Calcium-Mediated Mechanisms of Ischemic Injury and Protection

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Our understanding of calcium's role in cerebral ischemia continues to evolve from the initial recognition that it may be harmful to the ischemic cell. A multitude of experiments have supported the hypothesis that excessive influx of calcium into the cell under ischemic conditions is a major mechanism of cell injury and death. Pharmacological intervention to restore cellular calcium homeostasis is protective in many models of cell anoxia. Principle routes of calcium entry are the voltage-sensitive (VSCC) and *N*-methyl-D-aspartate linked receptor operated (ROCC) calcium channels. Regional variations in channel densities have been described and it is now known that these classes of channels are located in different regions of the neurons. Activation of both channel types has been identified in *in vivo* models of cerebral ischemia. Although the ROCC is predominant in number, the VSCC appears to activate at higher cerebral blood flow values suggesting that it is an earlier conduit for calcium than the glutamate-driven ROCC. Intracellular calcium is well recognized as a second messenger system and there is increasing appreciation that it induces immediate early genes (IEG). Since IEGs function as transcriptional regulating factors, the differential expression of specific target genes may be of importance for determining death or survival of the ischemic tissue.

Stroke remains a major source of mortality and disability in society, but our understanding of the

metabolic processes that render neurons vulnerable to ischemic cell death have much improved. In this context, Ca^{2+} has assumed major importance as the ion that brings about death of the ischemic cell and various efforts have been made to control its intracellular concentration. More recently the role of Ca^{2+} in signalling molecular responses in the ischemic cell and their potential neuroprotective role has become clearer. This review will emphasize our present understanding of Ca^{2+} homeostatic mechanisms, the role the voltage-sensitive and receptor operated Ca^{2+} channels (VSCC and ROCC) play *in vivo* and the gene responses that can be induced by moderate activation of these channels. Regarding the genomic responses, see the review by Kiessling and Gass in this symposium.

Loss of Calcium Homeostasis in Ischemia

Neurons, like other cells, maintain their resting intracellular free calcium concentration ($[Ca^{2+}]_i$) at extremely low levels (approximately 100 nM) despite being bathed in an extracellular fluid containing 1 mM Ca^{2+}. The electrochemical gradient across the neuronal membrane which tends to drive Ca^{2+} into the cells is maintained by the relative impermeability of the membrane to Ca^{2+} and by energy-dependent extrusion processes (Fig. 1). The $[Ca^{2+}]_i$ is the result of several opposing forces: (i) influx through Ca^{2+} channels, (ii) sequestration by internal storage vesicles and (iii) expulsion by Ca^{2+} pumps and exchangers (1).

A number of physiological stimuli use small and transient increases in $[Ca^{2+}]_i$ as signals for an array of metabolic, structural and functional processes

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Glossary for Abbreviations

4-VO 4-vessel occlusion; **AMPA** Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; **[Ca²⁺]ᵢ** Intracellular calcium ion concentration; **CBF** Cerebral blood flow; **CCA** Common carotid artery; **DHP** 1,4-dihydropyridine; **IEG** Immediate early gene; **IP₃** Inositol-1,4,5-trisphosphate; **KA** Kainate; **MCA** Middle cerebral artery; **NMDA** *N*-methyl-D-aspartate; **ROCC** Receptor operated calcium channels; **PLC** Phospholipase C; **VSCC** Voltage sensitive calcium channels

such as growth, learning, memory and neurotransmitter release (1). However, large, sustained increases in neuronal $[Ca^{2+}]_i$ initiate a cascade of destructive metabolic processes which culminate in the death of the neuron (2). It is widely believed that such a disruption of Ca^{2+} homeostasis and the resulting lethal Ca^{2+} overload is the major cause of damage in the ischemic brain (3-5). The initial effects of ischemia are the loss of cellular energy production resulting from the reduction in oxygen supply and a massive extracellular buildup of the excitatory neurotransmitter glutamate (3,6-8).

Membrane Ca^{2+} influx pathways. Energy depletion in ischemic neurons rapidly leads to a loss of sodium gradients which are normally maintained by the adenosine triphosphate (ATP)-dependent membrane Na^+-K^+ pump (6). The resulting depolarization triggers Ca^{2+} influx through voltage sensitive calcium channels (VSCCs) in the plasma membrane. Four types of neuronal VSCCs, designated as T, L, N and P, have been described based on their electrophysiological, pharmacological and physical characteristics (9,10). Temporary protection may be provided by the increased $[Ca^{2+}]_i$ activating Ca^{2+}-dependent K^+ channels which hyper-polarize the cell (11), but if the $[Ca^{2+}]_i$ remains elevated the destructive process progresses.

The oxygen-deprived, electrically impaired neurons release large amounts of glutamate in response to the depolarization of the plasma membrane. Glutamate is the major excitatory neurotransmitter in the central nervous system. Normally, excess extracellular glutamate is actively returned to presynaptic terminals and glial cells (12). In ischemia however, the mechanism for glutamate re-uptake is impaired due to the lack of energy and therefore, there is a prolonged and excessive activation of post-synaptic glutamate receptors. Glutamate activates several subtypes of ligand-gated cation channels which allow further Ca^{2+} influx into the neurons (3,13-16). These subtypes, the ionotropic glutamate receptors, are defined by their pharmacological profiles as the N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (KA) receptors (17). Glutamate also activates a metabotropic receptor, linked to phospholipase C (PLC) and G-proteins, which triggers the mobilization of Ca^{2+} from intracellular stores (18,19). Glutamate also induces further Ca^{2+} influx through the activation of VSCCs secondary to the transmitter-evoked depolarization of the membrane (20).

It was originally thought that glutamate over-stimulation of NMDA receptors during ischemia was the major pathway for the lethal influx of Ca^{2+}, but other routes may play secondary roles or amplify the NMDA-triggered influx and resulting injury. The relative contributions and significance of Ca^{2+} influx through VSCCs and NMDA-operated ion channels is not fully understood, however, it has

recently been demonstrated in hippocampal neurons that L-type Ca^{2+} channels and NMDA receptors transmit signals to the nucleus and regulate gene transcription through two distinct Ca^{2+} signalling pathways (21).

Although the functional role of Ca^{2+}-permeable non-NMDA-operated ion channels is still unclear, they also appear to be involved in glutamate-mediated toxicity. AMPA receptor channels may conduct Ca^{2+}, especially upon reperfusion. This is reflected by a post-ischemic decrease in the proportion of the normally dominant non-Ca^{2+} conducting AMPA receptor subunit GluR2 compared to the Ca^{2+}-conducting GluR1 and GluR3 subunits in ischemia-sensitive CA1 neurons, whereas little change in AMPA receptor subunits are seen in the less vulnerable CA3 neurons (22). The importance of these receptors is demonstrated by the ability of the AMPA antagonist 2,3-dihydro-6-nitro-7-sulfamoylbenzoquinoxaline (NBQX) to reduce damage of CA1 neurons after transient ischemia of rat brains (23).

Membrane ion pumps and exchangers normally maintain the electrochemical ion gradients and membrane potential of neurons. The Na^+-Ca^{2+} exchanger returns the $[Ca^{2+}]_i$ to the resting level following its elevation. It pumps Ca^{2+} ions out and Na^+ ions into the cell using the inwardly directed electrochemical gradient for Na^+ which is maintained by the Na^+-K^+ pump. In ischemia, the loss of neuronal ATP inhibits the Na^+-K^+ pump (6) and the resulting accumulation of Na^+ reverses the operation of the Na^+-Ca^{2+} exchanger (24-26). Inhibiting the Na^+-Ca^{2+} pump or removing Na^+ or Ca^{2+} ions from the extracellular milieu is neuroprotective, suggesting that a reversal of this exchanger during ischemia pumps Ca^{2+} into, instead of out of, the neuron and this contributes to the lethal elevation of $[Ca^{2+}]_i$ (27).

Intracellular Ca^{2+} stores. The metabotropic glutamate receptor is a G-protein coupled receptor which activates phospholipase C (PLC) and generates inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerols, leading to the release of Ca^{2+} from intracellular stores and activation of protein kinase C, respectively (18,19). Like other cells, neurons have a variety of internal Ca^{2+} stores. One is sensitive to IP$_3$ and others are sensitive to caffeine and ryanodine which may be involved in phenomena such as Ca^{2+}-induced Ca^{2+} release (28). Many physiological agents trigger the release of Ca^{2+} from intracellular stores in neurons, but the physiological function of the metabotropic glutamate receptor is not known. It is also not known if the biological effects of elevated $[Ca^{2+}]_i$ depend on whether the Ca^{2+} comes from inside or outside of the cell. Dubinsky and Rothman (29) have suggested that processes causing cell death may be linked to the cell membrane and be activated only by the translocation of Ca^{2+} across the plasma membrane.
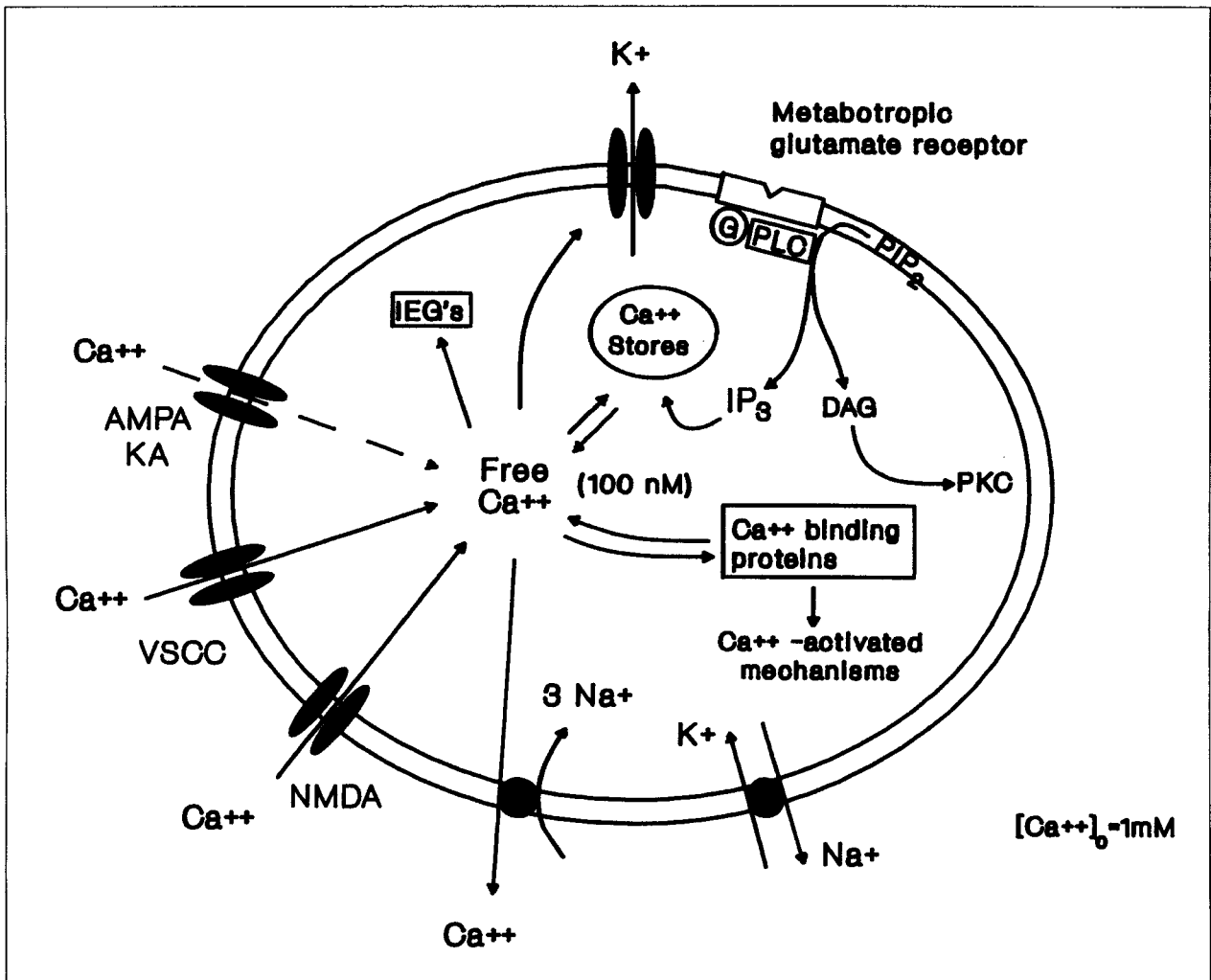
Figure 1 Under physiological conditions Ca^{2+} influx occurs mainly via voltage-sensitive (VSCC) and N-Methyl-D-aspartate (NMDA)-gated Ca^{2+} channels. After ischemia calcium currents may be gated additionally via the alpha-amino-3-hydroxy-5-methyl-4-iso-xazolepropionate (AMPA)/kainate-operated cation channel (broken arrows). Activation of the metabotropic glutamate receptor causes the release of Ca^{2+} from intracellular stores. Intracellular free Ca^{2+} is buffered by Ca^{2+}-binding proteins and is returned to resting levels by extrusion via the energy-dependent Na$^+$-Ca^{2+} exchanger. The Na$^+$ gradients which run the exchanger are maintained by the membrane Na$^+$-K$^+$ adenosine triphosphate (ATPase). The elevated [Ca^{2+}]$_i$ can activate many Ca^{2+}-mediated mechanisms, including activation of Ca^{2+}-sensitive K$^+$ channels and immediate early genes. Abbreviation: G, G-proteins; PLC, Phospholipase C; DAG, Diacylglycerols; IP$_3$, Inositol 1,4,5-trisphosphate; PKC, Protein kinase C; PIP$_2$, Phosphatidylinositol 4, 5-bisphosphate; IEG, Immediate early genes.

Recent studies have shown that in gerbil hippocampal neurons, two-thirds of the ischemia-induced Ca^{2+} surge is due to release from internal stores while only one-third is due to influx (30). Dantrolene, which blocks Ca^{2+} release from the ryanodine-sensitive stores, halved the ischemia-induced release of Ca^{2+} from the intracellular stores of hippocampal neurons, and significantly protected them following transient forebrain ischemia (31). Similar neuroprotective effects of dantrolene on glutamate-mediated toxicity in cultured neurons have been reported (32,33). In addition, the metabotropic receptor antagonist L-AP3 protects neurons from hypoxic injury in vitro (34) and the PLC inhibitor phenylmethylsulfonyl fluoride protects CA1 hippocampal neurons

from forebrain ischemia in vivo (35), suggesting that metabotropic receptor activation and mobilization of Ca^{2+} from intracellular stores are important in ischemic injury.

Changes in [Ca^{2+}]$_i$ in ischemia. Direct in vitro and in vivo measurements of [Ca^{2+}]$_i$ have shown that either ischemia (36-38) or glutamate raise [Ca^{2+}]$_i$ (29,39-41). In many systems, glutamate triggers a two-phase response. There is an initial transient [Ca^{2+}]$_i$ surge which is followed by a sustained rise which requires extracellular Ca^{2+}, but not the continued presence of the agonist (42). Neurons can maintain an elevated [Ca^{2+}]$_i$ long after the removal of glutamate (43). This secondary overload may indicate the irreversible loss of Ca^{2+} homeostasis

and is closely correlated with cell death (43,44). Basic fibroblast growth factor and nerve growth factor can protect neurons from ischemic insults by preventing this late rise in $[Ca^{2+}]_i$ (45,46). However, neurotoxicity does not depend solely on the size of the $[Ca^{2+}]_i$ response because large $[Ca^{2+}]_i$ surges induced by inhibiting oxidative metabolism are not toxic (29). Thus, it is likely that cell death, while responsive to alterations of $[Ca^{2+}]_i$, is not solely determined by it. According to the Ca^{2+} setpoint hypothesis, the survival of some neurons can be enhanced by small sustained elevations (i.e., less than 100 nM above resting level) of $[Ca^{2+}]_i$ that result from Ca^{2+} influx through VSCCs (47,48). Larger increases of $[Ca^{2+}]_i$ over resting levels are toxic. The mechanism by which Ca^{2+} promotes survival is unknown, but may be related to the induction of immediate early genes (IEG) as discussed below.

Neurons are complex cells, with specialized functions restricted to various parts of the cell and which require different local Ca^{2+} signals. Therefore, spatial as well as temporal variations of $[Ca^{2+}]_i$ are crucial for neuronal function. Although the $[Ca^{2+}]_i$ can rapidly rise and fall by an order of magnitude, the spatial localization of Ca^{2+} entry, release from internal stores, reuptake into stores and buffering by Ca^{2+}-binding proteins are all of potential importance. The spatial distribution of $[Ca^{2+}]_i$ responses is governed by the restriction of Ca^{2+} influx to certain parts of neurons due to the clustering of VSCCs and the non-uniform distribution of ligand-gated ion channels on the cell surface (49,50). For example, in response to depolarization the $[Ca^{2+}]_i$ of sympathetic neurons rises in the soma and growth cones, while the release of Ca^{2+} from internal stores by caffeine occurs only in the cell body (49). Selective localization of Ca^{2+} pumps and exchangers and Ca^{2+}-binding proteins could also play important roles in the regulation of $[Ca^{2+}]_i$. The relative importance of the restriction of the Ca^{2+} signal to specific regions of the neuron is not known, but it provides a means whereby Ca^{2+}-dependent processes can be switched on or off.

Intracellular Ca^{2+}-binding proteins are important regulators of $[Ca^{2+}]_i$ and their ability to buffer Ca^{2+} may be an important determinant of neuronal vulnerability in ischemia. Different populations of neurons contain variable concentrations of the Ca^{2+}-binding proteins calmodulin, parvalbumin, calbindin, calretinin, S100α and calcineurin (51,52). However, the data from studies postulating a protective role for Ca^{2+}-binding proteins in ischemia are inconsistent. Some authors report that hippocampal neurons immunoreactive to parvalbumin and calbindin are protected from ischemic damage, but others report the loss of neurons showing positive immunoreactivity for the Ca^{2+}-binding proteins (52,53). Although the protective role of Ca^{2+}-binding pro-

teins in ischemia is controversial, it clearly requires additional investigation.

The role of Ca^{2+} in selective vulnerability. Why some regions of the brain are more vulnerable to ischemia and Ca^{2+} overload than other regions is not known, but finding out why should enable the development of effective neuroprotective treatments. In the hippocampus, CA1 neurons are exquisitely sensitive to ischemia, whereas the neighbouring CA3 neurons and dentate gyrus neurons are relatively resistant to ischemic damage (54). Although vulnerability is associated with the differences in the number, type and localization of glutamate receptors in different brain regions (55), selective vulnerability to ischemia may also be related to the neurons' ability to manage the ischemia-triggered Ca^{2+} influx. Neurons of the CA3 region may survive ischemia because of their ability to conserve energy to deal with the Ca^{2+} load, whereas in the CA1 region metabolic failure renders the neurons incapable of dealing with the Ca^{2+} load and the cells die (56).

Neurotoxicity may be related to many factors including the kinds of receptors activated, the source of the surging Ca^{2+}, the severity and duration of the ischemic event or application of the agonist, the duration and persistence of the $[Ca^{2+}]_i$ surge after agonist withdrawal, the availability of nutrients and the intrinsic susceptibility of the neurons. Although Ca^{2+} is thought to trigger a cascade of destructive processes within minutes, the subsequent neuronal degeneration can begin within minutes or it can be delayed for many hours. The mechanism by which an elevated $[Ca^{2+}]_i$ kills the cell is not known and a link between increased $[Ca^{2+}]_i$ and neurotoxicity has not been proven conclusively.

In many experimental models of neurodegeneration, pharmacological intervention to block glutamate receptor activation prevents a lethal influx of Ca^{2+}. Similarly, removing extracellular Ca^{2+}, chelating intracellular Ca^{2+} or applying VSCC or ligand-gated ion channel antagonists can protect neurons from ischemic injury (57-66). Neuroprotection is proportional to the intervention's ability to restore normal Ca^{2+} homeostasis.

Voltage Sensitive Calcium Channel (VSCC) Activation in Cerebral Ischemia

Calcium channels play a central role in the changes in $[Ca^{2+}]_i$ noted during ischemia but there are few reports that directly observe the activation, or opening, of these channels *in vivo*. L-type VSCCs are ubiquitously distributed throughout the brain (67) and are present on neurons (68). The channels are located primarily in the proximal portion of the dendritic tree, adjacent to the cell body (69) and may normally serve to convert membrane electrical activity into cellular metabolic response (70). The L-type VSCC produces an inward calcium current with depolarization of the cell membrane (71) and may

be expected to open during ischemic cell membrane depolarization.

Pharmacology of L-type VSCCs. The L-type VSCC exists in at least three states (72); a resting state with a high probability of occurrence in polarized cell membrane, open and inactivated states with high probabilities of occurrence in depolarized cell membranes (73). These channels are antagonized by three main classes of Ca^{2+} channel antagonists; the phenylalkylamines, the benzothiazepines, and the 1,4 dihydropyridines (DHP) (74). The DHP class of channel ligands may act as either agonists or antagonists depending on structural and isomeric forms (72,75). The L-type VSCC exhibits state dependent binding having a much higher affinity for DHP antagonists in depolarized cell membranes (73,76). Thus, DHP binding may be expected to increase in ischemically depolarized tissue and this has been shown *in vivo* (77,78). Although this increased binding is to the inactive channel state in the depolarized membrane (73), it may be used to infer that activation, or opening of the L-type VSCC has occurred.

In vivo binding of [³H] nimodipine, a DHP Ca^{2+} channel antagonist to ischemic brain, has been used to identify activation of L-type VSCC in several models of cerebral ischemia in rats (77,79,80). Nimodipine is lipid soluble and readily crosses the blood-brain barrier (81). Following intravenous infusion, [³H] nimodipine reaches an equilibrium of distribution between plasma and brain within 30 minutes in all but the most severely ischemic brain regions (CBF < 5 ml / 100 g / min) (78). Under equilibrium conditions and using autoradiographic methods increased *in vivo* binding of [³H] nimodipine to acutely ischemic brain is observed and determined to be saturable and specific to the L-type VSCC (78). Thus, *in vivo* nimodipine binding may be used to identify ischemic regions in which activation of the L-type VSCC has occurred.

Response of the VSCC to irreversible ischemia. Binding of [³H] nimodipine *in vivo* was used to determine L-type VSCC activation at several times (5 minutes, 4, 24 and 48 hours, respectively) after the onset of irreversible focal cerebral ischemia in male Sprague Dawley rats (77). In this work, middle cerebral artery (MCA) territory ischemia was produced by simultaneous MCA and ipsilateral common carotid artery (CCA) occlusion. VSCC activation was observed in the most severely ischemic striatum five minutes after occlusion. The affected cortical regions (cerebral blood flow (CBF) 10 to 20 ml / 100 g / min) did not show a marked increase in binding at five minutes of occlusion but a significant increase was reported in this less severely ischemic region at four hours of ischemia. At this time, the striatum was showing histological evidence of infarction and was losing its ability to show increased nimodipine binding. At 24 hours, all tissue in the territory of the MCA territory had infarcted and increased [³H] nimodi-

pine binding started to slowly decline by 48 hours. It was concluded that: (i) VSCC activation, as determined by increased *in vivo* nimodipine binding, was a sensitive and early indicator of impending ischemic injury, and (ii) the rate of appearance of VSCC activation was dependent on the severity of the cerebral ischemia. All tissue that eventually infarcted in this model passed through a period of Ca^{2+} channel activation.

Response of the VSCC to reversible ischemia. Nimodipine binding *in vivo* has been used to investigate the response of L-type VSCC to a short period of reversible focal cerebral ischemia (79). In this study, MCA + CCA occlusion in the rat was performed using micro-aneurysm clips. Following 15 minutes of ischemia, the clips were removed and reperfusion for 45 minutes obtained. During occlusion, VSCC activation was observed in the ischemic MCA territory. With restoration of blood flow this increased activation returned to basal levels by 45 minutes of reperfusion in those regions in which CBF was reestablished. All regions showing persistent elevation of nimodipine binding in this model infarcted. The authors concluded that VSCC ischemic activation was initially reversible and could be used to identify potentially salvageable tissue in the setting of acute cerebral ischemia. The response of L-type VSCC activation has also been studied in forebrain ischemia (80). Nimodipine binding *in vivo* was determined 30 minutes, 2, 24 and 48 hours, respectively, following restoration of CBF after 30 minutes of four vessel occlusion (4-VO) in rats. At 30 minutes of reperfusion a general activation of cerebral VSCC was observed, presumably due to intense depolarization during ischemia. By two hours of reperfusion [³H] nimodipine binding had returned to control levels indicating that repolarization of cell membranes had occurred. A second peak of VSCC activation was subsequently observed in vulnerable brain regions undergoing delayed neuronal death. However, it was not possible to determine if this second peak in channel activation contributed to, or was a cause of, delayed neuronal death.

VSCC activation in spreading depression. Spreading depression is characterized by a slowly moving wave of depressed electrical activity and cell membrane depolarization (82). It is easily produced and observed in rat brain and under normal physiologic conditions pathological changes are not observed, although induction of IEG products will occur in response to spreading depression (83). Its importance in ischemia is due to the recent recognition that spreading depression may occur in focal cerebral ischemia and worsen ischemic damage. Control of this phenomenon is observed to lessen ischemically induced dysfunction following three hours of focal ischemia in the rat (84). Injury may be due to worsening of Ca^{2+} influx into ischemic neurons, potentially through calcium channel activation

secondary to membrane depolarization. In the face of inadequate energy stores the neuron cannot correct the resulting elevation of $[Ca^{2+}]_i$ and cell death results (85). A recent study of *in vivo* nimodipine binding in recurrent spreading depression in the rat has shown increased binding consistent with the occurrence of VSCC activation (86).

Receptor Operated Calcium Channel (ROCC) Activation in Cerebral Ischemia

The NMDA receptor-operated Ca^{2+} channels (ROCC) are also widely distributed throughout the brain but in particularly high numbers in CA1, hippocampus, striatum and to a lesser extent in the cortex (87). They open in response to glutamate binding to the NMDA receptor and may be the primary route of entry of Ca^{2+} during acute cerebral ischemia (85).

Pharmacology of the ROCC. Glutamate is a major excitatory neurotransmitter acting on both NMDA and non-NMDA receptors (88). Activation of the NMDA receptor produces increased neuronal intracellular calcium ion concentrations due to influx of extracellular calcium through NMDA receptor activated ion channels (89). Calcium current through the ROCC is blocked by Mg^{2+} ions within the channel at resting membrane potentials but this block is removed with cell membrane depolarization (90). The action of glutamate at the NMDA receptor may be antagonized both competitively and non-competitively by ligands binding to the NMDA-ROCC structure (88). MK-801 is a non-competitive NMDA receptor antagonist and binds to the activated, or open, NMDA linked ROCC in a saturable and specific manner (91,92). Agonists acting at the NMDA receptor site regulate binding of this class of non-competitive NMDA antagonists to the ROCC by changing the apparent association and dissociation rate constants (93). Enhancement of MK-801 binding by glutamate to the ROCC has been reported to be preserved at 12 hours following onset of focal cerebral ischemia (94).

ROCC activation in irreversible focal cerebral ischemia. Wallace et al. (95) have used the state dependent binding of MK-801 to the NMDA linked ROCC to identify activation of these calcium channels during focal cerebral ischemia. *In vivo* binding of [3H]MK-801 was measured autoradiographically 75 minutes after MCA occlusion. Following 60 minutes of circulation, increased *in vivo* [3H]MK-801 binding was observed in ischemic cortex and striatum. The authors conclude that the increased [3H] MK-801 uptake in ischemic brain had occurred due to glutamate activation of the NMDA receptors.

Comparison of VSCC and ROCC function in ischemia. As expected from observations of cell membrane depolarization and increased glutamate release during acute cerebral ischemia, radioligand binding studies performed *in vivo* have measured activation of both the L-type VSCC and the NMDA

linked ROCC early in the course of ischemia. However, the magnitude of Ca^{2+} influx through these ion channels may differ considerably. Using autoradiography and *in vitro* binding of [3H]nimodipine and [3H]CGS-19755 (a competitive NMDA receptor antagonist) we have determined regional VSCC and NMDA receptor densities after four hours of irreversible focal ischemia (96). The number of NMDA receptor sites in general was five to ten times greater than L-type VSCC binding sites supporting earlier suggestions that Ca^{2+} entry through the ROCC is the main route of influx in ischemia (85). Furthermore, the magnitude of Ca^{2+} influx may not be the only determinant of the cellular response. Neuronal cell culture studies have demonstrated that equal amounts of Ca^{2+} entering through either class of channel will have unequal effects with Ca^{2+} entry through the ROCC being much more detrimental (97).

The ability of the cell to regulate Ca^{2+} channel function in the setting of acute ischemia is not fully understood. We have observed no change in density of either L-type VSCCs or NMDA receptors following four hours of irreversible focal cerebral ischemia (96) but a decline in ROCC numbers may occur after 12 hours of MCA occlusion (94). However, channel function may not correlate with the quantity of channel protein. Prolonged depolarization of rat pituitary cells will result in rapid internalization of L-type VSCC (98) and a similar mechanism might occur in ischemic neurons, thus, preventing the channel from contributing further to calcium influx.

Finally, the ischemic threshold for Ca^{2+} channel activation is also not fully defined. In a cat model of global ischemia, extracellular glutamate was observed to increase in cortex with blood flows below 20 ml/100 g/min, but above the flow levels for ion pump failure (99). Recent observations by Osuga and Hakim (100) suggest that VSCC activation may start prior to the initial rise in glutamate. In view of the potential for VSCC activation to induce a genetic response in neurons this early activation may offer a mechanism for the brain to respond to an impending ischemic insult.

The Role of Calcium in Inducing Immediate Early Genes (IEG)

While it is likely that excessive Ca^{2+} accumulation can kill a neuron, the possibility that moderate reversible fluxes of Ca^{2+} may induce protective molecular responses is a challenging new proposition that receives a lot of interest. Cerebral ischemia rapidly and transiently increases the expression of a class of inducible genes known as IEGs because of their fast induction kinetics. Both c-*fos* and c-*jun* are members of the IEG group and their protein products, Fos and Jun, are thought to function as transcriptional regulating factors that couple extracellular signals to alterations in phenotype by regu-

lating the expression of specific target genes (101, 102). A large number of studies have demonstrated that ischemia, both focal and global, increases c-fos expression in the brain (103-107). This suggests that Fos may participate in those intracellular events which confer either protection or susceptibility to ischemic cell death.

Experimentation with the pheochromocytoma PC-12 cell line and primary cortical cell cultures have established that c-fos expression can be triggered by Ca^{2+} influx through VSCCs (108,109). The Ca^{2+} channel agonist BAY K 8644 increases c-fos expression in PC12 cells while c-fos, c-jun, NGFI-A and fos-B are induced by this compound in cortical cell cultures (109). Furthermore, stimulation of c-fos expression in PC12 cells by depolarization with either veratridine or high K$^+$ is blocked by the VSCC antagonist nisoldipine (108). Birnberg et al. (110), using a different cell system, have shown that nimodipine inhibits induction of c-fos and AP-1 by membrane depolarization. Activation of VSCCs also appears to be responsible for c-fos induction by excitatory amino acids. For example, kainate-induced c-fos expression in cultured cortical neurons is blocked by both the AMPA / kainate receptor antagonist NBQX and by VSCC antagonists (109) whereas VSCC antagonists fail to reduce c-fos expression evoked by stimulation of NMDA receptors (111). Thus, induction of c-fos expression by AMPA receptors is dependent on Ca^{++} influx through VSCC while NMDA-induced c-fos is considered to be mediated by the movement of Ca^{++} ions through the NMDA channel.

Ischemia and IEG induction. Focal and global ischemia produce brief increases in the expression of c-fos, c-jun, jun-B, NGFI-A and Krox-20 mRNAs, as well as their respective protein products, provided blood flow does not decline below the threshold of metabolic inhibition (103-107,112-116). In view of the wide array of IEGs induced by ischemia, it is difficult to establish whether the induction of a specific combination of IEGs is indicative of survival or cell death (for a detailed discussion see Kiessling and Gass' review, this symposium).

In a 4-VO model of global ischemia, two waves of c-fos induction have been observed prior to delayed cell death suggesting that c-fos may participate in this process (104). Because c-fos cannot act alone and it must first bind to the protein product of another IEG, usually a member of the jun family, to form an active complex (AP-1), Wessel et al. (115) examined the effects of ischemia on both c-fos and c-jun mRNA in the same 4-VO model. Like Jorgensen et al. (104), they reported two peaks in c-fos induction in CA1 neurons, the second peak occurring 24 to 48 hours after ischemia. Moreover, this pattern of c-fos induction was paralleled by two peaks in the expression of c-jun mRNA. Thus, jun may be the other component of AP-1 which participates in those intra-

cellular events culminating in either death or survival.

Enhancing the brain's resistance to ischemia. A brief ischemic insult can increase the brain's tolerance to the damaging effects of a subsequent longer episode of global ischemia. Initially, Kitagawa et al. (119) reported that two times two minute episodes of ischemia, spaced one day apart, offered complete protection against CA1 neuron damage produced by five minutes of ischemia occurring two days later. However, if the time between these two times two minute ischemic episodes was reduced to 12 hours, the protective effect was lost. These investigators concluded that a one day interval between the two short episodes of anoxia was necessary to allow for alterations in gene expression necessary for the production of protective protein(s). These results were confirmed and extended by Kato and collaborators (120) who showed that a single two minute period of ischemia protected CA1 neurons against necrosis produced by three minutes of ischemia if it occurred one to seven days previously. However, this protective effect was lost if the interval was reduced to six hours or increased to 14 days. Lastly, Kirino et al. (121) demonstrated that two minutes of ischemia prevented the delayed death of CA1 neurons produced by five minutes of ischemia if it was imposed one, two or four days earlier. These results indicate that not only can brief periods of anoxia be protective against the necrotic effects of a longer episode of ischemia, but that the time interval between the protective and noxious events is critical to the eventual outcome.

It is usually held that this complex relationship is related to the expression of heat stress proteins. However, since the time intervals studied are compatible with those needed for induction of IEG's, it is possible that alterations in IEG expression may also be involved in the development of "ischemic tolerance" in CA1 neurons.

Conclusion

Increased [Ca^{2+}]$_i$ is recognized as a principle mechanism of neuronal injury and death in cerebral ischemia. Extracellular calcium enters ischemic neurons through both VSCC and NMDA linked ROCC. Initially, channel activation is reversible and recent evidence would indicate that brief exposures to elevated [Ca^{2+}]$_i$ may be beneficial in the setting of impending ischemia. Calcium entry through activated ion channels results in a wide array of IEG production which may be of importance for the outcome of the ischemic insult.

Acknowledgments

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