Activation of protein kinase C-δ attenuates kainate-induced cell death of cortical neurons

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Received 28 January 2005; accepted 4 March 2005

We investigated the role of individual protein kinase C (PKC) isoforms during kainate toxicity in cortical neurons. Treatment with 50 μM kainate induced isoform-specific activation of PKC-δ according to the translocation from the soluble to the particulate fraction, while it caused remarkable decreases in PKC α, β, ε and ζ in both fractions. Kainate-induced neuronal death was significantly increased by pharmacological inhibition of PKC-δ with rottlerin, suggesting a protective role of PKC-δ against kainate toxicity. A PKC activator phorbol 12-myristate 13-acetate remarkably attenuated the kainate-induced neuronal death. Although phorbol 12-myristate 13-acetate activates PKC-ε and PKC-δ, the protective effect of phorbol 12-myristate 13-acetate was almost completely abolished by rottlerin, but not by εVI-2. These results suggest that activation of PKC-δ attenuates the kainate-induced cell death of cortical neurons. NeuroReport 16:741–744 © 2005 Lippincott Williams & Wilkins.

Key words: Cortical neurons; Kainate; Protein kinase C-δ

INTRODUCTION

Neuronal cell death occurs in various brain diseases, including stroke [1] and other neurodegenerative diseases [2]. During brain injury, toxic levels of glutamate are known to accumulate, which over-activate glutamate receptors and trigger neuronal death known as excitotoxicity. Kainate, a glutamate receptor agonist, has been reported to induce neuronal death (NMDA) (100%).

Members of the protein kinase C (PKC) family have been implicated in the regulation of various neuronal functions [6]. Substantial evidence has accumulated for the role of PKC in neuronal degeneration resulting from cerebral ischemia and/or excitotoxicity [7,8]. Although various isoforms of PKCs, including α, β, δ, ε and ζ, have been investigated to define their actions in the brain, their roles in neurotoxicity, and specifically in kainate toxicity, have not been fully characterized. The present study was designed to define the roles of PKC isoforms during kainate-induced cell death in primary cultured mouse cortical neurons. We investigated whether PKC isoforms are modulated by kainate, and if so, what the roles of PKC isoforms are during kainate toxicity.

MATERIALS AND METHODS

Mouse cortical neuron culture: Primary mouse cortical neurons were cultured, as previously described [9]. Fetal mouse brains were isolated and cortices were dissected. The cells from approximately 4.5 hemispheres were grown in 10 ml of culture media, consisting of Eagle’s minimum essential medium (MEM) (Earle’s salts, Gibco-BRL, Gaithersberg, Maryland, USA) supplemented with 21 mM glucose, 5% fetal bovine serum, 5% horse serum and 2 mM glutamine in 5% CO2 at 37°C. 10 μM cytosine arabinofuranoside (AraC) was added to the cultures at 7–9 days in vitro (DIV 7–9) to halt overgrowth of glial cells.

Assessment of cell death: Cell death was quantified by measuring levels of lactate dehydrogenase (LDH) released into bathing media, as previously described [10]. The values were scaled to the mean LDH value released after continuous exposure to 500 μM N-methyl-D-aspartic acid (NMDA) (100%).

Western blotting of protein kinase C: Cells were washed in ice-cold phosphate-buffered saline (PBS) and suspended in homogenization buffer [20 mM Tris-HCl, pH 7.4, 2 mM ethylenediamine tetraacetic acid (EDTA), 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 6 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin and 10 μg/ml aprotinin]. After sonication in buffer containing 0.01% Triton X-100 and centrifugation at 100 000 g for 1 h, the supernatant was recovered and was designated as the soluble fraction. The pellet was resuspended in homogenization buffer with 1% Triton X-100 and centrifuged at 10 000 g for 10 min, and the recovered supernatant from this step was designated as the particulate fraction. All steps were carried out at 4°C. The samples were resolved on 8% sodium dodecyl sulfate polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Massachusetts, USA). After incubation with antibodies against various PKC isoforms (Santa Cruz, California, USA), antibody binding was...
detected with an enhanced chemiluminescence kit (INTRON Biotechnology, Sungnam, Korea) and quantitated using a LAS1000 (Fuji Photo Film Co., Tokyo, Japan).

**Chemicals:** G69976 (an inhibitor for PKC-α and PKC-β), PKC-βII inhibitor (PKC-β C2-4, an inhibitor for PKC-β), εV1-2 (myristoylated PKC-εV1-2, an inhibitor for PKC-ε), ε inhibitor (myristoylated PKC-ε, a peptide inhibitor for PKC-ε), PMA and NMDA were purchased from Biomol (Plymouth Meeting, Pennsylvania, USA). Rottlerin and cyto- sine-β-arabinofuranoside (AraC) were purchased from Sigma (St Louis, Missouri, USA). Kainate was purchased from Tocris (Ballwin, Missouri, USA), and l-glutamine was from Bio-Whittaker (Walkersville, Maryland, USA).

**Statistical analysis:** All data were expressed as means± SD. Multiple comparisons were made with a one-way analysis of variance (ANOVA) post-hoc test. Two-group comparisons were made with the two-tailed Student’s t test. A p-value of <0.05 was considered significant.

**RESULTS**

**Expression of protein kinase C isotypes in cortical neurons exposed to kainate:** The expression levels of the PKC-α, β, δ, ε and ζ isotypes after treatment with 50 μM kainate were examined by immunoblotting of the soluble (S) and particulate (P) fractions from cortical neurons (Fig. 1). The expression of PKC-α, ε and ζ decreased in both soluble and particulate fractions with continuous exposure to kainate, while PKC-βII showed a brief initial increase in the particulate fraction (30, 60 min) followed by sequential degradation. In contrast, PKC-δ showed a remarkable translocation from the soluble to the particulate fraction following kainate treatment. The level of PKC-δ in the particulate fraction was increased as early as 15 min after kainate treatment and reached a maximum level at 1 h. This maximal level was maintained for 4 h and was decreased by 8 h following kainate exposure. In the soluble fraction, the level of PKC-δ decreased in a time-dependent manner.

**Effects of protein kinase C inhibitors on kainate-induced neuronal cell death:** To elucidate the roles of PKC isotypes in the process of kainate-induced neuronal death, we examined the effects of specific inhibitors for PKC isotypes on LDH release 24 h after treatment with kainate. Treatment with 50 μM kainate alone caused LDH release greater than 55% and cointreatment with rottlerin (3 μM) resulted in an increase in the LDH release of up to 91 ±3.7% (Fig. 2a). None of the other PKC inhibitors tested – G69976 (for PKC-α, PKC-βII), PKC-βII inhibitor, εV1-2 (for PKC-ε), and PKC-ζ inhibitor – had any significant effect on kainate-induced cell death. Figure 2b shows the time course of LDH release after kainate treatment in the absence or presence of 3 μM rottlerin. Kainate-induced LDH release was increased to greater than 60% by cotreatment with rottlerin 12 h after treatment and to its maximum level by 20 h. This suggests an aggravating role for rottlerin in kainate-induced neuronal death.

**Effects of phorbol 12-myristate 13-acetate on kainate-induced neuronal death and expression of protein kinase C:** To further investigate the role of PKCs during kainate-induced neuronal death, we examined the influence of PKC activation on kainate-treated neuronal cells using the general PKC activator PMA, because no isotype-specific PKC activator is available. Preliminary experiments were conducted to establish treatment intervals and concentrations of PMA (data not shown). After treatment with 0.1 μM PMA for different times (15, 30 min and 1 h), followed by a 3-h washout, cortical cells were exposed to 50 μM kainate for 24 h. As shown in Fig. 3a, kainate-induced LDH release was significantly decreased by pretreatment with 0.1 μM PMA, nearly to the control level. Treatment of cortical cells with PMA for 60 min resulted in a significant translocation of PKC-δ and PKC-ε, but not PKC-α and PKC-βII, from the soluble to the particulate fraction, indicating that PKC-δ and PKC-ε were activated by PMA under our conditions (Fig. 3b).

**Roles of protein kinase C-δ and ε in the protective effect of phorbol 12-myristate 13-acetate:** Because both PKC-δ and PKC-ε appeared to be activated by PMA, as judged by translocation, we examined the effects of their inhibitors on the protective effect of PMA against kainate-induced neuronal death. As shown in Fig. 3c, the PMA-induced decrease in LDH release was almost completely abolished by 3 μM rottlerin. On the other hand, the PKC-ε specific inhibitor εV1-2 (10 μM) did not affect LDH release (Fig. 3c, lower). These inhibitory activities of rottlerin and εV1-2 on PKC-δ and PKC-ε, respectively, were confirmed by Western blotting (Fig. 3c, upper).
**DISCUSSION**

In this study, we found that kainate induces isoform-specific translocation of PKC-δ from the soluble to the particulate fraction in cortical neurons, conferring protection against kainate-induced cell death. Despite the growing evidence that PKC becomes activated during glutamate toxicity [11,12], this is the first report to examine the modulation and roles of individual PKC isoforms during the exposure of cultured cortical neurons to the glutamate analog kainate. Our results show that kainate leads to translocation of PKC-δ from the soluble to the particulate...
fraction as early as 15 min following kainate exposure, with a maximum protein level in the particulate fraction achieved by 1 h (Fig. 1). The protein expression of the PKC-α, βII and ε remained unaltered until 1 h after kainate exposure, after which a sequential decrease in response to kainate was observed in both the soluble and particulate fractions. In contrast, the protein level of PKC-ζ, in the particulate fraction began to decrease as early as 30 min. Assuming that translocation to the membrane is essential for PKC activation, our results suggest that kainate induces the activation of PKC-ζ. A similar effect of kainate in inducing the translocation of PKC-ζ from the cytosol to the membrane was previously reported in the rat hippocampus [13]. Recently, the upregulation of PKC-ζ messenger RNA (mRNA) and protein has been demonstrated in the rat cortex and hippocampus after systemic administration of kainic acid [14], suggesting a modulating ability of kainate at the mRNA level of PKC-ζ. Because the present study did not include mRNA study, it remains to be further investigated whether kainate can modulate PKC-ζ at the level of mRNA in cultured cortical neurons. On the other hand, a previous report indicated that mRNA and protein expression of PKC-ζ other than PKC-ζ are selectively upregulated in granule cells after kainate-induced seizures in rats [15]. This apparent discrepancy in results may be attributable to differences between tissues and cell types.

Controversy exists about the role of PKC isoforms in neuronal [16] and non-neuronal cell death [17]. We have previously reported contrasting data about the role of PKC-ζ, showing detrimental and beneficial roles in oxidative stress-induced neuronal death [9] and hypoxia-induced cardiac cell death [18], respectively. As regards PKC-δ, we previously demonstrated its protective role during hypoxic injury in heart-derived H9c2 cells [19], while others have reported a proapoptotic role of PKC-δ in both nonneuronal [20] and neuronal cells [21]. A recent report implicates PKC-ζ [22] in NMDA-induced neuronal cell death. In this study, we investigated the role of individual PKC isoforms in the process of kainate-induced neuronal death by examining the effects of pharmacological inhibitors for PKC-α, β, δ, ε, ζ and η on kainate-induced cell death. Our results showed that the PKC-δ inhibitor rottlerin significantly increased neuronal death induced by kainate, while none of the inhibitors for PKC-α, βII, ε and ζ had any effect on kainate-induced cell death (Fig. 2). Taken together with our data for the kainate-induced isofrom-specific translocation of PKC-δ, we suggest that the activation of PKC-δ is specifically triggered by a compensatory mechanism during kainate toxicity and that it plays a preventive role against kainate-induced cell death. The protective role of PKC-δ was further confirmed by the findings that PMA, which activates both PKC-ζ and PKC-ε in cultured cortical neurons (Fig. 3b), prevented kainate-induced neuronal cell death and that this protective effect was completely abolished by rottlerin but not by εVI-2 (Fig. 3c).

In conclusion, our study implicates PKC-δ as a modulator of kainate-induced neuronal cell death and suggests that activation of PKC-δ can enhance neuronal survival during kainate toxicity. Further investigation is warranted to determine whether PKC-δ plays a protective role in an in vivo kainate-seizure model.

REFERENCES


