DELAYED EFFECTS OF ANOXIA UPON THE ELECTRICAL PROPERTIES OF AN IDENTIFIED COCKROACH MOTONEURONE

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SUMMARY

1. In nerve preparations isolated from cockroaches that had survived exposure to carbon dioxide or nitrogen for 1–3 h, the cell body of the metathoracic 'fast' coxal depressor motoneurone (Df) gained the ability to support all-or-none action potentials. Such responses were observed between 10 h and 5 days after the anoxic period.

2. Recordings from isolated cell bodies indicated that the action potentials were generated in the cell body itself rather than in adjacent regions of the neurone.

3. Action potentials were abolished in a sodium-free medium or in the presence of tetrodotoxin, but persisted in saline containing manganese ions, indicating that sodium ions are the major ions involved in the rising phase of these action potentials.

4. Elevation of the intracellular H\(^+\) concentration by acid injection or by bathing the preparation in saline containing 40 mmol\(\cdot\)l\(^{-1}\) ammonium chloride caused a marked depolarization of the membrane potential and action potentials could not be elicited. This indicates that a fall in intracellular pH during anoxia could not itself directly produce the enhancement in excitability reported here.

5. Histological examination of the neurone, after periods of anoxia sufficient to enable the cell body to generate the action potentials reported here, showed no detectable change in the perinuclear distribution of RNA and hence did not indicate any alteration in protein synthesis.

INTRODUCTION

Insect motoneurones are unipolar; synapses are absent from the cell body and are restricted to areas of the ganglionic neuropile. The cell body is, therefore, not on the direct route of information flow and is not normally actively invaded by action potentials (Pitman, Tweedle & Cohen, 1972; Hoyle & Burrows, 1973; Gwilliam & Burrows, 1980). Despite this, it appears that the somata of at least some motoneurones have voltage-dependent ion channels which can be unmasked by a reduction in the intracellular calcium concentration or by suppression of potassium ions.
conductance. Such procedures have demonstrated that calcium channels are normally present in the soma membrane of the fast coxal depressor motoneurone (Df) of the cockroach (Pitman, 1979), whereas the locust fast extensor tibiae motoneurone (FETi) possesses both sodium and calcium channels (Goodman & Heitler, 1979). Several days after the axotomy of these insect motoneurones, their cell bodies gain the ability to support all-or-none action potentials in which sodium carries most of the inward current (Pitman et al. 1972; Heitler & Goodman, 1979). Similar action potentials have also been observed several days after colchicine had been applied to the axons of cockroach motoneurones (Pitman, 1975). Both axotomy and colchicine treatment produce characteristic changes in the distribution of RNA around the nucleus of the neurone which appear with a similar time course to the changes in electrical properties of the neurones (Cohen & Jacklet, 1967; Jacklet & Cohen, 1967; Pitman et al. 1972). It has been suggested, therefore, that sodium-dependent action potentials develop in the soma after axotomy because sodium channels are incorporated into the soma membrane as a consequence of alterations in protein synthesis necessary for axonal repair. A similar relationship appears to exist in some crayfish neurones, since protein synthesis inhibitors prevent the appearance of action potentials in these neurones following axotomy (Kuwada, 1981).

I report here that exposure of cockroaches to carbon dioxide or nitrogen produces a delayed change in the excitability of an identified motoneurone soma; a number of hours after the period of anoxia, action potentials can be evoked by depolarization. These action potentials, like those recorded in this motoneurone after axotomy or treatment with colchicine, are sodium-dependent.

MATERIALS AND METHODS

Adult male cockroaches, Periplaneta americana, were obtained from a colony maintained in the laboratory. They were placed in a moisturized container, which was then gassed with either 100% CO2 or nitrogen, and sealed. After 1–3 h the animals were transferred to a fresh, aerated box. Nerve cords from animals which appeared healthy after recovery from gassing were then set up for recording at a measured interval after the anoxic period.

Electrophysiological recordings were made from the soma of the metathoracic fast coxal depressor motoneurone [known as cell 28 (Cohen & Jacklet, 1967) or Df (Pearson & Iles, 1970)] in an isolated preparation of the ventral nerve cord, consisting of three thoracic ganglia and three abdominal ganglia. The cord was first attached ventral surface upwards to a plastic slide, and the nerve sheath was removed from the ventral surface of the metathoracic ganglion using sharpened watchmaker’s forceps. The preparation was then placed in the recording chamber (volume 2 ml) and oxygenated with circulating saline. In all experiments the neurone soma was impaled with two microelectrodes filled with 2 mol l⁻¹ potassium acetate (7–15 MΩ) unless otherwise stated.
The normal physiological saline had the following composition (in mmol l⁻¹): NaCl, 214; KCl, 3.1; CaCl₂, 9.0; Tes buffer (pH 7.2), 10. In sodium-free saline solution, Tris-HCl replaced NaCl and TES of the normal solution. Manganese chloride and ammonium chloride were added to normal saline solution without compensatory adjustments of other ions.

For the isolation of nerve cell bodies, ganglia were prepared and placed in the experimental chamber as described above. The chosen soma was then freed from surrounding cells using a fine jet of physiological saline solution. To do this, a microelectrode was filled with saline solution and connected to a 2 ml syringe via a flexible plastic tube. The microelectrode tip was then broken to a diameter of approximately 5–20 μm and manually positioned under visual control, allowing the cell body to be gently loosened from surrounding tissue with the fine saline jet. When the soma remained attached by the axon hillock region only, it was ready to be isolated. This was done using a glass capillary tube with one end drawn out to the same diameter as that of the nerve cell body and flame-polished; the other end of the capillary was connected to flexible plastic tubing which allowed suction to be applied to the open end by mouth. The tip of the suction pipette was manipulated over the cell, which could then be isolated by gently sucking it into the capillary tube. Once drawn into the tube, the isolated soma was deposited onto a convenient substrate for making intracellular recordings. In most cases, the isolated soma was placed upon the surface of the nerve cord from which it had been removed (at a distance from its original position in the ganglion); cells adhered sufficiently strongly to the ganglion surface to prevent them from being washed away by the flow of saline solution over the preparation. Impalements of these cell bodies were normally made 10–30 min after isolation had been completed, at which time the membrane properties of the soma had stabilized.

For intracellular injection of acid, one microelectrode was mounted in a holder connected via plastic tubing to a Picospritzer II (General Valve Corporation) pneumatic system. Pressure was generated by a Polyspray 3 garden spray pressure vessel (Airflow Ltd). This arrangement allowed timed pressure pulses to be applied to the solution in the microelectrode.

For histology, ganglia were fixed, embedded in paraffin, sectioned at 10 μm and stained with pyronine–malachite green (Baker & Williams, 1965; Cohen & Jacklet, 1967).

RESULTS

Chronic effects of CO₂ or nitrogen on intrasomatically recorded responses

Depolarizing pulses applied to the soma of the metathoracic fast coxal depressor motoneurone (Df) from untreated cockroaches elicit a series of damped oscillations (Pitman, 1979). In contrast, this soma gained the capacity to generate action potentials with a distinct threshold in preparations from animals that had been
exposed, a few hours previously, to 100% CO₂ or nitrogen. Depolarizing current pulses of small magnitude produced little or no active membrane response (Fig. 1A); slightly larger pulses could generate one or more action potentials (Fig. 1B,C). In some neurones the action potential peak could overshoot zero potential.

There were no differences between the effects observed after treatment with 100% CO₂ and those following 100% nitrogen exposure, indicating that the results can be attributed to anoxia.

Action potentials were recorded from the cell body of motoneurone D₁ at periods ranging from 10 h to 5 days after animals had received a 1 h exposure to 100% CO₂ or nitrogen. However, in recordings made as soon as possible after the gassing period,
no action potentials were observed. It appears, therefore, that a delay of at least several hours is required for the change in electrical properties to develop.

**Recordings from isolated cell bodies**

Recordings were made from the freshly isolated soma of the motoneurone to determine whether the action potentials observed after exposure to CO₂ or nitrogen were actually generated in the soma or whether they reflected a change in some other part of the neurone. The soma was removed from its ganglionic environment by aspiration. In most cases a small amount of axon could be seen attached to the isolated cell body; the length of this was hard to determine accurately, as it was

![Figure 2](image)

*Fig. 2. Electrical responses of isolated motoneurone cell bodies. (Ai–iv) Responses of an isolated soma from a normal (non-gassed) animal. Horizontal bars beneath each trace indicate the duration of applied current pulses. Depolarization caused the cell to produce a series of oscillations, the amplitude of which increased with the magnitude of the current applied through the second intracellular microelectrode. Resting potential = −80 mV. Time calibration, 80 ms. (B) Effect of a depolarizing pulse upon an isolated soma from an animal that had received a 2 h exposure to CO₂, 27 h before preparation for recording. This neurone produced an all-or-none action potential on depolarization. Resting potential = −75 mV. Time calibration, 200 ms.*
stretched during the isolation procedure and then contracted following separation from the ganglion. However, in those cells that were selected for these studies, the axon stump formed a fine process that appeared to project less than 20 μm from the isolated soma.

In the isolated cell body from a non-gassed animal, depolarizing pulses evoked a series of oscillations, the amplitudes of which were dependent upon the magnitude of the applied current (Fig. 2Ai–iv). These responses are similar to those recorded normally from neurones in situ (Pitman, 1979). In the soma from an animal that had been treated with CO₂ 27 h before the cell was set up for recording, depolarization produced all-or-none action potentials (Fig. 2B). This response is similar to recordings made from cells in situ in the metathoracic ganglion after animals had been pretreated with CO₂ or nitrogen (cf. Fig. 1). These observations on isolated cell bodies support the hypothesis that the electrical changes induced by pretreatment with CO₂ or nitrogen actually take place in the cell body itself. If, instead, an

![Graph](image)

Fig. 3. Effect of Na⁺-free solution upon action potentials induced by prior CO₂ treatment. Horizontal bars beneath each trace indicate the duration of applied current pulses (5 nA). (A) Response in normal saline; (B) after 2 min in Na⁺-free saline; (C) after 7 min in Na⁺-free saline; (D) the action potential was restored within 8 min of returning to normal saline solution. Na⁺-free solution hyperpolarized the membrane potential by approximately 10 mV. However, this was insufficient to account for suppression of the action potential.
alteration in axonal properties were responsible, the small length of axon left attached to the soma in these experiments would have to be capable of producing these changes recorded from the soma.

Ionic dependence of action potentials observed after CO₂ or nitrogen treatment

Na⁺-free solution

Removal of sodium from the bathing medium caused a progressive reduction in the amplitude of action potentials evoked by depolarizing pulses (Fig. 3). The reduction started within 2 min (Fig. 3B) and action potentials were almost completely abolished within 10 min (Fig. 3C). Upon washing with normal saline solution, action potentials returned to their original amplitude (Fig. 3D).

Tetrodotoxin

Action potentials could also be abolished by exposure to the selective sodium channel blocking agent tetrodotoxin, at a concentration of 2 × 10⁻⁷ mol⁻¹ (Fig. 4). This block was irreversible within the time period over which recordings were maintained (up to 1 h after the onset of washing).

![Fig. 4](image-url)

Fig. 4. Effect of tetrodotoxin (TTX) upon action potentials induced by CO₂ treatment. Horizontal bars beneath each trace indicate the duration of applied current pulses (6 nA). (A) Normal response of the neurone to depolarization; (B–E) 1 min, 2 min, 4 min and 6 min after addition of 2 × 10⁻⁷ mol⁻¹ tetrodotoxin to the solution bathing the preparation. There was a progressive decline and abolition of the action potential. Block was irreversible up to 1 h after the onset of washing when the experiment was terminated. Resting potential = −66 mV.
Manganese ions

Perfusion with saline containing 40 mM Mn²⁺ caused no reduction in action potential amplitude (Fig. 5). Mn²⁺, however, did reversibly suppress the after-hyperpolarization of the action potential (Fig. 5B,C).

Elevation of intracellular hydrogen ion concentration

Anoxia may be expected to produce a fall in intracellular pH as a result of metabolic acidosis. Since intracellular acidification has been shown to enhance excitability in some other preparations (Moody, 1980, 1983, 1984), the effects upon motoneurone Df of reducing intracellular pH were studied. Two approaches were used to decrease intracellular pH: (1) intracellular injection of acid and (2) addition of ammonium chloride to the solution bathing the preparation.

Intracellular acid injection

One intracellular microelectrode was filled with 2 mol L⁻¹ potassium acetate, while a second microelectrode was filled with either 100 mM hydrochloric acid or acetic acid, containing 0.2% Fast Green; pressure pulses (34.5–103.5 Pa) were applied to the back of this second microelectrode to inject H⁺ into the neurone. The dye allowed visual monitoring of the acid injection; normally each pressure pulse produced a small spot of stain around the microelectrode tip. Thus, microelectrode blockage could be detected immediately, as no dye would be ejected. Control injections of Fast Green made up in 2 mol L⁻¹ potassium acetate had no effect upon membrane potential or excitability. Immediately after acid injections the membrane potential was depolarized by 7–12 mV and the amplitude of membrane oscillations evoked by depolarizing current pulses was reduced (Fig. 6). The membrane potential and the amplitude of oscillations produced by depolarizing pulses gradually returned over a period of 2–5 min (Fig. 6D). In no experiment did H⁺ injection enable impaled somata to generate all-or-none action potentials.

Exposure to ammonium chloride

Since intracellular pH was not monitored during H⁺ injection, it is possible that the changes observed were not a direct result of a fall in intracellular pH. Therefore,
Fig. 6. Effect of injecting acetic acid into the motoneurone soma. Acid was pressure-injected from a microelectrode containing 100 mmol l⁻¹ acetic acid. Membrane potential was monitored through a second intracellular microelectrode filled with 2 mol l⁻¹ potassium acetate. Horizontal bars beneath each trace indicate the duration of applied current pulses (13 nA). (A) Response of the neurone before injection of acid; (B–D) responses 30 s, 1 min and 5 min after acid had been injected by a single 400 ms, 69 kPa pressure pulse applied to the acid-containing microelectrode. Acid injection caused a transient depolarization of 11 mV and a decrease in the magnitude of membrane oscillations evoked by depolarizing current pulses. The resting potential and amplitude of oscillations returned to normal within 5 min.

An alternative method was also used to lower intracellular pH; ammonium chloride was perfused over the preparation. In solutions of ammonium chloride, an equilibrium exists such that some ammonia and H⁺ are present, in addition to ammonium ions. Uncharged ammonia can cross cell membranes relatively easily and will tend to enter the cell until its intracellular and extracellular concentrations become equal. Within the cell ammonia will associate with free H⁺ and produce a rise in intracellular pH. However, this rise in pH is only transient, since NH₄⁺ can also enter the cell (travelling down an electrochemical gradient) although it does so more slowly than ammonia. The electrochemical gradient for NH₄⁺ is such that, at equilibrium, its intracellular concentration will exceed the extracellular concentration by between 10- and 24-fold (for a range of membrane potentials between −58 mV and −80 mV), depending upon the membrane potential. Dissociation of intracellular NH₄⁺ will lead to a reversal of the transmembrane gradient of ammonia, so that ammonia will now diffuse out of the cell; an excess of H⁺ remains within the cell and lowers the intracellular pH. On removal of ammonium chloride from the bathing medium, NH₄⁺ that has accumulated intracellularly will leave the cell mainly as ammonia, causing a further increase in intracellular H⁺ concentration (Boron & de Weer, 1976a, b; Thomas, 1984).

Within 1 min of introducing 20 mmol l⁻¹ ammonium chloride to the solution bathing the preparation, the membrane potential of the neurone began to depolarize. There was also a progressive decline in the amplitude of membrane oscillations evoked by depolarizing current pulses (Fig. 7A–D); on washing the preparation with
normal saline solution, the membrane potential further depolarized (Fig. 7E) before recovering to approximately its original value (Fig. 7F). The effects of ammonium ions in the external solution were very similar to those of injected H⁺.

**Distribution of RNA in the perinuclear cytoplasm**

To determine whether the change in electrical properties produced by exposure to CO₂ or nitrogen was associated with a redistribution of RNA, similar to that associated with the production of action potentials by axotomy or colchicine treatment (Pitman et al. 1972), histological sections of ganglia were stained using the pyronine–malachite green method (Baker & Williams, 1965; Cohen & Jacklet, 1967). In ganglia prepared 1–2 days after animals had been exposed to CO₂, the distribution of staining in the cell body cytoplasm was indistinguishable from that of neurones in control ganglia. This indicates, therefore, that the change in electrical properties of the soma of motoneurone D₁ induced by pretreatment with CO₂ is not associated with a major change in distribution of RNA in the perinuclear cytoplasm.

**DISCUSSION**

The experiments presented here demonstrate that exposure of cockroaches to an atmosphere of either carbon dioxide or nitrogen causes the cell body of the fast coxal depressor motoneurone (D₁) to undergo a dramatic change in properties, such that it can produce action potentials; the effects appear a number of hours after treatment.
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and may persist for several days. Recordings made from isolated nerve cell bodies a short time after isolation indicate that the changes in excitability apparently take place in the soma membrane itself rather than in adjacent regions of the neurone. The changes reported here resemble those previously observed in this and some other insect neurones following axotomy or treatment with the drug colchicine (which disrupts microtubules and thus blocks axonal transport) (Pitman et al. 1972; Pitman, 1975; Goodman & Heitler, 1979).

The excitable properties of neurones may be altered via one of two general mechanisms. First, the experimental conditions may produce an immediate and direct effect either upon ion channels or upon transmembrane ion gradients. Second, the experimental conditions could produce long-term modifications in cellular regulation processes which themselves could cause (a) novel types of ion channel to appear in the membrane, (b) redistribution of the normal classes of ion channel among different regions of the neurone or (c) a change in the density or properties of membrane ion pumps, which could generate relatively long-term shifts in the intracellular activities of one or more species of ion. The motoneurone studied in the present investigation (Df) provides a preparation in which excitability can be enhanced by either short-term or long-term mechanisms.

Intracellular injection of EGTA or citrate ions enables the soma of the neurone to generate all-or-none calcium-dependent action potentials within minutes of the start of injection. External application of tetraethylammonium ions (TEA+) also rapidly enables the soma of this neurone to generate calcium-dependent action potentials (Pitman, 1979). It appears that these procedures enhance excitability by suppressing one or more classes of potassium current; normally calcium currents are shunted to such a degree by potassium conductances that depolarization produces graded responses in the nerve cell body. However, when potassium currents are blocked, calcium currents are large enough to produce all-or-none action potentials. A similar mechanism also underlies hyperexcitability observed in crustacean muscle fibres during anoxia or in the presence of inhibitors of oxidative phosphorylation (Moody, 1978). Under these conditions, the intracellular H+ concentration rises and causes suppression of delayed outward potassium currents, enabling the muscle fibres to generate all-or-none calcium-dependent action potentials instead of graded responses (Moody, 1980, 1983). Thus these examples of increased excitability do not involve the appearance of any novel class of ion channel.

An example of a long-term enhancement in excitability is provided by alterations in the electrical properties of axotomized somata. The cell bodies of some insect and crustacean neurones develop the ability to generate sodium-dependent action potentials after axotomy (Goodman & Heitler, 1979; Kuwada, 1981). There is evidence to link the development of this hyperexcitability with changes in protein synthesis associated with axonal repair. In cockroach neurones the period of hyperexcitability coincides with the appearance of a dense perinuclear shell of RNA (Pitman et al. 1972), whereas in crayfish neurones, it has been demonstrated that inhibition of protein synthesis prevents any change in excitability (Kuwada, 1981). The hyperexcitability seen after axotomy probably results from an increase in the
number of functional sodium channels in the soma membrane during axonal repair (Goodman & Heitler, 1979; Wiens & Atwood, 1982). It has been proposed that sodium channels may normally distribute themselves among the different regions of a neurone; if the axon has been severed, and so no longer operates as a 'sink' for these channels, the somatic channel density may undergo a consequent increase (Wiens & Atwood, 1982). However, two different observations in the literature make this proposal less attractive. First, resectioning regenerating crustacean axons does not cause reappearance of hyperexcitability once it has waned, even though this would reduce the area of axonal membrane available to accept new sodium channels (Kuwada & Wine, 1981). Second, treatment of the axonal region of motoneurone D\(_1\) with colchicine induces electrical changes similar to those seen after axotomy, even though this agent does not disrupt axonal conduction or neuromuscular transmission in this preparation (Pitman et al. 1972). The observations reported here are also hard to reconcile with the above proposal since anoxia, like colchicine, does not block axonal conduction or disrupt axonal integrity.

The effect of anoxia reported here represents another relatively long-term excitability change. Like the change in electrical properties associated with axotomy or colchicine treatment, it only appears after a significant delay. In the preparation studied here several days must elapse after axotomy or colchicine treatment before hyperexcitability develops; this then persists for 1–2 weeks. Following a period of anoxia events take place more rapidly; action potentials may be recorded within 10 h and may persist for several days. Action potentials observed after anoxia, like those recorded after axotomy or colchicine treatment, are apparently dependent upon the operation of a class of ion channel that is normally absent from the soma membrane.

There are several different ways in which anoxia may induce a change in soma excitability. Anoxia will produce a fall in intracellular pH as a result of metabolic acidosis. It may also do so indirectly by inhibition of metabolically dependent sodium pumping systems; in many preparations (Thomas, 1984), including crayfish motoneurones (Moody, 1981), hydrogen ions are extruded by Na\(^+\)-dependent exchange mechanisms. Sodium pump inhibition could cause a fall in the transmembrane sodium gradient leading to a failure of Na\(^+\)/H\(^+\) exchange and a consequent fall in intracellular pH. Carbon dioxide will, in addition, itself cause a direct fall in intracellular pH. However, a rise in intracellular [H\(^+\)] is unlikely to underlie the changes in electrical properties observed in the cockroach fast coxal depressor motoneurone after anoxia for the following reasons: the excitability of the neurone shows no short-term enhancement either (1) in the presence of the sodium pump inhibitor ouabain or of metabolic inhibitors such as cyanide (R. M. Pitman, unpublished observations) or (2) in experiments designed to lower intracellular pH (by direct injection of hydrogen ions or by adding NH\(_4\)Cl to the solution bathing the preparation; see Figs 6, 7). In addition, the excitability changes do not appear immediately after the anoxia; following a short recovery period, the electrical characteristics of the neurone return to normal and alterations in excitability only begin to develop after about 10 h. These observations suggest that the changes reported here do not result from an immediate direct effect of intracellular pH on
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electrical properties of the neurone. However, an increase in intracellular concentration may either directly trigger an alteration in electrical properties through a long-term change in cellular regulatory mechanisms or may operate indirectly. For example, both CO$_2$ (Clark & Eaton, 1983; R. M. Pitman, unpublished observation) and procedures which may be expected to lower intracellular pH (see Figs 6, 7) produce a marked and rapid depolarization of the membrane potential. It is possible that this membrane depolarization or some other response to a fall in intracellular pH may trigger the long-term alterations in electrical properties observed after anoxia.

Since there does not appear to be any detectable change in perinuclear RNA distribution during the period of hyperexcitability following anoxia, it does not seem likely that the appearance of Na$^+$-dependent action potentials under these conditions is dependent upon a major change in protein synthesis such as that which apparently takes place after axotomy or colchicine treatment (Cohen & Jacklet, 1967; Pitman et al. 1972). If a change in the pattern of protein synthesis is important for the development of action potentials after anoxia, it must be relatively small and unrelated to axonal repair.

The observations reported here indicate, therefore, that a period of anoxia can produce a relatively long-term increase in excitability of an identified cockroach motoneurone cell body. It thus seems that the appearance of sodium-dependent action potentials in the soma of this neurone may be triggered by several different mechanisms and can occur in the absence of any significant mechanical damage. Future chronic experiments on isolated nerve cords will be necessary to determine whether the excitability changes reported here can be produced in vitro.

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