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Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type β confers wide-ranging protection on rat hippocampal neurons

(excitotoxicity/apoptosis/necrosis/growth factor)

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ABSTRACT **Excessive activation of glutamate receptors accompanied by Ca^{2+} overloading is thought to be responsible for the death of neurons in various conditions including stroke and epilepsy. Neurons also die if deprived of important growth factors and trophic influences, conditions sensitive to certain oncogene products such as the Bcl2 protein. We now demonstrate that transforming growth factor type β (TGF-β) prevents neuronal Ca^{2+} overloading of rat hippocampal neurons in response to the glutamatergic agonist *N*-methyl-D-aspartate or the Ca^{2+} ionophore 4-Br-A23187 and, in addition, leads to a substantial increase in neuronal Bcl2 protein expression. Parallel cytotoxicity experiments demonstrate that treatment with TGF-β protects rat hippocampal neurons from death induced by excitotoxicity, trophic factor removal, and oxidative injury. Thus, TGF-β may protect against a wide range of toxic insults by regulating two factors with great importance for neuronal viability.**

Inappropriate activation of glutamate receptors is thought to be responsible for the death of neurons following brain insults such as ischemia, epilepsy, and trauma (1). It is widely believed that the deleterious effects of glutamate receptor overactivation (excitotoxicity) are mainly due to massive Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA) receptors, leading to toxic Ca^{2+} overloading and its sequelae (1). On the other hand, it is known that under some circumstances neurons can die due to activation of a process known as programmed cell death. Under these circumstances, death is frequently associated with apoptosis (2). In the central nervous system, this type of neuronal death commonly occurs during development (3). Pathophysiologically, apoptotic cell death may be involved in Alzheimer β-amyloid peptide toxicity (4), in the death of certain neuronal populations in response to cerebral ischemia (5), as well as in free radical-mediated neuronal damage (6). Programmed cell death is also under the control of a set of genes that have either positive or negative effects (7). One of these is the *Bcl2* gene (8, 9), which is expressed in large amounts in the central nervous system (10).

Growth factors and cytokines are promising agents for treatment of a wide variety of neurodegenerative diseases for which little or no alternative treatments exist. Although neurotrophic/neuroprotective effects of these peptides against both excitotoxic and apoptotic cell injury have been reported from numerous *in vitro* and *in vivo* studies, little is known about their potential mechanisms of action. In the present study, we demonstrate that the neuroprotective cytokine transforming growth factor type β (TGF-β; ref. 11) is able to acutely stabilize neuronal Ca^{2+} homeostasis under conditions of pathophysiological Ca^{2+} overloading and also produces a rapid induction of the Bcl2 oncoprotein.

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MATERIALS AND METHODS

Cell Culture. Primary hippocampal neurons were dissociated from embryonic day 17 Holtzman rat embryos and plated onto poly(L-lysine)-coated glass coverslips. The cells were maintained in serum-free medium [Dulbecco's modified Eagle's medium with modified N2 supplements (N2-DMEM)] above a layer of secondary astrocytes (12). For biochemistry, hippocampal neurons were plated on 35-mm tissue culture dishes with perforated plastic coverslips containing astrocytes directly opposing the neurons. Cells used in this study were between 10 and 16 days *in vitro*. Animal care followed university guidelines.

Induction of Neuronal Injury. Excitotoxic injury was induced by washing the coverslips in Hepes saline (146 mM NaCl/10 mM Hepes/2 mM $CaCl_2$/1 mM $MgCl_2$/5 mM KCl/10 mM D-glucose, pH 7.4 and 312 mosM) and exposing them subsequently to the glutamatergic agonist NMDA (100 μM) in Mg-free Hepes saline supplemented with 0.1 mM glycine. After 20 min, coverslips were washed in saline and returned to conditioned culture medium. Controls were exposed to Hepes saline only. Mortality was assayed 24 h later by fluorescein diacetate/propidium iodide double staining and trypan blue exclusion. A total of 400–500 neurons were counted per coverslip.

Apoptosis was induced by deprivation of trophic influences. For this purpose, coverslips with neurons were removed from the astrocyte layer, washed once in Hepes saline, and maintained in Hepes saline for a period of 24 h. Osmolarity of the saline was set to that of the culture medium. Cell viability was determined after 24 h of deprivation by morphological criteria. Viable neurons were identified having round to oval, smooth soma and intact neurites. Apoptotic death was verified morphologically and by using terminal deoxynucleotidyltransferase-based immunodetection of DNA fragmentation (Oncor) in conjunction with the diaminobenzidine staining method.

Free radical-mediated neuronal injury was induced by a 24-h exposure to 0.1–10 μM $Fe_2(NH_4)_2(SO_4)_2$ in N2-DMEM. Viability was determined by morphological criteria. TGF-β (recombinant human; R & D Systems) stocks (1 μg/ml) were made in saline containing 1 mg of ovalbumin per ml and 4 mM HCl. Controls were treated with vehicle.

Abbreviations: $[Ca^{2+}]_i$, internal free calcium concentration; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; NMDA, *N*-methyl-D-aspartate; R-123, rhodamine 123; TGF-β, transforming growth factor type β.

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Fluorimetric Measurements. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was calculated by digital video microfluorimetry (13). Cells were loaded with fura-2 acetoxymethyl ester (3 μM) for 15 min at 37°C and were allowed at least 30 min of wash for dye deesterification. Intracellular free sodium concentration ($[Na^+]_i$) was determined by the dye sodium-binding benzofuran isophthalate (SBFI; Molecular Probes) and fura-2 imaging methods (14, 15). Cultures were loaded with 10 μM SBFI plus 12.25% pluronic F-127 for 1 h at 37°C and washed 30 min. Mitochondrial potential was monitored by measuring rhodamine 123 (R-123) fluorescence by digital imaging microfluorimetry and methods similar to those described (16). Cells were incubated for 2 min in saline containing 10 μM R-123 (Molecular Probes), and fluorescence intensity monitoring began after 2 min of wash. Mitochondrial potential was dissipated by 2-min applications of 1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to the bath. Experiments were performed at 22–24°C.

Protein Gel Electrophoresis. For biochemistry, plastic coverslips containing the astrocytes were removed from the cultures after treatments; protein samples were quantitated (BCA protein assay; Pierce) and analyzed by SDS/12.5% PAGE. After electrophoresis, the proteins were transferred to nitrocellulose and probed with a polyclonal antibody (1:1000) specific for Bcl2 (17). Detection of the signal was performed with an enhanced chemiluminescence detection kit (Amersham). Densitometric measurements were performed by means of an Ultrascan XL enhanced laser densitometer.

Statistics. Statistical comparisons were made by *t* test or ANOVA followed by the Student–Newman–Keuls test. Nonparametric data were analyzed by the Mann–Whitney U test or the Kruskal–Wallis H test followed by Dunn's test.

RESULTS

TGF-β1 Protects Against Excitotoxic Injury and Death Induced by Deprivation of Trophic Influences. Injury to cultured rat hippocampal neurons induced by a 20-min exposure to the glutamatergic agonist NMDA was characterized by the appearance of a rough, darkened soma with pyknotic nuclei (Fig. 1*A*, arrows) and loss of phase brightness. Fragmentation of neurites was preceded by swelling and formation of phase-dark blebs (Fig. 1*A Right*). A 2-h pretreatment of cultures with TGF-β1 at concentrations between 1 and 10 ng/ml produced dose-dependent protection from neuronal death with complete protection occurring at 10 ng/ml (Fig. 1*B*). TGF-β1 also prevented neuronal death when added simultaneously with NMDA and even conferred a degree of protection when added 1 h after the NMDA exposure (Fig. 1*C*). Protection against NMDA-induced injury was also obtained with a 2-h pretreatment using TGF-β3 (10 ng/ml), whereas a variety of other growth factors including fibroblast growth factor 2 (50 ng/ml), brain-derived neurotrophic factor (50 ng/ml), and epidermal growth factor (20 ng/ml) were without an effect in this paradigm (data not shown).

Neuronal injury produced by depriving cells of trophic influences showed distinct morphological changes. After 24 h, these changes were characterized by roughening of the plasma membrane, blebbing, and occasionally large translucent swellings. In many cases, the nucleus moved to one side of the neuronal soma (Fig. 2*A*). DNA fragmentation was detectable 12 h after trophic factor withdrawal with more widespread and intense staining after 18 h (Fig. 2*A Right*). Pretreatment of the cultures with TGF-β1 (0.1–10 ng/ml) for 24 h afforded significant protection against death caused by trophic factor removal (Fig. 2*B*). While addition of TGF-β1 2 h before the insult still afforded significant protection, addition of TGF-β1 at the time of astrocyte removal produced no effect (Fig. 2*C*).

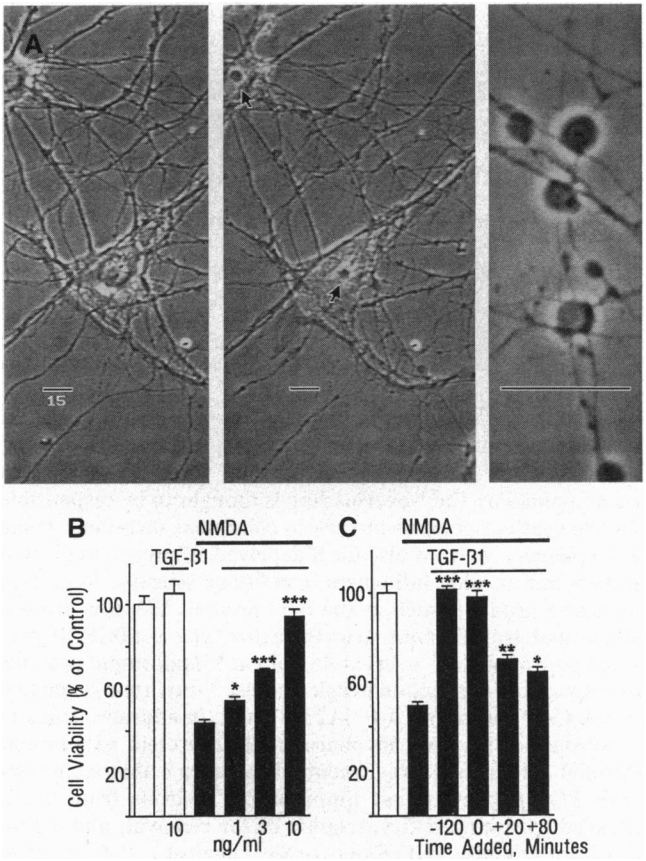

FIG. 1. TGF-β1 protects cultured rat hippocampal neurons from NMDA-mediated excitotoxicity. (*A*) (*Center*) Morphological changes caused by toxic exposure to NMDA typified by rough, darkened soma with pyknotic nuclei (arrows), loss of phase brightness, and fragmentation of neurites. (*Left*) Neuron before NMDA exposure. (*Right*) Fragmentation of neurites was preceded by formation of phase-dark blebs. (Bars = 15 μm). (*B*) Survival of neurons treated with 100 μM NMDA for 20 min was significantly enhanced by a 2-h pretreatment with TGF-β1. Toxicity data are given as means ± SEM from four or five coverslips. Triplicate experiments gave similar results. (*C*) Significant protection was obtained with 2-h TGF-β1 pretreatment (10 ng/ml), when coapplied with NMDA, when applied after the 20-min NMDA treatment, or even 1 h after NMDA exposure. Data are means ± SEM from four or five coverslips. Duplicate experiments gave similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TGF-β1 Prevents Pathophysiological Ca^{2+} Overloading. Fig. 3 shows the effect of NMDA on $[Ca^{2+}]_i$ in an experimental paradigm identical to that used for the toxicity experiments. NMDA produced sustained increases in $[Ca^{2+}]_i$ that often reached high plateaus and persisted even after agonist washout. Many cells also showed secondary increases in $[Ca^{2+}]_i$ after agonist removal, probably an indication of impending death (Fig. 3*A*). Cells pretreated with TGF-β1 for 2 h exhibited greatly reduced $[Ca^{2+}]_i$ responses to NMDA (Fig. 3 *B* and *C*) and returned rapidly to control levels after removal of the agonist. Secondary $[Ca^{2+}]_i$ increases and plateaus were rarely observed. TGF-β1 also had the capacity to lower NMDA-induced $[Ca^{2+}]_i$ increases when given acutely. Treatment with TGF-β1 (10 ng/ml) for 10 min reduced the peak increases caused by 10-sec application of 30 μM NMDA by 24.4% ± 4.6% compared to vehicle-perfused controls. These changes were specific for $[Ca^{2+}]_i$ since NMDA-induced increases in $[Na^+]_i$ were not altered by acute treatment with TGF-β1 (Fig. 3*D*). Using the whole-cell patch clamp technique, we found that NMDA-induced currents

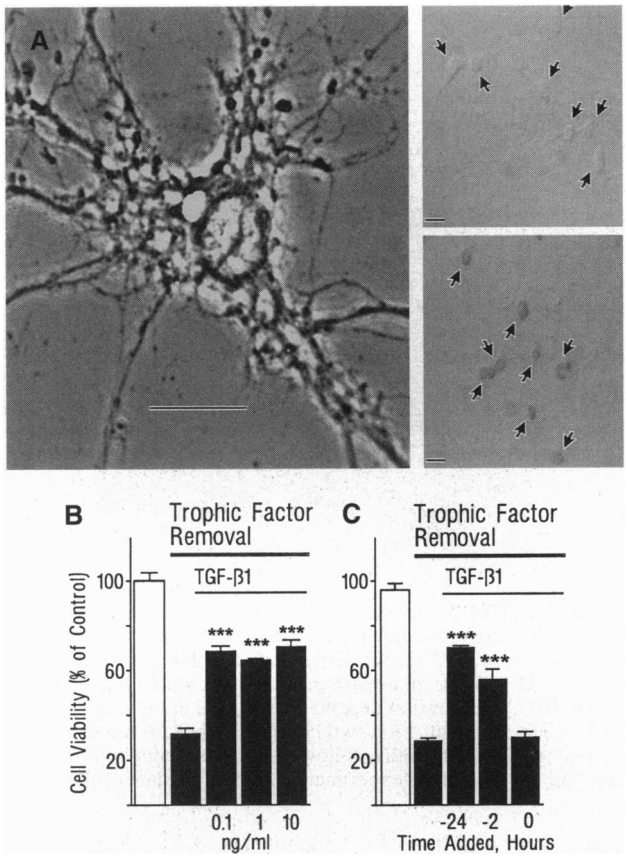

FIG. 2. TGF-β1 protects cultured rat hippocampal neurons from death induced by deprivation of trophic influences. (*A*) Morphology of neurons undergoing death induced by trophic factor removal was characterized by roughening of the plasma membrane, blebbing, and large translucent swellings. Note that the nucleus moved to one side of the neuronal soma (*Left*). DNA fragmentation was detectable after 18 h of deprivation (*Lower Right*), whereas control cultures showed no staining (*Upper Right*). (Bar = 15 μm.) (*B*) Deprivation of trophic influences resulted in 70% mortality at 24 h in untreated cultures. Significant protection resulted from a 24-h pretreatment with 0.1–10 ng of TGF-β1 per ml. Data are means ± SEM from four or five coverslips. Triplicate experiments gave similar results. (*C*) Highest protection was observed with 24-h pretreatment (time, −24 h) with TGF-β1 (10 ng/ml), less protection occurred with 2-h pretreatment (time, −2 h), and mortality was not altered if TGF-β1 was added only at the onset (time, 0) of trophic factor removal. Means ± SEM from four or five coverslips. Duplicate experiments gave similar results. ***, $P < 0.001$.

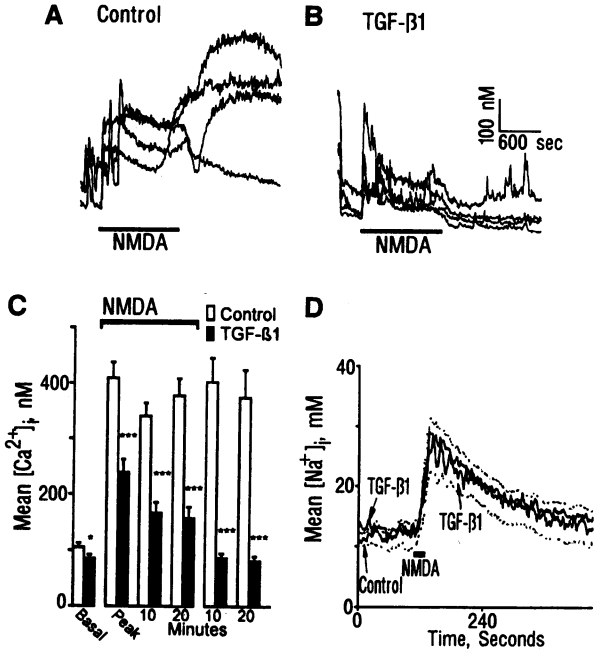

FIG. 3. TGF-β1 enhances $[Ca^{2+}]_i$ buffering in hippocampal neurons exposed to NMDA. (*A*) Baseline fluctuations in $[Ca^{2+}]_i$ represent ongoing network activity at excitatory synapses established between cultured neurons (12). Four representative neurons are shown. Application of NMDA (100 μM; 20 min) produced sustained elevations in $[Ca^{2+}]_i$ in most neurons that outlasted application. Note secondary increases in three of four neurons shown. (*B*) A 2-h pretreatment with 10 ng of TGF-β1 per ml reduced peak $[Ca^{2+}]_i$ and plateau level during NMDA application. TGF-β1 was present during NMDA exposure. Rapid recovery was evident in all four representative neurons. (*C*) Average $[Ca^{2+}]_i$ was significantly lower in TGF-β1-treated neurons at all time points measured, including basal $[Ca^{2+}]_i$. Data are from 25 control and 18 TGF-β1-treated neurons. Experiment was performed four times with similar results. (*D*) Peak increases and recovery time constants for $[Na^+]_i$ elevations induced by NMDA (30 μM; 10 sec) were not significantly altered ($P > 0.1$). Data are means ± SEM (dotted line) from $n = 27$ controls and $n = 28$ TGF-β1-treated neurons. Experiments performed in duplicate with similar results. *, $P < 0.05$; ***, $P < 0.001$.

also appeared unaltered in cells perfused with TGF-β1 (data not shown).

The ability of TGF-β1 to enhance Ca^{2+} buffering during glutamate receptor overactivation is a likely protective mechanism. To test this possibility further, we found that cells treated with TGF-β1 showed greatly reduced increases in $[Ca^{2+}]_i$ due to Ca^{2+} ionophore (4-Br-A23187) exposure. Fig. 4*A* demonstrates that 4-Br-A23187 produced a dose-dependent increase in neuronal $[Ca^{2+}]_i$. TGF-β1-treated cells exhibited greatly reduced increases in $[Ca^{2+}]_i$ after 4-Br-A23187 treatment (Fig. 4 *B* and *C*). At the highest ionophore concentrations used, $[Ca^{2+}]_i$ reached very high levels (plateaus), outlasting ionophore application. Despite increasing to high levels during application of 8 μM 4-Br-A23187, $[Ca^{2+}]_i$ frequently returned rapidly to baseline in TGF-β1-treated cells after washout of the ionophore, a result rarely observed in control neurons. Furthermore, smaller maximum slopes for $[Ca^{2+}]_i$ increases were typical in TGF-β1-treated cells (Fig. 4*D*).

To determine whether the Ca^{2+}-stabilizing effect of TGF-β1 may be associated with improved cellular energetics, mitochondrial potential was analyzed by using the mitochondrial dye R-123 (16). Mitochondrial staining with R-123 was significantly more intense in neurons pretreated for 24 h with TGF-β1 than in control neurons (initial intensity: TGF-β1 = 93.8 ± 6; control = 73.2 ± 3.9 intensity units; $P < 0.001$) (Fig. 5). The change in fluorescence produced by the mitochondrial uncoupler FCCP was significantly greater in TGF-β1-treated cells (for first FCCP application: TGF-β1, $\Delta = +35.2 \pm 5.4$; control, $\Delta = +15.5 \pm 2.6$ intensity units; $P < 0.01$), indicating a greater mitochondrial potential. Mitochondrial potential in TGF-β-treated cells also recovered more quickly after washout of FCCP, resulting in faster recovery time constants (TGF-β1 = 13.6 ± 2.0 sec; control = 51.2 ± 8.0 sec; $P < 0.001$) and greater retention of R-123.

TGF-β1 Induces Neuronal Expression of the Bcl2 Oncoprotein. Unlike NMDA toxicity, the protective effects of TGF-β1 on cell death caused by deprivation of trophic influences were manifest only after a period of pretreatment (Fig. 2*C*). It has been demonstrated that the 26-kDa product of the *Bcl2* gene can protect a variety of cells against certain types of cell death, often associated with withdrawal of important trophic factors (8, 9). Although Bcl2 is expressed in the mammalian brain (10), details of its regulation remain undetermined. We observed that neurons treated with

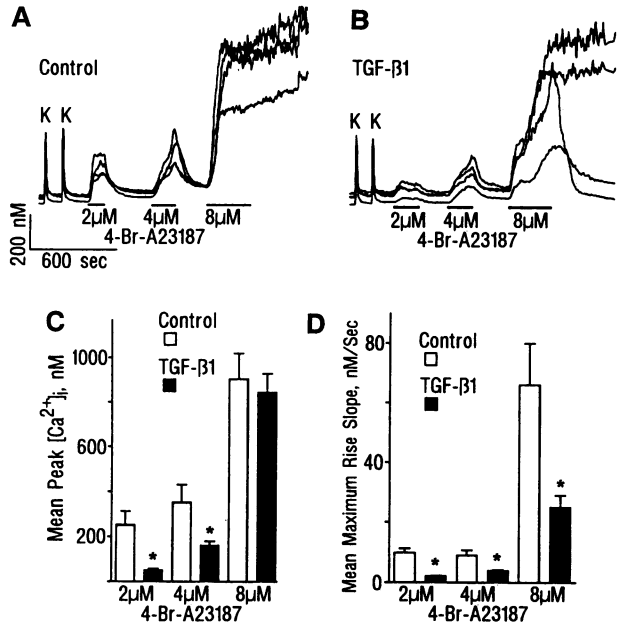

FIG. 4. TGF-β1 treatment enhances the ability of hippocampal neurons to buffer $[Ca^{2+}]_i$ during treatment with the Ca^{2+} ionophore 4-Br-A23187. (*A*) Representative records from four control neurons exposed to two 5-sec 50 mM KCl applications and applications of 2, 4, and 8 μM 4-Br-A23187. (*B*) Representative records from four neurons pretreated for 2 h with 10 ng of TGF-β1 per ml. (*C*) Peak increase in $[Ca^{2+}]_i$ was higher in control neurons, significantly ($P < 0.01$) at 2 and 4 μM ionophore concentrations ($n = 18$ controls and $n = 20$ TGF-β1-treated neurons). (*D*) Maximum slopes attained during increases to peak $[Ca^{2+}]_i$ were significantly faster in controls, even where peak $[Ca^{2+}]_i$ was not different (8 μM 4-Br-A23187). Reductions in $[Ca^{2+}]_i$ plateaus and/or significantly smaller maximum slopes were observed in seven of eight experiments. *, $P < 0.05$.

TGF-β1 or -β3 showed a greatly enhanced expression of the Bcl2 oncoprotein (Fig. 6*A*). Significant enhancement occurred after 2 h of TGF-β1 (10 ng/ml) treatment, with even larger effects after 24 h (increase of 239% ± 61% and 595% ± 176%, respectively; $n = 4$ experiments). Bcl2 protein induction occurred at low TGF-β1 concentrations with clear

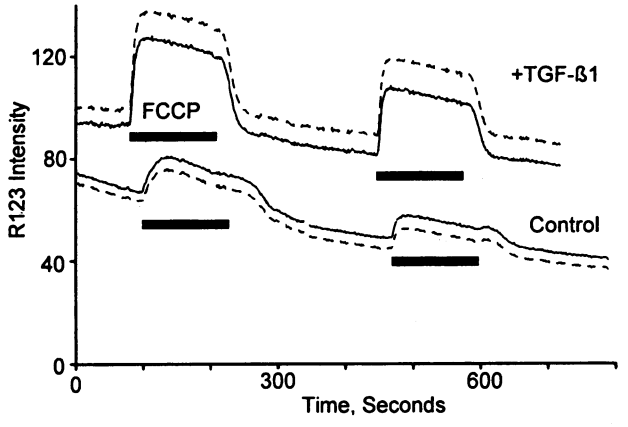

FIG. 5. TGF-β1 treatment improves mitochondrial energetics. Neurons were stained with R-123 2 min prior to time 0. Upper solid trace represents mean R-123 fluorescence intensity of 15 neurons after a 24-h pretreatment with 10 ng of TGF-β1 per ml; dashed trace is mean + SEM. Lower trace pairs correspond to control mean ($n = 25$) and mean − SEM. FCCP (1 μM) released more fluorescence in TGF-β-treated cells, indicating that those mitochondria were initially more polarized than those in control cells. After washout of FCCP, R-123 was resequestered faster and retained better in TGF-β-treated cells. Data are representative of four experiments.

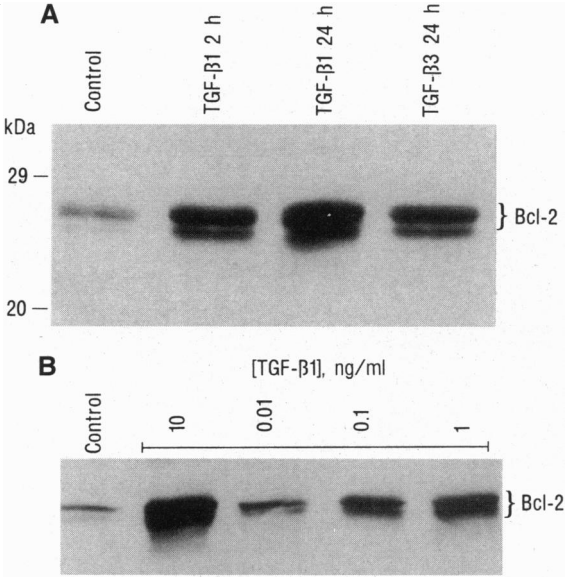

FIG. 6. TGF-β1 treatments induce expression of Bcl2 oncoprotein. (*A*) Western blots showing Bcl2 expression in untreated cultures and induction of Bcl2 oncoprotein after 2- and 24-h pretreatment with 10 ng of TGF-β1 per ml or 24-h pretreatment with 10 ng of TGF-β3 per ml. (*B*) Concentration dependence of Bcl2 induction by TGF-β1 for 24-h pretreatment. First two lanes are vehicle-treated and TGF-β1-treated (10 ng/ml) cells, followed by treatments with 0.01, 0.1, and 1 ng/ml. Triplicate experiments yielded similar results.

effects after addition of 0.1 ng/ml (Fig. 6*B*). Thus, the time course and concentration dependence of Bcl2 induction were similar to that observed for the protective effects of TGF-β1 after deprivation of trophic influences.

TGF-β1 Protects Against Free Radical-Mediated Neuronal Injury. While the mechanism of Bcl2 action is not yet fully elucidated, recent reports suggest that Bcl2 protects cells by inhibiting the generation or effects of reactive oxygen species (18, 19). We therefore evaluated whether TGF-β1 might also protect neurons against oxidative neuronal injury. For this purpose, the hippocampal cultures were exposed to ferrous ions that catalyze the formation of oxygen radicals (20). Exposure to iron resulted in a dose-dependent increase in neuronal mortality that was significantly reduced in TGF-β1 pretreated cells (Fig. 7).

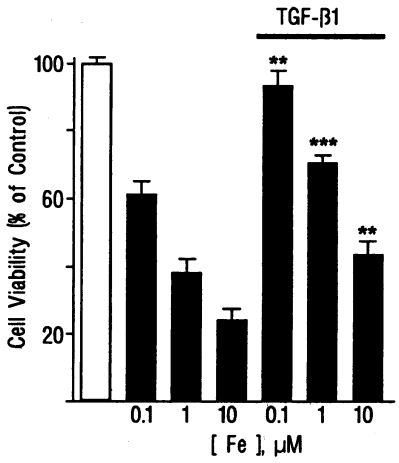

FIG. 7. Pretreatment with TGF-β1 (10 ng/ml) for 24 h protects against iron-induced oxidative injury. Cultures were exposed to iron as described. Data are means ± SEM from four coverslips. Experiment was performed in duplicate. **, $P < 0.01$; ***, $P < 0.001$.

DISCUSSION

The present study demonstrates that TGF-βs have the capacity to regulate two factors with great importance for neuronal viability and to protect neurons against a wide range of toxic insults. We suggest that the TGF-βs play an important role both in maintenance of neuronal viability in the unlesioned brain (21) and in limiting the destructive consequences of metabolic and traumatic insults to the central nervous system (22).

Treatment of rat hippocampal neurons with TGF-β1 protected against NMDA receptor-mediated excitotoxic injury and against injury induced by depriving the cells from trophic factors. While the death induced by deprivation of trophic factors showed many of the characteristics of apoptosis (ref. 2; Fig. 2), excitotoxic injury exhibited morphological changes that are typical of necrotic injury, including dendritosomatic swelling and an early loss of membrane integrity (refs. 1 and 2; Fig. 1). These processes are normally accompanied by failure of the cell to regulate ion homeostasis (refs. 1 and 2; Fig. 3*A*). In particular, overloading with Ca^{2+} is thought to play a key role in glutamate- and NMDA-induced neuronal injury (1, 23). We demonstrate here that treatment of hippocampal neurons with TGF-β1 leads to improved Ca^{2+} homeostasis in response to the glutamatergic agonist NMDA or the Ca^{2+} ionophore 4-Br-A23187, an effect that may well be related to the protective effects against NMDA-mediated injury.

TGF-β1 appears to influence the interaction between $[Ca^{2+}]_i$ and mitochondrial energetics. In support of this idea, we have found that mitochondria appear to be more hyperpolarized and are more able to maintain their membrane potential after TGF-β treatment (Fig. 5). This implies that mitochondrial function and, as a consequence, Ca^{2+} buffering and extrusion are enhanced. In turn, greater buffering of $[Ca^{2+}]_i$ during prolonged NMDA or calcium ionophore exposures implies that TGF-β-treated neurons would experience less Ca^{2+}-mediated mitochondrial uncoupling (16, 24). It is interesting to note that a previous study has demonstrated that TGF-β1 is localized to mitochondria in different cell types (25), but the significance of this remains unknown.

Treatment with TGF-β also produced a rapid and pronounced induction of the Bcl2 oncoprotein in neurons (Fig. 5). Microinjection of Bcl2 expression plasmids has been shown to protect neurons against certain types of apoptotic cell death (9). We now give evidence that this may also occur physiologically—in response to TGF-β1. Although it seems highly likely that the increased expression of Bcl2 may contribute to the protective effect against trophic factor withdrawal, we cannot exclude that effects of TGF-β1 other than, or in conjunction with, those on Bcl2 expression may be involved in the protective effect of this cytokine.

It is currently believed that the death of neurons and other cells can be categorized as necrosis or apoptosis according to several criteria (2). Nevertheless, many of the same factors might be involved in both phenomena. This would include a role for Ca^{2+} and also toxic free radicals (1, 2, 7, 18, 19). It seems that TGF-βs may be able to modulate the viability of neurons in a variety of situations by simultaneously orchestrating different key protective elements in the cell.

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