

Neurotoxicity Induced by Antiepileptic Drugs in Cultured Hippocampal Neurons: A Comparative Study between Carbamazepine, Oxcarbazepine, and Two New Putative Antiepileptic Drugs, BIA 2-024 and BIA 2-093

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Summary: *Purpose:* Newly designed antiepileptic drugs (AEDs) are being evaluated for their efficacy in preventing seizures and for their toxic profiles. We investigated and compared the toxic effects of two dibenz[*b,f*]azepine derivatives with anticonvulsant activity, 10,11-dihydro-10-hydroxyimino-5H-dibenz[*b,f*]azepine-5-carboxamide (BIA2-024) and (*S*)-(-)-10-acetoxy-10,11-dihydro-5H-dibenz[*b,f*]azepine-5-carboxamide (BIA2-093), with the structurally related compounds carbamazepine (CBZ) and oxcarbazepine (OXC), both in current use for the treatment of epilepsy.

Methods: Primary rat hippocampal neurons were used to evaluate neuronal morphology and biochemical changes induced by the AEDs used in this study. Immunocytochemical staining against MAP-2 was used to evaluate neuronal morphology. Reactive oxygen species (ROS) and changes in mitochondrial membrane potential (Ψ_m) were measured by fluorescence techniques. Intracellular adenosine triphosphate (ATP) levels were quantified by high-performance liquid chromatography (HPLC).

Results: Hippocampal neurons treated for 24 h with CBZ or OXC (300 μ M) showed degeneration and swelling of neurites, but this effect was not observed in neurons treated with BIA 2-024 or BIA 2-093 (300 μ M). ROS production also was increased in neurons treated with OXC, but not in neurons treated with the other AEDs. ATP levels were significantly decreased only in neurons treated with OXC, although the energy charge was not altered. Furthermore, OXC led to a decrease of Ψ_m .

Conclusions: In all parameters assayed, OXC was more toxic than the other AEDs used. Because the new putative AEDs have previously been shown to have an efficacy in preventing seizures similar to that of CBZ and OXC, and are less toxic to neuronal cells, they may be considered as alternatives to the current available therapies for the treatment of epilepsy. **Key Words:** Neurotoxicity—Carbamazepine—Oxcarbazepine—BIA 2-024—BIA 2-093.

Epilepsy is a common neurologic disease estimated to affect 50 million persons worldwide. The major antiepileptic drugs (AEDs) in current clinical use [e.g., phenytoin (PHT), carbamazepine (CBZ), valproate (VPA), and phenobarbital (PB), among others] have a high efficacy in reducing seizures. An ideal AED would prevent seizures without producing side effects that adversely affect the patient's quality of life. Unfortunately, patients taking AEDs display a broad spectrum of undesirable side effects. Particularly dizziness, ataxia, drowsiness, and re-

duction of alertness occur in therapy with CBZ, in the beginning of treatment and with increasing doses (1). Thus, extensive research has focused on the design of new drugs with anticonvulsant activity and fewer adverse effects. New drugs have progressed in terms of longer half-lives, greatly reduced potential for drug interactions, and general lack of hepatic enzyme induction (2–5).

CBZ is a dibenz[*b,f*]azepine derivative (5H-dibenz[*b,f*]azepine-5-carboxamide (6) that, since its introduction for clinical treatment of epileptic seizures, has become the most frequently prescribed first-line drug. Besides the fact that a significant percentage of affected individuals do not respond to treatment with CBZ, this drug has the disadvantage of inducing hepatic microsomal enzymes that cause self-induction of its

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own metabolism. Moreover, its metabolites (particularly epoxides) have been held responsible for the general and neuronal toxicity of CBZ. Oxcarbazepine (OXC) is an analogue of CBZ, with comparable anticonvulsant efficacy, in which a keto group has been added to the position 10 of the azepine ring. This modification results in important differences in the metabolism of the two drugs. It has the advantage of a lower incidence of allergic reactions and enzyme induction. In combined therapy with other AEDs, OXC is usually better tolerated than CBZ (7).

10,11-Dihydro-10-hydroxyimino-5H-dibenz[*b,f*]azepine-5-carboxamide (BIA 2-024) and (*S*)-(-)-10-acetoxy-10,11-dihydro-5H-dibenz[*b,f*]azepine-5-carboxamide (BIA 2-093) are representative of a new series of compounds (8,9), structurally related to CBZ and OXC, with anticonvulsant activity, as determined by maximal electroshock stimulation (9,10). As newly developed drugs should be less toxic than already existing drugs, these two compounds were specifically designed to circumvent their further degradation to toxic metabolites, such as epoxides, without losing anticonvulsant potency.

Several studies can be found in the literature regarding the toxicity of AEDs to neuronal cells. Some reports show that CBZ is able to induce apoptosis in cultured cerebellar granule cells (11–13). In a previous study, we showed that OXC and CBZ can be toxic to cultured hippocampal neurons (14), showing patterns of neuronal deterioration, mainly in cells exposed to high concentrations, and also showing increased activity of caspase-3-like enzymes, in the case of OXC. We also have seen some morphologic changes (nuclear condensation) in neurons exposed to high concentrations of either BIA 2-024 or BIA 2-093, suggesting apoptosis, although the extent of the insult was much less than that observed with CBZ or OXC. Recently, Pavone and Cardile (15) described the effects of CBZ and OXC on astrocyte cultures and showed decreased tolerance by cortical astrocytes to high concentrations of these and other AEDs.

Within this scenario, we found important to compare the toxicity profiles of the new putative AEDs, BIA 2-024 and BIA 2-093, with those of currently used and structurally similar AEDs, CBZ and OXC. We chose cultured hippocampal neurons as a model, given that the hippocampal formation is one of the most affected brain regions during status epilepticus. In this study, we further investigated the toxic effect caused by AEDs in cultured neurons. We focused on parameters related to apoptosis-like cell death and markers for mitochondrial dysfunction, which compromises the energy status of the cell and impairs neuronal survival.

MATERIALS AND METHODS

Cell culture

Hippocampal neurons were dissociated from hippocampi of E18–E19 Wistar rat embryos, after treatment

with trypsin (2.0 mg/ml, 15 min, 37°C) and deoxyribonuclease I (0.15 mg/ml) in Ca²⁺-free and Mg²⁺-free Hank's balanced salt solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH₂PO₄, 0.34 mM Na₂PO₄ 2H₂O, 4.16 mM NaHCO₃, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4). The cells were cultured in serum-free Neurobasal medium, supplemented with B27 supplement, glutamate (25 μM), glutamine (0.5 mM), and gentamicin (0.12 mg/ml), as described previously (16). Cultures were kept at 37°C in a humidified incubator in 5% CO₂/95% air, for 7 to 8 days before the experiments, the time required for maturation of hippocampal neurons.

For morphology studies and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) confocal microscopy analysis, cells were plated in coverslips at 0.045 × 10⁶ cells/cm². For reactive oxygen species (ROS) production experiments and JC-1 fluorimetric analysis, cells were plated in coverslips at 0.2 × 10⁶ cells/cm². For adenine nucleotide determinations, cells were plated in multiwell plates at 0.1 × 10⁶ cells/cm².

Exposure of hippocampal neurons to drugs

The hippocampal neurons were exposed to drugs for 24 h. The stock solutions of the drugs [prepared in dimethylsulfoxide (DMSO)] were diluted in small aliquots of conditioned medium (medium in which the cells were cultured until the experiment) and then added back to the corresponding well. All drugs were used at a concentration of 300 μM. The presence of DMSO did not cause any difference or toxicity compared with cultures that were not treated with DMSO. The final concentration of DMSO used (0.1%) was without consequences to cell viability.

Morphology studies

Neuronal morphology was evaluated after an immunocytochemical labelling of the hippocampal neurons by using an anti-microtubule-associated protein 2 (MAP-2) antibody. After incubation of cultured hippocampal neurons with the drugs for 24 h, the culture medium was removed, the cells were washed 3 times with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde, at room temperature, for 30 min. After three washes with PBS, nonspecific binding was blocked with 3% bovine serum albumin (BSA) in PBS. Cells were then incubated with anti-MAP-2 (1:200) at room temperature for 1 h. After three washes with PBS, cells were incubated with a secondary antibody, anti-mouse immunoglobulin G (IgG) labelled with Alexa Fluor 594 (1:200), at room temperature, for 1 h. Finally, coverslips were mounted by using a Prolong Antifade Kit and after drying, the neurons were visualized with confocal fluorescence microscopy (Bio-Rad MRC 600 B10-Rad Laboratories, Hercules, CA, U.S.A.).

Measurement of ROS production

ROS production in populations of hippocampal neurons was measured by using 2',7'-dichlorodihydrofluorescein

diacetate (DCFH₂-DA), a cell-permeant probe for ROS, which is deesterified within the cells to DCFH₂. The accumulated DCFH₂ becomes fluorescent when it is oxidized to dichlorofluorescein (DCF), thus detecting the formation of ROS or intracellular peroxides.

Hippocampal neurons plated on coverslips at a density of 0.2×10^6 cells/cm² were incubated with 5 μ M DCFH₂-DA in Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM MgCl₂, 1 mM CaCl₂, 6 mM glucose, 10 mM HEPES-Na, pH 7.4) for 30 min, at 37°C, in the dark. The coverslips were rinsed with Krebs buffer and mounted in a special holder (Perkin-Elmer L2250008), in a temperature-controlled cuvette with magnetic stirring. The fluorescence was monitored at 37°C with a Spex Fluoromax spectrofluorometer, with excitation at 502 nm and emission at 550 nm, by using 3 nm slits. The increase in fluorescence due to oxidation of DCFH₂ to DCF was recorded for 120 s, and the slope was calculated with the values of fluorescence arbitrary units at 20 and 100 s. Results are expressed as percentage of control.

Assessment of mitochondrial depolarization

JC-1 is a cationic carbocyanine dye that accumulates in mitochondria and is suitable for assessing changes in mitochondrial membrane potential (Ψ_m). The dye exists as a monomer at low concentrations and yields green fluorescence. At higher concentrations, JC-1 forms aggregates that exhibit a broad excitation spectrum and maximum emission at \sim 590 nm. The JC-1 red/green fluorescence ratio has been used as a tool to estimate changes in Ψ_m (17).

Neurons were loaded with 3 μ M JC-1 for 15 min, at 37°C in the dark, after exposure to the AEDs for 24 h. For spectrofluorimetric analysis of JC-1 red/green fluorescence ratio, the fluorescence was monitored during 700 s, with excitation at 490 nm and emission at 535 and 595 nm. In the end of each experiment, FCCP (2.5 μ M) plus oligomycin (1 μ g/ml) were added to completely depolarize mitochondria and validate the assay; FCCP is a proton ionophore known to uncouple the proton gradient across the inner mitochondrial membrane, and oligomycin, which inhibits proton transport through the ATP synthase, prevents maintenance of Ψ_m by ATP synthase reversal and glycolytic ATP hydrolysis (18), thus eliminating Ψ_m artifacts. Red/green ratios were obtained by dividing the signal at 595 nm (aggregate) by the signal at 535 nm (monomer) and normalizing to a starting value of 1, for comparison between the various conditions. Results are presented as the rate of decrease in red/green ratio signal (17). For microscopy analysis, after loading the cells with 3 μ M JC-1, for 15 min at 37°C in the dark, the coverslips were observed with laser scanning confocal microscopy in a Bio-Rad MRC 600 confocal micro-

scope. Neurons were excited at 488 nm, and emission was recorded at 530 and 590 nm.

Determination of adenine nucleotides

Intracellular adenine nucleotides, ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) from hippocampal neurons were determined by reversed-phase HPLC, as previously described (19), after cell extraction. After exposure of hippocampal neurons to drugs for 24 h, cells were lysed in 0.3 M perchloric acid and collected. Extraction was followed by centrifugation at 15,800 g for 5 min. The pellet was solubilized in 0.1 M NaOH for total protein analysis by the Bradford/Biorad method. The supernatant was neutralized with 3.3 M KOH in 1.67 M Tris and centrifuged at 15,800 g for 10 min. The potassium perchlorate precipitate was discarded, and the resulting supernatants were stored at -80°C . Adenine nucleotide content was determined by reverse-phase HPLC, by using a Beckman System Gold, consisting of a binary pump (126 Binary Pump Model) with a 166 Variable UV detector. A Lichrospher 100 Rp-18 column (Merck) was used. The mobile phase was composed of 100 mM KH₂PO₄, pH 6.5, 1% methanol, at a flow rate of 1.1 ml/min. Absorbance was monitored at 254 nm.

Peak identity was determined by the retention time compared with standards. The amounts of ATP, ADP, and AMP were determined by a concentration standard curve. ATP-content values were normalized according to the protein concentration for each sample. The energy charge (EC) of the hippocampal cells was calculated by using the following formula (20):

$$EC = (\text{ATP} + 0.5 \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$$

Chemicals

BIA 2-024, BIA 2-093, CBZ, and OXC were obtained from BIAL (S. Mamede do Coronado, Portugal). Neurobasal medium, B27 supplement, gentamicin and trypsin (USP grade) were purchased from GIBCO BRL (Life Technologies, Scotland). Glutamate, glutamine, DNase (DN-25), anti-MAP-2, and trypan blue were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). DCFH₂-DA, JC-1, and anti-mouse IgG labelled with Alexa Fluor 594 were purchased from Molecular Probes, Leiden, The Netherlands. BSA was purchased from Calbiochem-Boehringer (San Diego, CA, U.S.A.). All other reagents were from Sigma Chemical or from Merck-Schuchardt, Germany.

CBZ, OXC, BIA 2-024, BIA 2-093, JC-1, and DCFH₂-DA stock solutions were prepared in DMSO. The solutions of all other reagents were prepared in ultrapurified water.

Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance was determined by using an analysis of variance

(ANOVA), followed by Dunnett's posttest, as indicated in the figure legends.

RESULTS

Evaluation of the effect of antiepileptic drugs on the morphology of cultured rat hippocampal neurons

Previous studies showed that AEDs like PHT, CBZ, or OXC are toxic to neurons in culture (11–14) and also to cultured astrocytes (15). Indeed, our group previously tested different concentrations of CBZ and OXC (from 10 to 300 μM) and observed that cultured hippocampal neurons treated with 300 μM OXC, CBZ, BIA 2-024, or BIA 2-093 for 24 h show apoptosis-like features (e.g., condensed chromatin and nuclei), and OXC is clearly more toxic than the other AEDs tested (14). At these concentrations, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was significantly decreased by OXC and CBZ ($62.5 \pm 3.2\%$ and $75.4 \pm 2.3\%$, respectively; $p < 0.01$) and nonsignificantly decreased by BIA 2-024 or BIA 2-093 (14). The number of apoptotic neurons is increased by treatment with OXC or CBZ ($39.3 \pm 3\%$ or $31.3 \pm 3\%$; $p < 0.01$, respectively; above 13% in control cultures), whereas BIA 2-024 or BIA 2-093 only slightly increases the number of apoptotic neurons (14). In the present work, we further investigated the mechanisms of the toxic effect caused by these AEDs. The effects of high concentrations (300 μM) of OXC, CBZ, BIA 2-024, or BIA 2-093 on the morphology of cultured hippocampal neurons, for a 24 h exposure, as assessed by anti-MAP-2 immunocytochemistry (Fig. 1), confirm the previous results. Changes in neuronal morphology were notorious when cells were treated with OXC (compare Fig. 1A and B, and Fig. 1F and G): the fine neuritic network observed in untreated neurons was significantly affected in OXC-treated cultures, and the remaining neurites were swollen and with less punctate synaptic varicosities (Fig. 1B and G). CBZ was not as effective as OXC in inducing morphologic changes to hippocampal neurons, although some damage is evident, and neurites appear to be retracting (Fig. 1C). BIA 2-024 (Fig. 1D) and BIA 2-093 (Fig. 1E and H) did not induce any apparent neuronal damage, as the neuronal network appears to be well preserved.

OXC increases reactive oxygen species production

The formation of ROS in cultured hippocampal neurons was studied by following DCF fluorescence on exposure to AEDs for 24 h (Fig. 2). OXC (300 μM) induced a significant increase in ROS production to $221.7 \pm 40.0\%$ of the control ($p < 0.001$). Lower concentrations of OXC (50 and 100 μM) did not significantly increase ROS production (data not shown). ROS production in cells treated with CBZ (300 μM), BIA 2-093 (300 μM), or BIA 2-024 (300 μM) was also not increased, as compared with control (Fig. 2).

Effect of AEDs in intracellular ATP levels and energy charge of cultured hippocampal neurons

Adenine nucleotide (ATP, ADP, and AMP) levels were determined by reverse-phase HPLC (Fig. 3). Exposure to OXC (300 μM) for 24 h resulted in a significant decrease of intracellular ATP levels from 50.4 ± 2.6 nmol/mg protein (control cultures) to 35.7 ± 3.0 nmol/mg protein ($p < 0.05$). The other AEDs did not cause a significant variation in intracellular ATP levels. However, the EC was not significantly decreased in OXC-treated cultures (0.85 ± 0.034) relative to control cultures (0.88 ± 0.026) or with the other AEDs.

OXC decreases mitochondrial membrane potential

Confocal microscopy imaging showed that the red fluorescence of JC-1 aggregate form is decreased in OXC-treated cultures, whereas the green monomer fluorescence is greatly increased, indicative of mitochondrial depolarization (Fig. 4). In cultures treated with CBZ, BIA 2-024, or BIA 2-093, the aggregate fluorescence is similar to that of untreated cultures, and the monomer fluorescence is faint.

In attempting to quantify the JC-1 red/green fluorescence ratio, it was observed that this ratio decreased with time (400 s) in all situations, which was likely to be due to photobleaching. The addition of FCCP (2.5 μM) plus oligomycin (1 $\mu\text{g/ml}$) significantly decreased Ψ_m in all conditions. In cells treated with 300 μM OXC for 24 h, the JC-1 ratio decreased in a faster way, accounting for a significant decrease in Ψ_m during the time-drive fluorescence monitoring: the rate of decrease of the red/green fluorescence ratio over time was significantly higher in OXC-treated neurons ($133.4 \pm 10\%$ of the control; $p < 0.05$; Fig. 5) than in control conditions. Neither CBZ nor the new putative AEDs had a significant effect on Ψ_m .

DISCUSSION

In this work, we show that OXC, at high concentrations, is toxic to cultured hippocampal neurons, triggering mechanisms that result in decreased intracellular ATP levels and increased ROS, compromising cell survival. We observed significant neurotoxic changes after treatment with OXC, which are not observed with treatment with the two new putative AEDs, BIA 2-024 and BIA 2-093, or with CBZ, thus sustaining the concept that this new putative AEDs are less toxic than currently used AEDs. These results are in agreement with and extend the observations in previous studies by our group (14), in which it was shown that OXC is toxic to cultured hippocampal neurons and that BIA 2-024 and BIA 2-093 are less toxic than the related compounds, CBZ and OXC, which toxicity is detected at concentrations as low as 50–100 μM .

When dealing with a disease such as epilepsy (21), which is a life-long condition and requires continuous treatment after diagnosis, bioaccumulation of AEDs

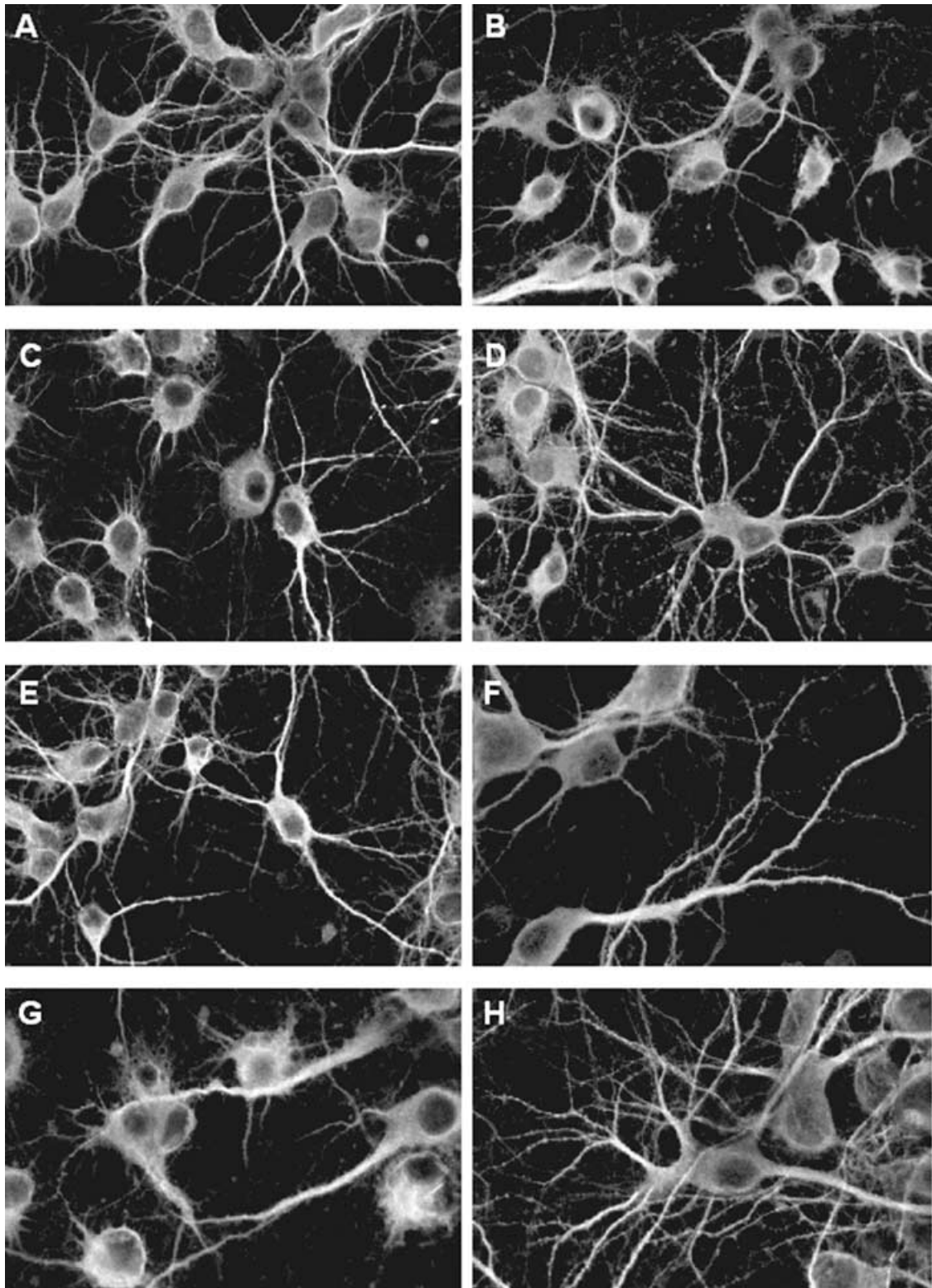


FIG. 1. Changes in the morphology of cultured hippocampal neurons treated with antiepileptic drugs for 24 h, as assessed by anti-MAP-2 immunocytochemistry. **A:** Control culture. **B:** 300 μ M oxcarbazepine (OXC). **C:** 300 μ M carbamazepine (CBZ). **D:** 300 μ M BIA 2-024. **E:** 300 μ M BIA 2-093. All at $\times 600$ magnification. **F:** Control culture. **G:** 300 μ M OXC. **H:** 300 μ M BIA-2-093. All at $\times 900$ magnification. At higher magnification (**F–H**), the toxic effect of OXC is clear in the swollen and shorter neurites (**G**) and in the loss of the fine neuritic network. BIA 2-024 (**D**) or BIA 2-093 (**E, H**) did not induce such modifications (similar to control culture).

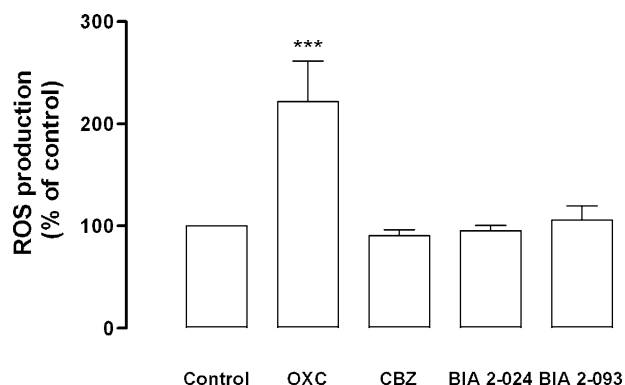


FIG. 2. Reactive oxygen species (ROS) production in cultured hippocampal neurons after treatment with antiepileptic drugs for 24 h. ROS production was assessed by following the oxidation of the cell-permeant fluorescent probe, DCFH₂-DA, to the fluorescent form DCF. Each drug was used at 300 μ M. The results are presented as percentage of control (no treatment), by using arbitrary fluorescence units at an excitation wavelength of 502 nm, and represent the mean \pm SEM of at least five independent experiments. *** p < 0.001, significantly different from control; Dunnett's posttest.

cannot be excluded, and the plasma levels of the drug can surpass the therapeutic dose; hence the interest of studying the effect in neuronal survival of high concentrations of AEDs. Pavone and Cardile (15) characterized the effect of various concentrations of CBZ and OXC (ranging from 1 to a 100 μ g/ml), as well as other AEDs, on the viability of cultured cortical astrocytes, showing increased cellular stress. To our knowledge, little is known about the toxicity triggered by AEDs in neurons. High concentrations of CBZ were shown to be neurotoxic in cultured cerebellar neurons (11–13) and in cultured hippocampal neurons (14). OXC also was described to be toxic to hippocampal neurons (14). It is very important to assess the neurotoxic profile of AEDs because metabolic failure due to drug interaction and/or hepatic dysfunction or renal failure might

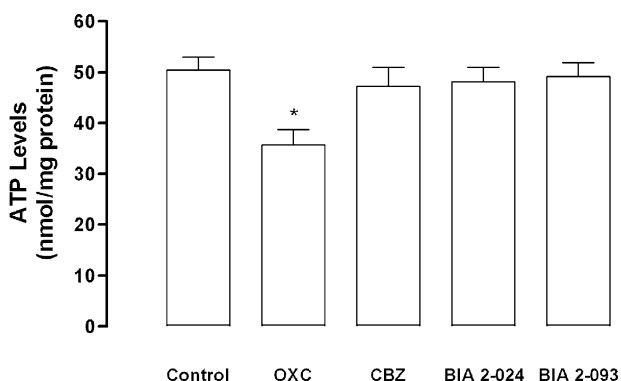


FIG. 3. Intracellular adenosine triphosphate (ATP) levels in cultured hippocampal neurons after treatment with antiepileptic drugs for 24 h. Each drug was used at 300 μ M. The results are presented as nanomoles per milligram protein and represent the mean \pm SEM of at least four independent experiments. * p < 0.05, significantly different from control; Dunnett's posttest.

lead to an increase in plasma levels of the drug, and thus the interest in choosing a therapy with as few toxic effects as possible to neuronal cells.

We found that OXC had a strong deleterious effect in the morphology of cultured hippocampal neurons: the neurites became swollen and shorter, and the network of fine processes that is observed in control cultures was greatly reduced. The CBZ effect was not as dramatic as that of OXC, although the damage in neuronal cultures was clear. Treatment of cultures with OXC (100 μ M) did not cause an apparent retraction and swelling of neurites (data not shown), although a decrease of MTT reduction and increase in the number of apoptotic-like nuclei were already observed at this concentration (14). Apparently, morphologic changes are secondary to biochemical detection of neurodegeneration, as when the MTT reduction is significantly decreased or the appearance of apoptotic-like nuclei is clearly higher, but still no morphologic changes at the neurite network level are yet visible. The hippocampal cultures treated with BIA 2-024 and BIA 2-093 did not show evident morphologic markers of neurodegeneration, suggesting that these drugs are not as toxic as OXC or CBZ. Previous work with BIA 2-024 and BIA 2-093 showed that at the same concentration used in the present study (300 μ M), BIA 2-093 significantly decreases MTT reduction by hippocampal neurons in culture, but BIA 2-024 did not (14). However, in the same study, the new putative AEDs did not cause an increase in caspase-3-like enzymes activity, as OXC or CBZ did, and neuronal morphology appears to be intact, as we observed.

Mitochondria are central players in the metabolic processes of eukaryotic cells, having a critical role in ATP synthesis. Additionally, mitochondria are highly involved in other cellular processes besides energy production, such as intracellular Ca²⁺ buffering and cell death. We screened for the effect of AEDs in intracellular ATP levels and the EC of cultured hippocampal neurons exposed to these drugs. Only treatment with OXC but not with the other AEDs caused a decrease in intracellular ATP levels. Although a consistent and significant decrease in intracellular ATP levels is caused by OXC exposure, the decrease observed is not massive, and thus this is not translated by a significant decrease in energy charge, which might be explained by the fact that apoptosis rather than necrosis is occurring. Our current data and previous work point to a situation of apoptosis. ATP is required for apoptotic cell death, and depletion of the intracellular ATP levels and a dramatic decrease of the cell energy charge would cause a shift from apoptosis to necrotic cell death (22), which does not seem to be the case. Likewise, an increase in ROS production as well as a decrease in Ψ_m , observed only in OXC-treated neurons, suggests that the toxic effects of OXC might be mediated by a mitochondrial pathway, because the majority of ROS generated in the cells are of mitochondrial origin.

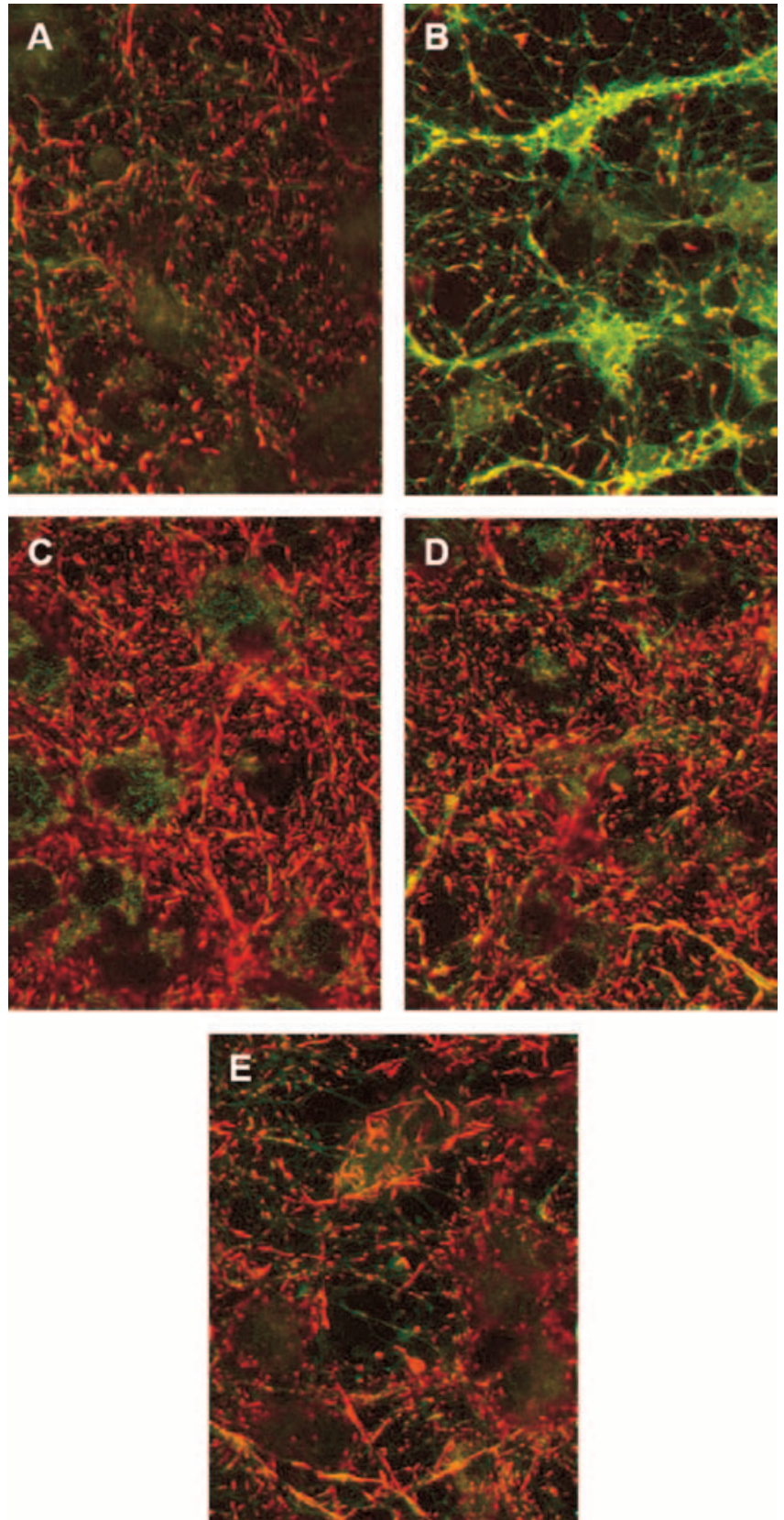


FIG. 4. Effect of antiepileptic drug treatment for 24 h on mitochondrial membrane potential (Ψ_m) in primary hippocampal cultures. Cultured hippocampal neurons were loaded with $3 \mu\text{M}$ JC-1, 15 min before imaging experiments. Under normal conditions, JC-1 aggregates emit red fluorescence; a decrease in Ψ_m results in an increase in the JC-1 monomer, which emits green fluorescence. **A:** Control culture. **B:** Oxcarbazepine (OXC). **C:** Carbamazepine (CBZ). **D:** BIA 2-024. **E:** BIA 2-093. $300 \mu\text{M}$ each; $\times 600$ magnification.

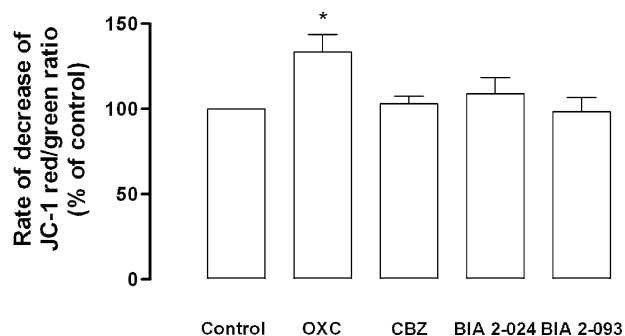


FIG. 5. Changes in mitochondrial membrane potential (Ψ_m) of cultured hippocampal neurons after treatment with antiepileptic drugs for 24 h. Each drug was used at 300 μ M. The figure represents changes in the rate of decrease of JC-1 red/green fluorescence ratio signal. The results are presented as percentage of control and represent the mean \pm SEM of at least four independent experiments. * $p < 0.05$, significantly different from control; Dunnett's posttest.

OXC rapidly undergoes reduction of the carbonyl group in the liver, to form the active agent 10,11-dihydro-10-hydroxycarbamazepine, and is thought to be a good alternative to CBZ in the treatment of epilepsy, because its metabolism does not involve formation of an epoxide metabolite, thus being less toxic. Compared with the parent compound (CBZ), hepatic microsomal enzyme induction and autoinduction are greatly reduced, and the clinical efficacy of OXC compares favorably with that of CBZ in clinical trials (5). OXC has fewer side effects and reduced potential for drug interaction in patients that did not respond well to CBZ therapy. However, of all the drugs tested in this study, OXC was the more toxic drug in all the parameters assayed. In vitro, OXC was not converted to other metabolites in cultured hippocampal neurons (data not shown), suggesting that the toxic effect of treatment with OXC in vitro is due to OXC itself and not to its metabolites. Being so, because in vivo OXC is rapidly metabolized to 10,11-dihydro-10-hydroxycarbamazepine, OXC will not reach the brain unless metabolic impairment occurs, and hepatic dysfunction is not uncommon in patients with epilepsy, which can often result from the AED therapy itself as an undesirable side effect. Thus care should be taken when administering OXC, because it proved to be toxic to cultured hippocampal neurons at concentrations not far from the therapeutic levels (14).

Within this scenario, because the two new putative AEDs have been shown to have efficacy in preventing seizures similar to that of CBZ and OXC (8,9), being less toxic to neuronal cells, there are advantages in considering these new drugs as possible alternatives to the current therapies available for the treatment of epilepsy. BIA 2-093 proved to be safe in a double-blind, placebo-controlled clinical trial in healthy male volunteers (23).

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