ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1998, 58: 247-252

Phosphorylation of Protein Kinase C substrate proteins in rat hippocampal slices - effect of calpain inhibition

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Abstract. Incubation of the acutely dissected rat hippocampal slices in calcium-containing media resulted in spontaneous activation-translocation of classical PKC isoforms and their subsequent (especially γ- type) proteolytic degradation. These changes were blocked by calpain inhibitor MDL 28 170 in 100 μM concentration. Rat hippocampal slices were metabolically prelabelled with ^{32} Pi and stimulated with NMDA/glycine, depolarization or phorbol dibutyrate (PDBu) treatment. The basal phosphorylation of specific PKC substrates (MARCKS, neuromodulin and neurogranin) was significantly reduced in non-stimulated slices by MDL pretreatment. In contrast, only the slices where calpain activity was inhibited responded to further NMDA or phorbol dibutyrate stimulation by a substantial increase of PKC-dependent protein phosphorylation. It is concluded that the PKC phosphorylation system is severely affected by non-specific activation and a subsequent, calpain-dependent proteolysis in the acutely prepared hippocampal slices. Calpain inhibition by 100 μM MDL partially prevented these changes and increased stimulus-dependent phosphorylation of PKC-specific protein substrates.

Key words: hippocampal slices, PKC, protein phosphorylation, calpain

INTRODUCTION

Current models of brain ischemic pathology implicate a disruption of calcium homeostasis leading to sustained increase of intracellular calcium (Siesjö and Bengtsson 1989) and activation of various related kinase and protease systems. The calcium-dependent protein kinase C (cPKC) is firstly stimulated/translocated (Domańska-Janik and Zabłocka 1993) and subsequently inhibited (Domańska-Janik and Zalewska 1992). In addition to translocation of PKC towards membranes, ischemia induces transient appearance of 50 kDa proteolytic fragment of PKC (Domańska--Janik and Zabłocka 1993), known as PKM which is a product of calpain activation (Kishimoto et al. 1989). Recently we have reevaluated the involvement of calpain-activated proteolysis in modification of PKC isoforms *in vitro* with comparison to that observed under cerebral ischemia *in vivo*. From this study and in contrast to our previous suggestions, it seems that protein kinase C can be a direct target of proteolytic attack by calpains during ischemia, with transient appearance of calcium-independent PKM isoform (Zalewska et al. 1997). However, further controversy regarding the function of PKC in cerebral ischemia may be compounded by the identification of the limited proteolysis of PKC by calpains with enzyme down-regulation and loss of its phosphorylating activity, which are probably dissociated and independent phenomenon, not directly related to calpain activation (Domańska-Janik and Zalewska 1992, Yavin et al. 1995).

In this study we evaluated the effect of membrane--permeable calpain inhibitor MDL 28 170 on the classical isoforms of PKC (α, β and γ) as well as on stimulus-dependent phosphorylation of the typical PKC substrates (MARCKS, neuromodulin and neurogranin) in hippocampal brain slices *in vitro*. The enzyme activity was stimulated directly by phorbol dibutyrate (PDBu), by depolarization or by activation of NMDA receptors, the events being involved in regulation of PKC activity under ischemia (Zabłocka et al. 1995, Domańska-Janik 1996). Assuming that acutely prepared hippocampal slices would be a model of tissue recovering after temporal ischemic stress rather than of that under stable, normal conditions, we directly demonstrated an involvement of calpain in deterioration of stimulus-dependent, PKC-specific phosphorylation system after brain ischemia.

METHODS

Three weeks old Wistar rats were cooled by keeping them on ice under deep pentobarbital anesthesia, to decrease their rectal temperature to 28°C. Then the animals were decapitated and the hippocampi dissected out from brains which were kept on ice all the time. Transverse slices, 400 μm thick, were cut on a vibroslice (Campden Instruments) in ice-cold bath containing PBS + 3 % dextran. Each slice (110-120 μg protein) was placed separately on porous plastic support coated with thin paper (lens clinex) in Eppendorf tube, submerged in 100 μl of medium and incubated at 34°C under continuous carbogen flow. The incubation medium was prepared according to the classical recommendations of Li and McIlwain (1957) but modified by decreased $CaCl_2$ concentration to 0.7 mM and increased $MgSO_4$ to 4.0 mM (Yamamoto and McIlwain 1966). During the first hour of incubation the medium did not contain calcium to preserve better slice integrity as was recommended by Schurr et al. 1995. Labeling was performed at second hour of incubation by changing medium to the fresh, phosphate-free, containing 5 μCi of ^{32}Pi/vessel (Amersham, s.a. 5 mCi/ml) with or without a calpain inhibitor, MDL 28 170 (100 μM in DMSO at 0.1% final concentration) or with a nonspecific PKC inhibitor: H7 (100 μM). Incorporation of ^{32}Pi into proteins extracted from slices was found to be linear during the 1.5 h incubation period. After 1 hour of metabolic labeling with ^{32}Pi, the slices were challenged with the appropriate stimulators for the next 15 min. The following compounds were added to the incubation medium: NMDA/glycine in concentration of 100μM each, 30 mM KCl or 0.5 μM PDBu in DMSO (at final concentration 0.1%).

The specific PKC substrate proteins: MARCKS, neuromodulin (GAP 43 or B50) and neurogranin were extracted from labeled homogenate proteins. They are known from their unusual solubility in 2.5% perchloric acid (PCA) (Baudier et al. 1989, Scholz 1994). Extraction procedure involved homogenization and sonication of individual slices in 0.1 ml of buffer A containing: 25 mM HEPES pH 7.5, 100 mM β-glycerophosphate, 15 mM $Na_4P_2O_7$, 25 mM NaF, 1 mM DTT, 10 mM EDTA. Then, 10 μl of homogenate was taken for PAGE analysis and the remaining part extracted 30 min. at 4°C by addition of 5% PCA (1:1 v/v). The nonsoluble proteins were removed by centrifugation, solubilized with 1M NaOH and determined by the method of Lowry (1951) or counted for radioactivity. Supernatant proteins were pre-

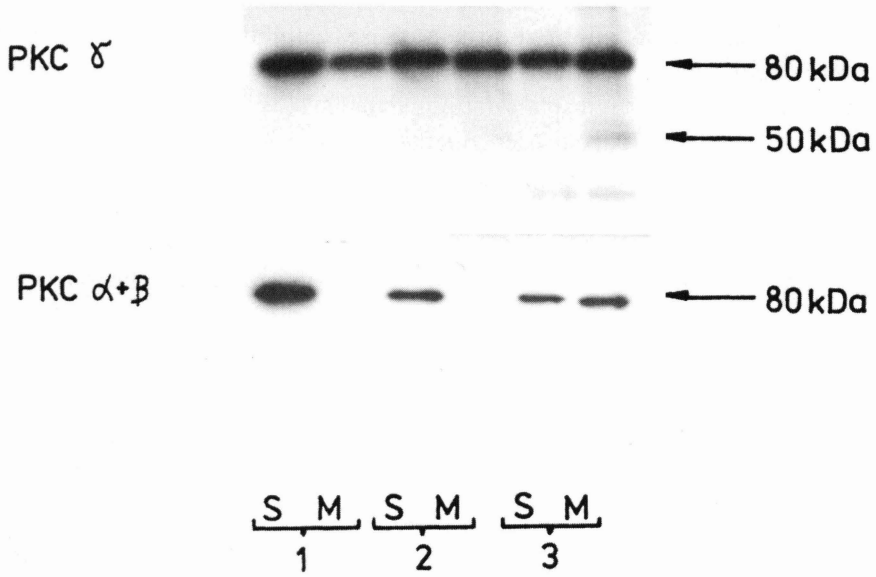

Fig. 1. Immunoblots of cPKC isoforms in soluble (S) and membraneous (M) fractions of rat brain hippocampal slices after incubation in calcium-free (1) and calcium containing (2,3) media with (2) and without (3) of MDL. Notice elevated content of cPKC isoenzymes and appearance of 50 kDa cleavage products in the slices incubated in the presence of calcium but without calpain inhibitor.

cipitated with trichloric acid (TCA) at 12% final concentration, washed twice with ethanol and boiled in sample buffer (250 mM Tris, 3 mM EDTA, 50 mM DTT, 4% SDS, 20% glycerol and bromophenol). The samples, equivalent to 100 μg of homogenate proteins, were resolved on 10% polyacrylamide gels. Radioactive proteins were revealed by autoradiography, identified according to their electrophoretic mobilities and determined by densitometry using a LKB Ultroscan XL densitometer.

Immunoblot analyses of classical isoforms of Protein Kinase C were performed on parallel incubated non--radioactive slices. After centrifugation of homogenate at 16,000 g for 20 min, the supernatant and pellet fractions, in quantities normalized to 20 μg of homogenate protein, were loaded on 10% polyacrylamide gels for SDS- PAGE. The proteins were electroblotted on nitrocellulose and probed with monoclonal anti- PKC antibodies specific for α+β and γ isoforms (Amersham). Immunoreaction was visualized with peroxidase-linked antimouse IgG and developed by enhanced chemiluminescence (ECL) system following the protocol provided by the manufacturer (Amersham, UK)

Proteins were determined using the method of Bradford (1979).

RESULTS

As stated in the Introduction, the purpose of this study was to determine the effect of calpain inhibition on PKC-mediated phosphorylation of the acidic substrates, occurring after stimulation by elevated extracellular potassium, NMDA receptor activation or PDBu treatment. As was reported previously (Domańska-Janik and Zalewska 1992), *in vitro* incubation of brain tissue in calcium containing media induces a profound PKC activity down-regulation as well as its extensive translocation to plasma membranes. In this study we have demonstrated that calpain inhibitor MDL 28170 in concentration of 100 μM can prevent translocation of α+β and γ isoforms

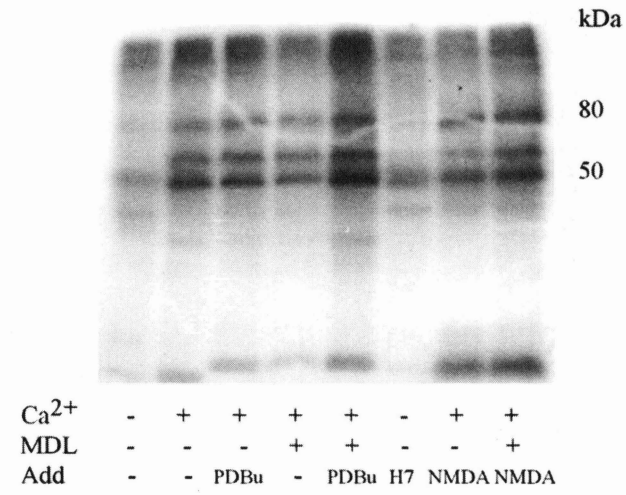

Fig. 2. The autoradiogram of ^{32}P incorporation into homogenate proteins after stimulation of rat brain hippocampal slices *in vitro*. Slices were incubated as described in Methods in media with and without calcium, H7, MDL, NMDA and PDBu.

of PKC as well as the appearance of its 50 kDa proteolytic fragment recognized by anti-γ antibody (Fig. 1) in non-stimulated slices incubated in the presence of calcium.

The phosphorylation of proteins estimated in further experiments relies on metabolically labeled [^{32}P]ATP, synthesized in slices during the second hour of incubation. The labeled phosphoproteins have been resolved on PAGE and autoradiographed (Fig. 2). The extent of phosphorylation of homogenate proteins was found to be dependent on extracellular calcium, substantially reduced by protein kinase C inhibitor- H7 and enhanced by stimulatory phorbol ester - PDBu. These indicate that the phosphorylating system in hippocampal slices is, at least in part, dependent on a classical, calcium-activated C kinase. This conclusion was further supported by the experiments showing that the main endogenous PKC substrates like MARCKS, neurogranin and neuromodulin (Baudier et al. 1989) were indeed phosphorylated

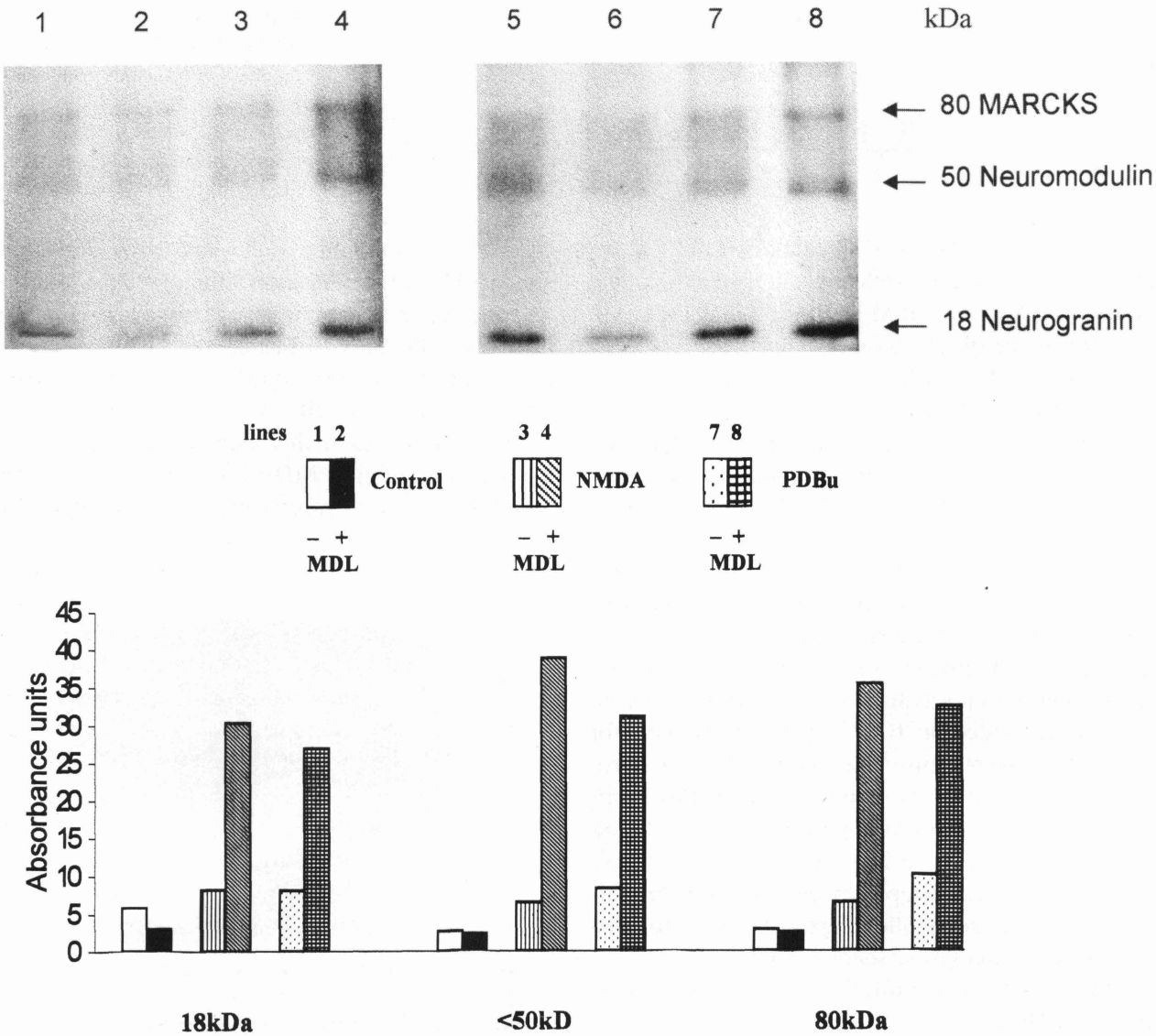

Fig. 3. Representative autoradiogram and densitometric analysis of ^{32}P-labeled acid-soluble PKC substrates in rat brain hippocampal slices stimulated with NMDA (3, 4), elevated K^+ (5, 6) or PDBu (7, 8) in the absence (1,3,5,7) or presence (2,4,6,8) of calpain inhibitor- MDL. The experiments were repeated 3 times with similar results. On densitogram the effect of K^+ stimulation (lines 5 and 6 on the autoradiogram) is not included, because of non-significant differences as comparing with non-stimulated control. The values are expressed in arbitrary units of absorbance.

under these circumstances (Fig. 3). However, when slices were incubated in the presence of calcium, their responses to further stimulation by NMDA or PDBu were negligible. Addition of MDL 28170 during the second hour of incubation had a dual effect on protein ^{32}Pi-labeling. It reduced basal radioactivity of acid-soluble PKC substrates and markedly increased their phosphorylation in response to NMDA and PDBu treatment. In contrast, the phosphorylating response to depolarization by elevated K^+ was still negligible even in the slices incubated in the presence of calpain inhibitor.

DISCUSSION

The results of this study are in line with our previous observations and with those reported by others demonstrating that acutely dissected brain slices are severely compromised by metabolic stress, which could be compared with transient ischemic insult. The ATP level is abnormally low in slices at the beginning but recover well within the first minutes of incubation in the presence of oxygen and glucose (Schurr et al. 1995). In these slices there is also transient elevation of intracellular calcium as well as signs of NMDA receptor activation (Feig and Lipton 1990). These are all factors contributing to substantial cellular damage usually observed in direct, morphological examination. Different protocols of slices preparation have been proposed to avoid these unwanted reactions. In general, several neuroprotective treatments, such as lowering incubation temperature, glutamatergic receptors antagonist application, elevating magnesium and lowering calcium are reported to be effective. In our hands the best results were achieved by using the Vibroslicer and procedure described in Methods section. However, even with these precautions we are obviously dealing with the tissue which differ in some respect from the intact brain. For example, there are studies reporting induction of c-fos, c-jun, and hsp 72 mRNA with accumulation of these gene-related proteins during routine incubation of hippocampal slices (Zhou et al. 1995). This reaction was comparable to that reported after transient ischemia. In our experiments, beside the substantial phosphorylation of homogenate proteins which is directly coupled to ATP synthesized during incubation (Fig. 2), the responses of PKC-connected phosphorylation system were still severely compromised (Fig. 3). For all these reasons acutely dissected and incubated brain slices might be treated as a model of recovery after a transient metabolic (ischemic) stress.

As reported previously for brain homogenate and synaptoneurosomes *in vitro* as well as under complete cerebral ischemia *in vivo* (Domańska-Janik and Zalewska 1992, Yavin et al. 1995), under all these experimental conditions a rapid, calcium-dependent down-regulation of PKC activity was observed. This was not associated with an increase of the Ca^{2+} independent phosphorylation (PKM) and protease inhibitors were not effective in preventing PKC inactivation. However, transient accumulation of the immunoreactive 50 kDa protein as well as the other, discrete, calpain-related cleavage products of PKC were recently found in the *ex vivo* ischemic brain (Domańska-Janik and Zabłocka 1993) as well as in brain homogenate incubated *in vitro* (Zalewska et al. 1997).

The mechanism of PKC down-regulation and inhibition during ischemia is still far from understood. Usually these events are accelerated by previous enzyme translocation into membranes. This study demonstrates that inhibition of calpain by membrane-permeable MDL28 170 is able to prevent spontaneous, stress-induced translocation of cPKC (due to slice preparation) as well as the subsequent accumulation of its proteolytic cleavage products in hippocampal slices. Moreover, the relatively weak responses of the PKC-dependent phosphorylation system to specific stimulators like NMDA or PDBu *in vitro* are also substantially improved after this treatment. Thus, calpain inhibition stabilized transduction of the membrane-generated signal (NMDA) as well as the action of the direct PKC activator (PDBu) on phosphorylation of its specific substrates (MARCKS, neuromodulin and neurogranin) in hippocampal slices. This mode of action, hypothetically, can mirror the neuroprotective effect of calpain inhibitors suggested in brain ischemia (Bartus et al. 1995). The relatively weak response of slices to depolarization observed here corresponds well with the postulated essential role of NMDA receptors in calpain as well as in cPKC activation in hippocampal neurons (Adamec et al. 1998). On the other hand, in this experiment K^+ -induced membrane depolarization, through stimulation of Na^+,K^+-ATP-ase system, can deplete endogenous energy stores and in this indirect way suppress all ATP-dependent processes.

In conclusion, the results allow us to consider activation of calpain as one of the most important triggering factors responsible for destabilization and attenuation of the PKC-connected protein phosphorylation system during routine preparation of hippocampal slices.

ABBREVIATIONS

cPKC - conventional isoforms of Ca^{2+} and phospholipids dependent protein kinase;
PKM - proteolytic form of PKC;
MDL - MDL 28170 Hoechst Marion Roussel Inc.;
NMDA - N-methyl-D-aspartate;
PDBu - β phorbol 12,13-dibutyrate;
H7 - 1-(5-isoquinolinesulfonyl)-2-methyl piperazine;
MARCKS - myristoylated alanine rich C-kinase substrate

ACKNOWLEDGEMENT

This work was partially supported by KBN grant # 4P05A02608

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Received 24 April 1998, accepted 8 September 1998