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REVERSIBLE CHANGES IN THE INTRACELLULAR POTASSIUM ION ACTIVITIES AND MEMBRANE POTENTIALS OF *APLYSIA* L_2–L_6 NEURONES IN RESPONSE TO NORMOXIA AND HYPOXIA

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SUMMARY

1. Exposure of 7 L_2–L_6 neurones to hypoxia for 65 min resulted in hyperpolarization of the membrane potential (E_M) from a mean of $-49{\cdot}1\pm2{\cdot}1$ to $-54{\cdot}1\pm3{\cdot}6$ mV (S.E.).

2. Intracellular potassium ion activities (a_K^i) increased significantly from $137{\cdot}7\pm4{\cdot}0$ to $155{\cdot}6\pm3{\cdot}4$ mM$-K^+$. This is equivalent to a change in E_K from $-74{\cdot}2$ mV commensurate with the observed hyperpolarization of 5 mV.

3. The reversibility of these responses was noted by reoxygenating the solution surrounding the ganglion for a period of 55 min.

4. In another group (n = 7) of L_2–L_6 neurones, the responses in a_K^i, E_M, and E_K were slower, although following hypoxia for 90–110 min, similar changes in the levels of these membrane phenomena were recorded.

5. P_{Na}/P_K ratios were computed for both L_2–L_6 groups of neurones using a modified version of the Goldman equation. There were only slight decreases in this ratio with hypoxia, which were not significantly different from the control (normoxia). Therefore, we conclude that this period of hypoxia is capable of stimulating the sodium pump of these cells since the membrane potentials seem to hyperpolarize according to the increase in a_K^i. However, tonic release of neurotransmitter, which could hyperpolarize these neurones and attract intracellular potassium, cannot be ruled out as an effect of hypoxia.

INTRODUCTION

In the isolated abdominal ganglion preparation of *Aplysia*, Chen, von Baumgarten & Harth (1973) and Chaplain (1976, 1979) have shown that signalling patterns of pacemaker cells are altered by compounds whose function is to regulate cellular respiration either by inhibiting glycolysis or by disrupting certain steps in this process, such as the catalysis of fructose-6-phosphate (F-6-P) by phosphofructokinase (PFK). Chen *et al.* (1973) reported that administration of glucose and dinitrophenol (DNP), two substances which affect glycolysis and uncouple oxidative phosphorylation respectively, also affects the frequencies of regular impulses in neurones occupying the

Key words: Potassium, hypoxia, membrane potential.

upper right rostral quadrant of the ganglion. In those studies, little effect of alteration in cellular metabolism upon the level of the membrane potential was demonstrated.

However, hyperpolarizations or depolarizations of the membrane potentials in abdominal ganglion neurones have been demonstrated by equilibration of the suffusate using CO_2, N_2, or O_2 gases (Chalazonitis, 1963; Chalazonitis & Takeuchi, 1964). The results of Coyer, Halsey & Strong (1981) showed that the membrane properties of neurones from the abdominal ganglion of *Aplysia* respond differentially to the oxygen tension present in the suffusate. In that paper, we concluded that one group of neurones remained 'resistant' to hypoxia while another one was 'non-resistant' based upon the following observations: the membrane potentials of 'resistant' neurones hyperpolarized while the membrane potentials of 'non-resistant' ones depolarized in response to hypoxia. At corresponding membrane potentials, there was also an apparent change in the membrane slope resistances of the two groups of neurones which was detected by injecting current in a linear, depolarizing manner and measuring the voltage change independently with a second microelectrode. For the 'resistant' group of neurones, hyperpolarizations of the membrane potentials and increases in the membrane slope resistance were reversible while for the 'non-resistant' group depolarizations of the membrane potentials and decreases in the slope resistance were irreversible with subsequent reoxygenation of the suffusate (Coyer *et al.* 1981). Other major differences, such as changes in a_K^i or the P_{Na}/P_K ratio, between these two groups of neurones were not explored.

In a series of experiments reported in this paper, the sensitivities of the 'resistant' L_2–L_6 pacemaker group have been investigated. In these experiments, simultaneous measurements of membrane potentials and intracellular potassium ion activities (a_K^i) were used in determining the relationship between the membrane potential (E_M) and the potassium equilibrium potential (E_K) as well as in computing the relative sodium/potassium permeability ratios (P_{Na}/P_K) based upon a modification of the Goldman equation.

Although the L_2–L_6 neurones were not physically isolated from others within the ganglion, thus precluding the possibility of synaptic intervention, intracellular recordings of pacemaker activity showed little evidence of synaptic potentials. However, the question of long-term alterations of these neurones' responses through synaptic activation, such as is the case of R_{15}'s prolonged hyperpolarization brought about by interneurones (Parnas, Armstrong & Strumwasser, 1974), has to be considered (see Discussion). Similar analyses have been applied to the responses of the 'non-resistant' group of neurones, and the results are the subject of a separate paper (P. E. Coyer, in preparation).

MATERIALS AND METHODS

Neurone preparation

Live *Aplysia californica* were supplied by the Pacific Bio-Marine Company, Venice, California. They were held in an aquarium containing filtered, circulating sea water (Instant Ocean, 1025 mosM) and fed boiled lettuce. For dissection the animals were pinned to a wax-bottomed tray, ventral incisions were made through the foot, and abdominal ganglia were removed under cold-treatment relaxation of the animals'

neuromuscular systems. Since it was felt that excessive cooling might lead to sodium loading during microdissection of the ganglionic sheath (Carpenter & Alving, 1968; Junge & Oritz, 1978), the ganglia were kept for a period of 30 min at 18 °C before being placed in a constantly-suffused, Sylgard-coated (Dupont), Corning dish (18 × 45 mm) and being desheathed.

The effects of hypoxia (see below) were tested in the L_2–L_6 group of neurones within the abdominal ganglion of *A. californica*. This distinct group of neurones was identified both anatomically and electrophysiologically. Neurones within this group were recognized using established criteria (Frazier *et al.* 1967; Kandel, Frazier, Waziri & Coggeshall, 1967; Koester & Kandel, 1977).

Experimental protocol

Microelectrodes and electrical recording

Membrane potentials (E_M, see inset Fig. 2A) and intracellular potassium ion activities (a^i_K) of 14 L_2–L_6 neurones were determined using double-barrelled, K^+-selective microelectrodes (Walker, 1971; Khuri, Hajjar & Agulian, 1972; Vyskočil & Kříž, 1972). The construction and use of these microelectrodes has been adequately described by Vyskočil & Kříž (1972), Schlue & Deitmer (1980), and Deitmer & Schlue (1981). The tip of the K^+-selective barrel was backfilled with potassium ion exchanger resin (Corning #477347) and the shaft with 0·5 M-KCl. The reference barrel of the double-barrelled microelectrode was filled with either 0·5 M-KCl or 0·5 M-NaCl. The K^+-selective barrel is known to have a slow electrical time response (Khuri *et al.* 1972; Fujimoto & Kubota, 1976). Therefore, the influence of a changing spike rate may affect the potential registered by this barrel. The response of the K^+-selective barrel was observed during independent stimulation using a second, intracellulary-positioned microelectrode (Fig. 2C). For further consideration of the influence of spike frequency and changes in the level of the membrane potential on the K^+-selective barrel, see the Discussion and Fig. 2C.

Constant-interference and constant-ionic strength calibration techniques were employed in obtaining the empirical slope (S), a constant (E_o), the selectivity coefficient (K_{KNa}), and their relationship to the electrode potential (E) given by equation 1.

$$E = E_o + S \log_{10} (a^i_K + K_{KNa}\, a^i_{Na}) \quad (1)$$

In each case, a slope of 53–55 mV was obtained for each 10-fold change in a^i_K and equation 1 could then be replaced by equation 2.

$$E = -138{\cdot}2 + 53{\cdot}0\,\text{mV} \log_{10}(a^i_K + K_{KNa}\, a^i_{Na}) \quad (2)$$

$K_{KNa} = 0{\cdot}0055$ for a selectivity ratio of 182 times for K^+ over Na^+.

A curve showing the results of constant-interference calibration using a background activity of 112 mM-Na^+ at various K^+ activities is presented in Fig. 1. A line relating the mV potential to the common logarithm of the K^+ activity has a slope of 53 mV. Confidence intervals for each mV value corresponding to a specific ionic activity were established using data from 8 electrodes. Although we found no statistically significant differences between these slopes and that of 57·7 mV as predicted by the Nernst equation, we recognize that substitution of a slightly smaller slope into equation 2 gives a higher value of a^i_K. The major findings reported in this paper are not

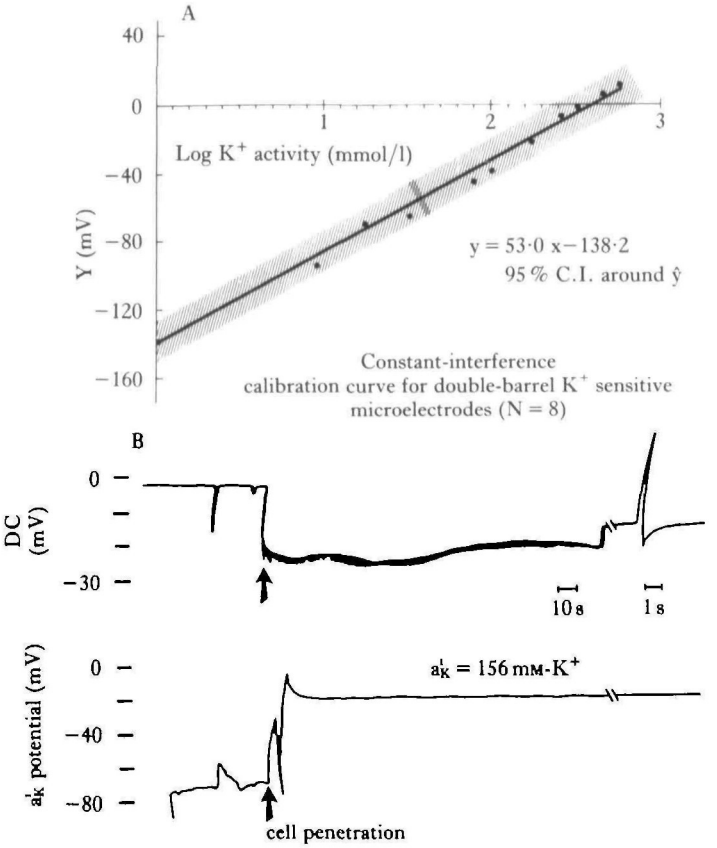

Fig. 1. Calibration curve of double-barrelled, K^+-selective microelectrodes (N = 8) under constant-interference conditions of a background Na^+ activity equal to 112 mM. A line having a slope of 53 mV exists for the relationship between the potential registered by the K^+-selective electrode and the logarithm of the K^+ activity (A). The results of insertion of K^+-selective microelectrode into an unidentified *Aplysia* neurone are shown (B). The activity potential responds by becoming more positive, indicative of a higher potassium ion activity. In the early part of the record of membrane potential, the spikes have been deleted to omit the blotchy appearance due to a slow chart speed.

contingent upon absolute numbers for a^i_K but rather upon differences between the values which we recorded during normoxic (control) and hypoxic (experimental) conditions in the same group of L_2–L_6 neurones. Our computations of a^i_K under control conditions of normoxia for the L_2–L_6 neurones are within the range which is reported in the literature (Kunze & Brown, 1974; see Discussion).

Extracellular potassium ion activities (a^o_K) were not measured simultaneously with intracellular potassium ion activity because of the limitations of electrode numbers and the availability of differential amplifier configurations. The K^+-activity potential registered by the double-barelled microelectrodes equalled 7·1–7·8 mM-K^+ when measured under a constant concentration of 10 mM-K^+. All electrophysiological measurements obtained from the 14 L_2–L_6 neurones were made during complete exposure of the cell bodies to the bathing solution.

Voltage changes corresponding to E_M, a^i_K, and a^o_K were displayed on a Tektronix 7623A oscilloscope, directed through either a low-level DC (Grass Model P1) amplifier for oscillograph recording (Grass polygraph Model 7D) or through a DC

amplifier for penwriter recording (Gould), and stored for future analysis on a 4-channel Hewlett-Packard FM tape recorder.

Construction of dynamic current-voltage curves

Double intracellular impalements of large, unpigmented L_2–L_6 neurones were accomplished with both voltage-measuring and current-passing micropipettes.

A linear, depolarizing ramp voltage provided a constant current source for stimulation (10^{-8} A/10 s). The level of the membrane potential was measured at a point of zero current passage (solid vertical line in Fig. 2B) and is shown by the inverted T-shaped line below 0 mV (Fig. 2B). The membrane slope resistance was calculated by measuring the slope of a line tangent to these curves (Fig. 2B), and the further details describing this procedure are contained in Coyer *et al.* (1981).

Solutions, gaseous equilibration, and PO_2 determinations

Normal *Aplysia* saline (NS) was delivered constantly at a rate of 10 ml/min to the Corning dish from a reservoir located above it. The normal solution consisted of: 425 mM-NaCl, 10 mM-KCl, 10 mM-$CaCl_2$, 22 mM-$MgCl_2$, 26 mM-$MgSO_4$, 2·5 mM-$NaHCO_3$, 10 mM-Tris-HCl (pH = 7·3). Hypoxia was achieved by equilibrating the suffusate with either 99·99% nitrogen or 95% nitrogen/5% oxygen mixtures. Hypoxia was arbitrarily defined as a suffusate PO_2 below 20 Torr which probably corresponds to an intracellular PO_2 of 5–7 Torr (Coyer *et al.* 1978). This contrasts with an air-equilibrated suffusate which has a PO_2 of 130–150 Torr, depending upon its relation to the surface of the suffusate, and an intracellular PO_2 of 20–30 Torr (Chalazonitis, 1963). Reoxygenation of the suffusate was accomplished by oxygen equilibration. The temperature at the outlet of the solution was monitored with a thermistor and was maintained at 18 ± 2 °C. The pH changed less than 0·1 unit during nitrogen bubbling (pH = 7·3 ± 0·1) at this constant temperature.

Oxygen microelectrodes were constructed according to the noble metal technique (Erdmann, Krell, Metzger & Nixdorff, 1969). A current to voltage amplifier having adjustable feedback compensation was used for these PO_2 determinations. Before and after use, oxygen microelectrodes were calibrated under high-grade nitrogen (purity 99·99%), 5% O_2, and ambient conditions of air-saturation at 18 °C. The oxygen partial pressure of the suffusate bordering the isolated ganglion was measured with these microelectrodes.

Computations of the potassium equilibrium potential (E_K) and the relative permeability ratios (P_{Na}/P_K)

E_K was computed from the Nernst equation using 18 °C or 291·16 °K for the temperature, 7·1–7·8 mM-K^+ for the numerator of the ratio of the potassium ion activities, whatever value was calculated from the potassium ion-sensitive measurement for the denominator, and the standard constants (RT/F = 25·09 mV)×2·303 equalling 57·7 mV/10-fold change in the potassium ratio.

The P_{Na}/P_K ratios were calculated from a modified version of the Goldman equation without consideration of the chloride ion permeability. The rationale for neglecting the contribution of this ion is the same that is contained in Moreton (1968, 1969)

and Gorman & Marmor (1970). Unlike the methodology used by them (Moreton 1968, 1969; Gorman & Marmor, 1970), a^i_K was measured directly in these experiments using potassium ion-selective microelectrodes. The P_{Na}/P_K ratio is given by equation 3:

$$P_{Na}/P_K = \{(a^iK^+)e^{E_M F/RT} - (a^oK^+)\}/\{(a^oNa^+) - (a^iNa^+)e^{E_M F/RT}\} \quad (3)$$

where a^i_K is the intracellular potassium ion activity; E_M, the membrane potential measured in mV; the ratio of the constants F/RT equals 0·03986 when expressing the membrane potential in mV and using a temperature equal to 18 °C or 291·16 °K. a^oNa^+ was calculated from a value of the extracellular sodium ion concentration equal to 425 mM-Na^+ using an activity coefficient. a^o_K and a^i_K were determined from the potentiometric measurements and conversions from the calibration curve. Substitution of known intracellular sodium values ($a^iNa^+ = 23$ mM) was used in calculating the P_{Na}/P_K ratios (Kunze & Brown, 1974). Preliminary results using this modification of the Goldman equation have been reported elsewhere (Coyer, 1981).

RESULTS

Determinations of a^i_K, E_M and P_{Na}/P_K ratios in L_2–L_6 neurones during normoxia, hypoxia and reoxygenation

Transient changes

Seven of the 14 L_2–L_6 neurones whose membrane potentials hyperpolarized during exposure to hypoxia showed reversible changes in a^i_K and E_M during hypoxia and reoxygenation. The typical response of a hyperpolarizing bursting pacemaker cell of this group is shown in Fig. 2A (A1, normoxia and hypoxia; A2, reoxygenation), and a summary of the data collected for all 7 neurones is listed in Table 1. These neurones showed a quick decrease in a^i_K followed by a return to pre-hypoxic levels of the membrane potential during reoxygenation of the suffusate. The results in Table 1 show that the values of E_M and a^i_K for these neurones increased (E_M, more negative) during hypoxia and subsequently decreased with reoxygenation. Moreover, paired *t*-tests for the mean differences for E_M and a^i_K during normoxia and hypoxia showed significant ($P < 0{\cdot}05$) increases following lowered PO_2's. The observed hyperpolarization and concomitant rise in a^i_K were not accompanied by a significant change in the P_{Na}/P_K ratios, which was also established by using paired *t*-tests. There were slight decreases in the ratio during hypoxia. Percent changes in the P_{Na}/P_K ratios from the control measurements were computed (see Table 1) and compared using the Student's *t*-test. Detectable decreases in the P_{Na}/P_K ratios were found not to be significant ($P > 0{\cdot}05$) using this statistical procedure.

Fig. 2B shows the method of determining the membrane slope resistance or $\Delta V/\Delta I$ during passage of a ramp-like current across the nerve cell membrane while monitoring the membrane potential independently with a second microelectrode. The membrane slope resistance increased with hypoxia as shown by the increase in the slope of the line while the current ramp is passed in a depolarizing direction. Subsequently, the slope became less steep with reoxygenation. Possible discrepancies between this observation and that of nearly constant P_{Na}/P_K ratios will be treated in the Discussion.

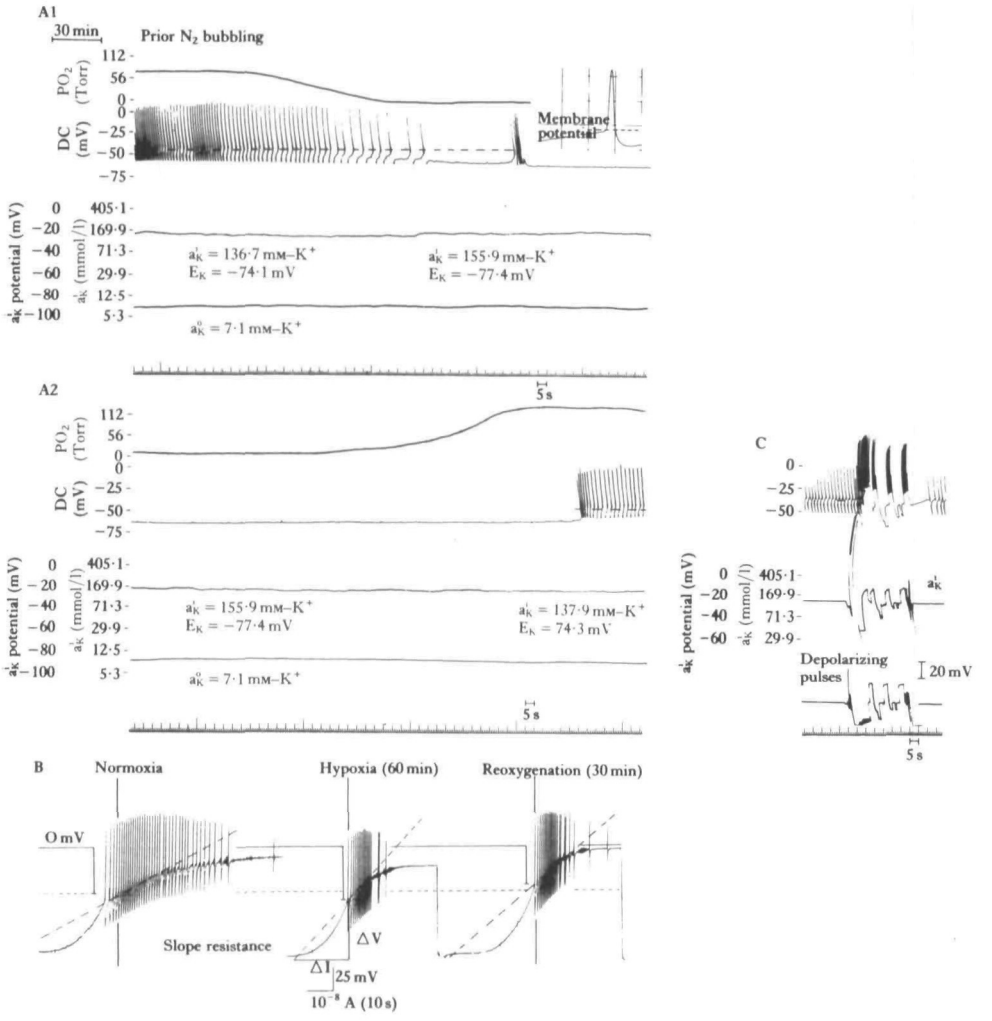

Fig. 2. Concomitant increases in a_K^i and membrane hyperpolarization in a L_2–L_6 neurone; return to prior levels of E_M and a_K^i with reoxygenation (A1 and A2). B. Changes in membrane slope resistance with hypoxia (middle curve). C. Response of K^+-selective microelectrode to depolarizing pulses (see text) through an independently inserted microelectrode.

Slower changes

For a second group of 7 L_2–L_6 neurones which we studied, the electrophysiological responses were slower. A typical record of the response in E_M and a_K^i is shown in Fig. 3. A longer period of hypoxia (90 min duration) was necessary to elicit comparable membrane hyperpolarizations and increases in a_K^i as observed in the first group. The summary data for 7 neurones is found in Table 2. Reversibility under subsequent re-oxygenation was not tested in these cells, and therefore only control and experimental data appear in this table since we were not able to maintain electrode penetrations in all of these cells during reoxygenation. Often the smooth muscle fibres, within the sheath (Mirolli & Gorman, 1968), contracted under longer durations of hypoxia, thus shifting

Table 1. *Data calculated for the L_2–L_6 neurones whose responses are shown in Fig. 1*

		L_2–L_6 Neurones			
		E_M (mV)	a'_K (mM)	P_{Na}/P_K Ratio	% Δ from control
Normoxia $\bar{X} \pm$ S.D.	1.	−49·6 ± 8·7	140·2 ± 14·5	0·032 ± 0·011	
	2.	−47·8 ± 8·9	142·8 ± 13·8	0·037 ± 0·011	
	3.	−47·9 ± 8·3	136·7 ± 12·0	0·035 ± 0·013	
	4.	−47·5 ± 9·3	137·0 ± 14·5	0·036 ± 0·018	
	5.	−49·1 ± 7·1	141·6 ± 13·8	0·028 ± 0·010	
	6.	−53·7 ± 5·3	132·6 ± 11·9	0·022 ± 0·009	
	7.	−48·4 ± 5·6	133·2 ± 22·7	0·030 ± 0·015	
$\bar{X} \pm$ S.E.		−49·1 ± 2·1	137·7 ± 4·0	0·032 ± 0·003	
		Mean $E_K = -74{\cdot}2$ mV			
Hypoxia (65 min) $\bar{X} \pm$ S.D.	1.	−53·1 ± 8·9	156·1 ± 8·9	0·031 ± 0·015	−3·1
	2.	−54·5 ± 8·0	162·7 ± 9·9	0·029 ± 0·014	−21·6
	3.	−55·0 ± 8·2	155·9 ± 7·0	0·026 ± 0·014	−25·7
	4.	−53·8 ± 7·1	155·0 ± 8.4	0·028 ± 0·013	−22·2
	5.	−54·0 ± 5·5	154·6 ± 11·5	0·028 ± 0·014	0·0
	6.	−53·0 ± 8·4	153·1 ± 10·6	0·029 ± 0·019	+31·8
	7.	−55·0 ± 8·2	152·1 ± 11·9	0·029 ± 0·001	−3·3
$\bar{X} \pm$ S.E.		−54·1 ± 3·6	155·6 ± 3·4	0·028 ± 0·002	−6·3 ± 7·5
		Mean $E_K = -77{\cdot}2$ mV			
Reoxygenation $\bar{X} \pm$ S.D.	1.	−48·3 ± 7·5	141·8 ± 14·8	0·036 ± 0·015	+12·5
	2.	−47·5 ± 8·2	145·8 ± 15·1	0·039 ± 0·001	+5.4
	3.	−47·8 ± 8·3	137·9 ± 13·0	0·035 ± 0·013	0·0
	4.	−49·0 ± 6·0	135·8 ± 12·8	0·032 ± 0·010	−11·1
	5.	−48·5 ± 6·5	140·8 ± 13·5	0·035 ± 005	+21·4
	6.	−52·0 ± 6·7	133·7 ± 12·8	0·025 ± 0·009	+13·6
	7.	−48·8 ± 5·8	138·9 ± 18·2	0·034 ± 0·018	+13·3
$\bar{X} \pm$ S.E.		−48·8 ± 1·5	139·2 ± 4·0	0.034 ± 0·004	+7·9 ± 4·1
		Mean $E_K = 74{\cdot}4$ mV			

Means ($\bar{X}$) ± standard deviations (S.D.) for the membrane potential (E_M), intracellular potassium ion activities (a_K^i), and the computed P_{Na}/P_K ratios for each of the 7 L_2-L_6 neurones whose responses are shown in Fig. 2 appear during normoxia (30 min), hypoxia (65 min), and reoxygenation (55 min). Data for each neurone are listed by a number. Group means ($\bar{X}$) ± their standard errors (S.E.) appear for each treatment, and mean values of the potassium equilibrium potential (E_K) were calculated from the mean value of a_K^i. $E_M = -49{\cdot}1 \pm 2{\cdot}1$ mV (normoxia); $-54{\cdot}1 \pm 3{\cdot}6$ mV (hypoxia); and $-48{\cdot}8 \pm 1{\cdot}5$ mV (reoxygenation). $a_K^i = 137{\cdot}7 \pm 4{\cdot}0$ mM (normoxia); $155{\cdot}6 \pm 3{\cdot}4$ mM (hypoxia); and $139{\cdot}2 \pm 4{\cdot}0$ mM (reoxygenation). $P_{Na}/P_K = 0{\cdot}032 \pm 0{\cdot}003$ (normoxia), $0{\cdot}028 \pm 0{\cdot}002$ (hypoxia), and $0{\cdot}034 \pm 0{\cdot}004$ (reoxygenation). Paired *t*-tests were performed among the mean differences of E_M, a_K^i, and the P_{Na}/P_K ratio during normoxia, hypoxia, and reoxygenation. E_M and ^i_L were significantly greater during hypoxia ($P < 0{\cdot}05$), and there were no differences between the normoxic and reoxygenated values ($P > 0{\cdot}05$). No significant differences were observed among the P_{Na}/P_K ratios or among the percent changes (hypoxia and reoxygenation) from the control ($P > 0{\cdot}05$).

the position of recording microelectrodes. Therefore, we have inconclusive data concerning the reversibility of E_M and a_K^i in these cells. Without showing this phenomenon, the importance of these experiments seems to lie in the observations of changes in E_M and a_K^i similar to those we saw in the first group of neurones although the difference in the length of hypoxia (65 versus 110 min of hypoxia) may not be critical for these neurones. Again, we computed the percent changes in the P_{Na}/P_K ratios between control (normoxic) and experimental (hypoxic) conditions and noted that there was a tendency for a decrease in the relative permeability ratio during

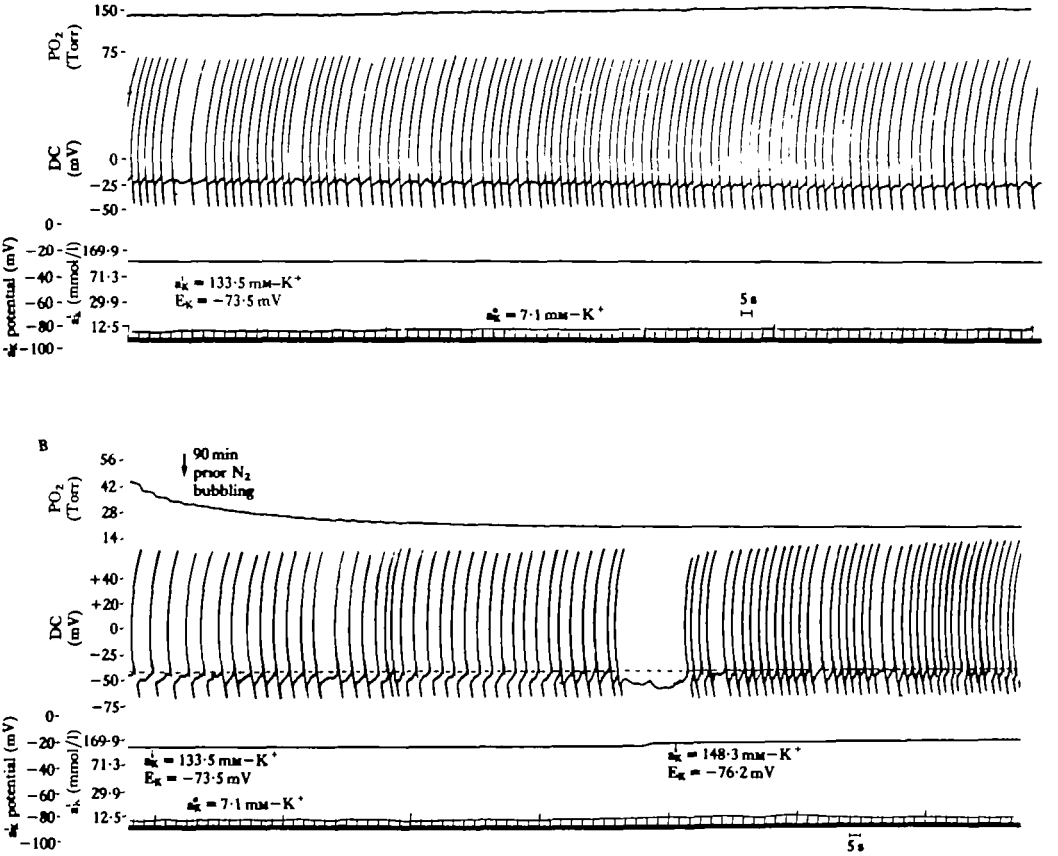

Fig. 3. Recordings of a_K^i and E_M during normoxia ($PO_2 \sim 150$) in A in another group ($N = 7$) of L_2–L_6 neurones. Slower changes in the observed membrane hyperpolarizations and concomitant increases in a_K^i were noted. After 90 min of hypoxia ($PO_2 < 20$ Torr), increases in a_K^i and membrane hyperpolarization were observed. The membrane potential hyperpolarized below the control (dotted line) level as shown in B.

hypoxia. The average percent change has a large standard deviation which included zero ($-13{\cdot}4 \pm 14{\cdot}3$) leading one again to conclude that the differences between control and experimental P_{Na}/P_K ratios are insignificant.

DISCUSSION

Possible sources of error in measurements of a_K^i and calculations of P_{Na}/P_K ratios

Alterations in spike frequency

Fig. 2C shows the response of the K^+-selective barrel to artificial depolarization resulting from current pulses passed through an independent microelectrode. The K^+-potential becomes more positive, thus indicating a higher level of a_K^i. Naturally, the reference barrel measures the depolarized membrane potential and the associated

Table 2. *Data calculated for the L_2-L_6 neurones whose responses are shown in Fig. 3*

		L_2-L_6 Neurones			
		E_M (mV)	a_K^i (mM)	P_{Na}/P_K	% Δ from control
Normoxia	1.	−47·5 ± 9·8	141·8 ± 13·8	0·038 ± 0·005	
$\bar{X}$ ± S.D.	2.	−48·9 ± 8·6	138·5 ± 8·9	0·033 ± 0·006	
	3.	−52·3 ± 5·8	137·0 ± 12·5	0·026 ± 0·010	
	4.	−47·5 ± 8·3	133·5 ± 15·0	0·034 ± 0·008	
	5.	−52·1 ± 7·5	140·2 ± 10·8	0·027 ± 0·006	
	6.	−49·3 ± 10·2	136·7 ± 9·8	0·032 ± 0·005	
	7.	−45·3 ± 8·3	137·0 ± 12·0	0·041 ± 0·009	
$\bar{X}$ ± S.E.		−49·0 ± 2·5	137·7 ± 2·7	0·033 ± 0·005	
			Mean E_K = −74·2 mV		
Hypoxia (110 min)	1.	−53·4 ± 15·8	152·3 ± 16·2	0.028 ± 0·010	−26·3
$\bar{X}$ ± S.D.	2.	−55·0 ± 16·8	168·4 ± 17·0	0·030 ± 0·005	−9.1
	3.	−56·2 ± 18·5	162·3 ± 18·0	0·026 ± 0·006	0·0
	4.	−50·5 ± 16·2	148·3 ± 16·9	0·033 ± 0·009	−2·9
	5.	−55·1 ± 18·2	158·5 ± 16·2	0·027 ± 0·004	0·0
	6.	−54·3 ± 13·8	148·6 ± 18·9	0·026 ± 0·008	−18·8
	7.	−48·2 ± 15·5	153·8 ± 7·8	0·026 ± 0·010	−36·6
$\bar{X}$ ± S.E.		−53·2 ± 2·9	156·0 ± 7·4	0·028 ± 0·003	−13·4 ± 5·4
			Mean E_K = −77·3 mV		

Means ($\bar{X}$) ± standard deviations (S.D.) for the membrane potential (E_M), intracellular potassium ion activities (a_K^i), and the computed P_{Na}/P_K ratios for 7 other L_2-L_6 neurones whose responses are shown in Fig. 3 during normoxia and prolonged hypoxia (110 min). Data for each neurone is listed by a number. Group means ($\bar{X}$) ± their standard errors (S.E.) appear for each treatment, and mean values of the potassium equilibrium potential (E_K) were calculated from the mean value of a_K^i. $E_M = -49{\cdot}0 \pm 2{\cdot}5$ mV (normoxia) and $-53{\cdot}2 \pm 2{\cdot}9$ mV (hypoxia). $a_K^i = 137{\cdot}7 \pm 2{\cdot}7$ mM (normoxia) and $156{\cdot}0 \pm 7{\cdot}4$ mM (hypoxia). $P_{Na}/P_K = 0{\cdot}033 \pm 0{\cdot}005$ (normoxia) and $0{\cdot}028 \pm 0{\cdot}003$ (hypoxia). Paired *t*-tests were performed among the mean differences of E_M, a_K^i and the P_{Na}/P_K ratios between normoxic and hypoxic conditions. E_M and a_K^i were significantly greater during hypoxia ($P < 0{\cdot}05$), but there was no significant difference betwee the P_{Na}/P_K ratios ($P > 0{\cdot}05$). The average (± S.D.) percent change is $-13{\cdot}4 \pm 14{\cdot}3$ (S.E. = 5·4)

increases in spike frequency. Increases in the spike frequency and concomitant increases in a_K^i are recorded in these experiments in which the cell is depolarized. Alternatively, during these artificially-induced changes in spike frequency, hyperpolarization and decreases in spike frequency would result in a more negative a_K^i potential indicative of less a_K^i. In experiments with the L_2–L_6 neurones, we recorded hyperpolarization and a reduced spike frequency. Obviously, the K^+-selective barrel responded to increases in a_K^i as shown by a more positive registered potential rather than to changes in the spike frequency (see Figs 2, 3). Also, the double-barrelled, K^+-selective microelectrode was capacity compensated by a positive feedback circuit before insertion into the cell, which should have minimized some of the errors in reading the potential developed by the K^+-selective barrel while the spike frequency was changing. We feel that this check on the system is compelling enough to lend credibility to the results of membrane hyperpolarization and increases in a_K^i.

Changes in a_K^o

A possible problem in the interpretation of these experiments is that increases in a_K^o in the intercellular areas might stimulate the pump and increase a_K^i. Changes in

a_K^o may have occurred within the deeper neuropile, but since these neurones were fully exposed and in contact with the flow-through bathing solution, it is doubtful whether a_K^o varied. Steady-state conditions were assumed to exist between inside and outside activities in calculating the P_{Na}/P_K ratios from the Goldman equation. Relatively high flow rates of 10 ml/min probably eliminated gradients existing between the solution and the surface of the neurones. Evidence for a constant value of a_K^o is indirect, but we feel that no transient increases in a_K^o occurred since we never recorded any spontaneous decreases in a_K^i during control (normoxic) monitoring, which might be expected to increase a_K^o. Every case of a change in a_K^i followed a typical pattern in which a_K^i first increased during hypoxia and subsequently returned to its pre-hypoxic level during reoxygenation.

Tonic release of a neurotransmitter

Tonic release of a neurotransmitter by a presynaptic neurone cannot be ruled out. This could result in membrane hyperpolarizations, which would naturally attract more positive K^+ ions. Pinsker & Kandel (1969) have demonstrated that under the presynaptic influence of the interneurone L_{10} the outward pump current of L_5 can be increased by the presence of post-synaptic potentials. These workers (Pinsker & Kandel, 1969) recorded synaptic potentials which were linked to outward pump currents having no reversal potential and concluded that pre-synaptic neurones could influence the metabolism of other post-synaptic neurones by altering the pump's activity. Presumably, these increases in extracellular potassium around L_5 were thought to stimulate its membrane pump.

Effects of hypoxia on membrane properties of other neurones

As mentioned above, hypoxia limits the amount of oxidative phosphorylation being carried out in mitochondria (providing there is direct coupling to aerobic pathways) resulting in a reduction in the amount of ATP available to supply active transport. Other studies, in which uncouplers of oxidative phosphorylation were used, report a depolarization of the resting potentials brought about by inhibition of the pump (Hodgkin & Keynes, 1955) or a change in the neurone's excitability due to an activation of a calcium-dependent potassium conductance (Godfraind, Kawamure, Krnjevic & Pumain, 1971). Godfraind *et al.* (1971) have reported a decrease in the input resistance during treatment of neurones with uncouplers of oxidative phosphorylation while Moody (1978, 1980) has found an increase in the excitability of crustacean tonic flexor fibres during treatments with anoxia or uncouplers of oxidative phosphorylation. A decrease in delayed rectification accounted for the calcium spike electrogenesis which resulted from interruption of cellular metabolism (Moody, 1978). For the *Aplysia* L_2–L_6 neurones, the data presented for relative permeability changes (see Results) suggest that the potassium permeability increases with hypoxia. This observation may seem to be supported in part by that of changes in the membrane slope resistance with hypoxia (Fig. 2B). We cannot single out specific ionic conductance changes from these experiments (Fig. 2B). Furthermore, we cannot necessarily relate results derived from steady-state calculations of the permeability ratios to these observations of changes in membrane conductance.

From the experiments reported here on the L_2–L_6 neurones of *Aplysia californica*,

it appears that hypoxia stimulates the metabolic pump which increases a_K^i and contributes to membrane hyperpolarization by making E_K more negative. Kerkut & York (1969) have demonstrated the oxygen sensitivity of the electrogenic sodium pump in brain neurones of the snail *Helix*. Sodium-injected neurones had membrane potentials which were more dependent upon PO_2 than were potassium-injected neurones. Kerkut & York (1969) concluded that the sodium pump relies heavily upon the process of oxidative phosphorylation to supply energy in the form of ATP or another high-energy phosphate-containing compound. It has been suggested that molluscan neurones have ATP reserves sufficient for 20–30 min of normal sodium pumping, providing the intracellular PO_2 is higher than 20 Torr, as exists under normal, aerated conditions of intracellular recording (Kerkut & York, 1969; Junge & Oritz, 1978). Chalazonitis, Gola & Arvanitaki (1966) suggested that the membrane potentials of *Aplysia* neurones were much more sensitive to PO_2 in the physiological range of 5–7 Torr (intracellular PO_2) at which most cytochromes are reduced. At extracellular PO_2's of less than 20 Torr, which were maintained in our experiments for 65–110 min, it is very likely that alterations in the synthesis of ATP are produced by low PO_2's (hypoxia). This change in the (ATP)/(ADP)(Pi) ratio, then, probably stimulates ATP synthesis via glycolytic feedback. The result is an increase in the sodium pump's activity. This accounts for the rapid increase in a_K^i and membrane hyperpolarization. Higher intracellular levels of ADP have been shown to stimulate respiration of neuroblastoma cells *in vivo* (Wilson, Erećinska, Drown & Silver, 1979; Wilson, Owen & Erećinska, 1979), and injections of ADP into squid axons stimulates $Na^+ : Na^+$ exchange (DeWeer, 1970). For myelinated neurones of *Xenopus*, incorporation of the (ATP)/(ADP)(Pi) ratio in describing the activity of the ionic pump, which underlies post-tetanic hyperpolarization, has been included in the Frankenhaeuser-Huxley constant field equations (Schoepfle & Tarvin, 1979). We speculate that in *Aplysia* decreases in this ratio brought about by depletion of ATP and accumulation of ADP may stimulate respiration and increase the pump's activity.

Calculations of P_{Na}/P_K ratios in L_2–L_6 neurones of Aplysia *and other molluscan neurones*

In the pulmonate *Helix aspera*, application of the 'constant-field' theory has been used to describe the behaviour of the resting membrane potential and to estimate intracellular potassium ion concentrations (Moreton, 1968). Moreton concluded that the mean P_{Na}/P_K ratio for neurones of the freshwater pulmonate was $0{\cdot}180 \pm 0{\cdot}015$ and the potassium concentration equalled $92{\cdot}9 \pm 4{\cdot}3$ mM.

The permeability ratios which are calculated in this paper (Tables 1, 2) are similar in magnitude to the ones Eaton, Russel & Brown (1975) computed for *Aplysia* using permeability measurements in response to each ion's contribution to the voltage change. From step-wise electrical changes, Eaton *et al.* (1975) computed a P_{Na}/P_K ratio equal to 0·012. Their chemical computations of the P_{Na}/P_K ratio were made from ion-selective electrode measurements (Eaton *et al.* 1975), and the chemical values are higher ($P_{Na}/P_K = 0{\cdot}13$), indicating that there was a higher sodium permeability than that shown by the electrical measurements. Using 'constant-field' conditions and ouabain or low temperatures to block the membrane pump, Gorman & Marmor (1970) calculated similar P_{Na}/P_K ratios in *Anisodoris* neurones to those reported here

for *Aplysia*. The ratios which are calculated here show a potassium permeability of approximately 30× greater than the sodium permeability. The agreement of observed membrane hyperpolarizations with the increases in a_K^i suggest that the potassium permeability of these neurones is relatively high. Our P_{Na}/P_K values changed slightly with hypoxia so that there may be a small but insignificant increase in the potassium permeability. Values of E_M and a_K^i reported in this paper for the L_2–L_6 neurones are also in agreement with those found by Kunze & Brown (1974), who reported the mean value of a^iK^+ for L_1–L_6 neurones to be 142·6 mM-K^+ and $E_K = -75{\cdot}7$ mV. The values reported in Tables 1 and 2 during normoxia are similar in magnitude. The increases in a_K^i and hyperpolarizations of E_M found during hypoxic exposures of the L_2–L_6 neurones lie just outside the normal values which are reported by Kunze & Brown (1974). These reversible changes in a_K^i, E_K and E_M seem to be brought about by augmentation of the pump at reduced PO_2's. Comparable increases in a_K^i, E_K and E_M (more negative) have been reported in this paper over a duration of 65 min hypoxia (Fig. 2, Table 1) and over long-term (110 min) hypoxia (Fig. 3, Table 2). These findings suggest that the kinetics involved in stimulating the pump, which are presumably linked to oxidative phosphorylation and the synthesis of high-energy nucleotides, are saturable within 90 min of hypoxia. Reversible changes observed in Fig. 2 and Table 1 following reoxygenation are more immediate and may reflect the sensitivity of the system to an increase in the (ATP)/(ADP)(Pi) ratio.

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