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Short communication

Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line

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Abstract

A neuronal cell line, HT-22, is sensitive to glutamate cytotoxicity via a non-receptor mediated oxidative pathway. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, blocks this glutamate-induced cell death. Down-regulation of protein kinase C eliminates the protection against glutamate cytotoxicity afforded by TPA. The data suggest that protein kinase C activation blocks an early step in the cytotoxic pathway.

Key words: 12-*O*-Tetradecanoylphorbol-13-acetate; Neurotoxicity; Oxidative stress; Glutathione; Anti-oxidant

Excitatory amino acids (EAAs) are toxic to neuronal cells in culture via two distinct processes. The classical pathway for EAA toxicity is mediated by glutamate receptors and is characterized by inhibition by specific glutamate receptor antagonists (for review see [2]). Evidence for the involvement of a second pathway in EAA neurotoxicity arose from studies on cultured cells where it was shown that competition by glutamate for a glutamate/cystine antiporter leads to an imbalance in cystine homeostasis, a reduction in cellular glutathione and ultimately, cell death [13]. The neuronal cell death brought about through this oxidative pathway is inhibited by antioxidants or high concentrations of extracellular cystine [11] and has been described in neuronal cell lines [13], primary neurons [14] and oligodendrocytes [16].

We recently reported that the toxic effect of glutamate on neurons could be modified by growth factors, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), and that the mechanism of glutamate toxicity in these cells appeared to involve oxidative stress since it could be inhibited by antioxidants [18]. Since the receptors for both EGF and bFGF are tyrosine kinases, the earliest response of cells to these growth factors is an increase in receptor tyrosine kinase activity. To better understand the mechanisms underlying glutamate toxicity in neuronal cells, we assayed the ability of various protein kinase activators and inhibitors to protect cells from glutamate-induced death. We show here that treatment with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, or other agents which activate PKC, reduces the toxicity of glutamate for neuronal cells. In contrast, activators of other kinases or phosphatases have little or no protective effect.

HT-4 cells, an immortalized mouse hippocampal cell line, were obtained from Morimoto and Koshland [12] and subcloned. The HT-22 clone was particularly sensitive to glutamate and was used in the following studies. Cells were maintained in DME/10% fetal calf serum (FCS) and passaged by trypsinization. Cell viability was assayed using the MTT assay [8] or a colony forming assay [3]. For the MTT assay, cells were plated into 96-well plates at 5×10^3 cells/well in complete medium and 24 h later the experimental agents added. The ability of cells to reduce MTT was assayed at various times, exactly as described previously [18]. To complement the MTT assay, a colony forming assay was used which measures the ability of cells to divide [3]. After treatment, cells were dissociated and serially diluted into complete medium and the number of colonies formed determined 7 days later [3].

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Chicken cortical neurons were prepared from 9-embryonic-day chicken brains as described [1] and cultured in DME/F12 containing 5 μg/ml insulin and 100 μg/ml transferrin. Glutamate toxicity was assayed after 4 days in culture. The cells, in 96-well dishes, were treated with glutamate and other agents in a buffer containing 120 mM NaCl, 5.4 mM KCl, 2.1 mM $CaCl_2$, 15 mM glucose, 25 mM Tris-HCl, pH 7.5. After 8 h the medium was replaced with DME/F12 containing 5 μg/ml insulin and 100 μg/ml transferrin and % survival was determined after 24 h by the MTT assay. 9-Embryonic-day chicken brains are composed of nearly pure populations of neuronal cells [1] so there is no interference in the MTT assay from non-neuronal cells.

To measure glutathione levels, the specific, glutathione-binding, fluorescent dye monochlorobimane was used [17]. 2×10^5 cells in DME, 10% FCS were exposed to glutamate and agents or vehicle for the required time. Cells were stained for 5 min with 10 μM monochlorobimane and both floating and adherent cells were harvested by trypsinization. Following washing in Phenol red-free/HEPES buffered/DME/2% FCS at 4°C, the cells were analyzed by FACS with UV excitation and emission at 485 nm as described [17].

Glutamate causes a dose-dependent killing of HT-22 cells, for which a prolonged (6–8 h) and continuous incubation is required (not shown). Similar to the PC-12 cells [18], glutamate, quisqualate and BOAA are toxic for the HT-22 cells, but kainate, NMDA, BMAA and aspartate are not (not shown). Furthermore, the glutamate receptor antagonists which block glutamate binding to NMDA or quisqualate receptors do not rescue the HT-22 cells from glutamate toxicity (not shown). These results, and the finding that antioxidants and high extracellular cystine protect the HT-22 cells from glutamate toxicity (not shown), indicate that cell death occurs via the oxidative pathway for glutamate toxicity [13].

To determine if protein phosphorylation plays a role in glutamate toxicity the cells were treated for 8 h simultaneously with glutamate and various agents which reduce or increase the levels of serine, threonine or tyrosine phosphorylation in proteins, and assayed for cell death 16 h later (Table 1). The concentrations in Table 1 are the highest concentrations of the agents that could be used without causing significant toxicity. Most of the agents did not protect the HT-22 cells from glutamate-mediated cell death. Only the phorbol ester TPA, an activator of PKC, and staurosporine, a protein kinase inhibitor, protected cells. Concentrations of TPA as low as 1 ng/ml were effective (Table 1), whereas the inactive phorbol derivative, 4-α phorbol had no effect on survival (Fig. 1A). Down-regu-

Table 1
Effect of agents that alter phosphorylation on glutamate toxicity

Treatment	% survival
Control	100
5 mM glutamate alone	11.1 ± 4
+ 0.1 ng/ml TPA	14.0 ± 2
+ 1 ng/ml TPA	40.7 ± 1
+ 100 ng/ml TPA	56.7 ± 6
+ 35 mM KCl	26.8 ± 4
+ 50 mM KCl	71.8 ± 7
+ 50 mM KCl + PKC-DR	42.7 ± 11
+ calphostin C	16.4 ± 3
+ 50 μM H-7	23.0 ± 0
+ 100 nM okadaic acid	8.7 ± 1
+ 10 μM Na_2VO_4	6.3 ± 2
+ 100 μM genistein	8.5 ± 5
+ 26 μM lavendustin	8.6 ± 4
+ 10 nM staurosporine	22.9 ± 5
+ 100 nM staurosporine	77.7 ± 13
+ 100 nM staurosporine + PKC-DR	70.6 ± 6
0.5 mg/ml dibutryl cAMP	5.4 ± 5
50 μM H-89	87.0 ± 4

HT-22 cells were untreated or treated for 8 h with 5 mM glutamate alone or with the various agents indicated above. The % survival was measured after 24 h using the MTT assay as described in the text and is presented relative to survival in the presence of the agent alone. PKC-DR indicates the cells were treated for 24 h with 1 μg/ml TPA to down-regulate PKC prior to the addition of KCl or staurosporine and glutamate. Values are given as means ± standard deviation. Similar results were obtained in 3–6 separate experiments.

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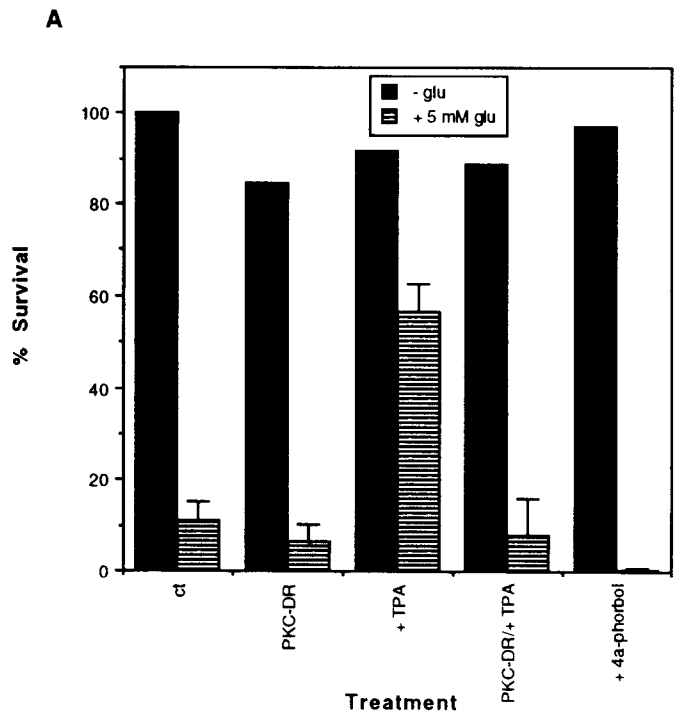
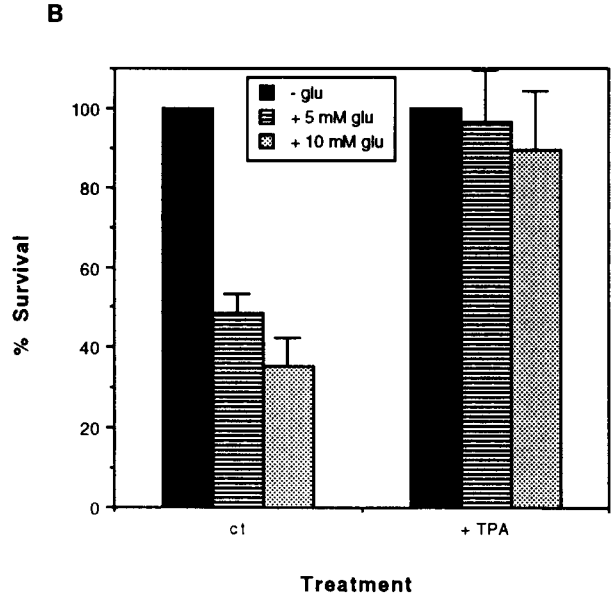
Fig. 1. A: effect of PKC down-regulation on glutamate toxicity. Cells were treated with 5 mM glutamate alone (ct) or in the presence of 100 ng/ml TPA (TPA) or 160 ng/ml 4α-phorbol (phorbol) for 8 h. % survival was measured after 24 h by the MTT assay [8]. In some cases, the cells were treated for 24 h with 1 μg/ml TPA to down-regulate PKC (PKC-DR) prior to the addition of glutamate and/or other agents. The results are the mean of quadruplicate determinations ± standard deviation. Similar results were obtained in four separate experiments. B: protection of primary chick cortical neurons from glutamate toxicity by TPA. Chick cortical neurons were prepared as described in materials and methods. After 4 days in culture the cells were treated for 8 h with 5 or 10 mM glutamate in the absence (ct) or presence (+TPA) of 100 ng/ml TPA. % survival was measured after 24 h using the MTT assay [8]. The results are the mean of quadruplicate determinations ± standard deviation. Similar results were obtained in 3 separate experiments. C: effect of glutamate and agents which protect from glutamate toxicity on intracellular glutathione levels. HT-22 cells were treated for 8 h with 5 mM glutamate alone (ct) or glutamate plus 100 μg/ml vitamin E (vit. E), 1 mM cystine (cys.) or 100 ng/ml TPA (TPA). The data are presented as changes in arbitrary fluorescence units. The results are the mean of triplicate determinations ± standard deviation. Similar results were obtained in 3 separate experiments. D: time dependence of protection from glutamate toxicity by 100 ng/ml TPA (TPA), 100 μg/ml vitamin E (vit. E) or 50 μM $CoCl_2$ (cobalt). HT-22 cells were treated with 5 mM glutamate and the various agents were added at the same time (0) or up to 7 h after the addition of glutamate. After 8 h the medium was changed and % survival was measured after 24 h by the MTT assay [8]. The results are presented relative to survival in the presence of the agent alone. The results are the mean of quadruplicate determinations ± standard deviation. Similar results were obtained in 4 separate experiments.

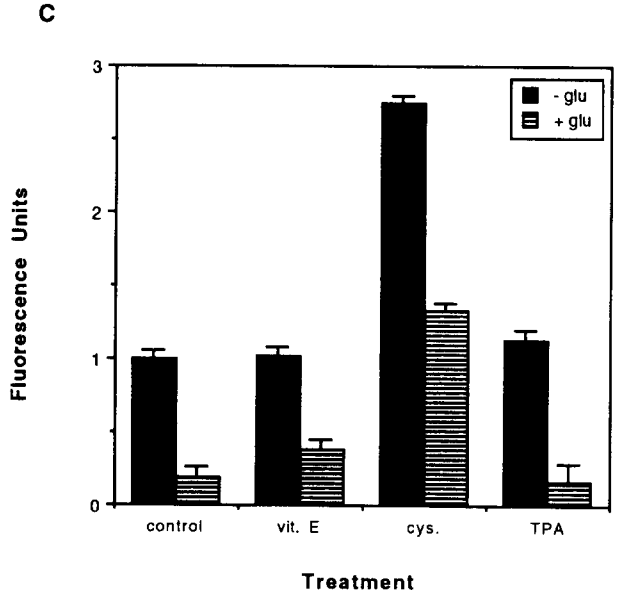
lation of PKC by a 24 h pre-incubation of the cells with a high (1 μM) concentration of TPA blocked the protective effect of TPA (Fig. 1A).

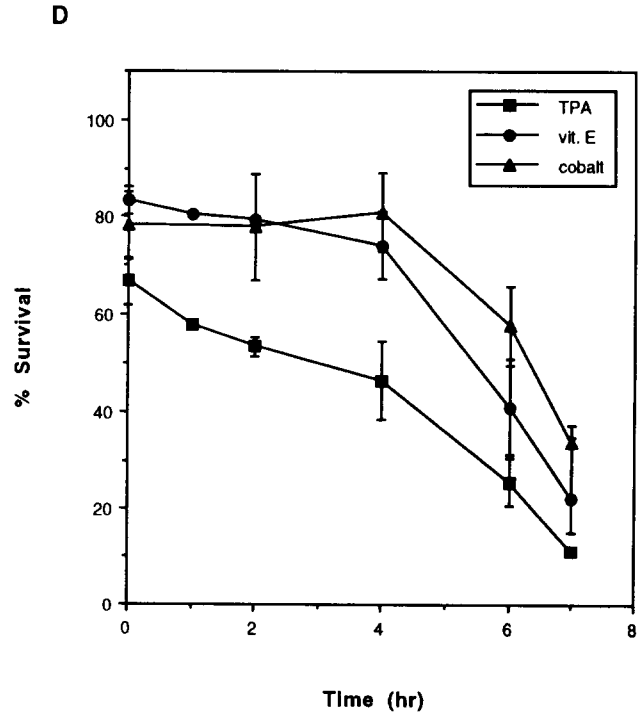
In addition to the studies with the clonal hippocampal cell line, we examined the effect of PKC activation by TPA on glutamate toxicity in primary cultures of chicken cortical neurons. Treatment of the primary cortical neurons with 5–10 mM glutamate lead to a dramatic reduction in survival (Fig. 1B). As with the clonal hippocampal cell line, the toxic effect of glutamate on the primary neurons was blocked by treatment with 100 ng/ml TPA (Fig. 1B).

To further characterize the role of PKC in glutamate toxicity, the effects of several other agents which









alter PKC activity were studied. Neither calphostin C, a recently described and highly specific inhibitor of protein kinase C, nor H-7, a widely used but less specific inhibitor of PKC, reduced glutamate toxicity (Table 1) over a wide range of concentrations. Potassium ions not only depolarize cell membranes but also activate PKC in primary sympathetic cultures [19]. K^+ protected the HT-22 cells from glutamate toxicity in a dose-dependent manner (Table 1). Down-regulation of PKC by prolonged incubation of the cells with a high concentration of TPA significantly reduced the protection afforded by K^+ (Table 1). Surprisingly, staurosporine, a non-specific protein kinase inhibitor, also protected the HT-22 cells from glutamate toxicity in a dose dependent manner (Table 1). Although staurosporine can inhibit PKC, it can also mimic the effects of TPA on cells [9,20]. In our experiments, the protective effect of staurosporine was not affected by prior down-regulation of PKC (Table 1). This suggests that its effects are independent of PKC and involve another pathway, possibly acting upon another kinase in the cascade. Relevant to this observation is the finding that inhibition of protein kinase A (PKA) with the specific inhibitor H-89 protects the HT-22 cells from glutamate toxicity (Table 1).

The first step in the oxidative pathway of glutamate toxicity is the inhibition of cystine uptake by extracellular glutamate [13], leading to a subsequent decrease in intracellular glutathione levels [13] and oxidative stress. To determine whether TPA reduced this effect of glutamate, we measured the intracellular levels of glutathione in the HT-22 cells. As shown in Fig. 1C, glutamate lowers glutathione levels in the cells. Although addition of extracellular cystine raises glutathione levels in the presence of glutamate, both TPA and vitamin E, fail to do so (Fig. 1C). This suggests that these agents are not protecting the cells from death by maintaining high levels of intracellular glutathione.

In order to determine where in the oxidative pathway of glutamate toxicity TPA was acting, we determined the latest time after glutamate addition at which TPA could be added to the HT-22 cells and still protect the cells from death (Fig. 1D). Protection by TPA decreased gradually over the first four hours following addition of glutamate and then dropped sharply so that little or no protection was seen if TPA was added after 6 h of glutamate treatment (Fig. 1D). In contrast, both vitamin E and the Ca^{+2} channel blocker, cobalt, afforded a constant, high level of protection if they were added during the first four hours of glutamate treatment (Fig. 1D). Protection by vitamin E fell off sharply between 4 and 6 h following glutamate addition whereas protection by cobalt fell off more gradually and was still significant, even if added after 7 h of glutamate treatment (Fig. 1D). These findings suggest that TPA blocks a relatively early step in the pathway leading to cell death.

The above data show that activation of PKC can protect hippocampal HT-22 cells from glutamate toxicity mediated by the oxidative pathway. Phorbol esters, which stimulate PKC activity, are protective while the down-regulation of the enzyme by long-term exposure to TPA eliminates any protective effect. Furthermore, excess extracellular K^+, which also protects the cells from glutamate toxicity, appears to be acting via activation of PKC, since down-regulation of PKC significantly reduces the protection afforded by K^+. However, the exact mechanism involved in the protective effect of PKC activation remains to be defined. Protein kinase C can block Ca^{2+} influx [4] and can also inhibit mobilization of intracellular Ca^{2+} [15]. Ca^{2+} is required for endonuclease and protease activity [2] and inhibition of Ca^{2+} influx can block glutamate neurotoxicity in the HT-22 cells (Fig. 1D). Thus, PKC may prevent the activation of nucleases and/or proteases required for cell death. The regulation of the activity of these enzymes by PKC is an area requiring investigation.

Alternatively or simultaneously, PKC may act on enzymes involved in free radical production. A number of enzymes active in neuronal cells, such as monoamine oxidase, tyrosine hydroxylase and L-amino oxidase, produce H_2O_2 [7]. Although the H_2O_2 produced by these reactions is not normally toxic to the neuronal cells, under conditions of oxidative stress it may become so. Thus, the inhibition of such an enzyme could alleviate the oxidative stress brought about by the glutamate-induced reduction in glutathione levels. Regardless of the site of PKC action, these results suggest how growth factors may affect glutamate toxicity. A number of growth factors, including EGF, NGF and bFGF, reduce or enhance glutamate toxicity in neuronal cell lines, primary cultures and brain slices (e.g. [10,18]). All of these growth factors activate PKC in some types of cells.

Our data on the role of PKC in glutamate toxicity are in contrast to those obtained with primary cultures of rat cerebellar neurons [5,6] where down regulation or inhibition of PKC protected the cells from glutamate toxicity. However, the glutamate toxicity observed in these earlier studies appears to be mediated via the receptor-linked pathway rather than the oxidative pathway, as in our studies. A further difference is reflected in our use of homogenous populations of neuronal cells rather than primary cultures containing a mixture of cell populations [5,6].

In summary, we show that PKC is a major regulator of glutamate neurotoxicity in the HT-22 hippocampal cell line. The involvement of PKC in the cytotoxic process may explain the modulatory actions of growth factors on glutamate toxicity.

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