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Regulation of Synaptic Transmission by Mitochondrial Ion Channels

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Mitochondria are abundant within neuronal presynaptic terminals, where they provide energy for sustained neurotransmitter secretion. Injection of Bcl-xL protein into squid giant presynaptic terminal potentiates neurotransmitter release, while a naturally occurring, proteolytic fragment of BCL-xL causes rundown of synaptic function. The cleaved form of BCL-xL generates large, multiconductance ion channel activity in synaptic mitochondrial outer membranes. A rapid onset of synaptic rundown can also be produced by depriving the synapse of oxygen, and hypoxia also induces large channel activity in mitochondrial outer membranes. Channel activity induced by cleaved BCL-xL or by hypoxia is attenuated by NADH, an inhibitor of the voltage-dependent anion channel (VDAC) of mitochondrial outer membranes. Finally, the large conductances elicited by hypoxia are prevented by the addition of a protease inhibitor that prevents cleavage of BCL-xL. The opposing activities of BCL-xL and its proteolytic fragment may regulate the release of ATP from mitochondria during synaptic transmission.

KEY WORDS: Neurotransmission; synapse; ischemia; mitochondria; VDAC; BCL-xL.

Regulation of events at the presynaptic terminal of a synapse is important for determining whether a neuronal pathway will become strengthened during such processes as learning and the making of new memories. Conversely, biochemical events at the synapse can cause the synapse to fail during neurodegeneration, such as in Alzheimer's disease, or in acute injury, such as during brain ischemia. Studies of the modulation of synaptic transmission comprise an important area of focus in the field of Neurobiology.

The squid giant presynaptic terminal is a well-established model system for studying neurotransmission. It has an extremely large (1 mm) presynaptic terminal that enables investigators to study synaptic properties with relative ease. Electron micrographs reveal collections of synaptic vesicles adjacent to the area of contact between pre- and postsynaptic cells (Jonas *et al*., 1999; Martin and Miledi, 1975). Deeper inside the terminal are arrays of neurofilaments and numerous mitochondria that are thought to provide the energy for neurotransmission and to manage the calcium that enters at the active zone. Most other types of organelles are absent. In mammalian synapses, presynaptic mitochondria have specific morphological features that differentiate them from other types of mitochondria (Tolbert and Morest, 1982). For example, some presynaptic mitochondria are tethered to active zones and physically linked to chains of vesicles (Rowland *et al*., 2000).

Previous studies have begun to shed light on the role of mitochondria in the synapse, and have hinted that, rather than simply making ATP constitutively to provide the energy for synaptic activity, they regulate the supply of ATP. Mitochondria are also known to buffer cytoplasmic calcium ions. Sustained elevations in presynaptic calcium following rapid, repetitive neuronal firing are not only correlated with enhancement of synaptic transmission (Swandulla *et al*., 1991; Wang and Kaczmarek, 1998), but also require intact mitochondria in secretory cells (Babcock and Hille, 1998) and in neurons (Billups and Forsyth, 2002; Friel and Tsien, 1994: Nguyen *et al*., 1997; Tang and Zucker, 1997). Nevertheless, the specific molecular mechanisms that define the role of mitochondria in calcium and metabolite management during high frequency presynaptic activity are not yet known.

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INTRACELLULAR ION CHANNEL RECORDING TECHNIQUE

The activity of mitochondrial ion channels is required for calcium buffering and release of energy metabolites. To characterize channels on mitochondrial membranes that might be important during synaptic transmission and during apoptosis, we developed a technique to record from intracellular ion channels in intact cells (Jonas *et al*., 1997, 1999). The intracellular organelle recording technique is a variant of the patch clamp technique but the patch electrode is contained within an outer, large bore microelectrode. The concentric electrodes can be manipulated past the plasma membrane, after which the outer electrode is withdrawn, exposing the inner tip. Negative pressure causes the inner tip to form a high resistance seal on intracellular membranes, after which single channel data is gathered either on the organelle, or after excision of the patch into the cytoplasm or bath. Lipophilic fluorescent dyes (Pagano *et al*., 1989) have been included in the patch pipette, and give information about the intracellular location of the pipette tip. In the squid presynaptic terminal, as in many other presynaptic terminals, mitochondria are the only internal organelles that are compatible with seal formation by the patch pipettes, which have tip diameters of approximately ~180–200 nm by scanning electron microscopy.

EFFECTS OF SYNAPTIC STIMULATION ON MITOCHONDRIAL ION CHANNEL ACTIVITY

Mitochondrial recordings inside the resting squid presynaptic terminal reveal small conductance activity. Very infrequently, much larger conductances occur spontaneously. Electrical stimulation of the squid presynaptic terminal to evoke synaptic transmission, however, causes a marked change in activity and conductance of mitochondrial patches. During a brief, high frequency, train of stimuli, mitochondrial ion channel activity increases inside the terminals, resulting in an approximately 60-fold enhancement of membrane conductance lasting up to 60 s (Jonas *et al*., 1999). Activity then gradually decreases in frequency and amplitude over the 5–30 s following the period of enhanced activity.

The delayed onset and the persistence of the mitochondrial channel activity after stimulation implies that this increased activity on the mitochondrial outer membrane is not simultaneous with the opening of plasma membrane channels, and suggests that this increase may depend on an intracellular second messenger. During synaptic stimulation, there is a build-up of calcium in the presynaptic terminal. This is thought to be responsible for a form of short-term synaptic plasticity termed posttetanic potentiation, and this persistent calcium elevation has been found previously to depend on mitochondria (Friel and Tsien, 1994; Tang and Zucker, 1997). In recordings from mitochondrial membranes inside the terminal in a calcium-deficient bathing medium, there is no response to stimulation of the presynaptic nerve, demonstrating that the evoked intracellular membrane channel activity is dependent on calcium influx. Intracellular membrane channel activity is also dependent on an intact mitochondrial membrane potential. Uncoupling mitochondria with FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) completely eliminates the increase in conductance during stimulation. FCCP also eliminates posttetanic potentiation. The timing of the changes in mitochondrial conductance and their dependence on calcium suggest that opening of a mitochondrial channel is important for short-term plasticity of the synapse.

The identities of the mitochondrial channels responsible for changes during synaptic transmission are not yet known. The channels of interest that may undergo regulation during synaptic events include those of both the inner and outer membranes. The calcium-selective uniporter (Kirichok *et al*., 2004), and the calcium-sensitive permeability transition pore (Bernardi, 1996) are candidates for inner membrane channels. Once calcium and metabolites are released from the matrix into the intermembrane space, they are released across the outer membrane to reach the cytosol. The voltage-dependent anion channel (VDAC), which is a ubiquitous protein in mitochondrial outer membrane (Colombini *et al*., 1996) may perform this function. More recently, channels formed by interactions of BCL-2 family proteins with mitochondrial membranes (Kroemer, 1997; Reed, 1997) have been found to release or inhibit the release of mitochondrial components such as cytochrome *c* into the cytosol. Many BCL-2 family proteins, such as BCL-xL, are endogenously present in mitochondrial membranes (Kaufmann *et al*., 2003). BCL-xL is a potent inhibitor of programmed cell death and is abundantly expressed in neurons of the adult brain (Blömer *et al*., 1998; Boise *et al*., 1993; Frankowski *et al*., 1995; González-Garcia *et al*., 1995; Krajewski *et al*., 1994) where its role in developmental apoptosis is obviously constrained. It has been suggested that the role of BCL-xL in adult neurons is to protect cells from death by regulating export of ATP from mitochondria and/or by blocking the activation of proapoptotic proteins (Basañez *et al*., 2002; Vander Heiden *et al*., 2000; Zong *et al*., 2001), but other roles for this important molecule have yet to be elucidated.

Although BCL-2 family proteins are able to conduct ions when reconstituted into artificial lipid bilayers (Antonsson *et al*., 1997; Minn *et al*., 1997; Schendel *et al*., 1998; Schlesinger *et al*., 1997), the precise biochemical mechanisms by which they regulate mitochondrial permeability and apoptosis in cells or whether they form channels in vivo was not previously known.

BCL-xL in the Presynaptic Terminal of the Squid Giant Synapse Enhances Synaptic Transmission and Induces Channel Activity in Mitochondria

BCL-xL is present on mitochondria in the stellate ganglion of the adult squid (Jonas *et al*., 2003), where it could play a role in protection against acute insults to the nervous system. To determine the action of BCL-xL on mitochondrial membrane conductances, we recorded from mitochondria inside the synapse with recombinant BCL-xL in the patch pipette solution (Jonas *et al*., 2003). Activity with multiple conductances was readily detected in these mitochondrial patches (Fig. 1). Activity typically switched between different conductance levels every few seconds, but a single conductance level could also occasionally be maintained for several minutes. Recordings made with BCL-xL in the patch pipette demonstrated larger conductances than those observed in control recordings.

Activity on mitochondrial membranes during synaptic transmission could be a consequence of, or an integral link in the chain of events that leads to posttetanic potentiation. Because BCL-xL produces a change in mitochondrial membrane conductance, it is a possible candidate for a mitochondrial membrane channel that could alter synaptic transmission. Indeed, injection of BCL-xL into presynaptic terminals enhances the rate of rise of postsynaptic responses in both healthy synapses and in those in which transmission has run down to the point that the postsynaptic potential no longer triggers postsynaptic action potentials.

ATP Enhances Synaptic Transmission

Work with nonneuronal cells has suggested that BCL-xL regulates the flux of metabolites across the outer mitochondrial membrane to facilitate transport of ATP into the cytosol following a death stimulus (Vander Heiden *et al*., 2000, 2001). Consistent with these findings, in squid synaptic terminals, direct microinjection of ATP into the presynaptic cell effectively enhances the postsynaptic responses, and injected ATP occludes the effects of BCL-xL. The findings raise the possibility that BCL-xL may enhance synaptic activity by triggering release of ATP from mitochondria, and support the idea that the ion channel function of antiapoptotic proteins may include regulation of the release of ATP. The moderate size of the conductance (200–500 pS) produced by the antiapoptotic protein in mitochondrial membranes suggests that it would be unable to release large components of mitochondria such as cytochrome *c* during apoptosis.

INDUCTION OF LARGE CONDUCTANCES DURING INSULTS TO THE SYNAPSE

In apoptosis or in insults to the nervous system, a different set of changes occur in the outer membrane. These are associated with the release of cytochrome *c* and

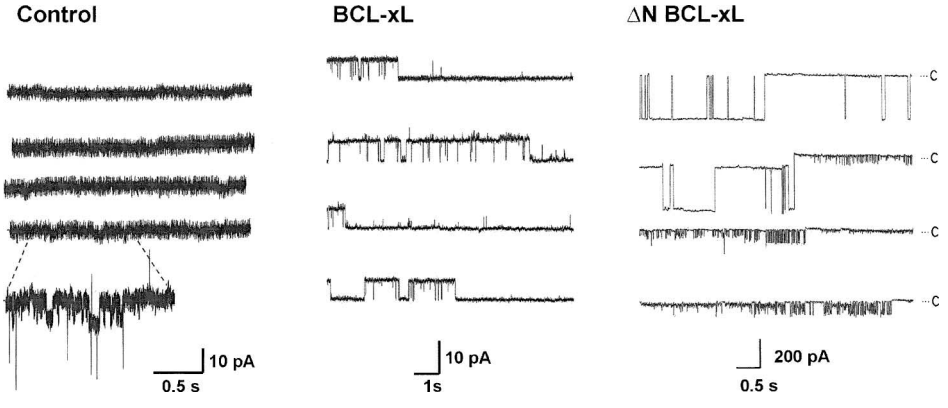

Fig. 1. Different forms of multiconductance channel activity are produced by the antiapoptotic protein BCL-xL and its proapoptotic cleavage fragment ΔN BCL-xL applied to mitochondria within the presynaptic terminal of the squid. Left panel shows small conductance channel activity in control mitochondria.

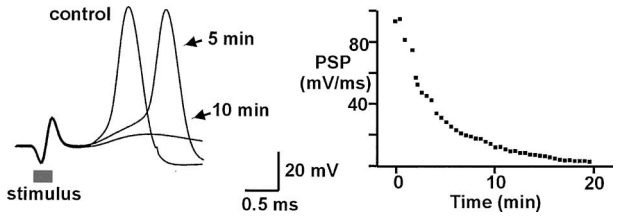

Fig. 2. Hypoxia causes synaptic rundown. Postsynaptic responses are shown at different times after the onset of oxygen deprivation. The second panel shows the change in slope of postsynaptic potentials over time after the onset of oxygen deprivation.

other factors from the intermembrane space (Gross *et al*., 1999). Under these conditions BCL-2 family proteins, either by interaction with VDAC or by independent mechanisms, may also contribute to channel activity in the outer membrane without any activation of the inner membrane (Polster *et al*., 2001). In order to study how mitochondria participate in pathological states of the synapse that might be similar to apoptotic conditions, and how the outer membrane channels VDAC and BCL-xL participate in these events, we studied the reverse of synaptic potentiation, synaptic rundown during hypoxia.

Hypoxia Induces Multiconductance Channel Activity in Synaptic Mitochondria

We used the giant synapse of the squid stellate ganglion as a model system to study the effects of hypoxia on mitochondrial ion channel activity. The presynaptic terminal of this synapse is very sensitive to hypoxia, which attenuates synaptic transmission over 10–30 min (Fig. 2). Hypoxia can be induced in squid giant stellate ganglia by eliminating perfusion of oxygenated sea water. Patch clamp recordings of channel activity on mitochondria during hypoxia show that, in contrast to controls, a new, large multiconductance channel appears on average within about 13 min after the start of hypoxia. Conductances ranging from 300 pS to 2.0 nS can be detected in these hypoxic neurons using invertebrate intracellular solution.

The channel activity produced by hypoxic conditions is much larger than that observed with application of full length BCL-xL to the patch, but closely resembles that produced by applying a proapoptotic version of BCL-xL to the mitochondrial membranes. Whether BCL-xL, which we have shown to be correlated with potentiation of the synapse, could also be responsible for synaptic rundown during hypoxia, is an interesting question. BCL-xL protein can be cleaved between the BH4 and BH3 domains by zVAD-sensitive proteases caspase-3 (Asp61, Asp76) and calpain (Ala60) to produce a proapoptotic C-terminal fragment, ΔN BCL-xL lacking amino acids 2–76 (Clem *et al*., 1998; Fujita *et al*., 1998; Nakagawa and Yuan, 2000). In the squid presynaptic terminal, hypoxia produces proteolysis of BCL-xL, an effect that can be blocked by the protease inhibitor zVAD.

In mammalian cells, overexpression of ΔN BCL-xL potently induces loss of mitochondrial membrane potential, cytochrome *c* release from mitochondria and apoptosis (Basanez *et al*., 2001; Kirsch *et al*., 1999). Injection of ΔN BCL-xL into the squid presynaptic terminal has the opposite effect from that of full length BCL-xL, it attenuates synaptic transmission (Jonas *et al*., 2003). In addition, hypoxic rundown has the same time course as that induced by ΔN BCL-xL and application of ΔN BCL-xL to mitochondrial membranes in intact terminals produces large multiconductance channel activity, similar to that seen during hypoxia. This activity is dependent on an intact BH3 domain and on interaction with mitochondrial membranes. Peak conductances in different recordings range between 300 pS and 3.8 nS, similar to channel activity observed during hypoxia. The large conductance of this channel could be responsible for the release of cytochrome *c* and other proapoptotic factors into the cytosol during hypoxic or degenerative synaptic rundown.

Formation of ΔN BCL-xL Mitochondrial Channels Requires VDAC

Because the gigaohm seals were formed directly on intracellular organelles within the presynaptic terminal, it is likely that the membrane contacted by the patch pipette is the outer mitochondrial membrane. The conductance of this membrane is known to be reduced by millimolar concentrations of NADH (Lee *et al*., 1994; Wunder and Colombini, 1991). In lipid bilayers, NADH has also been shown to reduce the conductance of VDAC, a relatively nonselective channel that is believed to be the major conductance pathway across the outer membrane. In squid, NADH specifically reduces the probability of large conductance activity induced by ΔN BCL-xL or hypoxia in mitochondrial membranes. It fails, however, to inhibit the actions of ΔN BCL-xL on the permeability of artificial lipid membranes. Taken together, the data suggest that NADH alters the conductance of hypoxia- or ΔN BCL-xL-induced channels by acting on a mitochondrial component other than the BCL-xL protein alone.

To further investigate the hypothesis that ΔN BCL-xL or hypoxia require VDAC to form channels, recordings were made on mitochondrial membranes prepared

from wild type yeast and from yeast that lack the *por1* gene, which encodes the VDAC-1 channel (YVDAC1) (Lee *et al*., 1998; Lohret and Kinnally, 1995).

Recording from the outer membrane of isolated wild type yeast mitochondria revealed a voltage-dependent behavior with properties similar to those previously described for VDAC in artificial membranes (Colombini *et al*., 1996). In a separate set of recordings on these wild type mitochondria, the inclusion of ΔN BCL-xL protein in the pipette solution resulted in a very different pattern of activity. The typical VDAC-like activity could no longer be detected, and significantly larger conductance activity was detected at both positive and negative potentials. These large conductances were similar to those observed after the addition of ΔN BCL-xL in squid presynaptic terminal, and were also markedly attenuated by the addition of NADH.

Large conductance activity could be recorded under control conditions in mitochondrial membranes from mutant ΔPOR1 yeast lacking the VDAC channel, but, in contrast to the wild type mitochondria, this activity was completely unaffected by ΔN BCL-xL and was not inhibited by NADH, suggesting that VDAC is required for formation of channels by the ΔN BCL-xL protein.

In summary, by using techniques that allow mitochondrial membrane recordings within living cells, we can begin to describe mitochondrial ion channel activities that play a role in synaptic transmission.

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