Neuroscience 128 (2004) 729–740

Na^+ AND Ca^{2+} HOMEOSTASIS PATHWAYS, CELL DEATH AND PROTECTION AFTER OXYGEN–GLUCOSE-DEPRIVATION IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

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Abstract—Intracellular ATP supply and ion homeostasis determine neuronal survival and degeneration after ischemic stroke. The present study provides a systematic investigation in organotypic hippocampal slice cultures of the influence of experimental ischemia, induced by oxygen-glucose-deprivation (OGD). The pathways controlling intracellular Na^+ and Ca^{2+} concentration ($[Na^+]_i$ and $[Ca^{2+}]_i$) and their inhibition were correlated with delayed cell death or protection. OGD induced a marked decrease in the ATP level and a transient elevation of $[Ca^{2+}]_i$ and $[Na^+]_i$ in cell soma of pyramidal neurons. ATP level, $[Na^+]_i$ and $[Ca^{2+}]_i$ rapidly recovered after reintroduction of oxygen and glucose. Pharmacological analysis showed that the OGD-induced $[Ca^{2+}]_i$ elevation in neuronal cell soma resulted from activation of both *N*-methyl-D-aspartate (NMDA)-glutamate receptors and Na^+/Ca^{2+} exchangers, while the abnormal $[Na^+]_i$ elevation during OGD was due to Na^+ influx through voltage-dependent Na^+ channels. In hippocampal slices, cellular degeneration occurring 24 h after OGD, selectively affected the pyramidal cell population through apoptotic and non-apoptotic cell death. OGD-induced cell loss was mediated by activation of ionotropic glutamate receptors, voltage-dependent Na^+ channels, and both plasma membrane and mitochondrial Na^+/Ca^{2+} exchangers. Thus, we show that neuroprotection induced by blockade of NMDA receptors and plasma membrane Na^+/Ca^{2+} exchangers is mediated by reduction of Ca^{2+} entry into neuronal soma, whereas neuroprotection induced by blockade of AMPA/kainate receptors and mitochondrial Na^+/Ca^{2+} exchangers might result from reduced Na^+ entry at dendrites level.

Key words: experimental ischemia, ATP level, intracellular ion homeostasis, glutamatergic transmission, neurodegeneration, neuroprotection.

The pathogenesis of cerebral ischemia involves massive energy depletion leading to loss of cellular ion homeostasis. The Ca^{2+} ion is an important intracellular messenger which modulates a variety of cellular processes. Cells regulate their Ca^{2+} homeostasis by a complex interplay between three cellular mechanisms, Ca^{2+} influx and efflux through the plasma membrane, Ca^{2+} uptake and release from intracellular stores and intracellular Ca^{2+} buffering. Ca^{2+} fluxes are directly or indirectly energy-dependent and can thus be drastically altered by cerebral ischemia. Intracellular Ca^{2+} is abnormally increased in neurons exposed to ischemic conditions. This effect has been described for *in vivo* conditions (Nakamura et al., 1999; Silver and Erecinska, 1992), during *in vitro* ischemia in hippocampal slices (Lobner and Lipton, 1993; Zhang and Lipton, 1999) and in neuronal cell cultures exposed to mitochondrial and glycolytic inhibitors (Dubinsky and Rothman, 1991). In these studies, Ca^{2+} rises during the first few minutes after ischemia and progressively returns close to the resting level after the end of the ischemic conditions.

Several lines of evidence demonstrate that the imbalance in the Ca^{2+} homeostasis leads to neurodegeneration by triggering a series of adverse reactions (Kristian and Siesjo, 1996; Sattler and Tymianski, 2000). Overactivated proteases, lipases, endonucleases and phosphatases have been proposed to mediate cell degeneration in vulnerable cell populations through necrotic and/or apoptotic cell death pathways. Also an intraneuronal Na^+ overload seems to be involved in the pathogenesis of cerebral ischemia, since inhibition of extracellular Na^+ influx has been shown to reduce neuronal ischemic vulnerability (Breder et al., 2000; Fried et al., 1995; Tasker et al., 1992). The exact mechanisms through which intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular Na^+ concentration ($[Na^+]_i$) are increased during ischemia, and the pathological processes culminating in neuronal cell loss are still poorly understood. So far, most studies focused on the role of postsynaptic glutamate receptors. During cerebral ischemia there is an excessive synaptic release of glutamate and a decrease in glutamate transporter activity. Glutamate binds to postsynaptic receptors, including the *N*-methyl-D-aspartate (NMDA) receptor and the 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA)/kainate receptor. Activation of these receptors results in

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Abbreviations: AMPA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CGP-37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)one; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPA, cyclopiazonic acid; IP_3, inositol trisphosphate; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea methanesulphonate; MEM, Minimal Essential Medium; MK-801: (5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate; $[Na^+]_i$, intracellular Na^+ concentration; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; NMDA, *N*-methyl-D-aspartate; OGD, oxygen–glucose-deprivation; PBS, phosphate buffered saline; PI, propidium iodide; SBFI, Na^+-binding benzofuran isophthalate; SERCA, sarcoendoplasmic reticulum Ca^{2+}ATPase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; 2-APB, 2-aminoethoxydiphenyl borate.

0306-4522/04$30.00+0.00
doi:10.1016/j.neuroscience.2004.06.074

opening of the associated ion channels and leads to the entry of extracellular Ca^{2+} and Na^{+} into the cells. NMDA-glutamate receptors are widely accepted as an important contributor to the $[Ca^{2+}]_i$ increase induced by ischemia (Kubo et al., 2001; Zhang and Lipton, 1999). Moreover, antagonists of this receptor type have been shown to protect neurons *in vitro* (Newell et al., 1995) and after transient global and focal ischemia (Foster et al., 1988; Hatfield et al., 1992). In contrast, the contribution of AMPA/kainate receptors to both intracellular Ca^{2+} and/or Na^{+} accumulation and neuronal cell loss is still controversial (Laake et al., 1999; Pringle et al., 1997; Tasker et al., 1992; Zhang and Lipton, 1999).

Besides ionotropic glutamate receptors, further routes contributing to the entry of extracellular Ca^{2+} and Na^{+} through the plasma membrane are the voltage-dependent Ca^{2+} and Na^{+} channels, which are expected to be activated upon plasma membrane depolarization induced by ischemia. Studies by Goldberg and Choi (1993) revealed a significant contribution of voltage-dependent Ca^{2+} channels to the ischemia-related $[Ca^{2+}]_i$ rise. Nevertheless, the involvement of these channels in the post-ischemic neurodegeneration is still a matter of debate (see Kobayashi and Mori, 1998 for review). The plasma membrane in addition contains Na^{+}/Ca^{2+} exchangers, which under normal conditions extrude Ca^{2+} from the intracellular space. This electrogenic exchanger can, however, reverse its mode of operation under certain pathological conditions and aggravate ischemic intracellular Ca^{2+} accumulation (Blaustein and Lederer, 1999). Blockade of this ionic pathway has clear neuroprotective effects in different models of ischemia (Breder et al., 2000; Matsuda et al., 2001; Schroder et al., 1999). This complex scenario is further complicated by the contribution of intracellular stores to the cytosolic Ca^{2+} elevation. Under ischemic conditions, mitochondria are thought to accumulate Ca^{2+}, to induce the generation of harmful reactive oxygen species and to release pro-apoptotic factors contributing to ischemic cell damage (Kristian and Siesjo, 1996). In acute hippocampal slices, mitochondrial Ca^{2+} release is involved in the ischemic Ca^{2+} elevation observed in dendrites of CA1 pyramidal neurons (Zhang and Lipton, 1999). In addition, Ca^{2+} release from the endoplasmic reticulum has been proposed as an important mediator of ischemic cell loss (Paschen, 2003). Cytosolic Ca^{2+} is pumped into the endoplasmic reticulum by a sarcoendoplasmic reticular Ca^{2+}-ATPase (SERCA) pump and released via two receptors, the inositol trisphosphate (IP_3)-sensitive receptor and the ryanodine-sensitive receptor. Different SERCA inhibitors have been described to trigger toxicity in neuronal cultures through an apoptotic pathway (Nguyen et al., 2002). By contrast, blockade of endoplasmic reticulum Ca^{2+} release via the ryanodine-sensitive receptors reduced the $[Ca^{2+}]_i$ rise induced by oxygen-glucose-deprivation (OGD) in hippocampal slices (Mitani et al., 1993) and the neuronal vulnerability in *in vivo* ischemic models (Nakayama et al., 2002; Zhang et al., 1993).

The complexity of the ionic processes implicated in ischemic neurotoxicity and the controversy of the role of the various ionic pathways involved requires a systematic analysis. The present study focuses on neurodegeneration caused by OGD and the connection to somatic and/or dendritic transient elevations of intraneuronal $[Ca^{2+}]_i$ and $[Na^{+}]_i$. We provide the first comprehensive and detailed investigation, using identical test conditions in one system, i.e. organotypic hippocampal slice cultures, a model that preserves the neuronal circuitry, to study the influence of experimental ischemia. We explored several pathways controlling intracellular Na^{+} and Ca^{2+} concentration, and aimed to correlate the inhibition of these pathways with delayed cell death or protection.

EXPERIMENTAL PROCEDURES

Preparation and maintenance of organotypic hippocampal slice cultures

Slice cultures were prepared according to Stoppini et al. (1991) from rat pups (8–10 days old Wistar rats) provided by Harlan Winkelmann (Borchen, Germany). After decapitation brains were removed aseptically and placed in sterile preparation medium containing 25% Minimal Essential Medium (MEM)-Hanks' (Gibco, Eggenstein, Germany), 25 mM HEPES and 2 mM L-glutamine, that was kept at 3 °C. After hippocampus isolation, 350 μm thick slices were prepared by using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Guildford, UK). Only slices with continuous bright transparent neuronal layers were selected and placed on membrane inserts in six-well plates (NUNC, Wiesbaden, Germany). Each well contained 1.2 ml of tissue culture medium consisting of 50% MEM (Biochrom, Berlin, Germany), 25% Hanks' balanced salt solution (Gibco), 25% horse serum (Gibco), 350 mg/ml $NaHCO_3$ (Sigma, St. Louis, MO, USA), pH 7.3–7.4. Cultures were maintained in a humidified incubator with 5% CO_2 at 36 °C changing the culture medium three times per week. All cultures used in this study were grown for 8–12 days. Animals were treated according the European Guidelines on Animal handling (86/609/EU) and experimental procedures were approved by the local Institutional Animal Care committee. All possible efforts were made to minimize animal suffering and the number of animals used.

ATP content determination

ATP content of slice cultures was determined by the use of the commercial bioluminescence assay (ATP Bioluminescence Assay Kit HS II; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. OGD of graded severity was induced by placing the cultures in a humidified anaerobic chamber (Almedica AG, Galmiz, Switzerland) aerated with an anoxic gas mix (95% N_2/5% CO_2) at 37 °C and incubated with buffer solution consisting of 124 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$, 2 mM $CaCl_2$ and 10 mM mannitol (OGD solution). Either immediately or 1 h following OGD, cultures were removed from the membrane insert, immersed in 2 ml of boiling solution containing Tris 100 mM and EDTA 4 mM, boiled for 3 min and centrifuged. Supernatant was diluted 1:10 in dilution buffer (Roche Diagnostics) and measurements were made using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Harzburg, Germany). Luciferase reagent (100 μl) was injected together with 100 μl of diluted sample. Data are expressed as percentage of ATP in untreated controls ±S.E.M. Mann-Whitney test was used to compare values. A probability level of 0.05 was considered as the limit of significance.

Intracellular ion measurements

Intracellular Ca^{2+} and Na^{+} ions were monitored by fluorescence microscopy using an environmentally regulated perfusion chamber mounted on the stage of an inverted fluorescence microscope (Axiovert 100 TV; Zeiss, Jena, Germany). Cultures on their supporting membranes were transferred to the flow-through chamber (2.2 ml/min) and were continuously superfused with buffer solution consisting of 124 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$, 2 mM $CaCl_2$ and 10 mM glucose equilibrated with 95% O_2/5% CO_2 (pH 7.4; normoxic solution). To reduce dye compartmentalization the temperature during the complete experimental procedure was maintained at 27 °C. $[Ca^{2+}]_i$ was measured by incubating slice cultures with either the high-affinity Ca^{2+} dye fura-2 (50 μM) or the low affinity Ca^{2+} dyes magfura-2 (5 μM) and fura-FF (5 μM). The Na^{+}-sensitive fluorescent dye Na^{+}-binding benzofuran isophthalate (SBFI, 10 μM) was used to monitor changes in the $[Na^{+}]_i$. All these dyes were used in their membrane permeant acetoxymethylester form. Dyes were dissolved in normoxic solution and supplemented with 0.5% of dimethyl sulfoxide and 10 μl/ml pluronic F-1270. The loading solution (1 ml) was added to the surface of the membranes and was allowed to permeate during 45 min in the case of Ca^{2+} dyes and during 120 min for SBFI.

After loading, the culture membrane was removed from the insert and put into the recording chamber. Perfusion with normoxic solution was applied to wash the excess of non-loaded dye. OGD was induced for 20 or 40 min by perfusing the cultures with OGD solution. The perfusate took approximately 2 min to go through the connecting tube and to completely replace the buffer in the recording chamber. After OGD, perfusion with normoxic solution for a further 20 min (reperfusion) was reinitiated to allow ion recovery. Computer-based image analysis software TillVision v3.3 (T.I.L.L. Photonics, Martinsried, Germany) was used to analyze changes in the fluorescence emission of the dyes during OGD and reperfusion. Experiments were performed in CA3 pyramidal neurons by outlining a region of interest around cell soma and applying these regions to subsequent images. Only neurons (10–20 per study) with clear smooth membranes that did not show any apparent soma swelling were selected. Fluorescence images were acquired every 20 s at 510 nm during alternate excitation at 340 and 380 nm with a Xenon Lamp (Ushio Inc, Japan). Results are expressed as magnitude of the increase in the fluorescence ratio (F340 nm/F380 nm) evoked by OGD above the preischemic ratio value (R_0). Data are expressed as means ±S.E.M. *t*-Test was used to compare the ratio increases (Δ ratio) after OGD insults of different severity. A probability level of 0.05 was considered as the limit of significance. In some experiments calibration of the ratio values obtained with fura-2 and fura-FF were performed as described by Grynkiewicz et al. (1985). Calibration of the magfura-2 and SBFI ratio signal was not possible.

Assessment of nerve cell injury

Hippocampal slice cultures were exposed to OGD at 37 °C lasting for either 20 or 40 min, as described for ATP content determination. Following OGD, culture plates were put back into the incubator. Neuronal damage was analyzed 24 h after OGD by using four separate criteria. Firstly, macroscopic estimation of the tissue damage using an inverted light microscope at a magnification of 20× (Eclipse TE 300; Nikon, Düsseldorf, Germany). Darkening of the tissue culture was considered as a feature of tissue damage. Secondly, Nissl staining to study cell morphology. Cultures were washed with 0.1 M phosphate buffered saline (PBS) and fixed with a solution of 4% paraformaldehyde in 0.1 M PBS for 30 min at 4 °C. Cultures were rinsed again in PBS, permeabilized with Triton X-100 (0.5%) in PBS for 5 min, incubated for 45 min with a solution of Neurotrace 500/525 (dilution 1:100 in PBS) and coverslipped with Antifade mounting media. Thirdly, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay to identify apoptotic cells. Cultures were fixed, permeabilized with 0.5% Triton X-100 in PBS for 5 min and incubated with the TUNEL reaction mixture for 60 min at 37 °C. After washing with PBS, cultures were mounted with Antifade media. Non-specific labeling was investigated by omitting the enzyme terminal deoxynucleotidyl transferase. Fourthly, propidium iodide (PI) staining was used to assess cellular loss. Live rather than fixed cultures were exposed to PI (10 ng/ml) dissolved in culture medium during 2 h at 37 °C.

Confocal laser scanning images were captured using an inverted microscope (Axiovert 100 M; Zeiss) equipped with an argon laser source and a confocal system (LSM 510; Zeiss). The following wavelengths were used: Nissl and TUNEL staining, excitation 488 nm, emission 510 nm; PI staining, excitation 530 nm, emission 610 nm.

Pharmacological manipulations

Intracellular Ca^{2+} and Na^{+} recordings. Contribution of potential ion sources to the OGD-induced $[Ca^{2+}]_i$ and $[Na^{+}]_i$ deregulation were investigated by drug-induced modulation of magfura-2 and SBFI recordings. A battery of known glutamate receptor antagonists and ion channel blockers was separately dissolved in the perfusate medium and applied by bath application starting from the dye loading procedure to the end of OGD lasting for 40 min. Drugs used were: the NMDA glutamate receptor antagonist (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), the AMPA (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate/kainate receptor antagonist NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide), the plasmalemma $3Na^{+}/Ca^{2+}$ exchanger blocker 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate (KB-R7943), the L-type Ca^{2+} channel blocker nimodipine, the broad spectrum Ca^{2+} channel blocker mibefradil, the voltage-dependent Na^{+} channel blocker lidocaine, the SERCA–ATPase inhibitor cyclopiazonic acid (CPA), the IP_3-sensitive receptor blocker 2-aminoethoxydiphenyl borate (2-APB), the ryanodine-sensitive receptor blocker dantrolene and the mitochondrial $2Na^{+}/Ca^{2+}$ exchanger inhibitor 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)1 (CGP-37157). None of these drugs affected the basal magfura-2 fluorescence under normoxic conditions. By contrast, in SBFI experiments performed with lidocaine and NBQX, lidocaine strongly reduced the basal SBFI fluorescence. Both Ca^{2+} and Na^{+} experiments used paired drug-perfused and control cultures from the same animal. Each paradigm consisted of four to seven such pairs each coming from a different rat. Four parameters were investigated (Fig. 3A): the *lag time* as the time needed to induce a significant rise of the fluorescence signal above R_0 (corresponds to pre-OGD value) and calculated by a stepwise series of one-tailed serial *t*-tests averaged to R_0; the *ratio rise* giving the difference between the maximal ratio value during OGD and reperfusion and R_0; the *recovery* of the ratio measured as the percentage of ratio return to R_0 at the end of reperfusion, and the *integrated response* of the ion signal or area under the curve, calculated by the sum of the semi-areas defined by the ion signal, starting from R_0 to the end of reperfusion (*integrated response*$=20\times[(R_n+R_{n+1})/2]$). All values are expressed as mean±S.E.M. Paired *t*-test was used to compare values in control and in drug-perfused experiments. A $P<0.05$ was considered significant.

Neuronal death. Neuroprotective properties of the drugs employed for intracellular ion recordings were tested in hippocampal slice cultures by quantifying PI uptake 24 h after OGD lasting for 40 min. Cultures were categorized in three conditions, either non drug-supplemented cultures exposed to OGD (OGD control), drug-supplemented cultures exposed to OGD and drug-supplemented cultures not exposed to OGD. The latter group was included to evaluate drug neurotoxicity. When in the results section this group is not specified, this

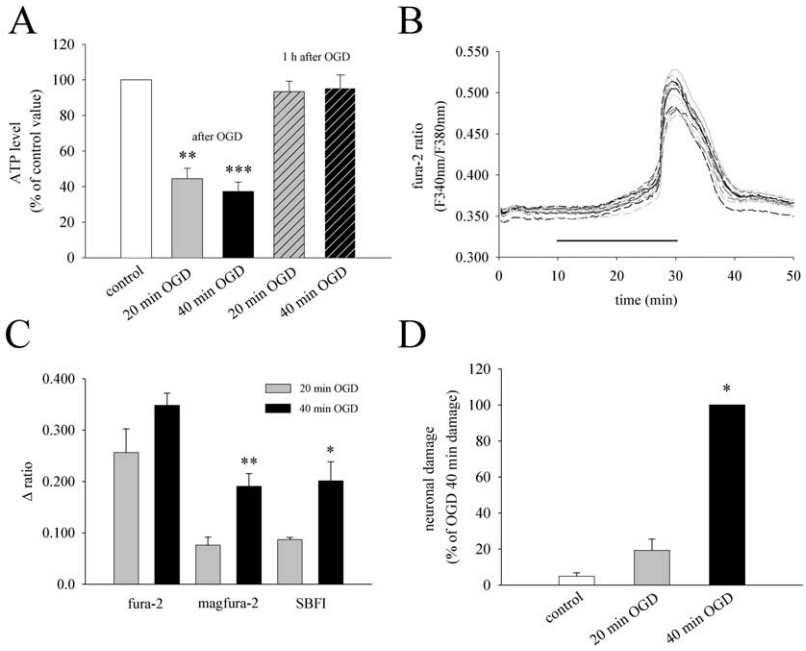

Fig. 1. Alterations in cellular energy, ion homeostasis and cellular viability induced by OGD in hippocampal slice cultures. (A) Modulation of ATP content by OGD and reperfusion in hippocampal slice cultures. ATP quantification was performed either immediately after OGD or 1 h after OGD which lasted for 20 or 40 min. Results are expressed as mean±S.E.M. Since cultures were from independent animals the significance of the difference was determined using the Mann-Whitney Test (** $P<0.01$; *** $P<0.001$). (B) Example of $[Ca^{2+}]_i$ rise induced by OGD in cell somata of CA3 hippocampal pyramidal neurons measured with fura-2. The horizontal bar indicates the duration of OGD (20 min). (C) Quantitative evaluation of intracellular Ca^{2+} and Na^+ elevations induced by OGD lasting for 20 and 40 min in hippocampal pyramidal neurons. Fura-2 and magfura-2 were used to monitor Ca^{2+} transients, while the Na^+-sensitive dye SBFI was used to investigate OGD-induced $[Na^+]_i$ rise in hippocampal neurons. Results are expressed as mean±S.E.M. The significance of the difference was determined using the *t*-test (* $P<0.05$; ** $P<0.01$). (D) Quantitative evaluation of OGD-induced cell damage by PI-uptake 24 h after OGD lasting for 20 and 40 min. Results are expressed as mean±S.E.M. Critical comparisons were made from cultures obtained from the same animal. Therefore, the Wilcoxon Test (* $P<0.05$) was chosen to determine the significance of the difference.

implies that the drug did not affect neuronal viability. Drugs were applied by two different protocols, either the *short protocol* by exposing cultures to a certain drug for 2 h prior to OGD, during and for 2 h following OGD; or the *long protocol* by exposing cultures to a drug 2 h before OGD, during and for 24 h following OGD. Cultures were incubated with PI as previously described. The probe was excited with a 510–560 nm light and the emitted fluorescence acquired at 610 nm using an inverted fluorescence microscope (Eclipse TE 300; Nikon). Simultaneously a transmission image was collected to identify the cell subfields within each culture. Images were captured using a CCD camera (Visitron Systems, Puchheim, Germany) stored and analyzed on a PC with the image analysis software LUCIA (Nikon). For the quantification of neuronal damage, the percentage of the CA1–CA3 subfields expressing PI fluorescence above background level was calculated in relation to the total area of each culture. This value obtained in slices exposed to 40 min OGD was set to 100% damage and was then compared with the fluorescence intensity following other experimental conditions. This gives % of control OGD damage. Values are expressed as means±S.E.M. The non-parametric Wilcoxon matched pair test was used to compare neuronal damage of drug-incubated cultures against standard damage of control cultures. $P<0.05$ was considered significant.

Chemicals

Fura-2, magfura-2, SBFI, Neurotrace 500/525, DMSO and Antifade were from Molecular Probes (Eugene, OR, USA). TUNEL reaction kit was from Roche Diagnostics. MK-801, NBQX, nimodipine, CGP-37157, CPA, and KB-R7943 were provided by Tocris (Ballwin, MO, USA). Dantrolene and 2-APB were obtained from Calbiochem (Darmstadt, Germany), lidocaine and pluronic F-127 were from Fluka Chemica (Steinheim, Germany). Mibefradil was a generous gift from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents were from Sigma (St. Louis, MO, USA) or from Merck (Schuchardt, Germany).

RESULTS

Effect of experimental ischemia on ATP content, $[Ca^{2+}]_i$, $[Na^+]_i$ and neuronal death

The effect of OGD on ATP content was investigated in organotypic hippocampal slice cultures by bioluminescence quantification. ATP content was reduced in proportion to OGD severity. OGD lasting for 10 min partially reduced the ATP content (data not shown). Significant reduction of the ATP content, when compared with control conditions, was achieved after 20 min of OGD (Fig. 1A, $n=22$). OGD lasting for 40 min further reduced the ATP content when compared with control values (Fig. 1A, $n=22$). After readmission of oxygen and glucose (reperfusion), ATP levels recovered back to control values within 1 h (Fig. 1A, $n=16$).

Intracellular Ca^{2+} transients induced by OGD and reperfusion were investigated in organotypic hippocampal cultures by fluorescence microscopy. One high affinity Ca^{2+} dye, fura-2, and two low affinity Ca^{2+} dyes, magfura-2 and fura-FF, were used to monitor $[Ca^{2+}]_i$ ranging from nanomolar to micromolar Ca^{2+} values. Intracellular

Ca^{2+} measurements with fura-2 (K_d=224 nM) revealed that at resting levels, the $[Ca^{2+}]_i$ in the cell soma of CA3 pyramidal neurons was 103.4±38.5 nM (n=8). OGD induced in this region a slow and progressive rise in the $[Ca^{2+}]_i$ at 11.2±1.6 min (n=13) after OGD induction (Fig. 1B). After OGD the fura-2 signal returned close to resting levels, independent of insult severity. However, the magnitude of the fura-2 ratio signal did not significantly vary between OGD insults of 20 and 40 min (Fig. 1C). Similarly, the peak of $[Ca^{2+}]_i$ was comparable between both OGD exposures, being 234.5±90.2 nM (n=4) at the end of 20 min OGD and of 345.5±89.3 nM (n=10) after 40 min OGD.

To investigate whether fura-2 underestimates the $[Ca^{2+}]_i$ during long lasting OGD, the experiments were repeated using Ca^{2+} dyes of lower affinity. When measured with magfura-2 (K_d=25 μM) Ca^{2+} transients induced by OGD started 21.3±0.8 min (n=10) after OGD induction. Magfura-2 measurements revealed ischemic Ca^{2+} alterations that were proportional to the duration of OGD (Fig. 1C, n=10). Since magfura-2 does not allow calibration of the Ca^{2+} signal, we also used fura-FF (K_d=5 μM), which reported at the end of 40 min OGD a $[Ca^{2+}]_i$ of 5.38±1.42 μM (n=4). By using the Na^+-sensitive fluorescent dye SBFI (K_d=11.3 mM) we observed a transient elevation of the $[Na^+]_i$ in cell somata of CA3 hippocampal neurons exposed to OGD conditions. The $[Na^+]_i$ rise showed a peak at 12.01±2.01 min (n=10) after induction of OGD and was proportional to the insult severity (Fig. 1C, n=5).

After showing that the duration of an OGD episode crucially modulates ATP level, as well as $[Ca^{2+}]_i$ and $[Na^+]_i$, we investigated the effects of OGD severity on neuronal viability. For this purpose, different staining procedures were performed 24 h after OGD. Fluorescence Nissl (Neurotrace) was used to investigate morphological characteristics of the cells, nuclear uptake of PI was employed to identify necrotic cells with impaired membrane integrity and TUNEL assay was used to identify apoptotic cells.

Control cultures displayed a healthy appearance with clear delimitation of all cell layers (Fig. 2A). Pyramidal neurons showed intact cell membranes, small and round cell soma and short dendritic processes (Fig. 2B). Under these conditions almost no TUNEL-positive cells (Fig. 2C) and very few PI-labeled cells (Fig. 2D) were distinguished with no specific distribution. OGD lasting for 20 min induced a weak darkening of the hippocampal tissue (Fig. 2E) although only minimal morphological signs of cell degeneration were evident (Fig. 2F). Consistently, negligible presence of TUNEL-positive cells (Fig. 2G) and a very moderate PI uptake were seen (Fig. 2H). These parameters were dramatically altered after exposing slice cultures to 40 min OGD. Cultures displayed a widespread darkening of their tissue (Fig. 2I). Degenerated cells identified by loss of cell cytoarchitecture and pycnotic appearance were observed in the pyramidal and granular cell layer (Fig. 2J). In these cell layers numerous apoptotic cells (Fig. 2K) were accompanied to a large extent by PI-stained cells (Fig. 2L). PI staining provided an index of cell death and confirmed the correlation between insult severity and degree of neurodegeneration induced by OGD. In hippocampal slice cultures at least 40 min of OGD are needed to cause a widespread and significant neuronal damage (Fig. 1D, n=5).

Intracellular Ca^{2+} and Na^+ overload and correlation with OGD-induced neurotoxicity

By the use of a well-known range of glutamate receptor antagonists and ion channel blockers we investigated the routes responsible for the ischemic intracellular Ca^{2+} and Na^+ overload and determined whether their blockade enhanced neuronal survival after OGD. Sufficient neuronal damage was ensured by OGD lasting for 40 min. Under these experimental conditions the possibility of Ca^{2+} signal underestimation was avoided by using magfura-2 for Ca^{2+} recording.

Role of NMDA receptors

The role of NMDA receptors in OGD-induced neuronal Ca^{2+} overload and neuronal loss was tested by the use of the non-competitive NMDA receptor antagonist MK-801. MK-801 (10 μM) markedly reduced the rise of $[Ca^{2+}]_i$ induced by OGD in cell somata of hippocampal pyramidal neurons (Fig. 3A, n=5). The drug increased the interval required to elevate $[Ca^{2+}]_i$ (lag time) significantly, reduced the amplitude of the maximal increase of the Ca^{2+} signal and improved the Ca^{2+} recovery to baseline during reperfusion. The mean values for these parameters are given in Table 1. MK-801 significantly reduced the integrated response of the Ca^{2+} signal when compared with control experiments (Fig. 3A, n=5). Moreover, we found that MK-801 added to the incubation medium from 2 h before OGD until 2 h after OGD (short protocol) drastically reduced the neuronal degeneration induced by OGD (Fig. 4, n=5). When the drug application was prolonged for the next 24 h after OGD (long protocol), the rate of neuroprotection was not different from that induced by the previous condition (Fig. 4, n=5).

Role of AMPA/kainate receptors

Contribution of AMPA/kainate glutamate receptors to the intracellular Ca^{2+} and Na^+ deregulation induced by experimental ischemia, and the subsequent neuronal cell loss was evaluated by using the competitive AMPA/kainate receptor antagonist NBQX. Presence of NBQX (10 μM) in the perfusion media did not significantly modify intracellular Ca^{2+} transients induced by OGD in the soma of pyramidal neurons (Fig. 3B, n=7, Table 1). We next investigated the involvement of AMPA/kainate receptors in the intracellular Na^+ overload induced by OGD. As in Ca^{2+} recordings, NBQX failed to show a clear effect on the SBFI signal when compared with control experiments (Fig. 3C; n=4, Table 1). Despite the lack of effectiveness of NBQX to alter ischemic Ca^{2+} and Na^+ transients, NBQX was clearly neuroprotective (Fig. 4, n=5).

Beneficial effects of NBQX when applied by the short protocol were even enhanced by prolonging the drug presence in the culture medium by the long protocol (Fig. 4, n=5).

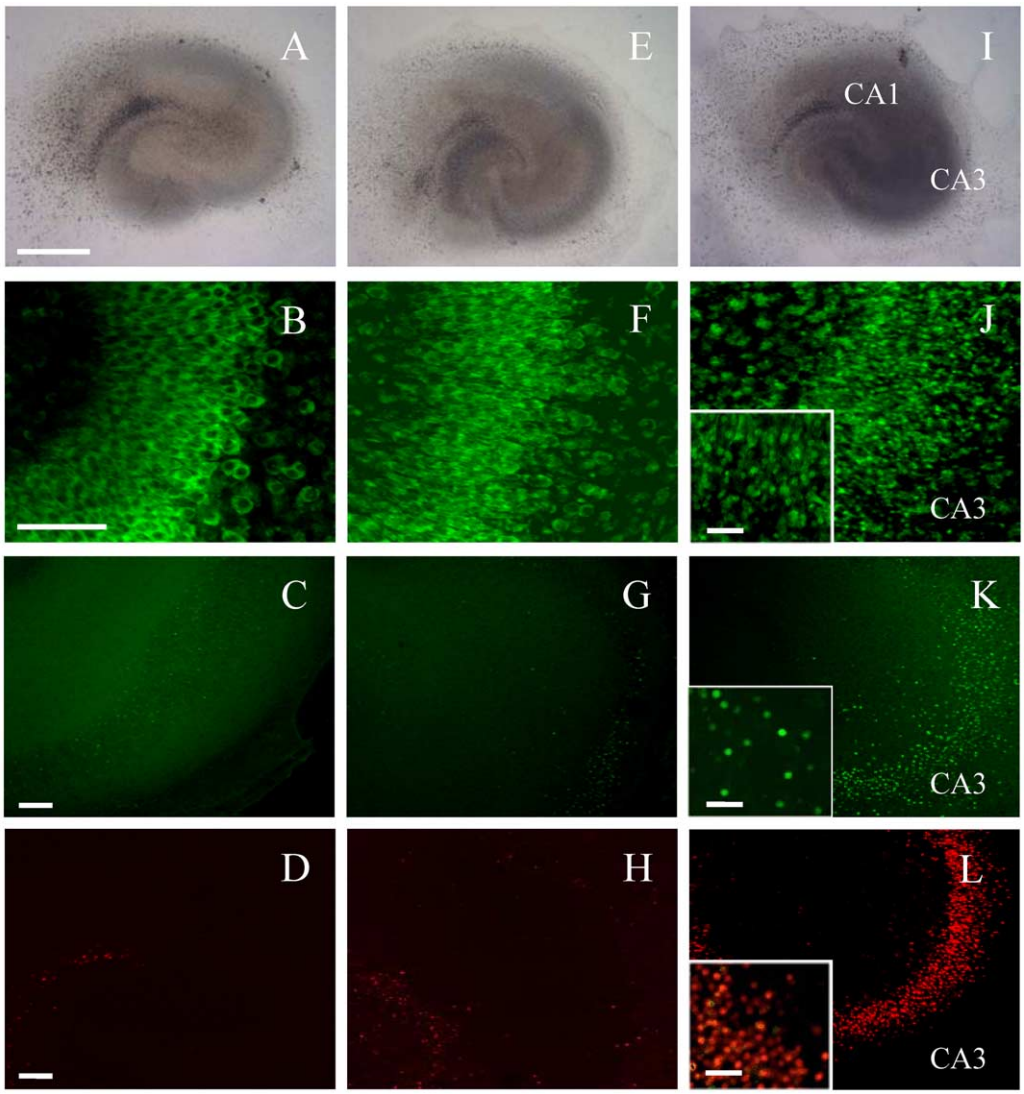

Fig. 2. Qualitative evaluation of OGD-induced cell damage in hippocampal slice cultures. Left column: control conditions; middle column: 24 h after 20 min OGD; right column: 24 h after 40 min OGD. Upper row: transmission light images; second row: Nissl staining; third row: TUNEL staining, fourth row: PI staining. Scale bars=500 μm (in A; also for E and I); in B=100 μm (also for F and J); in C=100 μm (also for G and K); in D=100 μm (also for H and L); scale bar=50 μm for insets of pictures J, K and L.

Role of voltage-dependent Ca^{2+} channels

Nimodipine a blocker of the L-type Ca^{2+} channels and mibefradil, a broad-spectrum Ca^{2+} channel blocker were used to investigate the involvement of voltage-sensitive Ca^{2+} channels to the abnormal $[Ca^{2+}]_i$ induced by OGD in cell somata of CA3 pyramidal neurons. In these cells, nimodipine (20 μM) only slightly inhibited the OGD-induced intracellular Ca^{2+} elevation (Fig. 3D, n=5). The drug effectively reduced the magnitude of the Ca^{2+} signal but failed to modify the time profile of the Ca^{2+} signal, the Ca^{2+} recovery, as well as the integrated response of the Ca^{2+} signal (Table 1). Mibefradil (10 μM) also failed to significantly modify any of the Ca^{2+} parameters investigated in the present study (Fig. 3E, n=6, Table 1). We next studied whether the modest amelioration of Ca^{2+} rise induced by nimodipine during OGD resulted in neuroprotection. Nimodipine failed to enhance neuronal survival when delivered to the culture medium until 2 h after OGD (Fig. 4, n=5).

Role of the plasmalemma Na^+/Ca^{2+} exchanger

The plasmalemma Na^+/Ca^{2+} exchanger induces Ca^{2+} extrusion from the cell under normal conditions, but causes intracellular Ca^{2+} accumulation by acting in the reverse mode under certain pathological circumstances (for review Blaustein and Lederer, 1999). The reverse mode of this exchanger can be specifically blocked by KB-R7943 (Iwamoto et al., 1996). Since previous studies in our laboratory had already found neuroprotective properties of this drug in the same model (Breder et al., 2000), only intracellular Ca^{2+} recordings were performed here.

KB-R7943 (10 μM) effectively reduced the intracellular Ca^{2+} elevation induced by OGD in the cell soma of hip-

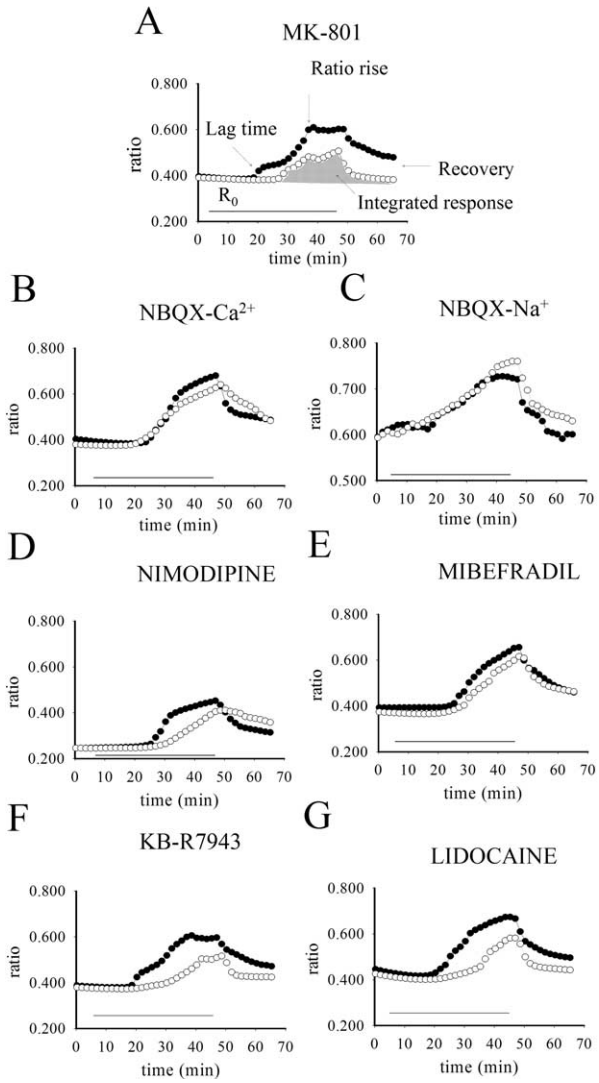

Fig. 3. Plasma membrane pathways implicated in the OGD-induced $[Ca^{2+}]_i$ elevation in hippocampal neurons. Cultures were loaded with magfura-2 and exposed to 40 min OGD followed by 20 min reperfusion. Drugs (A) MK-801 10 μM, (B) NBQX 10 μM, (D) nimodipine 20 μM, (E) mibefradil 10 μM, (F) KB-R7943 10 μM, (G) lidocaine 50 μM, added from loading procedure to end of OGD, were used to determine the role of ionotropic glutamate receptors, voltage-dependent Na^+ and Ca^{2+} channels and the plasma membrane Na^+/Ca^{2+} exchanger in the $[Ca^{2+}]_i$ elevation induced by OGD in cell somata of hippocampal neurons. Moreover NBQX 10 μM (C) was used to study the contribution of AMPA/kainate receptors to the $[Na^+]_i$ elevation induced by OGD in pyramidal neurons. Filled dotted curves are means of control experiments and empty dotted curves are means of drug-perfusion experiments. Every curve shows the mean of four to seven experiments; where in each one 10–20 hippocampal neurons were monitored. Horizontal bars indicate the duration of OGD (40 min). Fig. 3A indicates the ion signal parameters investigated in the present study: the lag time as the OGD period needed to increase significantly the dye signal over the basal ratio at the time of OGD induction (R_0), the ratio rise of the ion signal given by the difference between the largest ratio amplitude and R_0, the recovery of the ion signal, given by the percentage of ratio increase return to R_0, and the integrated response of the ion signal, given by the area under the curve of the dye signal from R_0 to the end of reperfusion.

pocampal pyramidal neurons (Fig. 3F, $n=6$). Although the onset of the Ca^{2+} signal was comparable in the presence of KB-R7943 with that seen in control OGD experiments, the drug was effective in causing an increase in the Ca^{2+} signal which was of a lesser extent and which recovered almost to baseline values during reperfusion (Fig. 3F). Moreover, KB-R7943 significantly reduced the integrated response of the Ca^{2+} signal, when compared with control OGD experiments (Table 1).

Role of voltage-dependent Na^+ channels

The involvement of the voltage-dependent Na^+ channels to the $[Ca^{2+}]_i$ and $[Na^+]_i$ increase and to the neurodegeneration induced by OGD was investigated in hippocampal slice cultures by using the Na^+ channel blocker lidocaine. Lidocaine (50 μM) resulted in effective reduction of the intracellular Ca^{2+} elevation induced by OGD (Fig. 3G, $n=6$). This effect was due to a significant reduction of the magnitude of the Ca^{2+} signal and of the integrated response when compared with control experiments (Table 1). To test whether the lidocaine effect on $[Ca^{2+}]_i$ is due to extracellular Na^+ entry blockade, Na^+ recordings were performed in the presence of lidocaine. Unfortunately, lidocaine strongly modified the basal SBFI fluorescence making neuronal localization impossible. Nevertheless, when tested for neuroprotection, lidocaine strongly reduced neuronal loss induced by OGD (Fig. 4, $n=5$). Neuroprotection achieved by the short incubation protocol was even enhanced by prolonging the presence of lidocaine in the culture media (Fig. 4, $n=5$).

Role of Ca^{2+} uptake into the endoplasmic reticulum

In order to determine the role of Ca^{2+} release from intracellular stores, the endoplasmic reticulum was depleted from Ca^{2+} by the use of the SERCA inhibitor CPA. In Ca^{2+} monitoring experiments, CPA (30 μM) was applied from dye loading until the end of reperfusion, due to reversibility of its effect. In the presence of CPA, the $[Ca^{2+}]_i$ rise induced by OGD was comparable to that induced in control experiments (Fig. 5A, $n=6$). Neither the lag time, nor the magnitude or the recovery of the Ca^{2+} signal was significantly modified after addition of CPA (Table 1). Moreover, CPA failed to enhance neuronal survival after OGD, when applied from 2 h before OGD until 2 h following OGD (Fig. 6, $n=5$).

Role of Ca^{2+} release from the endoplasmic reticulum

There are two families of intracellular receptors associated with Ca^{2+} channels and responsible for Ca^{2+} release from the endoplasmic reticulum, the ryanodine-sensitive receptors and the IP_3-sensitive receptors (Pozzan et al., 1994). Dantrolene was used as a blocker of the receptors sensitive to ryanodine, while the cell-permeable 2-APB, was used to study the contribution of IP_3-sensitive receptors to the $[Ca^{2+}]_i$ elevation and subsequent neuronal cell loss induced by OGD in hippocampal slice cultures.

Inhibition of the ryanodine-sensitive receptors with dantrolene (10 μM) significantly reduced the interval re-

Table 1. Drug modulation of the intraneuronal Ca^{2+} recordings (both Ca^{2+} and Na^{+} for NBQX) during OGD and reperfusion[a]

Drug (*n*)	Concentration		Lag time (min)	Ratio rise = (Δ F340nm/F380nm)	Recovery (%)	Integrated response (%)
MK-801 (5)	10 μM	C	22.1±2.9	**294.4±21.2**	**82.6±4.2**	**100**
		E	26.3±1.2	**154.7±28.8****	**101.8±1.6****	**85.1±1.7****
NBQX (7)	Ca^{2+}	C	23.4±1.3	289.3±21.1	81.9±3.2	100
	10 μM	E	20.8±2.5	284.3±38.5	79.9±4.5	97.1±6.5
NBQX (4)	Na^{+}	C	15.3±3.5	113.6±14.5	103.05±2.1	100
	10 μM	E	11.6±2.8	170.6±50.9	96.4±2.06	103.5±5.7
Nimodipine (5)	20 μM	C	25±1.7	**209.5±37.8**	80.7±5.9	100
		E	26.9±1.1	**172.8±29.1***	72.9±8.5	94.6±2.2
Mibefradil (6)	10 μM	C	19.8±1.4	279.7±50.6	87.2±5.1	100
		E	20.5±0.7	256.6±54.1	82.3±5.6	94.3±8.3
KB-R7943 (6)	10 μM	C	18±1.6	281.1±28.5	82.3±3.3	**100**
		E	21.7±2.6	163.3±58.2	91.6±6.3	**85.9±3.7***
Lidocaine (6)	50 μM	C	20.8±1.8	**254.5±35.6**	79±3.7	**100**
		E	24.1±2.1	**170.4±52.4***	95.3±5.4	**86.5±3.2****
CPA (6)	30 μM	C	22.4±1.1	332.8±40.4	74.8±5.7	100
		E	22.3±0.9	282.6±34.4	67.4±2.5	95.4±2.9
Dantrolene (5)	10 μM	C	**24.2±2.3**	282.9±29.4	80.5±1.7	100
		E	**13.3±1.6****	177.2±35.4	87.9±1.9	97.6±6.9
2-APB (6)	50 μM	C	17.4±0.9	382.5±31.8	73.7±6.2	100
		E	19.1±1.2	292.5±40.6	78.5±4.4	92.07±6.4
CGP-37157 (6)	10 μM	C	20.2±2.2	308.8±25.9	76.4±4.5	100
		E	20±2.1	300.5±71.7	72.7±7.1	102.2±5.6

[a] C, control OGD experiment; E, experimental drug-perfusion OGD experiment; *n,* number of paired experiments (C and E) obtained from the same animal. Boldface types and asterisks denote significance of differences between control and drug-perfusion experiments (paired *t*-test, * $P<0.05$; ** $P<0.01$). Fig. 3A exemplifies the parameters of the ionic signal used for analysis: lag time; ratio rise above R_0; recovery, and integrated response.

quired to elevate the $[Ca^{2+}]_i$ (Fig. 5B, $n=5$; Table 1). However, the drug turned out to be not effective to modify the magnitude and the recovery of the ischemic Ca^{2+} rise. Moreover, dantrolene did not reduce the integrated response of the Ca^{2+} signal when compared with control OGD experiments (Table 1). When assessed in neuronal viability studies, short protocol incubation with dantrolene did not protect the neuronal population from ischemic damage (Fig. 6, $n=5$).

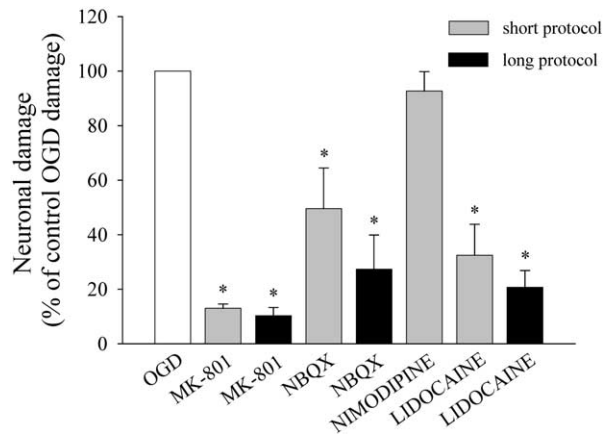

Fig. 4. Neuroprotection induced by pharmacological blockade of Ca^{2+} and Na^{+} entry pathways located in the plasma membrane. MK-801 10 μM, NBQX 10 μM, nimodipine 20 μM and lidocaine 50 μM were tested for neuroprotection in cultures exposed to a damaging OGD insult (40 min OGD) in comparison with the standard damage induced in untreated cultures by 40 min OGD (control OGD). Drugs were added by the short incubation protocol (2 h before OGD, during OGD and 2 h after OGD) and/or the long incubation protocol (2 h before OGD, during OGD and 24 h after OGD) by bath application. Each bar shows the mean PI staining of five experiments. In each experiment 40–50 cultures were used. Asterisks denote significant differences between treated and untreated cultures (Wilcoxon test, * $P<0.05$).

Blockade of the IP_3-sensitive receptors with 2-APB (50 μM) failed to change the ischemic Ca^{2+} rise in the cell somata of pyramidal neurons (Fig. 5C, $n=6$). In the presence of 2-APB, the Ca^{2+} signal peaked with a similar lag time, reached a comparable magnitude and behaved similarly during reperfusion. Consistently, the integrated response of the Ca^{2+} signal was comparable between both experimental conditions (Table 1). Surprisingly, as shown in Fig. 6, 2-APB (50 μM) not only failed to induce neuroprotection, but on the contrary increased to 50% the rate of neurodegeneration as compared with control OGD conditions (Fig. 6, $n=5$). Since neurotoxicity was also seen in cultures, which were not exposed to OGD but incubated with 50 μM 2-APB, experiments were repeated lowering the drug concentration. At 5 μM no deleterious effects of 2-APB were any longer detectable, but the drug similarly failed to induce significant neuroprotection (data not shown).

Role of mitochondrial Na^{+}/Ca^{2+} exchanger

A $2Na^{+}/Ca^{2+}$ exchanger controls the release of Ca^{2+} from the mitochondria. The effect of this exchanger can be inhibited by the diltiazem analog CGP-37157. By the use of CGP-37157 we investigated the contribution of the mitochondrial $2Na^{+}/Ca^{2+}$ exchanger to the neuronal Ca^{2+} overload and

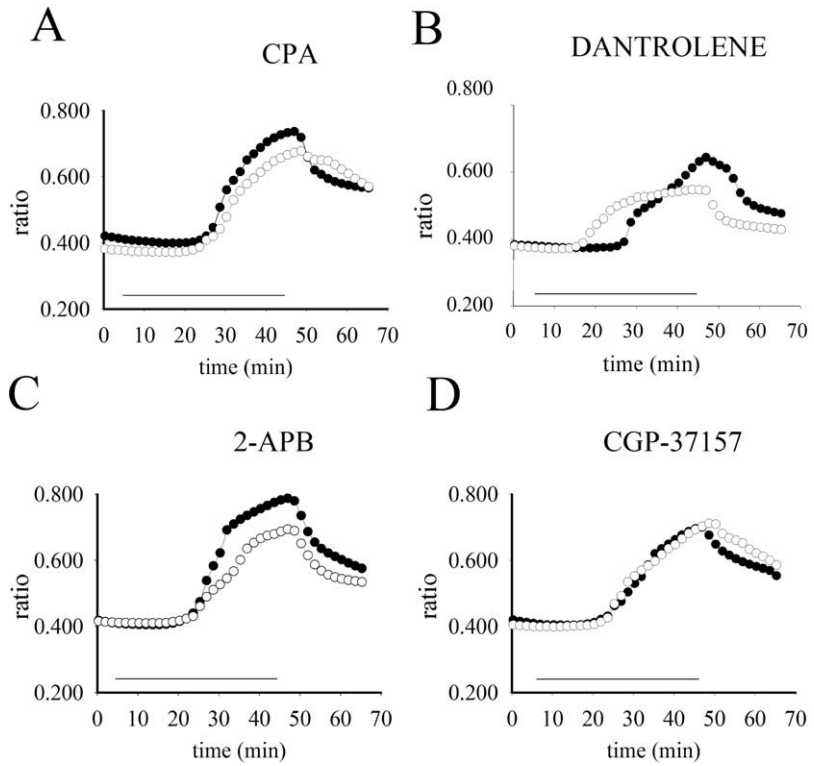

Fig. 5. Role of Ca^{2+} release from intracellular stores to the $[Ca^{2+}]_i$ elevation induced by OGD in hippocampal neurons. (A) CPA 30 μM, (B) dantrolene 10 μM, (C) 2-APB 50 μM, (D) CGP-37157 10 μM were used to study the role of SERCA-ATPase, ryanodine-sensitive receptors, IP_3-sensitive receptors and the mitochondrial Na^+/Ca^{2+} exchanger, respectively, in the $[Ca^{2+}]_i$ elevation induced by OGD in cell somata of hippocampal neurons. Drugs were added from loading period and were present until OGD cessation (except for CPA which was present until end of reperfusion). Filled dotted curves are means of control OGD experiments and empty dotted curves are means of drug-perfused experiments. Every curve shows the mean of five to six experiments where in each case 10–20 hippocampal neurons were monitored. Horizontal bars indicate the duration of OGD (40 min).

the related cell loss in hippocampal slice cultures. CGP-37157 (10 μM) failed to modify the Ca^{2+} signal induced by OGD in somata of hippocampal pyramidal neurons (Fig. 5D, $n=6$). The drug neither modified the time profile nor the amplitude or the integrated response of the Ca^{2+} signal nor enhanced Ca^{2+} recovery to baseline during reperfusion (Table 1). However, when tested for neuroprotection using the long drug-incubating protocol, CGP-37157 moderately, but significantly, reduced the amount of cell degeneration induced by OGD (Fig. 6, $n=5$).

DISCUSSION

Energy deficiency and loss of intracellular ion homeostasis correlates with neuronal degeneration induced by transient OGD

Cerebral ischemia results in a cascade of events with dramatic cellular changes. We used an *in vitro* preparation, which still maintains the hippocampal cellular organization to investigate the nature and characteristics of such changes. The present study reproduces in juvenile hippocampal slice cultures the energetic deficits and the pattern of neuronal vulnerability induced by ischemia in other experimental models (Folbergrova et al., 1992). However, in hippocampal slice cultures changes in ATP content are slower and less drastic than those observed in acutely isolated slices (Lobner and Lipton 1993; Zhang and Lipton 1999) or *in vivo* (Silver and Erecinska 1992; Katsura et al., 1993). This may be an inherent feature of organotypic slice cultures, but could also in part be due to the experimental conditions. We prove here that severity of OGD in hippocampal slice cultures correlates with ATP depletion, transient elevation of $[Ca^{2+}]_i$ in CA3 pyramidal neurons and with neuronal damage. Consistent with the delayed ATP reduction seen here, long-term OGD (40 min) induced a late increase of $[Ca^{2+}]_i$ into the micromolar range and a marked neurodegeneration in the pyramidal cell layer, whereas a shorter OGD (20 min) increased the $[Ca^{2+}]_i$ to the high nanomolar range, but failed to affect neuronal viability. Finally, we demonstrate that under OGD conditions, Ca^{2+} transients are associated with a transient elevation of the somatic $[Na^+]_i$, which modulates Ca^{2+} fluxes and contributes to the neurodegenerative response induced by *in vitro* ischemia. Therefore, the organotypic hippocampal slice culture does not suffer from the limitations of other ischemic models to correlate alterations in energy content with transmembrane ion changes and neuronal cell loss.

Glutamate receptors, voltage-dependent Na^+ channels and mitochondria: key mechanisms in ion deregulation and neuronal injury

Abnormal increase in intracellular free Ca^{2+}, one of the most important second messengers in the cells, has long been

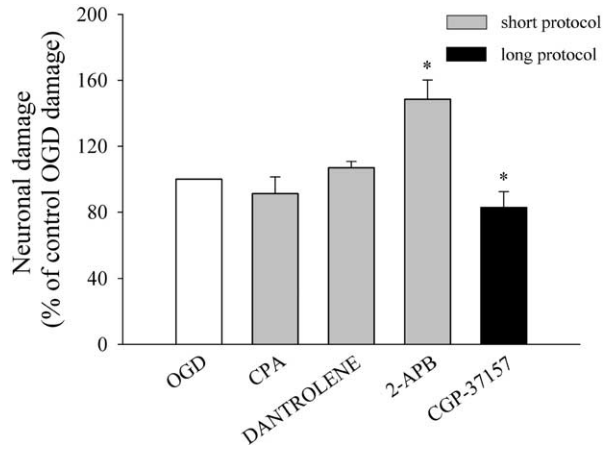

Fig. 6. Neuroprotection and/or neurotoxicity induced by pharmacological blockade of Ca^{2+} uptake and release pathways located in intracellular Ca^{2+} stores. CPA 30 μM, dantrolene 10 μM, 2-APB 50 μM, and CGP-37157 10 μM were tested for neuroprotection in cultures exposed to a damaging OGD insult (40 min OGD) in comparison with the standard damage induced in untreated cultures by 40 min OGD (control). Drugs were added by the short incubation protocol (2 h before OGD, during OGD and 2 h after OGD) or the long incubation protocol (2 h before OGD, during OGD and 24 h after OGD) by bath application. Each bar shows the mean of five experiments where in each experiment 40–50 cultures were used. Asterisks denote significant differences between treated and untreated cultures (Wilcoxon test, * $P<0.05$).

proposed as a crucial factor in the ischemia-induced neurotoxicity (Siesjo, 1988; Tymianski and Tator, 1996). Glutamate and its receptors have been recognized to play key roles in the pathology of ischemia, leading to the glutamate-Ca^{2+} hypothesis (see for review Rothman and Olney, 1986; Siesjo and Bengtsson, 1989). Subsequently, many studies (e.g. Breder et al., 2000; Hatfield et al., 1992; Lobner and Lipton, 1993; Pringle et al., 1997) including the present one, showed that inhibition of the NMDA receptor by MK-801 rescues especially vulnerable neurons from ischemic damage *in vivo* and *in vitro*. In addition, a sudden and large ischemia-induced, mainly NMDA receptor-mediated, increase in $[Ca^{2+}]_i$ in the CA1 area of gerbil hippocampal slices (Liu et al., 1997), rat hippocampal slices (Zhang and Lipton, 1999) as well as in cortical cell cultures (Goldberg and Choi, 1993) has been reported, further supporting the view that Ca^{2+} influx through NMDA receptors might determine neuronal vulnerability to ischemic damage. The present study shows that the NMDA receptor is a major pathway responsible for the intracellular Ca^{2+} elevation also in CA3 neurons.

Cerebral ischemia also results in activation of AMPA/kainate glutamate receptors. Blockade of these receptors has been proven to decrease injury in CA1 neurons following global cerebral ischemia and to decrease the infarct size in focal ischemia models (Sheardown et al., 1990). However, in *in vitro* models the situation is less clear. Our results are in line with those of Pringle et al. (1997), who reported neuroprotective effects of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), another antagonist of the non-NMDA glutamate receptor, after 60 min hypoxia/hypoglycemia. In contrast, Tasker et al. (1992) and Laake et al. (1999) did not find any protective effect of CNQX. The discrepancy may at least in part be due to differences in the severity of the insult, as in another study CNQX exerted a protective effect only when the ischemic period was short enough to result only in partial neuronal cell death, whereas it was not protective when the insult lasted longer (Strasser and Fischer, 1995). We observed that the neuroprotective effect of NBQX, which was seen when the drug is present during the insult, can be slightly increased by prolonging the length of drug application in the culture medium until the end of the reperfusion period. The underlying mechanism is less clear. Similarly to Lobner and Lipton (1993), who tested in hippocampal slices the related antagonist 6,7-dinitroquinoxaline-2,3-dione, we did not observe alterations of somatic $[Ca^{2+}]_i$ by NBQX during OGD, indicating that Ca^{2+}/Zn^{2+}-permeable AMPA receptors are not critically involved. In the stratum radiatum of hippocampal slices from adult rats, CNQX reduced the $[Ca^{2+}]_i$ rise during ischemia in the absence of extracellular Ca^{2+}, suggesting that AMPA/kainate receptors mediate their effects by increasing $[Na^+]_i$ at the dendritic level (Zhang and Lipton, 1999). In our study NBQX was neuroprotective, but did not affect the somatic $[Na^+]_i$ elevation induced by OGD. This supports the hypothesis of Zhang and Lipton (1999), which claims that the detrimental mechanisms activated by AMPA/kainate receptor-mediated Na^+ influx resulting in mitochondrial Ca^{2+} release, might be localized in the dendritic spines. Complementing the findings described in Zhang and Lipton (1999) that CNQX and CGP-37157 attenuate dendritic $[Ca^{2+}]_i$ increase, we show that NBQX and CGP-37157 were protective, but did not alter the rise in somatic $[Ca^{2+}]_i$. Thus, an excessive increase in dendritic $[Na^+]_i$ and/or $[Ca^{2+}]_i$ appears to be sufficient to induce neuronal damage.

Voltage-sensitive Na^+ channels constitute the main route for Na^+ influx into neurons. Similarly to other studies, which used lidocaine or tetrodotoxin (e.g. (Breder et al., 2000; Fried et al., 1995; Tasker et al., 1992)), we show here that lidocaine is strongly neuroprotective. A rise in somatic $[Na^+]_i$ will alter the driving force for Na^+-dependent transporters and activation of Na^+ channels will depolarize the plasma membrane. Thus, lidocaine can exert its protection by affecting the following mechanisms: the effect on membrane potential depolarisation can limit Ca^{2+} influx through voltage-gated Ca^{2+} channels and, by stabilizing the Mg^{2+} block, also through NMDA receptors. This process decreases the Ca^{2+}-dependent glutamate release. By simultaneously diminishing the somatic $[Na^+]_i$ load, lidocaine will also restrict the Na^+/Ca^{2+} exchanger-mediated Ca^{2+} entry. Accordingly, lidocaine slows down and reduces the somatic $[Ca^{2+}]_i$ rise. Protection against hypoxic injury by lidocaine has been observed in brain slices using lidocaine doses (10 μM) that do not attenuate the increase in $[Ca^{2+}]_i$ (Raley-Susman et al., 2001). Thus, prevention of Na^+ entry may have additional protective effects.

Voltage-dependent Ca^{2+} channels and the Na^+/Ca^{2+} exchanger

It is widely accepted that the membrane depolarization induced by ischemia results in the opening of voltage-gated Ca^{2+} channels and in the subsequent intracellular

Ca^{2+} overload (Silver and Erecinska, 1992). However, the importance of Ca^{2+} influx through these channels for neuronal damage is still a matter of debate. Some *in vivo* and *in vitro* studies show neuroprotection after inhibition of L-type or other types of Ca^{2+} channels (Bogaert et al., 2001; Krieglstein et al., 1996) while others observe no effect (Lingenhohl et al., 1997; Pringle et al., 1996). We find that in hippocampal pyramidal neurons Ca^{2+} influx through voltage-dependent Ca^{2+} channels is not significant for neurodegeneration, as mibefradil had no and nimodipine only a very modest effect on $[Ca^{2+}]_i$ and both did not reduce neuronal damage. Similarly, Pringle et al. (1996) did not observe a neuroprotective effect of N-type, L-type and broad spectrum Ca^{2+} channel blockers in hippocampal slice cultures from rats of similar age as used here. In the work of Krieglstein et al. (1996) on the other hand, a protective effect of nimodipine in primary cultures of rat hippocampal neurons was shown. This indicates that differences in degree of membrane depolarization, age of the animals, cellular environment and culture conditions might thus influence the relative importance of Ca^{2+} entry systems.

The electrogenic Na^+/Ca^{2+} exchanger under normal conditions extrudes Ca^{2+} from the intracellular space. However, at high $[Na^+]_i$ and/or with cell depolarization, the exchanger can revert its operation mode leading to intracellular Ca^{2+} accumulation (Blaustein and Lederer, 1999). In agreement with this view we show that KB-R7943, a blocker of the reverse mode of the exchanger, strongly reduces the somatic $[Ca^{2+}]_i$ rise induced by experimental ischemia in pyramidal neurons. We suggest that the somatic elevation of $[Na^+]_i$, which we observed by SBFI fluorescence, and the concomitant cellular depolarization reverse the driving force of the Na^+/Ca^{2+} exchanger and contribute to degeneration. In line with this explanation, our laboratory and others have already shown that the inhibition of the exchanger generates neuroprotection *in vivo* and *in vitro* (Breder et al., 2000; Matsuda et al., 2001; Schroder et al., 1999; Stys, 1996).

Intracellular Ca^{2+} stores

In the presence of activated reversed Na^+/Ca^{2+} exchangers and NMDA receptors, intracellular Ca^{2+} stores contribute little, if anything, to the OGD-induced $[Ca^{2+}]_i$ rise in the soma of CA3 neurons. Neither the SERCA-ATPase antagonist CPA, nor the ryanodine-sensitive receptor blocker dantrolene, nor the IP_3-sensitive receptor blocker 2-APB or the mitochondrial Na^+/Ca^{2+} exchange inhibitor CGP-37157 significantly altered the overall Ca^{2+} increase. Dantrolene accelerated the OGD-induced $[Ca^{2+}]_i$ rise by a yet-undetermined mechanism, although similarly to CPA had no effect on neuronal viability. The detrimental effect of 2-APB cannot be explained by its known inhibitory effects on SERCA pumps and mitochondrial Na^+/Ca^{2+} exchanger (Bootman et al., 2002), since the respective specific inhibitors were not toxic and 2-APB did not affect $[Ca^{2+}]_i$. The toxicity can perhaps be explained by the ability of 2-APB to cause a rapid, slowly reversible cytoplasmic acidification (Bootman et al., 2002).

In conclusion, we show that the OGD-induced $[Ca^{2+}]_i$ rise in cell soma of hippocampal CA3 neurons is caused by a concomitant activation of NMDA receptors, Na^+/Ca^{2+} exchangers and Na^+ channels, whereas voltage-sensitive Ca^{2+} channels, intracellular Ca^{2+} stores and AMPA/kainate receptors are not significantly involved. Our data suggest that the protective effects of MK-801, KB-R7943 and lidocaine are due to inhibition of somatic Ca^{2+} load, while NBQX and CGP-37157 might block detrimental ionic mechanisms in the dendrites.

Acknowledgments—The authors wish to thank Anke Böcker for excellent technical assistance. This study was supported by the DFG Graduiertenkolleg "Biologische Grundlagen von Erkrankungen des Nervensystems" (GRK 253/5-02) at Medizinische Fakultät, Otto-von-Guericke-Universität Magdeburg.

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(Accepted 28 June 2004)
(Available online 15 September 2004)