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THERMAL ACCLIMATION OF A CENTRAL NEURONE IN *HELIX ASPERSA*

III. IONIC SUBSTITUTION AND RELATED STUDIES

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SUMMARY

(1) Thermal acclimation of the F1 neurone does not appear to result from changes in the chemical composition of the haemolymph. This is deduced from the lack of effect on the electrical characteristics of control neurones of either pooled haemolymph from acclimated individuals, or variations in the experimental salines made in accordance with haemolymph analyses.

(2) Changes in $[Ca]_o$ tended to act cooperatively with temperature shifts to induce alterations in the electrical properties of the neurone, notably to increase excitability and lower membrane resistance.

(3) Warm acclimation was associated with increased resting conductance of the neuronal membrane to sodium and potassium, whereas chloride conductance appeared little affected.

INTRODUCTION

It was shown in a preceding paper (Langley, 1979*a*) that there is significant alteration in Na and K levels and calcium activity in the haemolymph of *Helix* in response to long-term thermal acclimation. This is especially pertinent to a study of excitable tissues, which are sensitive to such changes in their immediate surroundings (see Hodgkin & Huxley, 1952; Treherne & Moreton, 1970).

This paper describes and analyses the effects of variation in the medium bathing the F1 cell on the electrical activity of the cells from various acclimation regimes, to discover whether changes in the haemolymph composition could be responsible for thermal acclimation of the neurone.

MATERIALS AND METHODS

Adult specimens of *Helix* were kept, as previously described (Langley, 1979*a*) under four acclimation regimes: (1) 4 °C; (2) 12 °C; (3) laboratory temperature of 19 ± 2 °C and (4) 30 °C. The preparation and intracellular recording technique have been described previously (Langley, 1979*b*).

The composition of Ringer solutions used is shown in Table 1, change in one constituent being accommodated by a change in other major constituents. The ionic composition of the normal Ringer was based upon that of Kerkut & Meech (1967).

Ca-depleted medium was made by substituting Mg for Ca, since Ringers made

Table 1. *Ringer composition*

	Composition as mM										
Ringer	NaCl	KCl	$CaCl_2$	$MgCl_2$	Tris	Hepes	Pipes	BES	Bicine	Glucose	$NaHCO_3$
A	80	4	7	5	5	—	—	—	—	—	—
B	80	4	7	5	—	5	—	—	—	—	—
C	80	4	7	5	—	—	5	—	—	—	—
D	80	4	7	5	—	—	—	5	—	—	—
E	80	4	7	5	—	—	—	—	5	—	20
F	84	—	7	5	—	5	—	—	—	—	—
G	74	10	7	5	—	5	—	—	—	—	—
H	54	30	7	5	—	5	—	—	—	—	—
I	—	4	7	5	—	5	5	—	—	160	—
J	$NaMeSO_4$ 80	$KMeSO_4$ 4	7	5	—	5	5	—	—	—	—
K	69	4·75	4·85	2·47	—	5	—	—	—	34·54	—
L	49·20	4·62	4·90	2·77	—	5	—	—	—	78·35	—
M	54·60	2·54	5·24	4·10	—	5	—	—	—	66·70	—
N	76·21	6·87	4·94	3·55	—	5	—	—	—	17·37	—

A, using Tris as a buffer (Kerkut & Meech, 1967); B, using Hepes as a buffer; C, using Pipes as a buffer; D, using BES as a buffer; E, using BICINE as a buffer; F, using 2·5 % $CO_2/NaHCO_3$ as a buffer, 0·2 mM-phosphate was added to prevent precipitation; G, Ringer made up replacing K^+ ions with Na^+; H, Ringer made up with increasing $[K^+]_o$ and concomitant decrease in $[Na^+]_o$; I, Ringer with highest $[K^+]$; J, chloride-depleted Ringer; K, 4 °C-acclimated saline; L, 12 °C-acclimated saline; M, 19 °C-acclimated saline; N, 30 °C-acclimated saline. Salines K, L, M, N based on analyses of haemolymph of *Helix aspersa* (Langley, 1979*a*): osmolarity was preserved by adding equi-osmotic glucose.

up with 5 mM EGTA ((ethyleneglycol-bis-aminoethyl ether)-*N*,*N*′-tetracetic acid) led to irreversible depolarizations of the membrane potential of the cell.

Various buffers were used. The following were employed, at a concentration of 5 mM, to arrive at a pH of 7·5 (at 20 °C): PIPES (piperazine-*N*,*N*′-bis (2-ethanesulphonic acid)); BES (*N*,*N*-bis (2-hydroxyethyl)-2-aminoethane sulphonic acids): BICINE (*N*,*N*-bis (2-hydroxyethyl) glycine); HEPES (*N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulphonic acid); TRIS (Tris-hydroxymethyl-methylammonium chloride), all obtained from Sigma. A bicarbonate/CO_2 Ringer was also prepared, consisting of 2·5% CO_2, 20 mM-$NaHCO_3$ to which 0·2 mM-phosphate was added to retard HCO_3^- precipitation (Burton, 1975).

RESULTS

Selection of buffer

Bes and Bicine, although showing (1) a favourable – pKa/°C (−0·016 and −0·018 respectively, compared with −0·017 for 'ideal' ectotherm haemolymph; Burton, 1975); (2) negligible metal-buffer binding; (3) values of pKa within the range found for snail haemolymph (7·15 and 8·35 respectively), tended to depolarize the resting potential and led to a gradual deterioration of the F1 cell in all four groups. However, acceptable and reversible changes in resting potential and spikes as a result of temperature change were found when Hepes, Tris, Pipes or HCO_3^-/CO_2 buffers were used. Salines made with 5 mM Hepes or Pipes were found to most nearly resemble pooled haemolymph in their action in maintaining the frequency of spontaneous spike production, amplitude and profile (Fig. 1 *a*).

Both Pipes and Hepes have pKa/°C values well outside the 'ideal' ectotherm haemolymph range (namely −0·0085 and −0·014 respectively, Burton, 1975). Since salines using these buffers tended to mimic haemolymph there is a strong suggestion that H^+ buffering systems exist in the membrane or cell interior of F1 to maintain 'normal' functioning in the face of changed haemolymph pH. Salines made up with Tris appeared to stimulate the F1 cell since spontaneous spike production in all four groups at any temperature between 10 and 25 °C increased as compared with snail haemolymph and with Hepes and Pipes salines. Such a stimulatory role of Tris has been demonstrated previously [cf. in a vertebrate system, Gillespie & MacKnight (1976) and Wilson, Clark & Pellmar (1977) in *Aplysia*; and the comprehensive review of Nahas (1962)]. Hysteresis of spike discharge which follows rewarming after the block of spontaneous activity (Langley, 1979*b*) was less marked with salines using Hepes, Pipes or HCO_3^-/CO_2 as buffers when compared with Tris, and was similar to the effect obtained with snail haemolymph. Resting potentials observed using these three buffers were similar to those obtaining when snail haemolymph was used – and lower than when measured in Tris-buffered salines, namely 34 ± 0·5 mV in warm-acclimated cells, 48·2 ± 2·8 mV in control cells, and 45·95 ± 2·5 mV and 37·90 ± 0·6 mV in the 12 and 4 °C cold groups respectively.

Variation of $[Ca]_o$

In cells from control individuals (acclimated to 19 ± 2 °C) perfusion with the Ca^{2+}-deficient saline (Mg-substituted) led to a small but sustained depolarization

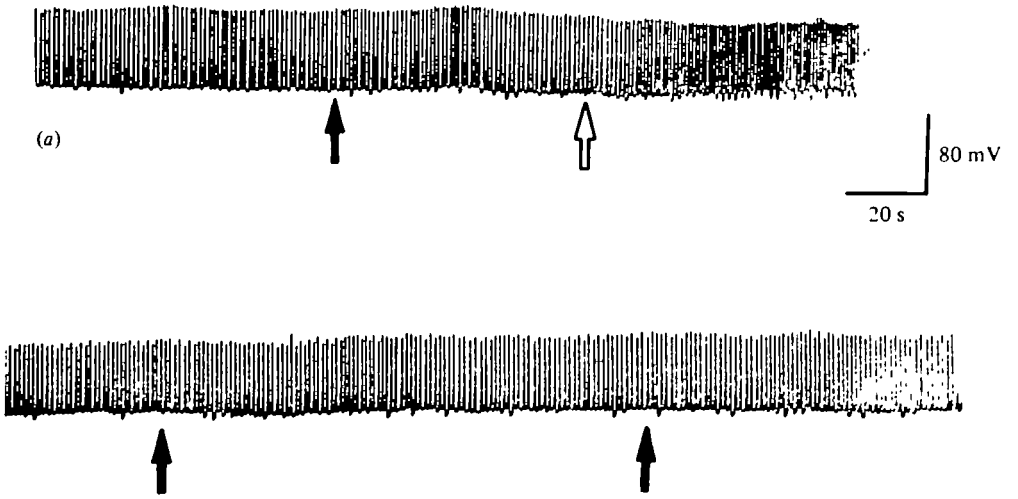

Fig. 1. Recordings of spontaneous spike activity of the F1 cell from control preparations in response to changes in composition of the bathing media. (*a*) The filled arrow indicates changes from Hepes-buffered saline to pooled, aerated haemolymph from control snails (living at 19 °C). When temperature was then changed from 19 to 24 °C (open arrow), spontaneous activity increased in a fashion similar to that recorded in Hepes-buffered saline (Langley, 1979*b*). (*b*) The filled arrow indicates changing from perfusion with Hepes saline to one based upon analyses of the haemolymph of control snails. The second arrow indicates changing back to original saline again. Recordings taken at 19 °C.

of $7{\cdot}50 \pm 1{\cdot}85$ mV ($n = 12$), together with a 12% reduction in the input resistance (from $3{\cdot}95 \pm 1{\cdot}05$ MΩ to $2{\cdot}98 \pm 0{\cdot}9$ MΩ, $n = 12$). When perfusion was continued for periods in excess of 40 min, a marked increase in excitability of the F1 cell occurred, manifested by short oscillations of the membrane potential, and decreased spike amplitude (from $80{\cdot}2 \pm 4{\cdot}30$ mV to $69{\cdot}70 \pm 2{\cdot}75$ mV, $n = 12$), the velocity of the falling phase of the spike showed a decrease to a value of 80% of the control situation. After 50 minutes perfusion the cell invariably became silent, with little further change in input resistance.

In comparison, cells from warm-acclimated snails were rapidly prone to the effects of Ca^{2+}-depleted media in less than 10 min with a marked drop in input resistance (from $0{\cdot}856 \pm 0{\cdot}03$ MΩ to $0{\cdot}625 \pm 0{\cdot}78$ MΩ). With less than 10 min incubation, the effects were fully reversible. Upon incubation for periods in excess of 15 min the F1 cell of the warm-acclimated groups appeared irreversibly damaged, with a marked depolarization of the resting potential (from $34{\cdot}20 \pm 0{\cdot}4$ to $20{\cdot}20 \pm 0{\cdot}8$ mV) (Fig. 3).

Cells from the two cold groups showed far less sensitivity to the effects of Ca^{2+}-deficient media than warm or control cells. The time taken for reversible changes in input resistance and membrane potential to become manifest was longer than for warm or control groups (periods in excess of 65 min being found for both 4° and 12 °C cold groups). Also, the magnitude of the changes were less; namely input resistance only dropped by 4% in both cold groups (from $5{\cdot}35 \pm 0{\cdot}98$ MΩ to $5{\cdot}136 \pm 0{\cdot}24$ MΩ for 12 °C, and from $7{\cdot}06 \pm 0{\cdot}90$ MΩ to $6{\cdot}76 \pm 0{\cdot}28$ MΩ in 4 °C individuals).

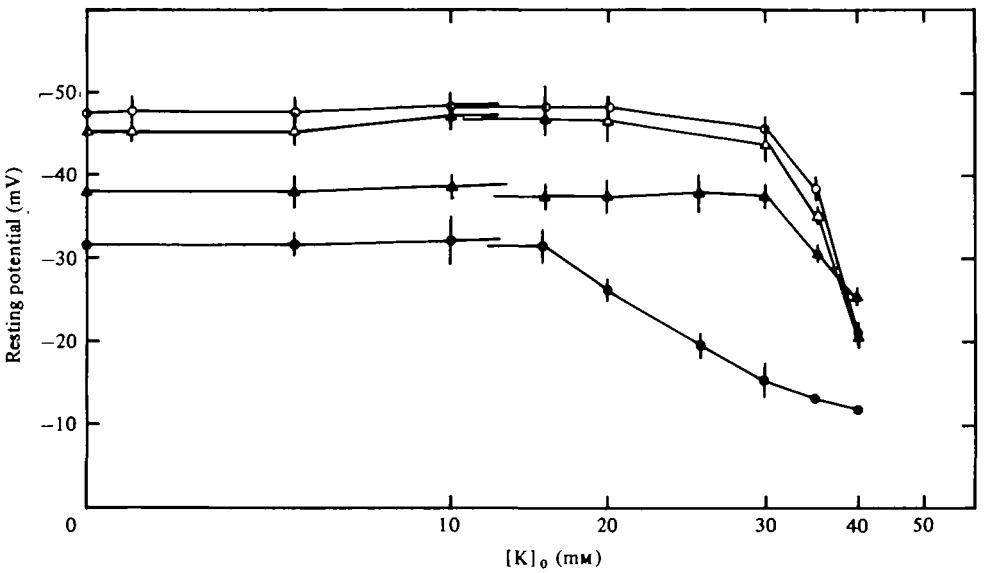

Fig. 2. Relationship between $[K]_o$ and membrane potential of the F1 cell. Each point is based upon at least 10 experiments – vertical lines through the points indicate standard errors. Note the discontinuities at $[K]_o$ of 6 mM – this represents the irreversibility of effects of 6 mM $[K]_o$ upon the membrane potential. ▲, Cold (4 °C)-acclimated cells; △, cold (12 °C)-acclimated cells; ○, control-cells; ●, warm-acclimated cells.

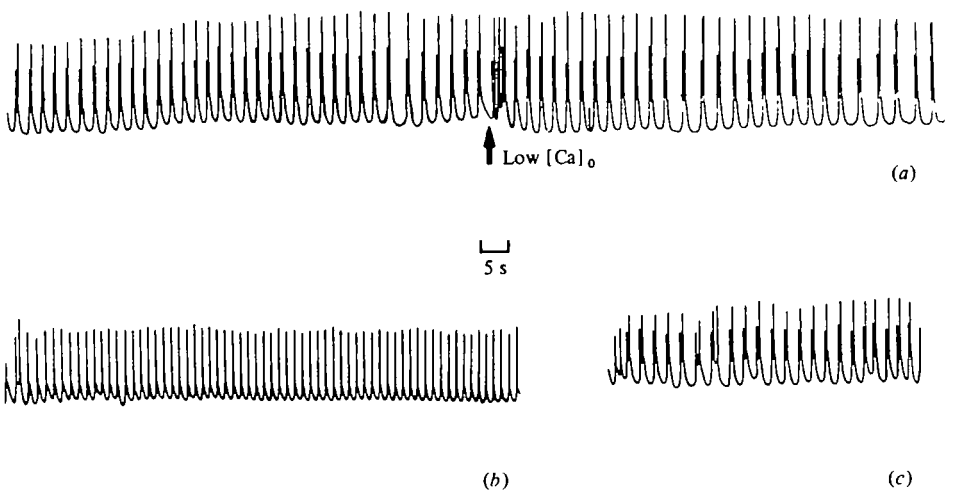

Fig. 3. Typical 'doublet' spikes found from the F1 cell of some specimens of warm acclimated snails. The record is continuous from *a* to *b*; from *b* to *c* there is a break of some 10 min. The record shows that bathing the cell in Ca-depleted saline (arrow), causes the cell to produce similar singlet spikes imposed on a small depolarizing shift as found in control preparations. Record *c* shows the response of the cell 10 min after returning to normal Ringer. All the recordings were carried out at a bath temperature of 20 ± 0·5 °C.

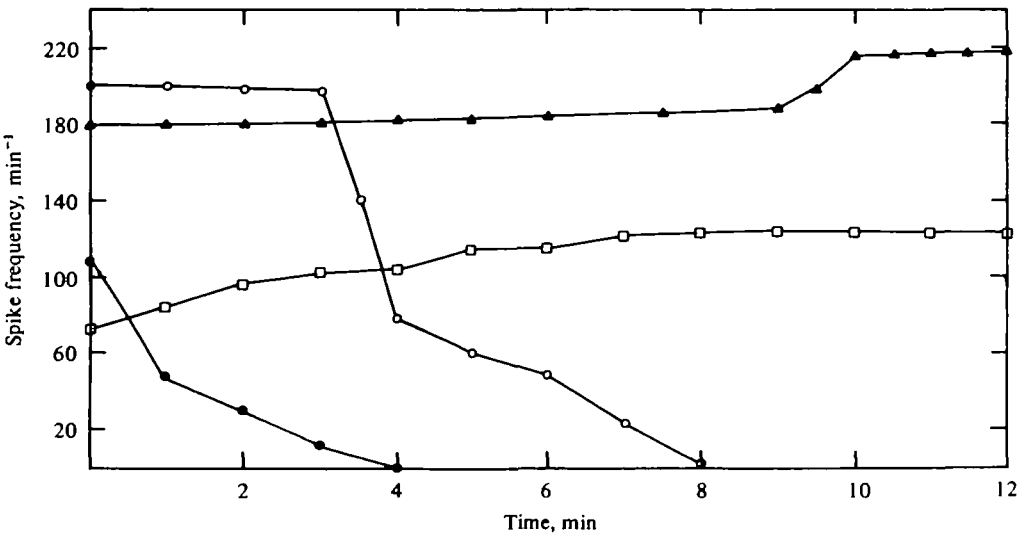

Fig. 4. The effect of variable incubation times in calcium-depleted media upon the frequency of spontaneously produced spikes from the F1 neurone at 20 °C. ○, Control cells; ●, warm acclimated cells; □, cells from animals collected and tested in March; ▲, cold (4 °C)-acclimated cells.

Even when cells from the cold regimes were incubated for periods of 2 h no significant change in the spike profile or electrical parameters of the cell could be found.

Spike frequency was reversibly changed by up to 10 min incubation in Ca^{2+}-deficient media in all groups (Fig. 4), with a decreased discharge in warm and control preparations, and an increase in both 4 and 12 °C cold cell spontaneous activity. The effects of low Ca^{2+} media could not be reversed with either hyperpolarizing or depolarizing current pulses. F1 cells from warm-acclimated snails frequently produced doublet spikes which could be reversibly abolished to yield the 'normal' beating pattern by incubation in Ca^{2+}-depleted saline (Fig. 3).

Increasing $[Ca]_o$ from 7 mM to 12 mM (with a corresponding decrease of $[Mg]_o$) led to significant neuronal hyperpolarization (up to $10 \pm 1{\cdot}50$ mV in control, $8{\cdot}55 \pm 1{\cdot}26$ mV in 4 °C cold groups, and $12 \pm 2{\cdot}4$ mV in warm groups), an increase in input resistance, and cessation of spontaneous activity. Exposure to enriched Ca saline (14 mM) also led to a decrease in the temperature range over which the F1 cell from all 4 groups operated after return to normal saline.

The effects of $[Ca]_o$ variation upon the F1 cell were identical in both whole ganglia and isolated cells – thereby indicating that synaptic involvement is not responsible for the observed effects. The proportion of the spike carried by Ca^{2+} ions was identical in all four groups and was not altered by thermal acclimation.

Variation of $[K]_o$

The relation between membrane potential and $[K]_o$ from 0 to 30 mM indicated that the membrane potential of the F1 cell does not behave in a manner predictable by the Nernst equation. This was true for all four groups of acclimated individuals (Fig. 2), the potential showing a small but significant hyperpolarization when $[K]_o$ was

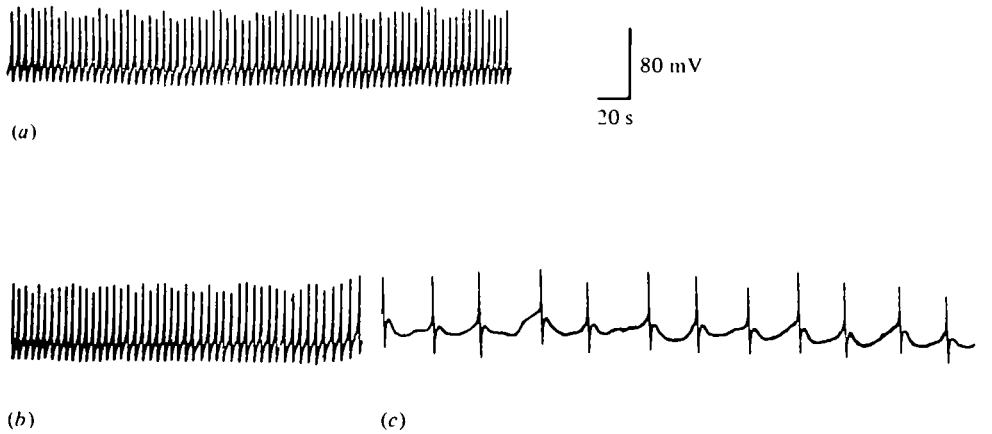

Fig. 5. Effects of Na-free saline upon the spike production of the F1 cell from control preparations. The record is continuous from *a* through to *b*. A 10 min gap intervenes between *b* and *c*.

increased from zero to 5 mM. Cells from warm-acclimated individuals showed a more marked sensitivity to the effects of increasing $[K]_o$ above 7·5 mM.

In the 'physiological' range of $[K]_o$ (i.e. 2–7 mM, see Langley, 1979*a*) there was no obvious change of resting potential.

Decrease in the speed of the repolarization phase of the autonomous spike induced by low temperature was facilitated by moderately high (up to 8 mM) concentrations of $[K]_o$ on the F1 cell from all the four groups. Injected depolarizing and hyperpolarizing current was capable of reversing these changes for $[K]_o$ up to 20 mM in control and cold groups but not warm cells.

Perfusion with Cs^+-substituted K^+-free salines produced cessation of spontaneous activity of F1 cells from all 4 groups. Injected current could not reverse this effect. The time of onset of silence varied between the four groups – with the shortest time (100–160 s ± 10·2 s, $n = 10$) being found in warm acclimated snails and the longest in the 4 °C-acclimated group (460–500 s ± 13 s, $n = 12$).

Variation of $[Na]_o$

In all cases of perfusion with Na^+-free (glucose) saline, the membrane was hyperpolarized to a level close to that of the positive after-potential. The degree of hyperpolarization varied between the 4 groups; being 7·5 ± 1·80 mV in warm snails, 10 ± 1·5 mV in control, 4 ± 1·20 mV in the 4 °C group and 6·0 ± 1·25 mV in the 12 °C group. There was also a cessation of spontaneous activity, which could not be re-started by appropriate injected square pulses of current (0·8–1·2 nA).

As with the K-induced effects, there was a progressive shortening of onset time with increasing acclimation temperature for all Na-free effects. Exposure to Li^+-substituted Na^+-free salines (at 20 °C) led to a membrane potential hyperpolarization and increase in input resistance, complete after 20 min in both cold groups, 15 min in control groups and 4½ min in warm acclimated cells. The magnitude of this hyperpolarization varied between the four groups, being largest in warm cells (10 ± 1·8 mV) and smallest in the 4 °C group (4·5 ± 1·75 mV). In cells silenced

with Na-free salines the membrane potential and input resistance showed none of the usual thermal sensitivity when temperature was altered (see Langley, 1979*b*).

Variation of $[Cl]_o$

Exposure to chloride-deficient salines after 10 minutes resulted in a moderate neuronal hyperpolarization in all groups ($5 \pm 1{\cdot}60$ mV). In 30° and 4 °C groups this was followed by a depolarization to give a net depolarization of $2{\cdot}5 \pm 1{\cdot}20$ mV and $5 \pm 1{\cdot}92$ mV respectively. In 12° and 19 °C groups there was further hyperpolarization to give a net hyperpolarization of $7 \pm 1{\cdot}3$ mV and $10{\cdot}2 \pm 1{\cdot}3$ mV respectively. Hyperpolarizations were accompanied by an increase in input resistance, depolarization by a decrease.

Incubation in chloride-free media led to no detectable modification of the thermal sensitivity of membrane potential in any of the four groups.

Salines made up in accordance with major haemolymph ion analyses

Salines modelled on haemolymph concentrations of Na, K, Ca, Mg in the four groups (Langley, 1979*a*), see Table 1, produced no significant ($P < 0{\cdot}001$) alteration of any of the spike parameters. Incubation times of up to $2\frac{1}{2}$ h were typically used.

Use of pooled haemolymph

Well-aerated haemolymph samples from the four acclimation regimes were used to bathe cells both from the same and different regimes. No significant differences from Hepes buffered solutions in effect upon electrical characteristics could be found except in the case of cells from warm-acclimated regimes which showed a lower frequency of spontaneous spike discharge when tested in 4 °C cold-acclimated haemolymph than in saline at the same temperatures.

DISCUSSION

The experiments described in this paper lead to three general conclusions regarding the effects of thermal acclimation. Firstly, as a result of acclimation there appears to be a marked alteration in the sensitivity of the F1 neurone to calcium ions. Secondly, there is no apparent 'factor' present in the haemolymph of acclimated individuals responsible for the changes in the electrical parameters of the neurone indicative of thermal acclimation. Thirdly, the resting conductance of the neurone to K is significantly altered by thermal acclimation.

Calcium ions have been shown to play a major role in modifying and controlling the behaviour of autoactive neurones in a variety of gastropod molluscs (Barker & Gainer, 1973, 1974*a*, *b*; Carpenter, 1973; Carpenter & Gunn, 1970; Kostyuk, 1968; Kostyuk, Krishtal & Doroshenko, 1974) where the ion acts both as a current carrying species and a modifier of membrane permeability, especially to K^+ ions (see Meech, 1976).

It is apparent that thermal acclimation results in a changed thermal sensitivity of the F1 cell to Ca, with the warmer acclimation temperature (30 °C) increasing, and the cooler (4 and 12 °C) reducing that sensitivity. It is significant in this con-

nection that thermal acclimation alters the Ca activity of the haemolymph (Langley, 1978*a*), with high temperature increasing activity and low reducing it.

Ambient bath temperature and $[Ca]_0$ tend to interact, such that high bath temperature increases the effects of both lowered and raised $[Ca]_0$, and conversely, low temperature decreases the Ca-induced effects in both acclimated and natural populations collected in early spring. Thermal acclimation accentuates these effects. Such interaction between $[Ca]_0$ and temperature has been noted previously in frog spinal ganglion and crayfish axons (Machne, Stoney & Nadell, 1967; Machne & Orozio, 1968).

It therefore appears conceivable that Ca variation and thermal acclimation involve alterations in a 'pacemaker locus' in the F1 neurone, which is responsible for the production of autonomous spikes. This effect may involve changes in the density of surface negative sites in the membrane, thereby altering the surface charge of the membrane (see Hille, 1968).

Since the F1 cell of *Helix aspersa* shows clear changes in neurosecretory activity as a result of thermal acclimation, the cell in warm-acclimated snails being heavily pigmented, whilst cells from both the two cold groups lack any such pigmentation, (C. K. Langley, unpublished results) it might be thought that the F1 cell is elaborating some factor, capable of modifying spike production in the varying thermal regimes. Such a factor has been found to accompany diapause and to influence the activity of an autoactive neurone in the mollusc *Otala lactea* (Barker & Gainer, 1975*a*, *b*; Gainer, 1972*a*, *b*, *c*; Gainer & Barker, 1975; Ifshin, Gainer & Barker, 1975; Loh, Sarne & Gainer, 1975; Peterson & Loh, 1973). But cells tested in either their own haemolymph or that of another acclimation regime showed no change in the acclimation-induced alteration of functioning of the F1 cell. It could, however, be that long incubation times are required to effect a change in the F1 cell, or that the presumed factor does not affect the neurone, but the target organ (kidney).

Changes in the resting permeability of the neurone to K and to a lesser extent Na have important consequences for autonomous spike-production (Connor & Stevens, 1971*a–c*; Langley, 1979*b*). In the present study the data clearly indicate an alteration of K permeability of the F1 neurone which influences spike profile and the autonomous production of the spike. Since K channels have been shown to be more temperature sensitive than Na (Kostyuk, 1975; Magura, Valeyev & Zamekhovsky, 1975; Owen, Brown & Saunders, 1975) it is highly likely that thermal acclimation influences K conductance to a larger extent that Na. Changes in P_k will also lead to changes in $[K]_i$, these features allowing the F1 cell to function in a wider spectrum of temperatures. The marked hysteresis of spike production after rewarming the cell from cold-block (Langley, 1979*b*) could be due to Na-loading of the cell in the cold.

Chloride concentration in the haemolymph of *H. aspersa* increased as a result of extremes of temperature (Langley, 1979*a*). Using chloride-reduced salines led to an initial hyperpolarization of the F1 neuronal membrane which is likely to be due to an altering of the junctional potential between the bathing solution and the 3 M-KCl bridge connecting the bath to earth. Following this hyperpolarization, responses were either a further neuronal hyperpolarization (in 12 °C and control preparations) or a depolarization in the 30° and 4 °C groups. Since these effects were

reversible it is unlikely that the neuronal depolarization indicates damage of the cell.

The most reasonable explanation for the observed effects of chloride-deficient salines involves alterations in the membrane surface charge, which varies with the level of the resting potential (the two thermal extremes having the lowest values), in a manner similar to the model of Hille (1968) which involves a 'titration effect' of reducing anion concentrations which thereby reduce the intra-membrane potential field. This will thereby alter the threshold for spontaneous activity of the F1 cell.

That the salines modelled on haemolymph analyses produced similar effects to normal Ringer is not too surprising, in view of the relative ionic insensitivity of the F1 neurone (see, for example, Kerkut & Meech, 1967; who designated F1 cell A). Wide fluctuations of ionic concentrations have been found in the haemolymph of a variety of natural populations of pulmonate molluscs (see Langley, 1979*a*) and would also seem to indicate a fairly wide ionic tolerance on the part of the F1 cell.

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