Glutamate and GABA dynamics in early ischaemia of rat hippocampal slices

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During brain ischaemia, the run-down of transmembrane ion gradients caused by the fall of ATP levels occurring leads to a rise in extracellular glutamate and GABA concentrations. The rise of glutamate concentration triggers neuronal death. In simulated ischaemia of hippocampal slices, taken from rats humanely killed in accordance with UK animal use legislation, we have used receptors in whole-cell patch-clamped CA1 pyramidal cells to sense released glutamate and GABA. E_{Cl} was set to 0 mV, so that currents mediated by ionotropic GABA receptors were inward, and membrane current was recorded at −30 mV to allow glutamate sensing by NMDA and AMPA receptors.

On applying superfusion solution mimicking the energy deprivation occurring during severe ischaemia (no oxygen and glucose, cyanide and iodoacetate present), a slow small increase of inward current occurred over the first few minutes, followed by a sudden massive inward current (nanoamps) which then sagged back to a less inward plateau (Rossi *et al.* 2000). The massive inward current corresponds to the 'anoxic depolarization' known to occur *in vivo*, when $[K^+]_o$ and [glutamate] rise to ~60 mM and 200 μM, respectively. Before this anoxic depolarization current, there was an increase in spontaneous EPSCs and IPSCs. Applying bicuculline or GABAzine during the maintained plateau current generated an outward current shift, demonstrating the occurrence of GABA release induced by the ischaemia. None of these events were affected by blocking action potentials with tetrodotoxin.

With bicuculline present throughout, a similar sequence of current changes was observed (Rossi *et al.* 2000). Both the initial anoxic depolarization current and the maintained plateau current were greatly reduced by glutamate receptor blockers, and so are produced by glutamate release. The glutamate-mediated current was not affected by blockers of Ca^{2+}-dependent transmitter release, but was blocked by preloading cells with a slowly transported glutamate analogue (PDC) to block glutamate release by reversal of plasma membrane glutamate transporters (Rossi *et al.* 2000). Blocking the glial glutamate transporter GLT-1 with dihydrokainate, or knocking it out in transgenic mice, had no significant effect on the rise of $[glutamate]_o$ in ischaemia, suggesting that glutamate release is by a neuronal transporter and that glia do not take up significant glutamate in ischaemia (Hamann *et al.* 2002). Reversal of neuronal transporters may occur more easily than reversal of glial transporters because $[glutamate]_i$ is normally lower in glia due to glutamate conversion to glutamine by glutamine synthetase.

One ATP-dependent mechanism that will be blocked in ischaemia is the Na/K pump. In non-ischaemic solution, blocking the Na/K pump with ouabain produced a large transient inward current like the glutamate-mediated current underlying the start of the anoxic depolarization in ischaemia, but produced less sustained glutamate release than did ischaemia. However, in the presence of bafilomycin to block the vesicular H^+-ATPase, or methionine sulfoximine to block glial glutamine synthetase, ouabain produced a maintained $[glutamate]_o$ rise more similar to ischaemia. This suggests that, following a fall of [ATP] in ischaemia, loss of glutamate from vesicles caused by inhibition of the H^+-ATPase may raise the cytoplasmic glutamate concentration and thus promote the release of glutamate which occurs by the reversal of plasma membrane transporters when the Na/K pump is inhibited. Furthermore, inhibition of glutamine synthetase by the fall of ATP occurring in ischaemia may prevent a protective uptake of glutamate into glia in early ischaemia: with glutamine synthetase inhibited, uptake of only a little glutamate released from neurons may be sufficient to raise $[glutamate]_i$ in glia sufficiently to inhibit further uptake.

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All procedures accord with current local guidelines.

Cation channels: a radical way of killing cells

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IL-1 and neurodegeneration

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Inflammation in the brain is now recognised as a major contributor in diverse CNS diseases. Amongst the inflammatory molecules, the cytokine interleukin-1 (IL-1) has been identified as a key mediator of neurodegeneration. IL-1 expression is induced rapidly in clinical and experimentally induced neurodegeneration, largely by glia. Administration of IL-1 exacerbates neuronal injury in rodents and, most importantly, inhibition of the expression, release or actions of endogenous IL-1 markedly reduces experimentally induced ischaemic, traumatic and excitotoxic brain injury. Understanding the mechanisms underlying the expression, release and actions of IL-1 is therefore of major scientific and clinical relevance.

Expression of IL-1 in the brain can be induced by many toxic insults, and is specifically increased in response to hypoxia, excessive neuronal activation and bacterial products. Release and activation of both forms of IL-1 (α and β) can be induced by activation of purinergic ($P2X_7$) receptors in microglia in response to extracellular ATP and possibly other stimuli. IL-1β has no leader sequence so its mechanism of release is not known, but depends on release of intracellular calcium stores and cleavage by caspase 1. IL-1 is believed to act via a single receptor (IL-1R1) though our data suggest the presence of additional signalling receptors. IL-1's mechanisms of action on neurodegeneration are largely unknown and probably multiple. IL-1 can protect neurones *in vitro* from excitotoxic insults, but induces the release of neurotoxins from glia. We have identified specific sites of action of IL-1 in the brain where it can induce or exacerbate extensive neuronal loss at distant sites in the brain, possibly due to enhancement of seizures. It may also influence neuronal susceptibility or death through its many physiological and pathophysiological actions, e.g. on body temperature, blood–brain barrier integrity, extracellular matrix, neurotoxin invasion of immune cells into the CNS, cerebral oedema and synaptic plasticity.

The endogenous inhibitor of IL-1, IL-1 receptor antagonist (IL-1ra), appears to act as a functional inhibitor of neurodegeneration, and is a potential clinical treatment for stroke, brain injury and related disease.

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Plasma membrane calcium pumps: molecular switches between apoptosis and necrosis

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Mitochondrial contribution to cell death in the ischaemic heart

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Although no doubt exists that the maintenance of tissue viability requires optimal mitochondrial function, the ensuing cell death is often determined by mitochondria. This concept is made dramatically clear by the sequence of events that characterizes the ischaemic damage in the heart. Three major phases can be described (Di Lisa *et al.* 1998). The first is associated with the onset of ischaemia, and changes mitochondria from ATP producers into powerful ATP utilizers (Di Lisa *et al.* 1995). During this phase, the inverse operation of F_0F_1 ATPase maintains the mitochondrial membrane potential by using the ATP made available by glycolysis. The second phase can be identified from the functional and structural alterations of mitochondria caused by prolongation of ischaemia, such as decreased utilization of NAD-linked substrates, release of cytochrome *c* and involvement of mitochondrial channels. These events indicate that the relationship between ischaemic damage and mitochondria is not limited to the failure in ATP production. Finally, the third phase links mitochondria to the destiny of the myocytes upon post-ischaemic reperfusion. Indeed, depending on the duration and the severity of ischaemia, not only is mitochondrial function necessary for cell recovery, but it can also exacerbate cell injury. Indeed, it is generally accepted that the rupture of sarcolemma results from an uncontrolled activation of contraction in cells lacking the possibility of relaxation. Such a condition results from a suboptimal recovery of mitochondrial function because reduced contents of ATP co-exist with elevated concentrations of intracellular Ca^{2+}.

A relevant link between intracellular Ca^{2+} overload and mitochondrial dysfunction is represented by the opening of the mitochondrial permeability transition pore (Bernardi, 1999). The permeability transition is a regulated permeability increase of the mitochondrial inner membrane to solutes with molecular masses up to 1500 Da mediated by opening of a high-conductance channel, the permeability transition pore (PTP), whose molecular nature remains debated. The PTP is modulated by a variety of effectors of cell death, including calcium, lipid mediators and reactive oxygen species (Bernardi, 1999). We have recently elucidated the role of PTP in the reperfusion damage by investigating NAD^+ metabolism (Di Lisa *et al.* 2001; Di Lisa & Ziegler, 2001). In fact, mitochondrial NAD^+ content, which is hardly affected during ischaemia, becomes almost depleted when coronary flow is restored after a prolonged period of ischaemia. The inhibition of mitochondrial NAD^+ depletion exerted by CsA suggests that upon reperfusion the rise in intracellular Ca^{2+}, along with the recovery of neutral pH and the boosting of oxyradical generation, promotes PTP opening, causing the release of intramitochondrial NAD^+ and its subsequent hydrolysis. Not only is the decrease of mitochondrial NAD^+ prevented when PTP is inhibited, but also tissue viability is significantly protected (Di Lisa *et al.* 2001). Besides affecting energy metabolism, the mitochondrial release of NAD^+ is likely to modify several intracellular processes triggered by ischaemia or other pathological conditions. Indeed, once released out of the mitochondrial matrix, NAD^+ could be transformed into cyclic ADP ribose which promoting Ca^{2+} release from intracellular stores may amplify and extend the effects exerted by an initial rise in intracellular $[Ca^{2+}]$. Thus the release of NAD^+ from mitochondria and its subsequent utilization within other cell compartments could be part of the mechanisms through which mitochondria transduce and amplify an initial trigger provided by reperfusion (Di Lisa & Ziegler, 2001).

Besides the involvement in necrosis, alterations of mitochondrial structure and function appear pivotal in the commitment of cells to apoptosis. Indeed, a severe reduction of the mitochondrial membrane potential, the opening of the permeability transition pore and/or the release of pro-apoptotic proteins often precede the appearance of other characteristic signs of apoptosis, such as phosphatidylserine or DNA fragmentation (Bernardi *et al.* 1999; Hengartner, 2000). The causal relationships between these processes are still debated (Hengartner, 2000; Bernardi *et al.* 2001). A key issue is to assess whether, and when, PTP opening occurs in the course of apoptosis and what are its consequences *in situ*. We investigated the relationship between PTP opening, mitochondrial depolarization, cytochrome *c* release and occurrence of cell death (Petronilli *et al.* 2001). Using a technique developed in our laboratory (Petronilli *et al.* 1999), we could detect both transient and long-lasting PTP openings. While cell viability is hardly affected by PTP openings of short duration, longer PTP openings cause mitochondrial depolarization followed by release of cytochrome *c* and apoptosis. Thus modulation of PTP open time appears to be the key element in determining the outcome of stimuli that converge on the PTP.

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All procedures accord with current UK legislation.

The role of synaptic and non-synaptic mitochondria in neuronal ischaemia

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Mitochondria are increasingly implicated in neurodegenerative mechanisms from several perspectives, including intracellular calcium regulation, free radical production, energy utilization, and release of apoptotic factors. Initially our group demonstrated that chelation of intracellular calcium by membrane-permeant calcium chelators was neuroprotective both in *in vitro* neuronal cultures and *in vivo*, significantly diminishing ischaemic stroke volume (Tymianski *et al.* 1993). All *in vitro* and *in vivo* experiments were performed according to local and national guidelines, including humane killing of animals. We then showed that an 8 min ischaemic (hypoxia/hypoglycaemia) insult to an organotypic hippocampal slice culture, promoted glutamate-mediated generation of free radicals with concomitant elevation of intracellular calcium (Perez Velazquez *et al.* 1997). In this same model, the mitochondrial complex I inhibitor, rotenone, the mitochondrial permeability transition blocker, cyclosporin A (CsA), and a blocker of NAD^+, nicotinamide, decreased ischaemia-induced free radical generation and increased mitochondrial calcium (Frantseva *et al.* 2001). Interestingly, CsA did not diminish the increase in the cytoplasmic calcium, but did reduce the increased mitochondrial calcium, suggesting that mitochondrial calcium could be the most important mediator of neurodegenerative processes.

Synaptic transmission could be an early and sensitive target for cerebral ischaemia. Membrane-permeant calcium chelators diminish synaptic transmission in the *in vitro* hippocampal CA1 region (Ouanounou *et al.* 1996). Brief hypoxia diminished stratum radiatum-evoked synaptic transmission, which was resistant to intracellular calcium chelation by a membrane-permeant calcium chelator (Ouanounou *et al.* 1999). Preliminary experiments in acutely prepared rat hippocampal slices have shown that during and following 8 min of hypoxia/hypoglycaemia, stratum radiatum evoked synaptic transmission in the CA1 subfield is depressed along with an increase in mitochondrial calcium which persists for up to 1 h following the ischaemic insult. We can now specifically load stratum radiatum presynaptic terminals with calcium fluorescent and mitochondrial dyes. Further experiments delineating the role of pre- and postsynaptic mitochondria in hypoxia/hypoglycaemia induced depression of synaptic transmission and the role of membrane-permeant calcium chelators will be discussed. We hypothesize that the neuroprotective actions of intracellular calcium chelation could be by limiting rises in intra-mitochondrial calcium and that the maintenance of 'healthy' mitochondria will be neuroprotective against ischaemic insults.

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Long-term effects of ischaemia on CA3 pyramidal neurons: permanent reduction of seizure threshold

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The effects of ischaemia were examined on CA3 pyramidal neurons recorded in hippocampal slices 2–4 months after a global forebrain insult. We show that CA3 post-ischaemic neurons had a more depolarized resting membrane potential but no change of the input resistance, spike threshold and amplitude, fast and slow AHP or ADP and firing properties in response to depolarizing pulses. Although there were no spontaneous network-driven discharges, the post-ischaemic synaptic network had a smaller threshold to generate evoked and spontaneous synchronized burst discharges. Thus lower concentrations of convulsive agents (kainate, high K^+) triggered all-or-none network-driven synaptic events in post-ischaemic neurons more readily than in control ones. Also, paired-pulse protocol generates, in post-ischaemics but not controls, synchronized field burst discharges when interpulse intervals ranged from 60 to 100 ms. In conclusion, 2–4 months after the insult, the post-ischaemic CA3 pyramidal cells are permanently depolarized and have a reduced threshold to generate synchronized bursts. This may explain some neuropathological and behavioural consequences of ischaemia as epileptic syndromes observed several months to several years after the ischaemic insult.