medium (Invitrogen, Carlsbad, CA). Patch pipettes had a resistance of 3 to 7 MΩ and were filled with 130 mM CsF, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP disodium salt, and 10 mM HEPES (pH 7.4). The holding potential typically was −100 mV. Agonist was applied with a fast solution switch system in which cells are exposed to a constant flow of the background solution that is momentarily interrupted during application of glutamate. The drugs were included in both background and agonist lines.

Extracellular Recording in Hippocampal Slices. Transverse hippocampal slices (400 μm) were prepared as described elsewhere (Arai et al., 1996c) and placed in an interface chamber, which was perfused at 0.5 ml/min with oxygenated ACSF containing 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 3.4 mM $CaCl_2$, 2.5 mM $MgSO_4$, 26 mM $NaHCO_3$, and 10 mM D-glucose and exposed to humidified 95% O_2/5% CO_2. Field EPSPs were recorded from the stratum radiatum in response to activation of Schaffer-commissural fibers in the same stratum. The input-output relation of the synaptic response was first established to determine the maximum EPSP amplitude without spike component, and the stimulation intensity was adjusted to 50% of the maximum EPSP amplitude. After establishing a stable baseline, the perfusion line was switched to one containing the drug.

Binding Assays. Binding tests were carried out with membranes from rat brain and from HEK 293 cells that express one of the AMPA receptor subunits. Membranes from rat brain were prepared according to conventional procedures (Kessler et al., 1996) involving homogenization in an isotonic sucrose solution and differential centrifugation to obtain a P_2 pellet fraction, followed by an osmotic lysis and repeated washing by centrifugation and resuspending in the binding assay buffer (100 mM HEPES/Tris and 50 μM EGTA, pH 7.4). Aliquots were frozen at −80°C; after thawing, the membranes were sonicated and washed twice by centrifugation. For tests with recombinant receptors, HEK 293 cells were collected into Tris-buffered saline (150 mM NaCl and 10 mM Tris/HCl, pH 7.4) containing in addition 2 mM EGTA and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cells were centrifuged at least four times (2000*g* for 10 min) and resuspended in the Tris-buffered saline without the additions; after the first centrifugation, 0.1% saponin was included in the medium to permeabilize the membranes, and the cell suspension was left at 25°C for 5 min. All other steps were carried out at 0–4°C. The cells were stored on ice and washed before use.

Binding tests with rat brain membranes were conducted with the centrifugation method, generally at 25°C. Aliquots of the membrane suspension containing 50 μg of protein were incubated for 45 min with the radioligand and appropriate additions in a final volume of 100 μl. Sets of 24 samples were then centrifuged for 15 min in a Beckman Coulter JA 18.1 rotor (Fullerton, CA) kept at the incubation temperature. The supernatant was aspirated, and the pellet was quickly rinsed with ice-cold buffered saline containing 50 mM KSCN (wash buffer). The pellets were dissolved in 20 μl of issue solubilizer (Beckman Coulter, Inc.) and counted after addition of acidified scintillation fluid. Binding incubations with HEK 293 cells were terminated by filtration through Whatman GF/C filters (Fisher Scientific, Pittsburgh, PA) after diluting the sample in 5 ml of ice-cold wash buffer; the filters were rapidly washed with three additional volumes of wash buffer. Drugs were dissolved in DMSO and diluted into the assay buffer at twice the highest final drug concentration; the assay buffer was heated to 60°C for the dilution and then quickly cooled to ambient temperature. Further dilutions were made with assay buffer containing the equivalent concentration of DMSO (0.1–1%; DMSO concentrations in this range did not have a significant effect on drug potency). Background values ("nonspecific binding") were measured by inclusion of 5 mM L-glutamate and subtracted from total binding; separate background values were determined for incubations with and without drug. Protein content was determined according to the Bradford method with the reagent available from Bio-Rad (Hercules, CA) and bovine serum albumin as standard. Binding curves were fitted to the data points with nonlinear regression (GraphPad Prism; GraphPad Software, San Diego, CA). (*R*,*S*)-[^{3}H]AMPA and [^{3}H]CNQX were purchased from PerkinElmer Life Sciences (Boston, MA), and [^{3}H]fluorowillardiine and (*S*)-[^{3}H]AMPA were obtained from Tocris Cookson Inc. (Ballwin, MO). Other reagents were from the usual commercial sources.

Results

Two of the more closely examined compounds of this study are shown in Fig. 1 along with three widely studied BTDs. The new compounds are derived from IDRA-21 and differ from it mainly by an alkyl substitution at the 5′ position. A comprehensive description of syntheses and structure-activity relations is given in Phillips et al. (2002), and drug names accord with those used therein.

AMPA Receptor Currents in Excised Patches. Figure 2A shows the effect of the 5′-ethyl-benzothiadiazide D1 on currents induced by 800-ms application of 1 mM glutamate in patches excised from hippocampal pyramidal cells. In the absence of drug, the agonist-induced current declined rapidly to a steady-state level that was 5 to 10% of the peak current. Increasing concentrations of D1 progressively raised the steady-state current and essentially blocked desensitization at concentrations above 100 μM. The increases in steady-state values occurred without changing the rate at which those values were reached, suggesting that D1 completely blocked desensitization in receptors that have bound the drug and that increasing the drug concentration in essence shifted the balance between drug-free and drug-bound receptors. Fitting a four-point logistic equation to the dose-effect data gives an EC_{50} estimate of 36 μM with a Hill coefficient of 1.6; the threshold concentration was around 2 μM (Fig. 2, inset).

Figure 2B summarizes data from experiments in which glutamate (10 mM) was applied for only 1 ms to measure the rate at which responses deactivate after glutamate offset. In the absence of drug, the response returned to baseline with a time constant of 3.4 ± 0.4 ms. In the presence of D1, the responses were greatly prolonged (Fig. 2, B and E). This effect is not the result of blocking desensitization, because no significant prolongation was observed with a near-saturating concentration of cyclothiazide in some of the same patches (12 ± 6%, $n = 4$) and in previous studies (Arai and Lynch, 1998; Arai et al., 2000). Deactivation in the presence of drug

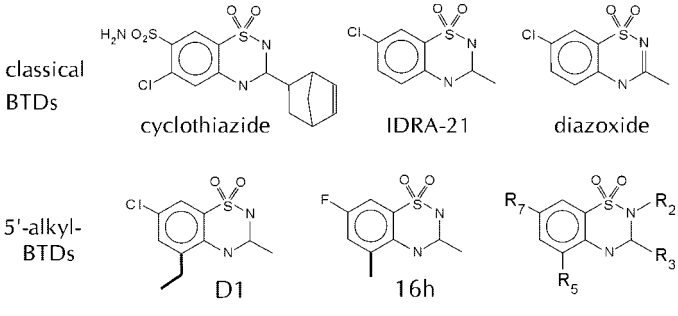

Fig. 1. Structure of benzothiadiazide compounds. Three conventionally used compounds of this drug family are shown in the upper row. The second row shows the structure of the two most extensively tested alkyl-BTDs. The benzothiadiazide core structure and the numbering of substituent locations are indicated on the right.

generally revealed two exponential components, as illustrated in Fig. 2E for the case of 200 μM D1. These components may reflect distinct kinetic processes and/or the presence of receptors with different degrees of drug occupancy at subsaturating drug concentrations. The dose-effect relation shown in Fig. 2B was constructed using a weighted average of the two time constants. Its value increased from 3.4 ± 0.4 ms in the absence of drug to 58.4 ± 8.1 ms at 240 μM D1, or by a factor of 17, providing an EC_{50} value of 77 μM with a Hill slope near 3. In Fig. 2C, two 1-ms pulses were applied in rapid succession (40-ms interpulse interval). Under these conditions, the second response is typically reduced by about 40%, as expected if a sizable proportion of the receptors activated by the first glutamate pulse desensitize and are not

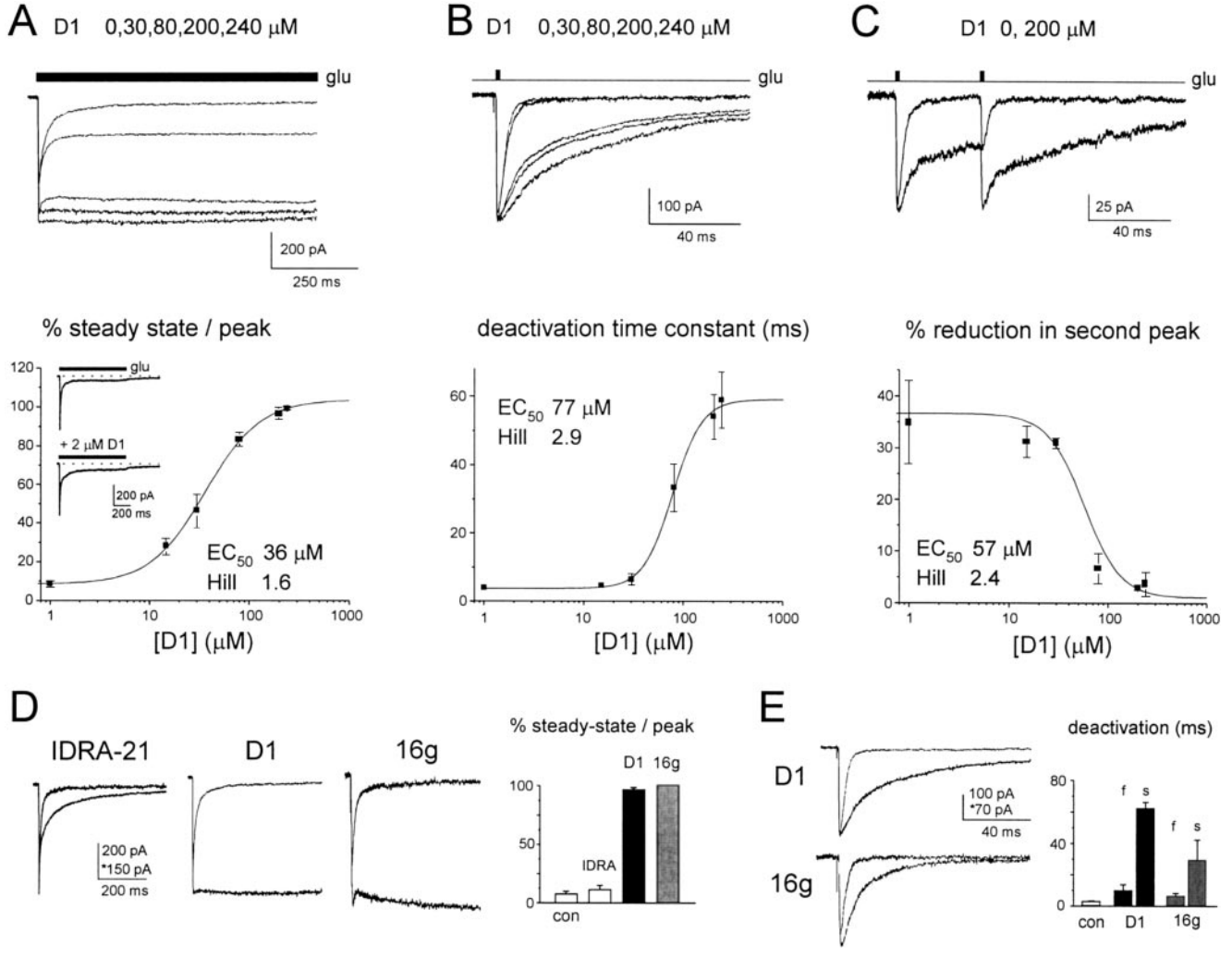

Fig. 2. Effects of D1 on AMPA receptor currents in patches from hippocampal pyramidal neurons. A, application of 1 mM glutamate for 800 ms to measure the effect of the drug on receptor desensitization. The upper graph shows a representative experiment from a single patch with responses recorded in the absence of drug and in the presence of the D1 concentrations shown above the graph. The horizontal bar indicates the duration of glutamate application. D1 was present before and during the glutamate pulse; the background medium was switched to one containing the desired drug concentrations at least 20 s before applying glutamate. The bottom graph shows the concentration-effect relation for the steady-state current expressed as percentage of the peak current (means and S.E.M. from 4–6 patches). Data points were fitted with a four-point logistic equation. The EC_{50} value and the Hill coefficient obtained from this curve fit are shown in the graph. Traces in the absence of drug and at 2 μM D1 are shown as insets. B, application of fast (1-ms) pulses of glutamate (10 mM) to mimic receptor activation during synaptic transmission. The upper graph shows a representative experiment from a single patch; the drug was equilibrated with the patch before glutamate application as described under A. The return of the response to baseline after glutamate offset is called deactivation. An approximate time constant for this deactivation phase was determined by fitting a two-exponential function to the data points and forming a weighted average of the two time constants by multiplying them with the fractional amplitude of each component. Its values are plotted in the lower graph (means and S.E.M. from 3–8 patches). Raising the drug concentration increased the time constant of the fast component (from 3.4 ms at 15 μM D1 to 9.5 ms at 240 μM), the slow component (from 15.5 to 60.8 ms, respectively), and the fractional contribution of the slow component (from 5 to 75%, respectively). C, two 1-ms pulses of 10 mM glutamate were applied at an interval of 40 ms. The upper graph shows representative records in the absence of drug and in the presence of 200 μM D1. In the absence of drug, the second response was reduced due to receptor desensitization. In the graph underneath, the percentage reduction in the amplitude of the second response relative to that of the first response was plotted against the concentration of D1. D, responses to prolonged application of glutamate in the absence of drug and in the presence of IDRA-21, D1, and the 5′-methyl analog 16g, each at 200 μM. Experimental procedures were the same as in A. Averaged values (mean and S.E.M.) for the steady-state/peak ratios are shown in the bar graph. The calibration marked by an asterisk refers to compound 16g. E, responses to 1-ms application of glutamate in the absence and presence of 200 μM D1 and 16g. Experimental procedures were as in B. The deactivation phase of the responses was fitted with single- and double-exponential equations. Control responses were in both cases fit best with the single-exponential function. Curve fits to responses in the presence of drug were superior according to F-tests when using two-exponential functions (D1: $F = 2618$, $P < 0.0001$; 16g: $F = 653$, $P < 0.001$; analysis by GraphPad Prism). Averaged time constants for the fast (f) and slow (s) component are shown in the bar graph (mean and S.E.M.; $n = 5$ for D1, $n = 4$ for 16g). The calibration marked by ⋆ refers to compound 16g.

available to respond to the second pulse. As seen in Fig. 2, this reduction was eliminated in the presence of 200 μM D1.

Effects similar to those of D1 were obtained with other alkyl-BTDs. The 5′-methyl analog 16g (see Fig. 5 for structure) had a lower potency than D1 (see Fig. 4, below) but was similarly efficacious in blocking desensitization (Fig. 2D). By comparison, IDRA-21 at 200 μM produced only a 2- to 3-fold increase in the steady-state current, which is in agreement with the data shown by Bertolino et al. (1993), and it slowed the rate at which the steady state was reached, suggesting that desensitization is attenuated but not completely blocked by this drug. Response deactivation was slowed by all active alkyl-BTDs, but the magnitude of the effect was generally smaller with 5′-methyl analogs than with the 5′-ethyl derivative D1. Thus, compound 16g at 200 μM increased the slow component of the deactivation phase to only 29 ± 13 ms (n = 4), and the weighted average at that concentration was 14.0 ms (Fig. 2E).

Effects on Agonist Binding. By changing receptor kinetics, most AMPA receptor modulators also enhance or reduce the binding of receptor agonists, such as [^{3}H]AMPA and [^{3}H]fluorowillardiine. The magnitude and direction of the binding effect depend to some extent on assay variables, such as temperature and buffer composition, but EC_{50} values for drug effects correlate well with those obtained from physiological recordings (e.g., Hall et al., 1993; Kessler et al., 1996; Arai et al., 1996b, 2000). Binding tests were therefore used together with excised patch recordings to screen and characterize the compounds developed in this project (Phillips et al., 2002). Figure 3 illustrates the typical effects of D1 on the binding of [^{3}H]fluorowillardiine (FW) (A) and [^{3}H]AMPA (B) under several commonly employed assay conditions. In all cases, D1 produced a robust increase in the binding of the agonist. At an assay temperature of 0°C, the EC_{50} value was 5.5 μM, which indicates a potency comparable with or higher than that of cyclothiazide (~30 μM; Hall et al., 1993). All other tests with brain membranes were conducted at ambient temperature to facilitate comparison with physiological measures. The EC_{50} at this temperature was 17 μM; the increase in [^{3}H]FW binding was 3.6 fold and thus considerably larger than that produced by any other modulator under those assay conditions (Arai et al., 2000). Drug potency depended only weakly on the choice of the agonist (AMPA versus fluorowillardiine) and was not affected by the chaotropic anion SCN^-, which is often used to augment the affinity for [^{3}H]AMPA (Fig. 3B). Thus, binding tests provide a consistent measure for the potency of D1 that matches within a factor of two the EC_{50} values obtained in patch experiments.

The enhanced binding seen in these assays could be either due to an increase in affinity or an increase in the B_{max}. This was examined more closely in Fig. 3C in which the affinity for glutamate was determined by measuring the degree to which the agonist displaces the high-affinity antagonist [^{3}H]CNQX. The latter radioligand was used because it has similar affinity at high- and low-affinity variants of the AMPA receptor and the different subunits and because its binding can be reliably measured without additions such as thiocyanate (see also Kessler et al., 1996). The affinity of glutamate was increased from 47 μM without modulator to less than 4 μM in the presence of saturating D1. Thus, [^{3}H]fluorowillardiine binding apparently underestimates the magnitude of the affinity increase for the endogenous ligand glutamate. [^{3}H]CNQX binding itself was slightly increased by D1; this stands in contrast to the 2-fold binding reduction observed with cyclothiazide (Kessler et al., 1996).

Figure 4 shows the effects on binding of other 5′-alkyl derivatives and of the parent compound IDRA-21. The isopropyl homolog 16c (which, like D1, extends a distance of two carbons from the ring) was similar to D1, but the longer chain

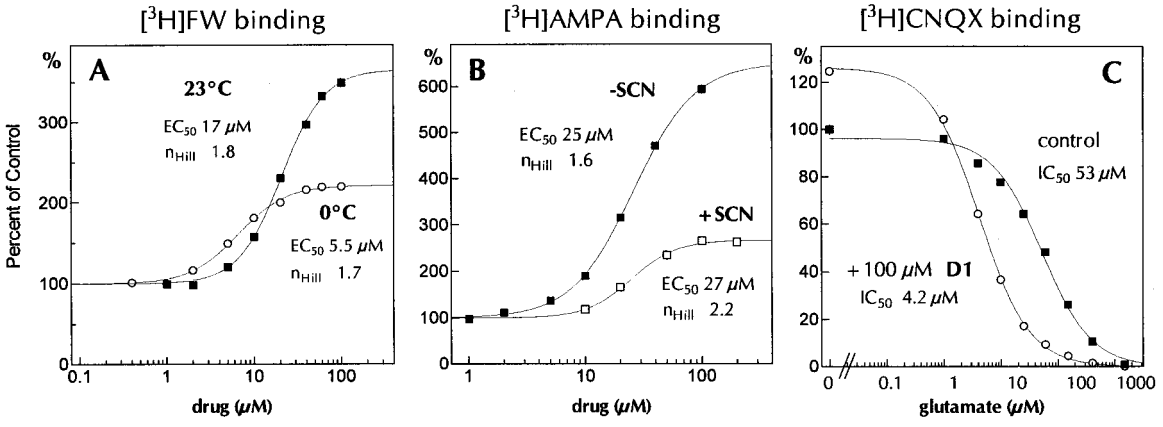

Fig. 3. Allosteric modulation of agonist binding by D1. A, effect of D1 on the binding of [^{3}H]FW (20 nM) to rat brain membranes at 0 and 23°C. Incubations were terminated by centrifugation. Control binding without drug was measured in quadruplicate; binding in the presence of drug was determined in duplicate or triplicate and normalized to that in the absence of drug. Data from typical experiments are shown as means and S.E.M.; the absence of an error bar indicates an error smaller than the size of the symbol. The data points were fitted with four-point logistic equations forced through 100% as the lower asymptote (GraphPad Prism). Binding in the absence of drug was 0.62 pmol/mg protein at 23°C and 0.98 pmol/mg protein at 0°C. Average values for drug potency (EC_{50}) and the Hill slope (n_{Hill}) obtained from these curve fits are shown as insets. Average values at 23°C were EC_{50}, 17.4 ± 1.8 μM; n_{Hill}, 1.8 ± 0.1; and maximal increase, 360 ± 15% (n = 9). Average values at 0°C were EC_{50}, 5.5 ± 1.1 μM; n_{Hill}, 1.7 ± 0.1; and maximal increase, 127 ± 4% (n = 2). B, effect of D1 on the binding of (*R*,*S*)-[^{3}H]AMPA (50 nM) in the absence and presence of the chaotropic ion SCN^- (50 mM; potassium salt). Binding was measured at 23°C. Average binding parameters in the presence of SCN^- were EC_{50}, 26.6 ± 1.8 μM; n_{Hill}, 2.2 ± 0.1; and maximal increase, 269 ± 6% (n = 11). Average values in the absence of SCN^- were EC_{50}, 25.4 ± 0.3 μM; n_{Hill}, 1.6 ± 0.1; and maximal increase, 673 ± 28% (n = 2). C, displacement of [^{3}H]CNQX binding (40 nM) by the glutamate concentrations indicated on the x-axis, measured in the absence and presence of 200 μM D1. Fitting of a sigmoidal curve (n_{Hill} = 1) gives an IC_{50} of 53 μM, which, after Cheng-Prusoff correction, provides an affinity estimate for glutamate of 47 μM. In the presence of D1, the IC_{50} was lowered to 4.2 μM (affinity estimate, 3.7 μM). A similar, approximately 10-fold increase in affinity was observed in two additional experiments.

propyl homolog 16e was much less potent with an EC_{50} of 170 μM. However, the maximum binding increase was larger than that produced by D1 for both drugs. In contrast, shortening the alkyl length reduced both drug potency and the maximal effect on binding. Thus the methyl-BTD 16g increased FW binding less than 2-fold, and IDRA-21 produced at most a 30% enhancement. Few of the earlier studies on IDRA-21 provided a potency estimate for this compound. Yamada et al. (1998) found an EC_{50} of 150 μM in cultured neurons, but extrapolation of the patch data in Bertolino et al. (1993) suggests an EC_{50} value on the order of 0.5 to 1 mM, and EPSP measurements in adult hippocampal slices (Arai et al., 1996a) and other physiological tests (Puia et al., 2000) are more consistent with the latter value. A similar EC_{50} estimate of about 1 mM was obtained here for the effect of IDRA-2 on FW binding. Thus, the ethyl substituent in D1 conferred an approximately 50-fold gain in potency over the parent compound IDRA-21. The potency of the methyl-compound 16g was intermediate between those of IDRA-21 and D1 with an EC_{50} of 100 μM. The differences between methyl and ethyl compounds extend to other compounds developed in this study. As shown in Fig. 5, the maximum increase in binding was always less than 2-fold for 5′ methyl substituents as opposed to the 260 to 430% seen with larger alkyl groups. Secondly, none of the 5′-methyl compounds had an EC_{50} lower than 90 μM, which confirms a progressive gain in potency with extension of the 5′-alkyl group. A further noteworthy aspect is that Hill coefficients of many compounds were in the vicinity of 2 and that all of them were significantly larger than unity.

Additional observations from binding experiments are illustrated in Fig. 6. GYKI 52466 is a selective downmodulator that effectively blocks AMPA receptor responses, probably by slowing channel opening (Arai, 2001). GYKI was previously reported to have no detectable impact on agonist binding, and it did not seem to interfere with the influence of cyclothiazide on [^{3}H]AMPA binding (Kessler et al., 1996). The effect of D1 was greatly altered, however, when GYKI was present (Fig. 6, A and B). In the presence of thiocyanate, [^{3}H]AMPA binding was reduced rather than increased, and in its absence, the binding increase caused by D1 was less extensive than without GYKI. The EC_{50} for GYKI in either case was around 20 μM (Fig. 6C), which is in the range commonly reported from physiological tests (Zorumski et al., 1993). More importantly, the EC_{50} for D1 changed by a factor of 2 or less, and the Hill coefficient remained virtually unaltered, indicating that the interaction between D1 and GYKI is not competitive.

Positive and negative allosteric modulators of the AMPA receptor in general seem to be very selective, with no detectable action at kainate receptors (Ito et al., 1990; Paternain et al., 1995). This was examined again for the case of D1 by testing whether the compound influences agonist binding to kainate receptors (Fig. 6, right). Unexpectedly, binding of [^{3}H]kainate was increased by 30%. However, the EC_{50} for this effect (27 μM) and the Hill slope (2.4) are close to those seen with FW and AMPA binding, and the effect was partially reversed by GYKI 52466, which does not act on kainate receptors (Paternain et al., 1995). It is thus likely that some of the [^{3}H]kainate binding occurs at AMPA receptors and that the observed enhancement by D1 is mediated entirely

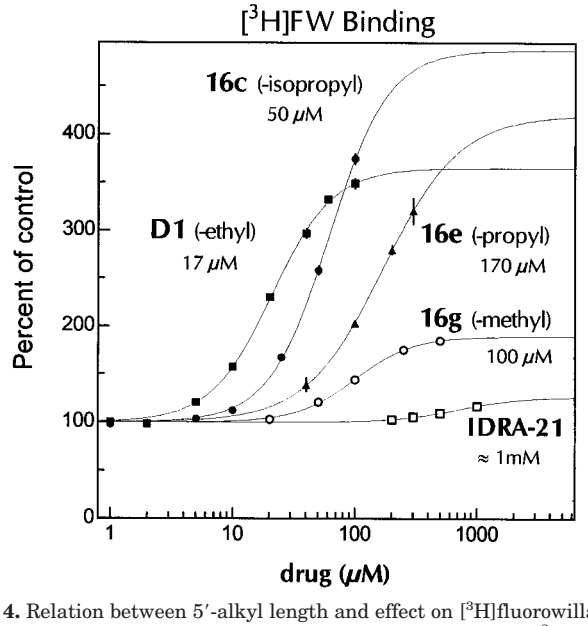

Fig. 4. Relation between 5′-alkyl length and effect on [^{3}H]fluorowillardiine binding. Binding was measured as in Fig. 3A with 20 nM [^{3}H]FW in the absence of SCN^-. The incubation temperature was 23–25°C. Data are from representative experiments. The nature of the alkyl group and average EC_{50} values are shown next to the drug name. Complete drug structures and additional binding parameters are compiled in Fig. 5. In the case of IDRA-21, data for each drug concentration were averaged from three experiments; limits in drug solubility prevented testing at concentrations above 1 mM, and thus the EC_{50} of 0.9 mM obtained from curve fitting is an approximation.

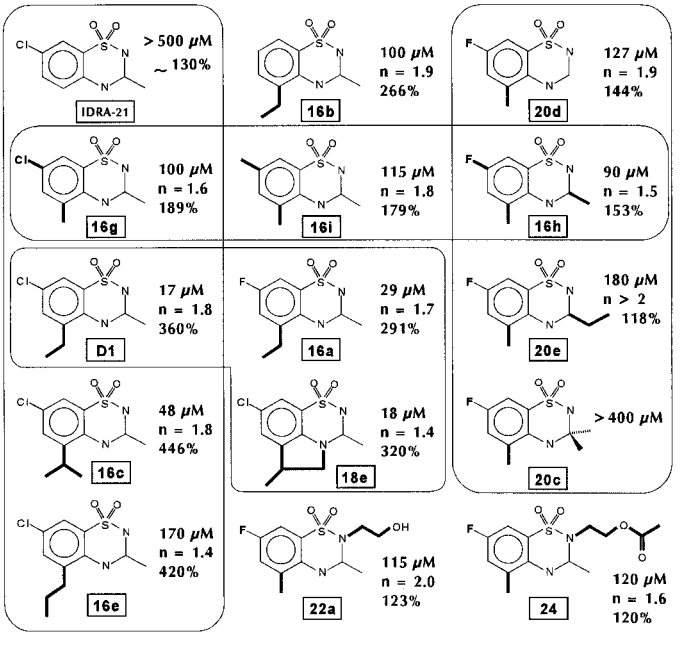

Fig. 5. Summary of structure-activity relations for 5′-alkyl-benzothiadiazides. Fifteen of about 50 compounds that were tested are shown together with their effects on agonist binding. Binding was measured as in Fig. 3A at 23–25°C with 20 nM [^{3}H]fluorowillardiine (without SCN^-) to construct dose-response relations. Data were fitted with four-point logistic equations; average values for the EC_{50}, Hill coefficient, and maximal increase in binding are shown next to the drug structure. Frames connect compounds with close structural relations. The left column shows compounds with progressively larger alkyl side chains at the 5′ position; the right column shows variations in the alkyl substituent at the 3′ position. The upper horizontal frame shows 5′ methyl compounds that differ only in their 7′ substituent.

through this component. This accords with our earlier observation that kainate binds with micromolar affinity to subpopulations of AMPA receptors (Hall et al., 1994).

Effects of Alkyl-BTDs on Synaptic Responses. Effects on synaptic responses were examined using field recording in area CA1 of hippocampal slices (Fig. 7) and whole-cell recording from pyramidal neurons of this region (Fig. 8). As shown at the top of Fig. 7, the methyl-BTD 16h and its ethyl analog 16a (the 7′-fluoro version of D1) significantly enhanced synaptic responses at concentrations of 50 and 20 μM, respectively. Onset of the effect was fast, and responses returned to baseline within 30 min of drug washout. Interestingly, response amplitude and response duration were affected in different ways by the two compounds. The 5′-methyl-BTD 16h produced a large increase in the amplitude with very small effects on the half width of the response, whereas the ethyl analog 16a caused a marked widening with modest amplitude effect. A similar differentiation was observed with other drugs with either methyl or longer chain alkyls, some of which are included in the diagram at the bottom of Fig. 7. As shown there, methyl-BTDs in all of 20 experiments enhanced the amplitude more than the response half width; in the 12 experiments involving compound 16h, the average ratio between the two effects was 4.6. Effects produced by the ethyl derivative 16a showed a nearly inverse pattern; in 9 of 13 experiments, the response duration was significantly increased, with little or no effect on amplitude, and only two experiments showed a preferred action on the amplitude.

The effects of the drugs on synaptic transmission were also examined in whole-cell recording with voltage-clamp configuration (Fig. 8). EPSCs recorded from CA1 pyramidal cells revealed a double dissociation between the drug effects similar to that seen with extracellular recording. The methyl compound 16h (100 μM) increased response amplitude by 83% ($\pm$36%, $n = 6$), with only 37% ($\pm$23%) increase in the response half width, and a similar amplitude preference was observed for compound 16*g* (97 $\pm$ 27%, $n = 6$ for amplitude versus 51 $\pm$ 20% for half width). Conversely, the ethyl derivatives D1 and 16a (50 μM) mainly increased response half width (83 $\pm$ 23%, $n = 9$ for D1 and 178 $\pm$ 63%, $n = 6$ for compound 16a) with less pronounced effects on amplitude (12 $\pm$ 21% and 53 $\pm$ 24%, respectively). The difference in the preference ratio (amplitude-effect/half width-effect) was highly significant between the two drug groups (Fig. 8, bottom). Thus, according to these observations, changes in the size of the 5′ substituent have consequences not only for drug potency but also for the way in which receptor kinetics is modulated by the drug.

Effects on Homomeric Receptors. The effects of D1 on recombinant homomeric AMPA receptors were examined in HEK 293 cells that stably express individual receptor subunits (Hennegriff et al., 1997). Measurements of whole-cell currents in such cells typically reveal small basal responses to glutamate because receptor desensitization proceeds faster than solution exchange. D1 entirely blocked this desensitization in GluR3 flop receptors (Fig. 9, left) and those made from other AMPA receptor subunits (data not shown) and thereby greatly increased the response. The compound was considerably more effective in this regard than cyclothiazide, which causes at most a slowing of desensitization in receptors of the flop variant (Partin et al., 1994; Arai et al., 2000). Dose-response relations for this effect yielded an EC_{50} of 38 μM, which is about the same as that obtained with native receptors (Fig. 2), and were characterized by a steep Hill slope of 3. Whether D1 has a preference among AMPA receptor subunits was examined in binding tests. Dose-effect curves were constructed at 0°C at a fixed concentration of (*S*)-[^{3}H]AMPA in the absence of thiocyanate. Under these conditions, D1 increased agonist binding to all subunits by 50 to 200% (Table 1). EC_{50} values were on the order of 5 μM (which corresponds to the value obtained with brain membranes at 0°C) with the exception of GluR4 flip, which exhibited an EC_{50} of 0.64 μM. It is also apparent, however, that D1 had a general preference for flip variants with selectivity ratios between 1.6 (GluR2) and 6 to 8 (GluR4). Most Hill coefficients were again between 1.5 and 2. Complete dose-

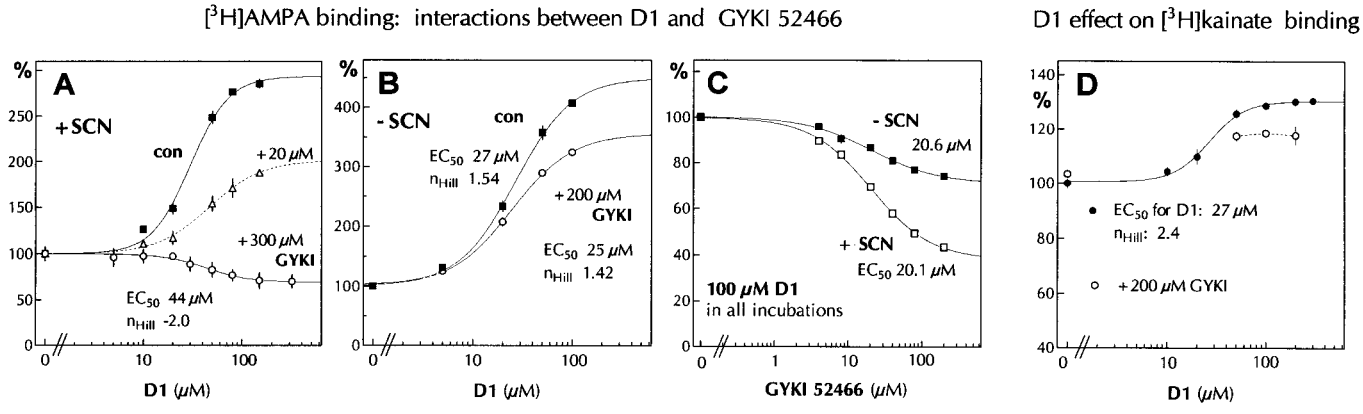

Fig. 6. D1 interaction with GYKI 52466 and D1 effect on [^{3}H]kainate binding. A, binding of (*S*)-[^{3}H]AMPA (50 nM) was measured at 25°C in the presence of the D1 concentrations indicated on the *x*-axis at ^{3}0, 20, or 300 μM GYKI 52466; all assays contained in addition 50 mM KSCN. Data for 0 and 300 μM GYKI are averages from three experiments each, EC_{50} and Hill slope were determined for the averaged data, and the curve for 20 μM GYKI is from a representative experiment. B, same as in A except that binding was measured in the absence of thiocyanate. Data points are averages of three separate experiments. C, dose-effect curves for GYKI 52466; binding of [^{3}H]AMPA was measured at a fixed concentration of D1 (100 μM) and the GYKI concentrations indicated on the *x*-axis. Binding at 0 μM GYKI was in this case set as 100%. Data shown in the graph are from a set of representative experiments with and without thiocyanate; nearly identical curves were obtained in a second set of experiments. Averaged binding constants were 17.2 $\pm$ 3.6 μM (S.D.), $n_{Hill} = -1.05 \pm 0.10$ in the absence of thiocyanate and 20.2 $\pm$ 0.1 μM, $n_{Hill} = -1.03 \pm 0.04$ in the presence of thiocyanate. D, effect of D1 on [^{3}H]kainate binding to rat brain membranes. Binding was measured at 25°C with 40 nM [^{3}H]kainate and the D1 concentrations shown on the *x*-axis. Some incubations contained in addition 200 μM GYKI 52466. Similar results were obtained in two additional experiments. The percentage increase in binding produced by D1 was lower at a [^{3}H]kainate concentration of 4 nM (data not shown).

effect curves are shown for GluR4 flop and flip in Fig. 9B. IDRA-21 exhibited a similar flip preference as did the 5′-methyl-BTD, which lacked the methyl group normally present at the 3′ position (compound 20d; Table 1). IDRA-21 enhanced [^{3}H]AMPA binding to GluR4 flip with an EC_{50} of 116 μM, indicating again two orders of magnitude difference in potencies between it and D1, but the Hill slope was the same for both compounds. This suggests that flip preference and a high value of the Hill coefficient are general properties of benzothiadiazides that contain the IDRA core structure.

Discussion

Cyclothiazide has been much more effective than IDRA-21 with regard to AMPA receptor currents in excised patches but not with regard to enhancing AMPA receptor-mediated field EPSPs. There is also evidence that the latter compound is behaviorally active while lacking the antidiuretic and antihypertensive qualities of the former. The goal of this study has therefore been to develop more potent and functionally distinct derivatives of IDRA-21 by modifying substituents around its bicyclic core. The most important findings have been 1) that an alkyl group at the 5′ position greatly potentiates drug-receptor interaction, whereas modifications at other positions generally were silent or disabling; 2) that D1 and cyclothiazide differ radically in their effect on AMPA receptor kinetics despite many commonalities in structure, potency, and subunit preferences; and 3) that the length of the 5′ substituent not only controls potency but also the manner in which the compounds affect the waveform of synaptic responses.

Potency estimates for IDRA-21 vary, but its effects on AMPA receptors in adult brain generally indicate an EC_{50} of about 1 mM. Introducing an ethyl group at the 5′ position increased the potency by at least one order of magnitude and resulted in a compound with a potency comparable with cyclothiazide. Binding tests and patch-clamp experiments with long glutamate applications produced similar EC_{50} values for D1 of about 20 to 40 μM. The EC_{50} obtained with 1-ms glutamate responses was somewhat higher and probably reflects a lower potency of the drug at receptors that are not

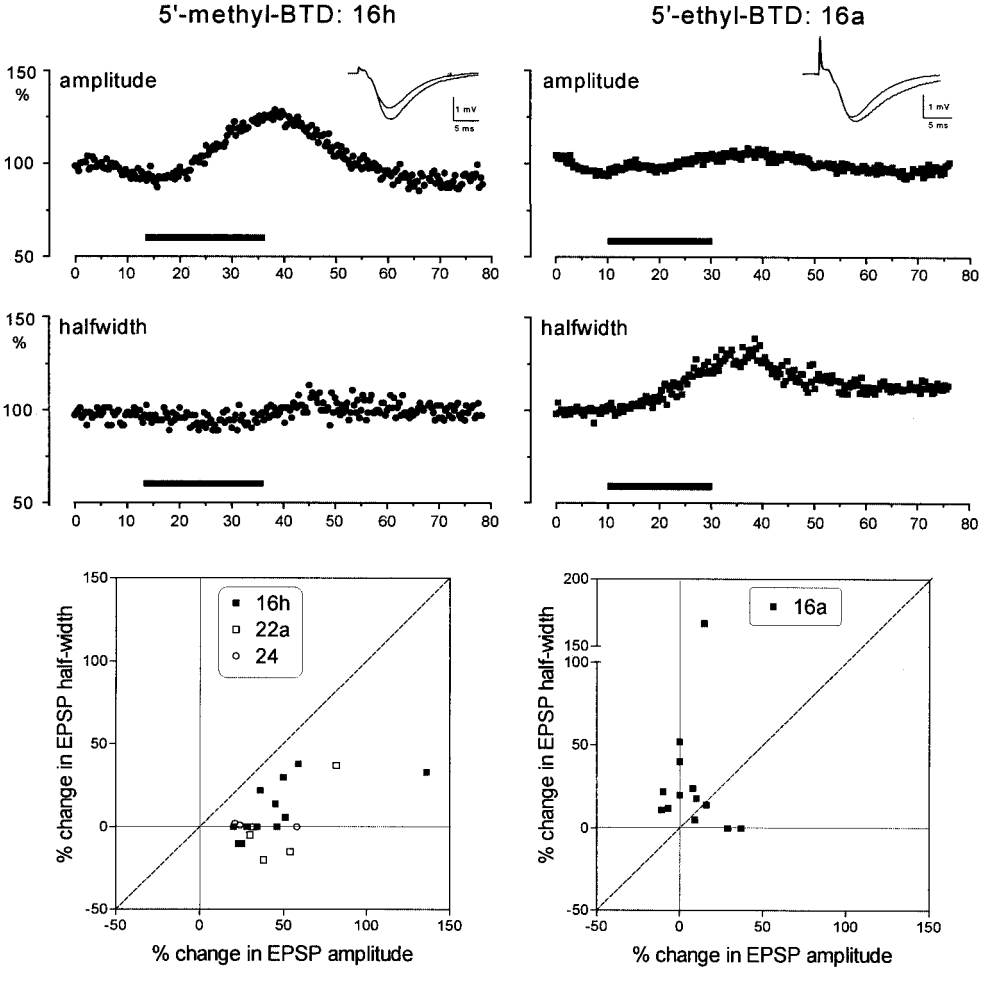

Fig. 7. Effects of 5′-methyl-BTDs and 5′-ethyl-BTDs on field EPSPs in CA1 of hippocampal slices. Top, representative experiments. Recording and stimulation electrodes were placed in stratum radiatum of the field CA1 of a hippocampal slice. After establishing a stable baseline, 50 μM of compound 16h or 20 μM of 16a was added to the perfusion line for the duration indicated by the horizontal bar. Amplitude and half width of the EPSP were normalized to those of the baseline responses and plotted against time. Traces taken before and during drug infusion are shown superimposed. Bottom, summary of drug effects. Each point represents one perfusion experiment. Percentage change in EPSP amplitude at the peak of the drug effect was plotted on the *x*-axis, and percentage change in response half width was plotted on the *y*-axis. Experiments at different drug concentrations are combined; the concentrations were 16h, 50 to 200 μM; 22a, 100 to 400 μM; 24, 50 to 100 μM; and 16a, 10–50 μM; only experiments in which responses returned to baseline were selected. Drug structures are shown in Fig. 5.

equilibrated with the agonist, suggesting that drug and agonists mutually increase their affinity. Insights into structure-activity relations for the alkyl-BTDs are discussed in greater detail elsewhere (Phillips et al., 2002). As shown there, the large gain in potency conferred by the ethyl group in D1 is close to the theoretical maximum for a hydrophobic substituent of this size, pointing to the conclusion that a major part of its surface is in contact with the receptor. This, and the abrupt loss in potency upon extending the alkyl group further, suggests that the 5′-ethyl group fits into a pocket of the receptor. Space around the R_3 substituent also seems to be confined insofar as extension beyond the methyl group at that position or introducing a second methyl substituent again reduced binding affinity. Larger substituents were however readily accommodated at the R_2 position. This suggests that the compounds described here bind to the receptor in such a way that their 3′-5′ edge is in close contact with the surface of the receptor, perhaps facing a groove.

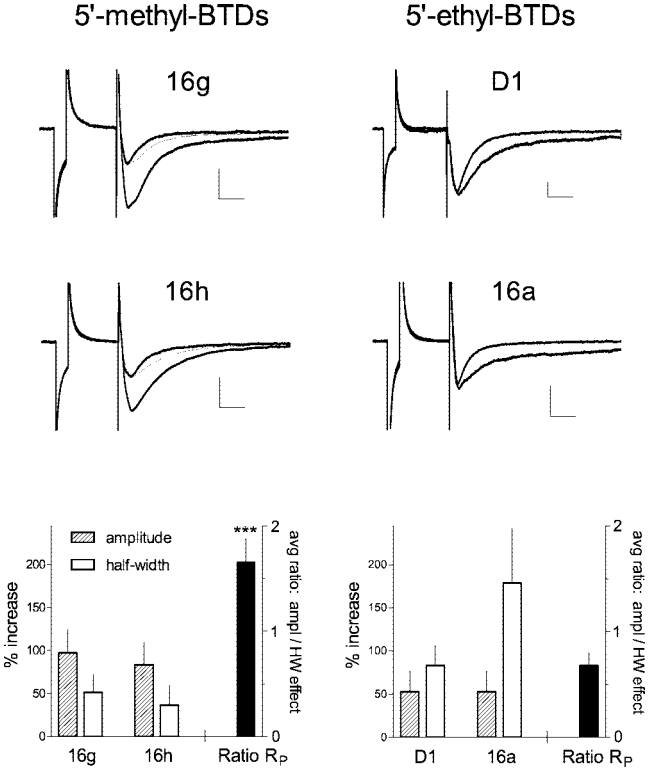

Fig. 8. Effects of 5′-methyl-BTDs and 5′-ethyl-BTDs on EPSCs recorded from hippocampal CA1 pyramidal neurons. Whole-cell recordings were made from stratum pyramidale of field CA1. Synaptic responses were evoked by stimulation of the Schaffer-commissural fibers. Compounds 16g and 16h were infused at a concentration of 100 μM, and D1 and 16a were infused at a concentration of 50 μM. Representative traces taken before and during drug application are shown at the top. The graphs for the 5′-methyl-BTDs also show the responses with drug normalized to the amplitude of the control responses. Holding potential: −70 mV. Calibration: 100 pA/200 ms. The hatched and clear columns in the bar graphs show the mean percentage increase (± S.E.M.) in the amplitude and half width over the predrug response (n = 9 for D1; n = 6 for all other compounds). The filled bars show the average preference for amplitude or half-width effect across all trials with either methyl- or ethyl-BTDs (experiments in which both amplitude and half width increased by less than 20% were excluded from this analysis). For this purpose, the ratios R_A (amplitude with drug)/(amplitude before drug) and R_{HW} (half width with drug)/(half width before drug) were calculated for each trial and then divided by each other to obtain a preference ratio $R_P = R_A/R_{HW}$, where $R_P = 1$ indicates equal increases in both amplitude and half width. The R_P values from all trials were then averaged and are shown as mean and S.E.M. (n = 10 for methyl-BTDs, n = 14 for ethyl-BTDs). ⋆⋆⋆, $P <$ 0.001 for the comparison between the two R_P values (t test).

Given that D1 and cyclothiazide belong to the same class of compounds, it was surprising that their effects on AMPA receptors differ in several fundamental ways. Deactivation of fast responses to millisecond glutamate pulses was slowed nearly 20-fold by D1, a value substantially greater than that reported for most other AMPA receptor modulators (Arai et al., 1996b, 2000), whereas cyclothiazide had almost no effect under the same test conditions (Arai and Lynch, 1998). Presumably related to this, the alkyl-BTDs markedly increased extracellular synaptic responses in CA1, whereas cyclothiazide is among the least effective of the AMPA receptor modulators in this measure. Furthermore, D1 caused an unprecedented increase in the equilibrium binding of agonists, even in assays containing the chaotropic ion thiocyanate (SCN^-) in which cyclothiazide reliably causes a 10-fold reduction in agonist binding (Hall et al., 1993; Kessler et al., 1996). Lastly, the effects of cyclothiazide in those tests could be adequately fitted with curves that had Hill slopes near 1, whereas D1 exhibited a large degree of cooperativity.

These observations suggest that D1 and cyclothiazide have very distinct effects on AMPA receptor kinetics, but it remains to be determined which aspects are preferentially targeted. Because we have previously shown that desensitization contributes minimally to response deactivation in hippocampal AMPA receptors (Arai and Lynch, 1998), the prolongation of fast glutamate responses by D1 indicates that this drug, unlike cyclothiazide, is also highly effective in slowing channel closing and/or dissociation of the agonist. Slowing of channel closing does by itself lead to a reduction in the macroscopic response desensitization, as seen during long glutamate applications (Ambros-Ingerson and Lynch, 1993). However, this is not likely to account for the complete blocking of desensitization caused by the alkyl-BTDs, because the methyl-BTDs were as effective as D1 in this regard despite lower efficacy in prolonging response deactivation. Also, the loss in paired-pulse inhibition in Fig. 2C indicates that the alkyl-BTDs, like cyclothiazide, directly block transition into the desensitized receptor state. In all, it seems that the alkyl-BTDs differ from cyclothiazide in being able to influence a much broader range of kinetic properties that encompasses both deactivation and desensitization.

The binding data support the above arguments. Equilibrium binding is mainly governed by the desensitized receptor states, but the dissociation constant K_D remains a mathematical function of all kinetic rate constants, including those for channel gating, as explicitly shown for the five-state receptor model by Ambros-Ingerson and Lynch (1993). Calculations with their equation indicate that slowing response deactivation will, under most conditions, increase equilibrium binding affinity, whereas blocking desensitization will have the opposite effect. Consistent with this, modulators that markedly slow deactivation have been found to produce larger increases in agonist binding. Thus, the methyl-BTDs were moderately effective in slowing the decay of fast responses and increased binding by less than 100%, whereas D1 had a very strong effect and produced the largest increases in binding affinity so far obtained with AMPA receptor modulators. Conversely, cyclothiazide has barely detectable effects on deactivation and generally reduces agonist binding.

It is also clear, however, that a simple scaling of drug effects with regard to effects on deactivation does not predict the full range of drug actions on synaptic responses. For example, the balance of effects on response amplitude versus duration differed substantially between methyl- and ethyl-BTDs in a manner than was not related in any obvious way to deactivation or desensitization. Thus, methyl-BTDs were more effective in enhancing the response amplitude despite having a lesser influence on response duration. Although it can not be ruled out from the present data that the drugs influenced other aspects of synaptic physiology, it seems more likely that the waveform of synaptic transmission is governed by subtle aspects of AMPA receptor kinetic that are not yet fully appreciated in their importance but happen to be affected differentially by minimal variations in drug structure. Whatever the underlying mechanism, the amplitude-versus-duration distinction could prove to be of practical significance, because the compounds predominantly affecting the former variable seemed to be less likely to cause epileptiform discharges. If so, then methyl-BTDs may have advantages over their ethyl counterparts in behavioral applications.

A consistent and unusual feature of the drugs described here is the size of the Hill coefficient. Binding effects of cyclothiazide, ampakines, and other agents are generally governed by Hill slopes near 1 (Arai et al., 1996c, 2000). By contrast, D1 effects consistently exhibited Hill coefficients larger than 1, usually on the order of 2, in binding tests and in recordings involving native and recombinant receptors. Hill slopes significantly higher than unity were found for all

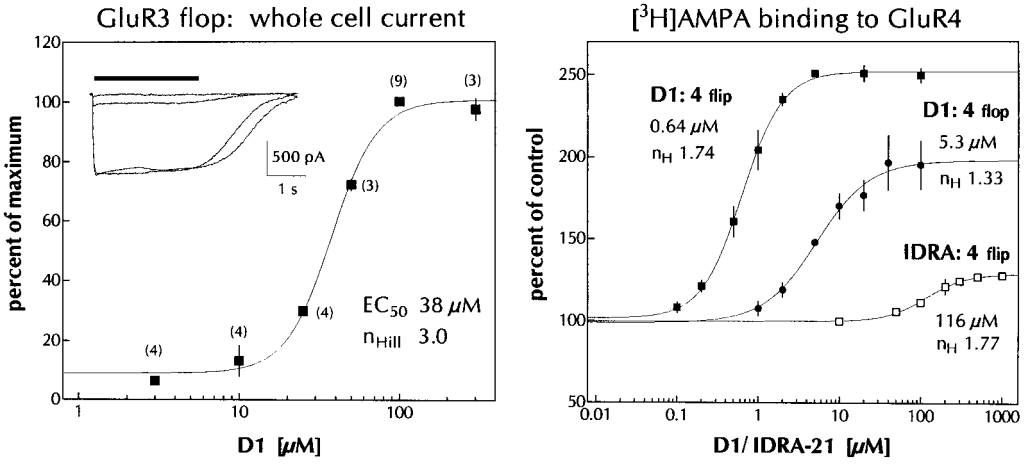

Fig. 9. Effect of D1 on homomeric recombinant AMPA receptors. Tests were carried out with HEK 293 cells that stably express homomeric AMPA receptors made of one of the subunits GluR1 through 4 in either the "flop" or the "flip" splice variant (Hennegriff et al., 1997). Left, whole-cell current mediated by GluR3 flop receptors during application of 10 mM glutamate. The drug was present at the indicated concentration in both glutamate containing solution and background flow solution; background flow lines were switched at least 30 s before the first glutamate pulse. The peak current at each drug concentration was normalized to that measured in the presence of 100 μM D1. Data (means and S.E.M.) were collected from a total of nine patches; the number of patches tested at each concentration is shown in parentheses. The data points were fitted with a four-point logistic equation. The inset shows traces from a typical experiment in which 3, 10, 100, and 300 μM D1 were applied alternatingly to the same patch; the duration of glutamate infusion is indicated by the horizontal bar. Right, effects on [^{3}H]AMPA binding to GluR4 flop and flip receptors. Binding was measured at 0°C without KSCN at an (*S*)-[^{3}H]AMPA concentration of 10 nM in the case of GluR4 flop and at 50 nM in the case of GluR4 flip; the (*S*)-AMPA concentrations were selected to give about 10% receptor occupancy. Binding at each drug concentration was normalized to that in the absence of drug, and data from four (D1) and three experiments (IDRA-21) were averaged (means and S.E.M.). The data were fitted with four-point logistic equations. Data for other subunits are summarized in Table 1.

TABLE 1

Binding affinities for homomeric AMPA receptors (0°C)

The selectivity for AMPA receptor subunits was determined as described in Fig. 9 by measuring the effect of the drug on the binding of (*S*)-[^{3}H]AMPA. Binding incubations were conducted at 0°C with a fixed concentration of (*S*)-[^{3}H]AMPA in the absence of KSCN. They were terminated by filtration through GF/C glass fiber filters. (*S*)-AMPA concentrations were selected between 10 and 50 nM to obtain approximately 10 to 20% receptor occupancy in the absence of drug. In each experiment, binding was measured at seven to nine drug concentrations and fitted with a four-point logistic equation. Binding constants from the indicated number of experiments were then averaged (means and S.E.M.). To obtain the binding parameters for the effect of IDRA-21, binding data from three experiments were first averaged and then fitted; its effect on GluR4 flop was fitted with an $n_{Hill} = 1$ because of the smallness of the binding change.

Compound	Subunit	EC_{50}	n_{Hill}	Max. %	n	EC_{50} Ratio Flop/Flip
		μM				
D1	GluR1flop	3.1 ± 0.5	2.5 ± 0.8	150 ± 5	3	
	GluR2flop	4.3 ± 0.3	2.0 ± 0.7	168 ± 12	2	
	GluR2 flip	2.7 ± 0.0*	1.6 ± 0.2	217 ± 7	2	1.6
	GluR3flop	7.7 ± 0.2	1.5 ± 0.1	273 ± 38	3	
	GluR3 flip	3.2 ± 0.6**	1.6 ± 0.2	288 ± 14	3	2.4
	GluR4flop	5.0 ± 1.3	1.4 ± 0.1	198 ± 16	4	
	GluR4 flip	0.8 ± 0.1*	1.9 ± 0.1	265 ± 15	4	6.3
20d	GluR4flop	104 ± 4		56 ± 10	2	
	GluR4 flip	20 ± 3**		132 ± 9	2	5.2
IDRA-21	GluR4flop	>500	N.D.	<90	3	
	GluR4 flip	117	1.8	129	3	>4

N.D., not determined.
Differences in EC_{50} values between flip and flop were significant as indicated; *, $P < 0.05$, **, $P < 0.01$.

analogs with the possible inclusion of IDRA-21; the significance of this observation remains unclear. Because AMPA receptors probably are tetrameric proteins, each functional unit contains four homologous drug sites in addition to the same number of agonist sites. It is thus possible that alkyl-BTDs, but not other modulators, invoke cooperativity between homologous sites on adjacent subunits. Alternatively, the subunits may have multiple modulatory sites, as indicated by competition studies with cyclothiazide and ampakines (Arai et al., 2000). Possibly, then, those alkyl-BTDs with Hill slopes near 2 bind to two sites with similar affinity, whereas those with smaller coefficients have various degrees of preference for one of the sites. The dramatic reduction in the effect of D1 on binding when GYKI was present raised the possibility of a common site, but further analysis showed that the D1 effect in the presence of saturating GYKI was still governed by a Hill slope larger than unity. Interactions with GYKI were also found in an earlier study with ampakines (Arai et al., 2000), and it was suggested there that the effect is due to opposing actions at the level of receptor kinetics rather than competition for a shared modulatory site.

The functional distinctions discussed above emphasize the importance of the structural differences between the alkyl-BTDs and cyclothiazide. It is of interest in this regard that IDRA-type compounds and cyclothiazide cannot be "connected" through conservative substitutions in the sense that compounds with intermediary structural features have little potency at AMPA receptors. Thus, introducing a norbornyl group into alkyl-BTDs at R_3 (Phillips et al., 2002) or replacing chloride at R_7 with the sulfonamide group present in cyclothiazide (Bertolino et al., 1993) caused a loss in potency. Similarly, several cyclothiazide analogs in which the norbornenyl group had been removed or replaced by other substituents were inactive at AMPA receptors (Yamada and Tang, 1993). It thus seems likely that the topology of drug-receptor association and the contact points responsible for bonding are different for IDRA-type compounds and cyclothiazide.

Another interesting aspect of comparison between D1 and cyclothiazide concerns the selectivity for the AMPA receptor subunits and their splice variants. Cyclothiazide has a large preference for the flip variant of all four subunits (Partin et al., 1994) that critically depends, however, on the norbornenyl moiety that is present in cyclothiazide but not other benzothiadiazides. In fact, replacing the norbornenyl substituent conservatively with a cyclohexyl ring was sufficient to switch the flip preference to flop at most subunits (Kessler et al., 2000). It was therefore quite unanticipated that IDRA-21 and D1 were again associated with a marked flip preference, at least at GluR4 subunits, and it suggests that this may be a rather widespread feature of this class of drugs despite the possibility of different docking modes mentioned above.

In conclusion, the drugs described here possess properties that differ in many aspects from those of other AMPA receptor modulators, including the ampakines, and thus provide new tools to study receptor function. Other compounds have recently been described that also exhibit greatly improved potency for the AMPA receptor. Some of them are structurally very similar to the compounds described here, such as S18986 (Desos et al., 1996) and the pyridothiadiazines (Pirotte et al., 1998). Others do not contain the characteristic bicyclic core but have a sulfonamide embedded in an elongated molecular structure, such as PEPA (Sekiguchi et al., 1997) and LY395153 and related compounds (Ornstein et al., 2000), the latter of which exhibited submicromolar EC_{50} values in various physiological tests. It will be of interest to compare these compounds with the alkyl-BTDs in the paradigms used here, because they have not yet been fully characterized regarding their effects on the synaptic waveform, receptor kinetics, or agonist binding.

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