Role of Protein Kinase C in Ca^{2+} Homeostasis Disorders in Cultured Rat Neurons during Hyperstimulation of Glutamate Receptors

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The primary culture of rat cerebellar neurons was used to study protein kinase C activity, intracellular variations in calcium concentration ($[Ca^{2+}]_i$), changes in the mitochondrial potential, and neuronal death during hyperstimulation of glutamate receptors and after 24-h incubation with phorbol ester. Prolonged exposure of neurons to glutamate (100 μM, 45 min) was followed by the development of delayed calcium dysregulation. Protein kinase C activity depended on the time of cell incubation with glutamate. Protein kinase C activity increased in response to application of glutamate for 15 min. However, protein kinase C activity decreased after 45-min exposure to glutamate and development of delayed calcium dysregulation. Protein kinase C activity was nearly undetected after 24-h preincubation of neurons with phorbol ester. Under these conditions, delayed calcium dysregulation developed more slowly and was observed in a smaller number of neurons. Neuronal death decreased to 2±1%. Our results suggest that protein kinase C plays an important role in death of neurons, which exhibit delayed calcium dysregulation during glutamate treatment.

Key Words: *protein kinase C; calcium; glutamate; neurons; phorbol ester*

Hyperstimulation of glutamate receptors is observed during cerebral ischemia/hypoxia or neurodegenerative diseases and results in alterations in intracellular Ca^{2+} homeostasis, mitochondrial dysfunction, increase in intracellular Na^+ concentration, and decrease in ATP content [2,7,11,12,14]. Prolonged exposure of cultured neurons from the cerebellum, cortex, and hippocampus to glutamate (Glu) is followed by a biphasic increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). It should be emphasized that phase 2 is accompanied by a significant simultaneous decrease in the mitochondrial potential (delayed calcium dysregulation, DCD) [2,7,11,12]. When $[Ca^{2+}]_i$ and mitochondrial potential do not return to the basal level after Glu withdrawal, the majority of neurons die due to cascade metabolic reactions [4,11]. An increase in $[Ca^{2+}]_i$ is followed by variations in activity of Ca^{2+}-dependent proteases, protein kinases, and protein phosphatases [10]. The role of protein kinase C (PKC) in neuronal damage during hyperstimulation of Glu receptors remains unknown. Some authors showed that decrease in activity and content of PKC in neurons protects these cells from Glu neurotoxicity [6]. Other authors reported that Glu neurotoxicity and neuronal death are associated with reduced activity of PKC during Glu treatment. Hence, prevention of PKC inactivation is necessary for cell protection [5].

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In our experiments, the culture of rat cerebellar neurons was used to study PKC activity after long-term treatment with Glu and development of DCD. The effect of PKC down-regulation on DCD and neuronal survival under hyperstimulation of Glu receptors was evaluated during 24-h preincubation with a PKC activator phorbol ester (phorbol 12-myristate 13-acetate, PMA).

MATERIALS AND METHODS

Experiments were performed on 7-9-day-old primary cultures of cerebellar granular cells from Wistar rats. The cell suspension was prepared as described elsewhere [2]. The cells were resuspended in neurobasal medium (NBM) with B-27, GlutaMax, penicillin/streptomycin, and 20 mM KCl. The number of neurons was 3.5×10^6 cells/ml. The cells (100 μl) were put on sterile cover slips coated with poly-D-lysine (10 μg/ml). NBM (1 ml) was added to Petri dishes after 1 h. Coverslips with cells were maintained in NBM medium using in incubator at 36°C and 5% CO_2 for 7-9 days. Cytosine arabinoside was added to the incubation medium on days 3-4 of culturing to inhibit differentiation of glial cells.

Neuronal survival was estimated 24 h after treatment. Morphological study was performed with fluorescent dyes Hoechst 33342 (1 mg/ml, 30 min at 37°C) and ethidium homodimer (10 mg/ml, 5 min) and included the evaluation of chromatin condensation and nuclear fragmentation (Hoechst 33342). The cells were washed with buffer, fixed with 4% formalin, and examined under a fluorescence microscope. Each experiment included examination of 10-15 independent optical fields (500 cells). The percentage of dead cells under control conditions did not exceed 7±1%.

The cells were loaded with Fura-2FF/AM (4 μM, 60 min) and Rhodamine 123 (3 μM, 15 min) to measure $[Ca^{2+}]_i$ and mitochondrial potential, respectively. A coverslip with cells was put in a dismountable perfusion chamber. The chamber was mounted on an objective table of an Axiovert 200 microscope (Zeiss). The neurons were washed 2-3 times with a buffer containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, and 20 mM HEPES (pH 7.4). All experiments were performed at room temperature. Fluorescence excitation was induced by light at 340 or 380 nm (Fura-2FF) and 485 nm (Rhodamine 123). Fluorescence emission was observed through a filter at 505-535 nm. The image was obtained using a CCD camera (Roper Scientific). The data were collected and analyzed by Metafluor 6.1. software (Universal Imaging Corp.). $[Ca^{2+}]_i$ variations were presented as the F_{340}/F_{380} ratio (F, fluorescence intensity). Changes in the mitochondrial potential were expressed in percents of baseline fluorescence (100%).

A special series was performed to study the survival of neurons with DCD. The cells were loaded with Fura-2FF/AM. Glu-induced variations in $[Ca^{2+}]_i$ were recorded for 45 min. The cells were stained with Syto-13 (1 μM, 5 min) and ethidium homodimer after 4 h to isolate living and dead cells.

Activity of membrane PKC in cell lysates was estimated using a PepTag®-analysis kit (Promega). Cell lysates were obtained using a buffer containing 1% Triton X-100. A mixture of reagents (buffer, fluorescent peptide C1 with the homologous sequence of PKC substrates, PKC activator, and protease inhibitors) was added to aliquots of lysates. After 30-min incubation at 37°C, the reaction of peptide C1 phosphorylation was stopped by kinase thermoinactivation on a water bath at 98°C for 10 min. Phosphorylated and nonphosphorylated peptides were identified. The samples were studied electrophoretically in 0.8% agarose gel (100 mA). The gel image was obtained on a DP-001.FDC device (Vilger Lourmat) at an excitation wavelength of 380 nm (UV light). The images were analyzed by Scan Array Express software (Perkin Elmer) to evaluate the degree of phosphorylation.

The results were analyzed by nonparametric test (ANOVA, PRIZM software). The data are expressed as *M±SEM*. Fluorescent probes were manufactured by Mol. Probes. Other reagents were from Sigma.

RESULTS

PKC activity increased by more than 5 times after 15-min exposure to Glu (magnesium-free buffer with 10 μM glycine, Fig. 1). PKC activity decreased 1-4 h after Glu treatment, but remained above the control level. The increase in PKC activity is associated with kinase translocation from the cytosol to the plasma membrane [6,9,10]. Phosphorylation of the NMDA receptor contributes to Ca^{2+} entry into the cytosol [8,10]. Our findings are consistent with published data [3,6,9]. However, the observed results are inconsistent with other data [5]. These differences are probably related to features of neuronal cultures. Experiments of J. Durkin *et al.* were performed on cortical neurons. The sensitivity of these neurons to Glu depended on the age of cultures. PKC activity remained unchanged after exposure of 7-9-day-old cultures to Glu. However, PKC activity in older neurons (14-18 days) decreased 4 h after 15-min exposure to Glu (as compared to the control).

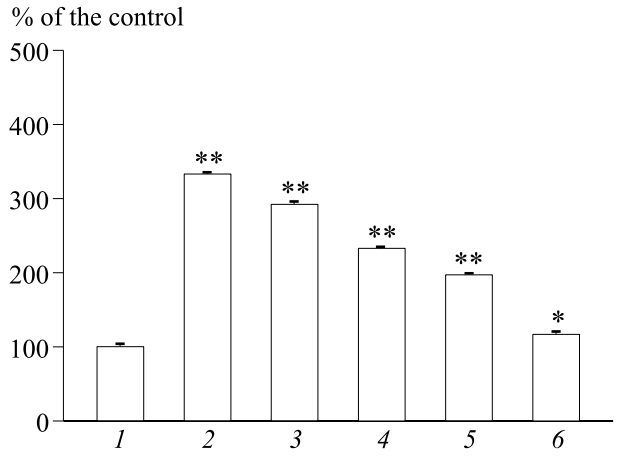

Fig. 1. PKC activity in cerebellar neurons after 15-min exposure to 100 μM Glu. Control (*1*); Glu, 15 min (*2*); Glu (15 min)+1 h in buffer (*3*); Glu (15 min)+2 h in buffer (*4*); Glu (15 min)+3 h in buffer (*5*); and Glu (15 min)+4 h after washing from Glu (*6*). **p*<0.05 and ***p*<0.01 compared to the control. Three independent experiments.

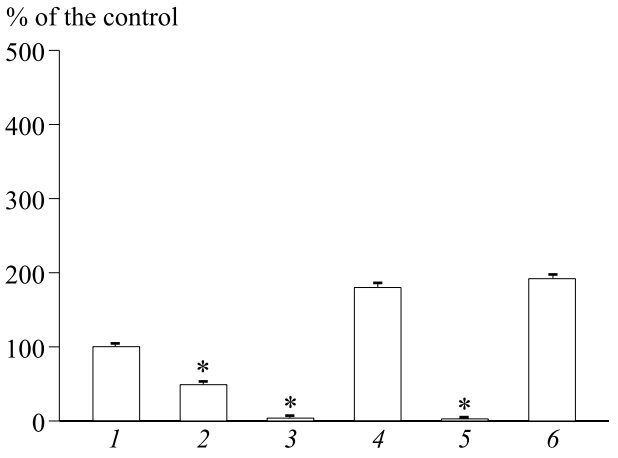

Fig. 2. PKC activity in cerebellar neurons. Control (*1*); Glu, 100 μM, 45 min (*2*); PMA, 500 nM, 24 h (*3*); PMA, 500 nM, 45 min (*4*); PMA (500 nM, 24 h)+Glu (45 min, *5*); and PMA (500 nM)+Glu (45 min, *6*). **p*<0.05 compared to the control. Three independent experiments.

PKC activity decreased to 49±5% after long-term treatment with Glu (45 min, $p<0.05$). Many neurons exhibited DCD (Fig. 2). The observed decrease in PKC activity is probably related to reverse translocation of PKC from the plasma membrane to the cytosol [13].

PKC was not detected after 24-h preincubation of neurons with PMA (Fig. 2). This sharp decrease in PKC activity after prolonged exposure to PMA was described as PKC down-regulation [6]. However, 45-min exposure to PMA (500 nM) was followed by an increase in PKC activity, which remained high after combined treatment with PMA and Glu.

Our previous studies and published data show that biphasic increase in $[Ca^{2+}]_i$ after prolonged exposure to glutamate is accompanied by a significant simultaneous decrease in the mitochondrial potential ($\Delta\Psi_m$) [2,7,11,12]. Similar results were obtained in the present study. Prolonged exposure to Glu (45 min) was followed by similar changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ (Fig. 3). DCD was observed in 83±7% neurons (n=975). The mean latency of DCD was 668±75 sec. $[Ca^{2+}]_i$ and $\Delta\Psi_m$ in the majority of phase 2 neurons did not return to normal after removal of Glu from the medium.

The next series was performed on neurons with PKC down-regulation. Glu treatment was also followed by DCD. However, DCD developed more slowly and was observed in a smaller number of neurons (Fig. 3). For example, the mean latency of DCD under conditions of PKC down-regulation was much higher compared to the control (947±157 and 668±75 sec, respectively, $p<0.05$). We revealed a 1.5-fold decrease in the ratio of cells with phase 2 of $[Ca^{2+}]_i$ elevation (57±9% *vs.* 83±7% in the control, $p<0.05$, n=945). The presence of PMA in the incubation medium during Glu treatment (45 min, Fig. 3) significantly increased $[Ca^{2+}]_i$ and accelerated the development of DCD (570±51 sec, $p<0.05$). The number of neurons with DCD was similar to the control (88±7%, n=1005). These variations in $[Ca^{2+}]_i$ are related to a greater entry of Ca^{2+} through NMDA channels under the influence of PMA and Glu [8,10].

PKC down-regulation was followed by a significant decrease in neuronal death (2±1% *vs.* 28±4% in the control). Combined treatment with PMA and

TABLE 1. Effect of PKC Down-Regulation on Delayed Ca^{2+} Dysregulation and Neuronal Death 4 h after 45-min Exposure to Glutamate

Treatment	Total number of neurons	Cells with DCD, %	Dead neurons, %
Control	290	0	3±1
Glu, 100 μM	871	60±4	43±4
PMA (500 nM, 24 h) and Glu (100 μM)	707	23±3*	2±1*

Note. Three independent experiments. **p*<0.05 compared to Glu.

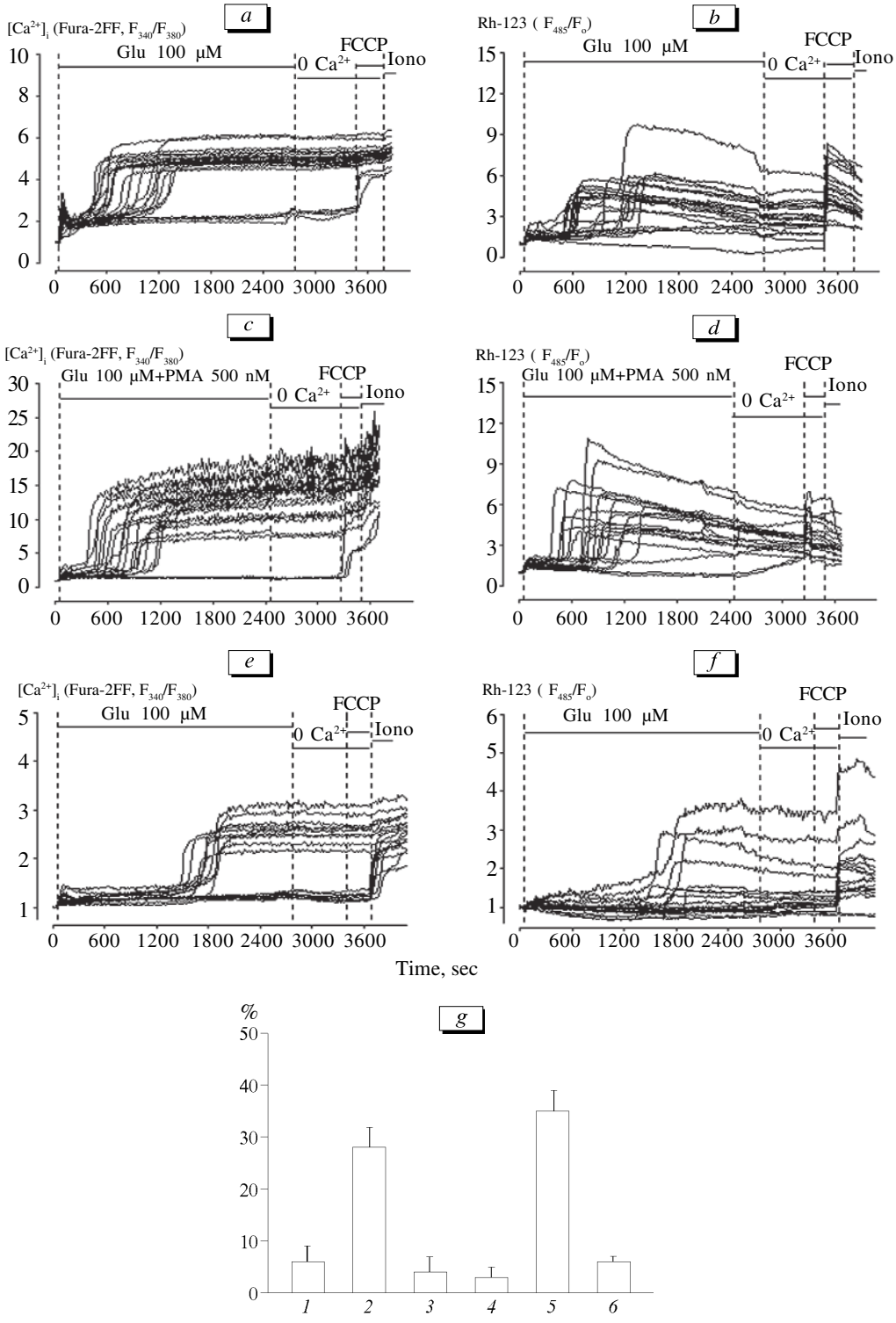

Fig. 3. $[Ca^{2+}]_i$ and mitochondrial potential of cerebellar neurons after prolonged exposure to Glu (100 μM) in a magnesium-free buffer with 10 μM glycine. (*a*, *b*) Control experiments (975 neurons); (*c*, *d*) effect of 500 nM PMA on Glu-induced variations in $[Ca^{2+}]_i$ and mitochondrial potential (1005 neurons); and (*e*, *f*) effect of PKC down-regulation on $[Ca^{2+}]_i$ and mitochondrial potential (945 neurons). (*a*, *c*, *e*) Change in the F_{340}/F_{380} ratio reflecting $[Ca^{2+}]_i$ variations; and (*b*, *d*, *f*) fluorescence of Rhodamine 123 (Rh-123), F_{490}. 0 Ca^{2+}, substitution of calcium in the outer solution for 100 μM EGTA. Maximum fluorescence of Fura-2FF and Rh-123 under the influence of 1 μM ionomycin and 1 μM FCCP, respectively. The figure illustrates similar results of a typical experiment with 5 cell preparations. (*g*) Effect of PMA on neuronal death 24 h after 45-min exposure to Glu: control (*1*); Glu, 100 μM, 45 min (*2*); PMA, 500 nM, 45 min (*3*); PMA, 500 nM, 24 h (*4*); PMA (500 nM)+Glu (45 min, *5*); and PMA (500 nM, 24 h)+Glu (45 min, *6*). Five independent experiments.

Glu for 45 min had no effect on this parameter (no differences from the Glu group, Fig. 3).

Incubation with Glu was followed by DCD in 60±4% neurons. The majority of these neurons died (Table 1). After PKC down-regulation, DCD was observed in a smaller number of neurons (2% dead cells).

Our results indicate that PKC play a role in Glu-induced death of neurons with DCD. DCD and $[Ca^{2+}]_i$ plateau are probably related to the increased Ca^{2+} release from mitochondria through an open mitochondrial pore and impairment of Ca^{2+} elimination from neurons by the plasma membrane Ca^{2+} pump [7,12]. Our experiments and published data show that the toxic effect of Glu is accompanied by the entry of Ca^{2+} and considerable amounts of Na^+ through NMDA-dependent channels [7,14]. The latter leads to activation of Na/K-ATPase due to phosphorylation of PKC [1]. Na/K-ATPase activation is associated with strong consumption of ATP. The appearance of a stable postglutamate $[Ca^{2+}]_i$ plateau during DCD is related to the decrease in ATP content under strong consumption by Na/K-ATPase. Hence, Ca^{2+} release by the plasma membrane Ca/H pump is impaired under these conditions. Previous studies revealed that prolonged exposure to Glu is followed by a decrease in ATP content in cerebellar granular cells [2,7,11,12]. It can be hypothesized that activation of Na/K-ATPase is not observed during PKC down-regulation. Greater amounts of ATP for the plasma membrane Ca/H pump contribute to a decrease in Glu neurotoxicity. A Na/K-ATPase inhibitor ouabain has the same effect [2,7].

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