Protein kinase Cδ-mediated proteasomal degradation of MAP kinase phosphatase-1 contributes to glutamate-induced neuronal cell death

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Summary

Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) is a dual-specificity phosphatase that is involved in the regulation of cell survival, differentiation and apoptosis through inactivating MAPKs by dephosphorylation. Here, we provide evidence for a role of MKP-1 in the glutamate-induced cell death of HT22 hippocampal cells and primary mouse cortical neurons. We suggest that, during glutamate-induced oxidative stress, protein kinase C (PKC) δ becomes activated and induces sustained activation of extracellular signal-regulated kinase 1/2 (ERK1/2) through a mechanism that involves degradation of MKP-1. Glutamate-induced activation of ERK1/2 was blocked by inhibition of PKCδ, confirming that ERK1/2 is regulated by PKCδ. Prolonged exposure to glutamate caused reduction in the protein level of MKP-1, which correlated with the sustained activation of ERK1/2. Furthermore, knockdown of endogenous MKP-1 by small interfering (si)RNA resulted in pronounced enhancement of ERK1/2 phosphorylation accompanied by increased cytotoxicity under glutamate exposure. In glutamate-treated cells, MKP-1 was polyubiquitylated and proteasome inhibitors markedly blocked the degradation of MKP-1. Moreover, inhibition of glutamate-induced PKCδ activation suppressed the downregulation and ubiquitylation of MKP-1. Taken together, these results demonstrate that activation of PKCδ triggers degradation of MKP-1 through the ubiquitin-proteasome pathway, thereby contributing to persistent activation of ERK1/2 under glutamate-induced oxidative toxicity.

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Key words: Glutamate, MKP-1, Immature cortical neuron, Neuronal cell death, Ubiquitylation, Proteasomal degradation

Introduction

Glutamate toxicity has been implicated in a variety of neurological disorders such as Parkinson's disease, Alzheimer's disease, seizures, stroke and trauma (Coyle and Puttfarcken, 1993). Glutamate has been shown to induce neuronal cell death through both receptor-mediated excitotoxicity and non-receptor-mediated oxidative stress. Glutamate-induced oxidative toxicity provides a model for studying the mechanism of oxidative stress in neurons and neuronal cells. In this model, glutamate induces oxidative stress by promoting cystine efflux and/or competitively blocking cystine uptake by inhibiting a glutamate-cystine antiporter. This results in the loss of intracellular cystine and glutathione, thereby inducing oxidative cell death (Murphy et al., 1989).

Mitogen-activated protein kinases (MAPKs) become phosphorylated and activated in response to a wide variety of external stimuli, resulting in the regulation of cell proliferation, differentiation, survival and apoptosis (Chen et al., 1995; Bhat and Zhang, 1999). They can be subdivided into at least three classes based on sequence homology: these include the extracellular signal-regulated kinases (ERK1/2), the Jun N-terminal kinases (JNKs) and the p38 kinases. MAPKs are activated by dual phosphorylation on Ser/Thr and Tyr residues in the TEY sites within the activation loop, whereas dephosphorylation of these residues by a family comprising about 11 dual-specificity phosphatases or MAP kinase phosphatases (MKPs) terminates such activation (Keyse, 2000). These phosphatases exhibit differential specificity towards MAPK substrates, show distinct subcellular localizations, and have different modes of regulation. Among these phosphatases, MKP-1 was originally identified as an ERK-specific phosphatase (Sun et al., 1993). However, it has also been shown to dephosphorylate and inactivate both JNK and p38 in a cell-type-specific and context-specific manner (Franklin and Kraft, 1997; Lasa et al., 2002). MKP-1 is overexpressed in many human tumors, and its overexpression has been shown to protect cells from apoptosis induced by the anticancer agent cisplatin, as well as ultraviolet (UV) irradiation and proteasome inhibitors (Franklin et al., 1998; Sanchez-Perez et al., 2000; Small et al., 2004). However, the role of MKP-1 in controlling cell survival, proliferation and apoptosis has been suggested through use of overexpression approaches in non-neuronal cells, and it has not been clearly

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demonstrated whether MKP-1 plays a role in the regulation of neuronal cell death.

Members of the protein kinase C (PKC) family of Ser/Thr protein kinases have been suggested to have important roles in the regulation of cell survival and programmed cell death (Whelan and parker, 1998; Maher, 2001). Several studies suggest that PKCδ is involved in apoptosis by acting as a pro-apoptotic signal. Many studies have shown that a selective inhibitor of PKCδ, rottlerin, and a dominant-negative mutant of PKCδ attenuate apoptosis induced by phorbol ester, H_2O_2, UV radiation, taxol and etoposide (Konishi et al., 1997; Denning et al., 1998; Majumder et al., 2000; Matassa et al., 2001). A previous study demonstrated that an apoptotic stimulus resulted in proteolytic activation of PKCδ, which was inhibited by treatment with caspase inhibitors and transfection with anti-apoptotic proteins such as Bcl-2 or Bcl-x_L (Emoto et al., 1995). This observation is in agreement with previous results demonstrating that expression of the catalytic fragment of PKCδ in HeLa cells was sufficient to induce cell death (Ghayur et al., 1996). Thus, PKCδ activity seems to have an important role in the regulation of apoptosis in many different cells. However, the mechanism by which activation of PKCδ drives cell death still remains largely unknown.

Recent studies have shown that persistent activation of ERK1/2 is strongly associated with glutamate-induced cell death in HT22 mouse hippocampal cells and immature primary cortical neurons (Stanciu et al., 2000). However, the mechanism by which prolonged activation of ERK1/2 occurs remains largely elusive. On the other hand, inhibition of PKCδ by rottlerin blocked glutamate-induced cell death in primary cortical neurons (Maher, 2001), suggesting that activation of PKCδ is implicated in oxidative stress. In this study, we investigated the roles of PKCδ and MKP-1 in glutamate-induced death of HT22 cells and immature primary cortical neurons. Our results show that glutamate treatment triggers activation of PKCδ, which causes degradation of MKP-1 through the ubiquitin-proteasome pathway, thereby contributing to the sustained activation of ERK1/2. Moreover, we show that downregulation of MKP-1 promotes glutamate-induced cell death, through an ERK1/2-mediated mechanism. Collectively, we suggest that MKP-1 is an important mediator in controlling neuronal cell death.

Results

ERK1/2 contributes to glutamate-induced cell death in HT22 cells

The role of ERK1/2 during glutamate-induced oxidative toxicity in HT22 cells has been subject to controversy (Stanciu et al., 2000; Maher, 2001). We thus first examined the effect of glutamate on activation of MAPKs in our culture system. As shown in Fig. 1A, phosphorylation of ERK1/2 was observed at 3 hours after glutamate treatment, and was sustained up to 9 hours. By contrast, phosphorylation of p38 and JNK kinases was detected only at 9 hours after glutamate treatment, and was undetectable at 6 hours. Next, we examined the involvement of MAPKs in glutamate-induced cell death by using specific inhibitors of MEK1/2 (U0126), p38 (SB202190) and JNK

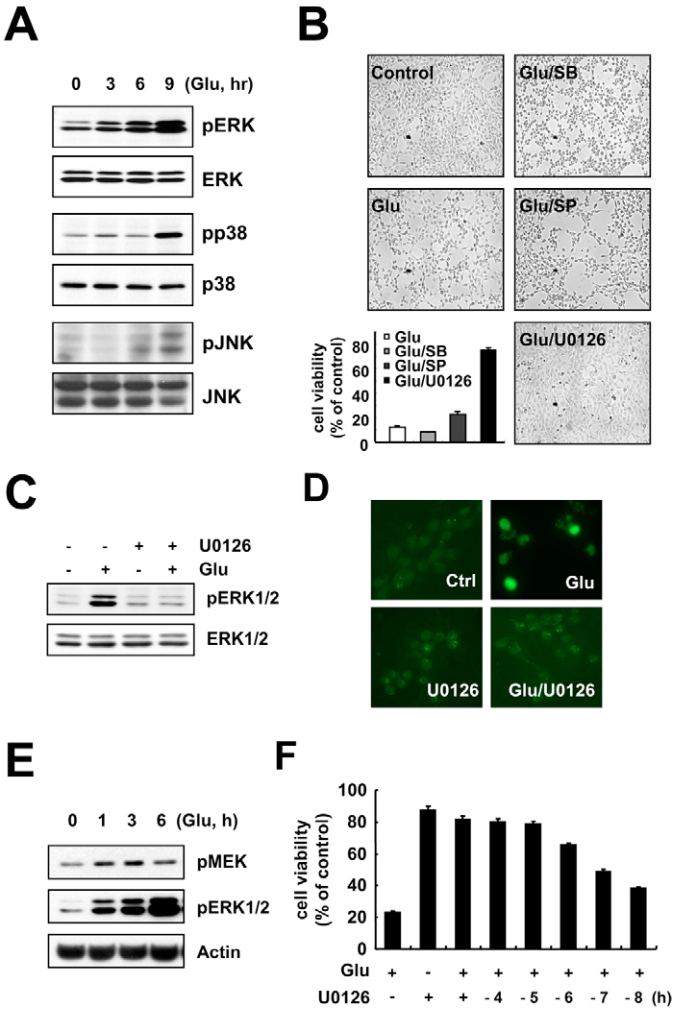

Fig. 1. ERK1/2 contributes to glutamate-induced cell death in HT22 cells. (A) HT22 cells were incubated with 5 mM glutamate (Glu) for the indicated times. Cell extracts were subjected to SDS-PAGE and immunoblotted with antibodies specific for phospho-ERK1/2 (pERK), phospho-JNK (pJNK), or phospho-p38 (pp38) MAPK. Membranes were stripped and reprobed for total ERK1/2, JNK or p38 as a control. Shown are blots from a representative experiment out of four separate experiments. (B) HT22 cells were incubated with 5 mM glutamate for 12 hours in the presence or absence of 20 μM U0126 (Glu/U0126), 10 μM SB202190 (Glu/SB) or 20 μM SP600125 (Glu/SP). Shown are representative light microscopic images. Results from the MTT assay are also presented. Each bar is a mean ± s.d. value from four separate experiments. (C) HT22 cells were exposed to 5 mM glutamate for 7 hours in the presence or absence of 20 μM U0126. Phosphorylation of ERK1/2 in whole cell extracts was determined by immunoblotting with an antibody against phospho-ERK1/2. Blots shown are representative of four separate experiments. (D) Representative photomicrographs of apoptotic cells treated with or without 5 mM glutamate for 12 hours in the presence or absence of 20 μM U0126. (E) HT22 cells were incubated with 5 mM glutamate for the indicated times. Cell extracts were immunoblotted with an antibody specific for phospho-ERK1/2 or phospho-MEK1/2. Membranes were stripped and reprobed for actin as a control. Results were reproducible, and blots shown are representative of three separate experiments. (F) HT22 cells were exposed to 5 mM glutamate, and then 20 μM U0126 was added either simultaneously or at the indicated times after the addition of glutamate. Cell viability was analyzed after 12 hours by the MTT conversion assay. Results are mean ± s.d. value from four separate experiments.

(SP600125). Using the MTT assay [which measures the amount of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide oxidized to purple formazan], we found that more than 80% of the cells underwent cell death in response to glutamate after 12 hours. U0126 substantially protected the cells from death (Fig. 1B), and similar results were obtained from terminal transferase dUTP nick-end labeling (TUNEL) assay (Fig. 1D), measurement of propidium iodide staining and fluorescence activated cell sorter (FACS) analysis (data not shown). The effect of U0126 was confirmed by its ability to reduce the level of phospho-ERK1/2 elevated by glutamate treatment (Fig. 1C). By contrast, SB202190- or SP600125-treated cells did not exhibit significant recovery from the glutamate-induced cell toxicity as compared with control cells (Fig. 1B). Neither SB202190 nor SP600125 had any effects in unstressed cells when treated alone (data not shown). The inhibitory effect of SP600125 on JNK was confirmed by its ability to block the glutamate-induced phosphorylation of c-Jun, which is a substrate of JNK (Fig. S1, supplementary material). To confirm further that p38 is not involved in glutamate-induced cell death, we used another specific inhibitor of p38, SB203580. MTT assay revealed that SB203580-treated cells did not exhibit any significant recovery from the glutamate-induced cell toxicity (data not shown) as in SB202190-treated cells. The inhibitory effect of SB203580 on activation of the p38 pathway was independently confirmed (data not shown). Taken together, these results suggest that activation of ERK by glutamate has a crucial role in regulating cell death, whereas JNK and p38 kinases do not.

Sustained activation of ERK1/2 might have resulted from elevated Ras-Raf-MEK signaling, but prolonged activation of ERK1/2 in some cases has been reported to be independent of MEK1/2 (Grammer and Blenis, 1997). We therefore examined the effect of glutamate on the activation of MEK1/2. As described above, glutamate increased phosphorylation of ERK1/2 in a time-dependent manner. Phosphorylation of MEK1/2 also increased up to 3 hours of glutamate treatment, but declined afterwards (Fig. 1E), implying that activation of MEK1/2 might not play a predominant role in the sustained phosphorylation of ERK1/2, which continued to increase up to 9 hours of glutamate treatment. To investigate the involvement of MEK1/2 further, we examined whether U0126 still had a protective effect when treated after glutamate had triggered cell death. The protective effect of U0126 started to decrease when added 4 hours after glutamate treatment, and a substantial amount of cell death was observed afterwards (Fig. 1F), supporting the notion that activation of MEK1/2 might not be the major cause for the sustained activation of ERK1/2 that was observed even after 9 hours of glutamate treatment.

Glutamate induces activation and tyrosine phosphorylation of PKCδ in HT22 cells

A recent study demonstrated that rottlerin, a selective inhibitor of PKCδ, blocked glutamate-induced oxidative toxicity (Maher, 2001). We thus examined whether PKCδ was involved in our system. For this purpose, we first examined the activity of PKCδ. As shown in Fig. 2A, PKCδ became highly activated by glutamate, and its activity reached maximum at 6 hours. The activity declined afterwards, but was sustained up to 9 hours. PKCδ can be activated by tyrosine phosphorylation or by enzymatic cleavage to a constitutively active catalytic fragment (Li et al., 1994; Emoto et al., 1995). To assess the mode of activation during glutamate-induced oxidative toxicity, we examined whether PKCδ was phosphorylated on tyrosine residues. As shown in Fig. 2B, a low basal level of tyrosine phosphorylation was detected in untreated cells. Maximal phosphorylation by glutamate was observed at 6 hours, and the phosphorylation decreased thereafter. The time course of tyrosine phosphorylation of PKCδ correlated with the window of time in which PKCδ was activated. We also examined whether glutamate induced proteolytic cleavage of PKCδ. We found that the full-length form of PKCδ decreased in a time-dependent manner, which was accompanied by a concomitant increase in a 40 kDa fragment (Fig. 2C). Although the 40 kDa fragment slightly increased, the time course of PKCδ cleavage did not match the mode of PKCδ activation. On the basis of

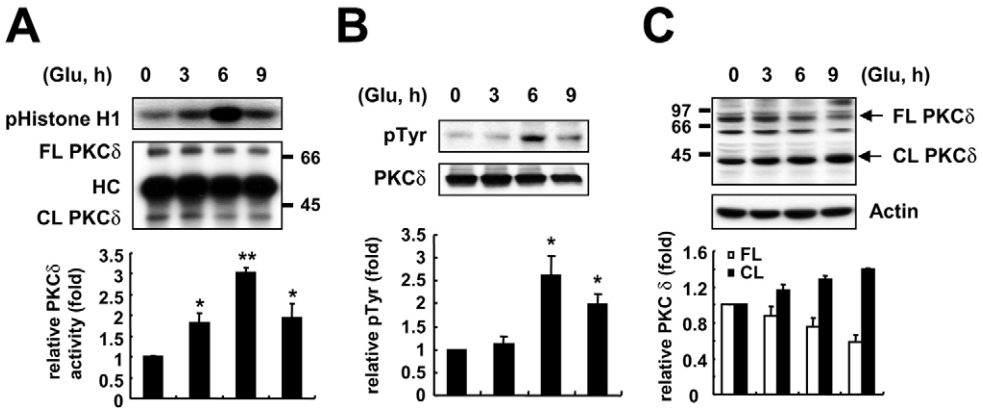

Fig. 2. Glutamate induces activation and tyrosine phosphorylation of PKCδ in HT22 cells. (A) HT22 cells were incubated with 5 mM glutamate (Glu) for the indicated times. Cell extracts were subjected to immunoprecipitation with anti-PKCδ. An in vitro immune complex kinase assay were performed using histone H1 as a substrate. The immunoprecipitates were also analyzed by immunoblotting with anti-PKCδ. (B) HT22 cells were incubated with 5 mM glutamate for the indicated times. Cells were then harvested, and immunoprecipitation of PKCδ was performed with an anti-PKCδ antibody as described in the Materials and Methods. Membranes were immunoblotted with anti-phosphotyrosine antibody (pTyr) and/or anti-PKCδ antibodies. The relative kinase activities and levels of tyrosine phosphorylation of PKCδ were quantified by densitometric analyses, and normalized to the level of total PKCδ (lower panel in A and B). Data represent an average fold increase as compared with controls (mean ± s.d.). $^{*}P<0.05$ and $^{**}P<0.01$, values compared with zero time. (C) HT22 cells were incubated with 5 mM glutamate for the indicated times, and the cleavage of PKCδ was determined by western blotting. The 40 kDa cleaved form is marked by an arrow. Results were reproducible, and blots shown are representative of three separate experiments. CL, cleaved form; FL, full-length form; HC, heavy chain.

these results, we concluded that the cleavage of PKCδ is unlikely to be the major mode of activation during glutamate-induced cell death. However, we cannot entirely exclude the possibility that this slight increase might have contributed to the activation of PKCδ.

Inhibition of PKCδ activity suppresses glutamate-induced ERK1/2 phosphorylation in HT22 cells

To elucidate whether PKCδ mediates the activation of ERK1/2 in response to glutamate, we first examined the effect of rottlerin. As shown in Fig. 3A, 5 μM rottlerin completely blocked the phosphorylation of ERK1/2 in response to glutamate. Since the inhibitory effect of rottlerin on PKCδ has been subject to controversy (Gschwendt et al., 1994; Soltoff, 2001), we further investigated the role of PKCδ in the glutamate-induced phosphorylation of ERK by using a dominant-negative mutant (DN) of PKCδ (K376R). Exogenous expression of PKCδ DN did not have any significant effects on the basal phosphorylation of ERK (Fig. 3B, lanes 1 and 2). However, it significantly inhibited the glutamate-induced phosphorylation of ERK (Fig. 3B, lanes 3 and 4). HT22 cells cultured at high densities (~80% or higher) were less sensitive to glutamate, i.e. glutamate-induced cell death was not as prominent as compared with cells cultured at lower densities (50-60%) (data not shown). For this reason, cells were transfected when the density reached 50-60%. This resulted in rather lower transfection efficiency (30-40%), which provides an explanation for the relatively less prominent inhibitory effect of PKCδ DN on ERK phosphorylation (Fig. 3B) as compared with the dramatic effect of rottlerin (Fig. 3A). Effects of rottlerin and exogenous expression of PKCδ DN on glutamate-induced activation of PKCδ were confirmed (Fig. 3A,B, lower panels). Together, these results suggest that activation of PKCδ mediates the glutamate-induced phosphorylation of ERK.

To determine whether PKCδ participated in the regulation of glutamate-induced neuronal cell death, we examined the effects of rottlerin and siRNA against PKCδ. The large number of apoptotic nuclei revealed by TUNEL assay, which was observed in cells 12 hours after glutamate treatment, did not appear when co-treated with rottlerin and glutamate (Fig. 3C). Since rottlerin has also been reported to inhibit other kinases such as calmodulin (CaM) kinase III (Parmer et al., 1997), we confirmed the effect of PKCδ on glutamate-induced cell death by using siRNA against PKCδ. As shown in Fig. 3C, glutamate-induced cell death was significantly recovered when cells were transfected with siRNA against PKCδ, suggesting that PKCδ plays an important role in the regulation of cell death in glutamate-treated cells.

Glutamate-induced ROS generation is involved in the activation of PKCδ and ERK1/2 in HT22 cells

Since exposure of HT22 cells to glutamate has been shown to increase the level of reactive oxygen species (ROS) (Tan et al., 1998) (Fig. S2A, supplementary material), we compared the kinetics between intracellular accumulation of ROS and PKCδ activation under glutamate treatment. The level of ROS slightly increased for the first 3 hours under glutamate exposure, followed by a much higher level. The time course of ROS production correlated with the window of time in which PKCδ was activated in response to glutamate treatment (Fig. S2A, supplementary material), suggesting that ROS might play a role in the activation of PKCδ. To test this possibility, cells

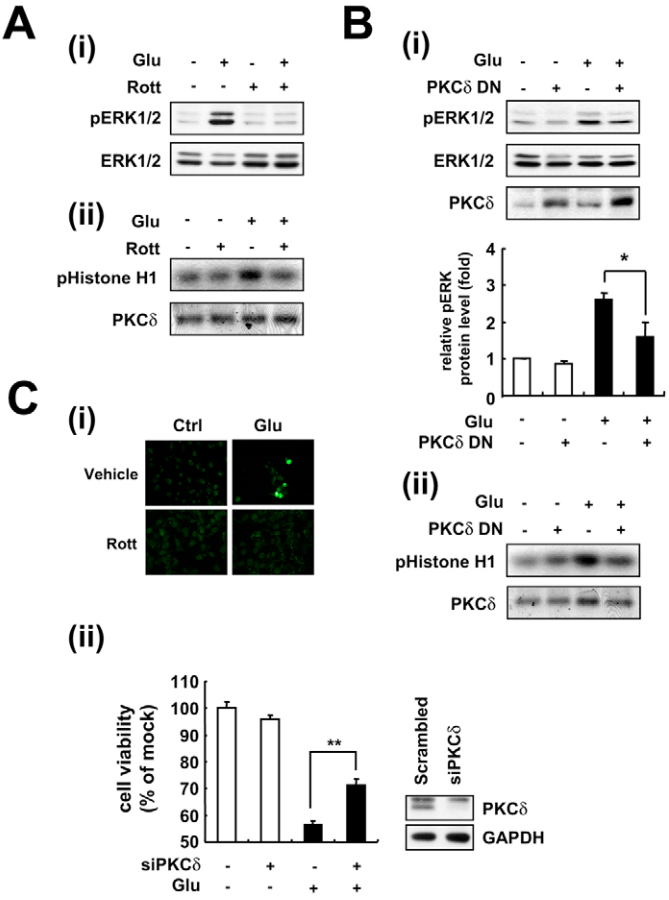

Fig. 3. Inhibition of PKCδ activity suppresses glutamate-induced ERK1/2 activation. (A) HT22 cells were incubated with 5 mM glutamate (Glu) for 7 hours in the presence or absence of 5 μM rottlerin (Rott). (i) Phosphorylation of ERK1/2 in whole cell extracts was examined by western blot analysis using a phospho-ERK1/2 (pERK1/2)-specific antibody, and (ii) PKCδ activity was determined by immune complex kinase assay as described in the Materials and Methods. Results were reproducible, and blots shown are representative of three separate experiments. (B) HT22 cells were transfected with either a mock vector or a dominant-negative mutant (DN) of PKCδ (PKCδ DN) vector. After 12 hours, the cells were incubated with or without 5 mM glutamate for 7 hours. (i) Phosphorylation of ERK1/2 was examined by western blot analysis using a phospho-ERK1/2-specific antibody, and (ii) PKCδ activity was determined by immune complex kinase assay. Relative phospho-ERK1/2 levels shown in the columns below were determined from densitometric scanning of enhanced chemiluminescence-exposed film. Each bar is a mean ± s.d. value of three independent experiments normalized by arbitrarily setting the mock-transfected cell densitometrics to 1. (C) (i) Representative photomicrographs of apoptotic cells treated with or without 5 mM glutamate for 12 hours in the presence or absence of 5 μM rottlerin. (ii) HT22 cells were transfected with either control or PKCδ siRNA (siPKCδ) and, after 24 hours, cells were exposed to 5 mM glutamate for 9 hours. Downregulation of PKCδ after transfection was confirmed by western blot analysis (right panel), and cell viability was measured by MTT assay (left panel). Each bar is a mean ± s.d. value from three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$.

were treated with N-acetyl cysteine (NAC), a scavenger of reactive oxygen intermediates. NAC treatment inhibited glutamate-induced activation of PKCδ and reduced the level of phospho-ERK1/2 elevated by glutamate treatment (Fig. S2B, supplementary material), suggesting that ROS generation is upstream of the activation of PKCδ and ERK1/2 in glutamate-treated HT22 cells.

MKP-1 is involved in the regulation of ERK1/2 phosphorylation

We then investigated the mechanism by which PKCδ induced sustained activation of ERK1/2. Dual-specificity phosphatases are an emerging subclass of the protein phosphatase gene superfamily, which appears to be selective for dephosphorylating the phosphotyrosine and phosphothreonine/serine residues within MAPKs (Keyse, 2000). To determine whether sustained activation of ERK1/2 induced by glutamate was associated with downregulation of MKPs, we examined the protein levels of MKPs in HT22 cells exposed to glutamate in a time-dependent manner. We observed that MKP-1 increased slightly during glutamate exposure until 1 hour, after which it substantially decreased (Fig. 4A). However, MKP-2 and -3 were not detected in unstressed and glutamate-treated cells up to 9 hours (data not shown), excluding the involvement of MKP-2 and -3 in glutamate-induced cell death.

To examine the role of MKP-1 in the regulation of ERK1/2 in HT22 cells, we transiently transfected the cells with either wild-type MKP-1 or its catalytically inactive mutant (MKP-1CS). Overexpression of MKP-1 reduced the level of phospho-ERK1/2 elevated by glutamate (Fig. 4B, lane 5), whereas overexpression of MKP-1CS significantly enhanced the glutamate-induced ERK1/2 phosphorylation (Fig. 4B, lane 6). Similarly, the basal level of phospho-ERK1/2 was reduced when cells were transfected with MKP-1, but was enhanced when cells were transfected with MKP-1CS (Fig. 4B, lanes 2 and 3).

We also applied siRNA against MKP-1. As shown in Fig. 4C, depletion of MKP-1 caused a pronounced enhancement in the basal level of phospho-ERK1/2. Moreover, glutamate-induced ERK1/2 phosphorylation was also increased in cells transfected with siRNA against MKP-1. Similar results were obtained when we used another siRNA that was designed against a different region of MKP-1 (Fig. 4D). We found that phosphorylation of both p38 and JNK in unstressed and glutamate-treated cells was not altered by siRNA against MKP-1 (Fig. 4D). These results suggest that MKP-1 plays an essential role in the regulation of both basal and glutamate-induced activation of ERK1/2.

Glutamate induces ubiquitylation and downregulation of MKP-1 through activation of PKCδ

We next examined whether PKCδ was involved in the downregulation of MKP-1 under glutamate exposure. Glutamate-induced downregulation of MKP-1 was not observed when cells were transfected with PKCδ DN (Fig. 5, lanes 3 and 4), and similar results were obtained when cells were treated with rottlerin (data not shown). These results suggest that activation of PKCδ significantly contributes to the downregulation of MKP-1 under long-term glutamate exposure.

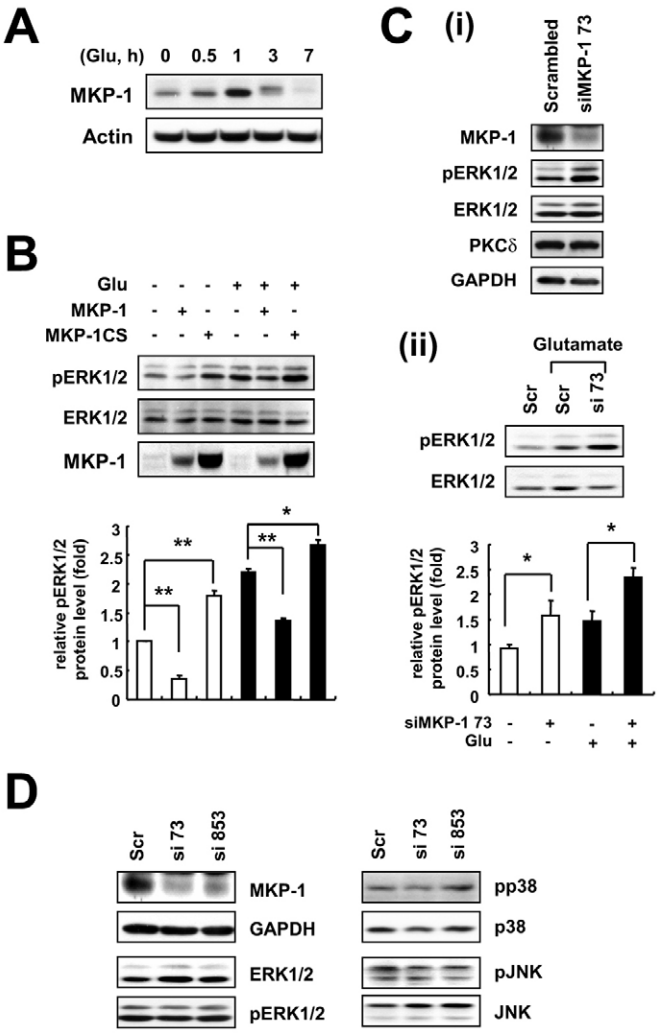

Fig. 4. MKP-1 regulates ERK1/2 phosphorylation during glutamate-induced oxidative toxicity. (A) HT22 cells were incubated with 5 mM glutamate (Glu) for the indicated times. The cells were then extracted to determine protein levels of MKP-1 by western blot analysis. Western blot analyses with anti-actin antibody in the same extracts are shown in the bottom panels. The results are from a representative experiment out of four separate experiments. (B) Cells were transfected with 2 μg of Myc-tagged vectors containing either wild-type MKP-1 or its catalytically inactive mutant (MKP-1CS), expression was allowed for 12 hours, and then cells were exposed to 5 mM glutamate for 9 hours. Whole cell extracts were subjected to western blot analysis using specific antibodies against phospho-ERK1/2 (pERK1/2), ERK1/2 and MKP-1. (C) HT22 cells were transfected with scrambled control (Scr) or MKP-1 siRNA (siMKP-1 73). (i) The level of MKP-1 at 24 hours after transfection was monitored by western blot analysis. The levels of phospho-ERK1/2, ERK1/2, PKCδ and GAPDH were also examined by western blot analysis. (ii) Phosphorylation of ERK1/2 under glutamate exposure for 7 hours was determined by western blot analysis. Relative phospho-ERK1/2 levels were determined by densitometric scanning of enhanced chemiluminescence-exposed film, and the levels were calculated by averaging the results obtained from three independent experiments. (D) Cells were transfected with either control (scrambled, Scr) or MKP-1 siRNAs [siMKP-1 73 (si 73) and siMKP-1 853 (si 853)]. After 24 hours, cells were treated with 5 mM glutamate for 7-9 hours, followed by western blot analysis. *$P<0.05$, **$P<0.01$.

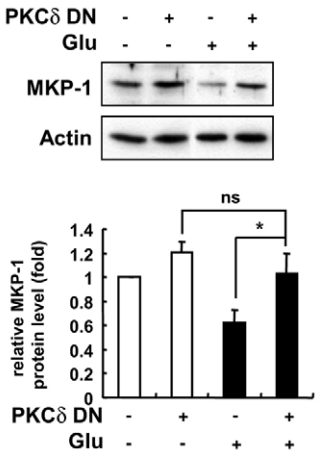

Fig. 5. Inhibition of PKCδ activity prevents glutamate-induced downregulation of MKP-1. HT22 cells were transfected with either a mock vector or a dominant-negative mutant (DN) of PKCδ (PKCδ DN) vector. After 12 hours, cells were exposed to 5 mM glutamate (Glu) for 7 hours. Western blot analysis was performed using specific antibodies against MKP-1 and actin. The relative MKP-1 protein levels were calculated by averaging the results obtained from three independent experiments. *$P<0.05$, ns, not significant.

MKP-1 has been reported to be a labile protein that is targeted for degradation by the ubiquitin-proteasome pathway (Brondello et al., 1999). To investigate whether the ubiquitin-mediated degradation pathway was involved in the glutamate-induced downregulation of MKP-1, we examined the effects of MG132, ALLN and lactacystin, which are common proteasome inhibitors. As shown in Fig. 6A, the level of MKP-1 in cells treated with proteasome inhibitors was dramatically higher as compared with untreated cells. Furthermore, a glutamate-induced decrease of MKP-1 was not observed in ALLN- or lactacystin-treated cells, indicating that proteasomal degradation of MKP-1 was involved in the downregulation of MKP-1 under glutamate exposure. We then examined whether MKP-1 was ubiquitylated in response to glutamate treatment. As shown in Fig. 6B, glutamate induced polyubiquitylation of MKP-1, suggesting that glutamate triggers degradation of MKP-1 through the ubiquitin-proteasomal pathway.

Since PKCδ contributed to the downregulation of MKP-1 during glutamate exposure (Fig. 5A,B), we examined whether ubiquitylation of MKP-1 was also regulated by PKCδ. As shown in Fig. 6C, rottlerin significantly prevented the polyubiquitylation of MKP-1 in glutamate-treated cells. Similar results were obtained by exogenous expression of PKCδ DN (data not shown). Since activation of PKCδ was regulated by ROS production (Fig. S2B, supplementary material), we examined whether generation of ROS was involved in the glutamate-induced downregulation of MKP-1. NAC blocked the glutamate-induced downregulation of MKP-1 as expected (Fig. S2B, supplementary material). Collectively, these results indicate that glutamate-induced activation of PKCδ contributes to the ubiquitylation and downregulation of MKP-1.

Inhibition of PKCδ activity suppressed the ubiquitylation-mediated degradation of MKP-1 in primary immature cortical neurons

In agreement with a previous study (Stanciu et al., 2000), primary cultures of rat immature cortical neurons underwent apoptotic cell death within 24 hours of exposure to glutamate, which was prevented by administration of U0126 (Fig. 7A). Similar results were obtained from the TUNEL assay (Fig. 7A). As observed in HT22 cells (Fig. 1A), treatment of mouse immature cortical cells with glutamate led to the activation of ERK1/2 (Fig. 7C, lane 4), and U0126 reduced both the basal and glutamate-induced elevation of phospho-ERK1/2 (Fig. 7C, lanes 3 and 6). Moreover, MKP-1 was significantly increased at 30 minutes after glutamate exposure, followed by a substantial decrease as observed in HT22 cells (data not shown and Fig. 4A).

To investigate the role of PKCδ during glutamate-induced toxicity in immature cortical cells, we examined the effect of rottlerin and siRNA against PKCδ. As shown in Fig. 7, both rottlerin and siRNA against PKCδ conferred significant

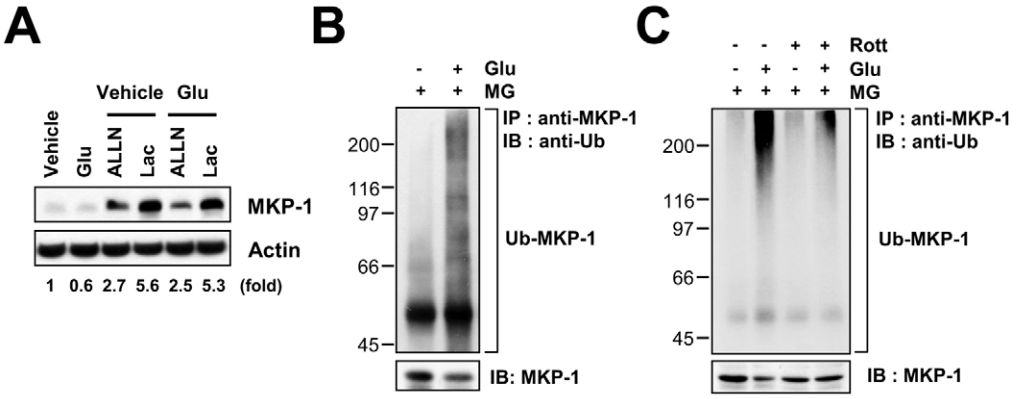

Fig. 6. PKCδ activation by glutamate induces ubiquitylation and downregulation of MKP-1 in HT22 cells. (A) HT22 cells were incubated with or without 5 μM ALLN or 5 μM lactacystine for 7 hours in the presence or absence of 5 mM glutamate (Glu). Western blot analysis was performed using specific antibodies against MKP-1 and actin. Relative MKP-1 protein levels were calculated by averaging the results obtained from three independent experiments. (B and C) Cells were treated with 5 mM glutamate in the presence or absence of 5 μM rottlerin (Rott) for 4 hours, followed by addition of 5 μM MG132 (MG) for 3 hours. Cells were then harvested, and subjected to immunoprecipitation (IP) of MKP-1 using anti-MKP-1 antibody. Following SDS-PAGE, membranes were immunoblotted (IB) with an anti-ubiquitin (anti-Ub) antibody (upper panel). The level of MKP-1 was determined by western blot analysis of the same cell lysates (lower panel). Results were reproducible, and blots shown are representative of three separate experiments.

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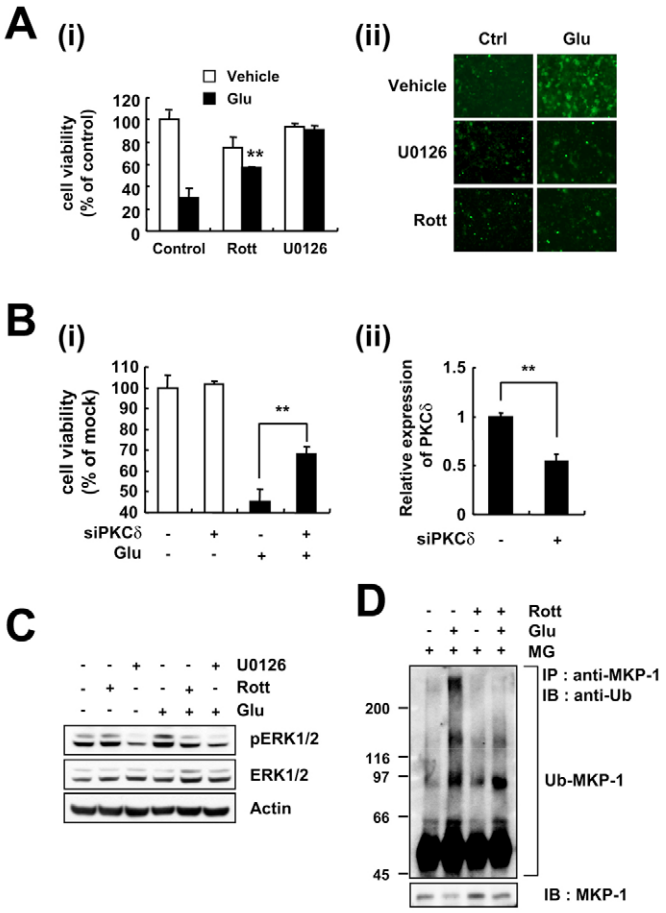

Fig. 7. Inhibition of PKCδ prevents ubiquitylation and degradation of MKP-1 in primary immature cortical neurons. (A) (i) Immature primary cortical neuron cultures prepared from day E13 mouse fetuses were seeded onto 24-well plates and grown for 24 hours. Cells were then incubated with or without 5 mM glutamate (Glu) in the presence or absence of 20 μM U0126 or 3 μM rottlerin (Rott) for 24 hours as indicated. Cell viability was analyzed by MTT assay. Results are mean ± s.d. from three separate experiments. $^{**}P<0.01$ compared with control cells exposed to glutamate alone. (ii) Representative photomicrographs of apoptotic cells incubated with or without 5 mM glutamate in the presence or absence of 20 μM U0126 or 3 μM rottlerin for 24 hours, detected by TUNEL staining. (B) Cells were transfected with either control or PKCδ siRNA (siPKCδ) and, after 24 hours, cells were exposed to 5 mM glutamate for 16 hours. Downregulation of PKCδ was confirmed by quantitative RT-PCR (ii), and cell viability was measured by MTT assay (i). Each bar is mean ± s.d. value from three separate experiments. $^{**}P<0.01$. (C) Cells were incubated with or without 5 mM glutamate in the presence or absence of 20 μM U0126 or 3 μM rottlerin for 12 hours. Cells were then harvested, lysed and phospho-(p)ERK1/2 or total ERK1/2 in equivalent amounts of total lysate protein (40 μg) were visualized by western blot analysis using specific antibodies against phospho-ERK1/2, ERK1/2 and actin. (D) Cells were incubated with or without 5 mM glutamate in the presence or absence of 5 μM rottlerin for 14 hours, followed by addition of 5 μM MG132 (MG) for 4 hours. Equal amounts of proteins in each cell extract were subjected to immunoprecipitation (IP) using 2 μg of anti-MKP-1 antibody. The immunoprecipitates were subjected to immunoblotting (IB) using antibody against ubiquitin (anti-Ub; upper panel). The level of MKP-1 was determined by western blot analysis of the same cell lysates (lower panel). Results were reproducible, and blots shown are representative of two independent experiments.

neuroprotection against glutamate-induced toxicity. Rottlerin caused a reduction in the level of phospho-ERK1/2 elevated by glutamate (Fig. 7C), and it also caused an increase in the level of MKP-1 (Fig. 7D). Glutamate evoked polyubiquitylation of MKP-1, which was significantly blocked by rottlerin (Fig. 7D), supporting the notion that the decrease of MKP-1 is strongly associated with the glutamate-induced activation of PKCδ in immature mouse cortical neurons as in HT22 cells.

Role of MKP-1 in the regulation of glutamate-induced cell death

Since downregulation of MKP-1 by glutamate was associated with persistent activation of ERK1/2, which contributes to oxidative cell death, we examined whether cell viability was affected by directly regulating the level of MKP-1. Glutamate-induced cell death was rescued by overexpression of MKP-1 (Fig. 8A), but was significantly increased when cells were transfected with siRNA against MKP-1 (Fig. 8B). The effect of siRNA against MKP-1 on glutamate-induced cell death was reversed by U0126, which prevents activation of ERK1/2 (Fig. 8D), supporting the notion that downregulation of MKP-1 is involved in glutamate-induced cell death through activation of ERK1/2. Cell viability was substantially decreased when cells were transfected with siRNA against MKP-1 even in the absence of glutamate, suggesting that MKP-1 plays an essential role in the regulation of cell survival both in unstressed and glutamate-treated cells.

To confirm the role of MKP-1 in glutamate-induced cell death, immature cortical cells were transfected with siRNA against MKP-1. As expected, cell viability was significantly decreased when cells were transfected with siRNA against MKP-1, both in the absence or presence of glutamate (Fig. 8C). Moreover, depletion of MKP-1 caused a pronounced enhancement in the basal level of phosho-ERK1/2 in immature cortical cells as in HT22 cells. Furthermore, glutamate-induced ERK1/2 phosphorylation was also increased in cells transfected with siRNA against MKP-1 (Fig. 8C), indicating that MKP-1 contributes to the glutamate-induced neuronal cell death through regulation of ERK1/2 phosphorylation.

Discussion

In the present study, we suggest that downregulation of MKP-1 is responsible for the sustained activation of ERK1/2, which plays an important role during glutamate-induced neuronal cell death. This study also provides evidence that downregulation of MKP-1 occurs through its ubiquitylation by activation of PKCδ.

ERK has been shown to be activated in response to oxidative toxicity. A previous study provided evidence for a protective role of ERK during oxidative stress, suggesting that H_2O_2-stimulated activation of ERK2 has a crucial role in preventing apoptosis (Guyton et al., 1996). By contrast, other studies demonstrated that the MEK/ERK pathway has a pro-apoptotic effect on neuronal cell death. For example, several neurological insults, such as ischemia and kainate-induced seizure, induce excessive release of excitatory amino acids and the subsequent

neuronal cell death resulted in the activation of ERK1/2 in vivo (Hu and Wieloch, 1994; Kim et al., 1994), and inhibition of the MEK/ERK pathway protected the brain from ischemic injury (Alessandrini et al., 1999). A recent study has demonstrated that oxidative toxicity in HT22 cells and immature embryonic rat cortical neurons was associated with the delayed activation of ERK1/2 and that inhibition of ERK1/2 activation protected the cells against glutamate-induced cell death (Stanciu et al., 2000). In agreement with that report, we found that glutamate led to persistent activation of

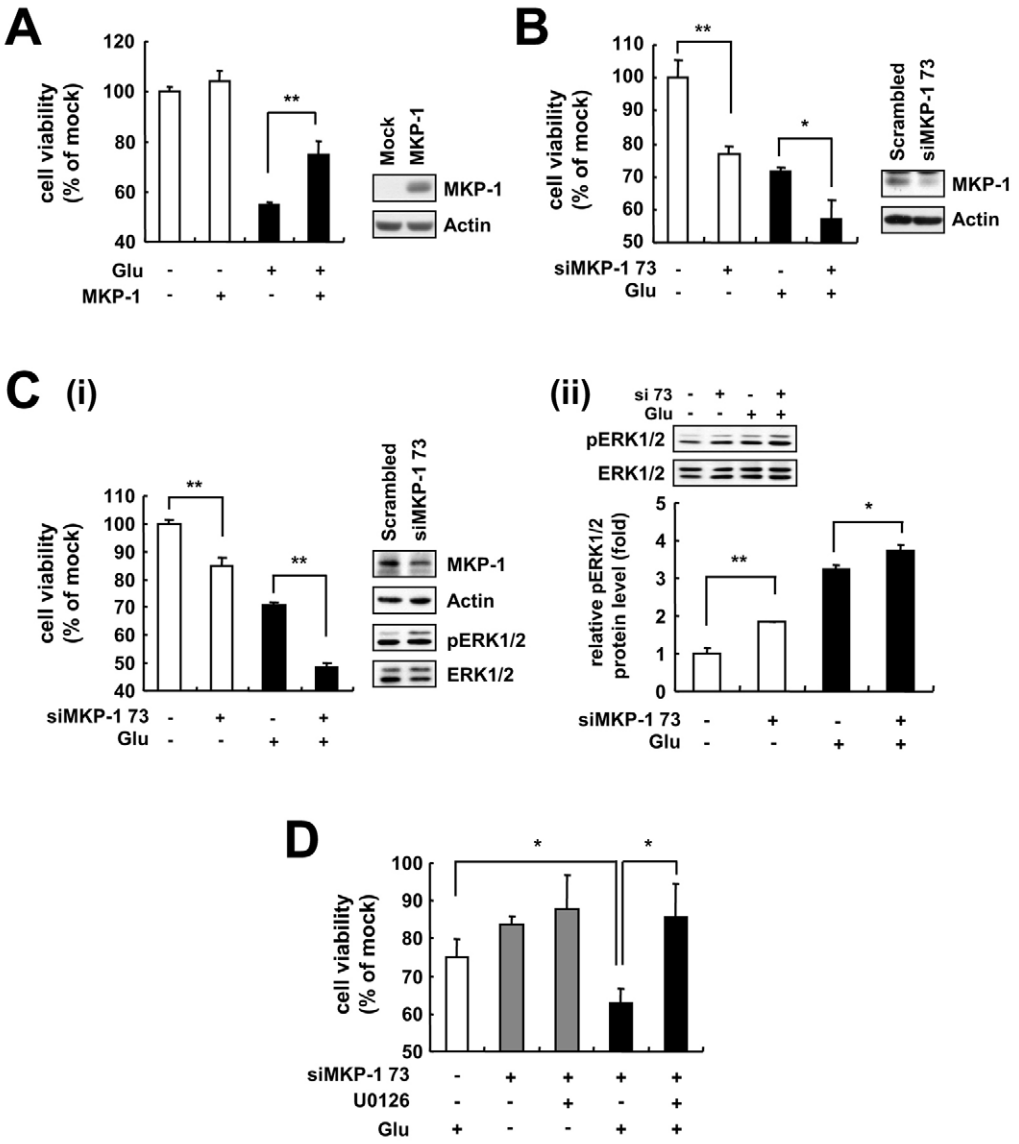

Fig. 8. Role of MKP-1 in the regulation of glutamate-induced oxidative toxicity. (A) Exogenous expression of MKP-1 increases cell viability during oxidative toxicity. Cells were transfected with 2 μg of Myc-tagged vectors containing either wild-type MKP-1 or mock. After 12 hours, cells were exposed to 5 mM glutamate (Glu) for 9 hours. MKP-1 protein levels were determined by western blot analysis (right panel), and cell viability was measured by MTT assay (left panel). Results are mean ± s.d. obtained from three separate experiments. $^{**}P<0.01$. (B) HT22 cells were transfected with control (Scrambled) or MKP-1 siRNA (siMKP-1 73) as indicated, expression was allowed for 24 hours, and then cells were exposed to 5 mM glutamate for 7 hours. Downregulation of MKP-1 after transfection was confirmed by western blot analysis (right panel) and cell viability was measured by MTT assay (left panel). Results are mean ± s.d. obtained from three separate experiments. (C) Immature primary cortical neuron cultures prepared from day E13 mouse fetuses were seeded onto 48-well plates and grown for 24 hours. (i) Cells were transfected with control or MKP-1 siRNA (siMKP-1 73; si 73) as indicated, expression was allowed for 24 hours, and then cells were exposed to 5 mM glutamate for 16 hours. Downregulation of MKP-1 after transfection was confirmed by western blot analysis (right panel), and cell viability was measured by MTT assay (left panel). (ii) Phosphorylation of ERK1/2 under glutamate exposure for 12 hours was determined by western blot analysis. Relative phospho-ERK1/2 (pERK1/2) levels were determined by densitometric scanning of enhanced chemiluminescence-exposed film, and the levels were calculated by averaging the results obtained from three independent experiments. (D) HT22 cells were transfected with either control or MKP-1 siRNA and, after 24 hours, cells were exposed to 5 mM glutamate in the presence or absence of 20 μM U0126 for 7 hours. Cell viability was measured by MTT assay. Results are mean ± s.d. obtained from three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$.

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ERK1/2, which was associated with neuronal cell death (Figs 1, 7). The mechanisms that underlie such diametric effects of ERK are unclear, but several lines of evidence suggest that cell death and survival might be related to the kinetics of its activation, i.e. rapid and transient activation of ERK1/2 has been shown to be associated with enhanced survival response, whereas delayed and sustained activation of ERK1/2 has a tendency to trigger cell death (Guyton et al., 1996; Wang et al., 2000). Therefore, regulating activation of ERK appears to be one of the key events that control cell death and survival.

Protein phosphatases have an important role in controlling the magnitude and duration of ERK1/2 activation. Thus, we focused on the possible role of MKP family members, which are known to be important for dephosphorylating specific MAPKs (Keyse, 2000). MKP-1, which was initially identified as an oxidative stress-induced protein phosphatase, was shown to dephosphorylate ERK, p38 MAPK and JNK (Sun et al., 1993; Franklin and Kraft, 1997; Lasa et al., 2002), depending on the cell type as well as the cellular context. Studies have demonstrated that the anti-apoptotic effect of MKP-1 was mainly associated with its ability to dephosphorylate p38 or JNK (Guo et al., 1998; Wu and Bennett, 2005). By contrast, it has also been reported that MKP-1 inactivates ERK, which resulted in inhibition of re-entry into the cell cycle (Brondello et al., 1995). Here, we suggest that glutamate-induced oxidative toxicity leads to downregulation of MKP-1, which results in persistent activation of ERK1/2. Overexpression of MKP-1 decreased the level of phospho-ERK1/2 (Fig. 4), whereas knockdown of MKP-1 using siRNA resulted in an enhanced phosphorylation of ERK1/2 (Fig. 4). MKP-1 has also been reported to regulate p38 MAPK and JNK activation (Lasa et al., 2002), and both kinases were previously shown to have crucial roles during oxidative stress in many cell types (Maher, 2001). However, we could not detect phosphorylation of p38 and JNK during glutamate exposure until 9 hours, in agreement with a previous report (Levinthal and DeFranco, 2005). Moreover, treatment with SB203580 and SP600125, specific inhibitors of p38 MAPK and JNK, respectively, failed to block the cell death induced by glutamate, excluding p38 MAPK and JNK in glutamate-induced cell death.

The suggested roles of MKP-1 in the regulation of cell death and survival have been controversial. A recent study showed that blocking dexamethasone-induced MKP-1 expression by siRNA protected cells from chemotherapy-induced apoptosis (Wu et al., 2005). By contrast, MKP-1 was also implicated in the anti-apoptotic effect of retinoids in mesangial cells incubated with H_2O_2 (Xu et al., 2002), and a recent report showed that MKP-1$^{+/-}$ and MKP-1$^{-/-}$ mouse embryonic fibroblasts (MEFs) proliferate at a slower rate compared with wild-type cells, owing to increased cell death (Wu and Bennett, 2005). Our results show that glutamate-induced cell death was rescued by overexpression of MKP-1 (Fig. 8A), but was significantly increased when cells were transfected with siRNA against MKP-1 (Fig. 8B,C). These results suggest that MKP-1 has a protective effect during oxidative toxicity.

It has been shown that degradation of MKP-1 protein is attenuated by inhibitors of the ubiquitin-directed proteasome complex (Brondello et al., 1999). Recently, Lin et al. suggested that degradation of MKP-1 was triggered by ERK signaling through activation of the ubiquitin-proteasome pathway, which contributes to the sustained activation of ERK1/2, thereby providing a positive-feedback mechanism (Lin et al., 2003). In the present study, we found that MKP-1 was downregulated after prolonged exposure to glutamate (Fig. 4), which was accompanied by its polyubiquitylation (Fig. 6). On the basis of these results, we concluded that long-term glutamate exposure induced degradation of MKP-1 through the ubiquitin-proteasome pathway. *MKP-1* mRNA is an early response gene, and induction of MKP-1 was shown to be associated with activation of ERK1/2 and PKCϵ (Brondello et al., 1997; Valledor et al., 2000). In HT22 cells, we observed an increase in the protein level of MKP-1 as well as transient activation of ERK1/2 within 1 hour of glutamate treatment (data not shown). Moreover, the induction of MKP-1 was blocked by U0126 (data not shown), suggesting that activation of ERK1/2 is involved in the induction of MKP-1 observed within 1 hour after glutamate treatment.

Recent studies have reported that the level of PKCδ increased during kainate-induced neuronal cell death (Kaasinen et al., 2002) and after transient global brain ischemia (Koponen et al., 2000). In addition, dieldrin-induced and 1-methyl-4-phenylpyridinium (MPP^+)-induced oxidative stress also caused persistent activation of PKCδ in dopaminergic neurons (Kaul et al., 2003; Kitazawa et al., 2003). Although several downstream targets of PKCδ have been described, the mechanism by which PKCδ regulates neuronal cell death is poorly understood. PKCδ has been reported to activate MAPK through several mechanisms. For example, a constitutively active mutant of PKCδ was reported to activate MAPK (Ueda et al., 1996), and neurogenic agents and estrogen induced PKCδ-dependent activation of MAPK (Corbit et al., 1999; Keshamouni et al., 2002). However, the molecular mechanism by which PKCδ activates MAPK has not been demonstrated. Here, we show that glutamate treatment resulted in a dramatic and persistent activation of PKCδ (Fig. 2), and that inhibition of PKCδ activity suppressed the glutamate-induced ERK1/2 phosphorylation (Fig. 3), demonstrating that PKCδ-dependent activation of ERK1/2 occurs during glutamate-induced neuronal cell death. Our data also show that glutamate-induced downregulation of MKP-1 was blocked by inhibition of PKCδ activation in HT22 cells (Fig. 5) as well as immature primary cortical neurons (Fig. 7). Furthermore, inhibition of PKCδ suppressed the polyubiquitylation of MKP-1 (Figs 6, 7). Therefore, we suggest that activation of PKCδ under glutamate exposure causes sustained activation of ERK1/2 through degradation of MKP-1.

Further studies are required to determine the exact mechanism by which activation of PKCδ leads to degradation of MKP-1. PKCδ might lead either to a direct or an indirect activation of the ubiquitin-proteasome pathway, targeting MKP-1 for degradation. Interestingly, PKC and ATM protein kinase were shown to phosphorylate Parkin and Mdm2, respectively, and modulation of the phosphorylation state of these proteins has been suggested to have a regulatory role on their E3 ubiquitin ligase activities (Yamamoto et al., 2005; Meulmeester et al., 2005). Therefore, it is plausible to suggest that activation of PKCδ results in phosphorylation of an E3 ubiquitin ligase and/or its upstream compartments involved in activating E3 ubiquitin ligase, which might participate in targeting MKP-1 for proteasomal degradation. An alternative mechanism might involve post-translational modification of MKP-1 itself by glutamate-induced PKCδ activation. In fact,

p42/44 MAPK-dependent phosphorylation of MKP family members, such as MKP-1, MKP-2 and MKP-3, affect the rate of degradation by the proteasome (Brondello et al., 1999; Marchetti et al., 2005). In particular, phosphorylation of MKP-3 resulted in enhanced degradation by the proteasome (Marchetti et al., 2005). It is thus possible to suggest that activation of PKCδ might induce phosphorylation of MKP-1 directly or through activation of another kinase, which might accelerate the ubiquitylation and degradation of MKP-1. However, it is also possible that activation of PKC might induce degradation of MKP-1 without affecting its phosphorylation status, as illustrated in the case of downregulation of the dopamine transporter, which was rapidly downregulated without being phosphorylated (Granas et al., 2003; Miranda et al., 2005). Thus, further studies are required to clarify whether glutamate-induced PKCδ activation induces the direct phosphorylation of MKP-1, and whether phosphorylation triggers its ubiquitylation and degradation.

Taken together, we conclude that MKP-1 is an important mediator during glutamate-induced oxidative toxicity. Its degradation through the ubiquitin-proteasome pathway is a crucial step regulating persistent activation of ERK1/2, which contributes to glutamate-induced cell death in HT22 mouse hippocampal cells and immature cortical neurons.

Materials and Methods

Plasmids and materials

The plasmids pSG5-MKP-1 and its catalytically inactive mutant, pSG5-MKP-1CS, containing a single copy of the Myc epitope tag were kindly provided by N. Tonks (Sun et al., 1993). The plasmids pHA.CE-PKCδ and its dominant-negative mutant, pHA.CE-PKCδ (K376R) were kindly provided by Y. S. Lee (Lee et al., 2002). L-glutamate was purchased from Sigma. U0126, SB202190, SP600125, rottlerin, lactacystin, MG132 and histone H1 were obtained from Calbiochem. [γ-^{32}P]ATP was purchased from NEN Life Science Products.

HT22 cell cultures and cell transfection

HT22 cells, a mouse hippocampal cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37°C. In a 6-well tissue culture plate (Falcon), HT22 cells were seeded at a density of about 2×10^5 cells per well. When the density of cells reached 50-60%, cells were transfected with expression plasmids for the dominant-negative mutant of PKCδ (K378R), or wild-type MKP-1, or its catalytically inactive mutant (MKP-1CS) using Lipofectamine™ reagent (Invitrogen). After incubation for 24 hours, the transfected cells were treated as indicated for analysis.

Primary cortical cell cultures

Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared from embryonic day 13-15 ICR (Institute Cancer Research) mice as described previously (Kim et al., 1999). In brief, dissociated cortical cells were plated on poly-L-lysine-coated 24-well dishes and maintained for 24 hours in DMEM (glutamine-free) supplemented with 20 mM glucose, 2 mM glutamine, 5% fetal calf serum and 5% horse serum. When cultured cortical cell preparations were subjected to immunocytochemistry, the astrocyte marker glial fibrillary acidic protein (GFAP) was barely detectable (data not shown).

Cell viability assay

Cell viability was assessed by measuring their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were seeded onto 96-well plates at a density of about 5×10^3 cells per well in growth medium and incubated until about 60-70% confluency, prior to the initiation of experimental treatment. Following the treatments as indicated, 15 μl of MTT solution (5 mg/ml) was added to each well, and cells were maintained for 4 hours at 37°C. 100 μl of solubilizing solution (50% dimethylformamide and 20% SDS, pH 4.8) was then added. After an overnight incubation at room temperature, absorbance at 570 nm was measured.

Visualization of apoptotic cells through TUNEL assay

After fixation in 4% paraformaldehyde, cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100, washed again with PBS, and incubated for 60 minutes at 37°C in the dark with the DeadEnd™ Fluorometric TUNEL System (Promega). In-situ-labeled nuclei were observed and photographed under a fluorescence microscope (Axioplan2, Zeiss).

Preparation of cell homogenate

In a 60 mm culture dish (Falcon), HT22 cells were seeded at a density of about 5×10^5 cells per dish. When the density of cells reached 60-70%, cells were treated with several stimulants for analysis. Cells were washed and resuspended in serum-free medium. The dishes were placed on ice, scraped with a rubber policeman, and centrifuged at 1000 ***g*** for 10 minutes. The supernatant was aspirated, and the cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF and 1 mM Na_3VO_4) on ice for 40 minutes. The cell lysates were centrifuged for 15 minutes at 22,250 ***g***, supernatants were removed, and 5× sample buffer was added.

Immunoblot analysis

Lysates (40 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in PBS-T and subsequently incubated with the primary antibody. The mouse monoclonal antibody to PKCδ was obtained from BD Transduction Laboratories. Rabbit polyclonal antibodies to pERK, ERK and MKP-1 were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, and mouse monoclonal antibody against actin was from NeoMarkers. Specific reactive bands were detected by using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase, and the immunoreactive bands were visualized by the SUPEX western blotting detection kit (Neuronex).

Immunoprecipitation

Briefly, cell lysates were prepared in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml aprotinin, 10 mM NaF and 1 mM Na_3VO_4). Equal amounts of proteins were immunoprecipitated using anti-PKCδ, anti-MKP-1 and anti-ubiquitin antibodies (Santa Cruz Biotechnology) and collected with protein A-Sepharose beads (Santa Cruz Biotechnology) at 4°C for 16 hours. The immunoprecipitate was then washed four times in cold lysis buffer and subjected to western blot analysis and PKCδ kinase assay.

PKCδ kinase assay

The activity of PKCδ was determined by immune complex kinase assay (Lee et al., 2003). The anti-PKCδ immunoprecipitate was washed in a kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate and 10 mM $MgCl_2$). The kinase assay was carried out in a total volume of 30 μl of a kinase reaction buffer containing 50 μM ATP, 1 μCi of [γ-^{32}P]ATP and 200 μg/ml histone H1 for 30 minutes incubation at 30°C. Phosphorylated histone H1 was resolved on 14% SDS-PAGE followed by autoradiography.

siRNA experiments

siRNA duplexes targeting MKP-1 (5′-AACGAGGCTATTGACTTCATA-3′ and 5′-CCGCACAAGATCGACCGACTT-3′) and PKCδ were purchased from Dharmacon. A scrambled non-targeting siRNA was used as a negative control. Transfections of siRNA duplexes at 50 nM of final concentrations were carried out using Metafectene (Biontex) according to the manufacturer's recommendations. After transfection, the cells were processed for western blotting and/or MTT assay as indicated. Effect of PKCδ siRNA in primary cortical cells was confirmed by quantitative RT-PCR, which was performed as described in detail elsewhere (Lee et al., 2005). Primers used in PCR were as follows: GAPDH forward 5′-GCC-ATCAATGACCCCTTCATT-3′ and reverse 5′-GCTCCTGGAAGATGGTGATGG-3′; PKCδ forward 5′-TCTGGGAGTGACATCCTAGACAACAACGGG-3′ and reverse 5′-CAGATGATCTCAGCTGCATAAAACGTAGCC-3′.

Statistical analysis

All experiments were independently repeated a minimum of three times. All quantitative data are presented as mean ± s.d. Comparisons between two groups were analyzed using Student's *t* test, and values of $P<0.05$ were considered to be significant.

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