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Inhibition of T-Type Calcium Channels Protects Neurons from Delayed Ischemia-Induced Damage

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ABSTRACT

Intracellular calcium increase is an early key event triggering ischemic neuronal cell damage. The role of T-type voltage-gated calcium channels in the neuronal response to ischemia, however, has never been studied. Using an in vitro model of ischemia-induced delayed cell death in rat organotypic hippocampal slice cultures, we show that T-type calcium channels inhibitors drastically reduce ischemic cell damage. Immunostaining studies reveal the existence of $Ca_V3.1$ and $Ca_V3.2$ types of low-voltage-activated calcium channels in rat organotypic hippocampal cultures. Low extracellular calcium (100 nM) or increase of intracellular calcium buffering ability by BAPTA-acetoxymethyl ester significantly reduced ischemia-induced neuronal damage. Pharmacological inhibition of the T-type calcium current by mibefradil, kurtoxin, nickel, zinc, and pimozide during the oxygen-glucose deprivation episode provided a significant protection against delayed neuronal death. Mibefradil and nickel exerted neuroprotective effects, not only if administrated during the oxygen-glucose deprivation episode but also in conditions of postischemic treatment. These data point to a role of T-type calcium currents in ischemia-induced, calcium-mediated neuronal cell damage and suggest a possible new pharmacological approach to stroke treatment.

Ischemic neuronal damage remains a common cause of severe neurological disability and death, thus making a search for neuroprotective drugs an extremely important issue. Among the early events in ischemia-induced cascades, extracellular calcium entry is of a critical importance as it triggers numerous mechanisms leading to cell damage and death (Fern, 1998; Nowicky and Duchen, 1998). Blocking of Ca^{2+}-permeable *N*-methyl-D-aspartate–sensitive glutamate receptors markedly reduces the rise in intracellular calcium during anoxia (Silver and Erecinska, 1990) and has been shown to be neuroprotective in neuronal cell cultures (Choi, 1988) and in animal models of focal brain ischemia, hypoglycemia, and trauma (Albers et al., 1989) but not in animal models of transient global ischemia (Buchan and Pulsinelli, 1990). Moreover, glutamate receptor antagonists were disappointing in clinical trials for short-term treatment of brain ischemia because of significant side effects (http://www.strokecenter.org/trials/index_cats.htm).

During ischemia, L-type voltage-gated calcium channels also ensure an early rise in intracellular calcium. Inhibiting L-type voltage-gated calcium channels with dihydropyridine abolished the intracellular calcium rise usually observed after 2 to 4 min. However, no effect was detected on a small very early increase in intracellular calcium concentration (Pisani et al., 1998). Phase III clinical trials on L-type calcium channels inhibitors all concluded that there was no efficacy (http://www.strokecenter.org/).

The role of low-voltage–activated calcium channels (LVAs) during brain ischemia has never been studied. Low-voltage–activated calcium channels constitute a family of three types of calcium channels commonly referred to as T-type calcium channels: α1G or $Ca_V3.1$, α1H or $Ca_V3.2$, and α1I or $Ca_V3.3$ (for review, see Perez-Reyes, 2003). Most brain regions express more than one isoform, and some neurons, such as olfactory granule cells and hippocampal pyramidal neurons, express all three genes (Craig et al., 1999; Kase et al., 1999; Talley et al., 1999). An interesting property of T-type calcium channels is their ability to sustain a continuous calcium influx in neurons and glia at rest by a window current mechanism. The activation and inactivation curves of T-type calcium channels overlap and cross at approximately −60 mV. For this reason, a fraction of the channels cycle through all molecular conformations when the membrane potential

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ABBREVIATIONS: LVA, low-voltage–activated calcium channels; HEK, human embryonic kidney; eGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; OGD, oxygen-glucose deprivation; PI, propidium iodide; EPSP, excitatory postsynaptic field potential; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

ranges from −80 to −40 mV. Therefore, when neurons are at rest, a small fraction of channels are continuously open and sustain a tiny continuous calcium entry. This property has been shown to produce input signal amplification and bistability in thalamocortical neurons (Williams et al., 1997). It is implicated in the differentiation of myoblasts (Bijlenga et al., 2000), neuroendocrine differentiation of human prostate cancer cells (Mariot et al., 2002), and steroidogenesis in adrenal glomerulosa cells (Cohen et al., 1988; Rossier et al., 1998). Overexpression of T-type calcium channels in human embryonic kidney (HEK)-293 cells has been shown to increase the intracellular calcium concentration (Chemin et al., 2000). The early intracellular calcium concentration increase observed after initiation of an ischemic insult could be the expression of a change in equilibrium between the calcium influx, through T-type calcium channels, and efflux sustained by active mechanisms that dysfunction under metabolic stress.

In the present report, we describe the neuroprotective effects of various LVA inhibitors in a model of in vitro ischemia on rat organotypic hippocampal cultures. These observations support the hypothesis of a critical role for the T-type calcium "window" current during ischemic insult.

Materials and Methods

Immunostaining of Transfected HEK-293T Cells and Hippocampal Organotypic Cultures. HEK-293T cells were transfected as described previously (Chemin et al., 2001) using 2.7 μg of different pBK-CMV plasmid constructs that encode for $Ca_V3.1$ (Chemin et al., 2001) and $Ca_V3.2$ (Cribbs et al., 1998). In some experiments, the cells were cotransfected with 0.3 μg of pBB14 plasmid encoding the reporter gene for enhanced green fluorescent protein (eGFP; Brideau et al., 1998) to make transfected cells visible by fluorescence. Two days later, cells were harvested and plated on the sterile coverslips. One day later, cells were fixed for 10 min at 4°C in 4% paraformaldehyde in PBS, preincubated in PBS with 10% fetal calf serum (Invitrogen, Carlsbad, CA), and 0.3% Triton X-100 for 30 min and incubated with rabbit polyclonal anti-$Ca_V3.1$ or anti-$Ca_V3.2$ antibodies (1:500; Brueggemann et al., 2005) kindly supplied by Dr. L. Cribbs (Loyola University Medical Center, Maywood, IL), for 24 h at 4°C. Secondary goat anti-rabbit IgG antibodies labeled with tetramethylrhodamine isothiocyanate or fluorescein isothiocyanate (1:100 in PBS containing 10% fetal calf serum; Chemicon International, Temecula, CA) were applied for 2 h at room temperature. Cell nuclei were revealed by Hoescht staining (0.5 mg/ml, 5 min at room temperature; Sigma-Aldrich Chemie GmbH Munich, Germany), and the cells were mounted onto Superfrost slides with FluorSave (Calbiochem, San Diego, CA). Hippocampal organotypic slices were fixed by immersion in 4% paraformaldehyde solution for 30 min at 4°C and processed for $Ca_V3.1$ or $Ca_V3.2$ immunostaining as described above.

Oxygen-Glucose Deprivation Experiments in Organotypic Hippocampal Slice Cultures. Organotypic hippocampal slice cultures were prepared from 7-day-old rats and maintained for 11 to 12 days in culture before the experiments (Stoppini et al., 1991). Ischemic insult was performed in an interface-type chamber as described previously (Bancila et al., 2004). In brief, after placing the cultures in the chamber, oxygen-glucose deprivation (OGD) was produced by filling the chamber with a gas mixture containing 95% N_2 and 5% CO_2 and perfusing with a medium containing sucrose instead of glucose (124 mM NaCl, 1.6 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10 mM sucrose, and 2 mM ascorbic acid, pH 7.4; temperature, 32°C) for 10 min (in the text, the term "ischemia" is also applied to this experimental condition). Except for the case in which postischemic effects of mibefradil and nickel were tested, all the drugs were always applied 5 min before (to insure diffusion of drugs into cultures) and during the OGD episode. After the challenge, the cultures were placed back in the incubator for recovery in the usual culture medium. To study postischemic protection by mibefradil or nickel, drugs were added to the culture medium either just after or 3 or 6 h after the OGD insult and maintained in the medium throughout the whole postischemic period.

A few hours before OGD experiments, organotypic slices were transiently treated with propidium iodide (PI; 5 μg/ml, 20 min incubation; Sigma-Aldrich) and observed in a fluorescence microscope (Axioscop 2; Carl Zeiss, Jena, Germany) to assess cell viability. Only cultures exhibiting no or very low PI labeling at that stage were selected for the experiments. After the OGD, PI staining was carried out again at 2, 24, and 48 h to assess the timing and degree of ischemia-induced cell damage. In all experimental groups and at all time points, the images of PI staining were taken at the same magnification and exposure time, using the same settings of the digital camera (Axiocam; Carl Zeiss). The images were analyzed by using Photoshop7 software (Adobe Systems Inc., Mountain View, CA) (Bancila et al., 2004). Images of the whole hippocampal slice culture were taken and converted to gray-scale. We then adjusted the contrast so that the fluorescence precisely matched the contours of labeled pyramidal cell nuclei and then summed the intensity values of all pixels. We verified that these values were proportional to the number of PI-labeled nuclei as tested by direct visual counting on specific samples. The result obtained for each slice was then normalized to the mean of eight slice cultures exposed to OGD under control conditions within the same experiment. All fluorescence data were thus expressed as percent of a control value and then averaged across experiments. Statistical significance was assessed using the unpaired Student's t test.

Evoked Transmission Experiments in Organotypic Hippocampal Slice Cultures. Slice cultures were placed in a recording chamber and continuously perfused with artificial cerebrospinal fluid containing 124 mM NaCl, 1.6 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 10 mM glucose, and 2 mM ascorbic acid, pH 7.4, saturated with 95% O_2/5% CO_2. Stimulating and recording electrodes were positioned in CA1 stratum radiatum. Paired pulse-evoked excitatory postsynaptic field potentials (EPSPs) were recorded before and during application of 10 mM mibefradil using an Axoclamp 2B (Axon Instruments, Foster City, CA). Initial slopes and amplitudes of all EPSPs and ratios of paired pulse EPSPs amplitudes were calculated. Data obtained in the presence of mibefradil were expressed as percentage of control baseline values. Statistical significance was assessed using the paired Student's t test.

Results

Experiments on HEK-293T expressing recombinant $Ca_V3.1$ or $Ca_V3.2$ channels have shown that approximately 50% of Hoescht-labeled cells were stained using antibodies directed against $Ca_V3.1$ or $Ca_V3.2$ types of LVAs (Brueggemann et al., 2005) 24 h after transfection. No immunostaining was observed in nontransfected HEK-293T cells (data not shown). No cross-staining was observed when using anti-$Ca_V3.1$ antibodies on $Ca_V3.2$-transfected HEK-293T or vice versa (Fig. 1, A–D). Thus, antibodies used in our experiments adequately revealed $Ca_V3.1$ and $Ca_V3.2$ types of LVAs. These antibodies, when applied to rat organotypic hippocampal cultures, clearly showed labeling in the dentate gyrus, CA3 and CA1 areas (Fig. 1, E and F).

After exposure of organotypic hippocampal slice cultures to a transient (10 min) OGD episode, almost no cell death was observed 2 h after the insult, whereas at 24 h and 48 h, PI staining showed significant cell damage (Fig. 2, insets). Delayed cell death was usually observed in the CA1 area and

occasionally in CA3 and DG regions, if the damage was massive. As revealed in the preliminary experiments by means of electron microscopy, the damage was predominantly attributed to neurons, although glial population was not impaired by this brief OGD episode. Even 30 to 45 min of OGD did not provoke glial cell death inside the cultures, whereas neurons in all hippocampal areas showed in these conditions the signs of massive immediate death (data not shown). Thus, our in vitro ischemia model reproduces a delayed neuronal cell death similar to that observed in the penumbra region during stroke.

To check whether the induction of this delayed death was calcium-dependent, we performed these experiments in conditions of low extracellular calcium (calcium-EGTA buffered at 100 nM calcium) or increased intracellular calcium buffer capacity with BAPTA-acetoxymethyl ester (10 μM). In both cases, we observed significant neuroprotection: less than 20% of propidium iodide fluorescence was detected at 48 h compared with control cultures (100%) (Fig. 2).

Application of the LVA blocker mibefradil during the OGD insult at a concentration of 10 μM protected the cultures very significantly, with 13 $\pm$ 5.5% of PI fluorescence at 48 h compared with untreated control cultures ($p < 0.002$). When tested in eGFP/Ca_V3.2-cotransfected HEK cells, 10 μM mibefradil reduced the calcium current by 93 $\pm$ 2.7%, confirming that at this concentration of the drug, the inhibition was almost complete (data not shown). In addition, 10 μM mibefradil applied to hippocampal slice cultures did not significantly affect evoked excitatory transmission and thus did not alter action potential-dependent synaptic mechanisms. Neither the slopes, the amplitudes, nor the ratio of paired pulse-evoked EPSPs was significantly affected by more then 15 min of mibefradil application (93 $\pm$ 6, 98 $\pm$ 5, and 106 $\pm$ 8%, respectively; $n = 4$–5). At 1 μM mibefradil concentration, the neuroprotective effect was reduced but was still significant, with 39 $\pm$ 11% of propidium iodide fluorescence compared with control cells ($p < 0.05$). No significant inhibition of the calcium current and no neuronal protection were observed with 0.2 μM mibefradil, showing a clear dose dependence of the effect. To confirm this, we further tested the effects of other LVA blockers in our ischemia model. The neuroprotective effect of 350 and 500 nM kurtoxin applied during the OGD episode was very significant, with 38 $\pm$ 12% and 10.4 $\pm$ 3.2%, respectively, PI fluorescence after the insult compared with control cultures. In tandem, we observed no effect on cell survival with application of 50 nM kurtoxin (Fig. 2). A strong protection was also obtained with 50 μM nickel, 1 μM zinc, and, to a lesser extent, 1 μM pimozide (16 $\pm$ 6, 23 $\pm$ 2, and 35 $\pm$ 8% of propidium iodide fluorescence, respectively, 48 h after the insult compared with control cultures) (Fig. 2). These doses of nickel and zinc were also effective in blocking Ca_V3.2 current recorded from eGFP/Ca_V3.2-cotransfected

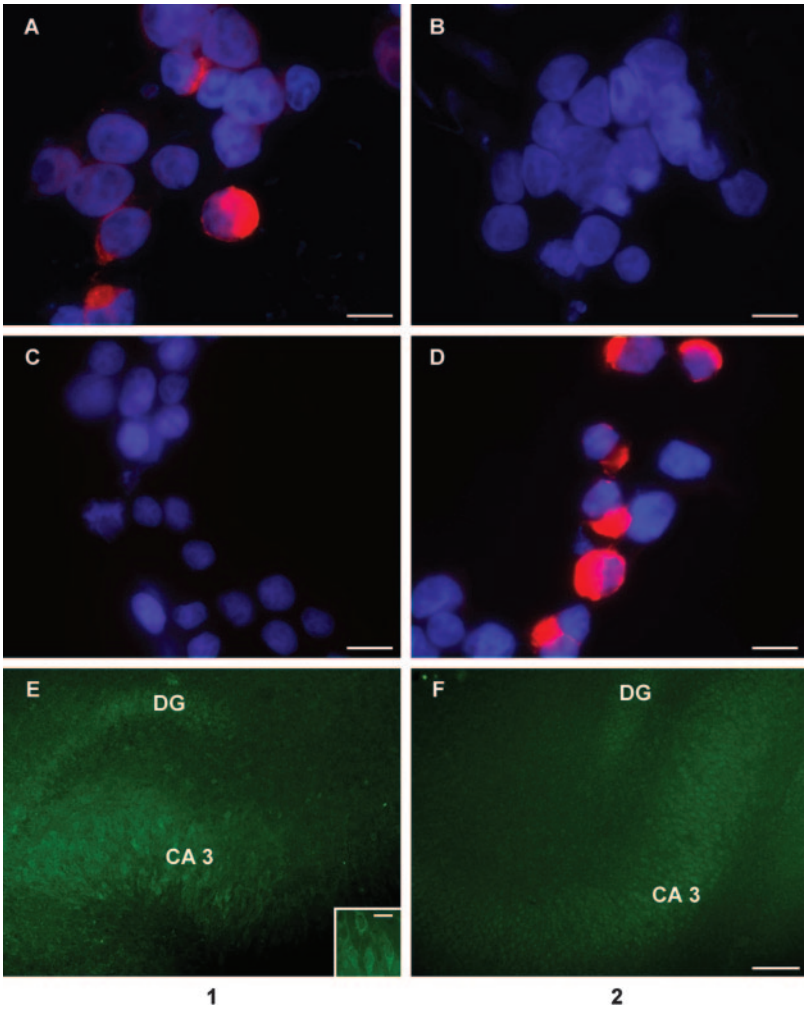

Fig. 1. Immunochemistry using antibodies against Ca_V3.1 (column 1) and Ca_V3.2 (column 2). A and B, Ca_V3.1-transfected HEK-293T cells. C and D, Ca_V3.2-transfected HEK-293T cells. Cell nuclei are blue (Hoechst staining); tetramethylrhodamine isothiocyanate-labeled secondary antibodies were used (red staining). E and F, LVA staining in organotypic hippocampal slices (fluorescein isothiocyanate-labeled secondary antibodies). CA3, CA3 pyramidal cell layer; DG, dentate gyrus. Inset, higher magnification in CA1 pyramidal layer. Scale bars: A–D, 10 μm; E and F, 100 μm; inset, 20 μm.

HEK-293T cells (85 ± 3.6 and 66 ± 8.5% inhibition, respectively; data not shown).

We measured the effect of the inhibition of high-voltage–activated calcium channels with 10 μM nifedipine on delayed neuronal death in the same conditions as used when inhibiting low-voltage–activated calcium channels. In this case, no significant reduction of PI fluorescence was observed (84 ± 22%, data not shown).

It is interesting that mibefradil or nickel (at a concentration of 10 or 50 μM, respectively) was also neuroprotective if applied not during the insult but immediately after and during the remaining 48 h of the experiment. Compared with control cultures, PI fluorescence at 48 h was reduced to 21 ± 5% in mibefradil-treated cultures and to 19 ± 3% in nickel-treated cultures ($p < 0.05$). A smaller but still significant protective effect was also observed when mibefradil or nickel was added to the culture medium 3 h after the OGD insult, with 53 ± 10% and 60 ± 15% of PI fluorescence at 48 h, respectively ($p < 0.03$). However, no significant protection was observed when mibefradil treatment was started 6 h after the OGD episode (Fig. 3).

Discussion

Much evidence indicates that a small and slow increase in intracellular calcium concentration takes place during the early phase corresponding to the first 2 to 3 min of ischemia. It starts just after cells transiently alkalinize secondarily to the hydrolysis of phosphocreatines (Silver and Erecinska, 1990). It has been observed in vivo and in vitro in acute

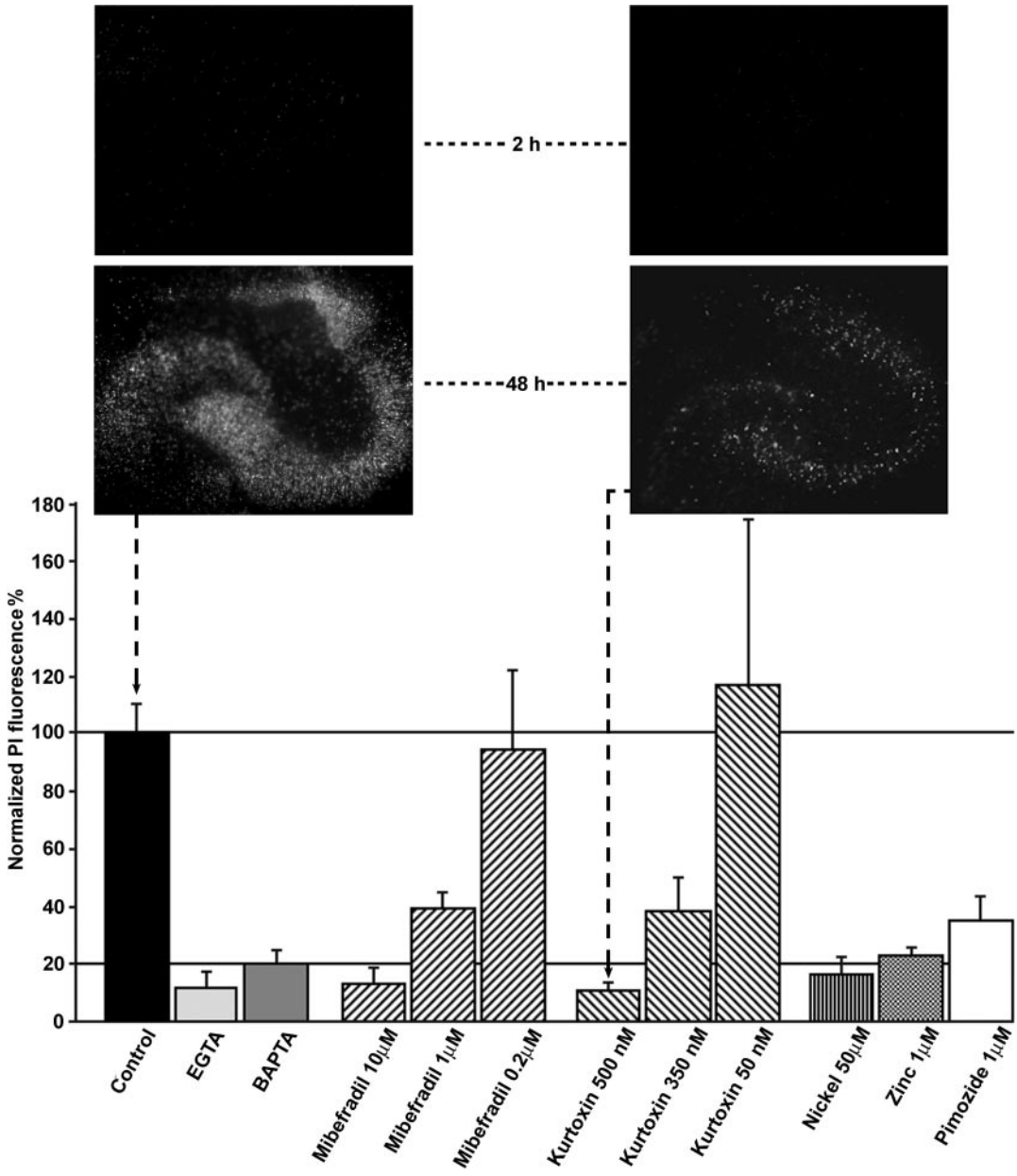

Fig. 2. Normalized propidium iodide fluorescence in organotypic hippocampal slices 48 h after transient OGD in the presence of low extracellular calcium concentration (EGTA), increased intracellular calcium buffering ability (BAPTA), or various concentrations of mibefradil, kurtoxin, nickel, zinc, and pimozide. Insets represent the examples of PI fluorescence images in organotypic hippocampal cultures 2 h and 48 h after insult in control condition and with 500 nM kurtoxin.

hippocampal slices and in dissociated neurons (Pisani et al., 1998). The origin of the calcium increase has been demonstrated to be extracellular, whereas intracellular and mitochondrial calcium stores seem not to be involved (Nowicky and Duchen, 1998). High-voltage–activated calcium channels have been proposed as a potential entry pathway for calcium, but application of dihydropyridine in animal studies (Silver and Erecinska, 1990) and in dissociated cortical neurons (Pisani et al., 1998) either did not modify the calcium concentration increase profile over time or acted only on a delayed component.

It has been suggested that low-voltage–activated calcium channels are an important factor for intracellular glial calcium increase and cell death during ischemia in neonatal rat optical nerve white matter (Fern, 1998). The aim of the present study was to evaluate the effect of inhibition of T-type calcium current on delayed postischemic neuronal death in hippocampal neurons.

In rat hippocampal organotypic cultures used in our in vitro ischemia model, low-voltage–activated calcium channels $Ca_V3.1$ and $Ca_V3.2$ are indeed expressed in CA1, CA3, and dentate gyrus areas, as shown by immunochemistry. Significantly fewer neurons suffer delayed neuronal death when exposed to low extracellular calcium or after increasing the intracellular calcium buffering ability during the OGD insult, which confirms the calcium dependence of delayed cell death and correlates with earlier observations (Abdel-Hamid and Tymianski, 1997).

In resting neurons and glia, the intracellular calcium concentration is maintained by the equilibrium between a constant passive calcium influx and an energy-consuming pumping. T-type voltage-gated calcium channels sustain a small but constant calcium current at membrane potential between −80 and −40 mV (Bijlenga et al., 2000). At rest, neurons and glia are hyperpolarized at nearly −70 mV. We expect that in resting neurons and glia, calcium continuously flows in through voltage-activated T-type calcium channels. During ischemia, the metabolic resources are challenged, and we hypothesize that calcium extrusion is affected. Reducing or abolishing the calcium influx would be beneficial by itself and would also spare energy for other essential cellular housekeeping activities. In line with this hypothesis, we show here

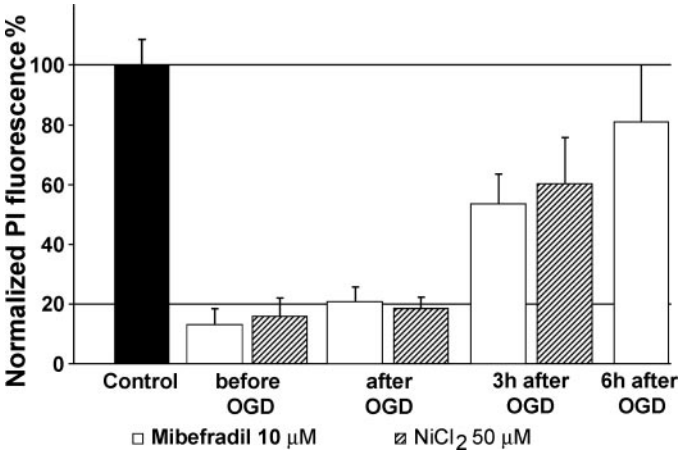

Fig. 3. Normalized PI fluorescence in organotypic hippocampal slices 48 h after transient OGD. Treatment with 10 μM mibefradil and 50 μM nickel occurred during OGD or was started at different times after the insult.

that pharmacological inhibition of the T-type calcium current by LVA blockers mibefradil, kurtoxin, nickel, zinc, and pimozide, at effective concentrations inhibiting more than 80% of the current, provides a very significant protection against delayed neuronal death. All these drugs are selective LVA inhibitors, although most of them have additional effects. Mibefradil was the first selective T-type calcium current blocker (Clozel et al., 1997), but it has been also shown to block delayed rectifier potassium channels (K_{dr}) at similar concentrations, to significantly inhibit L-type calcium channels (Liu et al., 1999), and to block sodium channels in a state-dependent manner (McNulty and Hanck, 2004). The effects observed here, however, are unlikely to have been caused by a block of sodium channels, because they could be reproduced by all other calcium channel antagonists used and because mibefradil did not significantly affect evoked action potential-dependent synaptic transmission under those conditions. Among other blockers tested, at the concentration used in our experiments, nickel is a traditional T-type calcium current blocker (Hille, 2001). Other divalent metals, such as Cu^{2+} and Zn^{2+}, have also recently been described to be selective T-type calcium inhibitors (Jeong et al., 2003). At small concentrations, as used in our experiments, zinc also activates ATP-sensitive potassium channels (K_{ATP}) (Bloc et al., 2000; Bancila et al., 2004). Kurtoxin is a peptide purified from scorpion venom and was reported to bind with high affinity to $Ca_V3.1$ type LVAs and to inhibit the T-type calcium current by modifying the channel gating. This toxin was shown to distinguish between T-type calcium channels and high-voltage–activated calcium channels (Chuang et al., 1998; Sidach and Mintz, 2002). At a concentration of 350 nM, kurtoxin was reported to inhibit almost totally the current of $Ca_V3.1$ channels and 80% of $Ca_V3.2$ channels current, whereas $Ca_V3.3$ channels seem to be resistant (Olamendi-Portugal et al., 2002). Pimozide, a neuroleptic drug, has also been described to be a selective T-type calcium channel blocker with a half-inhibition concentration estimated at ~100 nM for all Ca_V3 channels, although $Ca_V3.2$ is less sensitive (Santi et al., 2002). It is 10 times more selective for LVAs than for high-voltage–activated calcium channels. To exclude the possibility that neuroprotective effect observed with LVAs inhibitors is in fact a nonspecific effect caused by inhibition of high-voltage–activated calcium channels, we inhibited L-type calcium current with nifedipine and performed OGD. Under our experimental conditions, we observed no significant protection against delayed neuronal death.

Rekling (2003) has reported the neuroprotective effect of anticonvulsants on organotypic hippocampal cultures subjected to transient ischemia. It is interesting that ethosuximide, phenobarbital, and phenytoin, reported to be the most neuroprotective anticonvulsants, are also the drugs with the most potent T-type calcium current inhibitory activity (EC_{50} values of 23.7, 1.7, and 7.3 μM, respectively) (Todorovic and Lingle, 1998; Todorovic et al., 2000).

Despite the different pharmacological profiles of the LVAs blockers tested, all of them act as potent T-type calcium current inhibitors, and all of them ensured significant neuroprotection after a transient OGD in our experiments. The converging neuroprotective effects of these various drugs, which, at the concentrations used, exhibit selectivity for T-type calcium channels, support the hypothesis suggesting an

important role for LVAs in ischemia-induced cell damage. Because the effect may be neuroprotective not only when the drugs are applied during the OGD episode, as shown with mibefradil and nickel, but also when applied shortly after the insult, these data open new perspectives of possible pharmacological approaches to neuroprotection.

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