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Calcium transients in brain ischemia: role in neuronal injury

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Abstract. The involvement of calcium ions in mechanisms of ischemic brain injury has been suggested for several years. Our understanding of the role of intracellular Ca^{2+} as a trigger of acute neurotoxicity and in the induction of long lasting processes leading to necrotic and/or apoptotic postischemic delayed neuronal death or of compensatory, neuroprotective mechanisms has increased considerably. Still many questions concerning the generation of Ca^{2+} signal such as the nature of the main routes of ischemic Ca^{2+} influx to neurones, involvement of intracellular Ca^{2+} stores and Ca^{2+} buffers, spatial and temporal relations between ischemia-induced increases in intracellular Ca^{2+} concentration and neurotoxicity remain open. Some conclusions from experiments in cultured neurones concerning glutamate-evoked destabilization of Ca^{2+} homeostasis and neurotoxicity may be not relevant to *in vivo* ischemic conditions. This review, apart from emphasising generally proposed mechanisms of Ca^{2+} transients and toxicity in ischemic neurones, will discuss some of these controversial issues.

Review

Key words: brain ischemia, Ca^{2+}, neurotoxicity, mechanisms

INTRODUCTION

Several decades of studies on ischemic brain damage revealed a complex character of pathogenic mechanisms involved. A recent review by Siesjö (1993) points to the remarkable evolution of ischemic research, from early pathophysiological studies, through neurochemical research demonstrating metabolic and regulatory disturbances, to recent neurobiological studies on cellular and molecular levels. Different correlates of regulatory and metabolic disturbances in brain ischemia, such as energy deficit, acidosis, lipolysis and proteolysis, disruption of ion homeostasis, particularly concerning Ca^{2+}, overstimulation of excitatory amino acid (EAA) receptors, disorders in protein biosynthesis and phosphorylation and changes in gene expression have been suggested to play a key role in ischemic neuronal injury. In comparison to some other concepts, a relatively old, but continuously developing idea of a primary role of Ca^{2+} in ischemia-induced neurodegeneration occupies a unique place, since Ca^{2+} may trigger almost all secondary mechanisms which have been suggested to participate in this process. A calcium hypothesis is still vital and, in spite of many unclear points, remains relevant to our knowledge of ischemic pathology, including recent considerations on the molecular pathology of brain ischemia (Choi 1995).

The original calcium hypothesis was very quickly overtaken by the excitotoxic hypothesis pointing to a primary role of EAA neurotransmitters in brain ischemia. In fact Ca^{2+}-mediated neuronal damage plays an important role in excitotoxicity (Choi 1988 a,b). However, implicit understanding of the excitotoxic hypothesis of ischemic neuronal damage involves the opinion that in brain, at the neuronal level, practically all ischemia-evoked processes secondary to energy imbalance, including a cytotoxic increase of intracellular calcium concentration ($[Ca^{2+}]_i$), are mediated by EAA and their receptors in neurones, which is difficult to accept. The aim of this article is to discuss, free from presuppositions concerning the excitotoxic concept, selected key issues of the calcium hypothesis connected with generation of Ca^{2+} signal during and after ischemia. A hypothesis of Ca^{2+} involvement in brain ischemia and differences between various models of brain ischemia will be recalled. This will be followed by a review of suggested mechanisms leading to ischemia-evoked increases in $[Ca^{2+}]_i$ in different types and stages of this pathology.

CALCIUM HYPOTHESIS OF NEURONAL ISCHEMIC DAMAGE

Neuronal calcium homeostasis: mechanisms and importance

Calcium homeostasis in neurones and its disturbances in brain ischemia have been described in detail in several recent reviews (Siesjö 1988, 1993, 1994, Siesjö and Bengtsson 1989, Mayer 1989, Morley et al. 1994, Choi 1994, 1995). Briefly, the mechanism of neuronal Ca^{2+} homeostasis under normal conditions encompasses control of plasma membrane permeability to Ca^{2+}, its mobilization from intracellular stores, mainly in the endoplasmic reticulum, and the mechanisms releasing cellular Ca^{2+} outside or binding free Ca^{2+}, and thus limiting $[Ca^{2+}]_i$. Several classes of Ca^{2+}-permeable ionic channels including different voltage-dependent Ca^{2+} channels (VSCC) and receptor operated channels, particularly ionotropic EAA receptors sensitive to NMDA and AMPA/kainate, have been defined in neurones. Intracellular Ca^{2+} stores from endoplasmic reticulum may be released *via* inositol 1,4,5-trisphosphate- or Ca^{2+}- induced channels. Ca^{2+} may be released from neuronal cytosol via neurolemmal Ca^{2+}-ATPase or Na^+/Ca^{2+} exchanger, may be accumulated in the vesicles of endoplasmic reticulum and bound to various Ca^{2+}-binding proteins. Excessive increases in $[Ca^{2+}]_i$ may be buffered by mitochondrial Ca^{2+} uptake. Obviously, intracellular Ca^{2+} homeostasis is strictly related to energy state and neuronal activity.

A function of this sophisticated and still not completely clear machinery is to support the role of Ca^{2+} as a second messenger and to prevent potentially

toxic excessive increases in $[Ca^{2+}]_i$. In neurones Ca^{2+} triggers the release of neurotransmitters from presynaptic sites and takes part in signal transduction postsynaptically. Intracellular Ca^{2+} directly regulates activities of membrane proteins such as various ionic channels and phospholipases A and C. The most important cytosolic proteins regulated by Ca^{2+} are calmodulin, calpains and protein kinase C. This protein kinase and Ca^{2+}/calmodulin-dependent kinase II modulate membrane receptors and channels and are involved in transcriptional regulation of gene expression (Morgan and Curran 1989, Rasmussen and Means 1989, Campbell and Abdulla 1995).

Ischemic disturbances of neuronal calcium homeostasis: neurotoxic mechanism

It is easy to predict that well documented energy imbalance, membrane depolarisation and EAA release in brain ischemia should induce disturbances in ion homeostasis. Thus, both voltage and receptor mediated mechanisms of Ca^{2+} influx to neurones may be activated. Simultaneous inhibition of the activity of ATP-driven Ca^{2+} pumps and efflux of Ca^{2+} from intracellular stores may result in an increase in neuronal $[Ca^{2+}]_i$. In fact a decrease in extracellular calcium concentration ($[Ca^{2+}]_e$) and increase in $[Ca^{2+}]_i$ in brain during ischemia have been observed by several groups (see below).

Early speculations on the role of Ca^{2+} in ischemic neuronal damage suggested that mitochondria may suffer Ca^{2+}-related damage due to their Ca^{2+} overload (Shay 1973, Łazarewicz et al. 1978). This, together with Ca^{2+}-induced lipo- and proteolysis, may produce a rapid necrosis of neurones. However, the role of mitochondrial Ca^{2+} overload during reversible global cerebral ischemia may be challenged (Siesjö 1994), since translocation of extracellular Ca^{2+} to brain neurones provides only a limited source of Ca^{2+} which may not be sufficient to disrupt mitochondrial integrity. Moreover, this mechanism does not explain the phenomenon of delayed neuronal death after ischemia. It seems that mitochondrial dysfunction connected with Ca^{2+} may rather develop during the postischemic period (Sims and Pulsinelli 1987, Sims 1991, Dux et al. 1992).

A comprehensive hypothesis of Ca^{2+}-induced neuronal injury, published by Siesjö (1981), suggested that a common neurotoxic mechanism in brain ischemia, hypoglycaemia and epilepsy may be based on disturbances in Ca^{2+} homeostasis causing lipo- and proteolysis and membrane dysfunction. Additionally, the role of acidosis (Siesjö 1988) and free radicals (Majewska et al. 1978) in ischemia has been strongly suggested. This calcium hypothesis has been confirmed and extended by an excitotoxic hypothesis of ischemic brain damage (Benveniste et al. 1984, Simon et al. 1984, Hagberg et al. 1985), which suggests that excessive stimulation of EAA receptors evoked by ischemia induces neuronal injury. There is no inconsistency between both hypotheses explaining basic mechanisms of neurotoxicity. Ca^{2+}-evoked cytotoxicity plays a central role in the excitotoxic mechanisms of neuronal lesions (Choi 1988a,b). In conjunction with development of the excitotoxic hypothesis, great progress has been made during the last two decades in understanding the role of Ca^{2+} in signal transduction. In particular a regulatory role of Ca^{2+}-related protein phosphoryltions in neuronal functions, in the activity of membrane receptors, ionic channels and enzymes and in transcriptional regulation of gene expression has been recognized. This allowed speculations on possible long-lasting effects of disturbances in Ca^{2+}-dependent phosphorylations during and after ischemia (Wieloch et al. 1991, Aronowski et al. 1992, Domańska-Janik and Zalewska 1992, Zalewska et al. 1996), which may explain the phenomenon of delayed neuronal death. Nevertheless, a central point of the excitotoxic hypothesis of ischemic neuronal damage is the assumption that stimulation of EAA receptors is a prerequisite of the injurious increase in neuronal $[Ca^{2+}]_i$, and ionotropic glutamatergic receptors, particularly NMDA receptors, constitute a main route of ischemia-induced influx of extracellular Ca^{2+} to neurones, which is not always true (see below).

Regardless of the validity of the excitotoxic hypothesis in ischemia, the calcium hypothesis offers an explanation of many potentially injurious secondary effects of ischemic insult and is consistent with most recent speculations on mechanisms of ischemic neuronal damage. For instance, ischemia-evoked induction of peroxidative processes is not an alternative pathomechanism to Ca^{2+}-related injury, but is secondary to disturbances in Ca^{2+} homeostasis. Free radicals are side-products of such Ca^{2+}-mediated processes as stimulation of arachidonic acid release and metabolism, activation of NO synthase and NO production and xantine oxidation after Ca^{2+}- and calpain-mediated conversion of brain xantine dehydrogenase to xantine oxidase (Olanow 1993). Debates on possible compensatory and/or pathogenic changes in gene expression in neurones after brain ischemia and on involvement of necrotic *versus* apoptotic mechanisms of ischemic neuronal injury are also closely related to the calcium hypothesis (Bonfoco et al. 1995, Choi 1995), as Ca^{2+}-dependent kinases participate in regulation of gene transcription (Sheng and Greenberg 1991), whereas Ca^{2+}-activated endonucleases may be involved in DNA fragmentation (Arends et al. 1990, Li et al. 1995, Ueda et al. 1995).

The relationship between increases in neuronal $[Ca^{2+}]_i$ and neurotoxicity is complex and not fully understood. $[Ca^{2+}]_i$ is not a stable value, but it varies within rather broad regulatory limits (Campbell and Abdulla 1995). It may be influenced by several factors including various neurotransmitters, hormones, and neurotrophins. This is of course in agreement with a role of Ca^{2+} as an intracellular messenger (see above). Studies on survival of cultured neurones and its dependence on $[Ca^{2+}]_i$ led to a concept of survival set-point (Franklin and Johnson 1994). Neurones *in vitro* require a critical, slightly elevated level of intracellular Ca^{2+} to avoid apoptotic cell death. To maintain this optimal Ca^{2+} level and avoid apoptosis, neurones should be cultured in the presence of growth factors, with depolarising concentrations of K^+, or with subtoxic concentrations of EAA (Mattson et al. 1989, Johnson et al. 1992, Prehn et al. 1994). However, further increase in $[Ca^{2+}]_i$ to sustained micromolar levels leads to neuronal necrosis. Choi (1995) speculates that the fate of neurones after an acute insult (ischemia) may depend not only on the severity of injury but also on the intracellular set-point. Depending on the $[Ca^{2+}]_i$ level in neurones, apoptosis, necrosis or survival may be achieved. This interesting hypothesis should be taken with caution, however, since it has been founded on data from cultured neurones which differ from in situ neurones (see below).

MODELS OF BRAIN ISCHEMIA

Brain ischemia encountered in the clinic encompasses mainly, but not exclusively, stroke and cardiac arrest. *In vivo* models of brain ischemia in laboratory animals include various types of global (forebrain) and focal ischemia (Siesjö 1993, Hunter et al. 1995). It is important to differentiate between pathomechanisms and, in particular, mechanisms of disruption of Ca^{2+} homeostasis in global and focal ischemia. In global ischemia these changes are transient and last minutes rather than hours, and result in a selective, delayed neuronal death, whereas in the ischemic focus, ischemia and damage may be permanent. In the penumbra zone around the ischemic focus cerebral blood flow is severely reduced leading to complex pathophysiological events including destabilization of Ca^{2+} homeostasis, which may be reversible or may lead to Ca^{2+}-related cell death (Siesjö and Bengtsson 1989). This subject will be discussed below.

To overcome the complexity of brain ischemia *in vivo*, a number of *in vitro* models simulating ischemic disturbances in energy metabolism have been developed (Domańska-Janik and Zalewska 1979, Amagasa et al. 1990, Knöpfel et al. 1990, Mitani et al. 1990, 1994, Reiner et al. 1990, Murphy and Horrocks 1993, Bickler and Hansen 1994). In these models brain slices or cultured neurones are incubated in anoxic-aglycaemic or cyanide- and iodoacetate-containing media simulating ischemic conditions. Although these experiments brought many valuable findings, including data on the na-

ture of channels involved in ischemic Ca^{2+} influx to brain neurones (Bickler and Hansen 1994), conclusions from these studies should be drawn with particular caution. Under *in vitro* conditions a ratio between the extracellular and intracellular volume is extremely high as compared to *in vivo* ischemic brain. Thus, *in vivo* the amount of Ca^{2+} invading neurones is limited by its contents in the extracellular space, whereas in *in vitro* model experiments it is almost unlimited. Also changes in extracellular levels of such important factors as K^+ and EAA are different in *in vivo* and *in vitro* ischemia, particularly when superfusion of slices or cell cultures has been used (Salińska and Łazarewicz 1994). The other limitation is that neurones cultured without natural trophic factors differ from in situ brain neurones in the important characteristics of intracellular Ca^{2+} homeostasis (Johnson et al. 1992).

It is important to differentiate between excitotoxic models of neuronal Ca^{2+} overload evoked by *in vitro* or *in vivo* excessive stimulation of EAA receptors and genuine ischemia. Application of NMDA *in vivo*, especially in the hippocampus, leads to a significant decrease in extracellular calcium concentration ($[Ca^{2+}]_e$), due to influx of Ca^{2+} to neurones (Łazarewicz et al. 1993), and to functional lesions (Rogers et al. 1989). Also, proposed Ca^{2+}-dependent mechanisms of neuronal damage are similar in ischemia and excitotoxicity (Choi 1988a,b, 1994). Nevertheless, many characteristics of brain ischemia, such as energy imbalance and drop in pH, are not exactly simulated.

MECHANISMS OF ISCHEMIC INCREASES IN NEURONAL $[Ca^{2+}]_i$

Ca^{2+} influx to neurones

Ischemic changes in neuronal Ca^{2+} homeostasis should be considered, based on exact qualification of period of the ischemic episode and the type of ischemia in question. Using different methods Uematsu et al. (1988), Folbergrová et al. (1990) and Silver and Erecińska (1990) demonstrated that during the initial seconds of focal and global brain ischemia there is a temporary slight increase in $[Ca^{2+}]_i$. Although the mechanism of this increase is not clear, most probably it reflects Ca^{2+} mobilization from intracellular membranous stores. Some authors point to a coincidence and possibly a causal interrelationship between $[Ca^{2+}]_i$ increase and early ischemic lipolysis (Katsura et al. 1993). Early increase in $[Ca^{2+}]_i$ represents a compensatory mechanism leading to neuronal hyperpolarization and inhibition of these neurone's activity *via* stimulation of potassium channels.

Ischemic neuronal injury has been related to disturbances of Ca^{2+} homeostasis which fully develop during ischemia lasting only a few minutes. One of the central points of the calcium hypothesis is the assumption that the increase in $[Ca^{2+}]_i$ during the ischemic insult represents a primary event. This effect would trigger further changes leading to necrotic damage and/or selective delayed neuronal death. However, the answer to the fundamental question of the exact mechanisms of ischemic increase in neuronal Ca^{2+} concentration is not simple. Here a distinction between global and focal ischemia is essential. Decreases in $[Ca^{2+}]_e$ in the penumbra of focal ischemia, which have a very heterogeneous pattern and resemble spreading depression in its features and mechanism (Siesjö 1992a,b, Hossmann 1994), are sensitive to MK-801. Also the results of fluorimetric studies of Uematsu et al. (1991) in focal ischemia indicate that pretreatment of rats separately with MK-801 partially inhibited a rise in $[Ca^{2+}]_i$, whereas a mixed treatment with MK-801 and nimodipine completely prevented it. There is a consensus that both NMDA receptors and L-type VSCC are responsible for Ca^{2+} influx to neurones in the penumbra. This is supported by neuroprotective effects of corresponding inhibitors (Uematsu et al. 1991).

During global (forebrain) ischemia, $[Ca^{2+}]_e$ decreases rapidly and stabilises at a level reduced to 10% of the initial values. Silver and Erecińska (1990, 1992) and Kristián et al. (1994) report concentrations from 0.1 to 0.6 mM, depending prob-

ably on completeness of the ischemic model. Concomitantly $[Ca^{2+}]_i$ increases to the level of 4-5x10^{-5}M and in some neurones even rises to 10^{-4}M (Silver and Erecińska 1990). Using a model of forebrain ischemia in rats these authors noticed that NMDA receptor antagonists MK-801 and ketamine significantly slowed the rises in $[Ca^{2+}]_i$, particularly in CA1, but only slightly decreased the final level of intracellular Ca^{2+}. Inhibition of L-type VSCC did not influence ischemic increase in intracellular Ca^{2+}. Other laboratories confirm that MK-801 slows decreases in $[Ca^{2+}]_e$ in the ischemic brain but does not prevent it (Kristián et al. 1994). In the anoxic model of developing rats Puka-Sundvall et al. (1994) did not find any effect of MK-801 on decreases in $[Ca^{2+}]_e$ in the cortex. In turn Benveniste et al. (1988) observed inhibition of ischemia-evoked decreases in $[Ca^{2+}]_e$ in the rat hippocampus by glutamatergic denervation and its partial inhibition by pharmacological NMDA receptor blockade by 2-amino-5-phosphonovalerate (APV) applied *via* microdialysis. Our own former data from experiments in rabbit forebrain ischemia using microdialysis indicate that APV, a competitive NMDA receptor antagonist, significantly inhibits a decrease in $[Ca^{2+}]_e$ in the hippocampus, whereas nimodipine given locally remained ineffective (Salińska et al. 1991, Łazarewicz et al. 1993). Considering the different effects of NMDA receptor antagonism in our studies as compared to most other reports, we favour a hypothetical explanation that microdialysis interferes with local pH in ischemic brain tissue, preventing development of ischemic acidosis, which could inhibit the activity of NMDA channels (see below). To sum up, recent data indicate that antagonists of NMDA receptors may slow down, but do not prevent ischemia-induced decrease in $[Ca^{2+}]_e$ and corresponding increase in neuronal $[Ca^{2+}]_i$. This treatment failed to prevent delayed neuronal death in CA1 (for review see Paschen 1996). Also, *in vitro* fluorimetric detection of $[Ca^{2+}]_i$ in cortical slices incubated in ischemia-like conditions revealed that pharmacological inhibition of NMDA and non-NMDA receptors and VSCC may only slow but not prevent a high increase in $[Ca^{2+}]_i$ (Bickler and Hansen 1994). Thus, the role of alternative mechanisms such as activation of non-selective leak channels (Partridge and Swandulla 1993) and of a reverse mode Na^+/Ca^{2+} exchange (Siesjö and Bengtsson 1989) in Ca^{2+} uptake to neurones during ischemia may be postulated. In agreement with this limited effectiveness of NMDA and Ca^{2+} antagonists on ischemic Ca^{2+} fluxes, NMDA receptor antagonism does not provide protection to CA1 neurones in global ischemia (Buchan and Pulsinelli 1990). A reported nimodipine neuroprotection (Łazarewicz at al. 1989a, Nuglisch et al. 1990), which has been denyed by others (Tateishi et al. 1989), may be attributed to mechanisms unrelated to neuronal VSCC antagonism (Łazarewicz et al. 1990).

After ischemia there is a recovery of intracellular Ca^{2+} (within 20-30 min) followed by a complete return of $[Ca^{2+}]_e$ to the preischemic level within 1 h (Silver and Erecińska 1992). Then, 2 and 3 h after ischemia a secondary increase in $[Ca^{2+}]_i$ has been observed in CA1 but not in CA3 (Silver and Erecińska 1992). In these experiments after 8 min of four vessel occlusion in rats the average increase in $[Ca^{2+}]_i$ exceeded 10^{-7}M, but in 50% of CA1 neurones it reached the level of 0.3 - 0.4 μM. Andiné et al. (1988, 1992) observed in rats 4, 6, and 12 h after forebrain ischemia an enhanced uptake of Ca^{2+} to the CA1 neurones upon electrical stimulation, probably *via* NMDA and non-NMDA channels, without any symptoms of enhanced neuronal excitability. This Ca^{2+} uptake correlated with the extent of neuronal damage. Antagonists of non-NMDA receptors appeared to be highly neuroprotective when given several hours after ischemia (Diemer et al. 1992, Sheardown et al. 1993). It is not clear whether increased $[Ca^{2+}]_i$ and enhanced influx of extracellular Ca^{2+} to neurones, mainly via non-NMDA receptors observed several hours after ischemia, may be related to a sustained increase in $[Ca^{2+}]_i$ after prolonged or excessive glutamatergic stimulation that proceeds neuronal death, shown in experiments in cultured neurones (Manev et al. 1989, Mattson et al. 1989, Johnson et al. 1992). An alternative explanation is that increases in $[Ca^{2+}]_i$ in CA1 neurones dur-

ing the postischemic period may reflect a selective decrease in the expression of a completely edited GluR2 subunit of AMPA receptors, inhibiting Ca^{2+} influx through non-NMDA channels (Paschen 1996). However, a postischemic decrease in GluR2 mRNA levels was not observed until 2 days after the insult (Pellegrini-Giampetro et al. 1992). Thus, from the temporal point of view the latter phenomenon may be rather relevant to a secondary Ca^{2+} accumulation in CA1 neurones that coincides with their selective delayed death 2-3 days after insult (Dienel 1984). This late postischemic Ca^{2+} accumulation may be alternatively interpreted as an epiphenomenon, a terminal consequence of neuronal damage accompanying cell death rather than a primary trigger of their injury.

Thus, results of studies in various models of brain ischemia indicate that at different periods of ischemia or recovery diverse mechanisms may be responsible for excessive influx of extracellular Ca^{2+} to neurones leading to their death. NMDA receptors and L type VSCC are involved in Ca^{2+} entry to neurones in the penumbra of focal ischemia. In global (forebrain) ischemia mainly non-NMDA receptors mediate Ca^{2+} influx in the post-ischemic period, whereas the influx of Ca^{2+} during ischemic insult utilizes many mechanisms. In the initial phase NMDA receptors and probably VSCC, particularly L channels, are partially responsible for extracellular Ca^{2+} influx, but in ischemia lasting several minutes other ill-defined processes resistant to Ca^{2+} antagonists and blockers of EAA receptors predominate. Surprisingly, NMDA receptors representing a potential main route of Ca^{2+} accumulation in hippocampal neurones (Łazarewicz and Salińska 1993) seem to play a marginal role in Ca^{2+} fluxes in advanced forebrain ischemia.

Putative suppressors of NMDA receptors in global ischemia

It is known from electrophysiological studies that the activity of NMDA channels is highly sensitive to extracellular pH, and at pH close to 6.5 Ca^{2+} currents through NMDA receptors may be significantly inhibited (Tang et al. 1990, Vyklicky et al. 1990). Moreover, reduced pH in the incubation medium protects cultured neurones from NMDA receptor-mediated injury evoked by *in vitro* "ischemia" and glutamate neurotoxicity (Giffard et al. 1990, Tombeugh and Sapolsky 1990). This subject was addressed in the recent *in vivo* studies in rats subjected to a global ischemia and hyper- or hypoglycaemia (Kristián et al. 1993, 1994, 1995).

It has been demonstrated that enhanced brain acidosis to pH 6.4 in hyperglycaemic, and also in hypercapnic, rats slows ischemia-evoked decrease in extracellular Ca^{2+} in a similar way to MK-801 (see above), but *in vivo* acidosis exaggerates brain damage. In ischemic plus hypoglycaemic rats at brain pH 7.1, a decrease in extracellular Ca^{2+} was almost instant. In the control ischemia a drop in pH is substantial, thus one may conclude that the activity of NMDA channels in the ischemic brain neurones of normoglycaemic individuals is significantly suppressed by acidosis. A local prevention of this Ischemic fall in pH values may probably take place in microdialysis studies (Salińska et al. 1991). It may result in attenuation of the pH block of NMDA channels (see above). In fact our recent experiments indicate that although lowering of pH in the microdialysis medium to 6.5 was inactive, a decrease of pH to 6.0 greatly inhibited the NMDA-induced decrease in $[Ca^{2+}]_e$ in the rabbit hippocampus (Salińska and Łazarewicz, unpublished), which indicates that pH of the microdialysis medium may influence pH, and indirectly Ca^{2+} homeostasis, of the tissue.

The other putative suppressant of the activity of NMDA channels in brain ischemia is nitric oxide (NO). It is known that NO is generated in brain during ischemia (Nowicki et al. 1991, Malinski et al. 1993, Dalkara and Moskowitz 1994, Strosznajder and Chalimoniuk 1996) and that NMDA receptors/channels apart from many other modulatory sites possess the redox sites (Łazarewicz et al. 1989b, Levy et al. 1990). Their oxidation leads to inhibition of the channel activity, and according to Lei et al. (1992) and Lipton et al. (1993) NO may inhibit this channel *via* oxidation of redox sites. It has been suggested that NO may induce complex

effects in ischemia, participating *via* peroxidation processes in ischemic neuronal damage (Stamler et al. 1992), but also exerting compensatory effects through inhibition of NMDA channels (Lipton et al. 1993). However, the inhibition of NMDA channels and neuroprotection demonstrated in some *in vitro* experiments could be evoked by the NO donors rather than by nitric oxide itself (Kiedrowski et al. 1991, Wroblewski et al. 1991). Moreover, Akira et al. (1994) demonstrated that NO potentiates MK-801 binding to NMDA channels, which suggests their open configuration in the presence of NO. Thus the role of NO in modulation of the activity of NMDA receptors in brain ischemia remains controversial and requires more studies (Choi 1993).

These data indicate that NMDA receptors, which in normal conditions seem to represent a major pathway of Ca^{2+} influx to neurones in ischemia-sensitive hippocampal regions (Łazarewicz and Salińska 1993), may be significantly inhibited by a fall in pH and production of NO during ischemia. However, in light of sufficient Ca^{2+} influx via alternative Ca^{2+} ionophores and considering other harmful effects of acidosis and NO, it is hard to postulate their explicit neuroprotective or compensatory role in brain ischemia.

Mobilisation of Ca^{2+} pools in endoplasmic reticulum

Apart from extracellular Ca^{2+}, intracellular stores of Ca^{2+}, particularly in the endoplasmic reticulum (ER) may also participate in increases in $[Ca^{2+}]_i$ *via* inositol 1,4,5-trisphosphate (IP3)-induced and Ca^{2+}-induced Ca^{2+} release. ER is the major intracellular Ca^{2+} store that participates in depolarisation- or hormone-evoked increases and oscillations of $[Ca^{2+}]_i$. The importance of intracellular Ca^{2+} in brain ischemia may be suggested mainly based on *in vitro* data from experiments in cultured neurones (Frandsen and Schousboe 1993) reporting neuroprotective effects of dantrolene against excitotoxic neuronal damage. This known inhibitor of intracellular Ca^{2+} mobilization also significantly reduced increases in $[Ca^{2+}]_i$ in neurones evoked by EAA agonists. According to Frandsen and Schousboe (1993), in cultured neurones EAAs mobilise intracellular Ca^{2+} from dantrolene-sensitive pools, both dependent on and independent of extracellular Ca^{2+}, which constitute a great portion of the NMDA-induced increase in $[Ca^{2+}]_i$.

It is not clear if these *in vitro* data are relevant to brain ischemia *in vivo*. Dantrolene was shown to protect CA1 neurones in gerbil brain *in vivo* from forebrain ischemia-evoked injury (Zhang et al. 1993). However, dantrolene nonselectively inhibits intracellular Ca^{2+} mobilisation *via* both IP3- dependent and Ca^{2+}-dependent ryanodine-sensitive Ca^{2+} release (Frandsen and Schousboe 1993). In the rat brain ryanodine receptors are mainly localized in dentate granule neurones, while IP3 receptors are almost exclusively concentrated in CA1 neurones (Worley et al. 1989, Nio et al. 1991, Sharp et al. 1993). Thus, localization of IP3 receptors but not ryanodine receptors corresponds with the highest increases in intracellular Ca^{2+} in the ischemic brain in CA1 (Silver and Erecińska 1990) and with a well established highest sensitivity of the CA1 neurones to selective delayed neuronal death after ischemia. Dentate neurones are particularly resistant to ischemic damage.

Ca^{2+} binding proteins

The other potentially important elements of Ca^{2+} homeostasis in neurones influencing $[Ca^{2+}]_i$ are Ca^{2+} binding proteins (Andressen et al. 1993). A number of EF-hand proteins like calmodulin, calbindin D_{28k} and parvalbumin bind Ca^{2+} with high affinity and may function as cytosolic Ca^{2+} buffers. Calbindin D_{28k} is highly expressed in rat dentate neurones, but moderately in CA1 and CA2, and absent from CA3 (Sloviter 1989). Several examples of correlation between expression of this protein and neuronal survival after ischemia or excitotoxicity have been presented (Freund et al. 1992, Goodman et al. 1993, Luiten et al. 1994, Potier et al. 1994). It has been proposed that calbindin is an important factor for the survival of pyramidal neurones in the hippocampus after ischemia (Rami et al. 1992).

Ischemia and Ca^{2+} ionophore induced decreases of calbindin and parvalbumin contents in CA1 pyramidal cells (Johansen et al. 1990), indicating Ca^{2+}-induced destruction of these Ca^{2+}-binding proteins. Moreover, ischemia results in increased expression of mRNA encoding calbindin in brain neurones (Lowenstein et al. 1994), which may represent a cellular compensatory response to disturbed Ca^{2+} homeostasis. However, there are negative reports concerning a putative neuroprotecive role of calbindin (Tortosa and Ferrer 1994). Calbindin is absent from rat CA3 neurones which are relatively resistant to ischemia and NMDA neurotoxicity, in contrast to its presence in ischemia-sensitive CA1 neurones (Sloviter 1989, Freund et al. 1990).

Interrelationship between increases in neuronal $[Ca^{2+}]_i$ and cell damage: temporal and spatial aspects

Obviously a precise intraneuronal Ca^{2+} homeostasis includes spatial and temporal regulation of $[Ca^{2+}]_i$ (Smith and Augustine 1988). Disruption of these relations may induce neuronal injury. Although several putative mechanisms of Ca^{2+}-mediated neuronal damage have been identified, there is still inadequate information relating these processes to actual changes in $[Ca^{2+}]_i$ in defined subcellular sites at various periods of ischemic pathology. Data from *in vitro* experiments on high K^+-, excitotoxin- and model "ischemia"-evoked increases in $[Ca^{2+}]_i$ and neuronal injury indicate that Ca^{2+}-induced damage may not be related to an average $[Ca^{2+}]_i$, but rather to actual $[Ca^{2+}]_i$ in hypothetical restricted microdomains which are at present difficult to define and study. Recent papers of Tymianski et al. (1993a,b,c) addressing questions of intracellular Ca^{2+} buffering, and relationships between ways of Ca^{2+} influx, $[Ca^{2+}]_i$, absolute Ca^{2+} load and excitotoxic neuronal injury indicate that a final outcome (neurodegeneration or cell survival) depends on neuronal Ca^{2+} loading, $[Ca^{2+}]_e$, a mechanism of Ca^{2+} influx, activity of mechanisms releasing Ca^{2+} from neurones and binding intracellular Ca^{2+}. These data suggest that particular postsynaptic intraneuronal sites preferentially associated with NMDA receptors may be preferentially responsible for excitotoxic, Ca^{2+}-mediated neuronal injury.

CONCLUSIONS

Although the calcium hypothesis of ischemic neuronal injury remains in the centre of research concerning pathomechanisms of ischemic neuronal injury, our understanding of the mechanisms of disruption of Ca^{2+} homeostasis resulting in uncontrolled increase in $[Ca^{2+}]_i$ and generation of pathological Ca^{2+} signal is still incomplete. Also, exact Ca^{2+}-triggered processes leading to ischemic nerve cell death and their relationship to changes in neuronal $[Ca^{2+}]_i$ in spatially and temporally restricted microdomains in neurones remain to be elucidated.

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