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Role of Kainate Receptor Activation and Desensitization on the $[Ca^{2+}]_i$ Changes in Cultured Rat Hippocampal Neurons

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We investigated the role of kainate (KA) receptor activation and desensitization in inducing the increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cultured rat hippocampal neurons. The rat hippocampal neurons in the cultures were shown to express kainate receptor subunits, KA2 and GluR6/7, either by immunocytochemistry or by immunoblot analysis. The effect of LY303070, an α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptor antagonist, on the alterations in the $[Ca^{2+}]_i$ caused by kainate showed cell-to-cell variability. The $[Ca^{2+}]_i$ increase caused by kainate was mostly mediated by the activation of AMPA receptors because LY303070 inhibited the response to kainate in a high percentage of neurons. The response to kainate was potentiated by concanavalin A (Con A), which inhibits kainate receptor desensitization, in 82.1% of the neurons, and this potentiation was not reversed by LY303070 in about 38% of the neurons. Also, upon stimulation of the cells with 4-methylglutamate (MGA), a selective kainate receptor agonist, in the presence of Con A, it was possible to observe $[Ca^{2+}]_i$ changes induced by kainate receptor activation, because LY303070 did not inhibit the response in all neurons analyzed. In toxicity studies, cultured rat hippocampal neurons were exposed to the drugs for 30 min, and the cell viability was evaluated at 24 hr using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The selective activation of kainate receptors with MGA, in the presence of Con A, induced a toxic effect, which was not prevented by LY303070, revealing a contribution of a small subpopulation of neurons expressing kainate receptors that independently mediate cytotoxicity. Taken together, these results indicate that cultured hippocampal neurons express not only AMPA receptors, but also kainate receptors, which can modulate the $[Ca^{2+}]_i$ and toxicity. J. Neurosci. Res. 65:378–386, 2001.

Key words: concanavalin A; MGA (4-methylglutamate); LY303070; $[Ca^{2+}]_i$ changes; KA2; GluR6/7

Non-NMDA receptors have been extensively studied in the last years, but only more recently it was possible to establish that kainate receptors exist as independent receptors in neurons (Lerma, 1997). The cloning of glutamate receptors (Hollmann and Heinemann, 1994) and the discovery of new pharmacologic tools (Bleakman, 1999; Chittajallu et al., 1999) has allowed to better understand their physiological roles.

Several observations suggest that kainate receptors play an important role in epilepsy and excitotoxicity (Pollard et al., 1994; Mulle et al., 1998; Routbort et al., 1999; Moldrich et al., 2000). Although some studies show that kainate-induced toxicity is mainly due to the activation of AMPA receptors (Ohno et al., 1997; Ferreira et al., 1998; Ambrósio et al., 2000), other reports also demonstrate the presence of functional kainate receptors (Parternain et al., 1995) and their involvement in excitotoxicity (Sánchez-Gómez and Matute, 1999). Selective agonists and antagonists to AMPA and kainate receptors have been used to unmask the role of kainate receptors (Malva et al., 1995; Savidge et al., 1997; Bleakman et al., 1998). Thus, GYKI 53655, a selective non-competitive AMPA receptor antagonist, protects cultured neurons against AMPA toxicity and minimally affects kainate-induced responses at kainate receptors (Paternain et al., 1995; Kovács et al., 1997). Also, experiments carried out using homomeric human GluR5 receptors have demonstrated that LY293558 and LY294486 antagonize the actions of kainate in the CA3 area of the rat hippocampus (Vignes et al., 1997). The use of a selective kainate receptor antagonist, LY382884, has allowed to determine the involvement of these receptors in synaptic plasticity in the mossy fiber projection of the hippocampus (Bortolotto et al., 1999), although this conclusion has been challenged recently (Nicoll et al., 2000).

Many compounds that can interact with AMPA and kainate receptors to reduce desensitization have also been

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identified (Partin et al., 1993). Cyclothiazide (CTZ), which blocks AMPA receptor desensitization, enhanced both AMPA and kainate excitotoxicity in rat hippocampal neurons (Ohno et al., 1998; Ambrósio et al., 2000), and this effect was partially (24 hr exposure) or totally (30 min exposure) prevented by LY303070, the (−)enantiomer of GYKI 53655 (Ambrósio et al., 2000). Concerning the kainate receptor desensitization, concanavalin A (Con A), a plant lectin, is a potent pharmacologic tool in inhibiting the desensitization of kainate receptors (Partin et al., 1993). In Con A-treated cerebellar granule cells, currents evoked by low concentrations of kainate are not significantly reduced by GYKI 53655 (Pemberton et al., 1998). Also, the selective activation of kainate receptors, in the presence of Con A, can result in neuronal toxicity in cultured murine neocortical cells (Jensen et al., 1999).

The link between the $[Ca^{2+}]_i$ increase and cell death has been demonstrated in studies that show that the activation of non-NMDA receptors can increase the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Brorson et al., 1994), which can in turn lead to a cascade of intracellular events, and finally to cell death by necrosis or apoptosis (Choi, 1985; McGinnis et al., 1999). Also, it has been demonstrated that kainate- and AMPA-induced toxicity in cortical neurons is greatly attenuated by omission of external Ca^{2+}, and the increase in $[Ca^{2+}]_i$ is due to stimulation of Ca^{2+} influx rather than release from intracellular stores (Frandsen et al., 1993).

We have determined previously the existence of different populations of hippocampal neurons with different AMPA receptors, in terms of AMPA receptor-mediated $[Ca^{2+}]_i$ changes and subsequent toxicity (Ambrósio et al., 2000). Because AMPA and kainate receptors can coexist within a single cell as two independent entities (Partin et al., 1993), in this study, we examined the role of kainate receptors in the $[Ca^{2+}]_i$ increase, with particular attention to the desensitization of kainate-preferring receptors, and also in the putative neurotoxicity in cultured hippocampal neurons. A selective agonist of kainate receptors (MGA) (Jones et al., 1997; Donevau et al., 1998), and a blocker of receptor desensitization (Con A) were used to evaluate the contribution of kainate receptors to $[Ca^{2+}]_i$ and toxicity. The role of active kainate receptors in both $[Ca^{2+}]_i$ changes and excitotoxicity was also elucidated by the direct identification of KA2 and GluR6/7 subunits of kainate receptors by using immunocytochemistry and Western immunoblotting techniques. Preliminary results of this work were published previously in abstract form (Silva et al., 2000).

MATERIALS AND METHODS

Cell Culture

Hippocampal neurons were dissociated from hippocampi of E18–E19 Wistar rat embryos (the mothers were killed by rapid cervical dislocation), after treatment with trypsin (2.0 mg/ml, 15 min, 37°C) and deoxyribonuclease I (0.15 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4 2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4). The cells were cultured in serum-free Neurobasal medium, supplemented with B27 supplement, glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml), as described previously (Brewer et al., 1993). Cultures were kept at 37°C in a humidified incubator in 5% CO_2/95% air, for 7–8 days, the time required for maturation of hippocampal neurons.

For calcium imaging and immunocytochemistry studies, cells were plated on poly-D-lysine-coated (0.1 mg/ml) glass coverslips at a density of 45×10^3 cells/cm^2. For the assessment of neuronal injury with the MTT assay and for Western immunoblotting, cells were plated on poly-D-lysine-coated multiwells at a density of 0.1×10^6 cells/cm^2.

MAP-2 and Kainate Receptor Immunocytochemistry

Hippocampal neurons plated on coverslips at a density of 45×10^3 cells/cm^2 were washed twice with 0.15 M PBS, and fixed with 4% paraformaldehyde for 30 min at 37°C. Coverslip-mounted cells were then incubated with 0.2% Triton X-100 for 2 min at room temperature and non-specific binding subsequently blocked with 3% BSA for 30 min at room temperature. Cells were then incubated for 1 hr at room temperature with anti-mouse MAP-2 (1:500 dilution), and for double-immunolabeling studies hippocampal neurons were incubated with a mixture of both anti-mouse MAP-2 and anti-rabbit KA2 (1:100) or anti-mouse MAP-2 and anti-rabbit GluR6/7 (1:20). Furthermore, incubation with secondary antibodies (1:100; anti-mouse IgG for MAP-2; anti-rabbit IgG for KA2 and GluR6/7) was conducted for 1 hr at room temperature. Finally, cells were mounted using a Prolong Antifade Kit and after drying, the neurons were visualized in a Confocal Fluorescence Microscopic (Bio-Rad MRC 600, Cambridge, MA).

Western Immunoblotting Analysis

Hippocampal neurons plated on poly-D-lysine-coated multiwells at a density of 0.1×10^6 cells/cm^2 were washed in PBS medium (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), and then collected with a cell scraper in lysis medium (20 mM HEPES, 1 mM EDTA, pH 7.4) supplemented with a cocktail of protease inhibitors (100 μM phenylmethylsulfonyl fluoride, PMSF, 1 mM benzamidine, 50 μM leupeptin, 50 μM antipain and 1 μM pepstatin). After sonication (6×5 sec with 2 sec on-off pulses), the cell suspension was centrifuged in a refrigerated Eppendorf microfuge (3,500 rpm, for 2 min, followed by centrifugation of the supernatant at 14,000 rpm, for 12 min). The final pellet was then resuspended in PBS medium supplemented with the protease inhibitors cocktail.

Various aliquots of equal amount of membrane protein (10 μg) was loaded in a 4% running polyacrylamide gel and 7.5% spacer gel, subjected to electrophoresis, and then transferred to polyvinylidene (PVDF) membranes (Amersham, Arlington Heights, IL). Membranes were saturated with 5% (w/v) fat-free dry milk in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6) with 0.1% Tween 20 (TBS-T), for 1 hr 45 min at room temperature. Blots were then further incubated for 2 hr, at room temperature, with primary antibodies at 1:600 and 1:800 dilutions for KA2 and GluR6/7, respectively. After washing with 1% milk TBS-T solution, blots were further incubated overnight, at 4°C, with anti-rabbit IgG antibody coupled to alkaline phosphatase. The immune complex was detected by the ECF

System (Amersham) and visualized by scanning on a STORM 860 (Molecular Dynamics, Sunnyvale, CA), using blue excited fluorescence.

Fura-2 Fluorescence Measurements by Video Imaging

Hippocampal neurons plated on coverslips at a density of 45×10^3 cells/cm^2 were loaded by incubation with 5 μM fura-2/AM and 0.02% Pluronic F-127 for 45 min, at 37°C, in Krebs buffer supplemented with 0.1% BSA (w/v), essentially as described previously (Ambrósio et al., 2000). After incubation, the coverslips were washed and placed in a perfusion chamber on the stage of an inverted Nikon Diaphot fluorescence microscope. The cells were then perfused with Krebs buffer (37°C) for about 5 min before data acquisition. The experiments were conducted under continuous superfusion with Krebs buffer containing the drugs, as indicated in the figure legends. The neurons were stimulated 15 sec after starting image acquisition, and when required, preincubated with 250 μg/ml Con A for 5 min before stimulation with the receptor agonists. Fluorescence changes were recorded with a multiple excitation Magical imaging system (Applied Imaging, UK). Hippocampal neurons were alternately excited at 340 and 380 nm using a switching filter wheel, and the emitted fluorescence, collected with a 40× objective (Nikon, The Netherlands), was driven to a Photonics Science SIT camera, after passing through a 510 nm bandpass filter. Image analysis was carried out with the Magical system and software developed by Dr. Enrique Castro (Faculty of Veterinary Sciences, Complutense University of Madrid, Madrid, Spain). The background fluorescence at each wavelength was subtracted and fluorescence ratios calculated on a pixel-by-pixel basis, being ratio data stored as an 8-bit pseudocolored images. Areas of the cell bodies were drawn and the averaged value of pixel intensities was evaluated at each time point, to obtain ratio vs. time plots for all areas defined. The results were expressed as the ratio of fluorescence intensity with excitation at 340 nm and 380 nm.

Assessment of Neuronal Injury

Assessment of neuronal injury was carried out by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Krebs buffer with MTT (0.5 mg/ml) was added to the cell cultures and incubated for 1 hr at 37°C in the incubation chamber. MTT, when taken up by cells that remain alive, is converted from yellow to a water-insoluble blue-colored product (Mossman, 1983). The precipitated dye was dissolved in 0.04 M HCl in isopropanol, for 5 min, and colorimetrically (absorbance at 570 nm) quantitated. All experiments were carried out in triplicate.

Toxicity Experiments

Neurons cultured for 7 DIV were exposed to drugs for 30 min, and when using Con A, neurons were preincubated with 250 μg/ml (in HEPES-buffered saline solution) for 5 min at 37°C. Drug exposure for 30 min in conditioned medium was carried out after removal of the Con A solution (Jensen et al., 1999). Aliquots of 250 μl (conditioned medium) were taken from each well, the drugs were added, and homogenized. Then, the aliquots were again added to the correspondent well, and the medium gently mixed to preserve the integrity of neurons. After incubation with the drugs, cells were carefully washed two times with Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES-Na, pH 7.4), and then further incubated for 24 hr in Neurobasal medium supplemented with B27 supplement (GIBCO BRL, Gaithersburg, MD), glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml). In these experiments, Con A had effects on the control values (MTT reduction was 68.6 ± 2.1% of the control). Thus, the control conditions in all experiments with Con A treatment were a 5 min preincubation with this lectin, followed by the 30 min incubation with conditioned medium.

Chemicals

Neurobasal medium, B27 supplement, gentamicin and trypsin (USP grade) were purchased from GIBCO BRL (Life Technologies, Scotland, UK). Glutamate, DNase (DN-25), MTT, concanavalin A (Con A), protease inhibitors and anti-mouse MAP-2 were purchased from Sigma Chemical Company (St. Louis, MO), Kainate (KA), cyclothiazide (CTZ) and 4-methylglutamate (MGA) were purchased from TOCRIS (Ballwin, MO). LY 303070 was a kind gift of Lilly Research Laboratories (Indianapolis, IN). The acetoxymethyl ester of Fura-2 (Fura-2/AM) and Pluronic F-127 were purchased from Molecular Probes (Leiden, The Netherlands). KA2 and GluR6/7 antibodies were from Upstate Biotechnology Inc., (Lake Placid, NY). Goat anti-rabbit and anti-mouse IgG antibodies, anti-alkaline phosphatase linked antibodies and ECF substrate were purchased from Amersham (Buckinghamshire, England). All the other reagents were from Sigma, or from Merck-Schuchardt, Germany. LY 303070, CTZ, Fura-2/AM and Pluronic F-127 stock solutions were prepared in DMSO.

Statistical Analysis

The data are expressed as means ± SEM. Statistics were carried out using an analysis of variance (ANOVA), followed by Dunnett's post-test, as indicated in the figure legends. In the $[Ca^{2+}]_i$ measurements, due to the variability of the responses among cells, the 95% Confidence Intervals (95% CI) are indicated.

RESULTS

Expression of Kainate Receptor Subunits

Cell immunoreactivity with the neuronal marker Microtubule Associated Protein 2 (MAP-2) (1:500) indicated that ~95% of cells were neurons (Fig. 1A). The labeling seen in the presence of anti-MAP-2 antibody was localized all over the cell, but especially in the neurites (Fig. 1A). These observations suggest that a nearly pure neuronal population is obtained as glial growth is reduced in the cultured hippocampal neurons utilized in the present study (Brewer et al., 1993).

We could also identify the presence of kainate receptor subunits in the cultures by using either immunocytochemistry or immunoblotting. KA2- (1:100) (Fig. 1B) or GluR6/7-like (1:20) immunoreactivity (Fig. 1C) was detected especially within the cell bodies, but also with strong punctuate labeling in the neurites. Moreover, double-labeling immunocytochemical studies suggest a predominant localization of KA2 or GluR6/7 subunits at synaptic sites in the cultured neurons. Furthermore, West-

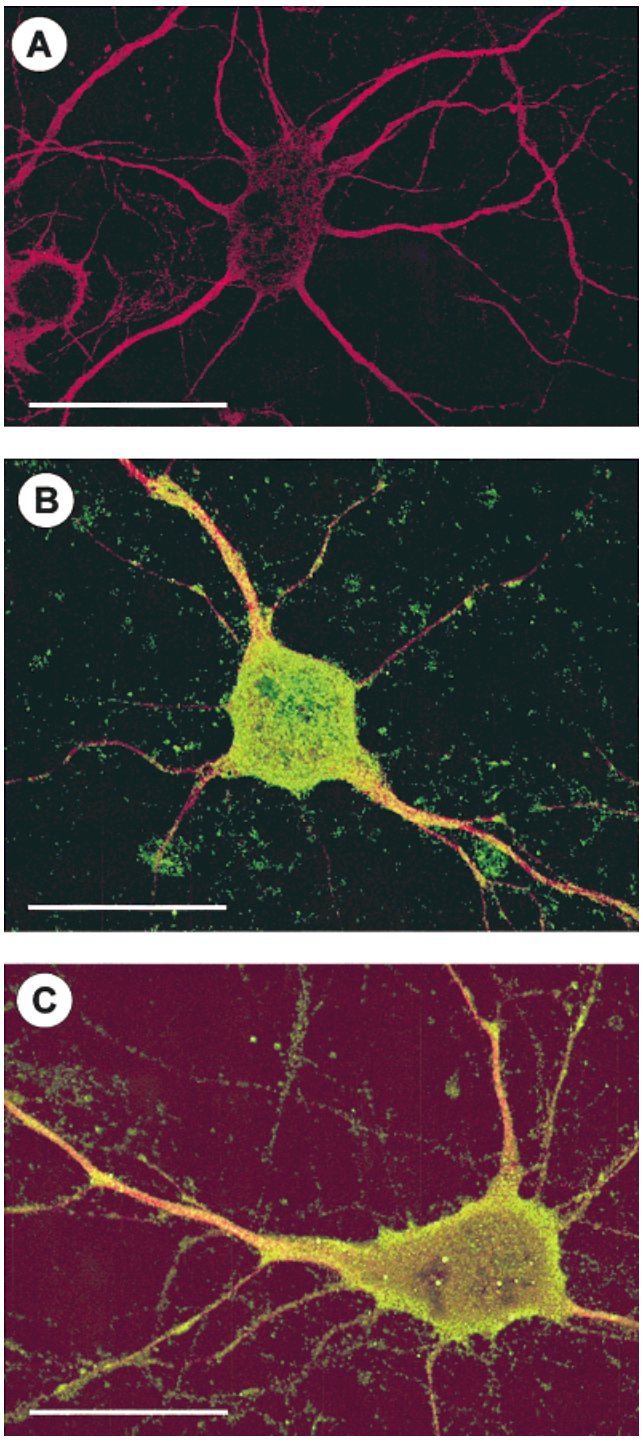

Fig. 1. Confocal image of cultured hippocampal cells labeled with (**A**) anti-microtubule associated protein 2 antibody (MAP-2) (1:500 dilution) (red) or a mixture of both (**B**) anti-MAP-2 and anti-KA2 (1:100) (green) or (**C**) anti-MAP-2 and anti-GluR6/7 (1:20) (green). The white bar below the figure represent 25 μm. Note the strong positive labeling of the MAP-2 antibody along the neurites and the KA2- or GluR6/7-like punctuate immunoreactivity. The figures shown are representative of the whole culture. The same pattern of labeling was obtained for two independent cultures.

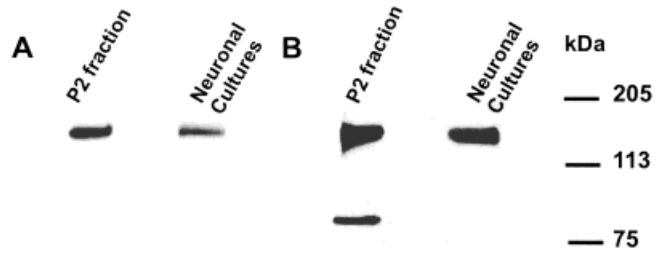

Fig. 2. Western blot analysis of kainate receptor subunits (**A**) KA2 and (**B**) GluR6/7 in membranes of P2 fraction obtained from 6-week-old Wistar rat hippocampus and in membranes isolated from cultured hippocampal neurons. The immune complex was detected by the ECF system and visualized by scanning on a STORM 860, using blue excited fluorescence. Standard proteins were used as a molecular weight markers (Broad Range; Bio-Rad Laboratories, Richmond), and the molecular weight standards are indicated (kDa). Similar results were obtained from two independent preparations.

ern immunoblotting analysis of KA2 and GluR6/7 subunits in membranes isolated from cultured hippocampal neurons identified a ~123 kDa and ~115 kDa band for KA2 and GluR6/7, respectively (Fig. 2A,B). For comparison, P2 membranes obtained from 6-week-old Wistar rat hippocampus were analyzed by Western immunoblotting, using the same antibodies, and we also identified the two bands. In addition, in the case of anti GluR6/7 antibodies, a band of ~99 kDa was also identified.

Kainate-Induced $[Ca^{2+}]_i$ Increase: Single Cell Imaging of Hippocampal Neurons

A prolonged increase in intracellular Ca^{2+} is regarded as one major trigger of the neurodegenerative processes. To better understand the role of kainate receptors in cell death, we analyzed $[Ca^{2+}]_i$ changes evoked by different kainate receptor agonists, in individual cells in the cultured neurons, and the results are illustrated in Figures 3–5. We observed that, when neurons were stimulated with kainate at low concentrations (10 μM), 33% of neurons did not show any response (49 neurons analyzed). In a higher percentage of cells (67%), however, we could observe an increase in the 340 nm/380 nm fluorescence ratio. In the cells that respond to 10 μM kainate with an increase in $[Ca^{2+}]_i$, we determined the inhibitory effect of LY303070 on kainate-induced responses, and three subpopulations of neurons were detected. In the first, second, and third population of cells, we considered neurons where the kainate-evoked response in the presence of LY303070 was between 10% and 50%, 50% and 90% or 90% and 110% of the control, respectively. In the first population of cells (33% of neurons) (Fig. 3A) the response to kainate was strongly inhibited to 33.7 ± 2.2% of the control (95% CI between 29.0 and 38.3%); in the second population (26% of neurons) (Fig. 3B) the response to kainate was moderately inhibited to 64.8 ± 3.0% of the control (95% CI between 58.6 and 71.1%); and in a third small population of cells (8.2% of neurons) LY303070 did not inhibit the response to kainate (101.0 ± 3.0% of the

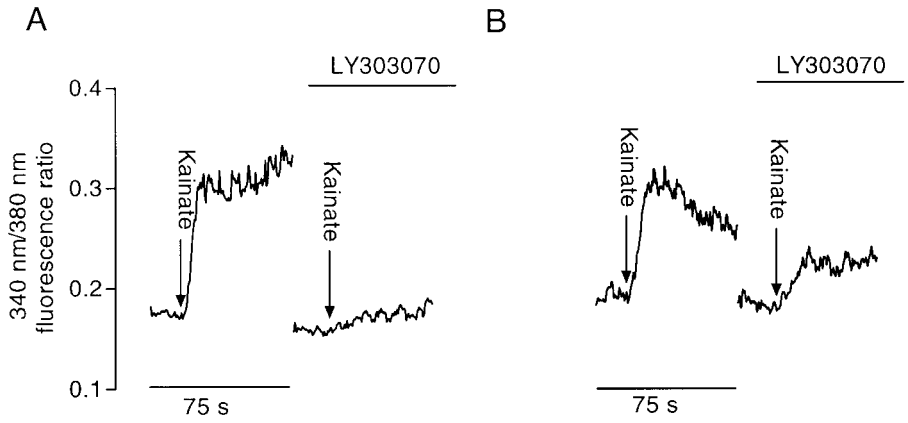

Fig. 3. Increase in the $[Ca^{2+}]_i$ upon stimulation of hippocampal neurons with 10 μM kainate. Representative recordings of two populations of neurons showing a (**A**) strong (33% of neurons) or (**B**) moderate inhibition (26% of neurons) of the $[Ca^{2+}]_i$ response in the presence of 15 μM LY303070. Neurons were stimulated 15 sec after data acquisition and each recording lasted 75 sec. The second recording in each experiment was carried out 15 min after the end of the first. The recordings are representative of data obtained from 49 cultured hippocampal neurons (two independent cultures).

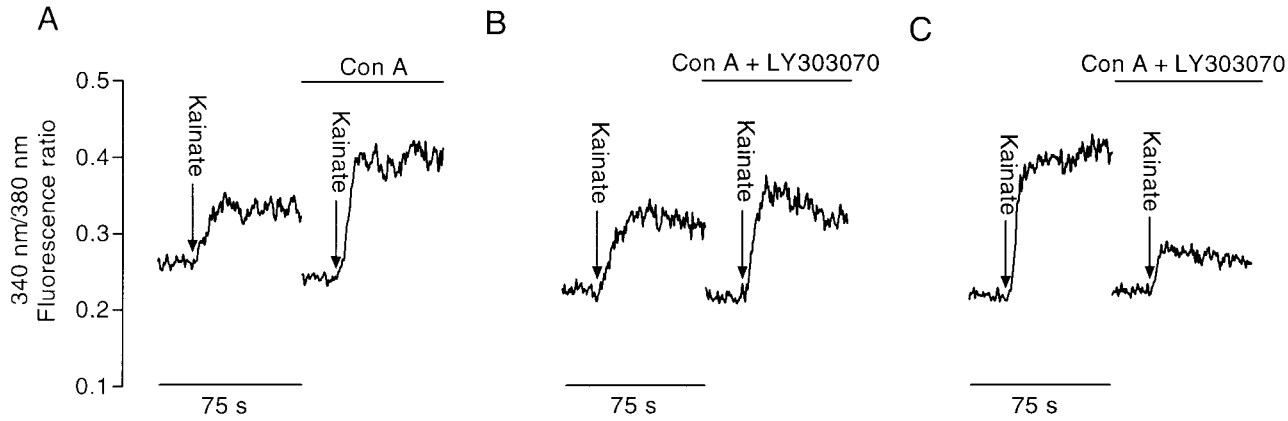

Fig. 4. Effect of non-desensitizing activation of kainate receptors on the $[Ca^{2+}]_i$ upon stimulation with 10 μM kainate. (**A**) Potentiation of $[Ca^{2+}]_i$ response by Con A (250 μg/ml). (**B**) and (**C**) Two populations of neurons showing (B) no effect of LY303070 (37.7% of neurons) or (C) a partial inhibition (37.7% of neurons) on the $[Ca^{2+}]_i$ response. Each recording (A–C) is representative of one of three subpopulations of neurons that responded differently in the presence of Con A. The results were obtained from 196 neurons (four independent cultures).

control) (95% CI between 93.4 and 108.7%) (not shown). The $[Ca^{2+}]_i$ increase in the 340 nm/380 nm fluorescence ratio in the cells of first (33% of neurons) (Fig. 3A) or second population of cells (26% of neurons) (Fig. 3B) was 0.06 ± 0.01 (95% CI between 0.05 and 0.07) or 0.07 ± 0.01 (95% CI between 0.06 and 0.09), respectively, in control conditions (first stimulus, without LY303070).

We then tested the effect of blocking desensitization of kainate receptors on the $[Ca^{2+}]_i$ changes evoked by kainate. We observed that the presence of Con A (250 μg/ml) significantly enhanced the $[Ca^{2+}]_i$ changes evoked by 10 μM kainate in 82.1% of neurons (196 neurons analyzed). The response was potentiated to 142.3 ± 2.1% of the control (95% CI between 138.1 and 146.5%) in 70.9% of neurons, as illustrated in Figure 4A, and to 245.2 ± 11.4% in a smaller percentage of neurons (11.2%) (95% CI between 221.5 and 268.9%) (not shown). In the first group of cells, we considered the response between 110% and 200% of the control, and above this value, we considered the second group. The increase in the fluorescence ratio in the cells from the first (70.9% of neurons) (Fig. 4A) or second group (11.2% of neurons) (not shown) was 0.17 ± 0.01 (95% CI between 0.15 and 0.20) or 0.07 ± 0.01 (95% CI between 0.05 and 0.08), respectively, in control conditions (first stimulus, without Con A). Contrarily to what happened when neurons were stimulated only with kainate, the presence of Con A induced a kainate receptor response in a higher percentage of neurons (82% versus 67% of neurons). Moreover, two distinct populations were found based on

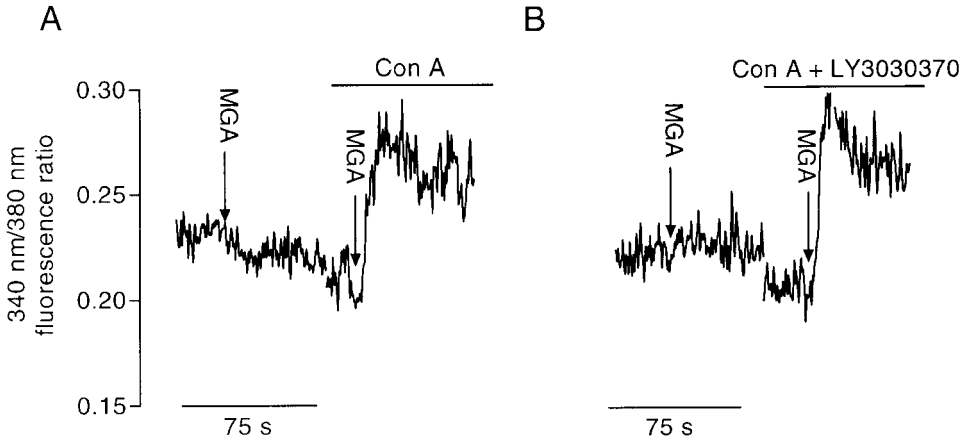

Fig. 5. Challenging neurons with the selective kainate receptor agonist MGA (10 μM) resulted in the $[Ca^{2+}]_i$ increase, only in the presence on Con A (250 μg/ml). (**A**) Effect of Con A (250 μg/ml) on the $[Ca^{2+}]_i$ responses induced by 10 μM MGA. (**B**) Effect of LY 303070 (15 μM) on the $[Ca^{2+}]_i$ responses induced by MGA in the presence of Con A. The traces in A and B are representative of two populations of neurons in terms of the $[Ca^{2+}]_i$ response in the presence of Con A or Con A plus LY303070, respectively. The results were obtained from 44 neurons (two independent cultures).

the inhibitory effect caused by LY303070. In the first population (37.7% of neurons) (Fig. 4B), the response induced by kainate in the presence of Con A was not inhibited by LY303070. In the second population (37.7% of neurons), LY303070 decreased the response to kainate plus Con A as follows: in 10.1% of neurons the response was greatly inhibited to 35.4 ± 3.9% of the control, as illustrated in Figure 4C (95% CI between 26.0 and 44.8%), whereas in 27.5% of total neurons, the response was moderately inhibited to 69.4 ± 2.8% of the control (95% CI between 63.6 and 75.1%) (not shown). The fluorescence ratio in the cells from the second group, in control conditions (first stimulus, without Con A and LY303070), was 0.31 ± 0.05 (95% CI between 0.20 and 0.42) in 10.1 % of total neurons, and 0.15 ± 0.02 (95% CI between 0.11 and 0.19) in 27.5% of total neurons.

Concanavalin A Unmasks Active Kainate Receptors Upon Stimulation With MGA

We also investigated the $[Ca^{2+}]_i$ alterations evoked by MGA, because it has been shown that this compound is a potent and selective agonist at kainate receptors, at least for the low concentrations (10 μM) utilized (Jones et al., 1997; Donevan et al., 1998). We analyzed 44 neurons in the cultures, and in 86.4% of the cells, the response induced by MGA (10 μM) was unmasked in the presence of Con A (Fig. 5A). So, in the presence of this lectin, MGA did mediate the $[Ca^{2+}]_i$ increase to 151.5 ± 4.8% of the control (95% CI between 141.7 and 161.2%) in 63.6% of total neurons (not shown), and to 271.3 ± 15.3% of the control, in 22.7% of neurons (95% CI between 236.8 and 305.8%) (Fig. 5A). Moreover, LY303070 did not inhibit the response to MGA plus Con A in all cells analyzed as illustrated in Fig. 5B. The increase in the fluorescence ratio in the first (63.6% of neurons) or second group (22.7% of neurons) was 0.06 ± 0.01 (95% CI between 0.05 and 0.10) or 0.04 ± 0.01 (95% CI between 0.03 and 0.04), respectively, in control conditions (first stimulus, without Con A). Thus, in these specific conditions of stimulation (MGA plus Con A), we could observe a clear role of kainate receptors in producing $[Ca^{2+}]_i$ changes in hippocampal neurons, independent of AMPA receptor contribution.

Neurotoxic Effect of MGA in the Presence of a Kainate Receptor Desensitization Blocker in Hippocampal Neurons

The selective kainate receptor agonist, 4-methylglutamate (MGA; 10 μM), was without effect in inducing cell death by itself (Fig. 6). When the cells were exposed to MGA in the presence of Con A for 30 min, however, we observed the unmasking of MGA toxicity, because the MTT reduction was 75.5 ± 3.2% of the control. Furthermore, this effect was not abolished by LY303070 (15 μM), a non-competitive AMPA receptor antagonist (73.2 ± 5.2% of control) (Fig. 6). In the presence of cyclothiazide (CTZ; 30 μM), a blocker of AMPA receptor desensitization, 10 μM MGA did not result in significant toxicity, because the MTT reduction was 99.7 ±1.9% of the control, suggesting that in this case AMPA receptors were not involved (Fig. 6). Thus, when using a selective agonist to kainate receptors, and in the presence of a receptor desensitization blocker, it was possible to unmask a consistent toxic effect insensitive to AMPA receptor antagonist.

DISCUSSION

The present work shows that cultured hippocampal neurons express functional kainate receptors that respond with different sensitivities to kainate and to MGA, in the presence of Con A. Moreover, the results also indicate that kainate receptor desensitization play an important role in kainate-induced increase in intracellular free Ca^{2+}.

The role of AMPA receptors desensitization on the neuronal viability has been extensively studied recently (Ohno et al., 1997; Ferreira at al., 1998; Ambrósio et al., 2000), but for several years the role of kainate receptors remained elusive. The specific role of kainate receptors as modulators of synaptic transmission has, however already been revealed (Paternain et al., 1995; Jones et al., 1997; Vignes and Collingridge, 1997; Wilding and Huetter, 1997; Malva et al., 1998). The pharmacologic differentiation between active AMPA and kainate receptors was possible due to the development of selective agonists to kainate receptors (Wong et al., 1994; Donevan et al., 1998; Swanson et al., 1998a) and selective AMPA receptor antagonists (Bleakman et al., 1996). Some kainate receptor

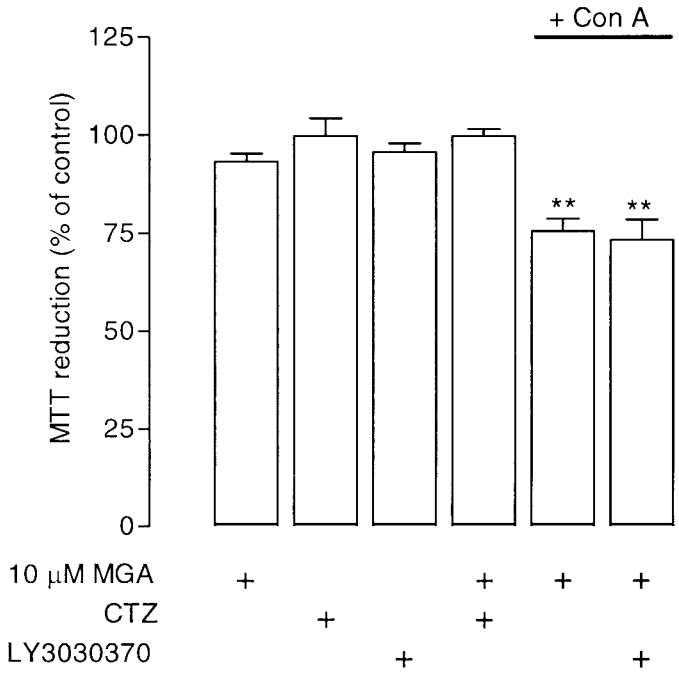

Fig. 6. Effect of exposure of cultured hippocampal neurons to the selective kainate receptor agonist, 4-methylglutamate (MGA). Neurons were treated with 10 μM MGA in the presence of cyclothiazide (CTZ; 30 μM) for 30 min, or preincubated with Con A (250 μg/ml) (5 min) and subsequently incubated with MGA (10 μM) for 30 min. The results are presented as percentage of MTT reduction in control conditions (no drug treatment or Con A alone), and represent the mean ± SEM of at least four independent experiments carried out in triplicate. $^{**}P > 0.01$, significantly different from control (Con A pretreatment); Dunnett's post-test.

agonists, like (5)-5-iodowillardiine (IW) (Swanson et al., 1998a), (R,S)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid (ATPA) (Chittajallu et al., 1999; Johansen et al., 1999) and domoate (Malva et al., 1996; Larm et al., 1997) are extensively used. Recent studies demonstrate, however that (2S,4R)-4-methylglutamate (MGA) is the most potent and selective agonist at kainate receptors (Jones et al., 1997; Donevan et al., 1998), and that it has no effect on AMPA-evoked inward currents in the presence of cyclothiazide (CTZ) in cerebellar granule cells (Savidge et al., 1999). Moreover, it has been shown that Ca^{2+} influx and subsequent elevation of $[Ca^{2+}]_i$ is crucial in triggering neuronal death after exposure to excitatory amino acids (Choi, 1985).

In the present work, the pharmacologic identification of kainate receptors in single cell Ca^{2+} imaging studies is evident. Kainate, even at low concentrations (10 μM), was not a selective agonist for these receptors, and the response could in part be mediated by active AMPA receptors. The blockade of kainate receptor desensitization by Con A, which is selective to these receptors (Huettner, 1990; Partin et al., 1993) allowed, however, to unmask the role of kainate receptors on the $[Ca^{2+}]_i$ changes. It was also very interesting to observe that hippocampal neurons showed variability in terms of the inhibitory effect caused by LY303070. In some cells the $[Ca^{2+}]_i$ changes were only mediated by kainate receptors and in others the response can be due to AMPA receptors, or to both AMPA and kainate receptors in the same cell. The variability of neuronal responses may be due to the possibility that Con A action depends on the receptor type (Yue et al., 1995; Everts et al., 1999; Lerma, 1999), different affinities to the agonist/antagonist or different expression levels of kainate and AMPA receptors. Previous studies also showed that CTZ enhanced AMPA-induced $[Ca^{2+}]_i$ responses with great variability (Hoyt et al., 1995; Sekiguchi et al., 1998; Ambrósio et al., 2000). Moreover, it is well established that the desensitization kinetics of kainate receptors depends of the subunit composition (Swanson et al., 1998b), and that brief exposure to the lectin Con A eliminates desensitization of current gated by kainate and domoate. Using the more selective agonist to kainate receptors (MGA) (Jones et al., 1997; Donevan et al., 1998), we observed that, in the presence of Con A, to eliminate receptor desensitization, MGA did mediate the $[Ca^{2+}]_i$ increase. Contrary to what happened when cells were stimulated with kainate, the response induced by MGA in the presence of Con A was never inhibited by the AMPA receptor antagonist, LY303070, which further supports the involvement of kainate receptors.

Previous studies have demonstrated that excitotoxicity induced by kainate stimulation is mediated by AMPA- but not by kainate-preferring receptors in hippocampal neurons and in amacrine-like neurons in culture (Ohno et al., 1997; Ferreira et al., 1998; Ambrósio et al., 2000). Kainate receptors can, however, mediate selective responses (Paternain et al., 1995), especially in the presence of Con A (Savidge et al., 1997; Jensen et al., 1998). In fact, this plant lectin (Con A) has been used as a tool to unmask the activity of kainate receptors (Partin et al., 1993; Savidge et al., 1997; Jensen et al., 1999), but may also induce neuronal death by itself (Kulkarni et al., 1998). Using MGA as a selective agonist for kainate receptors, we could observe a neurotoxic effect only in the presence of Con A. These results suggest a possible role of kainate receptors in neurotoxicity, because LY303070 did not protect neurons from the neurotoxic effect. Furthermore, MGA in the presence of CTZ, which blocks AMPA receptor desensitization (May and Robison, 1993), did not induce toxicity, further excluding the contribution of AMPA receptors. Concerning this toxic effect, however, we suggest that a subpopulation of neurons that express kainate receptors, may be susceptible to kainate receptor cytotoxicity in the cultured hippocampal neurons.

We have also demonstrated that kainate receptor subunits are present in the cultured hippocampal neurons. Immunocytochemistry with antibodies against KA2 and GluR6/7 subunits showed a high level of expression of kainate receptors throughout the neuronal population. Moreover, the same antibodies produced immunoreactive bands with apparent molecular weights of 123 and 115 kDa, which are similar to the expected bands of KA2 and

GluR6/7 subunits (Herb et al., 1992; Raymond et al., 1993; Roche et al., 1994). Thus, hippocampal cultures express functional kainate receptors, which may play a role in the excitotoxicity accordingly to previous observations in GluR6 knockout mice (Mulle et al., 1998). These results are also in agreement with some studies where an increase in $[Ca^{2+}]_i$ was found to be mediated through kainate-preferring receptors in cerebellar granule cells (Savidge et al., 1997), and also with the finding that activation of these receptors may cause oligodendroglial cell death (Sânchez-Gómez and Matute, 1999). We are currently investigating the coupling mechanisms between Ca^{2+} influx after kainate receptor activation and neurotoxicity.

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