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Apoptosis Induction by a Dopaminergic Neurotoxin, 1-Methyl-4-Phenylpyridinium Ion (MPP^+), and Inhibition by Epidermal Growth Factor in GH3 Cells

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ABSTRACT. A dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can induce dopaminergic denervation and Parkinsonism in humans. The active metabolite of MPTP is the 1-methyl-4-phenylpyridinium ion (MPP^+). Previously we reported that MPP^+ is incorporated via the dopamine transport system and causes delayed cell death in GH3 cells, a clonal strain from the rat anterior pituitary. In this study, we investigated whether MPP^+ induces apoptosis. GH3 cells cultured with MPP^+ exhibited DNA laddering and fragmentation in a time- and concentration-dependent manner. The effect of MPP^+ was inhibited in GH3 cells treated with a pan-caspase inhibitor (100 μM ZVAD-fmk), an antioxidant (25 mM *N*-acetyl-l-cysteine), or epidermal growth factor (EGF; 50 ng/mL). Because EGF stimulated tyrosine phosphorylation of the EGF receptor and tyrphostin AG1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline; 5 μM, a specific inhibitor of EGF receptor kinase] abolished EGF inhibition, involvement of EGF receptor kinase is assumed. Protein kinase C-dependent processes and Bcl-2 protein expression were shown not to be involved in EGF inhibition. MPP^+ increased cytochrome *c* immunoreactivity in cytosolic fractions in GH3 cells. The addition of 200 μM MPP^+ to isolated mitochondrial fractions from GH3 cells stimulated the release of a 13-kDa protein that cross-reacted with anti-cytochrome *c* antibody. The release was inhibited in EGF-treated GH3 cells. Our findings demonstrated that (i) MPP^+ induces apoptosis of GH3 cells via cytochrome *c* release and caspase activation, and (ii) apoptosis by MPP^+ can be blocked by *N*-acetyl-l-cysteine or EGF treatment. BIOCHEM PHARMACOL **60**;1: 111–120, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. dopaminergic neurotoxin; MPP^+; apoptosis; caspase; cytochrome *c*; EGF; GH3 cells

Neuronal cells may die by either necrosis or apoptosis. Apoptosis is a naturally occurring process of cell suicide that plays a decisive role in normal development and is integral to the pathogenesis of many diseases (for review, see Refs. 1 and 2). Among the characteristic events that occur during apoptosis are the cleavage of certain proteins by caspases, a set of cysteine proteases that are activated during apoptosis, and the destruction of the genome, which results in the release of nucleosome-sized fragments of DNA.

The selective dopaminergic neurotoxin MPTP† can induce Parkinsonism in primates and humans and is used to create models of dopaminergic denervation and Parkinson's disease (for review, see Refs. 3–5). MPP^+, the active metabolite of MPTP, inhibits mitochondrial respiratory chain complexes and depletes cellular ATP levels, resulting in cell death [3, 6]. Studies have reported apoptosis in some neuronal cells exposed to MPP^+ [7–9], and features of apoptotic cell death also have been observed in neurons of the substantia nigra in MPTP-treated mice [10]. However, higher concentrations of MPP^+ have been shown to result in necrosis of neuronal cells [8, 9]. Injection of certain neurotrophic factors into the brain has been shown to protect MPTP-treated mice [11–14]. MPP^+-induced cell death of dopaminergic neurons in culture [15] and SH-SY5Y cells [16] is prevented by BDNF. These studies show that MPP^+-induced death of neuronal cells can be blocked by some neurotrophic factors, but it is unknown whether these factors block apoptosis or necrosis.

We used GH3 cells, a clonal strain from the rat anterior pituitary, as a model for studying the regulation of neuronal peptide/hormone secretion in the anterior pituitary. In GH3 cells, the addition of protein phosphatase inhibitors has been shown to induce apoptosis [17, 18]. We reported previously that MPP^+ is incorporated via nomifensine-sensitive dopamine transporters and causes LDH leakage in GH3 cells [19]. Cell death induced by MPP^+ was blocked in GH3 cells by treatment with EGF, but not with BDNF. In this study, we investigated whether GH3 cell death

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† *Abbreviations*: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium ion; BDNF, brain-derived neurotrophic factor; LDH, lactate dehydrogenase; NAC, *N*-acetyl-L-cysteine; EGF, epidermal growth factor; HS, horse serum; FBS, fetal bovine serum; PMA, phorbol myristate acetate; PKC, protein kinase C; PI 3-kinase, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; and GDNF, glial cell line-derived neurotrophic factor.

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induced by MPP^+ is apoptotic, and whether EGF can protect against MPP^+-induced apoptosis. MPP^+ caused apoptosis of GH3 cells, cytochrome *c* release from mitochondria, caspase activation, and DNA fragmentation. Apoptosis of GH3 cells by MPP^+ was blocked by pretreatment with the antioxidant NAC or the neurotrophic factor EGF.

MATERIALS AND METHODS

GH3 Cells

A GH3 cell clone was obtained from the American Type Culture Collection. GH3 cells were cultured in Ham's F10 medium supplemented with heat-inactivated 15% HS and 2.5% FBS, as previously reported [19]. Forty-eight hours before the experiment, GH3 cells were plated in dishes and grown as monolayers in F10 medium (HS/FBS-containing). In some experiments, agents such as EGF were added to the F10 medium.

Materials

MPP^+ iodide was purchased from Research Biochemicals Inc. [*Methyl*-^{3}H]thymidine [74 GBq/mmol (2 Ci/mmol)] was obtained from NEN^{TM} Life Science. EGF, H_2O_2, dl-α-tocopherol, and ascorbic acid were obtained from Wako Pure Chemicals. Anti-phosphotyrosine antibody was purchased from Transduction Lab. Anti-Bcl-2 antibody (N-19, Cat. No. sc-492-G) and anti-EGF receptor antibody (Cat. No. 1005) were purchased from Santa Cruz Biotech. The EGF receptor kinase inhibitor tyrphostin AG1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline] and the pan-caspase inhibitor ZVAD-fmk (Z-Val-Ala-dl-Asp-fluoromethylketone) were purchased from Calbiochem and Bachem AG, respectively. NAC, deferoxamine mesylate, cytochrome *c* [from horse heart (C2506), molecular weight 12,384], PMA (an activator of PKC), wortmannin (an inhibitor of PI 3-kinase), and mastoparan were purchased from the Sigma Chemical Co. GF109203X (3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione) was obtained from BIOMOL Res. An LDH assay kit and recombinant human BDNF were purchased from Boehringer Mannheim and Pepro, respectively. PD98059 (2′-amino-3′-methoxyflavone; an inhibitor of ERK kinase) was obtained from New England Biolabs. Anti-cytochrome *c* antibody was obtained from Pharmingen.

Detection of DNA Laddering

DNA laddering was assayed as previously reported [20] with minor modifications. GH3 cells on 100-mm dishes ($1–2 \times 10^6$ cells/dish) were suspended in lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100) for 15 min at 4°. The samples were centrifuged at 15,000 *g* for 15 min at 4°. The supernatants were treated with 50 μg/mL of proteinase K at 37° for 30 min and extracted with an equal volume of phenol, phenol:chloroform (1:1, v/v), and chloroform. The DNA was precipitated with a 1/10 vol. of 3 M sodium acetate (pH 5.2) and 2 vol. of ethanol. The DNA was suspended in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) and treated with 40 μg/mL of RNase A for 1 hr at 37°. The concentrations of DNA were determined by absorbance at 260 nm. DNA was subjected to agarose gel electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Quantification of DNA Fragmentation

DNA fragmentation was quantified as described previously [21] with minor modifications. Growing GH3 cells on 22-mm dishes (12-well plate, $1–2 \times 10^5$ cells/well) were labeled with F10 medium containing HS/FBS and 1 μCi/mL (37 KBq/mL, 2.9 μM) of [*methyl*-^{3}H]thymidine for 24 hr and washed three times with fresh F10 medium. In some experiments, 50 ng/mL of EGF or other agents was added to the F10 medium for labeling. Because of the high concentrations of HS/FBS, the amount of incorporated [^{3}H]thymidine in EGF-treated GH3 cells was almost the same as that in control GH3 cells without EGF. Labeled cells were exposed to MPP^+ in F10 medium (HS/FBS-free) for 24 hr for DNA fragmentation assays. In additional experiments, cells were preincubated with various agents or inhibitors for a given period before MPP^+ addition. Cells on dishes and detached cells in medium were pooled and suspended in lysis buffer as described above. After 15 min at 4°, samples were centrifuged at 12,000 *g* for 15 min at 4°, and the pellets were dissolved with detergent solution (0.2 N NaOH, 2% SDS). The radioactivity present in the supernatant (detergent-soluble low molecular weight DNA) and in the pellet (intact chromatin DNA or large chromatin fragments, > 50 kbp) was determined by liquid scintillation counting. Apoptosis was measured as the ratio of radioactivity in the supernatant to total radioactivity [% = (supernatant radioactivity)/(supernatant activity + remaining activity in pellet)] per dish. In control GH3 cells cultured with F10 medium containing HS/FBS, DNA fragmentation was 1–4%, and in GH3 cells cultured with F10 medium (HS/FBS-free), it was 3–8% of total incorporated [^{3}H]thymidine.

Immunoblotting Analysis of EGF Receptor

GH3 cells on 100-mm dishes were cultured with F10 medium (HS/FBS-free) for 3 hr, and cells were stimulated with 50 ng/mL of EGF for 10 min at 37°. Cells were washed twice with ice-cold PBS and detached from dishes. Cells (2×10^6/tube) were suspended with 1 mL of ice-cold buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 0.1 mM Na_3VO_4, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, and 1 mM phenylmethanesulfonyl fluoride). Lysates were centrifuged at 10,000 *g* for 10 min. The supernatants were solubilized in Laemmli

sample buffer and loaded on 8% SDS gels. Transfer to nitrocellulose paper, immunoblotting with antibodies [anti-EGF receptor antibody (diluted to 1:1000) or anti-phosphotyrosine antibody (diluted to 1:1000)], and washing were performed as described [19, 22].

Immunoblotting Analysis of Bcl-2 Protein

GH3 cells were cultured with F10 medium (HS/FBS-containing) for 24 or 48 hr in the presence of 50 ng/mL of EGF and 100 μM MPP^+ or 1 μM PMA. Cells were washed twice with PBS and detached from dishes. Cells (5×10^6/tube) were suspended in 200 μL of ice-cold buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 0.1 mM phenylmethanesulfonyl fluoride) and homogenized at 4° by sonication for 30 sec at a power output of 20 W [TAITEC handy Sonic (Vp-5S)]. After centrifugation at 4° for 5 min at 8000 *g*, supernatants were solubilized and loaded on 12% SDS gels. Transfer to nitrocellulose paper, immunoblotting with anti-Bcl-2 antibody (diluted to 1:2000), and washing were performed as described [20, 23]. Images of reactive bands were analyzed by laser densitometry, and band intensities were shown to increase in proportion to protein concentrations.

Detection of a Cytochrome c-Like Molecule in GH3 Cells

Control and MPP^+-treated GH3 cells were suspended with 1 mL of ice-cold buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose, 10 μg/mL of leupeptin, and 1 mM phenylmethanesulfonyl fluoride) and homogenized using a glass–Teflon homogenizer (10 strokes) at 4°. The homogenates were centrifuged at 20,000 *g* for 15 min. Equal amounts of protein in the cytosolic fractions were subjected to immunoblotting analysis using anti-cytochrome *c* antibody (diluted to 1:1000).

Mitochondria from GH3 cells were prepared as described [24], with minor modifications. Control and EGF-treated GH3 cells were suspended with mitochondrial preparation buffer (MPB: 5 mM HEPES–KOH, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 1 mM phenylmethanesulfonyl fluoride). Small aliquots (5 μL) of digitonin solution (5%) were added to the cell suspensions (1 mL, 1×10^8 cells/tube; the final concentration of digitonin was 0.025%), and incubated for 5 min at 4°. After the addition of 1 mL of MPB, samples were centrifuged at 4° for 3 min at 3000 *g*. Cell pellets were suspended with 1 mL of MPB and homogenized using a glass–Teflon homogenizer (10 strokes) at 4°. After the addition of 2 mL of MPB, lysates were centrifuged at 4° for 5 min at 625 *g*, and mitochondria in supernatant fractions were obtained. Pellets were re-homogenized, and a second mitochondrial fraction was obtained and pooled with the first fractions. The combined supernatants were centrifuged at 4° for 10 min at 9800 *g*, and the pellets were used as isolated mitochondria.

Mitochondrial fractions (50 mg protein/tube) were suspended with buffer (5 mM HEPES–KOH, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM sodium pyruvate, 10 mM phosphocreatine, 2 mM ATP, 0.5 mM phenylmethanesulfonyl fluoride), and incubated for 4 hr at 37° with the agents. After incubation, samples were centrifuged at 4° for 3 min at 12,000 *g*, and the supernatants were concentrated with an ULTRAFREE-MC filter (<10,000 molecular weight, Millipore Corp.), then dissolved with SDS–sample buffer and loaded in 14% SDS gels. Purified cytochrome *c* was used as a standard. The gels were stained with Coomassie brilliant blue or a silver staining kit.

Statistics

Data are the means ± SEM of 3–5 independent experiments done in triplicate, and were analyzed using the unpaired *t*-test. For multiple comparisons, the significance of difference was determined by ANOVA followed by Dunnett's or Tukey's test. *P* values of < 0.01 were considered to be significant. In some experiments, data are the means ± SD of 3 determinations in a typical experiment and are representative of 2 or 3 independent experiments.

RESULTS

MPP^+-Induced DNA Laddering and Fragmentation in GH3 Cells and Its Inhibition by EGF Treatment

Previously we reported that 200 μM MPP^+ induces cell death, as indicated by LDH leakage, 48 hr after addition to GH3 cells [19]. In this study, we investigated whether this MPP^+-induced GH3 cell death is apoptotic. Figure 1 shows DNA laddering in GH3 cells. Culture with F10 medium (HS/FBS-free) for 24 hr induced DNA laddering slightly (data not shown), and addition of 200 μM MPP^+ induced DNA laddering remarkably (panel A, lane 2). DNA laddering was detectable at 12 hr and marked at 24 hr after 200 μM MPP^+ addition (panel B, lanes 3 and 4). Although clear DNA laddering induced by MPP^+ was observed in some experiments, broad DNA fragmentation was observed in other experiments. Chromosomal condensation of GH3 cells was assessed using the chromatin dye Hoechst 33258 (5 μg/mL in PBS). At 24 hr, treatment with 200 μM MPP^+ resulted in nuclear chromatin condensation (data not shown), as previously reported in GH3 cells treated with apoptotic agents such as okadaic acid [18]. MPP^+-induced DNA laddering was inhibited in GH3 cells treated for 24 hr with 50 ng/mL of EGF before the addition of MPP^+ (panel B).

Next, we analyzed the effect of MPP^+ by quantification of DNA fragmentation using the [^{3}H]thymidine labeling assay (Fig. 2). As mentioned in Materials and Methods, DNA fragmentation after 24 hr in control GH3 cells (cultured in F10 medium containing HS/FBS) was 2.4 ± 0.6% (N = 6, in the range of 1–4%) of total incorporated

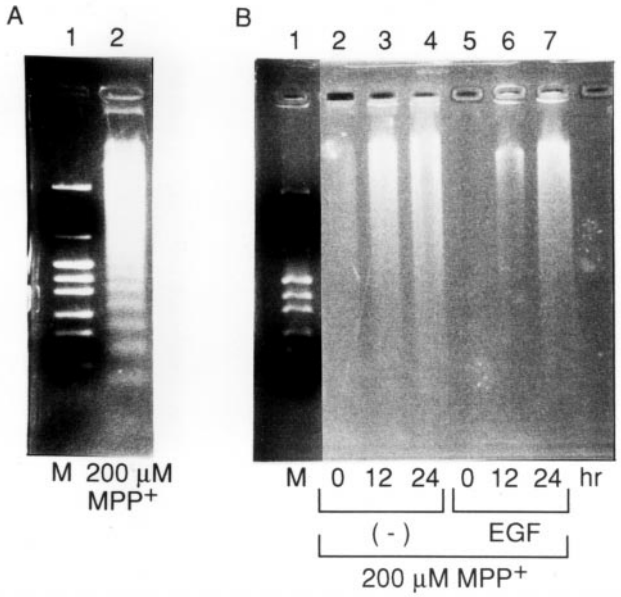

FIG. 1. DNA laddering induced by MPP^+ in GH3 cells. (A) DNA laddering induced by MPP^+ in a typical experiment. GH3 cells were cultured in fresh F10 medium (HS/FBS-free) for 24 hr with 200 μM MPP^+. (B) GH3 cells were cultured for 24 hr in F10 medium (HS/FBS-containing) with (lanes 5–7) or without (lanes 2–4) 50 ng/mL of EGF. The washed cells were cultured further for 12 hr (lanes 3 and 6) or 24 hr (lanes 4 and 7) in fresh F10 medium (HS/FBS-free) in the presence of 200 μM MPP^+. In lanes 5–7, 50 ng/mL of EGF was added again to the EGF-treated cells. In lanes 2 and 5, DNA was obtained from the washed cells without further culture. DNA markers are shown in lane 1 (A and B). Data are representative of 3–5 independent experiments.

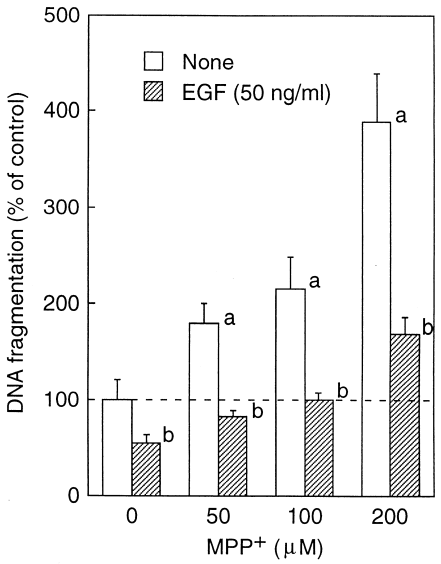

FIG. 2. Quantification of DNA fragmentation by MPP^+ and its prevention by EGF treatment in GH3 cells. GH3 cells were labeled with [^{3}H]thymidine in F10 medium (HS/FBS-containing) with (hatched column) or without (None, open column) 50 ng/mL of EGF for 24 hr. The washed cells were cultured further for 24 hr in fresh F10 medium (HS/FBS-free) with the indicated concentrations of MPP^+. An identical concentration of EGF was added again to the EGF-treated cells. DNA fragmentation was quantified as described in Materials and Methods. Data are presented as percentages of DNA fragmentation in control GH3 cells, which were cultured for 24 hr with F10 medium (HS/FBS- and MPP^+-free). Each experiment was done in triplicate, and data are the means ± SEM of 3–4 independent experiments. In a typical experiment, the absolute values of DNA fragmentation at 24 hr were (means ± SD of 3 determinations) 4.5 ± 0.3 and 1.8 ± 0.8% of total incorporated [^{3}H]thymidine in control (4.2×10^5 dpm/well) and EGF-treated GH3 cells (4.5×10^5 dpm/well), respectively, in the absence of MPP^+; and 25.1 ± 2.2 and 10.5 ± 1.7% in control and EGF-treated GH3 cells, respectively, in the presence of 200 μM MPP^+. The total incorporated [^{3}H]thymidine in MPP^+-treated cells was almost the same as that in control cells. Key: (a) $P < 0.01$, significantly different from the value without MPP^+; and (b) $P < 0.01$, significantly different from the value without EGF treatment.

[^{3}H]thymidine, and that in serum-depleted GH3 cells (cultured in HS/FBS-free F10 medium) was 4.5 ± 1.6% (N = 8, in the range of 3–8%) of the total. To avoid effects by contaminating growth factors in HS/FBS, the effect of MPP^+ on DNA fragmentation was investigated using HS/FBS-free F10 medium in the following experiments. At first we compared DNA fragmentation induced by serum withdrawal in control and EGF-treated (50 ng/mL, 24 hr) GH3 cells. The DNA fragmentation induced by serum withdrawal in EGF-treated GH3 cells was significantly less extensive than that in control cells (Fig. 2).

The addition of 200 μM MPP^+ did not induce DNA fragmentation at 6 or 12 hr. Only 1–4% of total incorporated [^{3}H]thymidine was detected in the supernatant, similar to cultures without MPP^+. At 24 hr after MPP^+ addition, DNA fragmentation was obvious in GH3 cells and occurred in an MPP^+ concentration-dependent manner starting at 50 μM (Fig. 2). EGF treatment (50 ng/mL for 24 hr) inhibited MPP^+-induced DNA fragmentation significantly at all concentrations examined. At 48 hr after 200 μM MPP^+ addition, the degree of DNA fragmentation was much greater than at 24 hr, but showed wide variation (data not shown). The DNA fragmentation by MPP^+ at 48 hr in EGF-treated GH3 cells was much less extensive than that in control cells. Neither treatment with nerve growth factor nor treatment with BDNF (100 ng/mL) affected serum withdrawal- or MPP^+-induced DNA laddering and fragmentation in GH3 cells, in accordance with our previous report measuring LDH leakage [19].

Characteristics of MPP^+-Induced Apoptosis in GH3 Cells

Du *et al.* [9] reported that MPP^+ induces apoptosis in cultured cerebellar granule neurons via a caspase-3-like protease. Treatment with 100 μM ZVAD-fmk, a pan-caspase inhibitor, inhibited DNA fragmentation induced by serum withdrawal or by 200 μM MPP^+ in GH3 cells (Table 1). ZVAD-fmk inhibited DNA fragmentation in a concentration-dependent manner starting at 50 μM. MPP^+ induces mitochondrial formation of superoxide radicals, and radical damage has been shown to contribute to

TABLE 1. Effect of ZVAD-fmk on MPP^+-induced DNA fragmentation in GH3 cells

	DNA fragmentation (% of control)	
Addition	**None**	**100 μM ZVAD-fmk**
None	100	57 ± 14*
MPP^+ (200 μM)	331 ± 57	122 ± 53*

GH3 cells were cultured in fresh F10 medium (HS/FBS-free) with vehicle or 100 μM ZVAD-fmk for 2 hr, and then were cultured for 24 hr with or without 200 μM MPP^+. Data are presented as percentages of DNA fragmentation in control GH3 cells, which were cultured for 26 hr with F10 medium (HS/FBS- and MPP^+-free). Data are the means ± SEM of 3 independent experiments. In a typical experiment, the absolute values of DNA fragmentation at 26 hr were (means ± SD of 3 determinations) 7.8 ± 0.9 and 3.8 ± 0.6% of total incorporated [^{3}H] thymidine in control and ZVAD-fmk-treated GH3 cells, respectively, in the absence of MPP^+; and 39.9 ± 2.3 and 19.8 ± 1.7% in control and ZVAD-fmk-treated GH3 cells, respectively, in the presence of 200 μM MPP^+. The total incorporated [^{3}H] thymidine was 4.5 to 4.9 × 10^5 dpm/well in the control and the treated cells.

* $P < 0.01$, significantly different from values without ZVAD-fmk.

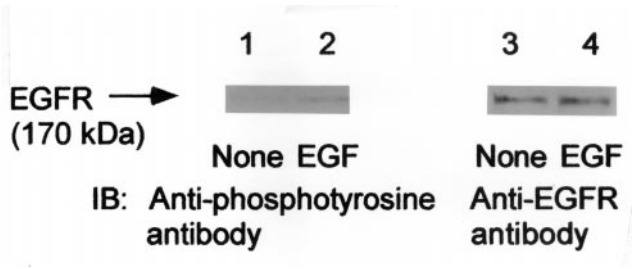

FIG. 3. Tyrosine phosphorylation of the EGF receptor in GH3 cells. GH3 cells were incubated with vehicle (None, lanes 1 and 3) or 50 ng/mL of EGF (lanes 2 and 4) for 10 min at 37°. The cell extracts were obtained as described in Materials and Methods. Identical amounts of protein in the extracts were analyzed by SDS–PAGE followed by western blotting with anti-phosphotyrosine (lanes 1 and 2) and anti-EGF receptor (lanes 3 and 4) antibodies. Data are representative of 2 independent experiments.

the neurotoxicity of MPP^+ [5, 25, 26]. Antioxidants have been reported to prevent cell death induced by oxidative stress in various cells [27]. Next, we investigated the effects of antioxidants on MPP^+-induced apoptosis of GH3 cells. As shown in Table 2, 25 μM α-tocopherol and 1 mM ascorbic acid had no effect on serum withdrawal- and MPP^+-induced DNA fragmentation. Deferoxamine (1 mM), an iron chelator, induced DNA fragmentation by itself and enhanced MPP^+-induced DNA fragmentation in GH3 cells. NAC is an antioxidant that increases intracellular glutathione, one of the pathways by which cells are protected from oxidative stress [5, 28]. Treatment with NAC inhibited 200 μM MPP^+-induced DNA fragmentation in a concentration-dependent manner, and the effect of both 25 and 50 mM NAC was significant. Interestingly, not even 50 mM NAC inhibited serum withdrawal-induced DNA fragmentation, in contrast to the effects of EGF and ZVAD-fmk.

TABLE 2. Effects of various antioxidants on MPP^+-induced DNA fragmentation in GH3 cells

Addition	**None**	**200μM MPP^+**
Experiment 1*	DNA fragmentation (%)	
None	4.8 ± 0.2	31.9 ± 2.2
Deferoxamine (1 mM)	17.5 ± 2.3	39.2 ± 1.8
α-Tocopherol (25 μM)	7.4 ± 0.4	44.8 ± 4.4
Ascorbic acid (1 mM)	7.2 ± 1.2	34.3 ± 0.3
Experiment 2†	DNA fragmentation (% of control)	
None	100	288 ± 21
NAC (12 mM)	100 ± 8	229 ± 25
(25 mM)	100 ± 4	206 ± 22‡
(50 mM)	108 ± 8	173 ± 14‡

* Experiment 1: GH3 cells were labeled with [^{3}H] thymidine in F10 medium (HS/FBS-containing) for 24 hr. The washed cells were cultured for 2 hr in fresh F10 medium (HS/FBS-free) with the indicated concentrations of antioxidants, and were cultured further for 24 hr in the presence or absence of 200 μM MPP^+. Data are presented as percentages of total incorporated [^{3}H] thymidine. Data are the means ± SD of 3 determinations in a typical experiment and are representative of 2 independent experiments.

† Experiment 2: The labeled and washed GH3 cells were cultured for 2 hr in fresh F10 medium (HS/FBS-free) with the indicated concentrations of NAC, and further cultured for 24 hr in the presence or absence of 200 μM MPP^+. Data are presented as percentages of DNA fragmentation in control GH3 cells, which were cultured for 26 hr with F10 medium (HS/FBS- and MPP^+-free). The absolute value of DNA fragmentation in control GH3 cells was 4.7 ± 0.2% of total incorporated [^{3}H]thymidine. Data are the means ± SEM of 3 independent experiments. The total incorporated [^{3}H]thymidine was 5.2 to 5.4 × 10^5 and 4.8 to 5.0 × 10^5 dpm/well in the control and the MPP^+-treated cells, respectively. The value was not modified by the antioxidants.

‡ $P < 0.01$, significantly different from the value without NAC.

Involvement of EGF Receptor Kinase in the Protective Effect of EGF

GH3 cells are endowed with receptors for EGF, which can regulate the expression of specific genes (for review, see Ref. 29), so we investigated whether the intrinsic EGF receptor tyrosine kinase is involved in the protective effects of EGF. First, we analyzed the phosphotyrosine content of the EGF receptor in GH3 cells. The addition of EGF induced tyrosine phosphorylation of EGF receptors in GH3 cells (Fig. 3). Tyrphostin AG1478 acts as a specific inhibitor of EGF receptor kinase activity, but not of other tyrosine kinases such as Src and insulin receptor kinases [30, 31]. The addition of 5 μM AG1478 completely inhibited the tyrosine phosphorylation of EGF receptors by EGF (data not shown), and the protective effects of EGF on serum withdrawal- and MPP^+-induced DNA fragmentation were almost completely abolished in GH3 cells treated with AG1478 (Table 3). The effect of AG1478 was concentration-dependent and significant from 1 μM. Addition of AG1478 had no effect by itself, and did not change serum withdrawal- and MPP^+-induced DNA fragmentation. These findings suggest that EGF inhibition of DNA fragmentation is mediated by EGF receptor kinase activity. Stimulation of EGF receptor couples with the activation of PI 3-kinase in various cells [32], and EGF stimulates ERK in GH3 cells [33]. However, neither treatment with 100 nM wortmannin (an inhibitor of PI 3-kinase) nor treatment with 25 μM PD98059 (an inhibitor of the upstream kinase of ERK) affected EGF inhibition (data not shown).

TABLE 3. Inhibition of the protective effects of EGF on MPP^+-induced DNA fragmentation by AG1478

	DNA fragmentation (% of control)			
	None		EGF treatment	
Addition	None	200 μM MPP^+	None	200 μM MPP^+
None	100	451 ± 95	41 ± 7	187 ± 29
AG1478 (5 μM)	103 ± 9	466 ± 100	112 ± 3*	529 ± 144*

GH3 cells were cultured in F10 medium (HS/FSB-containing) with vehicle or 5 μM AG1478 for 2 hr, and then labeled for 24 hr with [^{3}H]thymidine in the presence or absence of 50 ng/mL of EGF. The washed cells were cultured further for 24 hr in fresh F10 medium (HS/FBS-free) with or without 200 μM MPP^+. Identical concentrations of EGF or AG1478 were added again to the indicated cells. Data are presented as percentages of DNA fragmentation in control GH3 cells, which were cultured for 24 hr with F10 medium (HS/FBS- and MPP^+-free). In a typical experiment, the absolute value of DNA fragmentation in control GH3 cells was 5.5 ± 0.5% of total incorporated [^{3}H]thymidine. The total incorporated [^{3}H]thymidine was 5.6 to 5.9 × 10^5 dpm/well, and was not modified by the treatments and additions. Data are the means ± SEM of 3 independent experiments.

* $P < 0.01$, significantly different from values without AG1478.

Lack of Involvement of PKC or Bcl-2 Expression in EGF Inhibition of Apoptosis

EGF receptor kinase phosphorylates phospholipase C-γ, resulting in activation of PKC, and various biological effects of EGF are decreased in PKC-depleted cells (for review, see Ref. 34). Thus, we investigated whether EGF inhibition of apoptosis is mediated by PKC. Table 4 shows the effect of PMA on DNA fragmentation. Under the conditions where EGF treatment decreased 200 μM MPP^+-induced DNA fragmentation, 1 μM PMA slightly enhanced the effect of serum withdrawal but did not influence the effect of MPP^+ (Experiment 1). In Experiment 2, GH3 cells were treated with 1 μM PMA for 24 hr, and then cultured further with or without EGF for 24 hr. PMA treatment (total 48 hr) did not block serum withdrawal- and MPP^+-induced DNA fragmentation. PMA treatment did not affect EGF inhibition of DNA fragmentation. Simultaneous addition of 1 μM PMA with MPP^+ to fresh F10 medium (HS/FBS-free) also had no effect (data not shown). It was difficult to determine the effect of GF109203X, an inhibitor of PKC, on EGF inhibition of serum withdrawal- and MPP^+-induced DNA fragmentation, because GF109203X had an apoptotic effect. In a typical experiment, treatment with 5 μM GF109203X enhanced DNA fragmentation; the values at 24 hr were 6.2 ± 1.5 and 13.2 ± 0.6% of total incorporated [^{3}H]thymidine in the absence and presence of 5 μM GF109203X in control (without MPP^+) cells, and 31.1 ± 4.9 and 51.2 ± 5.4% in the absence and presence of GF109203X in 200 μM MPP^+-treated cells, respectively.

Apoptosis is negatively regulated by antiapoptotic proteins, such as Bcl-2 family proteins. The expression of Bcl-2 is regulated at least partially through a PKC-dependent pathway [20, 35, 36]. Although the levels of Bcl-2 protein increased in SH-SY5Y cells treated with MPP^+ [23] or PMA [20], there was no increase in GH3 cells treated with 200 μM MPP^+ (Fig. 4, lanes 5 and 7) or 100 nM PMA (data not shown) for 48 hr. EGF treatment for 24 or 48 hr did not enhance the expression of Bcl-2 (lanes 1–4) in either the presence or absence of MPP^+ (lanes 5–8). Although it is probable that Bcl-2 protein translocates to the cytosolic fractions from the membrane fractions, including mitochondria, by MPP^+ or EGF treatment, we could not detect the changes of immunoreactivity (data not shown). These findings suggested that neither activation of PKC nor Bcl-2 expression is involved in EGF inhibition of apoptosis in GH3 cells.

Release of a Cytochrome c-Like Molecule from Mitochondria by MPP^+

The release of cytochrome *c* from mitochondria is thought to play a central role in some types of apoptosis (for review, see Ref. 37). The addition of MPP^+ to the mitochondrial

TABLE 4. Effect of PMA treatment on MPP^+-induced DNA fragmentation in GH3 cells

	DNA fragmentation (%)		
Experiment 1*	None	PMA	EGF
None	11.6 ± 1.4	15.4 ± 0.8	4.6 ± 0.2
MPP^+ (200 μM)	39.5 ± 1.9	39.2 ± 2.0	14.7 ± 1.5

	None		PMA	
Experiment 2†	None	EGF	None	EGF
None	8.4 ± 0.9	4.1 ± 0.9	8.2 ± 1.2	4.5 ± 0.5
MPP^+ (200 μM)	39.1 ± 0.8	16.2 ± 1.5	31.6 ± 7.2	16.0 ± 3.3

Data are the means ± SD of 3 determinations in a typical experiment and are representative of 2 independent experiments.

* Experiment 1: GH3 cells were labeled with [^{3}H]thymidine in F10 medium (HS/FBS-containing) with vehicle, 1 μM PMA or 50 ng/mL of EGF for 24 hr. The washed cells were cultured further for 24 hr in fresh F10 medium (HS/FBS-free) with or without 200 μM MPP^+. Identical concentrations of PMA or EGF were added again to the indicated cells.

† Experiment 2: GH3 cells were cultured for 24 hr in F10 medium (HS/FBS-containing) with or without 1 μM PMA. the cells were cultured for 24 hr in fresh F10 medium ([^{3}H]thymidine- and HS/FBS-containing) supplemented with or without 50 ng/mL of EGF. Identical concentrations of PMA were added again to the PMA-treated cells. The washed cells were cultured further for 24 hr in fresh F10 medium (HS/FBS-free) with or without 200 μM MPP^+. Data are presented as percentages of total incorporated [^{3}H]thymidine. The total value was 5.2 to 5.8 × 10^5 dpm/well in the control and the treated cells.

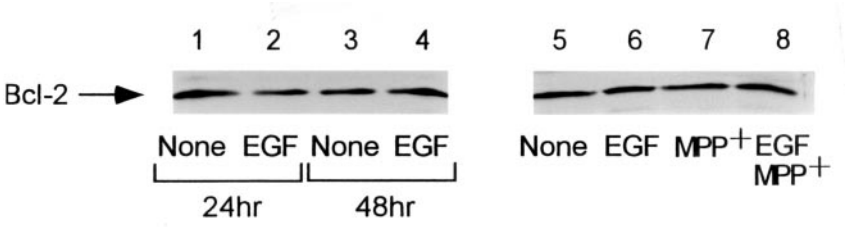

FIG. 4. Effects of MPP+ and EGF on Bcl-2 protein expression in GH3 cells. GH3 cells were cultured in F10 medium (HS/FBS-containing) for 24 hr (lanes 1 and 2) or 48 hr (lanes 3 and 4) with (lanes 2 and 4) or without (None, lanes 1 and 3) 50 ng/mL of EGF. In lanes 5–8, GH3 cells were cultured in F10 medium (HS/FBS-free) for 48 hr. The medium was supplemented further with vehicle (None, lane 5), 50 ng/mL of EGF (lane 6), 200 μM MPP+ (lane 7), or identical concentrations of EGF and MPP+ (lane 8). The cell extracts were obtained as described in Materials and Methods. Extracts were analyzed by SDS–PAGE followed by western blotting with anti-Bcl-2 antibody. Data are representative of 2 independent experiments.

fraction showed an inhibition of respiration at complex I [38], Ca^{2+} efflux and depolarization [26], and a stimulation of the transition pore and cytochrome *c* release [39]. However, the effects of MPP^+ and EGF on cytochrome *c* release to the cytosolic fractions have not been studied in GH3 cells. Addition of 200 μM MPP^+ to GH3 cells induced an increase of cytochrome *c* in the cytosolic fractions (Fig. 5, top panel). Basal (serum withdrawal-) and MPP^+-induced cytochrome *c* release was inhibited in cytosolic fractions from GH3 cells treated for 24 hr with 50 ng/mL of EGF. The level of cytochrome *c* in the cytosolic fraction from MPP^+-treated GH3 cells increased to 350 ± 45% (N = 3) compared with that in the control cells. In EGF-treated GH3 cells, the basal and MPP^+-induced release was 56 ± 25 and 125 ± 30% (N = 3), respectively.

Next, we prepared mitochondrial fractions from control and EGF-treated GH3 cells, and the mitochondrial fractions were incubated with 200 μM MPP^+ for 4 hr. After centrifugation, the released proteins in the supernatant were condensed and separated by SDS–PAGE (Fig. 5, bottom panel). Incubation with MPP^+ stimulated the release of a protein with the same molecular size as cytochrome *c*. This 13-kDa band cross-reacted with anti-cytochrome *c* antibody (data not shown), and its immunoreactivity in the supernatant increased with MPP^+ treatment in a concentration-dependent manner. The effect of 200 μM MPP^+ was significant, with over a 4-fold increase in expression (Table 5). Incubation with 20 μM mastoparan, a wasp venom toxin, also remarkably stimulated expression of this molecule from mitochondrial fractions of GH3 cells, as reported in rat cerebellar neurons [24]. In mitochondrial fractions from GH3 cells treated for 24 hr

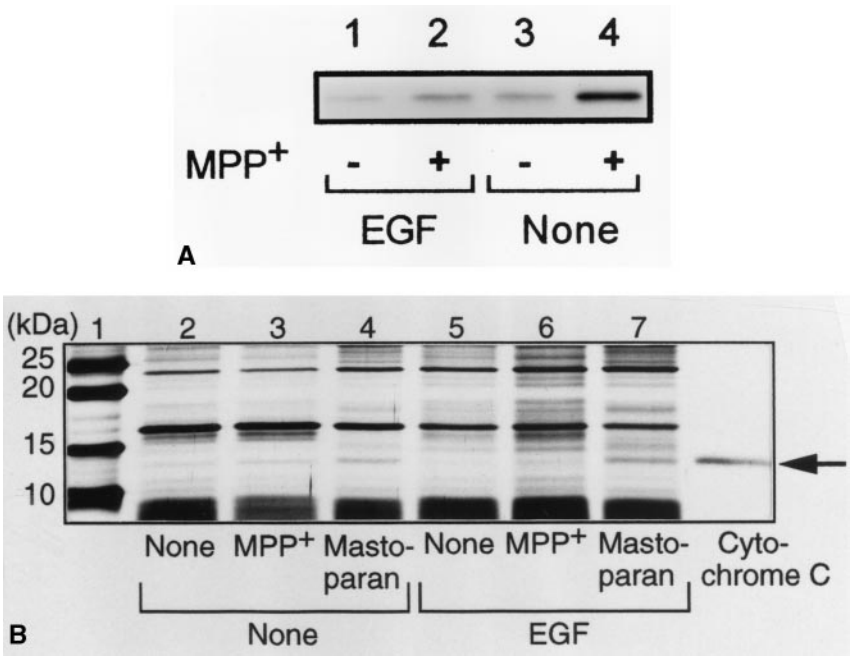

FIG. 5. Inhibition of MPP^+-induced cytochrome *c* release to the cytosolic fractions in GH3 cells by EGF treatment. (Top panel) GH3 cells were cultured for 24 hr in F10 medium (HS/FBS-containing) with (lanes 1 and 2) or without (lanes 3 and 4) 50 ng/mL of EGF. The washed cells were cultured further for 24 hr in fresh F10 medium (HS/FBS-free) with vehicle (lanes 1 and 3) or 200 μM MPP^+ (lanes 2 and 4). The cytosolic fractions were analyzed by SDS–PAGE followed by western blotting with anti-cytochrome *c* antibody. Data are representative of 2 independent experiments. (Bottom panel) Mitochondrial fractions were obtained from control (None, lanes 2–4) and EGF-treated (lanes 5–7) cells, and then incubated with vehicle (None, lanes 2 and 5), 200 μM MPP^+ (lanes 3 and 6) or 20 μM mastoparan (lanes 4 and 7) for 4 hr. After centrifugation, supernatant fractions were concentrated and analyzed by SDS–PAGE followed by silver staining. Lane 1 shows molecular markers, and the right lane shows purified bovine cytochrome *c* as a standard. Data are representative of 3–4 independent experiments. Quantitative analysis of the band intensity is represented in Table 5.

TABLE 5. Release of putative cytochrome *c* from isolated mitochondria fractions in GH3 cells and its inhibition in EGF-treated GH3 cells

	Release of putative cytochrome *c* (%)	
Addition	Control	EGF-treated
None	100	95 ± 9
200 μM MPP^+	450 ± 35*	120 ± 40†

Mitochondrial fractions were obtained from control and EGF-treated (50 ng/ml, 24 hr) GH3 cells, as described in Fig. 5. The fractions were incubated with vehicle or 200 μM MPP^+ for 4 hr. The densities of the 13-kDa band were analyzed by laser densitometry. Under the conditions, the densities of standard cytochrome *c* were linear in proportion to the amount of the protein. Data are presented as percentages of the density in basal (non-stimulated) release from mitochondria in control GH3 cells. Data are the means ± SEM of 3–4 independent experiments.

* $P < 0.01$, significantly different from the value without MPP^+.

† $P < 0.01$, significantly different from the value in control GH3 cells.

with 50 ng/mL of EGF, release by MPP^+ was inhibited to the level in control cells (Fig. 5, bottom panel), and the effect of MPP^+ was not significant in EGF-treated GH3 cells (Table 5). These findings show that (i) MPP^+ can directly stimulate the release of cytochrome *c* from mitochondria, and (ii) EGF treatment inhibits cytochrome *c* release from mitochondria in GH3 cells.

DISCUSSION

Induction of Apoptosis of GH3 Cells by MPP^+

Treatment with MPTP *in vivo* or MPP^+ *in vitro* induces neuronal cell death. Although some neurotrophic factors are reported to protect against MPP^+-induced death of neuronal cells, it has not been established whether neurotrophic factors protect against MPP^+-induced apoptosis or necrosis. Previously, we reported that the GH3 cell line is an appropriate model for dopaminergic neurons and that MPP^+ induces delayed cell death of GH3 cells [19]. In the present study, we showed that MPP^+ induces apoptosis of GH3 cells. The reasons for this are the following: (i) culture with MPP^+ induced DNA laddering (Fig. 1), fragmentation (Fig. 2), and nuclear chromatin condensation in GH3 cells; (ii) culture with MPP^+ induced an increase of cytochrome *c* in the cytosolic fractions (Fig. 5, top panel), (iii) addition of MPP^+ to the mitochondrial fraction from GH3 cells induced cytochrome *c* release (Fig. 5, bottom panel, and Table 5), and (iv) treatment with a caspase inhibitor, ZVAD-fmk, decreased MPP^+-induced DNA fragmentation (Table 1). In addition, DNA laddering and fragmentation were detectable from 12 hr and remarkable at 24 hr after MPP^+ addition (Fig. 1 and Table 1). LDH leakage induced by MPP^+ is also remarkable at 48 hr after MPP^+ addition [19]. In cultured cerebellar granule neurons, addition of MPP^+ induces cytochrome *c* release to the cytosolic fraction, activation of caspase 3-like activity, and apoptosis of the cells [9].

Although MPP^+-induced inhibition of NADH-CoQ_1 reductase activity and cell death was prevented by antioxidants and/or free radical scavengers [5, 38], it has not been established whether antioxidants protect against MPP^+-induced DNA fragmentation and/or apoptosis. Treatment with NAC reduced MPP^+-induced DNA fragmentation in GH3 cells, whereas the other antioxidants used were slightly toxic (Table 2). Interestingly, treatment with NAC did not protect against serum withdrawal-induced DNA fragmentation. In contrast, treatment with ZVAD-fmk or EGF blocked serum withdrawal- and MPP^+-induced apoptosis (Tables 1 and 3). Previous reports suggest that there are several apoptotic pathways that are triggered by different stimulants in the same type of cells (for review, see Refs. 1 and 2). Thus, glutathione may be involved in the MPP^+-induced apoptosis signalling pathway but not the serum withdrawal pathway.

Protection Against MPP^+-Induced Apoptosis in GH3 Cells by EGF Treatment

In vivo treatment with basic FGF [11], EGF [13], or GDNF [14] reversed the loss of dopaminergic neurons in MPTP-treated mice. In addition, BDNF protected SH-SY5Y dopaminergic neuroblastoma cells against MPP^+ [16]. We previously reported that treatment with EGF protected against GH3 cell death induced by MPP^+ [19], but whether these factors protect against MPP^+-induced apoptosis is unknown. In this study, we showed the protective effect of EGF against MPP^+-induced DNA laddering (Fig. 1), fragmentation (Fig. 2), and cytochrome *c* release (Fig. 5 and Table 5) in GH3 cells. To our knowledge, this is the first report to show that MPP^+-induced apoptosis of neuronal cells can be blocked by EGF, a neurotrophic factor.

In GH3 cells, EGF stimulated tyrosine phosphorylation of EGF receptors (Fig. 3), and EGF inhibition of serum withdrawal- and MPP^+-induced apoptosis was abolished completely by an inhibitor of EGF receptors (Table 3). These findings suggest that the tyrosine kinase activity of EGF receptors is involved in EGF inhibition in GH3 cells. Binding of neurotrophic factors to their receptors elicits a variety of signalling pathways (for review, see Ref. 2). One of the pathways that have been implicated in neuronal survival is the ERK kinase (MEK)-ERK pathway, although the involvement of this pathway differs from one cell type to another. EGF phosphorylates and stimulates ERK in GH3 cells [33]. However, EGF inhibition in GH3 cells does not seem to involve the ERK kinase–ERK pathway, as treatment with an inhibitor of ERK kinase did not affect the inhibition. The PI 3-kinase pathway is another pathway for neuronal survival, and EGF has been suggested to activate PI 3-kinase indirectly in several types of cells [32, 40]. However, PI 3-kinase is also not involved in EGF inhibition, since wortmannin did not affect it.

Lack of Involvement of PKC or Bcl-2 Protein Expression in EGF Inhibition of Apoptosis

The proto-oncogene *bcl-2* encodes an antiapoptotic 26-kDa protein (Bcl-2) involved in programmed cell death, and the

release of cytochrome *c* from mitochondria in cells undergoing apoptosis is prevented by Bcl-2 family proteins [2, 37]. The neurotoxicity of MPTP is attenuated in mice overexpressing Bcl-2 protein [41]. Recent reports suggest that apoptosis and/or the action of Bcl-2 is regulated at least partially through a PKC-dependent pathway(s). For instance, (i) phorbol esters such as PMA antagonize apoptosis [42], (ii) PKC inhibitors induce apoptosis [43] and promote agent-induced apoptosis [44], and (iii) the function and/or phosphorylation of Bcl-2 is probably regulated by the PKC-dependent pathway [35, 36]. Previously we reported that levels of *bcl-2* mRNA and Bcl-2 protein increase in human neuroblastoma SH-SY5Y cells treated with PMA for over 24 hr [20]. In addition, various PKC subtypes have biological functions in GH3 cells [33, 45]. Thus, it is probable that PKC activity and/or Bcl-2 expression are involved in EGF inhibition of apoptosis. Although addition of the PKC inhibitor GF109203X slightly induced DNA fragmentation itself and enhanced MPP^+-induced DNA fragmentation, co-addition of PMA had no effect on MPP^+-induced DNA fragmentation. MPP^+-induced apoptosis (Table 4) or expression of Bcl-2 protein (Fig. 4) was not affected in PKC-depleted GH3 cells. Also, pretreatment with PMA did not modify the EGF inhibition of apoptosis of GH3 cells. These findings show that PKC-dependent systems do not play a role in (i) Bcl-2 expression and apoptosis in GH3 cells, and (ii) EGF inhibition of serum withdrawal- and MPP^+-induced apoptosis. The phosphorylation of Bcl-2 by MPP^+ or EGF should be examined in the future.

In conclusion, EGF decreased MPP^+-induced cytochrome *c* release from the mitochondrial fraction and protected against serum withdrawal- and MPP^+-induced apoptosis of GH3 cells. The involvement of PKC-dependent systems and Bcl-2 protein expression was excluded in EGF inhibition of apoptosis. The antioxidant NAC also inhibited MPP^+-induced apoptosis. The effects of EGF and NAC on mitochondrial functions, including mitochondrial potential and the phosphorylation state of Bcl-2 protein, should be determined in the future. Also, the bridge molecule(s) between the EGF receptor tyrosine kinase and subsequent signalling pathways should be determined, as well as the role of other Bcl-2-related proteins.

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