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Exposure to Hypoxia Rapidly Induces Mitochondrial Channel Activity within a Living Synapse*

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One of the earliest effects of hypoxia on neuronal function is to produce a run-down of synaptic transmission, and more prolonged hypoxia results in neuronal death. An increase in the permeability of the outer mitochondrial membrane, controlled by BCL-2 family proteins, occurs in response to stimuli that trigger cell death. By patch clamping mitochondrial membranes inside the presynaptic terminal of a squid giant synapse, we have now found that several minutes of hypoxia trigger the opening of large multiconductance channels. The channel activity is induced concurrently with the attenuation of synaptic responses that occurs under hypoxic conditions. Hypoxia-induced channels are inhibited by NADH, an agent that inhibits large conductance channels produced by a pro-apoptotic fragment of BCL-xL in these synaptic mitochondria. The appearance of hypoxia-induced channels was also prevented by the caspase/cysteine protease inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (Z-VAD-fmk), which inhibits proteolysis of BCL-xL during hypoxia. Both NADH and Z-VAD-fmk reduced significantly the rate of decline of synaptic responses during hypoxia. Our results indicate that an increase in outer mitochondrial channel activity is a very early event in the response of neurons to hypoxia and suggest that this increase in activity may contribute to the decline in synaptic function during hypoxia.

Changes in the permeability of outer mitochondrial membranes occur during normal and pathological events in cellular physiology and are also associated with the onset of programmed cell death. Mitochondria release death-promoting factors into the cytosol (1–4). Although in many cells, ischemic insult can lead to programmed cell death (apoptosis or necrosis), neurons are particularly sensitive to oxygen deprivation (5), perhaps because of the high energy demands of synaptic transmission. Changes in synaptic efficacy occur very early during hypoxia and may, indeed, be the first response of the neuron to ischemic insult (6–10). The rapid onset of the effects of ischemia on synaptic transmission suggests that these events could play a role in determining whether the cell will survive (11–13) or die by necrosis or apoptosis (14–15). The synaptic mitochondrion, as the oxygen-sensing organelle, could control this decision.

A variety of lines of evidence indicate that proteins found in, or associated with, mitochondrial membranes participate in responses of a cell to environmental stimuli. BCL-2 family proteins that are either activators or inhibitors of programmed cell death are believed to exert their actions, at least in part, by controlling the permeability of the outer mitochondrial membrane to cytochrome *c* and other apoptosis-inducing factors, perhaps by regulating the opening of ion channels (16–17). Mitochondrial ion channels, such as those regulated by the BCL-2 family protein BCL-xL, may also control the export of metabolites including ATP under normal physiological conditions (18, 2, 19). Consistent with this idea, injection of ATP into run-down synapses causes the return of synaptic transmission (18).

Previous work has shown that BCL-xL is present in mitochondrial membranes of the giant presynaptic terminal of adult squid (18). When recombinant human BCL-xL is applied directly to the outer mitochondrial membrane, it results in the induction of intermediate size channels with conductances of ~200–500 pS.[1] In general, proteases are activated during ischemia (20) and other types of brain injury (21), and inhibition of caspase activity protects against ischemic brain injury (22–23). Consistent with these findings in mammalian brain, levels of BCL-xL decline in squid synaptic mitochondria during hypoxia, and this decline is blocked by the pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (Z-VAD-fmk) (24). Application of a naturally occurring proteolytic cleavage fragment of BCL-xL to mitochondria in the squid presynaptic terminal results in the formation of large conductance channels. The conductance of these channels (up to 3.8 nS) is larger than those produced by BCL-xL itself (24), and they are inhibited by NADH, a known inhibitor of outer mitochondrial membrane permeability (25). These findings suggest that specific proteolytic cleavage of BCL-xL could play a role in regulating synaptic function during hypoxia. Direct evidence for the activation of BCL-2 family proteins or other mitochondrial channel activity in neurons after a pathological insult such as ischemia, however, is still lacking.

In this study, we used an intracellular patch clamp technique to record the activity of mitochondrial membranes in an intact presynaptic terminal. We found that several minutes of

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[1] The abbreviations used are: pS, picosiemens; nS, nanosiemens; Z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone.

hypoxia caused the appearance of novel multiconductance channel activity. The appearance of hypoxia-induced channels was correlated in time with the deterioration of synaptic responses, suggesting that the two events may be linked functionally. NADH attenuated the hypoxia-induced channel activity and potentiated synaptic transmission under hypoxic conditions. The appearance of the hypoxia-induced channels was also attenuated by Z-VAD-fmk, suggesting that proteolysis contributes to the induction of channel activity during hypoxia. In addition, Z-VAD-fmk attenuated the hypoxia-induced decline in synaptic function. Our experiments support the hypothesis that an increase in outer mitochondrial channel activity is a very early event in the response of a neuron to hypoxia.

EXPERIMENTAL PROCEDURES

Intracellular Membrane Patch Clamp Recordings—Experiments were performed on small *Loligo pealii* at the Marine Biological Laboratory, Woods Hole, MA, as described previously (26, 18). In brief, isolated squid stellate ganglia were pinned to Sylgard in a Lucite chamber. The bathing solution (in mM: 466 NaCl, 54 $MgCl_2$, 11 $CaCl_2$, 10 KCl, 3 $NaHCO_3$, 10 HEPES, pH 7.2) was cooled, oxygenated with 99.5% O_2, 0.5% CO_2, and perfused over the ganglia. During experiments to induce hypoxia, perfusion was stopped. Intracellular membrane pipettes (20–80 megohms) were filled with intracellular solution containing (in mM): 570 KCl, 1.2 $MgCl_2$, 10 HEPES, 0.07 EGTA, 0.046 $CaCl_2$, and 2 ATP, pH 7.2. The mitochondrial patch electrode was contained in an outer, ensheathing electrode that was used to enter the terminal, after which the outer electrode was retracted, exposing the patch pipette tip (for more detailed methods, see Ref. 26). Gigaohm seals formed either spontaneously or in response to slight negative pressure. The polarities of potentials reported here refer to those of the patch pipette relative to that of the ground electrode, which was placed in the external medium.

Injection of Presynaptic Terminal and Measurement of Postsynaptic Responses—Intracellular microinjection pipettes were filled with intracellular squid solution and were inserted into the presynaptic terminal. Synaptic transmission was evoked by stimulating an external suction electrode attached to the presynaptic nerve. The nerve was stimulated at 0.033 Hz, 20 V, 0.01 ms to elicit single action potentials. The postsynaptic responses were recorded by an electrode containing 3 M KCl inserted into the postsynaptic nerve. Transmitter release was measured by recording the initial rate of rise of the postsynaptic response (27, 28, 18). The initial rates of increase of the postsynaptic responses were calculated using pClamp 8.0 Clampfit software (Axon Instruments) by placing a cursor at the first onset of the synaptic response, determined by eye, and a second cursor at a time point 100–300 μs later, before any detectable regenerative response occurred. For experiments in which NADH was injected into the presynaptic terminal, the microelectrodes were filled with 140 mM NADH in intracellular solution. Pulses of positive pressure (20–40 p.s.i., 100 ms) were given with a Picospritzer (General Valve, Parker Hannifin Corporation).

To produce hypoxic conditions, the perfusion of the stellate ganglion with oxygenated seawater was stopped. To determine the levels of oxygen in the medium under these conditions, measurements of oxygen levels were made with an oxygen electrode (Hansatech, Norfolk, UK). In the presence of ganglia, the oxygen content of the medium decreased by 48.1 ± 1.1% (n = 3) from 320 to 166 nmol/ml in 20 min (24).

RESULTS

Hypoxia Produces Run-down of Synaptic Transmission—To determine whether hypoxia alters mitochondrial membrane activity in a living neuron, we used the giant synapse of the squid stellate ganglion as a model system. The dissected synaptic preparation is very sensitive to hypoxia and requires a continuous flow of oxygenated seawater to maintain a responsive synapse. Cessation of the seawater perfusion usually causes an attenuation of synaptic transmission over the next 10–30 min (Fig. 1*a*). To show this, recordings were made from the postsynaptic axon while stimulating the presynaptic nerve once every 30 s under hypoxic conditions. The amount of neurotransmitter released from the presynaptic neuron was estimated by measuring the rate of increase of the postsynaptic potential that precedes a triggered action potential. In response to hypoxia, the mean half-time for the decrease in postsynaptic responses was 9.75 ± 1.18 min (n = 17). After re-oxygenation, recovery from this hypoxia-induced synaptic run-down was variable from synapse to synapse. After 40 min or more of hypoxia, many synapses failed to recover after restoration of oxygenation. The mean percentage recovery of synaptic responses, measured 20 min after the start of re-oxygenation, was 2.4 ± 2.4%, compared with values measured prior to the onset of hypoxia (n = 8).

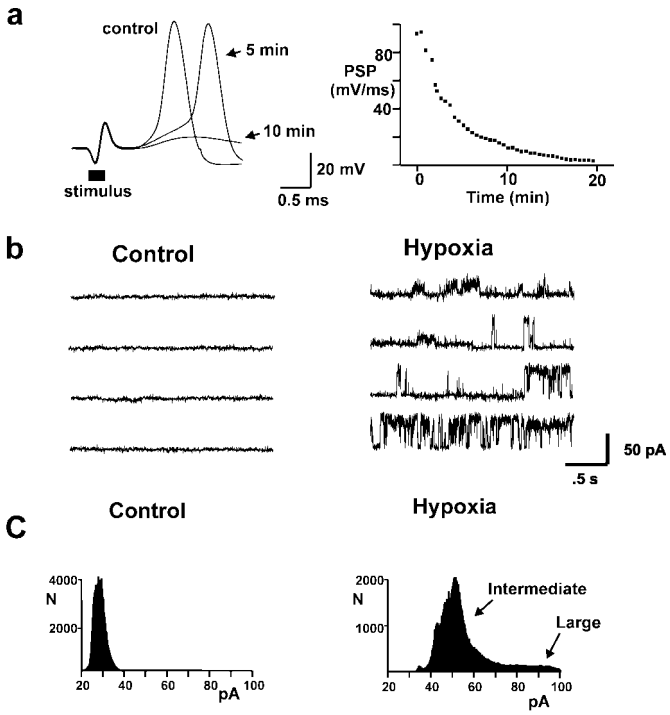

FIG. 1. **Induction of multiconductance channel activity on mitochondrial membranes by hypoxia.** *a*, loss of synaptic responses at the squid stellate ganglion giant synapse during hypoxia. *Left panel* shows representative traces of postsynaptic responses recorded prior to (*control*) and 5 min and 10 min after cessation of perfusion with oxygenated medium. The initial rate of rise of the postsynaptic potential (*PSP*) declines over time and, by 10 min, fails to reach threshold for an action potential. The time course in the *right panel* shows an example of rapid decline in the rate of rise of the postsynaptic response after cessation of perfusion of the preparation with oxygenated medium. *b*, a comparison of channel activity on mitochondrial membranes recorded at +100 mV before and 40 min after cessation of perfusion of oxygenated seawater. The recording shows that large conductance channel activity is induced in this mitochondrial outer membrane patch after cessation of perfusion with oxygenated seawater. *c*, all points amplitude histograms for a 1-min recording period immediately before (*left*) and 40 min after (*right*) cessation of perfusion. *N* represents the number of events (*y* axis) detected at the indicated current amplitude (*x* axis). In the control (*left*), the peak represents the amplitude of the base-line (leak) current. In the *right panel*, the base line is now a small proportion of the total points that make up the histogram and can be seen as a *small peak* at the *left* of the trace. The *major peak* in the histogram represents an intermediate conductance, and the *smaller peak* at the *right side* of the histogram represents those events contributed by large conductance openings.

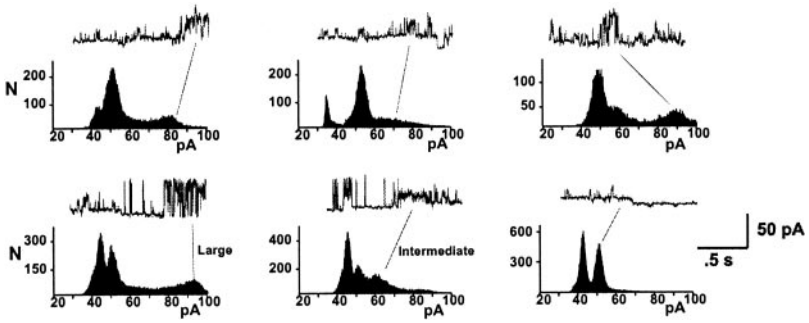

FIG. 2. **Current traces and associated amplitude histograms for six consecutive 10-s recording periods (+100 mV pipette potential) after induction of hypoxia.** *Lines* link peaks in the histograms with associated current events and indicate examples of intermediate (>180 pS, <760 pS) and large (>760 pS) conductances. *N* represents the number of events (*y* axis) detected at the indicated current amplitude (*x* axis).

terminals we found that discrete increases in current across the mitochondrial membrane were relatively infrequent and, when they occurred, typically had amplitudes of less than 18 pA (at a pipette potential of −100 or +100 mV). By recording these events at different potentials, it was found that the most prevalent activity in controls had a conductance of <180 pS (18, 29). Similar to these earlier results, we found that the most prevalent ion channel activity in control recordings followed for up to 1 h in ganglia perfused with oxygenated seawater had a conductance of <180 pS ($n = 16$) (Fig. 1*b*, *left*). The overall probability of opening of larger conductances (>180 pS) was very small (<0.02).

In contrast to controls, hypoxic treatment induced mitochondrial channel activities with a much greater amplitude in 10 of the 16 cells tested ($p < 0.0004$, Fisher's exact test) (Fig. 1*b*, *right*). Typically, these larger events had several different current amplitudes that changed throughout the recording. The mean delay from the time of withdrawal of oxygenated medium to the onset of the larger multiconductance channel activity was 12.8 ± 2.7 min, and, in some experiments, large channel activity could be detected as early as 5 min after the onset of hypoxia.

To better characterize the induced channel activity, the size distribution of the various channel openings in these recordings was determined and represented as amplitude histograms (Fig. 1*c*). Discrete peaks in the amplitude histograms represent the closed state (the peak found at ~30 pA in Fig. 1*c*, *left panel*) and various levels of channel openings in the mitochondrial membranes of the control and hypoxic neurons. Fig. 1*c* shows amplitude histograms for 1-min periods of recording before and after induction of hypoxia. Two major peaks in the amplitude histogram from the recording made after induction of hypoxia are labeled "intermediate" and "large." The amplitudes of the peaks in these histograms were not integral multiples of a single unitary conductance. This suggests that the openings do not represent the openings of many channels all of which have the same conductance (30). Instead, the finding suggests that one channel may be able to produce different levels of current amplitude or that several different types of channel activity are induced by hypoxia within each patch.

Our data support the hypothesis that hypoxia induces channels with multiple different conductances rather than the opening of several independent types of channels. In particular, we found that the conductance of a channel could remain stable for periods of time lasting for tens of seconds and then could change abruptly to a new level concomitantly with the disappearance of the previous level (Fig. 2). Such events are very unlikely to occur simultaneously for several independent channels in a patch. Thus, in 1 min of continuous recording, different conductance levels could be detected in successive amplitude histograms covering each 10 s of the recording (Fig. 2).

To provide an accurate measure of the hypoxia-induced channel conductances and to compare them with those of channels induced by proapoptotic BCL-2 family proteins (24), we determined the current-voltage relationships for the hypoxia-induced channels. This relationship between the amplitude of discrete channel openings and the membrane voltage was obtained by rapidly stepping the holding potential across a range of voltages during a series of openings at one conductance level. The majority of openings had current-voltage relationships that were approximately linear, with a reversal potential close to 0 mV (Fig. 3*a*). No systematic dependence of the probability of channel opening on the holding voltage was detected. In a minority of cases, however, smaller amplitude openings induced by hypoxia, corresponding to conductances less than ~300 pS, had current-voltage relationships that were outwardly rectifying (Fig. 3*b*).

In general, amplitude histograms of channel activity in the hypoxic neurons demonstrated the induction of discrete conductances between 180 pS and 2.0 nS. To compare all of the independent experiments, we divided channel openings into three groups according to their conductance (18, 24). These groups were selected based on peaks of amplitude histograms such as those shown in Fig. 1*c*. Small openings were defined as those with conductances <180 pS, and these comprise the majority of openings in control recordings. Intermediate conductances were defined as those between 180 pS and 760 pS, whereas large conductances were defined as greater than 760 pS. Channel activity produced by hypoxia underwent reversible transitions between intermediate and large conductance levels. Frequently, one channel opened and closed repeatedly to a single amplitude level for periods of time lasting tens of seconds (Fig. 3*c*). For each experiment, the proportion of time spent at these conductance levels was determined prior to hypoxia and at the time of maximally induced channel activity within 30 min of the onset of hypoxia. Prior to the onset of hypoxia, the majority of channel openings were <180 pS (small). In contrast, after the induction of channel activity, the majority of the openings were intermediate or large, and channels were less likely to remain closed (Fig. 3*d*).

Channels Induced by Hypoxia Are Attenuated by NADH—It is likely that the membrane contacted by the patch pipette in these squid preparations is the outer mitochondrial membrane because the gigaohm seals were formed directly on intracellular organelles within the presynaptic nerve cell endings. To provide further evidence, the effect of NADH addition was determined. The conductance of mitochondrial outer membranes has been shown previously to be reduced by millimolar concentrations of NADH (25, 31). In lipid bilayers, NADH has also been shown to reduce the conductance of the voltage-dependent anion channel, a relatively non-selective channel that is believed to be the major conductance pathway across the outer membrane (31). In addition, a pro-apoptotic proteolytic cleavage fragment of BCL-xL produces large conductance mitochondrial channel openings that are attenuated by NADH both in the squid presynaptic terminal and in isolated yeast mitochondria (24). We therefore tested the effect of this agent on the induction of channel activity by hypoxia in the presyn-

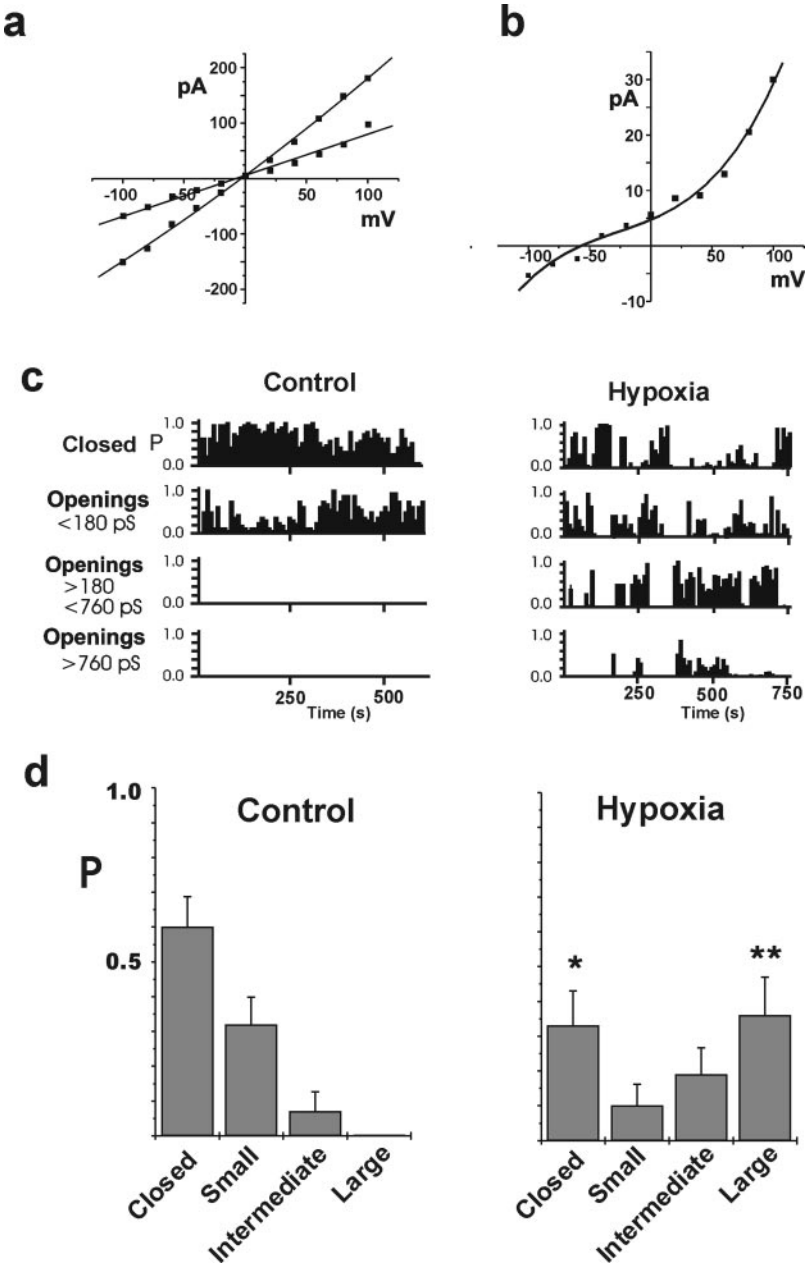

FIG. 3. **Characterization of hypoxia-induced channel activity.** *a*, current-voltage relationships for hypoxia-induced channel activity show that the channel activity has multiple conductances. Two different conductance levels of a channel induced by cessation of perfusion of oxygenated seawater (740 pS and 1.64 nS) are shown. *b*, current-voltage relationships for a lower-conductance outwardly rectifying channel observed after induction of hypoxia. *c*, time course of transitions of channel opening to different conductance levels obtained in a single patch shows that intermediate and large channel activity appears and disappears reversibly after the onset of hypoxia. *Left panel* shows transitions in a control recording; *right panel* shows transitions on the same membrane after induction of hypoxia. Openings were divided into three groups: <180 pS, >180 pS, <760 pS), and >760 pS. Probability of occurrence of openings in each group within successive 10-s recording periods is plotted as a function of time (*P* represents probability). *d*, bar graphs combining all experiments show that the probability of large channel activity is greater in patches after the onset of hypoxia. Bar graphs show the relative contribution of closed channels (*Closed*), activity at less than 180 pS (*Small*), activity between 180 and 760 pS (*Intermediate*), and activity greater than 760 pS (*Large*). Probabilities (*P*) were calculated for 10-s recording periods. The panels show the maximum channel activity seen during the control recording period (*left*), and within 30 min of the induction of multiconductance activity (*right*) ($n = 16$) (*, $p < 0.04$; **, $p < 0.008$).

aptic terminal. Inclusion of 1 mM NADH in the patch pipette before and after induction of hypoxia greatly reduced the probability of occurrence of the large conductance activity ($n = 5$, Fig. 4*a*).

NADH Inhibits Synaptic Run-down and Restores Synaptic Responses during Hypoxia—Because NADH alters the ability of the outer mitochondrial membrane to flux ions and metabolites, we reasoned that NADH may affect the strength of synaptic transmission. Release of neurotransmitter from the presynaptic terminal is known be influenced by the functional state of mitochondria. Mitochondria buffer intracellular calcium and other ions and produce the ATP necessary for repetitive synaptic events, activities that could be substantially altered in the presence of an agent that changes mitochondrial membrane properties. If the large conductance channels induced by hypoxia contribute to the mitochondrial dysfunction that results in decline in synaptic activity, then inhibition of these channels by NADH should alter the rate of synaptic run-down. Indeed, we found that intracellular injection of NADH (140 mM in the pipette) into the presynaptic terminal during the onset of hypoxia caused a transient enhancement of synaptic responses (39.6 ± 13.4% increase, $n = 8$) that endured for several minutes (10.15 ± 3.43 min) (Fig. 4*b*). The NADH injections delayed, but did not fully block, the ultimate elimination of synaptic responses caused by oxygen deprivation when stimulating at 0.033 Hz.

Because of the transient nature of the effect of NADH injection on synaptic responses, we assayed the effects of NADH injection on the rate of synaptic run-down in a paradigm that produces depression more rapidly. The presynaptic terminal was stimulated at a frequency of 2 Hz, which produces a depression in synaptic responses even under normoxic conditions (27). After cessation of oxygenation, the mean half-time for run-down of synaptic responses at this frequency was 1.76 ± 1.13 min ($n = 4$). If the presynaptic terminal was preinjected with NADH immediately prior to cessation of oxygenation, the mean half-time for run-down was lengthened to almost 7 min ($n = 5$, $p < 0.05$, Fig. 4*e*). In addition, as at 0.033 Hz, repeated injection of NADH during stimulation at 2 Hz produced an enhancement of synaptic responses even under hypoxic condi-

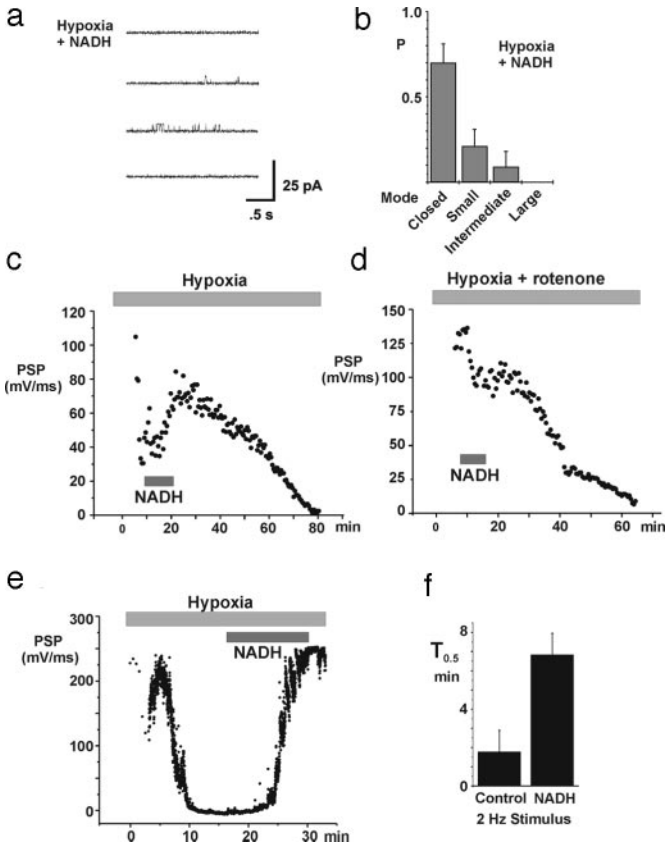

FIG. 4. **Effects of NADH on multiconductance channel activity produced by hypoxia and on synaptic responses.** *a*, example of channel activity recorded at +100 mV using a patch pipette solution containing 1 mM NADH, 21 min after cessation of perfusion of oxygenated seawater. Only small conductance activity occurs in the patch. *b*, bar graphs show the distribution of channel open probabilities (*P*) (*Small*, <180 pS; *Intermediate*, 180–760 pS; or *Large*, >760 pS) for patches recorded in the presence of 1 mM NADH between 20 and 30 min after cessation of perfusion of oxygenated seawater ($n = 5$). A comparison of the bar graphs obtained during hypoxia with and without NADH (see Fig. 3*d*) indicates that large conductances are suppressed in NADH-treated patches. *c*, example of the effect of injection of NADH into the presynaptic terminal on synaptic responses during hypoxia. NADH caused transient recovery of synaptic transmission. Synaptic responses were evoked every 30 s (0.033 Hz). The injection pipette contained 140 mM NADH in intracellular solution, which was injected by repeated pulse of pressure (100 ms, 40 p.s.i.). *d*, injection of NADH into the presynaptic terminal during synaptic run-down produced by hypoxia in the presence of 10 μM rotenone (0.033-Hz stimulation) produces transient recovery of synaptic responses. *e*, NADH injection into the presynaptic terminal after the onset of hypoxia (2-Hz stimulation) produces recovery of synaptic transmission. *f*, bar graphs showing the mean half-time ($T_{0.5}$) for the decrease in synaptic responses produced by hypoxia during stimulation at 2 Hz, with ($n = 5$) or without ($n = 5$) prior injection of NADH into the presynaptic terminal. *PSP*, postsynaptic potential.

tions (Fig. 4*d*). In a further study at 0.033 Hz, NADH injections also affected the reversibility of hypoxic run-down. At 20 min of reperfusion of synaptic preparations with oxygenated seawater, there was a significant increase in the extent of recovery of the rate of increase of postsynaptic potentials (57.6 ± 38.2%, $n = 5$) compared with non-injected synapses (2.4 + 2.4%, $n = 8$, $p < 0.03$).

Injection of NADH may have direct metabolic effects as well as effects on the permeability of the outer mitochondrial membrane. We, therefore, tested the effects of NADH injection in the presence of rotenone (10 μM). This agent blocks the metabolic functions of NADH by inhibiting mitochondrial complex I (NADH dehydrogenase) (32). During synaptic run-down produced by hypoxia in the presence of rotenone, NADH injection into the presynaptic terminal caused a transient enhancement of synaptic responses that endured for several minutes ($n = 3$, Fig. 4*c*). These findings imply that NADH can affect synaptic function independently of its actions at complex I, possibly by inhibiting the increase in outer membrane conductance that occurs with hypoxia.

Z-VAD-fmk Inhibits Induction of the Hypoxia Channel and Slows Synaptic Run-down—Increases in the permeability of the outer mitochondrial membrane occur during programmed cell death and are controlled by BCL-2 family proteins. It is known that cleavage of several BCL-2 family proteins, including BCL-xL, by proteases during programmed cell death enhances or activates their pro-death function (33–40). In the squid stellate ganglion, we showed previously that hypoxia induces proteolysis of native BCL-xL and that a proteolytic fragment of this protein produces large conductance channel activity when applied to mitochondrial membranes (24).

To determine whether proteases facilitate the large channel openings that are triggered by hypoxia, the ganglion was pretreated with Z-VAD-fmk (100 μM), a caspase/calpain inhibitor that inhibits the hypoxia-induced cleavage of BCL-xL. Z-VAD-fmk (100 μM) was included in the recording pipette. Treatment with Z-VAD-fmk potently inhibited the appearance of the large hypoxia-induced channels on mitochondria (Fig. 5*a*). In eight of eight experiments, no large conductance (>760 pS) channels were detected during recordings lasting for 27–47 min, except in one experiment in which such channel activity was observed only transiently at 22 min after the onset of hypoxia.

We also tested the effects of Z-VAD-fmk on the rate of run-down of synaptic responses produced by hypoxia. Treatment of ganglia with 100 μM Z-VAD-fmk during hypoxia significantly slowed the rate of run-down of synaptic responses, lengthening the mean half-time for the decrease in responses from 9.75 ± 1.18 min to 34.35 ± 12.71 min ($n = 6$, $p < 0.004$, Fig. 5, *b* and *c*).

DISCUSSION

Understanding the basic cellular mechanisms that operate during hypoxic-ischemic brain injury is of utmost importance in designing preventative treatments. Previous work has indicated that protease activation and increased levels of pro-death BCL-2 family members occur during ischemic injury and that run-down of neurotransmission is one of the earliest events in hypoxic injury to a neuron (41–45). By recording intact mitochondria in a living terminal, we have now found that within a few minutes of the onset of hypoxia, a death stimulus to neurons, multiconductance channel activity is induced in mitochondrial membranes and that this occurs during the run-down of synaptic transmission. It is likely that our recordings represent channel events primarily occurring in the outer mitochondrial membrane, because no steps were taken to disrupt the outer membrane.

Findings from a previous study also indicate that mitochondrial channel activity may be induced by a death stimulus (46). It was found that the macroscopic conductance of isolated mitochondria prepared from hematopoietic cells undergoing serum deprivation was larger than that of control cells. Although unitary channel activity could not be discerned in that study, large multiconductance activity could be detected in proteoliposomes prepared from the apoptotic cells, raising the possibility that such activity also occurs in live cells.

We have found previously that the BCL-2 family protein BCL-xL is present on mitochondria in the adult squid presynaptic terminal (18). Injection of this anti-apoptotic protein into the terminal produces an enhancement of synaptic transmission. Conversely, injection of ΔN BCL-xL, the naturally occurring cleavage product of BCL-xL, causes run-down of synaptic transmission, similar to that shown here during hypoxia (18).

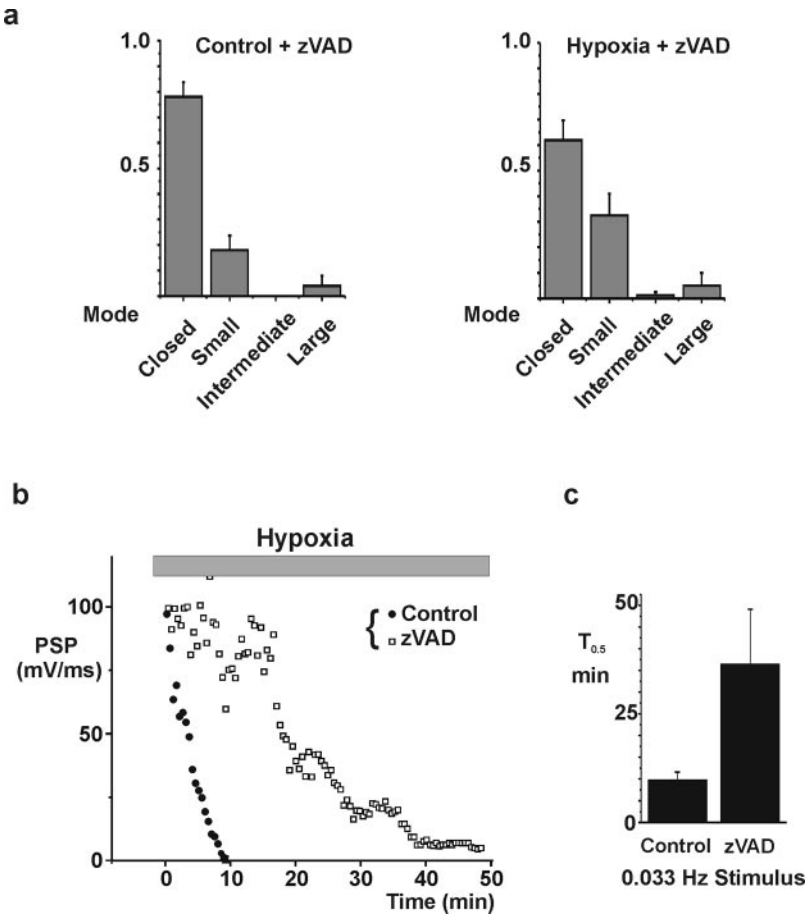

FIG. 5. **Effects of Z-VAD-fmk on multiconductance channel activity produced by hypoxia and on synaptic responses.** *a*, Z-VAD-fmk prevents hypoxia-induced mitochondrial channel activity. Bar graphs show the probability of closed channels, and small, intermediate, and large conductance for intracellular mitochondrial membrane channel activity during Z-VAD-fmk treatment (100 μM) before (*left*) and after (*right*) the onset of hypoxia ($n = 8$). *b*, the time courses of run-down of synaptic responses after cessation of perfusion of oxygenated medium in the presence or absence (*Control*) of 100 μM Z-VAD-fmk. Z-VAD-fmk greatly slows synaptic run-down. *c*, bar graphs showing the mean half-time ($T_{0.5}$) for the decrease in synaptic responses produced by hypoxia during stimulation at 0.033 Hz, in the presence ($n = 6$) or absence ($n = 17$) of 100 μM Z-VAD-fmk in the bathing medium. *PSP*, postsynaptic potential.

Proteolytic cleavage of BCL-xL occurs during hypoxia, and application of ΔN BCL-xL to mitochondria produces large conductance channels (300 pS–3.4 nS) similar to those produced by hypoxia. Thus, it is possible that the large conductance channels detected during hypoxia represent the action of endogenous BCL-xL that has undergone proteolytic cleavage on mitochondrial membranes. The facts that the appearance of hypoxia-induced channel activity is attenuated by pretreatment with Z-VAD-fmk, which attenuates BCL-xL cleavage, and that the channel activity is inhibited by NADH, which also inhibits ΔN BCL-xL-induced large conductance channel activity, are consistent with this hypothesis. Nevertheless, we cannot eliminate the possibility that hypoxia induces large conductance channel activity through an entirely different mechanism with similar pharmacological properties.

The findings that NADH enhances synaptic responses and that NADH and Z-VAD-fmk attenuate the run-down of synaptic transmission during hypoxia are consistent with the hypothesis that the induction of large conductance mitochondrial channel activity is causally linked to the decrement in neurotransmission. This could occur, for example, if the channel activity were associated with a loss of membrane potential across the inner mitochondrial membrane, leading to a loss of ATP production. Alternatively, the efflux of cytochrome *c* or other factors in the intermembrane space through a large conductance pathway in the outer membrane could compromise mitochondrial function or trigger biochemical reactions in the presynaptic cytoplasm. We attempted to determine whether the actions of NADH on enhancing synaptic responses during hypoxia were consistent with its actions on mitochondrial membrane permeability rather than resulting from the metabolism of NADH. We injected NADH in the presence of rotenone, an agent that prevents metabolism of NADH by inhibiting complex I of the mitochondrial respiratory chain. Our finding that enhancement of transmission by NADH could still be detected in the presence of rotenone is consistent with the hypothesis that NADH acts directly, perhaps by inhibiting mitochondrial outer membrane conductance. Nevertheless, although the correlation of the effects of both Z-VAD-fmk and NADH on mitochondrial channel activity and on synaptic transmission during hypoxia is a striking one, we cannot at present definitively establish the mode of action of these agents on neurotransmitter release during hypoxia.

Previous work has shown that certain forms of synaptic plasticity are associated with changes in mitochondrial membrane conductance (29). Studies on mammalian synapses have indicated that hypoxia may produce its effects on synaptic depression, in part, by altering the release of ATP and adenosine from presynaptic terminals (47–49). Our present findings suggest that a change in conductance of mitochondrial membranes is one of the earliest events in the neuronal response to hypoxia and suggest that the induced channel activity is closely linked to the change in transmission, perhaps by altering the flux of ATP, calcium, or other factors close to sites of exocytosis. A change in the permeability of mitochondrial membranes produced by hypoxia may also be linked to the apoptosis pathways that will eventually determine if a neuron will live or die after a hypoxic-ischemic insult.

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REFERENCES

1. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312
2. Vander Heiden, M. G., and Thompson, C. B. (1999) *Nat. Cell Biol.* **1,** E209–E216
3. Zamzami, N., and Kroemer, G. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 67–71
4. Kaczmarek, L. K., and Jonas, E. A. (2003) *Adv. Mol. Cell. Biol.* **32,** 431–457
5. Banasiak, K. J, Xia, Y., and Haddad, G. G. (2000) *Prog. Neurobiol.* **62,** 215–249

6. Aoyagi, A., Saito, H., Abe, K., and Nishiyama, N. (1998) *Brain Res.* **799,** 130–137
7. Howard, E. M., Gao, T. M., Pulsinelli, W. A, and Xu, Z. C. (1998) *Brain Res.* **798,** 109–118
8. Tian, G. F., and Baker, A. J. (2000) *J. Neurophysiol.* **83,** 1830–1839
9. Fleidervish, I. A, Gebhardt, C., Astman, N., Gutnick, M. J., and Heinemann, U. (2001) *J. Neurosci.* **21,** 4600–4608
10. Bolay, H., Gursoy-Ozdemir, Y., Sara, Y., Onur, R., Can, A., and Dalkara, T. (2002) *Stroke* **33,** 1369–1375
11. Lee, J.-M., Zipfel, G. J., and Choi, D. W. (1999) *Nature* **399,** Suppl. 6738, A7–A14
12. Sattler, R., and Tyminanski, M. (2000) *J. Mol. Med.* **78,** 3–13
13. Schwab, B. L., Guerini, D., Didszun, C., Bano, D., Ferrando-May, E., Fava, E., Tam, J., Xu, D., Xanthoudakis, S., Nicholson, D. W., Carafoli, E., and Nicotera, P. (2002) *Cell Death Differ.* **9,** 818–831
14. Sugawara, T., Fujimura, M., Morita-Fujimura, Y., Kawase, M., and Chan, P. H. (1999) *J. Neurosci.* **19,** RC38–RC45
15. Northington, F. J., Ferriero, D. M., Flock, D. L., and Martin, L. J. (2001) *J. Neurosci.* **21,** 1931–1938
16. Kluck, R. M, Bossy-Wetzel, E, Green, D. R, and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136
17. Schendel, S. L, Montal, M., and Reed, J. C. (1998) *Cell Death Differ.* **5,** 372–380
18. Jonas, E. A., Hoit, D., Hickman, J. A., Zhang, J., Brandt, T. A., Yin, D., Ivanovska, I., Fannjiang Y, McCarthy, E., Hardwick, J. M., and Kaczmarek, L. K. (2003) J. *Neurosci.* **23,** 8423–8431
19. Gottlieb, E., Armour, S. M., and Thompson, C. B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 12801–12806
20. Tanaka, H., Yokota, H., Jover, T., Cappuccio, I., Calderone, A., Simionescu, M., Bennett, M. V., and Zukin, R. S. (2004) *J. Neurosci.* **24,** 2750–2759
21. Sullivan, P. G., Keller, J. N., Bussen, W. L., and Scheff, S. W. (2002) *Brain Res.* **949,** 88–96
22. Gillardon, F., Kiprianova, I., Sandkuhler, J., Hossmann, K. A., and Spranger, M. (1999) *Neuroscience* **93,** 1219–1222
23. Inoue, S., Drummond, J. C., Davis, D. P., Cole, D. J., and Patel, P. M. (2004) *Anesthesiology* **101,** 75–81
24. Jonas, E. A., Hickman, J. A., Mushtaque, C., Polster, B. M., Brandt, T. A., Yihru Fannjiang, I., Ivanovska, G. B., Kinnally, K., Zimmberg, J, Hardwick, J., and Kaczmarek, L. K. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 13590–13595
25. Lee, A. C., Zizi, M., and Colombini, M. (1994) *J. Biol. Chem.* **269,** 30974–30980
26. Jonas, E. A., Knox, R. J., and Kaczmarek, L. K. (1997) *Neuron* **19,** 7–13
27. Swandulla, D., Hans, M., Zipser, K., and Augustine, G. J. (1991) *Neuron* **7,** 915–926
28. Morgan, J. R, Prasad, K., Jin, S., Augustine, G. J., and Lafer, E. M. (2001) *Neuron,* **32,** 289–300
29. Jonas, E. A., Buchanan, J., and Kaczmarek, L. K. (1999) *Science* **286**,1347–1350
30. Sakmann, B., and Neher, E. (1995) *Single Channel Recording*, Plenum Press, New York
31. Colombini, M., Blachly-Dyson, E., and Forte, M. (1996) *Ion Channels* **4,** 169–202
32. Sherer, T. B., Betarbet, R., Testa, C. M., Seo, B. B., Richardson, J. R., Kim, J. H., Miller, G. W., Yagi, T., Matsuno-Yagi, A., and Greenamyre, J. T. (2003) *J. Neurosci.* **23,** 10756–10764
33. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) *Science* **278,** 1966–1968
34. Li, H., Zhu, H., Xu, C., and Yuan, J. (1998) *Cell* **94,** 491–501
35. Fujita, N., Nagahashi, A., Nagashima, K., Rokudai, S., and Tsuruo, T. (1998) *Oncogene* **17,** 1295–1304
36. Clem, R. J., Cheng, E. H. Y., Karp, C. L., Kirsch, D.G, Ueno, K, Takahashi, A., Kastan, M. B., Griffin, D. E., Earnshaw, W. C., Veliuona, M. A., and Hardwick, J. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 554–559
37. Kirsch, D. G., Doseff, A., Chau, B., Lin, D. S., de Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A, and Hardwick, J. M. (1999) *J. Biol. Chem.* **274,** 21155–21161
38. Wood, D. E., and Newcomb, E. W. (2000) *Exp. Cell Res.* **256,** 375–382
39. Condorelli, F., Salomoni, P., Cotteret, S., Cesi, V., Srinivasula, S. M., Elnemri, E. S., and Calabretta, B. (2001) *Mol. Cell. Biol.* **21,** 3025–3036
40. Seo, S. Y., Chen, Y. B., Ivanovska, I., Ranger, A.M., Hong, S. J., Dawson, V. L., Korsmeyer, S. J., Bellows, D. S., Fannjiang, Y., and Hardwick, J. M. (2004) *J. Biol. Chem.* **279,** 42240–42249
41. Martinou, J. C., Dubois-Dauphin, M., Staple, J. K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C., and Huarte, J. (1994) *Neuron* **13,** 1017–1030
42. Kitagawa, K., Matsumoto, M., Tsujimoto, Y., Ohtsuki, T., Kuwabara, K, Matsushita, K., Yang, G., Tanabe, H., Martinou, J. C., Hori, M., and Yanagihara, T. (1998) *Stroke* **29,** 2616–2621
43. Alkayed, N. J., Goto, S., Sugo, N., Joh, H. D., Klaus, J., Crain, B. J., Bernard, O., Traystman, R. J., and Hurn, P. D. (2001) *J. Neurosci.* **21,** 7543–7550
44. Pang, Z. P., Deng, P, Ruan, Y. W., and Xu, Z. C. (2002) *J. Neurosci.* **22,** 10948–10957
45. Tekkok, S. B., Godfraind, J. M., and Krnjevic, K. (2002) *Neuroscience* **113,** 11–21
46. Pavlov, E. V., Priault, M., Pietkiewicz, D., Cheng, E. H., Antonsson, B., Manon, S., Korsmeyer, S. J., Mannella, C. A., and Kinnally, K. W. (2001) *J. Cell Biol.* **155,** 725–731
47. Sebastiao, A. M., de Mendonca, A., Moreira, T., and Ribeiro, J. A. (2001) *J. Neurosci.* **21,** 8564–8571
48. Aihara, H., Fujiwara, S., Mizuta, I., Tada, H., Kanno, T., Tozaki, H., Nagai, K., Yajima, Y., Inoue, K., Kondoh, T., Motooka, Y., and Nishizaki, T. (2002) *Brain Res.* **952,** 31–37
49. Frenguelli, B. G., Llaudet, E., and Dale, N. (2003) *J. Neurochem.* **86,** 1506–1515