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Early and delayed glutamate effects in rat primary cortical neurons Changes in the subcellular distribution of protein kinase C isoforms and in intracellular calcium concentration

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Abstract

Glutamate-induced changes in the subcellular distribution of protein kinase C isoforms and in the intracellular calcium concentration were investigated in rat primary cortical neurons. Western blot analysis of protein kinase C isoforms (α, β_1, β_2, γ, δ, ε, ζ and θ), performed 30 min after a 10 min treatment with 30 μM glutamate, revealed a decrease in the total β_1 (−24%) and β_2 (−40%) isoform levels, without any significant change in any of the other isozymes. All conventional isoforms translocated to the membrane compartment, while δ, ε, ζ and θ maintained their initial subcellular distribution. Twenty-four hours after glutamate treatment, the total protein kinase C labelling had increased, particularly the ε isoform, which accounted for 34% of the total densitometric signal. At this time, protein kinase C β_1, δ, ε and ζ isoforms were mainly detected in the membrane compartment, while γ and θ signals were displayed almost solely in the cytosol. Basal intracellular calcium concentration (FURA 2 assay) was concentration-dependently increased (maximum effect +77%) 30 min, but not 24 h after a 10 min glutamate (10–100 μM) treatment, while the net increase induced by electrical stimulation (10 Hz, 10 s) was consistently reduced (maximum effect –64%). The *N*-methyl-D-aspartate receptor antagonist, MK-801, 1 μM, prevented glutamate action both 30 min and 24 h after treatment, while non-selective protein kinase C inhibitors, ineffective at 30 min, potentiated it at 24 h. These findings show that protein kinase C isoforms are differently activated and involved in the early and delayed glutamate actions, and that the prevailing effect of their activation is neuroprotective.

Keywords: Neurotoxicity; Neuron; Cell culture; Calcium; Protein kinase C isozymes

1. Introduction

A massive release of excitatory amino acids occurs during cerebral ischemia (Choi, 1990; Phillis and O'Regan, 2003); thus, glutamate (Glu) treatment of cultured rat neurons has been widely used to study the initial cellular events triggering the delayed neuronal death induced by an ischemic insult (Budd, 1998; Chakravarthy et al., 1998). Overstimulation of Glu receptors induces the intracellular accumulation of several ions and second messenger molecules (Choi, 1990; Lee et al., 2000), among which Ca^{2+} ions have been shown to play a critical role in ischemic cell death (Choi, 1995; Kristian and Siesjo, 1998). The increase in cytosolic Ca^{2+} concentration, largely mediated by *N*-methyl-D-aspartate (NMDA) receptors (Lipton, 1999; Hardingham and Bading, 2003), activates intracellular cascades leading to excitotoxicity (Lee et al., 2000; Orrenius

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et al., 2003). Protein kinases, most importantly protein kinases C (PKC), are critically involved in alterations detected in the postischemic brain (Cheung et al., 2001; Zablocka et al., 2001) — a rapid increase in PKC activity followed by a dramatic loss in enzyme activity is characteristic of cerebral ischemia in a variety of in vivo and in vitro models (Cardell and Wieloch, 1993; Selvatici et al., 2003). Structural, biophysical, kinetic and molecular biological studies over the past few years have detailed the mechanisms determining the specificity in PKC activation (Newton and Johnson, 1998). The members of the PKC family identified to date share an amino-terminal membrane-targeting (regulatory) moiety linked to a carboxy-terminal kinase (catalytic) domain. They have been subdivided into three classes based on their cofactor-dependence: (i) conventional PKCs (cPKCs) – α, β_1, β_2 and γ – regulated by Ca^{2+}, diacylglycerol (DAG) and phosphatidylserine (PS); (ii) novel PKCs (nPKCs) – δ, ε, θ and η – regulated by DAG and PS; and (iii) atypical PKCs (aPKCs) – ζ, ι and λ – regulated by PS. The diversity in PKC isozyme structural and enzymological properties, together with their differential tissue expression, suggests that they play distinct functions in the processing and modulation of various physiological processes (Dekker and Parker, 1997; Way et al., 2000).

A large body of evidence supports a link between Glu, intracellular Ca^{2+} levels, PKC activation and neuronal death, although the sequence of events and the involvement of the individual PKC isoforms have yet to be fully clarified (Hardingham and Bading, 2003; Uemura et al., 2003).

This study was carried out in primary cultures of rat cortical neurons exposed to a brief excitotoxic insult. Changes in subcellular distribution of PKC isoforms and in intracellular Ca^{2+} levels were investigated to compare the early (30 min after) and late (24 h after) intracellular events, also in view of a possible PKC involvement in the mechanisms of endogenous neuroprotection and ischemic tolerance (Kirino, 2002).

2. Experimental procedures

2.1. Neuronal cell cultures and glutamate exposure

The experiments were carried out in accordance with protocols approved by the Ethic Committee of the University of Ferrara. Cortical neuronal cultures were prepared from 1-day-old Sprague Dawley rats, as described by Alho et al. (1988). Neurons (1.5×10^6) were plated in 35 mm culture dishes (NUNC) or in 24-well culture vessels (Falcon) coated with 20 μg/ml poly-L-lysine. For calcium measurements, glass coverslips (28 mm× 12 mm) were laid on the bottom of the dishes. Cells were cultured in modified neurobasal medium (2 ml/dish, 1 ml/well), supplemented with gentamycin sulfate 50 mg/ml, B27 2% and L-alanyl-L-glutamine 500 μM. Cultures were kept at 37 °C in a humidified incubator gassed with 5% CO_2 and air. After 24–48 h, 5 μM cytosin arabinoside (ARA-C) was added to prevent glial cell replication.

At the 7th–9th day in vitro, one half of the medium was withdrawn and set aside and neurotoxic insult was induced by adding 10–100 μM glutamate (Glu) for 10 min; then the cells were rinsed with fresh medium and returned to the incubation medium set aside. Drug treatments (PKC inhibitors and Glu receptor antagonists) were added during Glu treatment and maintained throughout the entire experiment. In experiments with the peptide εV1-2, cells were permeabilized by saponin 10 μg/ml (Di Capua et al., 2003).

The cells cultured on dishes were processed either for PKC isoform Western blotting or for calcium concentration measurement. The cells cultured on the multiwell system were processed for viability.

2.2. Western blot analysis

Either 30 min or 24 h after the neurotoxic insult, neuronal cells cultures (three dishes for each sample) were lysed using ice-cold lysis buffer containing: 5 mM Hepes, 320 mM sucrose, 5 mM glycerolphosphate, 5 mM KF, 2 mM 2-mercaptoethanol (2-ME), 3 mM EGTA, 0.5 mM $MgSO_4$, 2 mM phenyl-methyl-sulphonylfluoride (PMSF), 0.01% leupeptin and 10 μg/ml aprotinin. Cells were centrifuged at 14,000 rpm for 5 min to separate nuclei and unbroken cells (pellet), which were discarded. The supernatant was recovered in a separate tube, sonicated six times with 10 s bursts and ultracentrifuged at 100,000×g for 1 h at 4 °C to separate the cytosolic fraction (supernatant) from the membrane fraction (pellet). The latter was resuspended in the same buffer described above, to which 0.1% Triton X-100 was added and then re-sonicated (two pulses).

Protein contents of both cytosol and membrane fractions were determined by Bradford (1976) method.

Samples containing equal amounts of proteins (20 μg) were diluted with one-third of the loading buffer (187.5 mM Tris–HCl, pH 6.8, 15% 2-ME, 0.1% sodium dodecylsulfate, 30% glycerol, 0.003% bromophenol blue), subjected to gel electrophoresis on a 10% gel and then transferred to PVDF membranes (Bio-Rad, 0.2 μm) by electroblotting. Membranes were immersed overnight in a Tris buffered saline solution (TBS: 20 mM Tris and 137 mM NaCl) pH 7.6 containing 5% Blotting grade blotter (Bio-Rad) at 4 °C, washed three times with TBS plus 0.1% Tween 20 (TBS–T) and incubated for 2 h at room temperature with rabbit polyclonal antibodies against α, β_1, β_2, γ, δ, ε, ζ and θ PKC isoforms (Santa Cruz), used at dilution 0.3 μg/ml in TBS–T buffer. Following washes with TBS–T buffer, a 1:6000 dilution of horseradish peroxidase-labelled anti-rabbit IgG was added at room temperature for 1 h.

ECL Western blotting detection reagents (Amersham) were used to visualize specific hybridization signals. Densitometric analysis of autoradiographic bands was