Ionic Mechanisms Underlying Depolarizing Responses of an Identified Insect Motor Neuron to Short Periods of Hypoxia

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Le Corronc, Hervé, Bernard Hue, and Robert M. Pitman. Ionic mechanisms underlying depolarizing responses of an identified insect motor neuron to short periods of hypoxia. J. Neurophysiol. 81: 307–318, 1999. Hypoxia can dramatically disrupt neural processing because energy-dependent homeostatic mechanisms are necessary to support normal neuronal function. In a human context, the long-term effects of such disruption may become too apparent after a “stroke,” in which blood-flow to part of the brain is compromised. We used an insect preparation to investigate the effects of hypoxia on neuron membrane properties. The preparation is particularly suitable for such studies because insects respond rapidly to hypoxia, but can recover when they are restored to normoxic conditions, whereas many of their neurons are large, identifiable, and robust. Experiments were performed on the “fast” coxal depressor motoneuron (D1) of cockroach (Periplaneta americana). Five-minute periods of hypoxia caused reversible multiphasic depolarizations (10–25 mV; n = 88), consisting of an initial transient depolarization followed by a partial repolarization and then a slower phase of further depolarization. During the initial depolarizing phase, spontaneous plateau potentials normally occurred, and inhibitory postsynaptic potential frequency increased considerably; 2–3 min after the onset of hypoxia all electrical activity ceased and membrane resistance was depressed. On reoxygenation, the membrane potential began to repolarize almost immediately, becoming briefly more negative than the normal resting potential. All phases of the hypoxia response declined with repeated periods of hypoxia. Blockade of ATP-dependent Na/K pump by 30 µM ouabain suppressed only the initial transient depolarization and the reoxygenation-induced hyperpolarization. Reduction of aerobic metabolism between hypoxic periods (produced by bubbling air through the chamber instead of oxygen) had a similar effect to that of ouabain. Although the depolarization seen during hypoxia was not reduced by tetrodotoxin (TTX; 2 µM), lowering extracellular Na+ concentration or addition of 500 µM Cd2+ greatly reduced all phases of the hypoxia-induced response, suggesting that Na influx occurs through a TTX-insensitive Cd2+-sensitive channel. Exposure to 20 mM tetraethylammonium and 1 mM 3,4-diaminopyridine reduced the amplitude of the hypoxia-induced depolarization, suggesting that activation of K channels may normally limit the amplitude of the hypoxia response. In conclusion we suggest that the slow hypoxia-induced depolarization on motoneuron D1 is mainly carried by a TTX-resistant, Cd2+-sensitive sodium influx. Ca2+ entry may also make a direct or indirect contribution to the hypoxia response. The fast transient depolarization appears to result from block of the Na/K pump, whereas the reoxygenation-induced hyperpolarization is largely caused by its subsequent reactivation.

INTRODUCTION

The ability of a neuron to transmit signals is highly dependent on its ability to regulate its transmembrane ionic gradients and resting potential. Because such ionic gradients are maintained by energy-dependent ion pumps, suppression of ATP synthesis can have serious consequences. It is well known that mammalian central neurons cannot incur an oxygen (O2) debt and, as a consequence, are particularly susceptible to hypoxia (reduction of O2 supply to tissues below physiological levels) or ischemia (Fujinawa et al. 1987; Glötzner 1967; Godfraind et al. 1971; Grossman and Williams 1971; Hansen et al. 1982; Misgeld and Frotscher 1982; Negishi and Svætichin 1966; Speckmann et al. 1970). A brief fall in O2 tension can cause a rapid and complete loss of excitability, which is fully reversible; longer periods of hypoxia, however, rapidly cause cell death (Farooqui et al. 1994; Haddad and Jiang 1993a; Kristián and Siesjö 1997; Somjen et al. 1993).

By studying the effects of hypoxia in a variety of species including those that have developed protective mechanisms to resist the effects of hypoxia, a more profound understanding of the role of metabolism in maintenance of normal electrical activity can be gained. This approach may lead to clinical strategies to limit the impact of hypoxia on the human CNS. Certain species of turtle and carp have the capacity to survive periods of anoxia (total lack of O2) lasting days or weeks. Survival of these anoxia-tolerant animals depends on their ability to maintain ATP levels at or near normal levels through glycolysis, the only energy source during anoxia (Lutz et al. 1996; Lutz and Nilsson 1997; Sick et al. 1993). The energy consumption of the brain is reduced by increasing the level of inhibitory neurotransmitters such as γ-aminobutyric acid and adenosine (Buck and Bickler 1995; Nilsson and Lutz 1993; Pérez-Pinzón et al. 1993). In turtle, Na+ channels are also down-regulated (Pérez-Pinzón et al. 1992), and excitatory synaptic transmission is reduced (Nilsson and Lutz 1993).

Adult insects are tolerant to low levels of O2, but they appear to have developed a survival strategy that differs from those of the anoxia-tolerant vertebrates discussed previously. Within 2 min of being placed in an atmosphere of 100% nitrogen (N2) or 100% carbon dioxide (CO2), insects such as cockroaches convulse and then become paralyzed because they are unable to maintain normoxic levels of ATP under these conditions, but they can recover fully from periods of hypoxia lasting several hours (Pitman 1988; Wegener 1993). Biochemical and electrophysiological approaches were used to understand the response of insect tissues to acute O2 deprivation and subsequent reoxygenation. Insects have a level of aerobic metabolism that, for a given mass...
of tissue, exceeds that of mammals but have an extremely low capability for anaerobic metabolism. Thus it was found that insect flight muscles (Wegener 1993, 1996; Wegener et al. 1996) and drone retina (Coles and Tsacopoulos 1987) do not produce detectable concentrations of lactate. To support this high rate of aerobic metabolism, gaseous air is carried extremely close to cells through a network of ducts (tracheae). Hypoxia blocks virtually all energy-generating metabolic pathways and depletes the intracellular content of ATP in <10 min (Brazitikos and Tsacopoulos 1991; Walter and Nelson 1975; Wegener 1993). Hypoxia causes a depolarization of neurons of the cockroach nerve cord that is initially associated with an increase in electrical activity (Walter and Nelson 1975) and cholinergic synaptic transmission (Mony et al. 1986); after a few minutes, however, both decrease and finally cease for the remainder of the period of hypoxia (Mony et al. 1986). Hypoxia also depolarizes neurons of the drone retina (Dimitracos and Tsacopoulos 1985) and blocks impulses in mechanosensory neurons (Hamon and Guillet 1996; Hamon et al. 1988). During reoxygenation, the ATP stores are rapidly resynthesized and return to normal (Brazitikos and Tsacopoulos 1991; Walter and Nelson 1975; Wegener 1993). At the same time synaptic transmission, cellular membrane potential, and impulse thresholds return to control values with no apparent long-term alteration (Hamon et al. 1988; Mony et al. 1986). Although the electrical activity of the insect nervous system rapidly returns to normal after periods of hypoxia lasting a few minutes, longer anoxia periods can have chronic effects on excitability; between 10 h and 5 days after a 1- to 3-h period in CO₂ or N₂, the soma of the metathoracic fast coxal depressor motoneuron (D₁) of cockroach can generate fast sodium action potentials (Pitman 1988) in place of calcium-dependent action potentials or plateau potentials that are normally recorded from this preparation (Hancox and Pitman 1991). The reasons for such long-term alterations are still not clear. However, they may reflect a widespread phenomenon because similar changes in excitability and expression of ion channels were reported after anoxia in neurons of a number of invertebrates (Kuwada and Wine 1981; Pellegrino et al. 1984; Pitman et al. 1972) and vertebrates (Kuno and Llinas 1970; Titmus and Faber 1986; Waxman et al. 1994).

Although there is a considerable body of knowledge about the metabolic strategies used by different animals to minimize the effects of hypoxia, little is known about the electrical changes that occur in individual neurons of such animals during and after periods of hypoxia. The aim of the work presented here is to use an identified insect neuron, the properties of which are known in detail, to characterize its response to brief periods of hypoxia. A brief preliminary report of some of this work was already published (Le Corronc et al. 1997).

**Methods**

**Preparation**

All experiments were performed at room temperature (20–23°C) on adult male cockroaches (*Periplaneta americana*) teared at 28°C in the laboratory. The cockroaches were dissected ventrally, and the three thoracic ganglia and the first part of the abdominal nerve cord were isolated. The preparation was placed in the following saline (in mM): 210 NaCl, 3.1 KCl, 9 CaCl₂, 60 sucrose, 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; pH was adjusted to 7.2 with NaOH. The preparation was set up as described previously (Pitman 1988). The bathing solution superfused at a rate of 0.3 ml/min and was gassed continuously with 100% O₂ at a rate of ~60 ml/min through a vertical column connected to the chamber containing the isolated metathoracic ganglion. As shown in Fig. 1A the experimental chamber was designed to allow fast recirculation. Excess liquid was removed by a vacuum pump.

**Intracellular recordings**

One of the paired D₁ motoneurons was visually located, and its membrane potential was recorded with microelectrodes pulled from borosilicate glass capillary tubes (GC150F-15, Clark Electromedical Instruments, Reading, UK). Microelectrodes were filled with 1 M K-acetate and had a resistance of 15–20 MΩ. Recordings were made with an Axoclamp-2B intracellular amplifier (Axon instruments, Foster City, CA), the output of which was passed to a digital oscilloscope and a chart recorder. Data were digitized via an IEEE connection to an A/D converting interface (HO-79, Haeg, Frankfurt, Germany) and stored on a personal computer with software developed in our own laboratory.
Induction of hypoxia

Hypoxia was induced by switching from 100% O₂ to 100% N₂ (≈60 ml/min) for 5 min once every 30 min, allowing 25 min of oxygenation in between episodes. These gases were introduced by bubbling them into the chamber via separate plastic tubes (Fig. 1A). O₂ rather than air was bubbled through the saline because the Po₂ level attained with air was judged too low and because Hamon and Guillet (1986) previously demonstrated that the electrical activity of oxygenated cockroach nerve cords recorded in vitro is similar to that recorded in situ. To determine both the steady-state Po₂ values when either 100% O₂ or 100% N₂ was bubbled into the saline and the rate of change that occurred after switch-over, a polarimetric method was used. The microsensor (Chemical Microsystems 1201, Diamond Electro-Tech, Ann Harbor, MI) was first calibrated with O₂ (100% O₂) and N₂ (0% O₂) in a small tube with saline, as recommended, and then moved to the chamber. Under the conditions of our physiological experiments, the steady-state O₂ level in the chamber indicated 100% when O₂ was bubbled, and, during 5-min periods in which 100% N₂ was bubbled, the percentage of O₂ quickly decreased to a value closed to 0% (Fig. 1B). The Po₂ drop was rapidly reversed when O₂ was reintroduced. In some experiments air rather than 100% O₂ was bubbled through the chamber. Under these circumstances, the microsensor registered a Po₂ value of 20–21%, as might be expected if complete equilibration with the saline occurred. When air was replaced by N₂ for 5 min the Po₂ rapidly and reversibility dropped to a value closed to 0% (Fig. 1C).

Solutions and drugs

In experiments carried out in low-Na⁺ (50 mM) saline, tris(hydroxymethyl)aminomethane (Tris) was substituted at equal molarity (pH was adjusted to 7.2 with HCl) (cf. Pitman 1975, 1979). For 20 mM tetraethylammonium chloride (TEA) plus 1 mM 3,4-diaminopyridine (3,4-DAP) saline, an equal molarity of sucrose to normal saline had the same composition as normal saline increased during hypoxia but can fall below control values except that it contained 0.3 mM Ca²⁺ and 12 mM Mg²⁺. Nominally zero calcium solution contained 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 12 mM Mg²⁺. Ouabain, Ni²⁺, tetrodotoxin (TTX), and Cd²⁺ were added to normal saline at concentration used. Verapamil hydrochloride was first dissolved in 200 μl of dimethyl sulfoxide. The stock solution (50 mM) was diluted in saline to final concentration of 100 μM. All compounds were purchased from Sigma Chemicals.

Statistical data are expressed as means ± SE. On graphs, error bars are shown when larger than symbols. When necessary, statistical significance is assessed with analysis of variance in which a P value <0.05 (Dunnett test) was regarded as significant.

RESULTS

Effects of hypoxia

Figure 2 shows a typical response of motoneuron D₁ to a 5-minute period of hypoxia, consisting of a multiphasic membrane depolarization (10–25 mV) from the resting membrane potential of −78.2 ± 0.4 mV (n = 88). The depolarization started 45–75 s after the interruption of oxygenation and consisted of an initial transient depolarization (Fig. 2Ab) followed by partial repolarization (Fig. 2Ac). This, in turn, gave way to a slower phase of further depolarization (Fig. 2Ad). Early in the initial phase of the hypoxia-induced depolarization, spontaneous plateau potentials were produced (Fig. 2Ab), and the frequency of inhibitory post-synaptic potentials increased considerably beyond control levels (cf. Fig. 2, Aa and Ac). The second phase of depolarization developed more slowly than the first and was marked by the absence of plateau potentials or postsynaptic potentials (Fig. 2Ad). On reoxygenation the membrane potential began to repolarize almost immediately and reached a value more negative than the normal resting potential. In 58% (n = 51) of neurons, this hyperpolarization was followed by a transient depolarization (3–7 mV) before the membrane potential settled to a steady level (Fig. 2, A and B). In some instances, the late depolarization was sufficiently large to evoke plateau potentials (Fig. 2B), but this was not always the case (Fig. 2A); 42% (n = 37) of cells lacked the late depolarizing phase of recovery from hypoxia (Fig. 2C). We were unable to establish any characteristic of neurons that would predict whether they would show the late depolarizing phase during their recovery from hypoxia.

Membrane resistance was determined by measuring the amplitude of membrane potential excursions produced by applying regular hyperpolarizing current pulses (−1.1– to −1.7 nA intensity; 500-ms duration) through the recording electrode. Hypoxia produced a fall in membrane resistance, the magnitude of which varied during the course of the hypoxic period (Fig. 3A). Membrane resistance dropped to 70 ± 3% (5.63 ± 0.62 MΩ; n = 4) of its control value (100%; 8.16 ± 0.80 MΩ; n = 4) during the initial rapid phase of depolarization and continued to fall throughout the period of hypoxia, reaching 50 ± 5% (4.06 ± 0.60 MΩ; n = 4) of control when maximal depolarization was attained. During reoxygenation the membrane resistance increased beyond the control value during the late transient depolarizing phase (115 ± 3%; 9.35 ± 1.35 MΩ; n = 4) before returning to normal (Fig. 3A). The previously described changes in membrane resistance suggest that ionic conductances are increased during hypoxia but can fall below control values during the transient depolarizing phase of recovery.

Motoneuron D₁ is able to generate calcium-dependent plateau potentials when long depolarizing current pulses are applied to the soma (Hancox and Pitman 1991). To investigate the effect of hypoxia on the excitable properties of motoneuron D₁, regular depolarizing current pulses were delivered through the intracellular recording microelectrode (Fig. 3B). As shown in Fig. 3Ba, plateau potentials are prolonged depolarizing events, which can trigger (presumably Na⁺-dependent) axonal action potentials that appear in soma recordings as attenuated deflections superimposed on plateau potential (Fig. 3, Ba, Bb, and Bd–Bf). Near the beginning of the hypoxia response, the plateau potential developed more rapidly after the onset of each depolarizing pulse (Fig. 3Bb) than under control conditions. During the slow sustained depolarizing phase of the hypoxia response the ability to generate plateau potentials was lost and was not restored when the membrane potential was returned to its original level by hyperpolarizing current (Fig. 3Bc). Approximately 4 min after reoxygenation, cells regained the ability to generate plateau potentials (Fig. 3Bd). The late transient depolarizing recovery phase, plateau potential duration was increased beyond that observed under control conditions (Fig. 3Be). This increase probably resulted from the fall in membrane conductance, which occurs at this time. This would both enhance the excitability of the neuron and
FIG. 2. Effects of hypoxia on membrane potential of motoneurone D_f. A, left: 5-min period of hypoxia causes a multiphasic depolarization. The activity seen during the phases of the response marked a–d are shown in more detail in the correspondingly lettered panels. During the early phase of depolarization, plateau potentials were produced (b). Because the chart recorder had slow response characteristics, the amplitude of plateau potentials is attenuated. This does not apply to detailed panels a–d because they provided from the digital interface. Inhibitory postsynaptic potentials were also evoked during the first phase of hypoxia (c) but, like plateau potentials, were absent during the slow depolarizing phase (d). The resting membrane potential is shown by dotted lines. During reoxygenation a transient hyperpolarization appeared, which was followed by a transient depolarizing phase (e). The traces C and D are both shown on the same scale.

increase the depolarization evoked by applied current pulses of fixed magnitude. Eventually, the characteristics of plateau potentials returned to those observed before the period of hypoxia (Fig. 3Bf).

Effects of repetitive periods of hypoxia

When motoneuron D_f was exposed to five successive periods of hypoxia, each separated by a 25-min recovery interval, all phases of the hypoxia response underwent a progressive decline. Figure 4 shows responses to the first, the third, and the fifth hypoxia periods in one preparation that exhibited a late transient depolarization after reintroduction of oxygen (Fig. 4A) and another that did not (Fig. 4B). The magnitudes of the sustained depolarizing phase of successive hypoxia responses (measured relative to the resting membrane potential of the neuron, dotted line), showed a decline, the extent of which was similar from one neuron to another; this was also true for the hyperpolarization seen during recovery. The beginning of the first hypoxia period served as the reference time zero, and the amplitudes of depolarizing (filled star in Fig. 4, A and B) and hyperpolarizing phases (open star in A and B) of this first hypoxia response were taken as 100%. Data points on the graphs in Fig. 4C represent average values taken from successive hypoxia responses recorded from different neurons. Graphs combine data from responses similar to those shown in both Fig. 4, A and B. During the fifth hypoxia period, the magnitude of the sus-

FIG. 3. Membrane resistance and excitability of motoneuron D_f during hypoxia. A: voltage responses produced by hyperpolarizing current pulses (1.7 nA; 500-ms duration) were reversibly reduced during hypoxia, indicating a fall in membrane resistance. The lower box was the percentage change in membrane resistance (% of Rmb) taken from the record shown in the top panel. Hypoxia began at 0 min. B: plateau potentials evoked by depolarizing current pulses (3.6 nA; 3-s duration) applied during those phases of the hypoxia response lettered a–f are shown on an expanded time scale in the correspondingly lettered panels. Plateau potentials developed with less delay (b) during the initial phase of hypoxia compared with control (a). Plateau potentials could not be evoked during the second phase of depolarization even if the cell was manually clamped to its original resting potential (c). During reoxygenation, plateau potentials were restored (d) and were longer (e) during a time corresponding to the transient depolarization. The lower trace on the expanded scale of a shows the timing of the depolarizing current pulse.
Effects of repeated periods of hypoxia. A and B: all phases of the hypoxia-induced response progressively declined when 5 hypoxia periods (5-min duration) were repeated every 30 min. Only alternate responses (first, third, and fifth) are shown. Responses with (A) and without (B) reoxygenation-induced transient depolarizing phases are shown. The dotted line indicated the resting potential. C: effect of interval between successive hypoxia periods (5 min each) on the decline in size of the slow depolarization evoked during hypoxia (filled star in A and B; left box in C) and the hyperpolarization after reoxygenation (open star in A and B; right box in C). Values are expressed as percentages of the amplitudes of these components in the first hypoxia response. The first hypoxia period began at 0 min. Filled circles represent the normal experimental protocol (i.e., hypoxia periods repeated every 30 min; n = 8); open circles (n = 3) show the decline seen when hypoxia was administered every 60 min, and open squares (n = 9) that when hypoxia was repeated every 15 min.

Effects of ouabain and low oxygen level

To study whether an interruption of Na-K pump (or the Na^+-K^+ ATPase) activity contributes to the cellular response to hypoxia, we performed experiments with ouabain, a blocker of this pump. Bath application of 30 μM ouabain blocked the fast transient depolarization and the reoxygenation-induced hyperpolarization (Fig. 5A) but had little effect on the amplitude of the slow depolarization (which, even in the absence of ouabain, declines with successive periods of hypoxia). After reintroduction of oxygen, the cell repolarized more slowly than under control conditions. Although ouabain initially had little effect on the resting potential, after 25–30 min, the membrane potential gradually depolarized irreversibly to a new stable level 10–15 mV more positive than the normal resting potential.

An alternative strategy we used to investigate the role of metabolism in the maintenance of the electrochemical gradient was to reduce O_2 in the chamber by switching from pure O_2 (P_O2 = 100%) to air (P_O2 = 20%, low aerobic metabolism protocol) as used by Coles et al. (1996) on drone retina. Equilibration with air caused a small (5–10 mV), transient membrane depolarization, followed a return to its initial value (Fig. 5B). When the gas bubbling through the experimental chamber was switched from air to nitrogen, a hypoxia response was evoked that closely resembled that observed in the presence of ouabain but had the advantage that it was not complicated by a background membrane depolarization (Fig. 5B). After recovery from this type of response, reintroduction of O_2 caused an immediate hyperpolarization (7.8 ± 1.9 mV; n = 4). This is consistent with a transient increase of metabolism beyond the maximum level obtainable when the preparation is equilibrated with air. In two of four experiments in which O_2 was reintroduced after a period of exposure to air, cells produced spontaneous plateau potentials (Fig. 5B). All preparations in which the effects of equilibration with air were studied showed normal hypoxia responses, once they were subsequently equilibrated with O_2 before a period of hypoxia (Fig. 5B).

The previous results suggest that blockade of the sodium...
pump is probably not primarily responsible for the hypoxia-induced slow depolarization because this component of the response was not blocked by ouabain or low aerobic metabolism. In contrast the initial phase of depolarization and the rapid repolarization and subsequent hyperpolarization observed when O$_2$ is reintroduced may well, at least in part, respectively result from inhibition and reactivation of the Na-K pump. A net increase in transmembrane Na$^+$ and Ca$^{2+}$ influx and K$^+$ efflux through cationic channels could therefore contribute to the depolarization. Ionic channel blockers and solutions of altered ionic composition were applied to characterize the ionic dependence of the hypoxia-induced depolarization.

Effect of verapamil, Ni$^{2+}$ and low and zero Ca$^{2+}$ solutions

In these experiments we tested the hypothesis that membrane potential alterations seen during hypoxia resulted from an influx of Ca$^{2+}$ through the surface membrane. In a medium containing 100 μM verapamil ($n = 6$), the hypoxia response and the hyperpolarization seen on reoxygenation were unaffected (not shown); 600 μM Ni$^{2+}$ ($n = 6$) had no effect on the response to hypoxia but completely blocked the hyperpolarization seen on reintroduction of oxygen (Fig. 6, A and C). After treatment with Ni$^{2+}$, the progressive decline in the amplitude of the hypoxia depolarization that occurs in control conditions was reversed. In most preparations, Ni$^{2+}$ at the concentrations used caused the threshold for plateau potentials to become less negative to such an extent that these events occurred spontaneously (Fig. 6A). In the presence of a low Ca$^{2+}$ (0.5 mM) and high Mg$^{2+}$ (12 mM) saline, the only effect observed was a significant reduction in the rate of decline in the hypoxia-induced depolarization (Fig. 6C; $n = 3$; $P < 0.05$ for data measured 65 min from the start of the experiment). In a solution containing 2 mM Ca$^{2+}$ and 7 mM Mg$^{2+}$ ($n = 5$) (not shown) the results obtained were the same as those described for 0.3 mM Ca$^{2+}$ and 12 mM Mg$^{2+}$. In nominally zero Ca$^{2+}$ saline ($n = 4$), the amplitude of plateau potentials at the start of the hypoxia response was reduced, whereas the slow phase of the response was augmented ($P < 0.01$ at 65 min). This bathing solution reduced the amplitude of the hyperpolarization seen on reoxygenation (Fig. 6, B and C; $P < 0.01$ at 65 min). The results obtained in low or zero Ca$^{2+}$ salines indicated that there is a complex relationship between [Ca$^{2+}$]$_o$ and its effect on the slow phase of the hypoxia response.

Because Ca$^{2+}$ channel blockers did not depress either phase of depolarization during hypoxia, it appears that Ca$^{2+}$ channels do not play a major role in the response of the neuron to reduced oxygen tension. It appears, however, that Ca$^{2+}$ may play a modulatory role because low Ca$^{2+}$ solutions enhance rather than reduce the slow depolarizing phase of the response. The involvement of Ca$^{2+}$ in the posthypoxia hyperpolarization remains enigmatic because it is blocked by Ni$^{2+}$ but not in nominally zero Ca$^{2+}$ bathing solution or by verapamil.
Effect of TTX and low Na$^+$-containing solution

In the presence of 2 μM TTX ($n = 4$), the depolarizing phase of the hypoxia response was not significantly altered (Fig. 7, A and C; $P > 0.05$ at 65 min). It appears that this dose was effective in blocking Na$^+$ channels because both spontaneous postsynaptic potentials (not shown) and Na$^+$-dependent action potentials normally superimposed on plateau potentials were abolished (not illustrated). TTX, however, did cause a delayed and long-term reduction in the hyperpolarization seen on reoxygenation (Fig. 7C). To investigate the possibility that a TTX-resistant Na$^+$ influx is involved in the hypoxia response, the preparation was exposed to low-Na$^+$ (23% of normal) solution. After changing to low-Na$^+$ saline, the resting potential of the neuron hyperpolarized (by ~10–15 mV) and then gradually returned to its original value over a period of ~30–40 min. As a consequence, the membrane potential would have been more negative during the second hypoxia response than at the start of the experiment; to compensate for this the membrane was artificially returned to its starting value by passing current through the recording microelectrode. In such experiments ($n = 3$), all phases of the hypoxia response were greatly reduced (Fig. 7, B and C; $P < 0.01$ at 65 min). In low-Na$^+$ solution, return of the membrane potential to the resting level on reoxygenation was much faster than in normal saline. Hypoxia responses recorded after returning the preparation to normal saline solution showed that, although the slow phase of depolarization recovered, neither the early transient depolarizing phase nor the posthypoxia hyperpolarization reappeared (Fig. 7B).

The previous results combined with those presented in the previous section strongly support the conclusion that the depolarizing phase of the hypoxia response results from an influx of Na$^+$ through TTX-insensitive Na$^+$ channels.
were indistinguishable from those obtained in Cd\(^{2+}\) alone. This provides evidence that Na\(^{+}\) influx occurs via a Cd\(^{2+}\)-sensitive, TTX-resistant channel of a type not previously described in insect neurons.

**Effect of K\(^{+}\) channel blockers**

To test the hypothesis that the hypoxic response involved a K\(^{+}\) efflux, experiments were performed in the presence of the K\(^{+}\) channel blockers TEA (20 mM) and 3,4-DAP (1 mM). Under these conditions the hypoxia-induced depolarization was significantly increased (\(P < 0.01\) at 65 min; \(n = 3\)), and the hyperpolarization after reoxygenation was significantly reduced (\(P < 0.01\) at 65 min; \(n = 3\); Fig. 9, A and B). A dramatic increase in the electrical activity of the neuron was also observed. Because of the powerful effect of K\(^{+}\) channel blockers, the period of exposure to these agents was restricted to one period of hypoxia (rather than two for all other drugs) to minimize damage to the neuron. When these drugs were washed from the preparation, the reoxygenation hyperpolarization reappeared and reached a greater amplitude than normal. Abolition of the hyperpolarizing phase of recovery was not a consequence of the high level of spontaneous activity in the neuron because this persisted during the wash at a time when the hyperpolarizing phase had reappeared.

**DISCUSSION**

We have shown that brief periods of hypoxia cause a multiphasic response in the soma of the fast coxal depressor neurons of **H. Le CORRONC, B. HUE, AND R. M. PITMAN**

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**FIG. 8.** Effect of Cd\(^{2+}\) on the hypoxia response. Bath application of 500 \(\mu\)M Cd\(^{2+}\) (A) and 500 \(\mu\)M Cd\(^{2+}\) plus 2 \(\mu\)M TTX (B) reduced the same percentage (C) the slow depolarizing phase and the hyperpolarization induced by reoxygenation. TTX and Cd\(^{2+}\) are applied simultaneously to demonstrate Cd\(^{2+}\)-sensitive TTX-resistant conductance. The rapid transient depolarizing phase of the response persisted during application of drugs but was lost after washing the drugs from the preparation. Only the slow depolarization recovered. In C the horizontal bar indicates the period over which drugs were applied to the bath. ● control (\(n = 8\)); ○: 500 \(\mu\)M Cd\(^{2+}\) (\(n = 3\)); □: 500 \(\mu\)M Cd\(^{2+}\) plus 2 \(\mu\)M TTX (\(n = 4\)).

**FIG. 9.** In the presence of both 20 mM tetraethylammonium chloride (TEA) and 1 mM 3,4-DAP the amplitude of the hypoxia-induced depolarization was increased, whereas the hyperpolarization was reduced (A). These effects were probably not initiated by the large increase of electrical activity evoked by potassium blockers because this persisted during the wash at a time when the hyperpolarizing phase had reappeared. To minimize damage to motoneuron D\(_4\), K\(^{+}\) channel blockers were applied during only one hypoxia period then washed off (other drugs and altered solutions remained on the preparation during 2 hypoxia responses). The first, the second, and the fourth hypoxia response were shown. B: plots show the quantitative effects of drugs (horizontal bar) on the slow depolarization (left panel) and on hyperpolarization (right panel). ● control (\(n = 8\)); ○: 20 mM TEA plus 1 mM 3,4-diaminopyridine (\(n = 3\)).
motoneuron (\(D_f\)) of the cockroach. Our results demonstrate for the first time in an insect neuron that the different phases response to hypoxia are produced by different mechanisms.

**Features of the response to hypoxia**

Our observations clearly show that motoneuron \(D_f\) is highly sensitive to hypoxia to which it responds with a multiphasic membrane depolarization; initially spontaneous synaptic activity and excitability are increased, and then there is a fall in membrane resistance accompanied by a loss of excitability. Reoxygenation induces a fast hyperpolarization that is sometimes followed by a transient depolarization. During this latter phase, membrane resistance and excitability are higher than normal. Similar responses were seen in the cell bodies of some other identified cockroach neurons (personal observations), including giant interneurons (Mony et al. 1986). Our results are also generally comparable with observations on the effects of oxygen deprivation on some mammalian neurons; striatal (Calabresi et al. 1995), hypoglossal (Donnelly et al. 1992; Haddad and Donnelly 1990; Haddad and Jiang 1993b), vagal (Donnelly et al. 1992; Haddad and Jiang 1993b), and neocortical (O’Reilly et al. 1995; Rosen and Morris 1993) neurons depolarize, and their membrane resistance drops during hypoxia. Initially, the frequency of evoked action potentials in these neurons increases, but a few minutes later such activity is completely abolished. Hypoglossal neurons undergo a transient increase in presumed synaptic potentials in the early phase of hypoxia (Haddad and Donnelly 1990). When reoxygenated, striatal, hypoglossal, vagal, and neocortical neurons, unlike \(D_f\), only slowly repolarize to their normal resting potential and do not undergo any transient hyperpolarization (Calabresi et al. 1995; Donnelly et al. 1992; Haddad and Jiang 1993b; O’Reilly et al. 1995; Rosen and Morris 1993). During recovery from hypoxia, the membrane resistance of both vagal motoneurons and neocortical neurons, like that of \(D_f\), increases above normal (O’Reilly et al. 1995), presumably accounting for the period of hyperexcitability. If the period of hypoxia to which CA1 hippocampal (Fujiwara et al. 1987; Krnjevic and Leblond 1989; Leblond and Krnjevic 1989) or substantia nigra pars compacta (Mercuri et al. 1994) neurons are exposed is sufficiently brief, only membrane hyperpolarization is observed; if the preparation is reoxygenated at this point, an immediate transient further hyperpolarization occurs before the membrane potential returns to its normal value. This response may have a basis similar to that observed when \(D_f\) is reoxygenated.

**ATP-dependent processes**

Both the initial fast transient ouabain-sensitive and the slow depolarizing components of the hypoxia response are suppressed in a reduction in the external Na\(^+\) concentration, suggesting that both are caused by an influx of this ion. Because TTX has no effect, while Cd\(^{2+}\) has no effect, it is likely that the depolarization induced by hypoxia is mainly caused by flow of Na\(^+\) ions through a TTX-resistant Cd\(^{2+}\)-sensitive Na\(^+\) channel. That the effects of low Na\(^+\) and Cd\(^{2+}\) on both the first transient depolarization and the hyperpolarization induced by reoxyenation are irreversible suggests that they may be acting at the same site, although this cannot be considered certain because the cause of this irreversibility is unknown. Although Cd\(^{2+}\) blocks voltage-dependent Ca\(^{2+}\) currents in motoneuron \(D_f\) (Mills and Pitman 1997), it would be difficult to attribute the effects reported here to such an action. If this were the case, the effects of Cd\(^{2+}\) should be mimicked by low or zero Ca\(^{2+}\) saline but not by those of low Na\(^+\) solutions. However, the effect of Cd\(^{2+}\) was similar to that of low Na\(^+\) but different from that of low or zero Ca\(^{2+}\) saline. We suggest therefore that hypoxia activates a TTX-resistant Cd\(^{2+}\)-sensitive Na\(^+\) influx in \(D_f\). Our findings are similar to those obtained with brain stem neurons (Haddad and Donnelly 1990; Haddad and Jiang 1993b) and in striatal neurons (Calabresi et al. 1995) in which membrane depolarization is reduced by lowering external Na\(^+\) concentration but not application of TTX. With the exception of somatically recorded axon spikes the normal electrical activity in \(D_f\) was TTX insensi-
ductive (Hancock and Pitman 1991). Although we conclude that a Na\(^+\) influx is the major cause of the slow membrane depolarization seen during hypoxia, we cannot exclude the possibility that a small Ca\(^{2+}\) influx does make some contribution to the hypoxia response. The delay in the effect of Cd\(^{2+}\) on the first transient depolarizing phase of the hypoxia response could result from a relatively low sensitivity to this agent. However, it is more likely to occur because this phase of the hypoxia response is generated in a site within the ganglioncell that is relatively inaccessible of Cd\(^{2+}\). Because this phase of the response continued to decline after Cd\(^{2+}\) had been washed from the experimental chamber. To investigate this, Cd\(^{2+}\) was applied during three hypoxia periods rather than two. In such experiments, the initial transient depolarization of the third response was blocked (not shown), supporting the conclusion that Cd\(^{2+}\) does have a site of action that is relatively inaccessible.

In low and nominally zero Ca\(^{2+}\) solutions, the amplitude of the hypoxia-induced depolarization was increased. Calabresi et al. (1995) found a similar enhancement in the amplitude of the hypoxia-induced depolarization recorded from nigral neurons bathed in a medium containing low Ca\(^{2+}\) (0.5 mM) plus high Mg\(^{2+}\) (10 mM). We suggest the following mechanism for this enhancement: normally, sufficient Ca\(^{2+}\) influx occurs during hypoxia to activate a Ca\(^{2+}\)-dependent (g\(K_{\text{Ca}}\)) K conductance. In motoneuron D\(_1\), this conductance has been shown to be considerably larger than the inward Ca currents that generated them (David and Pitman 1995a,b; Mills and Pitman 1997; Thomas 1984). The effect of this current therefore would be to limit the size of the hypoxia-induced depolarization. When the preparation is bathed in low or nominally zero Ca\(^{2+}\) saline, I\(_{\text{Ca}}\) and hence I\(_{\text{KCa}}\) will fall, so increasing the overall amplitude of the hypoxia-induced depolarization. Ca influx may not be the only mechanism by which K currents are activated; activation and modulation of g\(K_{\text{Ca}}\) in D\(_1\) may be brought about by a rise in [Ca\(^{2+}\)], resulting not only from influx across the surface membrane but also by release from intracellular stores (David and Pitman 1995a,b, 1996). Both mechanisms may contribute to any rise in [Ca\(^{2+}\)], that occurs during hypoxia. Enhancement of the hypoxia-induced depolarization by K channel blockers (TEA plus 3,4-DAP) provides further support for the role of K current activation during hypoxia. It appears that K channels are also activated in other preparations; in drone retina and in mammalian neurons it was found that hypoxia increases the extracellular K\(^+\) concentration (Dimitracos and Tsacopoulos 1985; Donnelly et al. 1992), whereas K\(^+\) blockers augment the hypoxia response in mammalian neurons (Jiang and Haddad 1991). In mammalian neurons, g\(K_{\text{Ca}}\) (Leblond and Krnjevic 1989; Yamamoto et al. 1997) and ATP-sensitive K\(^+\) channels (g\(K_{\text{ATP}}\)) (Fujimura et al. 1997; Jiang and Haddad 1991) are the main route by which intracellular K\(^+\) is lost during hypoxia. In D\(_1\), depolarization caused by Na\(^+\) influx, a rise in intracellular Ca\(^{2+}\) and a fall in ATP could all contribute, in principle, to activation of gK and limitation in amplitude of the hypoxia-induced depolarization. The contribution of g\(K_{\text{ATP}}\) to the hypoxia response in D\(_1\) is not clear, however, because glibenclamide has little or no effect on hypoxia response (Pitman, personal observations).

Although we indicated previously that reactivation of Na\(^+\)-K\(^+\) pump appears to be the primary cause of the posthypoxic hyperpolarization, it appears that other processes also may be involved because this component is depressed by K\(^+\) channel blockers, TTX, or by bathing the preparation in nominally zero Ca\(^{2+}\) saline. One contributor may be Na\(^+\)-Ca\(^{2+}\) exchange because the posthypoxic hyperpolarization is blocked by Ni\(^{2+}\) (600 \(\mu\)M), which was used to selectively block Na\(^+\)-Ca\(^{2+}\) exchange in guinea-pig ventricular myocytes (Kimura et al. 1987). It has been shown previously that Ni\(^{2+}\) does not block voltage-dependent Ca\(^{2+}\) currents in D\(_1\) (Mills and Pitman 1997).

The reoxygenation-induced hyperpolarization was sometimes followed by a transient depolarization. Although we have not elucidated the mechanism of this latter phase, the associated decrease in membrane conductance suggests that closure of ion channels may be involved. In hippocampal CA1 neurons, the duration of the reoxygenation-induced hyperpolarization is longer at potentials close to the K\(^+\) equilibrium potential (Fujimura et al. 1987). These observations were attributed to a block of voltage-independent K\(^+\) currents occurring concurrently with the reoxygenation-induced hyperpolarization at the resting membrane potential. We suggest that a similar reduction in K conductance may account for the appearance of the late depolarizing component recorded from D\(_1\) when it is reoxygenated. This could account for the increase of membrane resistance and excitability seen during this phase.

**Repetitive hypoxia**

Under our experimental conditions, there was a progressive decline in all phases of responses to successive periods of hypoxia. This was not caused by a deterioration in preparations caused by loss of microelectrode impalement or by washout of some vital factor from the environment of the neuron because the decline was decreased by increasing the interval between hypoxia. Moreover, between periods of hypoxia, neurons regained normal electrical characteristics (e.g., resting potential, input resistance, and excitability), indicating they were undamaged. A reduction in extracellular Ca\(^{2+}\) concentration specifically blocks the decline of the depolarizing phase but not the transient hyperpolarization seen on reoxygenation. However, the relationship between the extracellular Ca\(^{2+}\) concentration and the decline is complex. We suggest that, in D\(_1\), the progressive decline of the reoxygenation-induced hyperpolarization is related to metabolism. Support for this suggestion comes from work on the drone retina, in which long recovery intervals between successive hypoxia periods are needed to replenish the stores of energy-rich substrates and to enable identical hypoxia responses to occur (Dimitracos and Tsacopoulos 1985).

This study is the first report of the acute effect of hypoxia on an insect motoneuron, showing that the response of this preparation has similarities with those of mammalian neurons. We conclude that, in this insect neuron, as in some mammalian neurons, hypoxia causes an increase in Na\(^+\) and Ca\(^{2+}\) influx and a K\(^+\) efflux associated with block of the Na-K pump, caused by a fall in intracellular ATP. In many studies on the irreversible damage to the mammalian brain induced by O\(_2\) deprivation, the main goal is a deeper understanding of events that lead to cell death. Increases in intra-
cerebral Ca\(^{2+}\) and Na\(^{+}\) are the two main hypothethical causes of hypoxia-induced injury (Farooqui et al. 1994; Friedman and Haddad 1993, 1994; Haddad and Jiang 1993a). It is thought that these ions may trigger a cascade of cellular events, the final outcome of which is the neuronal death. Unlike mammals, insects recover from hypoxia, showing that irreversible anoxic damage is not an inevitable consequence of metabolic block and loss ionic homeostasis.

Because we already provided evidence that the hypoxia response is associated with an Na\(^{+}\) influx in D\(_{1}\), we need to determine whether \([\text{Ca}^{2+}]_i\) also changes and makes a significant contribution. If both these cations are greatly increased in D\(_{1}\) during hypoxia, it is important to establish why they do not trigger irreversible neuronal injury or death.

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REFERENCES


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