REVERSIBLE CHANGES IN THE INTRACELLULAR POTASSIUM ION ACTIVITIES AND MEMBRANE POTENTIALS OF APLYSIA L₂–L₆ NEURONES IN RESPONSE TO NORMOXIA AND HYPOXIA

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SUMMARY

1. Exposure of 7 L₂–L₆ neurones to hypoxia for 65 min resulted in hyperpolarization of the membrane potential (E_M) from a mean of —49.1±2.1 to —54.1±3.6 mV (s.e.).

2. Intracellular potassium ion activities (a_K) increased significantly from 137.7±4.0 to 155.6±4.6 mM—K⁺. This is equivalent to a change in E_K from —74.2 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 L₂–L₆ neurones, the responses in a_K, 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 L₂–L₆ 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. 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 CO₂, N₂, or O₂ 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 or the PNa/PK ratio, between these two groups of neurones were not explored.

In a series of experiments reported in this paper, the sensitivities of the 'resistant' L₂-L₆ pacemaker group have been investigated. In these experiments, simultaneous measurements of membrane potentials and intracellular potassium ion activities (a'K) were used in determining the relationship between the membrane potential (EM) and the potassium equilibrium potential (EK) as well as in computing the relative sodium/potassium permeability ratios (PNa/PK) based upon a modification of the Goldman equation.

Although the L₂-L₆ 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 R₁₅'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'
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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 L2-L6 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 ($a_K$) of 14 L2-L6 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, intracellularly-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 ($E_0$), the selectivity coefficient ($K_{KNa}$), and their relationship to the electrode potential ($E$) given by equation 1.

$$E = E_0 + S \log_{10} \left( a_K^1 + K_{KNa} a_{Na} \right)$$  \hspace{1cm} (1)

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

$$E = -138.2 + 53.0 \text{mV} \log_{10} \left( a_K^1 + K_{KNa} a_{Na} \right)$$  \hspace{1cm} (2)

$K_{KNa} = 0.0055$ 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 $a_K^1$. 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.

Extracellular potassium ion activities (a_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 L2–L6 neurones were made during complete exposure of the cell bodies to the bathing solution.

Voltage changes corresponding to Ε_M, a_K, and a_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...
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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 (EK) 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).
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and Gorman & Marmor (1970). Unlike the methodology used by them (Moreton 1968, 1969; Gorman & Marmor, 1970), $aK$ was measured directly in these experiments using potassium ion-selective microelectrodes. The $PNa/PK$ ratio is given by equation 3:

$$PNa/PK = \{(aK^+)e^{EMF/RT} - (a^0K^+)\}/\{(a^0Na^+) - (aNa^+)e^{EMF/RT}\} \tag{3}$$

where $aK$ is the intracellular potassium ion activity; $EM$, 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. $a^0Na^+$ was calculated from a value of the extracellular sodium ion concentration equal to 425 mm-Na$^+$ using an activity coefficient. $a^0$ and $aK$ were determined from the potentiometric measurements and conversions from the calibration curve. Substitution of known intracellular sodium values ($aNa^+ = 23$ mm) was used in calculating the $PNa/PK$ ratios (Kunze & Brown, 1974). Preliminary results using this modification of the Goldman equation have been reported elsewhere (Coyer, 1981).

RESULTS

Determinations of $aK$, $EM$ and $PNa/PK$ 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 $aK$ and $EM$ 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 $aK$ 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 $EM$ and $aK$ for these neurones increased ($EM$, more negative) during hypoxia and subsequently decreased with reoxygenation. Moreover, paired $t$-tests for the mean differences for $EM$ and $aK$ during normoxia and hypoxia showed significant ($P<0.05$) increases following lowered PO$_2$'s. The observed hyperpolarization and concomitant rise in $aK$ were not accompanied by a significant change in the $PNa/PK$ ratios, which was also established by using paired $t$-tests. There were slight decreases in the ratio during hypoxia. Percent changes in the $PNa/PK$ ratios from the control measurements were computed (see Table 1) and compared using the Student’s $t$-test. Detectable decreases in the $PNa/PK$ ratios were found not to be significant ($P>0.05$) 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 $PNa/PK$ ratios will be treated in the Discussion.
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For a second group of 7 L₂—L₆ neurones which we studied, the electrophysiological responses were slower. A typical record of the response in $E_M$ and $a_K^*$ is shown in Fig. 3. A longer period of hypoxia (90 min duration) was necessary to elicit comparable membrane hyperpolarizations and increases in $a_K^*$ as observed in the first group. The summary data for 7 neurones is found in Table 2. Reversibility under subsequent reoxygenation 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 L2-L6 neurones whose responses are shown in Fig. 1

<table>
<thead>
<tr>
<th>L2-L6 Neurones</th>
<th>$E_M$ (mV)</th>
<th>$a'_K$ (mM)</th>
<th>$P_{Na}/P_K$ Ratio</th>
<th>% Δ from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia $\bar{X} \pm s.d.$</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>-49.6 ± 8.7</td>
<td>140.2 ± 14.5</td>
<td>0.032 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>-47.8 ± 8.9</td>
<td>142.8 ± 13.8</td>
<td>0.037 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>-47.9 ± 8.3</td>
<td>136.7 ± 12.0</td>
<td>0.035 ± 0.013</td>
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</tr>
<tr>
<td>4.</td>
<td>-47.5 ± 9.3</td>
<td>137.0 ± 14.5</td>
<td>0.036 ± 0.018</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>-49.1 ± 7.1</td>
<td>141.6 ± 13.8</td>
<td>0.028 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>-53.7 ± 5.3</td>
<td>132.6 ± 11.9</td>
<td>0.022 ± 0.009</td>
<td></td>
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<tr>
<td>7.</td>
<td>-48.4 ± 5.6</td>
<td>133.2 ± 22.7</td>
<td>0.030 ± 0.015</td>
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</tr>
<tr>
<td>$\bar{X} \pm s.e.$</td>
<td>-49.1 ± 2.1</td>
<td>137.7 ± 4.0</td>
<td>0.032 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>Hypoxia (65 min) $\bar{X} \pm s.d.$</td>
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<td></td>
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</tr>
<tr>
<td>1.</td>
<td>-53.1 ± 8.9</td>
<td>156.1 ± 8.9</td>
<td>0.031 ± 0.015</td>
<td>-3.1</td>
</tr>
<tr>
<td>2.</td>
<td>-54.5 ± 8.0</td>
<td>162.7 ± 9.9</td>
<td>0.029 ± 0.014</td>
<td>-2.1</td>
</tr>
<tr>
<td>3.</td>
<td>-55.0 ± 8.2</td>
<td>155.9 ± 7.0</td>
<td>0.026 ± 0.014</td>
<td>-25.7</td>
</tr>
<tr>
<td>4.</td>
<td>-53.8 ± 7.1</td>
<td>155.0 ± 8.4</td>
<td>0.028 ± 0.013</td>
<td>-22.2</td>
</tr>
<tr>
<td>5.</td>
<td>-54.0 ± 5.5</td>
<td>154.6 ± 11.5</td>
<td>0.028 ± 0.014</td>
<td>0.0</td>
</tr>
<tr>
<td>6.</td>
<td>-53.0 ± 8.4</td>
<td>153.1 ± 10.6</td>
<td>0.029 ± 0.019</td>
<td>+31.8</td>
</tr>
<tr>
<td>7.</td>
<td>-55.0 ± 8.2</td>
<td>152.1 ± 11.9</td>
<td>0.029 ± 0.001</td>
<td>-3.3</td>
</tr>
<tr>
<td>$\bar{X} \pm s.e.$</td>
<td>-54.1 ± 3.6</td>
<td>155.6 ± 3.4</td>
<td>0.028 ± 0.002</td>
<td>-6.3 ± 7.5</td>
</tr>
<tr>
<td>Reoxygenation $\bar{X} \pm s.d.$</td>
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</tr>
<tr>
<td>1.</td>
<td>-48.3 ± 7.5</td>
<td>141.8 ± 14.8</td>
<td>0.036 ± 0.015</td>
<td>+12.5</td>
</tr>
<tr>
<td>2.</td>
<td>-47.5 ± 8.2</td>
<td>145.8 ± 15.1</td>
<td>0.039 ± 0.001</td>
<td>+5.4</td>
</tr>
<tr>
<td>3.</td>
<td>-47.8 ± 8.3</td>
<td>137.9 ± 13.0</td>
<td>0.035 ± 0.013</td>
<td>0.0</td>
</tr>
<tr>
<td>4.</td>
<td>-49.0 ± 6.0</td>
<td>135.8 ± 12.8</td>
<td>0.032 ± 0.010</td>
<td>-11.1</td>
</tr>
<tr>
<td>5.</td>
<td>-48.5 ± 6.5</td>
<td>140.8 ± 13.5</td>
<td>0.035 ± 0.005</td>
<td>+21.4</td>
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<tr>
<td>6.</td>
<td>-52.0 ± 6.7</td>
<td>133.7 ± 12.8</td>
<td>0.025 ± 0.009</td>
<td>+13.6</td>
</tr>
<tr>
<td>7.</td>
<td>-48.8 ± 5.8</td>
<td>138.9 ± 18.2</td>
<td>0.034 ± 0.018</td>
<td>+13.3</td>
</tr>
<tr>
<td>$\bar{X} \pm s.e.$</td>
<td>-48.8 ± 1.5</td>
<td>139.2 ± 4.0</td>
<td>0.034 ± 0.004</td>
<td>+7.9 ± 4.1</td>
</tr>
<tr>
<td>Mean $E_K = -74.4$ mV</td>
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</table>

Means ($\bar{X}$) ± standard deviations (s.d.) for the membrane potential ($E_M$), intracellular potassium ion activities ($a'_K$), and the computed $P_{Na}/P_K$ ratios for each of the 7 L2-L6 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 $a'_K$. $E_M = -49.1 ± 2.1$ mV (normoxia); $-54.1 ± 3.6$ mV (hypoxia); and $-48.8 ± 1.5$ mV (reoxygenation). $E_K = 74.4$ mV. The importance of these experiments seems to lie in the observations of changes in $E_M$ and $a'_K$ 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
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Fig. 3. Recordings of 𝑎_𝐾 and 𝐸_𝑀 during normoxia (PO₂ ~ 150) in A in another group (N = 7) of L₂–L₆ neurones. Slower changes in the observed membrane hyperpolarizations and concomitant increases in 𝑎_𝐾 were noted. After 90 min of hypoxia (PO₂ < 20 Torr), increases in 𝑎_𝐾 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 (−13.4 ± 14.3) leading one again to conclude that the differences between control and experimental PNₐ/PK ratios are insignificant.

**DISCUSSION**

Possible sources of error in measurements of 𝑎_𝐾 and calculations of PNₐ/PK 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. 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

<table>
<thead>
<tr>
<th>L2–L6 Neurones</th>
<th>$E_M$ (mV)</th>
<th>$a_K$ (mm)</th>
<th>$P_{Na}/P_K$</th>
<th>% $\Delta$ from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>1. $-47.5 \pm 9.8$</td>
<td>$141.8 \pm 13.6$</td>
<td>$0.038 \pm 0.005$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. $-48.9 \pm 9.6$</td>
<td>$138.5 \pm 8.9$</td>
<td>$0.033 \pm 0.006$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. $-52.3 \pm 5.8$</td>
<td>$137.0 \pm 12.5$</td>
<td>$0.026 \pm 0.010$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. $-47.5 \pm 8.3$</td>
<td>$133.5 \pm 15.0$</td>
<td>$0.034 \pm 0.008$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. $-52.1 \pm 7.5$</td>
<td>$140.2 \pm 10.8$</td>
<td>$0.027 \pm 0.006$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6. $-49.3 \pm 10.2$</td>
<td>$136.7 \pm 9.8$</td>
<td>$0.032 \pm 0.005$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7. $-45.3 \pm 8.3$</td>
<td>$137.0 \pm 12.0$</td>
<td>$0.041 \pm 0.009$</td>
<td></td>
</tr>
<tr>
<td>Hypoxia (110 min)</td>
<td>1. $-53.4 \pm 15.8$</td>
<td>$152.3 \pm 16.2$</td>
<td>$0.028 \pm 0.010$</td>
<td>-26.3</td>
</tr>
<tr>
<td></td>
<td>2. $-55.0 \pm 16.8$</td>
<td>$168.4 \pm 17.0$</td>
<td>$0.030 \pm 0.005$</td>
<td>-9.1</td>
</tr>
<tr>
<td></td>
<td>3. $-56.2 \pm 18.5$</td>
<td>$162.3 \pm 18.0$</td>
<td>$0.026 \pm 0.006$</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4. $-50.5 \pm 16.2$</td>
<td>$148.3 \pm 16.9$</td>
<td>$0.033 \pm 0.009$</td>
<td>-2.9</td>
</tr>
<tr>
<td></td>
<td>5. $-55.1 \pm 18.2$</td>
<td>$158.5 \pm 16.2$</td>
<td>$0.027 \pm 0.004$</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>6. $-54.3 \pm 13.8$</td>
<td>$148.6 \pm 18.9$</td>
<td>$0.026 \pm 0.008$</td>
<td>-18.8</td>
</tr>
<tr>
<td></td>
<td>7. $-48.2 \pm 15.5$</td>
<td>$153.8 \pm 7.8$</td>
<td>$0.026 \pm 0.010$</td>
<td>-36.6</td>
</tr>
<tr>
<td></td>
<td>$-53.2 \pm 2.9$</td>
<td>$156.0 \pm 7.4$</td>
<td>$0.028 \pm 0.003$</td>
<td>-13.4 ± 5.4</td>
</tr>
</tbody>
</table>

Mean $E_K = -74.2$ mV

$\bar{X} \pm s.d.$

Mean $E_K = -77.3$ mV

Means ($\bar{X}$) ± standard deviations (s.d.) for the membrane potential ($E_M$), intracellular potassium ion activities ($a_K$), 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 $a_K$. $E_M = -49.0 \pm 2.5$ mV (normoxia) and $-53.2 \pm 2.9$ mV (hypoxia). $a_K = 137.7 \pm 2.7$ mM (normoxia) and $156.0 \pm 7.4$ mM (hypoxia). $P_{Na}/P_K = 0.033 \pm 0.005$ (normoxia) and $0.028 \pm 0.003$ (hypoxia). Paired t-tests were performed among the mean differences of $E_M$, $a_K$ and the $P_{Na}/P_K$ ratios between normoxic and hypoxic conditions. $E_M$ and $a_K$ were significantly greater during hypoxia ($P < 0.05$), but there was no significant difference between the $P_{Na}/P_K$ ratios ($P > 0.05$). The average ($\pm s.e.$) percent change is $-13.4 \pm 14.3$ ($s.e. = 5.4$)

Increases in spike frequency. Increases in the spike frequency and concomitant increases in $a_K$ 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 $a_K$ potential indicative of less $a_K$. 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 $a_K$ 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 $a_K$.

**Changes in $a_K$**

A possible problem in the interpretation of these experiments is that increases in $a_K$ in the intercellular areas might stimulate the pump and increase $a_K$. Changes in
PO₂ effects on L₂–L₆ neurones of Aplysia


\[ a^K \] 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 \( a^K \) varied. Steady-state conditions were assumed to exist between inside and outside activities in calculating the \( \frac{P_{Na}}{P_K} \) 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 \( a^K \) is indirect, but we feel that no transient increases in \( a^K \) occurred since we never recorded any spontaneous decreases in \( a^K \) during control (normoxic) monitoring, which might be expected to increase \( a^K \). Every case of a change in \( a^K \) followed a typical pattern in which \( a^K \) 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 L₁₀ the outward pump current of L₅ 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 L₅ 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* L₂–L₆ 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 L₂–L₆ neurones of *Aplysia californica,*
it appears that hypoxia stimulates the metabolic pump which increases \( a_K \) 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 \textit{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 mollusc 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 \textit{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 \( a_K \) and membrane hyperpolarization. Higher intracellular levels of \( ADP \) have been shown to stimulate respiration of neuroblastoma cells \textit{in vivo} (Wilson, Erečinska, Drown & Silver, 1979; Wilson, Owen & Erečinska, 1979), and injections of \( ADP \) into squid axons stimulates \( Na^+:Na^+ \) exchange (DeWeer, 1970). For myelinated neurones of \textit{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 \textit{Aplysia} decreases in this ratio brought about by depletion of ATP and accumulation of \( ADP \) may stimulate respiration and increase the pump's activity.

\textit{Calculations of } \( P_{Na}/P_K \) \textit{ratios in } \textit{L}_{2−L_6} \textit{neurones of Aplysia and other molluscan neurones}

In the pulmonate \textit{Helix aspersa}, 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 0.180 ± 0.015 and the potassium concentration equalled 92.9 ± 4.3 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 \textit{Aplysia} using permeability measurements in response to each ion's contribution to the voltage change. From step-wise electrical changes, Eaton \textit{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 \textit{et al.} 1975), and the chemical values are higher (\( P_{Na}/P_K = 0.13 \)), 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 \textit{Anisodoris} neurones to those reported here.
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For Aplysia. The ratios which are calculated here show a potassium permeability of approximately 30X greater than the sodium permeability. The agreement of observed membrane hyperpolarizations with the increases in a⁺K suggest that the potassium permeability of these neurones is relatively high. Our PNa/PK values changed slightly with hypoxia so that there may be a small but insignificant increase in the potassium permeability. Values of EM and a⁺K reported in this paper for the L₂–L₆ neurones are also in agreement with those found by Kunze & Brown (1974), who reported the mean value of a⁺K⁺ for L₁–L₆ neurones to be 142.6 mM-K⁺ and EK = -75.7 mV. The values reported in Tables 1 and 2 during normoxia are similar in magnitude. The increases in a⁺K and hyperpolarizations of EM found during hypoxic exposures of the L₂–L₆ neurones lie just outside the normal values which are reported by Kunze & Brown (1974). These reversible changes in a⁺K, EK and EM seem to be brought about by augmentation of the pump at reduced PO₂'s. Comparable increases in a⁺K, EK and EM (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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