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Protein serine/threonine kinases (PKA, PKC and CaMKII) involved in ischemic brain pathology

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Abstract. The protein serine/threonine kinases which are highly expressed in the central nervous system (CNS) are severely affected by brain ischemia. Irrespective of substantial differences among the particular members of this group of kinases, their responses to ischemic stress show a lot of similarities. Initially they are switched on by facilitated interaction with their specific activators/second messengers like cyclic AMP, 1,2,-sn-diacylglicerol and particularly Ca^{2+}, then they are translocated to highly specific regions of plasma membranes. After phosphorylation of target proteins, the kinases are deactivated by means of different routes. Activity of PKA is regulated by its direct access to cAMP. In the case of CaMKII, it is probably achieved by its extensive, inhibitory autophosphorylations, while PKC seems to be proteolytically degraded. These biphasic changes in serine/threonine kinases activity may play a critical role in the evolution of postischemic brain injury and provide a mechanism for a variety of short- and long-term signalling events.

Review

Key words: brain ischemia, PKC, PKA, CaMKII, signal transduction

INTRODUCTION

Brain ischemia produces an enormous metabolic stress which, if not directly lethal, initiates a number of potentially cytotoxic effects. Excessive release of glutamate and increase of cytosolic Ca^{2+} can activate many degrading enzymes including a protease calpain, endonucleases and phospholipases, leading to disintegration of cell membranes and production of free radicals, unsaturated fatty acids, prostaglandins and so on. The final outcome of the ischemic insult would depend on the fine interplay between these (and probably others) neurotoxic factors generated directly by ischemia and/or evoked by them secondary tissue reaction (reviewed by Hara 1993). Some of these responses seem to participate in the chain of events leading to delayed neuronal necrosis in affected brain regions, while the others would determine survival and ability of the tissue to repair neuronal circuitry in response to cell loses.

One of the most important systems that links the rapid tissue responses with long-lasting reprogramming of the cell metabolism and function, is the protein phosphorylation cascade. Protein phosphorylation modulates on the short-term scale, membrane excitability, release of nerotransmitters, permeability of ion channels and membrane receptors, while on a longer scale it can modify expression of critical genes by induction of nuclear transcriptional factors (rev. by Bading et al. 1993, Tanaka and Nishizuka 1994, Schulman et al. 1995).

The extent of protein phosphorylation depends on the activity of specific kinases and phosphatases. Protein kinases, highly expressed in CNS, can be divided into two main groups: one phosphorylating tyrosine and the other responsible for serine/threonine phosphorylation. This review will be restricted to the main members of the serine/threonine kinases of the brain: 1- cyclic AMP-dependent kinase A (PKA), 2- calcium/phospholipid-dependent kinase C (PKC), 3- calcium/calmodulin-dependent kinase II (CaMKII), with the special focus on their role in brain ischemia and post-ischemic recovery.

All of these kinases are highly homologous in their catalytic domains and are regulated by similar regulatory mechanisms. For each enzyme, the second messenger (cAMP, 1,2,-sn-diacylglicerol (DAG)/phospholipid or Ca^{2+}/calmodulin) activates its catalytic activity by displacing an autoinhibitory domain and de-inhibiting the kinase. Then the catalytic subunits of the kinase transfers the γ-phosphate of ATP to an appropriate serine or threonine residue on their specific substrate protein. In addition to this mode of activation, evidence has accumulated that stimulation of the kinases is accompanied by changes in their subcellular localization. Kinases translocate from the cytosol to the plasma membranes, nucleus, perinuclear region and cell periphery, depending on cell types and their function. Moreover, the same stimulation can shift individual isoenzymes to different cellular compartments. Intracellular translocation probably plays a critical role in relaying signals to the correct down-stream molecules.

CYCLIC AMP-DEPENDENT KINASE A (PKA)

The enzyme occurs as a tetramer consisting of two regulatory subunits, each containing two cAMP binding sites and two catalytic subunits. In the absence of cAMP, these subunits bind to each other and the activity of the complex is low. When cAMP binds to the regulatory subunit, it promotes the dissociation of the tetramer. Upon removal of the regulatory, autoinhibitory subunit, the catalytic activity of PKA markedly increases.

The brain cAMP-dependent protein kinase, whose activity could be recovered in Triton X-100 soluble fraction, shows generally no change after exposure of the animals (rats and gerbils) to ischemia (Aronowski et al. 1992). However, we have repeatedly reported that postischemic tissue *ex vivo* produces above normal quantities of cAMP in response to a variety of stimuli such as adenosine agonists, active phorbol esters, forskolin as well as histaminergic and adrenergic agonists (Domańska-Janik and Pylowa 1989, 1992, Domańska-Janik et al. 1993). The finding that the responses to all of these agents had been potentiated by ischemia, without directly affecting adenylate cyclase activ-

ity, suggested involvement of a multipotent indirect activating mechanism in cAMP generation. Indeed it was further demonstrated that ischemia temporarily attenuates A1-adenosine receptor-mediated inhibition of adenylate cyclase in cerebral cortical membranes (Domańska-Janik et al. 1993). Ischemic suppression of the inhibitory adenosine signal on cAMP generation involved both down-regulation of A1-adenosine receptors and dysfunction of signal transduction beyond the receptor level. The inhibition of A1-agonist binding after ischemia in many respects is similar to that evoked by unsaturated fatty acids, in particular by arachidonic acid in experiments performed *in vitro* (Domańska-Janik 1993). Perhaps, when the concentration of arachidonate (but also other unsaturated fatty acids) reaches the critical levels reported in ischemic brain, A1-receptor reduction would attenuate the inhibitory signal transduced from NMDA receptor activation-dependent release of adenosine (Manzoni et al. 1994). This mechanism may directly contribute to the observed hypersensitivity of cAMP-generating system after ischemia.

In accordance, a marked increase in cAMP levels in cerebral cortex has been found at 1 to 30 min (Kabayashi et al. 1977) and during the first hours after ischemia (Blomqvist et al. 1985).

In summary it may be concluded that, in spite of unchanged PKA catalytic activity under brain ischemia the cAMP-dependent protein phosphorylations could be greatly facilitated in ischemic brain by increased access of the kinase to its specific activator, cAMP. In support to this conclusion it has been demonstrated that NMDA receptor activation and transient increase of the cAMP concentration in CA1 region of the hippocampus correlates well with phosphorylation of the supposed PKA substrates: the voltage-gated Ca^{2+} channel protein (Chetkowich et al. 1991) and transcription factor CREB, which, in turn, can induce cAMP responsive genes (Ginty et al. 1993). However it remains still unclear whether the activation of the cAMP second messenger system with subsequent functional stimulation of PKA under ischemia may be beneficial or detrimental to the postischemic outcome.

CALCIUM/PHOSPHOLIPID--DEPENDENT KINASE C (PKC)

This kinase exists as a family of multiple species having closely related structures (reviewed by Parker 1992). Calcium-dependent PKC activity in brain tissue can be clearly separated into three main subspecies the α, β (I and II) and γ with primary structures highly homologous and conserved. The enzyme with the γ-sequence is expressed solely in the brain and spinal cord. The two others are found also in other types of cells and tissues. Physiological activation of these PKC subspecies most probably involves 1,2-sn-diacylglicerol (DAG) acting in the presence of a phospholipid (phosphatidylserine) and Ca^{2+} ions. It is worth noting however, that the dependency of PKC on Ca^{2+}, DAG as well as on other phospholipids and fatty acids in *in vitro* systems, varies markedly with the isoenzyme-specific sequences and protein acceptors employed (reviewed by Tanaka and Nishizuka 1994). The particular members of PKC family are widely and differently distributed in brain tissue, showing well-defined subcellular localisation (Gajkowska et al. 1994). Physiological activation of PKC may be mimicked by tumor-promoting phorbol esters which bind to the regulatory domain of the enzyme.

The mechanism of protein kinase C translocation in signalling has gained increasing attention. Enzyme proteins that exist in the cytosol are, upon activation, shifted to the particulate fraction and bind to specific protein termed "receptors for activated C kinase" (*RACKs*, Ron et al. 1994). This occurs only in the presence of the enzyme activators and does not involve the catalytic site. Thus, the extent of enzyme translocation toward membranes is coupled with the degree of its activation and parallels each other in the brain *in situ*.

Most reports published so far indicate that the increase of intracellular calcium, evoked mainly by the glutamatergic receptors, phosphatidylinositides breakdown products and voltage-dependent Ca^{2+} channels activation (Manev et al. 1990), mediates the development of a postischemic brain injury. In contrast to that, the total calcium and phospholipid-

dependent activity of PKC has been reported to be reduced in various brain regions after transient or complete cerebral ischemia (Zalewska and Domańska-Janik 1990, Cardell and Wieloch 1991, 1993, Domańska-Janik and Zalewska 1992). In spite of this reduction, electrophoresis/ blotting experiments indicate an extensive postischemic translocation followed by proteolytic cleavage of all membranous PKC subspecies (α, β, and γ isoforms) to 30 kD regulatory fragment and 50 kD catalytic fragment (Domańska-Janik and Zabłocka 1993 and unpublished recent data). The latter fragment, called PKM, is no longer dependent on calcium and is generated also during TPA-induced PKC down--regulation. TPA-dependent PKC down-regulation *in vitro* has been shown to involve proteolysis of membrane-bound (or stimulus-shifted) kinase by calpains (Kishimoto et al. 1983). After ischemia, the proteolysis of PKC to PKM coincides with calpain activation (Zalewska and Domańska-Janik 1990) and enhancement of [^{3}H]-phorbol binding (a specific ligand to 30 kD PKC-regulatory fragment) to membranous fraction (Zabłocka and Domańska--Janik 1993, Zabłocka et al. 1995). Hence brain ischemia, like phorbols, can cause translocation of PKC from the cytosol to the membranes. In consequence the enzyme is transiently activated, then, by means of protease-dependent cleavage, it is successively inactivated with concomitant induction of the calcium-independent kinase activity (Louise et al. 1988, Zalewska and Domańska-Janik 1990).

Several experiments were devoted to elucidate the relationship between cell function and kinase C behaviour after ischemia. Recently we have found that some agents which have been shown to reduce nerve cells damage and improve recovery following ischemia (i.e., NMDA-receptor antagonist MK801, a presynaptic PAF receptor antagonist, BN52021, and a nitric oxide synthase inhibitor L-NAME), simultaneously prevented or reduced postischemic PKC translocation with lowering of labelled phorbol-12,13-dibutyrate (^{3}H-PDBu) binding to isolated postischemic membranes (Zabłocka et al. 1995). A protective effect of a direct PKC inhibitor staurosporine has also been reported (Hara et al. 1990). In addition, hypothermia and gangliosides prevented or delayed both PKC translocation/activation and postischemic brain injury (Carolei et al.1991, Cardell and Wieloch 1993).

Early translocation/activation of PKC in the acute phase of ischemia may therefore be associated with induction of neuronal damage and enhanced glutamate excitotoxicity. In accordance, we have demonstrated that PKC activation contributes significantly to post-ischemic enhancement of K^+-evoked release of potentially excitotoxic amino acids *in vitro* (Zabłocka and Domańska-Janik 1994). There is also evidence that PKC enhances postsynaptic NMDA-receptor-mediated glutamate responses by reducing the voltage-dependent Mg^{2+} block of NMDA channels (Chen and Huang 1992).

The pathophysiological significance of the subsequent postischemic PKC deactivation should be elucidated in further studies. There are several lines of evidence that PKC may alternatively play a certain protective role for neurones, inhibiting Ca^{2+} influx (Di Virgilio et al. 1986) and mobilization (Nishizuka 1988) or even reducing glutamate and free radicals-induced cytotoxicity *in vitro* (Davis and Maher 1994). The above biphasic changes of the enzyme behaviour, i.e. an initial activation and subsequent down-regulation, may play a critical role in the evolution of postischemic brain injury.

CALCIUM/CALMODULIN--DEPENDENT KINASE II (CaMKII)

CaM kinase is a large multimeric enzyme whose subspecies are derived from four genes consisting of 6-12 kinase subunits each. The α and β genes give rise to neuronal isoforms while the γ and δ are broadly distributed in the body. Many cells contain several CaM kinase isoforms which are generated by alternative splicing in the variable region of the kinase situated between the catalytic and regulatory domain. Calmodulin binds to regulatory domain and activates the kinase by enabling the binding of ATP and protein substrate to the catalytic region. The binding of calmodulin is further facilitated by autophosphorylation of a critical threonine residue

(Thr^{286} in α) in the autoinhibitory domain. This autophosphorylation increases by several hundred folds the affinity for calmodulin binding at low as well as high calcium concentrations (Meyer et al. 1992). Moreover, the Thr^{286} autophosphorylated kinase remains permanently active even after calmodulin dissociates. Further autophosphorylation of the enzyme molecule on different threonine or serine residues finally results in kinase deactivation (Colbran 1992). Thus, the steady phosphorylation/dephosphorylation cycle of endogenous CaMKII would be essential for regulation of its catalytic activity.

Multifunctional Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is the most abundant kinase isoform in brain, particularly enriched in neurones. The enzyme seems to be selectively vulnerable to pathological states associated with a massive calcium influx such as epilepsy/kindling (Wu et al. 1990) and ischemia (Churn et al. 1990). Cerebral ischemia produces a profound decrease in CaMKII activity that occurs very rapidly following the insult. The reasons why ischemia leads that rapidly to the decrease of the enzyme catalytic activity are unknown at present. Our own study strongly suggests that among various possible mechanisms the most probable would be that involving abnormal or irreversible phosphorylation of the enzyme molecule (Zalewska et al. 1996). Alternatively, this could be due to the changes in enzymatic protein conformation, whose in effect would make the certain sites inaccessible to phosphorylation (Shackelford et al. 1995).

In contrast to ischemic enzyme deactivation, total amount of CaMKII protein is essentially stable but extensively and reversibly translocated to the membranes probably in an autophosphorylation-dependent mechanism (Zalewska et al. 1996). Most interestingly, as we have reported recently, ischemia of very short duration (less than 2 min) significantly stimulated Ca^{2+} independent, so-called autonomous kinase activity measured *ex vivo* (Zalewska and Domańska-Janik 1996). As mentioned above, autophosphorylation of CaM kinase on Thr^{286} is responsible for both activation and, after dissociation of calmodulin, generation of a Ca^{2+}-independent form of the enzyme. Thereby conversion of the kinase to the autonomous species *in vitro* provides a way of assessing the stage of CaMKII activation *in situ*. The relative contribution of this autonomous form with respect to total CaMKII activity was found significantly elevated from 2% in control up to 20-30% in all ischemic and recovering animals. In effect, even considering the total CaMKII inhibition after ischemia, its autonomous, thus *in situ* active component, remains more abundant than in control.

The apparent reversibility of CaMKII translocation and inhibition (Zalewska et al. 1996) together with the increase of its active (autonomous) component (Zalewska and Domańska-Janik 1996) supports our suggestion that abnormal phosphorylation/ dephosphorylation cycle may determine the enzyme behaviour during and after ischemia. However, this must be contrasted with recently reported persistent decrease of CaMKII message and protein noticed only in certain, discrete, ischemia-vulnerable regions of hippocampus (Hu et al. 1995). This would indicate possible contribution of an additional, enzyme-degrading mechanism, restricted to the defined cells or structures. Moreover, as in our study we have not observed any correlation between vulnerability of the selected brain regions to ischemia and their responsiveness concerning CaMKII, it seems that only these more subtle changes, involving protein degradation, would be pathognomic for irreversible ischemic injury. Whether ischemia-induced altered function of CaMKII (i.e., apparent changes in catalytic activity and subcellular localization) is an actual part of the ischemic cell death program or alternatively triggers the substantial neuroprotection against it, is for the present moment unknown.

In conclusion, in spite of the substantial differences among the particular members of serine/threonine kinases, their general reaction to ischemic stress seems to have a lot in common. Initially they are switched on by facilitated interactions with their specific, activating second messengers like cAMP, DAG and particularly Ca^{2+}, then are translocated to

highly specific regions of plasma membranes. Some of them, i.e., CaMKII are concomitantly and obligatorily phosphorylated. The others, like PKC, are translocated in a ligand/Ca^{2+}-dependent manner. Stimulus-dependent translocation of PKA was also reported (Hagiwara et al. 1993). Then, after phosphorylation of target proteins, the kinases are deactivated by means of different routes. In the case of CaMKII, it is probably achieved by its further, inhibitory autophosphorylation, while PKC seems to be proteolytically degraded. The PKA would be rather regulated by the access to its direct activator cAMP whose production is initially increased and then suppressed in the later phases of postischemic recovery.

The whole tale about the role of serine/threonine kinases in ischemic brain injury remains still incomplete in that we have no clarity to which extent the changes of each of these enzymes are parts of cellular death or defence program.

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