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EFFECTS OF DECAHYDROISOQUINOLINE-3-CARBOXYLIC ACID MONOHYDRATE, A NOVEL AMPA RECEPTOR ANTAGONIST, ON GLUTAMATE-INDUCED CA^{2+} RESPONSES AND NEUROTOXICITY IN RAT CORTICAL AND CEREBELLAR GRANULE NEURONS

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Abstract—In this study, we examined the effects of a novel water-soluble, putative AMPA receptor antagonist, (-)(3S,4aR,6R,8aR)-6-[2-(1(2)H-tetrazole-5-yl)ethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid monohydrate (LY326325), on glutamate-, N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, and kainic acid (KA)-induced elevations of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and $^{45}Ca^{2+}$ uptake, as well as glutamate agonist-induced neurotoxicity in primary cultures of intact rat cortical and cerebellar granule neurons. In some experiments, the actions of LY326325 were tested in the presence of cyclothiazide, a compound that is known to block glutamate-induced desensitization of AMPA-preferring subtypes of glutamate receptors, thereby largely potentiating the functional effects of AMPA. LY326325 fully blocked the elevations of $[Ca^{2+}]_i$ induced by NMDA and non-NMDA glutamate receptor agonists in both cortical and cerebellar granule neurons. The application of increasing concentrations of cyclothiazide was not able to reverse the LY326325-induced blockade of glutamate receptors in cortical neurons. In contrast, the same cyclothiazide treatment fully reversed the blockade produced by the noncompetitive AMPA/KA receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine HCl (GYKI 52466). In $^{45}Ca^{2+}$ uptake studies. LY325325 inhibited the NMDA-, AMPA-, and KA-induced enhancement of $^{45}Ca^{2+}$ uptake in a concentration-dependent fashion in both cortical and cerebellar granule cells. In analogy to the results obtained with $[Ca^{2+}]_i$ recordings, cyclothiazide failed to counteract the LY326325-induced blockade of KA-stimulated $^{45}Ca^{2+}$ uptake in cerebellar granule neurons, whereas the blockade induced by the noncompetitive AMPA/KA receptor blocking agent GYKI 52466 was fully reversed by cyclothiazide. Because a similar, although not identical pattern of actions was seen following the application of the competitive AMPA/KA receptor antagonist 6-nitro-7-sulphamoyl-benzo(f)quinoxaline-2-3-dione (NBQX), it is suggested that the inhibitory actions of LY326325 are similar to those produced by NBQX but clearly differ from those caused by the noncompetitive AMPA/KA receptor antagonist GYKI 52466. Finally, when the neuroprotective actions of LY326325 on glutamate agonist-induced neurotoxicity were examined in cerebellar granule neurons, we found that LY326325 almost completely blocked the neurotoxic actions of NMDA, AMPA, and KA, respectively, whereas it produced only a partial blockade of glutamate-induced neurotoxicity. Taken together, our current results suggest that although LY326325 blocked both nonNMDA and NMDA-induced Ca^{2+} responses, it still displayed a preferential affinity for nonNMDA receptors as compared to NMDA receptors. However, LY326325 appears to be a less selective AMPA/KA receptor antagonist than NBQX and GYKI52466, respectively.

Key words: calcium fluxes; glutamate; NMDA; AMPA; kainate; cyclothiazide; LY326325; GYKI 52466; receptor desensitization; neurotoxicity; cortical neurons; cerebellar granule neurons

Ionotropic glutamate receptors constitute a family of excitatory amino acid receptors that form ion channels with subtype selectivity for the receptor agonists NMDA‖, AMPA, and KA [1, 2]. Experimental work concerning the functional properties of these receptor systems was initially, mainly due to a better availability of specific receptor agonists and antagonists, primarily on NMDA receptor-regulated ion channels [3, 4]. The access to more selective AMPA/KA antagonists, represented by compounds such as CNQX, and DNQX [5], facilitated attempts to delineate the functional role of AMPA and KA receptors although these receptor antagonists still displayed limited receptor selectivity, in particular with regard to some unwanted interaction with NMDA receptors. The discovery of the competitive AMPA receptor antagonist NBQX, which showed a 30-fold greater af-

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‖ Abbreviations: AMPA, α-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid; ATPA, (RS)-2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid; KA, kainic acid; NMDA, N-methyl-D-aspartic acid; CGS19755, cis-4-(phosphonomethyl)-2-piperidine-carboxylic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GYKI52466, (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine HCl); LDH, lactate dehydrogenase; LY215490 (3RS,4aRS,6RS,8aRS)-6-[2-(1(2)H-tetrazole-5-yl)-ethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carcarboxylic acid); LY326325, (-)(3S,4aR,6R,8aR)-6-[2-(1(2)H-tetrazole-5-yl)ethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid monohydrate; NBQX, 6-nitro-7-sulphamoyl-benzo(f)quinoxaline-2-3-dione; $[Ca^{2+}]_i$, intracellular calcium concentrations; EPSP, excitatory postsynaptic potential.

finity for AMPA than KA receptors and was devoid of disturbing side effects on glycine recognition sites at the NMDA receptor [6], finally made it possible to convincingly separate biochemical and physiological events mediated through an activation of NMDA and nonNMDA receptors, respectively. Recently, another potent AMPA/KA receptor antagonist, GYKI 52466 [7], was found to inhibit AMPA/KA receptor activity in a noncompetitive manner [8] and to influence nonNMDA receptor kinetics in a different manner than NBQX [9, 10]. Furthermore, several other novel, structurally different, and potentially useful AMPA receptor agonists and antagonists are currently under development [11–13]. An increasing body of knowledge now indicates that AMPA receptors may play a crucial role in many glutamate-activated physiological and pathophysiological processes in the brain. For example, in electrophysiological experiments it has been demonstrated that stimulation of AMPA receptors enhances EPSPs [14–16]. Data obtained in biochemical investigations have shown that AMPA stimulates the release of various neurotransmitters [17, 18]. Although AMPA receptors initially were considered almost impermeable to divalent ions, in particular Ca^{2+}, rapidly accumulating biochemical evidence suggests that at least certain subpopulations of AMPA receptors display high Ca^{2+}-permeability, in some situations comparable to the Ca^{2+} permeability seen at NMDA receptors (for refs. and discussion, see [2]). Recent results from many behavioral pharmacology assays indicate that AMPA receptors may modulate neuronal processes involved in phenomena such as locomotor activity [19], anxiety and neophobia [20–21], electrically- and chemically-induced convulsions [22], as well as learning and memory [23–25]. Furthermore, much interest has been focused on the fact that AMPA receptor antagonists seem to have strong neuroprotective actions on ischemia-induced brain lesions studied in a variety of animal models for stroke [26]. These preclinical findings have created an increasing awareness of the potential usefulness of selective AMPA receptor ligands in the management of stroke, epilepsy, and Parkinson's disease in humans [26, 27].

The therapeutic potential of currently available AMPA receptor antagonists, for example NBQX and GYKI 52466, is weakened by their poor solubility and relatively short duration. The introduction of a structurally novel series of putatively selective AMPA receptor antagonists which, in preliminary investigations, were found to be water soluble and to display preferential affinity for brain AMPA receptors as compared to KA and NMDA may, thus, represent an important step towards the development of clinically useful AMPA receptor antagonists. Currently available results indicate that these new AMPA receptor antagonists show anxiolytic, anticonvulsant, and neuroprotective actions in several different behavioral, electrophysiological, and biochemical assays [12, 20, 22, 28, 29]. No anxiolytic properties could be demonstrated in mice, however, following the administration of the presently examined compound, LY326325, in a series of plus-maze behavioral tests performed in this laboratory [30]. It has been suggested that these structurally novel AMPA receptor antagonists, LY326325 in the present study, possess strong neuroprotective actions against AMPA-induced neuronal degeneration [31] and it is now more generally accepted that at least certain AMPA receptors display a considerable permeability to Ca^{2+}, which could be involved in glutamate-induced neurotoxicity. For these reasons, we decided to evaluate the effects of LY326325, a water-soluble derivative of the competitive AMPA receptor antagonist LY215490 [22], on glutamate agonist-produced Ca^{2+} fluxes and neurotoxicity in primary cultures of intact rat cortical and cerebellar granule neurons. Although there are currently no receptor binding data available concerning LY326325, it has been reported that LY215490 has IC_{50}-values against [^{3}H]AMPA, [^{3}H]CGS19755, and [^{3}H]kainate of 4.8, 26.4, and 247 nM, respectively [22].

MATERIALS AND METHODS

Cerebellar granule cell cultures

All experiments were approved by the Ethical Committee for animal experiments in Stockholm, Sweden. Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rat (B&K Universal, Sollentuna, Sweden) cerebellum as previously described by Levi *et al.* [32]. Briefly, after dissection, 8 cerebelli were pooled and sliced with a McIlwain tissue chopper in two orthogonal directions (slices were 0.4 mm thick), incubated in 0.25% trypsin solution, and dispersed by trituration in a DNase and soybean trypsin inhibitor-containing solution (0.01% and 0.05%, respectively). Cells were plated (2×10^6 cells/2 mL/dish) onto either precleaned (soaked in 95% ethanol and flamed) glass coverslips placed in 35-mm Petri dishes (Nunc AC, Roskilde, Denmark) or 24-well plates coated with 5 μg/mL of poly-L-lysine (M_w = 70,000–150,000), for microfluorimetric and $^{45}Ca^{2+}$ uptake assays, respectively. Cells were cultured for 8 days at 37°C in an atmosphere of 5% CO_2/95% air in a Basal Eagle's Medium supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/mL gentamicin. The addition of cytosine-β-arabinofuranoside (10 μM), 24 hr after plating limited the number of nonneuronal cells to less than 5%. The medium was not changed until the cultures were used in the experiments.

Cortical cell cultures

Cortical cell cultures were prepared from Sprague-Dawley 1-day-old rat (B&K Universal, Sollentuna, Sweden) cerebral cortex and neostriatum according to the method of Alho *et al.* [33] with some slight modifications [34, 35]. After careful dissection of diencephalic structures and hippocampus, the cerebral hemispheres and striata of 12–14 rats were pooled and treated as described in the protocol for cerebellar granule cell cultures (see above). Cells were plated with a density (1.25×10^6 cells/2 mL) and cultured for 10 days without changing the medium. Cytosine-β-arabinofuranoside (2 μM) was added 48 hr after plating to inhibit the replication of nonneuronal cells.

Microfluorimetric intracellular calcium ($[Ca^{2+}]_i$) measurements

Cells from both cortical and cerebellar granule cell cultures that had been grown onto precleaned poly-L-lysine coated glass coverslips (0.25 mm) were used. On the day of the experiment, coverslips with cell monolayers were washed twice with Locke's buffer containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5.5 mM D-Glucose, and 5 mM HEPES (pH 7.4). The same buffer was used throughout the experi-

ments. The cells were then loaded with 2 μM fura-2-AM and incubated for 60 min at 37°C to ensure that de-esterification was complete. The coverslips were removed from the dish, rinsed once with Locke's buffer, and mounted as the bottom of an open chamber placed on the temperature-controlled stage of an inverted epi-fluorescence microscope (Zeiss, Axiovert 35M). The volume of the perifusate was 0.2 mL, the perfusion rate 0.4 mL/min. The temperature of the perfusing Locke's buffer was maintained at 37°C. The test solutions were applied by switching from a drug-free perfusion buffer to a buffer containing the final concentrations of the test drugs. Each recording was made from a virgin coverslip and completed within 15 min of the start of the recordings. Single neurons were optically selected by means of a manual diaphragm fitted to the photomultiplier housing. The microscope was connected to a SPEX fluorolog-2 CM1T11I system. The excitation wavelengths generated by two monochromators (340 nM and 380 nM) were directed to the cell by a dichronic mirror. The emitted light, selected by a 510 nM filter, was monitored by a photomultiplier attached to the microscope. The excitation wavelengths were alternated at a frequency of 1 Hz and the length of time for data collection at each wavelength was 0.33 sec. For estimation of relative magnitudes and time courses of the Ca^{2+} transients the fluorescence images were rationed by dividing the 340 nm image (F_{340}) by 380 nm image (F_{380}) on a pixel-by-pixel basis and presented as changes in the ratio ($R_{340/380}$) fluorescence (y-axis) against time (x-axis) according to Grynkiewicz *et al.* [36]. Fluorescence ratios were not calibrated to $[Ca^{2+}]_i$ because of the variability of the results obtained using different calibration methods. The range of ratios found in these experiments are within the range closely resembling that of $[Ca^{2+}]_i$. Background fluorescence was measured after quenching of fura-2 fluorescence with manganese and was subtracted from all the traces before calculation of $R_{340/380}$. To compensate for variations in output light intensity from the two monochromators, all experiments were corrected for by the inclusion of a fluorescence ratio with both monochromators set at 360 nm. No corrections were made for interference of fura-2 fluorescence by drugs added to the incubation media.

$^{45}Ca^{2+}$ uptake measurements

Cell monolayers in 24-well plates were used for $^{45}Ca^{2+}$ measurements. Cultures were washed twice with prewarmed (37°C) Mg^{2+}-free Locke's buffer and preincubated for 10 min at 37°C. Calcium uptake was initiated by replacing the preincubation buffer with prewarmed Locke's buffer containing 1 μCi/mL $^{45}Ca^{2+}$ and the combination of test drugs. Incubation was terminated after 5 min by aspiration of $^{45}Ca^{2+}$-containing incubation media followed by a rapid wash with 0.5 mL ice-cold Locke's containing 1 mM Mg^{2+}. Cultures were washed 4 more times using the same washing buffer. Cells were dissolved in 0.5 mL/well of NaOH (0.1 N). The next day, 250 μL aliquots were pipetted into scintillation vials and the radioactivity determined in the presence of 4 mL of Ready-Safe (Beckman Instruments AB, Stockholm, Sweden) scintillation fluid by conventional techniques using a Hewlett Packard scintillation counter. An additional aliquot (200 μL) was taken for protein determination according to Lowry's method [37]. All data points were run in triplicate and the experiments repeated in at least 2–3 separate cell culture preparations.

Determination of lactate dehydrogenase (LDH) activity

Neuronal cell injury estimated by examination of cultures under phase-contrast microscopy and quantitatively assessed by measuring LDH released from damaged cells into the extracellular medium after exposure to test drugs, was demonstrated to correlate linearly with the degree of cellular damage assessed using morphological criteria [38]. To start the experiment, culture medium was removed and replaced with one mL prewarmed filtration-sterilised Mg^{2+}-free HEPES-buffered saline (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2*H_2O$, 2.3 mM; $NaHCO_3$, 3.6 mM; D-glucose, 5.5 mM; HEPES, 5 mM; pH = 7.4) containing final concentrations of test drugs, and returned to the 37°C culture incubator. Two hr later the toxic exposure was terminated by washing out the drug solution with 2 mL prewarmed saline and replacing it with one mL serum-free culture medium, prior to returning the dishes to the incubator. After 24 hr, 200 μL aliquots of media were taken from each dish and the LDH content measured spectrophotometrically by a standard kinetic assay for pyruvate (Sigma Kit 340-LD). Briefly, medium sample (150 μL) was added to 1.1 mL phosphate buffer (0.1 mol/L, pH 7.5) and 200 μL of NADH solution (0.25 mg/mL). 20 min later the 'reverse' reaction was started by adding 50 μL phosphate buffer containing sodium pyruvate (22.7 mmol/L). The decrease in absorbance at 340 nm was estimated over a 3-min time period at 30-sec intervals in a Pharmacia spectrophotometer. LDH content (U/mL) was calculated from the slope of the linear portion of the absorbance curve. To compensate for slightly different cell densities and responsiveness to excitatory amino acids, data from each separate cell preparation were scaled to the mean LDH values (U/mL) obtained in control mock-treated cultures (=0%) and cultures exposed to 100 μM glutamate (=100%). Data presented reflect results from 3–5 independent experiments performed in triplicate sister cultures from different rat cerebellar granule cell preparations. Effects of drug treatment were assessed by ANOVA and, if significant, group means were compared by the Bonferroni multiple comparisons test.

Chemicals and drugs

Reagents of analytical grade and double-distilled water were used throughout the experiments. Cell culture media, sera, and gentamicin were from Gibco BRL (Life Technologies/Labdesign, Täby, Sweden). $^{45}Ca^{2+}$ (30 mCi/mg) was purchased from DuPont Scandinavia AB (Stockholm, Sweden). Glutamic acid, KA, N-methyl-D-aspartic acid, and glycine were purchased from Sigma (St. Louis, MO, U.S.A.). AMPA was purchased from Tocris Neuramin (Bristol, England). LY215490 and cyclothiazide were generous gifts from Eli Lilly Research Laboratories (Indianapolis, IN, U.S.A.), NBQX was a generous gift from Novo Nordisk A/S (Målöv, Denmark), whereas GYK152466 was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Statistical analysis

Statistical analysis of data was performed by one-way repeated measures analysis of variance (ANOVA) followed by Bonferroni multiple comparison or Dunnett's tests. Concentration response curves were built using the

curve-fitting program of GraphPad (Intuitive Software for Science, San Diego, CA, U.S.A.) based on a four-parameter logistic equation to describe sigmoidal concentration-response curves [39] and analyzed by means of a nonlinear least-squares regression analysis. The curve that produced the smallest residual variance between the actual data and the logistic equation was used to estimate the parameters of maximal enhancement of $^{45}Ca^{2+}$, concentration of drug eliciting half-maximal effect (EC_{50}), and curve slope factor in each experiment.

RESULTS

Effects of LY326325 on $[Ca^{2+}]_i$ in single cortical and cerebellar granule neurons

In the first part of our study, we examined the effects of LY326325 on $[Ca^{2+}]_i$ in single cortical and cerebellar granule neurons on glutamate agonist-induced elevations of $[Ca^{2+}]_i$. As shown in Fig. 1, LY326325 blocked the enhancement of $[Ca^{2+}]_i$ produced by NMDA, glutamate, KA, and AMPA, respectively, in single cortical neurons. Similarly, LY326325 was an effective antagonist of glutamate agonist-induced elevations of $[Ca^{2+}]_i$ in single cerebellar granule neurons (Fig. 2). (Please note that because AMPA alone does not increase $[Ca^{2+}]_i$ in cerebellar granule cells, the effects of LY326325 on AMPA-activated Ca^{2+} responses were investigated in the presence of 30 μM cyclothiazide, a concentration which by itself does not alter $[Ca^{2+}]_i$ in cerebellar granule cells [35]). No attempt was made to evaluate the relative potency of LY326325 at the different glutamate receptor subtypes in the microfluorimetric experiments because quantitative estimations in these assays are largely influenced by existing inter-assay variability in calibration measures.

In the next series of microfluorimetric experiments, we investigated the effects of increasing concentrations of cyclothiazide on the LY326325-induced blockade of AMPA and KA receptors, respectively, in single cerebellar granule neurons. As shown in Fig. 3, a low concentration of cyclothiazide (10 μM) fully reversed the blockade of the KA-stimulated increase in $[Ca^{2+}]_i$ produced by the noncompetitive AMPA/KA receptor antagonist GYKI 52466. In contrast, increasing concentrations of cyclothiazide (up to 100 μM) had no influence on the LY326325-produced blockade of KA-activated Ca^{2+} responses. Furthermore, when similar experiments were performed in the presence of 100 μM AMPA, high concentrations of cyclothiazide (100 μM) were able to reverse the GYKI52466- and LY326325-induced blockade of AMPA-activated Ca^{2+} responses. In a previous study from this laboratory, we showed that this concentration of cyclothiazide (100 μM) by itself had no effect on $[Ca^{2+}]_i$ in cerebellar granule cells [35].

Effects of LY326325 on $^{45}Ca^{2+}$ uptake in cortical and cerebellar cell cultures

There was no significant difference in the basal $^{45}Ca^{2+}$ uptake between cortical (3.9 ± 0.3 nmoles/mg protein/min) and cerebellar granule (4.1 ± 0.4 nmoles/mg protein/min). Application of NMDA (50 μM in the presence of 10 μM) produced a marked increase in the uptake of $^{45}Ca^{2+}$ in monolayer cell cultures of both cortical (8.4 ± 0.1 nmoles/mg protein/min) and cerebellar granule (7.9 ± 0.4 nmoles/mg protein/min) neurons. As shown in Fig. 4 (panel A), NMDA-stimulated $^{45}Ca^{2+}$ uptake was, in the presence of glycine (10 μM), dose-dependently inhibited by LY326325 in both cortical and cerebellar granule neurons. However, the putative AMPA antagonist was much more potent in inhibiting the NMDA response in cerebellar granule cells (IC_{50} = 63.2 ± 1.2 μM) than in cortical neurons (IC_{50} = 272.7 ± 31.3 μM). Results presented in panel B of Fig. 4 illustrate LY326325-produced inhibition of AMPA-stimulated (100 μM, in the presence of cyclothiazide; 30 μM) enhancement of $^{45}Ca^{2+}$ uptake in cortical (20.4 ± 1.0 nmoles/mg protein/min) and cerebellar granule (11.5 ± 0.3 nmoles/mg protein/min) neurons. As shown, LY326325 was slightly more potent in inhibiting the AMPA-induced response in cortical neurons (IC_{50} = 24.6 ± 1.1 μM) as compared to that in granule neurons (IC_{50} = 54.6 ± 1.3 μM). When corresponding experiments were conducted with regard to the inhibitory properties of LY326325 on KA-induced enhancement of Ca^{2+} uptake, they revealed a marked difference between the LY326325-produced inhibition in cortical and cerebellar granule neurons, respectively (see Fig. 4, panels C and D). Thus, LY326325 was considerably more potent in inhibiting KA-induced $^{45}Ca^{2+}$ uptake in cortical (IC_{50} = 0.55 ± 1.1 μM) neurons as compared to cerebellar granule (IC_{50} = 17.4 ± 1.1 μM) neurons. Furthermore, when similar experiments with KA were conducted in the presence of cyclothiazide (30 μM), there was a clear, almost 20-fold shift in the dose-response curve obtained in cortical cell cultures (from IC_{50} = 0.55 ± 1.1 μM, to IC_{50} = 13.2 ± 1.1 μM in the presence of cyclothiazide), whereas no shift in the dose-response curve was observed in cerebellar granule neurons (from IC_{50} = 17.4 ± 1.1 μM, to IC_{50} = 23.7 ± 1.3 μM in the presence of cyclothiazide).

To analyze further the interaction between LY326325 and nonNMDA-sensitive recognition sites, we compared the effects of LY326325, NBXQ, and GYKI 52466, respectively, on AMPA- and KA-stimulated Ca^{2+} uptake in the presence of increasing concentrations of cyclothiazide (Fig. 5). (We have previously shown that cyclothiazide is able to fully reverse the effects of GYKI 52466 in this assay; data not shown.) As depicted in Fig. 5 (panels A and C), the application of increasing concentrations of cyclothiazide fully antagonized the GYKI 52466-produced blockade of AMPA- and KA-stimulated Ca^{2+} responses in cortical neurons. In contrast, cyclothiazide produced only a partial reversal of the LY326325-produced blockade of AMPA and KA responses, whereas only a partial (in the presence of AMPA) or no (in the presence of KA) reversal was obtained following the application of NBQX. Furthermore, when the same series of experiments were performed in cerebellar granule cells, our results indicate that the various nonNMDA receptor agonists and antagonists interact with cyclothiazide-sensitive recognition sites in a more complex and, thus, less clearly defined fashion (Fig. 5; panels B and D).

Effects of LY326325 on glutamate agonist-induced neurotoxicity in cultures of intact cortical and granule neurons

We have recently presented an extensive characterization of the neurotoxic actions of various glutamate receptor agonists in our current tissue culture preparations (unpublished manuscript). In the present investigation, we examined the neuroprotective effects of

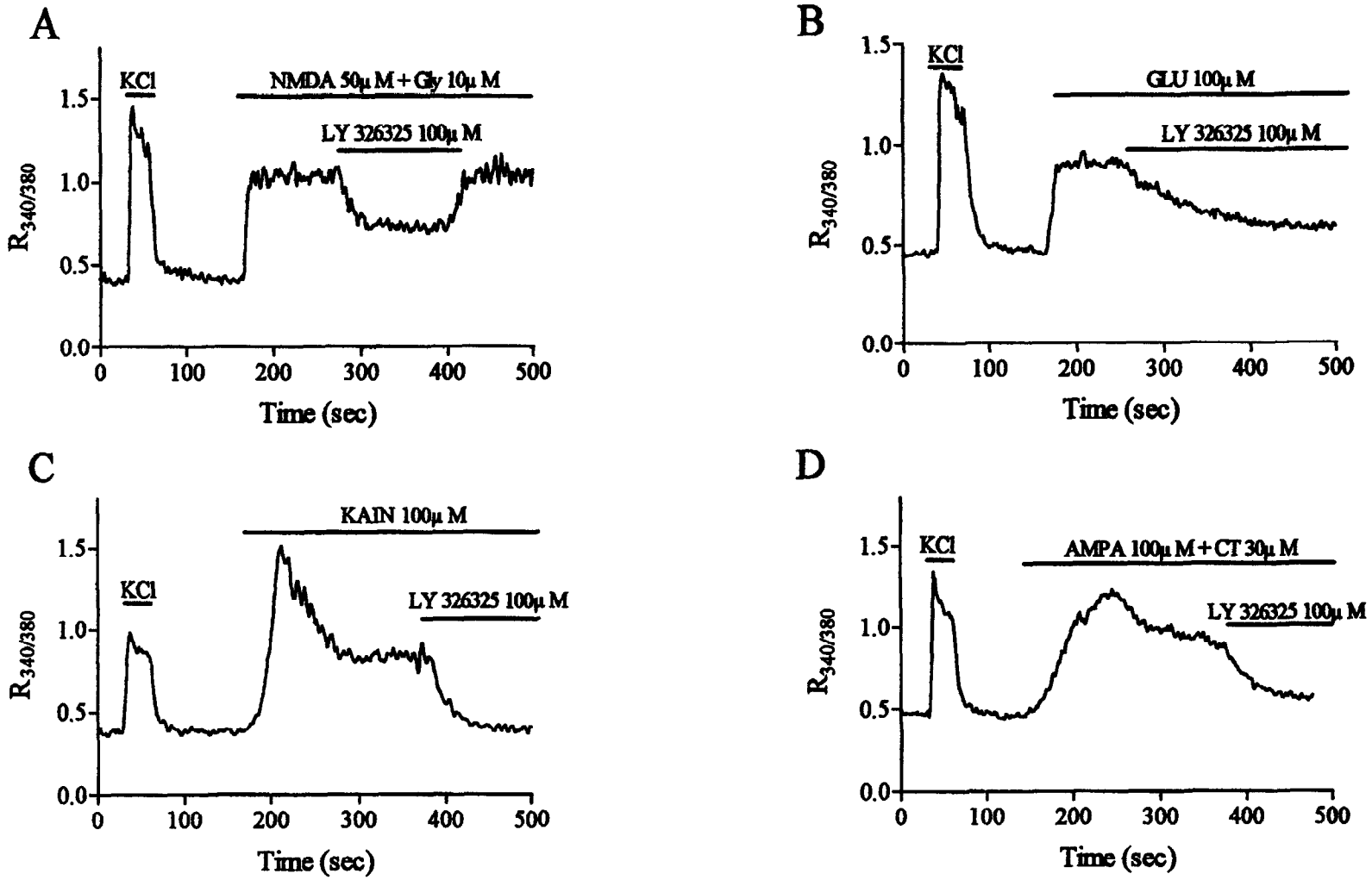

Fig. 1. Representative traces showing the effect of LY326325 on A: NMDA- (50 μM, in the presence of 10 μM glycine), B: glutamate- (100 μM), C: kainate- (100 μM), and D: AMPA-induced (100 μM, in the presence of 30 μM cyclothiazide, CT) elevations of $[Ca^{2+}]_i$ in single, intact cerebellar granule cells. $[Ca^{2+}]_i$ was determined as an increase in the fluorescence ratio $R_{340/380}$ in cells preloaded with the fluorescence agent fura-2 as previously described [34]. Prior to the administration of the test drugs, the neurons were stimulated once with 30 mM KCl to test the viability and responsiveness of the cell. Horizontal bars indicate the introduction to drugs of interest dissolved in Locke's buffer at concentrations indicated. Shown are typical recordings for results obtained from at least 6–8 cells from at least 3 different cell preparations.

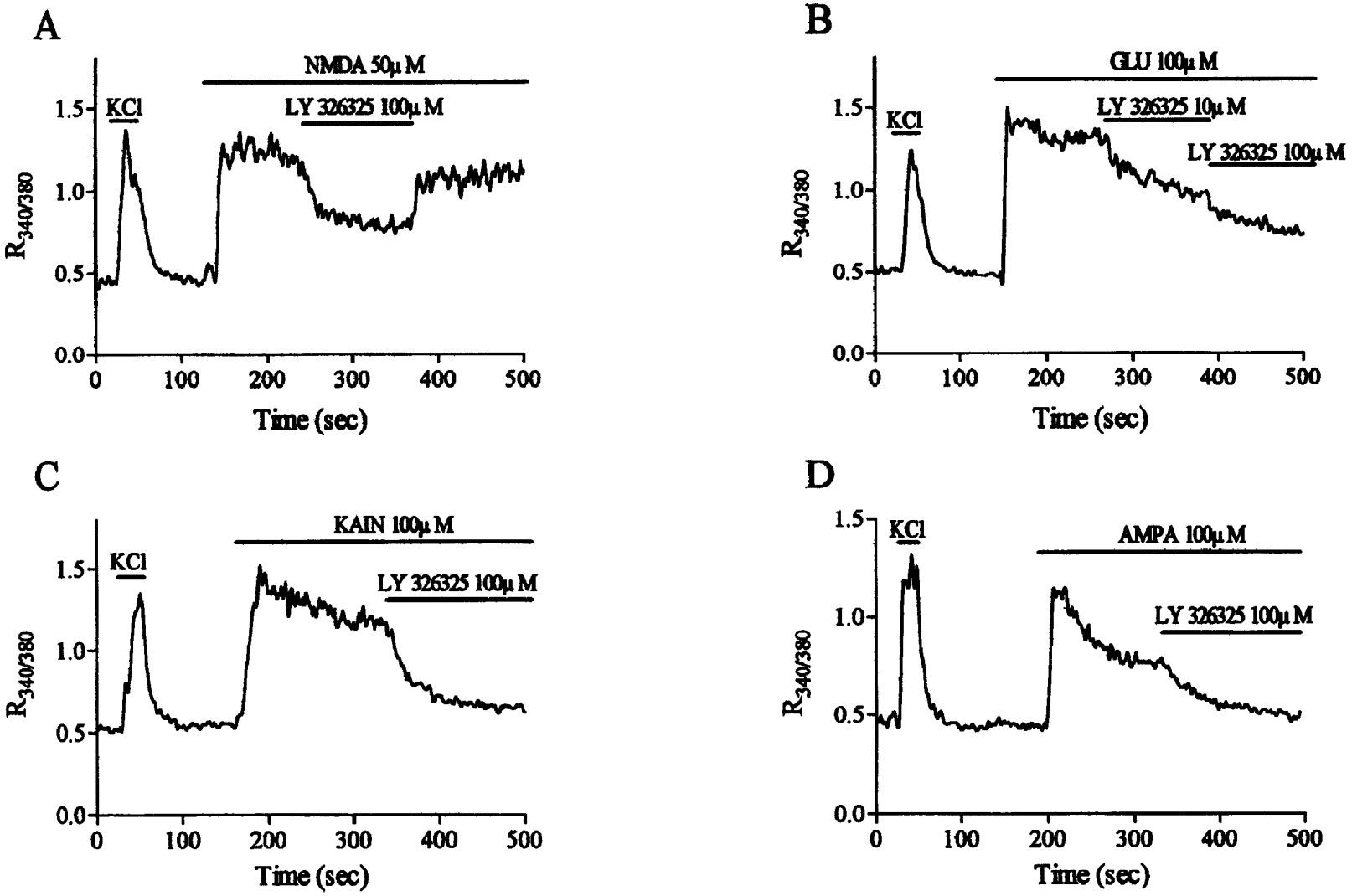

Fig. 2. Representative traces showing the effect of LY326325 on, A: NMDA- (50 μM), B: glutamate- (100 μM), C: kainate- (100 μM), and D: AMPA-induced (100 μM) elevations of $[Ca^{2+}]_i$ in single, intact cortical neurons. For further details, see legend to Fig. 1.

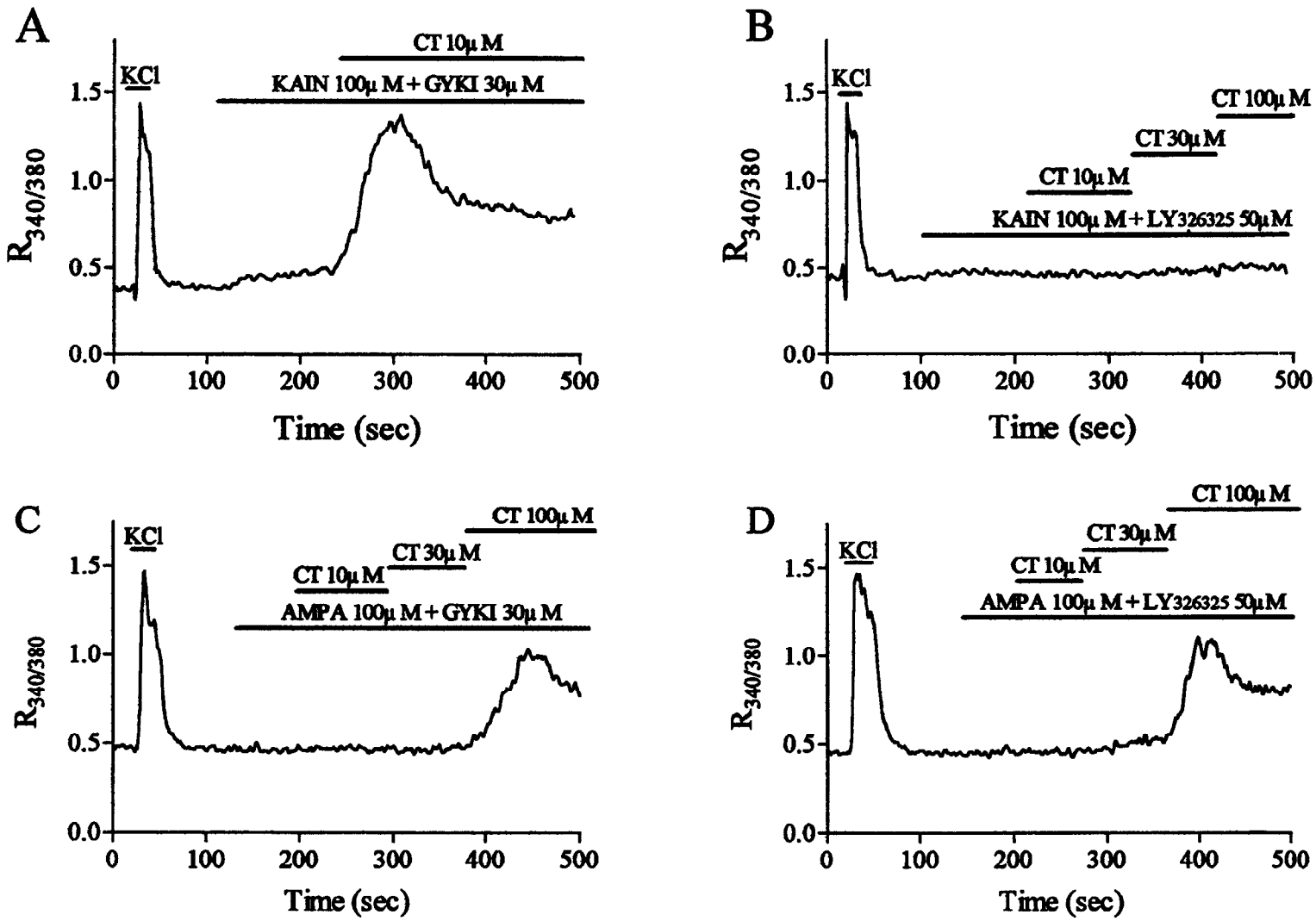

Fig. 3. (A, B) Cyclothiazide reverses the GYKI-produced (30 μM; A) blockade of kainate-stimulated (100 μM) elevation of $[Ca^{2+}]_i$ in single cerebellar granule cells, but fails to affect the LY326325-produced (50 μM; B) blockade. (C, D) In the presence of either GYKI (30 μM; C) or LY326325 (50 μM; D), cyclothiazide is able to reveal the stimulating effect of AMPA (100 μM) on $[Ca^{2+}]_i$ only when the concentration of cyclothiazide is raised to 100 μM. For further details, see legend to Fig. 1.

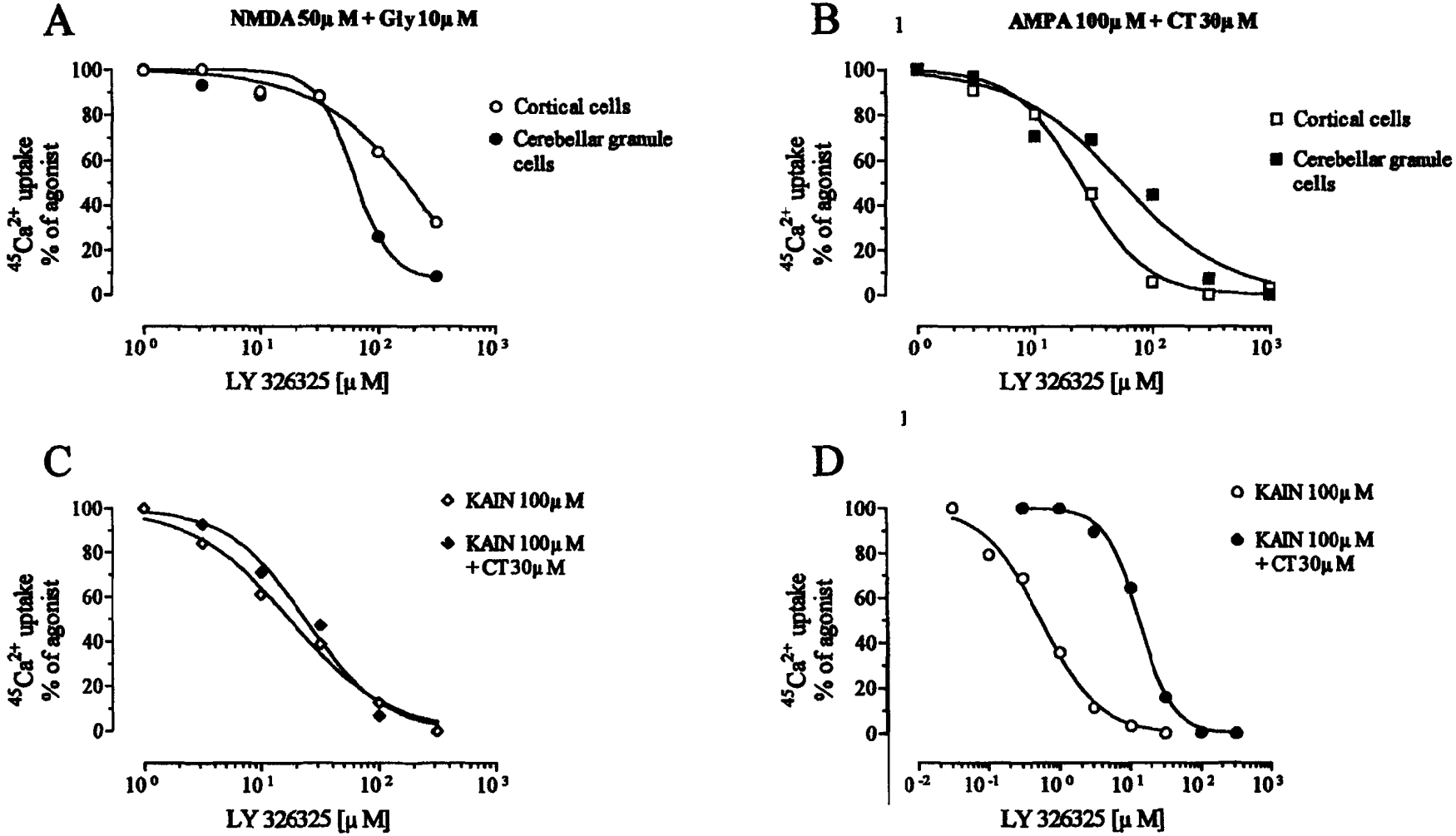

Fig. 4. Effect of increasing concentrations of LY 326325 on $^{45}Ca^{2+}$ uptake induced by A: NMDA (50 μM; in the presence of glycine 10 μM), and B: AMPA (100 μM; in the presence of cyclothiazide 30 μM) in monolayer primary cultures of intact rat cortical (open symbols) and cerebellar granule (closed symbols) neurons. (C, D) Effect of increasing concentrations of LY 326325 on $^{45}Ca^{2+}$ uptake induced by kainate (100 μM) in the absence (open symbols) or presence (closed symbols) of cyclothiazide (CT; 30 μM), in monolayer primary cultures of intact rat cortical (C; diamonds) and cerebellar granule (D; circles) neurons. $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. Data are normalized with respect to the maximal agonist-induced response (100%); each point is the mean of 6–12 determinations from different cell culture preparations. Standard errors did not exceed 10% of the means and, for illustrative purposes, were omitted from the figures. Solid lines in the graph represent the best theoretical fit to data as determined by nonlinear least-squares regression analysis. The IC_{50} values for LY326325 are given in the text.

LY326325 on glutamate-, NMDA-, AMPA-, and KA-induced neurotoxicity in monolayer cell cultures of intact cortical and cerebellar granule neurons. Application of glutamate (100 μM), NMDA (50 μM, in the presence of glycine; 10 μM), AMPA (100 μM, in the presence of cyclothiazide; 30 μM), and KA (100 μM) produced a marked enhancement of LDH activity, indicative of neurotoxicity caused by all glutamate receptor agonists (Fig. 6). As indicated in Fig. 6 (panel A), the neurotoxic actions of NMDA and glutamate in cerebellar granule cells were effectively blocked by LY326325. However, NMDA-induced toxicity was blocked equally well at all concentrations of NMDA tested (up to 100 μM), whereas inhibition of glutamate-induced cell damage by LY326325 tended to decrease with increasing concentrations of glutamate. On the other hand, in cortical cell cultures (Fig. 6, panel C) neither glutamate- nor NMDA-induced neurotoxicity was antagonized by LY326325 (100 μM), probably indicating a higher susceptibility of cortical cells to toxic effects of excitatory amino acids as compared to cerebellar granule cells. To elucidate whether or not LY326325 displayed a selective action on various nonNMDA glutamate receptor subtypes, the neuroprotective effects of LY326325 were compared to the corresponding actions of the more established AMPA/KA receptor antagonist NBQX (30 μM) in both cerebellar granule (Fig. 6, panel B) and cortical (Fig. 6, panel D) neurons. As seen, although NBQX appeared to possess a slightly higher potency than LY326325, both compounds were able to completely antagonize KA- and AMPA-induced neurotoxicity in both cell preparations. However, it should be noted that the AMPA/KA receptor antagonists had only weak (granule cells) or no (cortical cells) neuroprotective actions against glutamate-induced neurotoxicity, an event that we have recently shown to be fully inhibited by the specific NMDA receptor antagonist CGS19755 (100 μM; data not shown).

DISCUSSION

Based upon the recent observations that LY215490 blocked the depolarizing effects of AMPA (40 μM), KA (10 μM), and NMDA (40 μM) with IC_{50}-values 6, 32, and 61 μM, respectively, in rat cortical wedge preparations, it was suggested that LY215490 acts as a selective competitive AMPA receptor-preferring blocking agent with reduced (5-fold lower) affinity for KA-preferring receptors and low (10-fold lower) affinity for NMDA receptors in the brain [12]. These findings were further supported by results obtained in functional studies showing that LY215490 not only acted as an anticonvulsant against electrically induced seizures, but blocked muscular rigidity produced by the AMPA agonist ATPA [22] and displayed neuroprotective actions against neuronal degeneration induced by intrastriatal application of AMPA. No protective effects against NMDA-induced cell loss were observed in the same brain area [28]. In line with these findings, it has even more recently been reported that LY293558, a derivative of LY215490, reduced brain damage following focal brain ischemia in cats [29].

Our current data show that the LY215490 derivative, LY326325, is a potent blocker of AMPA- and KA-induced Ca^{2+} responses as well as of AMPA- and KA-produced neurotoxic actions in primary cultures of rat cortical and cerebellar granule neurons, with less effect on NMDA-produced effects. These data are in essential agreement with the contention that LY326325 acts as a potent blocker of AMPA and KA receptors in the brain [22, 28]. Although previously reported data indicated only neglible effects on NMDA receptors, our current observations suggest that LY326325, at least in certain situations, may act as a potent inhibitor of NMDA-induced responses. This pattern of pharmacological actions was witnessed in 3 different assays for assessing the functional consequences of glutamate receptor activation. First, in single neuron recordings of $[Ca^{2+}]_i$, LY326325 produced full inhibition of AMPA- and KA-induced elevations of $[Ca^{2+}]_i$ elevations. Corresponding Ca^{2+} responses induced by NMDA were also effectively inhibited by LY326325 in both cortical and cerebellar granule neurons. Second, LY326325 was a potent blocker of AMPA- and KA- and NMDA-produced enhancement of $^{45}Ca^{2+}$ uptake, although in this assay the blocking effects of LY326325 appeared to be weaker at NMDA receptors with an IC_{50}-value above 50 μM. Third, a similar pattern of effects was also seen in our assays concerning the neurotoxic effects of AMPA, KA, and NMDA, respectively. Taken together, our present findings clearly suggest that the pharmacological profile of LY326325 is different from the well-established AMPA/KA receptor antagonists NBQX and GYKI52466 which, in our currently used assays of Ca^{2+} fluxes and neurotoxicity tests, are devoid of any effects on either NMDA-induced Ca^{2+} fluxes or NMDA-produced neurotoxicity [35].

It is now widely accepted that agonist-induced activation of nonNMDA receptors causes a concomitantly occurring fast desensitization of AMPA as well as KA receptors, an event that results in a rapidly progressing decrease in the nonAMPA receptor-stimulated functional response (see e.g. [40]). The functional significance of an experimentally induced disturbance in the desensitization of nonNMDA receptors can be successfully examined following the application of cyclothiazide, a carbonic anhydrase inhibitor shown by several authors to be a potent blocker of AMPA and KA receptor desensitization [15, 41]. Initially these effects of cyclothiazide were demonstrated in various electrophysiological preparations [15, 41, 42]. More recently, it has been found that a cyclothiazide-induced blockade of nonNMDA receptor desensitization results in a dramatic potentiation of other AMPA- and KA-activated functional responses including enhancement of EPSPs [14–16], release of various neurotransmitters [17, 18], and glutamate-induced neurotoxicity [44]. Furthermore, in a recent study from this laboratory we presented evidence indicating that cyclothiazide is able to unmask nonNMDA receptor-activated Ca^{2+} fluxes, and that these functional events can be successfully used for a detailed characterization of how various competitive and noncompetitive receptor antagonists interact with the recognition sites for AMPA and KA, respectively [35]. Thus, we noted that cyclothiazide was able to reverse the inhibitory actions of the noncompetitive AMPA receptor antagonist GYKI 52466 in a concentration-dependent fashion. In contrast, increasing concentrations of cyclothiazide were only able to partially restore a NBQX-produced blockade of AMPA-induced stimulation of $^{45}Ca^{2+}$ uptake with no effects on the NBQX-produced blockade of KA-induced increases of $^{45}Ca^{2+}$ uptake. In analogy with our earlier findings, we now show that the

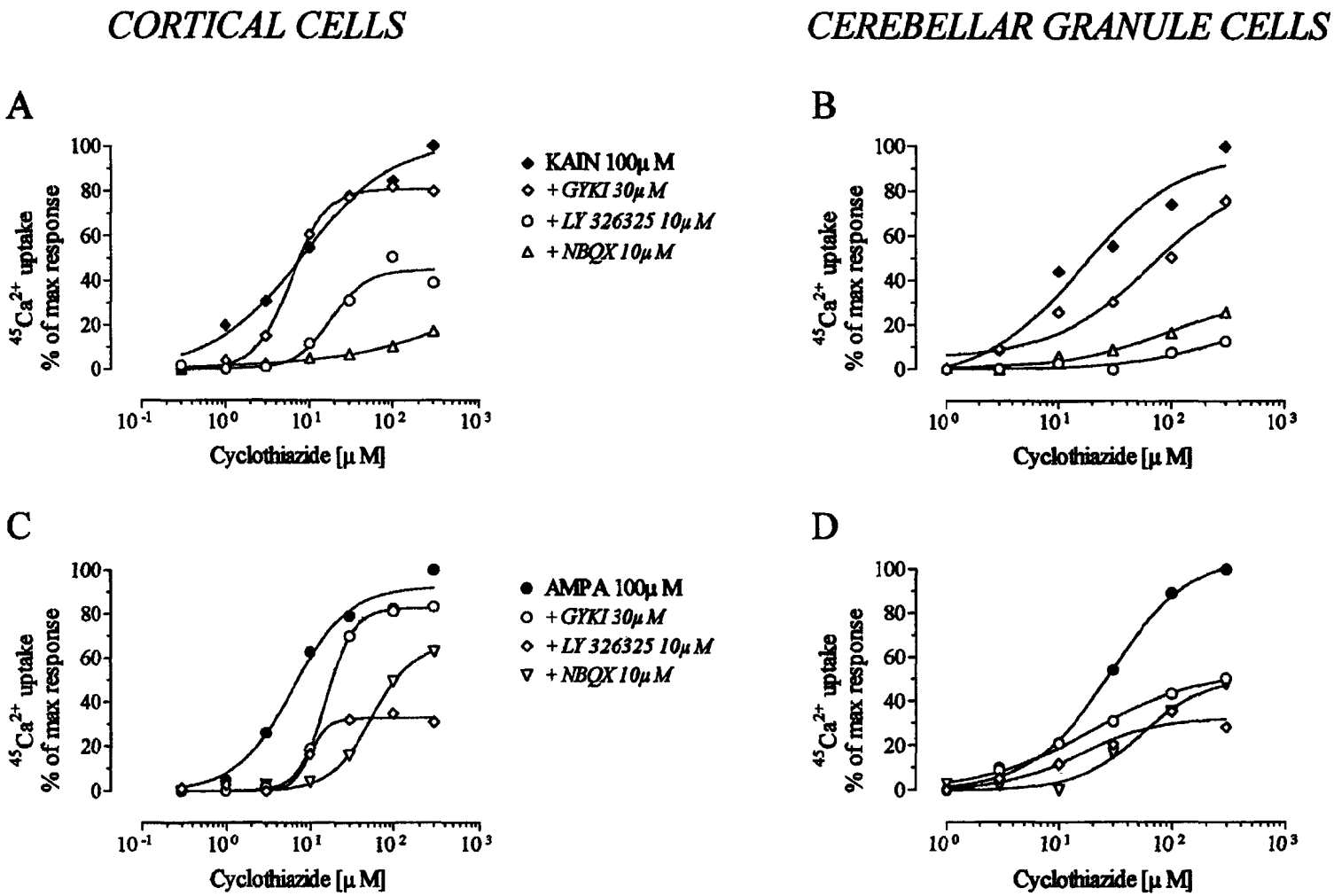

Fig. 5. Effects of increasing concentrations of cyclothiazide on NBQX- (10 μM), GYKI- (30 μM) and LY 326325-produced (10 μM) inhibition of kainate- and AMPA-stimulated $^{45}Ca^{2+}$ uptake in monolayer primary cultures of intact rat cortical (A, C) and cerebellar granule (B, D) neurons. Data are normalized with respect to the maximal cyclothiazide-induced potentiation of agonist response in the absence of antagonists (100%); each point is the mean of 6–12 determinations from different cell culture preparations. For further details, see legend to Fig. 4.

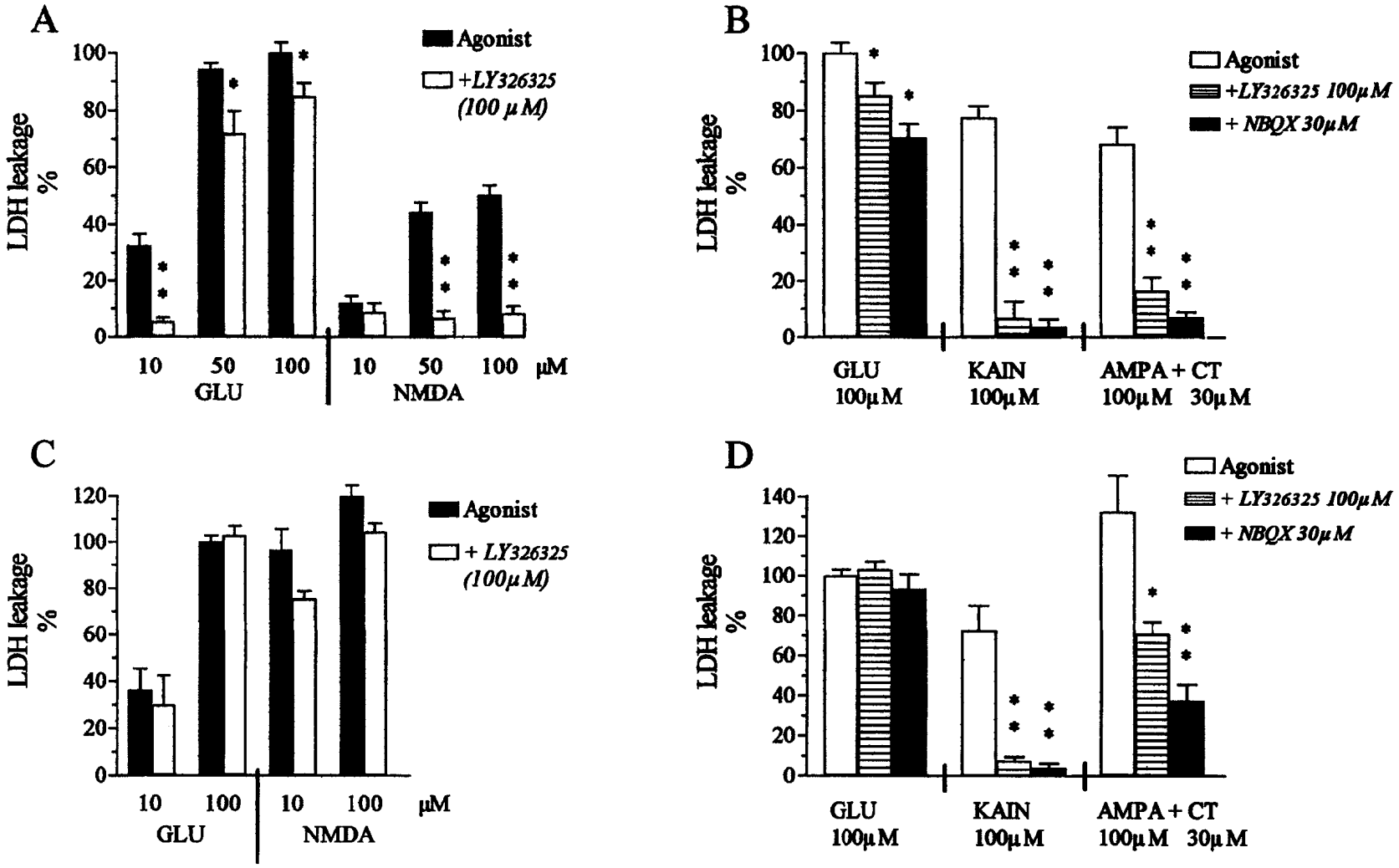

Fig. 6. (A, C) Concentration-dependent neurotoxic effect of glutamate and NMDA in the absence (closed bars) or presence (open bars) of LY 326325 (100 μM), in monolayer primary cultures of intact cerebellar granule (A) and cortical (C) neurons. (B, D) Effect of AMPA/kainate receptor antagonists LY 326325 (100 μM) and NBQX (30 μM) on glutamate- (100 μM), kainate- (100 μM), and AMPA-induced (100 μM, in the presence of 30 μM cyclothiazide) neurotoxicity in monolayer primary cultures of intact cerebellar granule (B) and cortical (D) neurons. Data from each separate cell preparation were scaled to the mean LDH values (U/mL) obtained in control cultures (=0%) and in sister cultures exposed to 100 μM glutamate (=100%). Shown are means ± SEM from 6–9 sister cultures from at least 3 different cell culture preparations. See also Materials and Methods for details. *$P < 0.05$; **$P < 0.01$; as determined by Bonferroni multiple comparisons test.

effects of cyclothiazide on the blocking properties of LY326325 on AMPA and KA receptors appear to be quite similar to those previously observed following application of NBQX. Thus, cyclothiazide fully reversed the effects of the noncompetitive AMPA/KA receptor antagonist GYKI 52466 on AMPA- and KA-induced enhancement of Ca^{2+} uptake, whereas only a partial (cortical cells) or none (granule cells) reversal was obtained in the presence of LY326325. These data are in good agreement with our current results from microfluorimetric measurements of $[Ca^{2+}]_i$ showing that cyclothiazide was unable to counteract the blocking properties of LY326325 on KA-produced increases of $[Ca^{2+}]_i$, whereas at least a partial reversal was noted following the application of LY326325 in the presence of AMPA. Furthermore, these findings are in line with earlier reports indicating that NBQX, as well as LY326325, alters the kinetics of AMPA/KA receptors in a different manner than GYKI52466 [9, 10]; they also strongly suggest that LY326325, like NBQX, acts as a competitive inhibitor rather than as a noncompetitive receptor antagonist at brain AMPA/KA receptors. Given these results, it is of interest to note that although primary cultures of cortical neurons are often considered as a typical GABAergic preparation [33, 44], our current data clearly suggest that the demonstrated interactions between AMPA and KA receptor agonists and antagonists in our preparation of cortical neurons, assessed by biochemical determinations, appear to be quite similar to those electrophysiological data previously obtained in the cortical wedge preparation largely held as the most representative for interactions at native glutamate receptors [45]. Furthermore, it should be pointed out that the interactions between both NMDA and nonNMDA receptor ligands and recognition sites clearly differ in cortical and cerebellar granule neurons, respectively, and that cortical neurons appear to be more useful for the examination of more complex interactions between various glutamate receptor subtypes. Further investigations are needed to elucidate to which extent these observations indicate that glutamate receptors are composed of different glutamate subunit combinations that are considered to be of critical importance for the Ca^{2+} permeability of various glutamate receptor channels, as has been shown by several investigators [46–48]. Furthermore, although cerebellar granule neurons often are considered as a powerful model for the study of glutamatergic receptor interactions, it should be pointed out that these neurons also contain GABAergic interneurons that may have contributed to the complex interplay between receptor agonists and antagonists observed in our present investigations [49].

With the increasing awareness that glutamate-activated biochemical and physiological processes are mediated not only through NMDA, but also *via* an activation of nonNMDA receptors, there has been constant need for more selective nonNMDA receptor antagonists to make it possible to clarify the relative role of various glutamate receptor subtypes. For instance, it is now generally accepted that many NMDA receptor antagonists possess anticonvulsant and neuroprotective properties, but that the clinical usefulness of these compounds may be restricted by their strong psychomimetic properties although species differences may exist [50]. Results from the first clinical trials aiming to evaluate the neuroprotective properties of NMDA receptor antagonists following stroke are forthcoming [51] and may further elucidate this important issue with regard to the use of NMDA receptor antagonists in the human situation. In contrast to many NMDA receptor antagonists, there is currently no evidence that nonNMDA receptor antagonists, such as NBQX, GYKI52466, and the currently examined LY326325, induce behavioral activation in experimental animals [31, 52]. Furthermore, preliminary evidence from behavioral studies suggests that these compounds may have antianxiety, anticonvulsant, and neuroprotective properties in mice and rats [20, 29], although at least some of these findings could not be confirmed in this laboratory [31]. NonNMDA receptor antagonists, including LY326325, also differ from NMDA receptor antagonists, in that they, in contrast to NMDA receptor antagonists, do not block the development of ethanol tolerance in mice [53]. However, with the increasing availability of systemically active nonNMDA receptor antagonists, further functional studies are now needed to elucidate the pharmacological profile(s) of these compounds in more detail.

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