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Ca^{2+} Entry Via AMPA/KA Receptors and Excitotoxicity in Cultured Cerebellar Purkinje Cells

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Initial studies of glutamate receptors activated by kainate (KA) found them to be Ca^{2+} impermeable. Activation of these receptors was thought to produce Ca^{2+} influx into neurons only indirectly by Na^+-dependent depolarization. However, Ca^{2+} entry via AMPA/KA receptors has now been demonstrated in several neuronal types, including cerebellar Purkinje cells. We have investigated whether such Ca^{2+} influx is sufficient to induce excitotoxicity in cultures of cerebellar neurons enriched for Purkinje cells. Agonists at non-NMDA receptors induced Ca^{2+} influx in the majority of these cells, as measured by whole-cell voltage clamp and by fura-2 $[Ca^{2+}]_i$ microfluorimetry. To assess excitotoxicity, neurons were exposed to agonists for 20 min and cell survival was evaluated by a fluorescence assay 24 hr later. KA (100 μM) reduced neuronal survival relative to controls to 43 ± 3% when applied in Na^+-containing solution and to 45 ± 3% in Na^+-free solution. This toxicity was blocked completely by CNQX but only slightly by 100 μM Cd^{2+} and 50 μM D-(−)-2-amino-5-phosphonovaleric acid. Both Purkinje neurons and non-Purkinje cell types present in the cultures were similarly vulnerable to toxic KA exposure, but the population marked by KA-induced Co^{2+} uptake was selectively diminished by the excitotoxicity. Na^+-independent excitotoxicity could also be induced by domoate, AMPA, or glutamate. Compared to KA, NMDA was relatively ineffective in inducing cell death. Most of the KA-induced excitotoxicity could be blocked by removal of extracellular Ca^{2+} during the KA exposure and for a 5 min period thereafter. Furthermore, antagonists of the Ca^{2+}-activated enzymes nitric oxide synthase and calpain significantly reduced the KA-induced cell death. These results show that non-NMDA receptor activation can cause excitotoxicity in cerebellar Purkinje neurons by mechanisms not involving Na^+ influx, but rather depending on direct Ca^{2+} permeation and activation of Ca^{2+}-dependent enzymatic processes.

[Key words: calcium, kainate, AMPA, glutamate receptors, excitotoxicity, calpain I, nitric oxide, Purkinje cells]

Glutamate receptor activation can increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neurons, and this is thought to contribute to Ca^{2+}-mediated excitotoxic neuronal cell death in certain disease processes (Rothman and Olney, 1987). NMDA receptors are ion channels that are highly Ca^{2+} permeable, whereas non-NMDA ionotropic receptors, activated by the agonists kainate (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), have traditionally been thought to be Ca^{2+} impermeable (Ascher and Nowak, 1988; Mayer et al., 1988). For this reason, non-NMDA receptors have been thought to cause Ca^{2+} influx only indirectly due to Na^+-dependent depolarization and the subsequent opening of voltage-gated Ca^{2+} channels. However, it is now clear that several types of AMPA/KA receptors are also directly Ca^{2+} permeable, and that these receptors can be important sources of Ca^{2+} influx in some types of neurons and astrocytes. Murphy and Miller (1989) observed that the Ca^{2+} influx induced by KA in cultured striatal neurons could not be entirely blocked by antagonists of voltage-gated Ca^{2+} channels, and suggested that KA receptors might be directly permeable to Ca^{2+}. Iino et al. (1990) showed that some cultured hippocampal neurons expressed KA-activated receptors which were Ca^{2+}-permeable, giving rise to an inwardly rectifying current–voltage (*I–V*) relationship. Other authors have also reported evidence for Ca^{2+}-permeable AMPA/KA receptors in various types of cultured neurons (Holopainen et al., 1989; Ogura et al., 1990; Gilbertson et al., 1991; Pruss et al., 1991) and glia (Glaum et al., 1990; Jensen and Chiu, 1991; Burnashev et al., 1992b). Additionally, the cloning of several non-NMDA glutamate receptor subunits has revealed that when combinations of these are expressed in oocytes or cell lines, they can give rise to glutamate receptors that are either Ca^{2+}-permeable or Ca^{2+}-impermeable (Hollmann et al., 1991; reviewed in Miller, 1991). Finally, we have provided evidence that cultured cerebellar Purkinje neurons simultaneously express both Ca^{2+}-permeable and Ca^{2+}-impermeable KA-activated receptors (Brorson et al., 1992a).

Work from a number of laboratories has implicated NMDA receptors in *in vitro* models of glutamate excitotoxicity (Goldberg et al., 1987; Rothman and Olney, 1987; Choi, 1988), and NMDA receptor activation seems to be an essential component of neuronal damage in several *in vivo* models of ischemia and trauma (Faden and Simon, 1988; Park et al., 1988). However, the NMDA receptor apparently does not account for all types of glutamate-mediated neurotoxicity. In certain models of global ischemia, specific antagonists of non-NMDA receptors alone (Sheardown et al., 1990) or in combination with NMDA antagonists (Kaku et al., 1991) are neuroprotective. Domoate poisoning following the ingestion of contaminated mussels causes a clinical syndrome resulting from the activation of non-NMDA glutamate receptors (Teitelbaum et al., 1990). The dietary toxins that have been postulated as the causative agents in lathyrism

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(Spencer and Schaumburg, 1983) and the Guam amyotrophic lateral sclerosis-Parkinsonism-dementia complex can also activate non-NMDA receptors (Spencer et al., 1987; Meldrum and Garthwaite, 1990). Thus, the action of glutamate at Ca^{2+}-permeable AMPA/KA receptors might also be expected to play an important role in some forms of experimental and clinical excitotoxicity.

Several properties of cerebellar Purkinje neurons indicate that Ca^{2+}-mediated processes might be particularly important in these cells. First, they have among the highest concentrations in the brain of several of the Ca^{2+}-binding proteins, including calbindin D_{28K} and parvalbumin (Celio, 1990), which are thought to be involved in Ca^{2+} buffering. Second, they exhibit high concentrations of markers for intracellular Ca^{2+} stores such as the inositol trisphosphate and ryanodine receptors (Brorson et al., 1991). They are known to have profuse glutamatergic synaptic inputs and to exhibit Ca^{2+}-dependent action potentials (Llinas and Sugimori, 1980). Activation of glutamate receptors in these neurons produces large increases in dendritic $[Ca^{2+}]_i$ due to both Ca^{2+} influx (Connor and Tseng, 1988; Llano et al., 1991) and Ca^{2+} mobilization from intracellular stores (Llano et al., 1991). Finally, Purkinje cells are known to be extremely vulnerable to hypoxic and ischemic neuronal death. They are often selectively damaged after asphyxiation or cardiac arrest. However, most studies have found that in adult tissues, Purkinje neurons lack NMDA receptors (e.g., Audinat et al., 1990), although they seem to be present on Purkinje cells during development (Rosenmund et al., 1992; Yuzaki and Mikoshiba, 1992). It would seem that excitotoxicity mediated by pathways other than the NMDA receptor might be particularly important to the vulnerability of Purkinje neurons. For these reasons, we have chosen to study the role of Ca^{2+}-permeable non-NMDA receptors in excitotoxicity in cultured cerebellar Purkinje neurons.

A preliminary report of this work has been published as an abstract (Brorson et al., 1992b).

Materials and Methods

Cell culture and staining techniques. Cultures of cerebellar neurons taken from embryonic day 16 Holtzman rats were prepared as previously described in detail (Brorson et al., 1991), except that 15 mM HEPES was added to the culture medium. Trypsin-dissociated neurons were plated on 15 mm round glass coverslips (Carolina Biological Supply) and suspended over a feeding layer of astrocytes in a serum-free defined medium (N2.1). Animal care was in accordance with University of Chicago Animal Care Committee protocols. Maternal rats were anesthetized irreversibly with ether prior to dissection. Neurons were used for physiological experiments when of age 12–39 d *in vitro* (DIV) and for excitotoxin exposures at ages 6–15 DIV. Immunocytochemistry was performed as previously described (Brorson et al., 1991). Co^{2+}-uptake staining was also previously described (Brorson et al., 1992a); in brief, cells were stimulated with KA for 20 min in the presence of 5 mM $CoCl_2$, and the Co^{2+} taken up by the cells was then precipitated and visualized with silver enhancement. Comparisons between counts of cells stained in parallel were evaluated using paired two-tailed Student's *t* tests (STATWORKS, Cricket Software).

$[Ca^{2+}]_i$ measurements and electrophysiology. Cells were incubated in a solution containing fura-2 acetoxymethyl ester (5 mM) for 30 min at 37°C in a buffered solution containing (in mM) NaCl, 140; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; and glucose, 10; pH to 7.4 with NaOH. Cells were then rinsed and incubated for a further 30 min at 37°C in buffer, to wash out remaining fura-2 ester. Fura-2 fluorescence was determined as previously described (Thayer et al., 1988). Background fluorescence was measured from a cell-free region of the coverslip. The above solution was used as an Na^+-containing solution. Na^+-free solutions contained (in mM) *N*-methyl-D-glucamine (NMDG), 140; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; and glucose, 10; pH to 7.4 with HCl. Ca^{2+}-free solutions contained 0.1 mM EGTA and no added Ca^{2+}. All solutions for Ca^{2+} microfluorimetry, for whole-cell voltage-clamp measurements, and for excitotoxicity assays contained tetrodotoxin (0.5 μM) and bicuculline (20 μM) in order to eliminate any synaptic contributions to the measured effects.

Whole-cell patch-clamp measurements of ligand-gated Ca^{2+} currents were performed as previously described (Brorson et al., 1992a). Cells were accepted for study if a stable seal formed with a whole-cell resistance of at least 200 MΩ. Intracellular solutions contained (in mM) NMDG, 145; and BAPTA, 10; pH to 7.2 with HF (ATP was omitted to allow rundown of the voltage-gated Ca^{2+} currents). Na^+-containing extracellular solutions were as above. Na^+-free solutions contained (in mM) NMDG, 145; $CaCl_2$, 2; HEPES, 10; and glucose, 10; pH to 7.4 with HCl (Mg^{2+} and K^+ were omitted to eliminate all non-Ca^{2+} currents). Cells were held at a membrane potential of −100 mV and agonists applied by bath perfusion of the recording chamber at a rate of 1–1.5 ml/min. Figures have been corrected for the perfusion delay of approximately 20 sec. All experiments were performed at room temperature. Data were recorded on an IBM PC–compatible system using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) and also on magnetic tape using a digital data recorder (VR-10A, Instrutech Corp., Elmont, NY). Current traces displayed have been replayed with electronic filtering at 1 Hz. Because there was a slow rundown of receptor-gated currents in the whole-cell mode, relative current magnitudes evoked by different agonists or under different conditions were always compared to the average of flanking control responses by paired two-tailed Student's *t* tests.

Excitotoxicity assays. Cell death and survival were assayed using fluorescein diacetate and propidium iodide, as described by Abele et al. (1990). To reduce background staining of cellular debris, prior to agonist exposure the coverslips of cultured neurons were placed for 24 hr in medium to which 10% horse serum (GIBCO) had been added. The coverslips were then washed and exposed at 37°C to various agents in buffers of ionic compositions as described above. After 20 min exposures, the coverslips were again washed in the same ionic solutions to remove the agonists, and returned to incubation in astrocyte-conditioned, serum-free medium. When indicated, an additional 5 min wash in Ca^{2+}-free solution preceded the return to the Ca^{2+}-containing N2.1 medium. For experiments involving calpain antagonists, the cells were placed in culture medium containing the antagonist 20 min prior to agonist exposures. The antagonist was also added to the buffer in which the KA exposure was performed, and after exposure the coverslips were returned to the medium containing the antagonists.

After 24 hr, cell survival was assayed. The coverslips were washed in buffer, and then exposed for 4 min to fluorescein diacetate (15 μg/ml) and propidium iodide (5 μg/ml). The stained cells were examined on an epifluorescence microscope (Leitz Diaplan) at wavelengths appropriate for each fluorophore. Surviving neurons contained fluorescein, and propidium iodide's red fluorescence marked the nuclei of dead neurons. These groups did not overlap. Living and dead neurons were counted on adjacent fields of each coverslip to totals of at least 100. The percentage of neurons surviving was determined on three or four coverslips for each condition in each experiment. The average percentage survival or average relative percentage survival (normalized to controls performed in the same ionic conditions) from at least three separate experiments for each condition is expressed in the text as the mean ± SEM. Statistical significance was evaluated comparing absolute survivals using unpaired two-tailed Student's *t* tests. None of the control survival rates in various ionic conditions significantly differed from that in the standard Na^+-containing buffer.

AMPA was obtained from Research Biochemicals, Inc. (Natick, MA). The calpain antagonist E-64 was obtained from Calbiochem (San Diego, CA), and MDL-28170 was the gift of Dr. Shujaath Mehdi (Marion Merrell Dow, Cincinnati, OH). GYKI 53655 was the gift of Dr. David Lodge (Eli Lilly and Co., Windlesham, UK). Propidium iodide was obtained from Aldrich Biochemicals (Milwaukee, WI). All other agents came from Sigma Chemical Co. (St. Louis, MO).

Results

KA-induced excitotoxicity

We explored the role of non-NMDA receptors in excitotoxicity, examining the effects of 20 min exposures to KA on cultured cerebellar neurons. The cultures were prepared from 16-d-old

rat embryos and included a 40–60% fraction of cells that expressed the Ca^{2+}-binding protein calbindin D_{28K}, a specific marker for Purkinje neurons among cerebellar cells (Celio, 1990; Brorson et al., 1991). In the bilaminar culture method employed (Brorson et al. 1991), the neurons were grown directly on glass coverslips, largely free of astrocytes. They were removed from the medium and exposed to agonists under defined ionic conditions. After 24 hr, the fraction of neurons surviving was determined using the fluorescent markers fluorescein diacetate and propidium iodide. Purkinje cells are quite fragile *in vitro* just as they are *in vivo*. Thus, even under optimal conditions, there was found to be some background neuronal death. In control coverslips exposed to buffer containing 140 mM Na^+ (Na^+-containing), cell survival was found to be 76 ± 2% (n = 20) at 24 hr. KA (100 μM) induced substantial additional cell death, reducing absolute survival to 32 ± 2% (n = 18). The KA-induced death was completely blocked by 10 or 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (n = 3). If this KA excitotoxicity was a result of Na^+ influx through the conventional Ca^{2+}-impermeable AMPA/KA receptors, one would expect it to be reduced or blocked by removal of extracellular Na^+. Accordingly, we performed similar experiments in an Na^+-free buffer, in which the Na^+ was replaced with the impermeant cation NMDG. Survival in the Na^+-free controls was 79 ± 1% (n = 26) and in the 100 μM KA-exposed cells 35 ± 2% (n = 19), similar to the results in Na^+-containing conditions. CNQX again fully blocked the KA-induced cell death (n = 3) in Na^+-free solution. For clarity, all excitotoxicity data were normalized by dividing the absolute survival in agonist-treated coverslips by the survival in parallel controls, to give a measure of relative survival. Expressed in this fashion, KA reduced relative survival to 43 ± 3% in Na^+-containing buffer (n = 17) and to 45 ± 3% in Na^+-free buffer (n = 19) (Fig. 1*A*). Thus, extracellular Na^+ did not seem to be essential to the excitotoxicity induced by KA.

Concentration–response data for the Na^+-independent excitotoxicity of KA revealed half-maximal effects at concentrations of KA between 30 μM and 100 μM (n = 3). Maximal effects occurred at 1000 μM or more, where relative survivals were reduced to less than 30% (Fig. 1*B*).

The percentage of cells staining for calbindin D_{28K} was found to be similar in control and KA-treated coverslips at 24 hr (41 ± 6% vs 44 ± 4%, n = 3), indicating that both Purkinje cells and non-Purkinje cell types were similarly reduced in number by the KA treatment (Fig. 1*C*). We have previously found that both cell types are represented among cells expressing Ca^{2+}-permeable non-NMDA receptors as identified by Co^{2+}-uptake staining (Brorson et al., 1992a). The Ca^{2+}-permeable non-NMDA receptors, unlike other ligand- or voltage-gated Ca^{2+} channels, have been found to be nonselective in their divalent cation permeability and are permeable to Co^{2+} as well as Ca^{2+} (Pruss et al., 1991). Therefore, KA-induced Co^{2+} uptake, visualized with silver enhancement, can be used to mark the cells expressing the Ca^{2+}-permeable non-NMDA receptors. We employed this technique on cells that had been treated 24 hr prior with 100 mM KA, and found a selective depletion of Co^{2+}-uptake staining cells (Fig. 1*D*). Among the cells subjected to an excitotoxic exposure to KA, only 14 ± 4% stained for Co^{2+} uptake, and most of those stained only lightly, while 54 ± 2% stained among controls (n = 4, p = 0.002). Thus, the subset of cells expressing a greater KA-induced Co^{2+} permeability, which included both Purkinje cells and non-Purkinje cells, seemed particularly vulnerable to KA toxicity.

Other glutamate agonists

Other agonists at non-NMDA glutamate receptors were also applied to these neurons. Domoate and AMPA also produced $[Ca^{2+}]_i$ increases and inward currents carried by Ca^{2+} in Na^+-free solutions. Domoate (100 μM) caused larger Ca^{2+} influxes than those produced by 100 μM KA, while those produced by 30 μM AMPA were smaller (Fig. 2*A*). Glutamate and AMPA are known to generally produce rapid inactivation of the AMPA/KA receptors, with plateau currents being only a small fraction of the transient peak currents (Patneau and Mayer, 1990). With the slow bath application of agonists that was utilized, the receptors would be largely inactivated by the time the AMPA was completely washed in. Thus, the maximal inward currents carried by Ca^{2+} evoked by AMPA were quite small compared to those evoked by KA or domoate (Fig. 2*B*). Nevertheless, concentration–response curves revealed that AMPA, though less efficacious, was more potent than either KA or domoate in activating ligand-gated Ca^{2+} currents, with an EC_{50} of 4.3 μM, compared to 19 μM for domoate and 171 μM for KA (Fig. 2*C*). Therefore, in terms of activation of ligand-gated Ca^{2+} currents, the agonist rank potency was AMPA > domoate > KA, suggesting an action at AMPA/KA receptors.

The various agonists of glutamate receptors were also compared when applied in the excitotoxicity assay. Under Na^+-free conditions, both the non-NMDA agonists AMPA (30 μM) and domoate (100 μM) also produced significant excitotoxicity, although to lesser degrees than 100 μM KA (Fig. 3*A*). The relative survivals were 75 ± 1% for 30 μM AMPA (n = 3) and 57 ± 2% for 100 μM domoate (n = 5). The combination of 30 μM AMPA with 100 μM KA resulted in a relative survival of 54 ± 3% (n = 4), increased compared to KA alone (p = 0.03). Thus, the reduced toxicity of AMPA compared to KA might be related to the receptor inactivation produced by AMPA. The reduced sensitivity of the neurons to domoate relative to KA does not correlate with the greater effect of 100 μM domoate as an agonist of non-NMDA receptor-gated Ca^{2+} currents.

In several *in vitro* systems, NMDA receptors have been found to be the predominant type involved in excitotoxicity. However, in the cerebellar neurons, NMDA-evoked Ca^{2+} currents were often entirely absent in cells with the Purkinje cell morphology, and were generally smaller than the Ca^{2+} currents evoked by KA. In Na^+-free, Mg^{2+}-free, 10 μM glycine–supplemented solutions, the average ratio of the currents evoked by 100 μM NMDA to those evoked by 100 μM KA was 0.28 ± 0.09 (n = 9). This is in keeping with the reported loss of NMDA receptor expression in developing Purkinje cells (e.g., Rosenmund et al., 1992). Nevertheless, it is conceivable that some direct activity of KA at NMDA receptors or indirect activation of NMDA receptors via induced glutamate release could have accounted for the Na^+-independent KA excitotoxicity. We therefore applied NMDA under Mg^{2+}-free, 10 μM glycine-supplemented conditions and assayed for excitotoxicity (Fig. 3*B*). In Na^+-containing solutions, 100 mM NMDA resulted in a relative survival of 68 ± 2% (n = 3) and of 80 ± 4% (n = 6) in Na^+-free solutions. Even maximally toxic concentrations of 3000 μM NMDA only reduced survival to 70 ± 2% (n = 3). Adding 100 μM KA to 300 μM NMDA substantially increased the toxicity to 45 ± 2% survival, equivalent to that of KA alone but significantly greater than 300 μM NMDA alone or even 3000 μM NMDA alone (p < 0.001). A portion of the toxicity of NMDA may have been indirect, since including the non-NMDA recep-

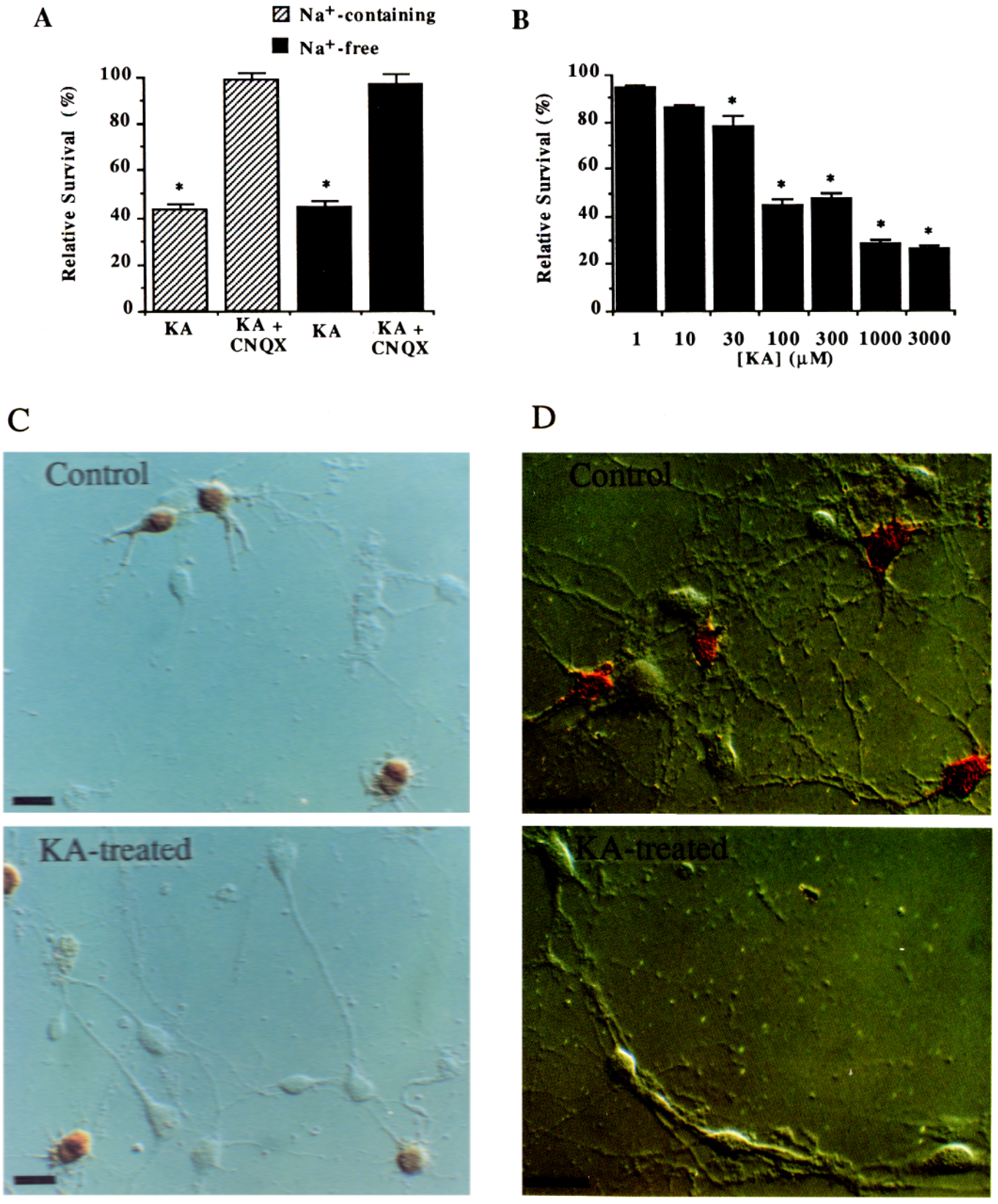

Figure 1. Na^+-independent KA excitotoxicity. *A,* Relative survivals, normalized to parallel controls, averaged over all experiments for each condition. KA (100 μM) reduced survival of neurons compared to controls by similar amounts whether in Na^+-containing or Na^+-free solutions. These effects were blocked by CNQX (10 or 20 μM). *, $p < 0.001$ compared to controls. *B,* Concentration–response data for the KA-induced toxicity in Na^+-free solutions. *, $p < 0.001$ compared to controls. *C,* Immunocytochemical staining for calbindin D_{28K} 24 hr after treatment with either control (*upper panel*) or 100 μM KA-containing (*lower panel*) Na^+-free solutions, indicating similar rates of survival of both calbindin-positive Purkinje cells and non-Purkinje cell types (cells of age 8 DIV; monoclonal anti–calbindin D_{28K}, 1:20,000, Sigma Chemical Co.). *D,* KA-induced Co^{2+}-uptake staining in cells subjected 24 hr prior to either control (*upper panel*) or 100 μM KA-containing (*lower panel*) Na^+-free solutions. Few Co^{2+}-uptake staining neurons remained on the coverslips previously exposed to KA, whereas a majority of control-treated neurons were stained (cells of age 9 DIV). Scale bars (*C* and *D*), 20 μm.

tor antagonist CNQX at 10 μM during the 100 mM NMDA exposure increased the relative survival to 86 ± 2% (n = 4). We have reported a similar involvement of indirect responses to NMDA, blocked by CNQX, using $[Ca^{2+}]_i$ microfluorimetry in these neurons, with less than one-half of the cells exhibiting direct responses to NMDA (Brorson et al., 1991). These actions may explain the modest excitotoxic effects that NMDA has in these cultures. Thus, in this system NMDA was considerably less effective in inducing excitotoxicity than was KA.

Since the toxicity of KA might also be in part indirect, we examined the effects on KA excitotoxicity in Na^+-free solutions of the NMDA antagonist D-(−)-2-amino-5-phosphonovaleric acid (D-AP5) (50 or 100 μM) and of 100 μM Cd^{2+}, which blocks all sustained voltage-gated Ca^{2+} currents (Brorson et al., 1992a) (Fig. 3C). Neither Cd^{2+} alone nor 50 μM D-AP5 alone produced significant toxicity. The toxicity of 100 μM KA was not significantly blocked by Cd^{2+} (relative survival 49 ± 4%, n = 4), but was slightly reduced in the presence of 50 μM D-AP5, resulting in a relative survival of 53 ± 3% (n = 3, p = 0.04 compared to KA alone). When cells were exposed to KA in the combined presence of Cd^{2+} and D-AP5, the relative survival was further increased to 64 ± 2% (n = 3). However, this result must be interpreted with caution, since combined Cd^{2+} and D-AP5 caused slight toxicity even in the absence of KA, reducing survival to 90 ± 4% (n = 3) of that in control solutions. Thus the excitotoxic actions of KA were partially blocked by Cd^{2+} and D-AP5. However, even in the presence of these antagonists, 100 μM KA was significantly more toxic than NMDA (p = 0.02 compared to NMDA alone). When KA was applied under conditions maximizing NMDA receptor activation (0 Mg^{2+}, 10 μM glycine), even a concentration of 100 μM D-AP5 failed to reverse most of the toxicity of KA, resulting in 64 ± 7% relative survival. To contrast this with the effect of a highly selective non-NMDA antagonist, the 2,3-benzodiazepine GYKI 53655 (Palmer and Lodge, 1993) was utilized at 3 μM, which concentration blocked 98 ± 1% of the KA-evoked currents (n = 3) but did not significantly affect the NMDA-evoked Ca^{2+} currents (n = 6). GYKI 53655 reversed the KA toxicity to a relative survival of 94 ± 3% (n = 4). Therefore, the direct action of KA upon non-NMDA receptors was substantially more toxic than was NMDA in these neurons.

It might still be questioned whether non-NMDA receptors can play an important role in the excitotoxicity of prolonged exposures to glutamate, the physiological agonist, since unlike KA, glutamate produces rapid inactivation of AMPA/KA receptors, whereas its action at NMDA receptors is sustained (Patneau and Mayer, 1990). We found that 100 μM glutamate in Na^+-free solutions induced significant toxicity, reducing survival to 70 ± 1% (n = 3) (Fig. 3D). This toxicity could be partially blocked to similar degrees by either 10 μM CNQX (to 85 ± 1%, n = 3) or 50 μM D-AP5 (to 82 ± 3%, n = 3), suggesting that in this mixed cerebellar neuron population, NMDA and non-NMDA receptors both play important roles in excitotoxicity when glutamate is the agonist.

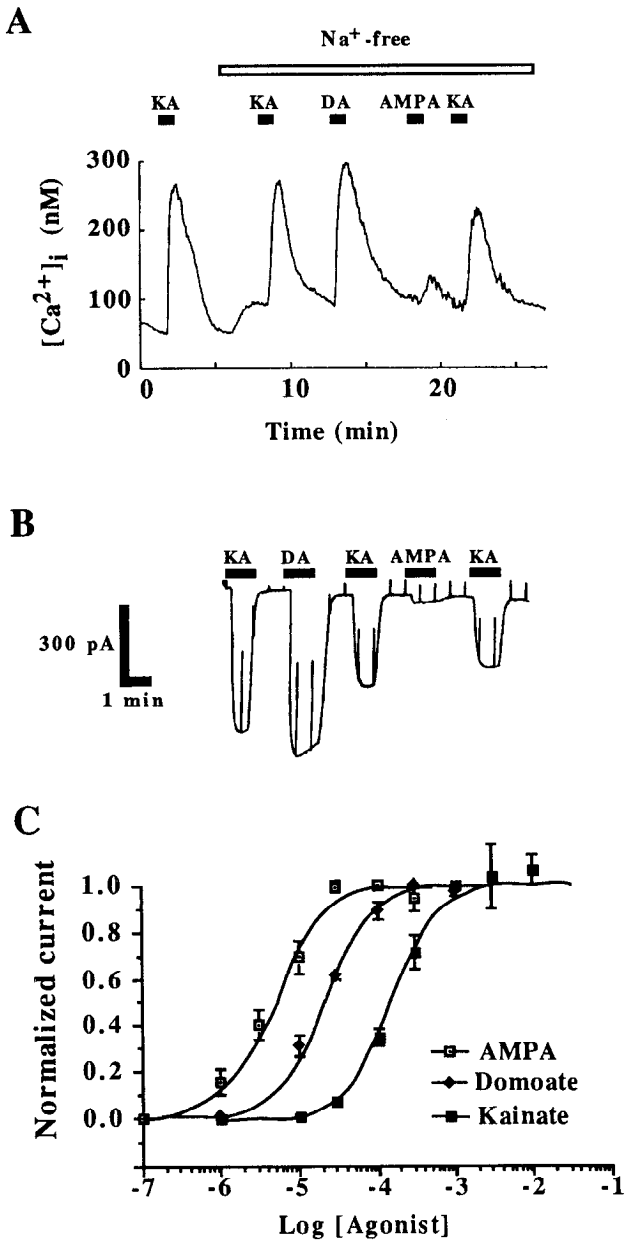

Figure 2. Ca^{2+}-permeable AMPA/KA receptors. *A*, Fura-2 microfluorimetry showing the Ca^{2+} influx induced by KA (100 μM) in Na^+-containing or Na^+-free solutions, and by domoate (*DA*) (100 μM), and AMPA (30 μM) in Na^+-free solution in a single cultured cerebellar neuron. *B*, Whole-cell inward currents induced by KA (100 μM), domoate (100 μM), and AMPA (30 μM) in Na^+-free solution in a cultured cerebellar neuron. The cell was held at −100 mV with periodic depolarizations to 0 mV every 30 sec (marked by *vertical deflections* in the chart record). The Ca^{2+} currents evoked by 100 μM DA were 153 ± 10% of those evoked in the same cells by 100 μM KA (n = 9). *C*, Concentration–response curves for AMPA, DA, and KA induction of Ca^{2+} currents normalized to maximal currents (n = 3 to 6 cells at each point).

Role of Ca^{2+} in the excitotoxicity

An excessive increase of $[Ca^{2+}]_i$ has been found to be an important mediator of excitotoxicity in several models. We examined the role of Ca^{2+} influx in causing the KA-induced excitotoxicity by removing the Ca^{2+} from the extracellular solutions. In Na^+-free solutions, this partially reduced the KA toxicity, resulting in a relative survival of 65 ± 3% (n = 8). However, simply removing the Ca^{2+} during KA exposure in Na^+-containing solutions did *not* block the toxicity (relative survival 47 ± 3%, n = 5; Fig. 4A), despite the washing of KA from the coverslips with Ca^{2+}-free solution prior to return to the Ca^{2+}-containing medium. We explored the mechanism of this phenomenon using $[Ca^{2+}]_i$ microfluorimetry (Fig. 4B). Removal of extracellular Ca^{2+} from Na^+-containing solutions always elim-

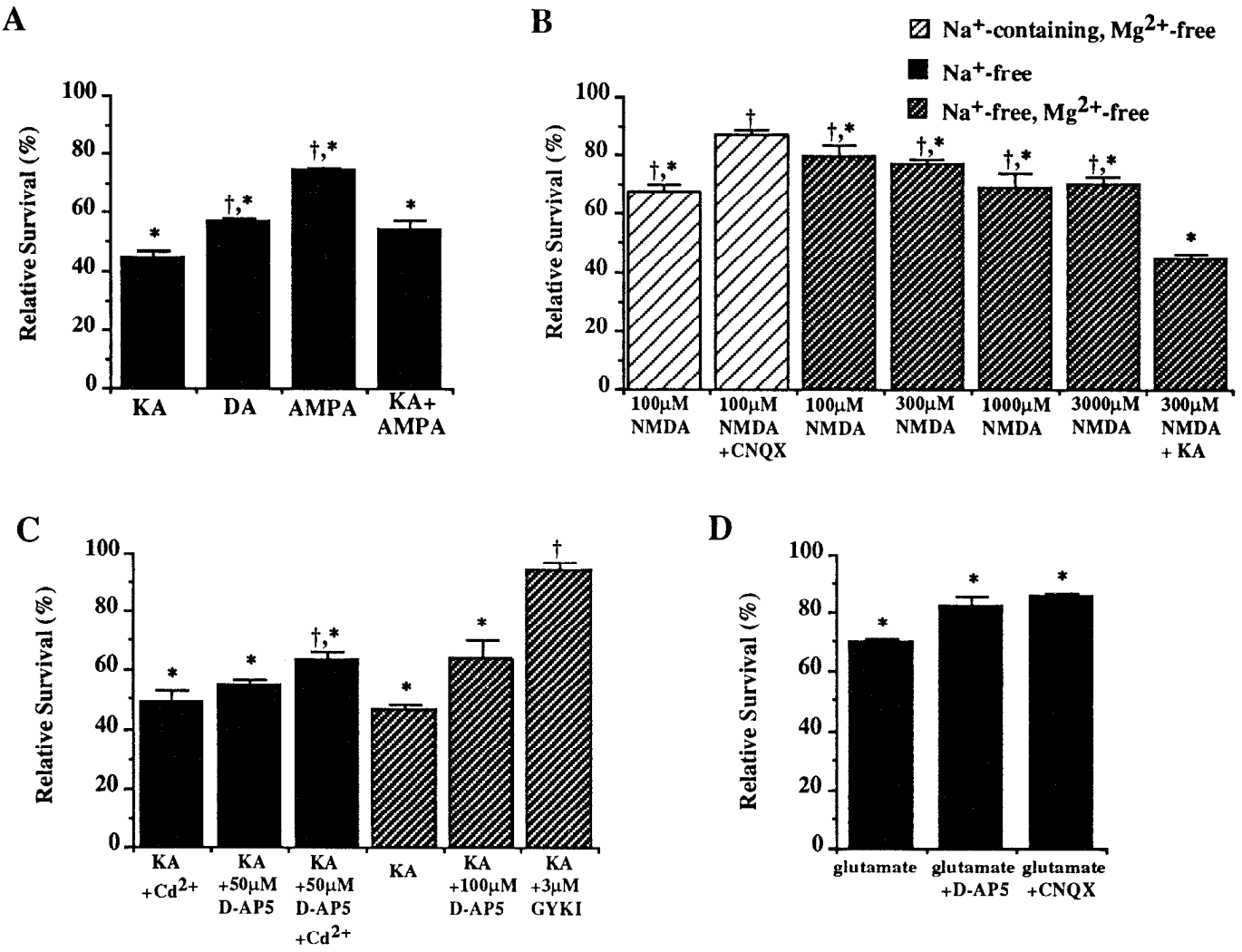

Figure 3. Agonist profile of Na^{+}-independent excitotoxicity. *A*, Average relative survivals after treatment with domoate (*DA*; 100 μM), AMPA (30 μM), or 100 μM KA plus 30 μM AMPA. The relative survival produced by 100 μM KA is repeated here and in subsequent figures for comparison. Each of these agonists produced significant cell death in Na^{+}-free medium (*, $p < 0.001$ compared to controls; †, $p < 0.01$ compared to 100 μM KA). *B*, The excitotoxicity produced by NMDA in Mg^{2+}-free, 10 μM glycine-supplemented solutions. The toxicity of 100 μM NMDA was less than that of 100 μM KA, whether in Na^{+}-containing or Na^{+}-free solutions, and was partially blocked by 10 μM CNQX, suggesting indirect activation of non-NMDA receptors. The addition of KA to 300 μM NMDA produced significantly greater toxicity than the maximal NMDA toxicity. *C*, The effects of Cd^{2+} (100 μM) and of D-AP5 on the toxicity produced by 100 μM KA in Na^{+}-free solutions. D-AP5 alone (50 μM) and in combination with Cd^{2+} produced small but significant increases in the relative survival. The toxicity of KA was not enhanced in Mg^{2+}-free, glycine-supplemented solution, and under these conditions even 100 μM D-AP5 blocked only a minor portion of the cell death, whereas the selective non-NMDA antagonist GYKI 53655 (GYKI) at 3 μM blocked nearly all of the KA toxicity. *D*, The toxicity of glutamate (100 μM), which could be partially blocked by either 50 μM D-AP5 or 10 μM CNQX.

inated the immediate Ca^{2+} influxes induced by KA ($n = 8$). However, after prolonged exposures to KA of 5 min or more, neuronal swelling was visible, and a return to Ca^{2+}-containing solutions within 2 min of the KA exposure resulted in substantial Ca^{2+} influxes ($n = 5$). These delayed Ca^{2+} influxes could be prevented by a 5 min wash period in Ca^{2+}-free solution ($n = 6$), and they were apparently Na^{+}-dependent, since they were not seen in three of four cells in which the prolonged KA exposures were performed in Ca^{2+}- and Na^{+}-free solutions.

Having found that KA could induce a delayed Ca^{2+} influx upon return to Ca^{2+}-containing solutions, and that the delayed influx could be prevented by a 5 min wash period in Ca^{2+}-free solutions, we performed 5 min Ca^{2+}-free washes after KA exposures in Ca^{2+}-free solutions for the excitotoxicity assays. When this procedure was followed, most of the KA toxicity was prevented, resulting in a relative survival of 84 ± 1% ($n = 3$) in Na^{+}-containing solutions and of 88 ± 1% ($n = 3$) in Na^{+}-free solutions (Fig. 4*A*). It seems that either direct Ca^{2+} influx during the KA exposure or delayed Ca^{2+} influx after KA exposure was capable of inducing damage. A small portion of the toxicity may also result directly from osmotic damage, since it was not blocked even with the 5 min wash in Ca^{2+}-free solution.

Mediators of Ca^{2+}-induced damage

Ca^{2+}-mediated excitotoxicity is thought to result from the ability of Ca^{2+} to activate a number of enzymatic processes which cause structural damage to the cell. One of the Ca^{2+}-dependent enzymes thought to be important is nitric oxide synthase, which produces nitric oxide (NO), a diffusible second messenger. N-ω-nitro-arginine (NArg) competitively inhibits NO synthase and

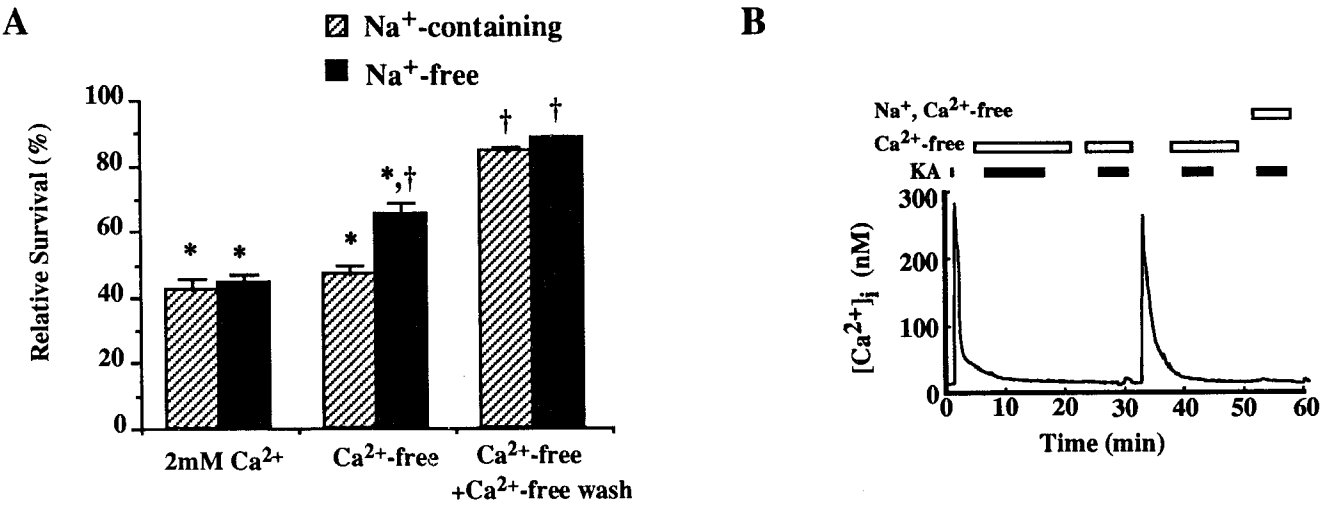

Figure 4. Ca^{2+} dependence of KA excitotoxicity. *A*, Average relative survivals after 100 μM KA exposures for 20 min in various ionic conditions. Removal of extracellular Ca^{2+} partially prevented the KA-induced excitotoxicity in Na^+-free solutions but did not block the toxicity of KA in Na^+-containing solutions. However, if neurons were washed for 5 min in Ca^{2+}-free, Na^+-containing solutions after the KA exposures, most of the toxicity was prevented (*, $p < 0.001$ compared to controls; †, $p < 0.001$ compared to 100 μM KA). *B*, Fura-2 microfluorimetric recording of $[Ca^{2+}]_i$ in a single cell. The immediate large Ca^{2+} influxes produced by 100 μM KA were prevented by removal of extracellular Ca^{2+}, even during prolonged (here, 10 min and 5 min) KA exposures. Following a 5 min KA exposure and a 1 min wash in Ca^{2+}-free solution to wash out the KA, a return to Ca^{2+}-containing solution produced a large Ca^{2+} influx; this influx could be prevented by 5 min washes in Ca^{2+}-free solutions after KA exposures. A 5 min exposure to KA in Na^+- and Ca^{2+}-free solution did not produce a similar Ca^{2+} influx upon return to Ca^{2+}-containing solution.

has been shown to block NO-mediated cell death in neocortical neurons (Dawson et al., 1991). NArg (100 μM) did not block KA-induced whole-cell inward Ca^{2+} currents ($n = 3$, Fig. 5*A*). However, in excitotoxicity experiments, 100 μM NArg partially blocked KA-induced toxicity, resulting in relative survivals of 78 ± 3% ($n = 8$) in Na^+-containing solutions and 74 ± 3% ($n = 4$) in Na^+-free solutions (Fig. 5*C*). The addition of excess substrate (1 mM L-arginine) reversed most of the protective effect of NArg, resulting in a relative survival of 55 ± 4% ($n = 3$) in Na^+-containing solution.

Another enzyme thought to play a role in excitotoxic cell death (Siman et al., 1989) and in ischemic neuronal damage (Lee et al., 1991) is the Ca^{2+}-activated protease calpain I. We examined the effects of several calpain inhibitors including leupeptin (100 μM), E-64 (10 μM) (Komatsu et al., 1986), and the highly cell-permeable agent MDL-28170 (10 μM) (Mehdi et al., 1988). These agents had at most minor nonspecific effects on KA-activated Ca^{2+} currents; only MDL-28170 significantly reduced the KA-induced currents, to 84 ± 2% of control values ($n = 6$; Fig. 5*B*). They were added to the excitotoxicity assay from 20 min before until 24 hr after 100 μM KA exposure. Each of the calpain antagonists was found to block most of the KA-induced cell death (Fig. 5*C*). The relative survival after KA exposure in Na^+-free solution with E-64 was 94 ± 8% ($n = 3$), with MDL-28170 81 ± 1% ($n = 3$), and with leupeptin 82 ± 4% ($n = 3$). Similarly, in Na^+-containing solutions, the calpain inhibitors also had highly significant protective effects. The relative survivals after KA exposures were 92 ± 5% in E-64 ($n = 3$), 91 ± 2% in MDL-28170 ($n = 4$), and 76 ± 4% in leupeptin ($n = 3$). In summary, all three antagonists of calpain inhibited KA-induced cell death, whether in Na^+-containing or in Na^+-free solutions. These results corroborate the involvement of Ca^{2+} as a mediator of KA-induced excitotoxicity, and suggest that activation of the Ca^{2+}-dependent enzyme calpain may be involved in the pathogenic process.

Discussion

Most standard paradigms from the study of excitotoxicity have implicated the action of glutamate at the highly Ca^{2+}-permeable NMDA receptor in the resulting delayed Ca^{2+}-mediated neuronal death (Choi, 1988). Non-NMDA receptors have been thought to play an indirect role, based on initial studies which showed that they were largely Ca^{2+}-impermeable (Mayer and Westbrook, 1987). However, recent evidence has suggested that non-NMDA receptors may also be important mediators of excitotoxicity (Sheardown et al., 1990). Furthermore, Ca^{2+}-permeable non-NMDA receptors have been demonstrated in a number of neuronal types (Holopainen et al., 1989; Murphy and Miller, 1989; Iino et al., 1990; Brorson et al., 1992a). The cloning of a number of non-NMDA receptor subunits has indicated the molecular basis for their permeability or impermeability to Ca^{2+}. A single amino acid change in the GluR2 receptor subunit, resulting from posttranscriptional editing of the RNA message, renders the resulting AMPA/KA receptor Ca^{2+} impermeable; unedited versions of the subunits produce Ca^{2+}-permeable receptors (Hume et al., 1991; Sommer et al., 1991). A mosaic of Ca^{2+}-permeable and Ca^{2+}-impermeable receptors can be coexpressed in the same cells when edited GluR2 is expressed with GluR4 or when editing of the GluR2 mRNA is partial (Burnashev et al., 1992a). For these reasons, it should perhaps come as no surprise that non-NMDA receptors might play a dominant role in Ca^{2+}-mediated cell death in certain neurons, particularly those in which there is relatively little expression of NMDA receptors, such as cerebellar Purkinje neurons (Audinat et al., 1990; Rosenmund et al., 1992; Yuzaki and Mikoshiba, 1992).

Excitotoxicity mediated by non-NMDA receptors

We have shown in this report that in cultured cerebellar Purkinje cells, sustained activation of non-NMDA receptors by KA was

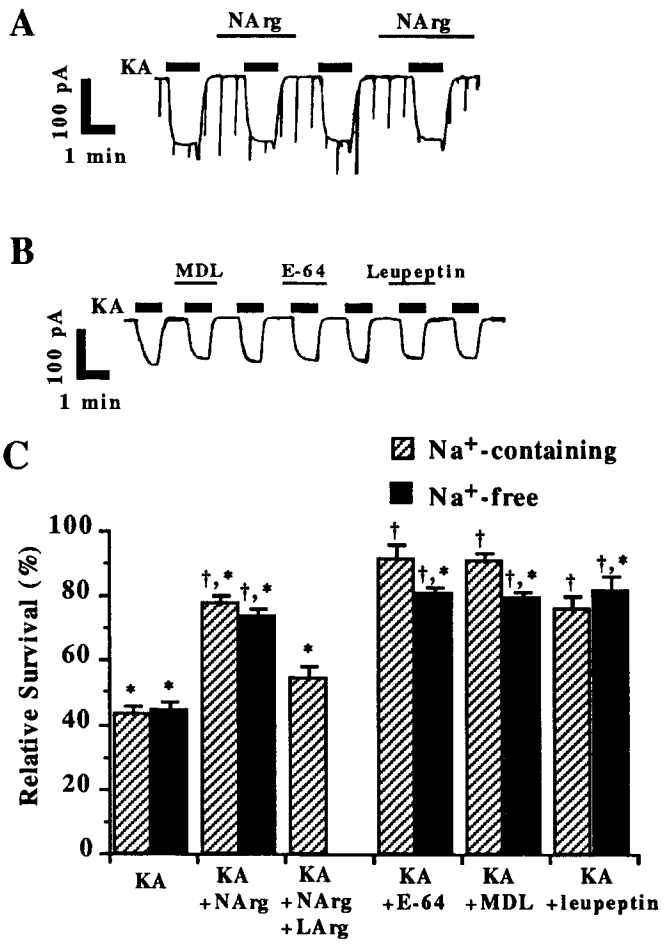

Figure 5. Prevention of KA excitotoxicity by NO synthase and calpain I antagonists. *A,* The whole-cell inward currents in Na^+-free solution in a voltage-clamped cell (holding potential, −100 mV) induced by 100 μM KA with or without NArg (100 μM). *B,* Similar whole-cell currents induced by 100 μM KA alone or in the presence of the calpain inhibitors E-64 (10 μM), MDL-28170 (10 μM), and leupeptin (100 μM). *C,* Average relative survivals after treatment with KA (100 μM) in the presence of the enzyme antagonists NArg (100 μM), NArg + 1 mM L-arginine (*LArg*), E-64 (10 μM), MDL-28170 (10 μM), and leupeptin (100 μM). The values for KA alone are again included for reference. *, $p < 0.01$ compared to controls; †, $p < 0.001$ compared to 100 μM KA alone.

a much more effective excitotoxic insult than NMDA receptor activation. Voltage-gated Ca^{2+} channels and indirect activation of NMDA receptors were shown to play at most minor roles in the KA-induced toxicity. In support of this conclusion, two non-NMDA antagonists acting at different sites, CNQX and GYKI, both effectively blocked the toxicity induced by KA, whereas high concentrations of the NMDA antagonist D-AP5 failed to block most of the toxicity. Also, 100 mM KA caused additional toxicity to that caused by maximally toxic NMDA concentrations. In addition, it can be shown that cyclothiazide, which specifically enhances AMPA/KA receptor responses, increases the toxicity of KA but not that of NMDA (J. R. Brorson, P. A. Manzolillo, and R. J. Miller, unpublished observations). Previous work in cerebellar slices also has demonstrated the selective vulnerability of Purkinje cells to the toxicities of KA and quisqualate over NMDA (Garthwaite and Garthwaite, 1986). Here we further demonstrate that the excitotoxic action of KA occurred independently of extracellular Na^+, and was largely blocked by the removal of extracellular Ca^{2+} (see below). These results suggest that in these cells Ca^{2+} permeation through non-NMDA receptors induced excitotoxic cell death.

We have previously shown that these cells seem to express both Na^+-permeable, Ca^{2+}-impermeable (type I), as well as Ca^{2+}-permeable (type II) receptors activated by KA, so KA induces large Na^+ currents and, in some cells, smaller Ca^{2+} currents (Brorson et al., 1992a). Even type I receptors may have a small Ca^{2+} permeability (Mayer and Westbrook, 1987). Without selective antagonists to distinguish the two classes of receptors, it may be impossible to evaluate the relative contributions to toxicity of Ca^{2+} entry via a small permeability through the large number of type I receptors versus a large permeability through a fewer number of type II receptors. However, we have previously presented evidence that a subset of the neurons in these cultures exhibits relatively larger KA-induced divalent cation permeabilities that allow Co^{2+} entry as well as Ca^{2+} influx (Brorson et al., 1992a), suggesting a greater representation of receptors with a higher divalent permeability on some of the neurons. We have now shown that this population was selectively depleted when KA-induced Co^{2+}-uptake staining was performed following toxic KA exposure in Na^+-free solution. A similar result has been reported in murine cortical neurons (Turetsky et al., 1992). The simplest explanation for this finding is that the greater divalent permeability that allowed for KA-induced Co^{2+} uptake also rendered those cells particularly sensitive to KA toxicity. It is also possible that a small permeability of Ca^{2+} through type I receptors might account for part of the toxic Ca^{2+} influx, but it would seem plausible to ascribe most of the toxicity to a separate set of receptors with greater divalent cation permeability, more numerous on certain cells.

Although it may have been expected that the Purkinje cells in the cultures would be particularly susceptible to the KA-induced toxicity, this did not prove to be the case. Both Purkinje cells, as marked by calbindin staining, and non-Purkinje cells within the cultures were eliminated by KA exposure. This is consistent with our earlier finding that KA-induced Ca^{2+} currents and Co^{2+} uptake were found both in the Purkinje cells and in the non-Purkinje cells (Brorson et al., 1992a), so the preferential expression of divalent permeability that conferred vulnerability to KA was not confined to the Purkinje cells. The mechanism of Ca^{2+}-dependent KA toxicity seems to apply to both the Purkinje cells and to the other cerebellar neurons represented.

Ionic dependence of toxicity

Although the toxicity of KA was partially blocked by Ca^{2+} removal in Na^+-free solutions, KA remained toxic when Ca^{2+} was removed from Na^+-containing solutions. To explain this finding, changes in $[Ca^{2+}]_i$ were studied during long exposures to KA in Ca^{2+}-free solutions, and a delayed Ca^{2+} influx was observed upon return to Ca^{2+}-containing solutions. This delayed Ca^{2+} influx could be blocked by a 5 min wash in Ca^{2+}-free solution following the KA exposure, as could most of the toxicity of KA in Ca^{2+}-free solutions. Thus, most of the excitotoxicity induced by KA was dependent on Ca^{2+} influx, whether immediate or delayed. The delayed Ca^{2+} influx could possibly have resulted from KA-induced Na^+ loading and reversal of membrane Na^+/Ca^{2+} exchange following prolonged KA exposure. However, the cell death resulting directly from osmotic damage would seem to be limited to the small portion that was not demonstrably Ca^{2+}-dependent. That calpain inhibitors were effective in blocking most of the KA-induced cell death even in

Na^+-containing solutions also suggests that osmotic damage contributed little to the KA-induced death.

Regarding the toxicity of KA in the absence of both Na^+ and Ca^{2+}, previous work in cerebellar granule cells also found that KA toxicity could occur in Na^+-free and in Ca^{2+}-free conditions (Kato et al., 1991). It should be noted that the Ca^{2+}-permeable AMPA/KA receptor ionophore is nonselective with respect to cationic permeability. Thus, other monovalent and divalent cations such as K^+ and Mg^{2+} could permeate KA-activated receptors (Iino et al., 1990). It may be that ionic or osmotic derangements due to efflux of these cations account for the residual toxicity of KA in Na^+- and Ca^{2+}-free conditions. In particular, a cytoplasmic acidification may accompany Purkinje cell stimulation (Chesler and Kaila, 1992). The hyperpolarization due to K^+ efflux via the KA-activated receptors in the absence of any depolarizing inward currents would exacerbate the acidification by increasing the driving force for proton entry via cation channels.

Non-NMDA receptor subtypes involved in excitotoxicity

In situ hybridization experiments have revealed the expression of at least the GluR1, -2, and -3 subunits in Purkinje cells (Keinanen et al., 1990). In the cultured cerebellar neurons, the concentration–response relationships of the ligand-gated currents for AMPA, domoate, and KA (Fig. 2*C*) suggest that these agonists are acting at AMPA-preferring receptors such as are formed by combinations of glutamate receptor subunits GluR1–GluR4. The concentration–response data for the KA-induced excitotoxicity also suggests an action at low-affinity KA receptors. The partial block by AMPA of KA toxicity, as well as lesser efficacies of AMPA and glutamate in inducing toxicity, all point to a primary role for AMPA/KA receptors in mediating these toxic effects of non-NMDA agonists as well. In keeping with these observations, we have found that cyclothiazide, which blocks the inactivation of AMPA/KA receptors (Patneau et al., 1992), enhances the AMPA-evoked currents and AMPA-induced excitotoxicity (Brorson, Manzolillo, and Miller, unpublished observations).

Thus far there is no positive evidence for functional participation of other non-NMDA receptor subunits in these cells. The GluR5–GluR7 subunits have higher reported affinities for KA and exhibit lesser sensitivities to AMPA and to CNQX (Egebjerg et al., 1991; Bettler et al., 1992; Sommer et al., 1992). Thus, the pharmacology of the toxicity of non-NMDA receptors described here does not point to a major role for these subunits. Domoate, while activating larger inward Ca^{2+} currents at 100 μM than KA, was less effective than KA in inducing excitotoxicity. Domoate has generally been found to be a more potent agonist at neuronal AMPA/KA receptors than KA, and the receptors formed from the cloned subunits GluR5 and -6 also are selective for domoate over KA (Herb et al., 1992). In contrast, the so-called KA binding proteins have affinities for KA in the nanomolar range (Werner et al., 1991; Herb et al., 1992) and are selective for KA over domoate. However, the low affinity of the KA toxicity does not suggest a role for these high-affinity receptors. It may be that differences in inactivation by domoate and KA will account for the lesser efficacy of domoate as a toxin.

Intracellular effectors of Ca^{2+} toxicity

Our results suggest that both NO production by NO synthase and calpain activation may be secondary mediators of the KA-induced cell death, since antagonists of these enzymes inhibited the excitotoxicity without blocking the KA-induced Ca^{2+} influx. Inhibition of NO synthase by NArg blocked about one-half of the specific cell death, and calpain inhibition blocked the majority of the cell death. Given the overlapping protective effects of inhibiting either enzyme alone, the actions of these Ca^{2+}-activated enzymes may be synergistic in causing cell death. That is, some neurons that succumb to the combined activation of both enzymes can survive if either enzyme system is inhibited. The antagonists employed may conceivably affect other processes involved in KA toxicity, so that one must be cautious in interpreting these data. Nevertheless, the consistent action of three different agents all known to inhibit calpain activity strongly supports a role for this Ca^{2+}-activated enzyme in the excitotoxicity induced by KA, and corroborates the role of Ca^{2+} as a mediator of this toxicity.

Other investigators have also found that these enzymes mediate glutamate toxicity in neurons (Siman et al., 1989; Dawson et al., 1991; East and Garthwaite, 1991; Lee et al., 1991). Both enzymes have been localized immunohistochemically to discrete neuronal populations in the brain (Siman et al., 1985; Bredt et al., 1990). Interestingly, NO synthase was less prominent in Purkinje cells than in other cerebellar neurons such as basket cells and granule cells. The NO-producing neurons themselves are thought to be resistant to the toxic effects of NO (Snyder, 1992). In this regard, KA toxicity in cultured granule cells was not found to be mediated by NO (Puttfarcken et al., 1992). Since NO is thought to act in a transmembrane fashion, in the present experiments NO synthase–negative neurons including the Purkinje cells could be vulnerable to its toxic effects. With regard to calpain, immunohistochemical studies showed that staining for calpain I was particularly strong in Purkinje cells and deep cerebellar nuclear neurons, but not prominent in hippocampal neurons (Siman et al., 1985). Nevertheless, previous demonstrations of the role of calpain in neuronal degeneration have concentrated on the hippocampus (Siman et al., 1989; Lee et al., 1991). To these results we may now add the finding that calpain and NO synthase seem to be involved in KA-induced excitotoxicity in cultured cerebellar neurons.

These results have implications for the pharmacological treatment of insults to the CNS in which glutamate-mediated excitotoxicity plays a role, such as stroke, global ischemia, and trauma. Attempts to treat these diseases by blockade of Ca^{2+} entry through the NMDA receptor alone may be inadequate, since glutamate activation of non-NMDA receptors can also lead to Ca^{2+}-mediated excitotoxicity. Combined antagonism of all classes of Ca^{2+}-permeable glutamate receptors may be necessary to provide more effective protection against neuronal damage. Alternatively, strategies aimed at antagonizing the actions of the secondary mediators of Ca^{2+}-induced injury, such as calpain I or NO synthase, may be found to have advantages over receptor blockade, as these act at points common to all pathways of neuronal injury, while avoiding effects on the fast synaptic activity that underlies normal brain function.

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