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METABOLIC CONTROL OF NEURONAL PACEMAKER ACTIVITY AND THE RHYTHMIC ORGANIZATION OF CENTRAL NERVOUS FUNCTIONS*

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SUMMARY

The endogenous rhythmic activity of isolated pacemaker neurones of *Aplysia californica* appears to be controlled by the operation of a substrate cycle. The recycling of fructose-6-phosphate is mediated by two membrane-bound enzymes: phosphofructokinase (PFK) and fructose-1,6-diphosphatase (FDPase). Allosteric effectors which promote the PFK-FDPase system either increase the regular beating activity or induce bursting discharges, while inhibitory effectors reduce pacemaker activity. Associated with the PFK–FDPase cycle are slow oscillations in membrane potential, the postulate being that changes in amplitude and time period of the waves are brought about by the cyclic fluctuations of H^+ ions and ATP in the immediate vicinity of the membrane. Other enzyme reactions which affect the concentrations of gluconeogenic substrates or PFK effectors can modulate the oscillatory driving input, a good example being the neurogenic amino acid glutamate. Modifiers of FDPase and PFK are equally effective in changing pacemaker activity within the intact neuronal network and, hence, the rhythmic body function connected to this network. This has been demonstrated with pacemaker neurones governing cardiovascular activity in *Aplysia*, blood pressure or heart beat in the cat, and respiration or thermoregulation in the rabbit. Nature appears to have achieved a functional differentiation between different pacemaker neurones by altering their response to at least one or two of the PFK and FDPase effectors. New periodicities can be entrained by current stimuli on the pre-existing rhythms of isolated *Aplysia* pacemaker neurones. Stimulus-induced resetting of the discharges is in fact accompanied by a redistribution between two kinetically distinct forms of PFK, and modifiers of this enzyme can stabilize the new periodicities or facilitate the conditioning effect of a stimulus. Memory facilitation and consolidation under PFK modifiers could also be demonstrated in avoidance and discrimination learning trials with honey bees and rats, which are consistent with the metabolic nature of the slow-wave rhythmicity in vertebrate microneurones thought to be the site of memory storage.

INTRODUCTION

Iberall & Cardon (1964) have suggested that the complex interaction between the many control systems within an organism requires the existence of some reference values which are continuously adjusted by some 'master signals' from higher centres

* Dedicated to Professor Sir Hans Krebs to whom I owe so much.

of the brain. In this way, essential rhythmic and homoeostatic functions of the body such as blood pressure, respiration and thermoregulation could be controlled by neuronal pacemakers.

To test this hypothesis it would be useful to find ways and means to change the activity of the 'master elements' in the central command programmes. This would allow the signals communicated to follower neurones and ultimately to peripheral effectors to be manipulated. While many neurones are silent until driven by synaptic inputs, pacemaker neurones exhibit endogeneous rhythmic activity (von Baumgarten, 1970). A considerable number of such circadian and faster oscillating pacemakers has been identified in specialized parts of the marine mollusc *Aplysia californica*. In particular, the exact localization of large-sized pacemaker neurones exhibiting characteristic firing patterns has been well established in the abdominal ganglion (Frazier *et al.* 1967; Strumwasser, 1967). These pacemaker neurones can be completely isolated from their synaptic, ephaptic and humoral inputs without impairing endogeneous rhythmic activity (Chen, Baumgarten & Harth, 1973); even circadian rhythms still persist after isolation (Strumwasser, 1967). Such isolated pacemaker neurones are, therefore, ideally suited for elucidation of the 'molecular clock' responsible for endogeneous rhythmic activity.

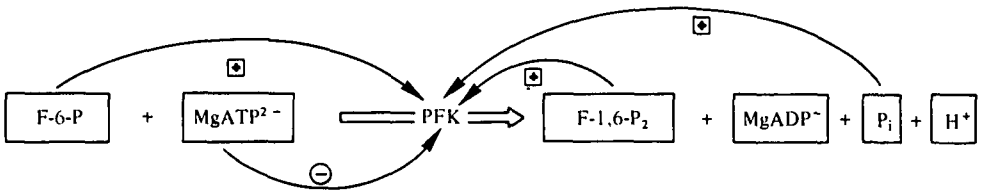
Metabolic regulation of rhythmic activity in isolated Aplysia *pacemaker neurones*

To understand the cellular mechanism leading to rhythmical impulse activity of pacemaker neurones, completely isolated neurones from *Aplysia californica*, either of the regular beating type (The R_3–R_{14} cells) or the bursting type (L_3 and L_6 neurones) have been used. The regular beating and bursting activity persists unaltered for at least 12 h (Chaplain, 1976).

An intimate coupling between the endogeneous rhythms and glycolysis was originally suggested by the known oscillations in cellular glycolysis (Betz, 1968; Hess & Boiteux, 1968) and the disappearance of all pacemaker activity in the presence of characteristic metabolic inhibitors (Chen *et al.* 1973). Oxidative phosphorylation or the pentose phosphate cycle do not constitute a driving input for the rhythmic activity and *de novo* protein synthesis has previously been excluded as being important for the pacemaker discharges (Schwartz, Castelluci & Kandel, 1971). An investigation of the effects of a variety of normal cellular metabolites revealed that rhythmic activity increased following the administration of characteristic gluconeogenic substrates such as pyruvate, malate, serine or glutamine (compare Scrutton & Utter, 1968). Thus gluconeogenesis, via a reversal of the glycolytic pathway, appeared to be implicated in the substrate supply for endogeneous rhythmic activity. As a large body of evidence has already been presented on earlier occasions (cf. Chaplain, 1976), only the most important findings will be summarized here and new data added where it helps to further clarify the emerging picture.

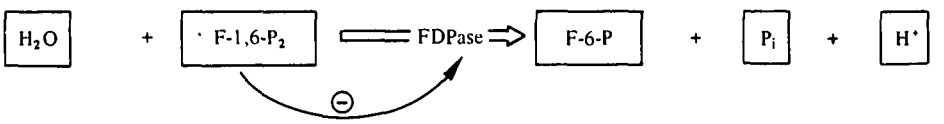
Localization and regulatory properties of phosphofructokinase and fructose 1,6-*diphosphatase.* In the search for an oscillatory driving system attention has been focused on one of the major regulatory sites determining the direction and rate of gluconeogenesis and glycolysis – the enzymes phosphofructokinase (PFK) and fructose 1,6-diphosphatase (FDPase). FDPase catalyses the hydrolysis of fructose-1,6

Positive effectors	F-6-P	G-6-P	P_i	AMP	F-1,6-P_2
$A_{0.5}$ (mM)	1·5	2	1·6	0·5	1



Negative effectors	Citrate	3-PGA	ATP
$I_{0.5}$ (mM)	1·5	3	1·5

Positive effectors	Citrate	3-PGA	ATP
$A_{0.5}$ (mM)	0·5	1	2·5



Negative effectors	AMP	F-1,6-P_2
$I_{0.5}$ (mM)	4	3·5

Fig. 1. Effector concentration for half-maximal activation ($A_{0.5}$) or inhibition ($I_{0.5}$) of neuronal phosphofructokinase (PFK) and fructose-1,6-diphosphatase (FDPase) activity studied *in vitro*.

diphosphate (F-1,6-P_2) which is formed in these neurones from gluconeogenic precursors to yield fructose-6-phosphate (F-6-P) and inorganic phosphate (P_i). PFK catalyses at the expense of ATP the rephosphorylation of the F-6-P produced in the FDPase step (Fig. 1).

Both enzymes are present in pacemaker neurones at high and nearly equal activity, being 3·4 and 2·8 μmol min g^{-1} for PFK and FDPase respectively (as assayed in the absence of any enzyme activators or inhibitors). When the distribution between cytosol and the plasma membrane pellet was determined, following differential centrifugation, 50–65% of the PFK and 40–55% of the FDPase remain bound to the cell membrane, even after extraction under mild conditions with a salt-mannitol medium. When the FDPase and PFK activities were assayed, at 14 °C under the conditions first introduced by Clark *et al.* (1973*a*, *b*) in their studies on bumble-bee flight muscle, it became clear that both enzymes are affected by a similar range of allosteric modifiers as the PFK and FDPase from other invertebrate and vertebrate sources (Scrutton &

Utter, 1968; Datta *et al.* 1974; Uyeda & Luby, 1974; Pogell, 1975). It is of particular interest that AMP and F-1,6-P_2 activate PFK, but at somewhat higher concentrations actually inhibit FDPase (Fig. 1). At the same time, 3-phosphoglycerate (3-PGA) and citrate initially stimulate FDPase even though, before, at higher concentrations these cell metabolites start to reduce PFK activity. With ATP the situation is rather similar, although fairly high concentrations are needed to activate the neuronal FDPase. Inorganic phosphate (P_i), ADP, glucose-6-phosphate (G-6-P), F-6-P, F-1,6-P_2 and cyclic 3′,5′-AMP only increased the PFK activity. Under no conditions was it possible to inhibit neuronal PFK by more than 80–85%. When the kinetic effects of modifier combinations were investigated in the FDPase assay, it was noted that the activator 3-PGA (3 mM) decreased the effectiveness of the AMP (5 mM) inhibition. AMP and F-1,6-P_2 at 5 mM appeared to act synergistically with the resulting 76% inhibition being overcome only by a combination of 3 mM 3-PGA plus 2 mM ATP. As far as the PFK activity was concerned, the inhibitory effect of 3 mM ATP (70% inhibition) could be overcome by 2 mM AMP or 3 mM F-6-P.

The reciprocal relationship is illustrated in Fig. 1; the positive effector for one of the two enzymes becomes at higher concentrations inhibitory for the coupled enzyme so that the actual activity of the PFK and FDPase will be critically dependent on the local substrate and effector concentration close to the enzyme molecules. The effector concentrations for half-maximal activation or inhibition are summarized in Fig. 1.

Evidence in favour of a PFK–FDPase-mediated substrate cycle. Simultaneous metabolism of F-6-P through reactions catalysed by PFK and FDPase constitutes a substrate cycle in which ATP is hydrolyzed and protons are produced (see Fig. 1). Such substrate cycles have been considered to be important for the control of glycolysis and gluconeogenesis (Newsholme & Gevers, 1967; Williamson, Jakob & Scholz, 1971) and certainly play a major role in the generation of heat in non-shivering thermogenesis (Clark *et al.* 1973*a*).

An estimate of the PFK–FDPase-mediated cycling rate becomes possible when changes in the $^3H/^{14}C$ ratio of hexose-6-phosphate are determined following administration of [^{3}H,U-^{14}C]fructose-6-phosphate to the medium. In the course of glycolysis, ^3H is lost to the cell water when [3-^{3}H,U-^{14}C]F-1,6-P_2 formed through the PFK reaction equilibrates rapidly with glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in presence of the excess activity of neuronal aldolase and triosephosphate isomerase. Consequently, a recycling of F-1,6-P_2 to F-6-P in the FDPase reaction would result in a F-6-P species of lower $^3H/^{14}C$ ratio (compare Clark *et al.* 1973*a*). Although one should be careful about drawing quantitative conclusions from these data on pooled neuronal populations, the average cycling rate was 0·95 μmol min^{-1} g^{-1} (compared with a glycolytic rate of 0·47 μmol min^{-1} g^{-1}), becoming almost undetectable in silent neurones. Thus for every molecule of F-6-P metabolized through the glycolytic sequence two out of three F-6-P molecules may possibly be recycled in regular pacemaker neurones. Substrate cycling became very low when 5 mM citrate was present in the medium.

Frequency-setting and interconversion between regular beating and bursting activity by metabolic modifiers of the neuronal PFK and FDPase. It should be emphasized that similar changes in spike activity have been observed for a given metabolite, which are independent of whether the modifiers of the PFK–FDPase system was added to the

medium or administered intracellularly under gentle pressure. However, the effective concentrations of metabolites were somewhat lower when applied intracellularly (compare Fig. $2l_1+l_2$). To compare the effects of the metabolic modifier on pacemaker neurones in intact neuronal networks, described in a later section, only the effects of extracellular additions are illustrated in Fig. 2. Upon extracellular administration of the metabolite there was an initial lag period of 1–4 min prior to the establishment of the new discharge pattern, which was then maintained for 80 min or longer, depending on the metabolite investigated (Chaplain, 1976, table 1). All modifier effects could be readily reversed on washing the neurone in normal sea water.

For a given R_3–R_{14} neurone there exists a marked concentration dependence for the action of F-1,6-P_2. Raising the F-1,6-P_2 level in the medium from 0·5 to 3 mM more than doubled the impulse frequency of the regular beating spike trains (Fig. $2a_1$). However, at 4 mM or even higher concentrations of F-1,6-P_2, there was a decrease (Fig. 2) often leading to a complete cessation of spike activity. The absolute magnitude of the stimulation depends of course on the original discharge frequency of the neurone under control conditions, being highest when the original frequency prior to the addition of F-1,6-P_2 was only 7–9 impulses/min. The maximal regular beating frequency which could be induced by F-1,6-P_2 was 40 impulses/min.

When AMP, another potent effector of the PFK–FDPase system, was investigated, a concentration dependence similar to that found with F-1,6-P_2 was observed. This is not altogether unexpected in view of the very similar modifying effects observed for both F-1,6-P_2 and AMP on neuronal PFK and FDPase (Fig. 1). AMP at concentrations between 1–3 mM in the immersion medium greatly enhanced spike activity, while at higher concentrations the spike activity declined and the discharge pattern became irregular (Fig. $2b_1$). The R_7, R_8 and R_{14} neurones deviated from this general pattern in that addition of 5–10 mM AMP led to the appearance of double spikes, an early configuration in the establishment of a bursting pattern (Fig. $2b_2$).

With F-1,6-P_2 and AMP activators of PFK have been used which at higher concentrations become inhibitory to the FDPase. Stimulating effects on the regular beating activity could also be elicited by low levels of citrate, ATP and 3-PGA which activate the FDPase (Fig. 1), but the discharge frequency was reduced at concentrations of 1·5–3 mM, which are expected to inhibit the PFK.

To discover to what extent the metabolic modifiers were interacting with each other as in the isolated PFK–FDPase system, their mutual effects on the endogeneous rhythmic discharges were studied. In presence of 3 mM AMP, at least 2 mM ATP plus 2 mM citrate had to be added to R_3, R_9 and R_{13} cells to obtain a significant reversal of the increase in frequency induced by AMP. As far as the inhibitory effect of ATP on the spike activity is concerned, new impulse discharges could be elicited in R_7 and R_8 neurones at a concentration of 4 mM when 7·5 mM F-6-P and 1·5 mM 3-PGA were added together, an effect which could not be induced by either of these compounds alone. Addition of 3 mM 3-PGA relieved the inhibition in R_{10} and R_{12} neurones following the application of 10 mM AMP, while the inhibition of R_6 and R_{14} neurones could be prevented only partially by 3 mM F-6-P.

Most important seems the observation that F-6-P and cyclic AMP, selective activators of PFK, actually induce interconversions from a beating to a bursting discharge pattern (Fig. 2*d*, *e*). The same has been observed for such neurones in which only the

activating effects of the FDPase modifiers (but not their inhibitory action on PFK) could be observed, either in the R_9 and R_{12} neurone in the presence of 0·5–1·5 mM citrate or the R_{10} cell in the presence of 6 mM 3-PGA. A hypothesis that it may be the high-activity state of the PFK–FDPase cycle which is related to the bursting discharges is supported by the finding that cyclic AMP in combination with citrate only brings about an alternation of short bursts with intervals of beating activity (Fig. 2*f*).

Since it might be argued that the addition of all these modifiers could provide precursors and substrates for other secondary reactions, 1-deoxy-D-fructose-6-phosphate (deoxy-F-6-P), a non-metabolizable analogue of F-6-P, was tested. This analogue, however, was found to mimic the modifier action of F-6-P on PFK. It seems noteworthy that deoxy-F-6-P induced a bursting pattern that was similar to that for F-6-P, except for a longer time lag probably due to its lower binding affinity to PKF (Fig. 2*d*+*g*). Experiments with characteristic burster neurones (L_3 and L_6), provide additional evidence in favour of an intricate coupling between the discharge pattern and the activity of the PFK–FDPase system. Citrate administration at 2–4 mM reduced the spike density of the bursts from 18 imp./min to 8 imp./min (Fig. 2k_3), with beating discharges appearing after 10–15 min. The PFK inhibitor ATP only decreased the spike density of the bursts, an effect which could actually be reversed by AMP (Fig. 2k_{33}). Space does not permit elaboration on an interesting finding, which is very likely to have considerable functional and evolutionary significance. Although there is a wide range of modifiers that act rather similarly on all pacemaker neurones of the abdominal ganglion of *Aplysia*, the identified pacemaker neurones (Frazier *et al.* 1967) can also be distinguished by their specific response to at least one or two of the various metabolic modifiers (Chaplain, 1976, table 1), while e.g. R_{15} bursters and follower neurones do not respond to the modifiers.

Changes in the free ion concentration of the medium owing to the presence of the

Fig. 2. Metabolic control of pacemaker properties. Concentration-dependence or time course of the pattern changes characteristic for regularly beating R_3–R_{14} pacemaker neurones from *Aplysia* (if not otherwise indicated) are illustrated from *top to bottom*. (a_1) F-1,6-P_2: control, 0·5, 1, 1·5, 2, 3 and 4 mM added; (a_2) 3 mM F-1,6-P_2 plus 5×10^{-6} M tetrodotoxin; (b_1) AMP: 0·5, 1, 2, 3, 4·5 and 6 mM; (b_2) appearance of double spikes in R_7, R_8 and R_{14} neurones in presence of 5–10 mM AMP; (*c*) 7·5 mM F-6-P plus 2 mM 3-PGA with recordings shown for 5 min, 10 min (expanded time scale), and 30 min after administration; (d_1) 1·5 mM F-6-P after 10, 30, 60, 120 and 150 min; (d_2) effect of additional administration of 4·5 mM F-1,6-P_2 to (d_1) as shown after a further 15 and 60 min; (*e*) 3 mM cAMP after 15 and 30 min; (*f*) 3 mM cAMP plus 2·5 mM citrate, arrow indicates time of addition; (*g*) 5 mM deoxy-F-6-P at 90, 120, 150, 200 and 250 min after administration; (k_1) citrate: 1·5, 2, 3, 4 mM; (k_2) 2·5 mM citrate in absence and presence of 5×10^{-6} M tetrodotoxin; (k_3) addition of 2 mM ATP (arrow) to L_6 burster neurones – and reversal of the ATP-effect by 3 mM AMP (see k_{33} with the time-scale expanded to demonstrate the doubling of the spike density within the burst); (l_1) effect of 0·75 mM-Na-glutamate recorded 30 and 45 min after addition to the medium; (l_2) immediate effects of intracellular pressure injection of 0·5 mM-K-glutamate (see arrow) and the discharge pattern established after 30 min; (*i*) changes in ion composition of the medium: $[Ca^{2+}]_e$ lowered to 1·5 mM, recordings after 10 min (i_1) and 30 min (i_2); increase in $[K^+]_e$ to 40 mM (i_3); $[Ca^{2+}]_e$ lowered to 0·1 mM (i_4); 0·1 mM-Ca^{2+} plus 40 mM-K^+ in the medium (i_5). For comparison intracellular recordings from *microneurones* of the rabbit bulbus olfactorius are included, in m_1 and m_2 characteristic rhythmic patterns are displayed, with the m_2 pattern modified in m_3 first by 2 mM pyruvate and additionally (see arrow) by 2 mM F-1,6-P_2. The time scale on the right-hand side corresponds to 60 s in the case of a_1–i_5 (respectively to 12 s for the expanded time scales in *c* and k_{33}) and to 200 msec for the recordings in m_1–m_3. The voltage scale is equal to 100 mV for a_1–l_2, the exceptions being *g*, k_3 and *i*, where it is only 85 mV, and k_2 (bottom half) and m_1–m_2 where it corresponds to 30 mV.

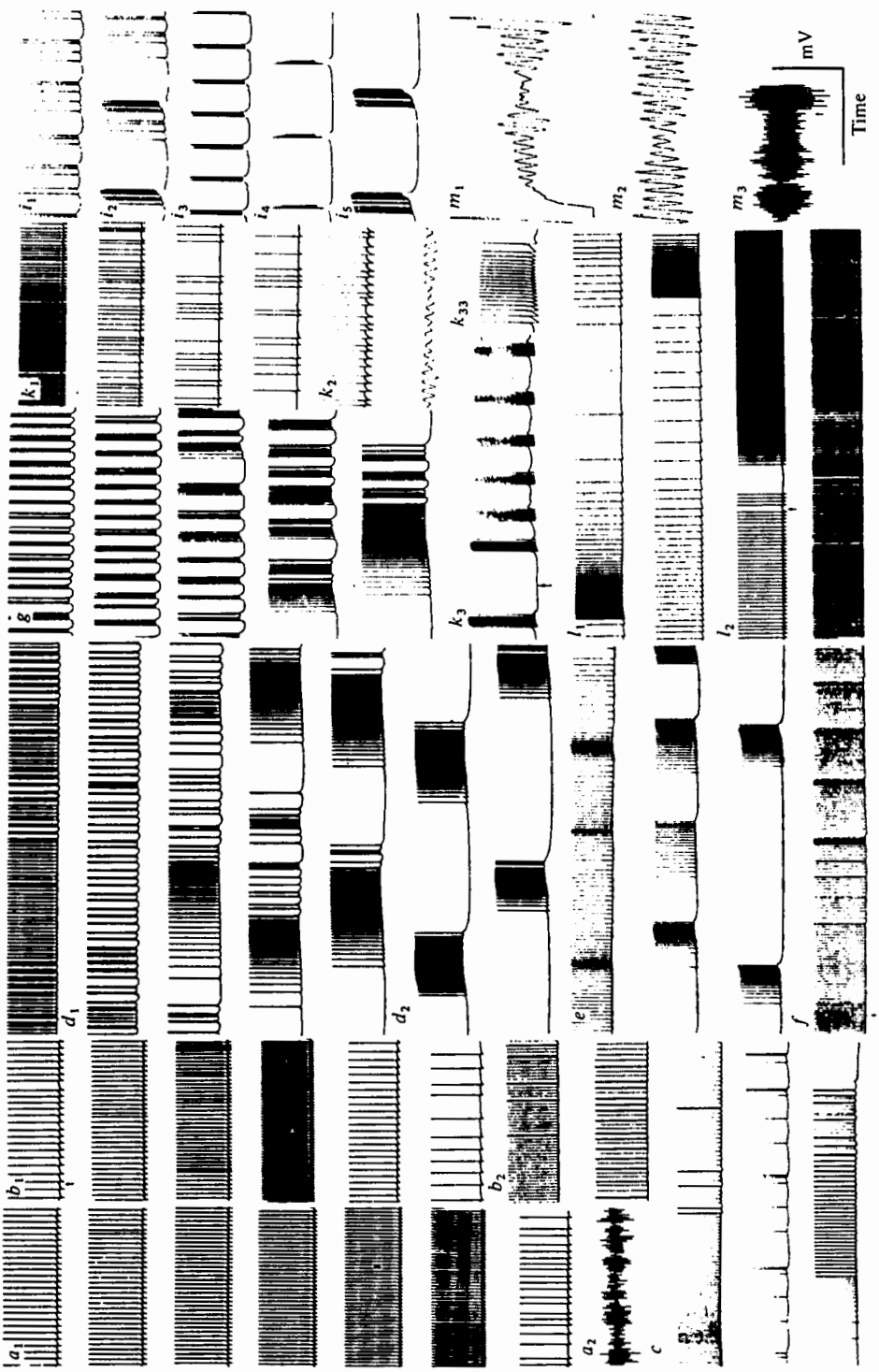


Fig. 2. For legend see opposite.

metabolites have been excluded by adjusting the ionic composition (based on the known association constants of the organo-metal complexes). The observed metabolic modifier effects could be partially duplicated only by extreme changes in the external Ca^{2+} ion concentration (Chaplain, 1976). The Ca^{2+} level had to be lowered from 10 to 1·5 mM to induce the appearance of spike triplets and burst-like activity (Fig. 2 i_1+i_2). Lowering the Ca^{2+} content even further resulted at 0·1 mM only in regular short bursts, with the spike amplitudes decreasing progressively between the first and last impulse (Fig. 2i_4). While even 200 mM changes in the 425 mM-Na^+ concentration proved ineffective, neither an increase in K^+ from 10 to 40 nM (Fig. 2i_4) nor a concomitant lowering of the Ca^{2+} level to 0·1 mM (Fig. 2i_5) were able to mimic the bursting pattern characteristic for *Aplysia* pacemaker neurones (Frazier *et al.* 1967; Strumwasser, 1967) or the metabolically induced conversion of regular beating to bursting activity. Therefore an intracellular regulatory mechanism which governs the excitability of the neuronal membrane is clearly suggested.

A possible mechanism for coupling the substrate cycle to changes in ionic currents across the neuronal membrane. The location of PFK and FDPase on the plasma membrane in *Aplysia* pacemaker neurones may be a pre-requisite for the dynamic interaction of the two enzymes with each other and with the ion transport processes across the membrane. For example, F-1,6-P_2 bound to PFK, which is accessible to FDPase (Uyeda & Luby, 1974), may provide a 'recognition site' for FDPase to interact with PFK. On the other hand, FDPase is known to enhance the inhibition of PFK by such negative effectors as ATP, citrate or 3-PGA (Kono & Uyeda, 1974). Further, it is known for FDPase that at physiological concentrations of AMP and ATP (an activator and an inhibitor of PFK, see Fig. 1) small variations in the F-1,6-P_2 level can regulate the release or binding of this enzyme to free phosphoryl groups of a membrane-like cellulose matrix (Kratovich & Mendicino, 1974). On the basis of the magnitude of the apparent association constants summarized in Fig. 1, these metabolic effectors would become functionally significant, especially if both enzymes are tightly packed. Small localized fluctuations in effector concentrations would result in dynamic changes of the PFK–FDPase interaction and, hence, the substrate cycling rate. If the substrate cycle state is indeed reflected by the organization pattern of the PFK–FDPase–membrane complex, the recycling rate of the common metabolite may in fact be much higher than can be measured by the dilution of label following the dissociation of F-6-P off the enzyme surface.

The immediate effect of the metabolic modifiers governing the proton-producing and ATP-splitting substrate cycle (Fig. 1) seems to be directed towards the slow oscillatory waves which become uncovered when spike initiation is blocked by tetrodotoxin (TTX). In beating neurones, at least, these slow depolarization–hyperpolarization cycles correspond in time period to the frequency of spike trains in the absence of TTX. The slow waves are speeded up by stimulators of the endogeneous spike activity such as F-1,6-P_2 or AMP and are slowed down by the inhibitors citrate or 3-PGA (Fig. $2a_2+k_2$). At the same time, the amplitude of the waves is equally affected, varying between 3 and 5 mV in the presence of inhibitory citrate levels and 12–18 mV at stimulating F-1,6-P_2 concentrations. Thus under conditions where the H^+ concentration in the immediate vicinity of the membrane rises, with a concomitant fall in the level of ATP, the slow potential changes display high amplitudes. The effectors

deoxyF-6-P, cyclic AMP and F-6-P plus F-1,6-P_2 converted regular beating into bursting discharges, increased the wave amplitudes even further and extended their periods; amplitudes of 20 mV and periods longer than 1 min have been observed under certain bursting conditions. Voltage-clamp experiments under these conditions revealed marked quasi-sinusoidal oscillations in the inward current conductance. A possible explanation would be that during regular spike activity (between 7 and 42 imp./min) only stochastic inputs may be provided by localized PFK–FDPase units, which at higher substrate cycling rates fuse into oscillatory waves.

The inhibitory effect observed with *Aplysia* pacemaker neurones in a medium containing 50% deuterium oxide (Chen *et al.* 1973) is consistent with the hypothesis that proton transfer processes are involved in these changes in neuronal membrane permeability (compare Bass & Moore, 1973). The main effect of any protons produced in the substrate cycle may be directed towards a change in sodium conductance as similar slow waves as those shown in Fig. 2 have been observed in TTX-treated R_{15} neurones in Ca^{2+}-free but not in Na^+-free media, with the waves remaining unaffected by tetraethylammonium ions (Strumwasser & Kim, 1969; Barker & Gainer, 1975*a*). As TTX only blocks the electrically excitable Na^+ channel but has apparently no affinity for other kinds of Na^+ channels such as in excitatory synapses (Strumwasser, 1968), the protons generated by the PFK-FDPase-mediated substrate cycle may activate a chemically excitable Na^+ channel in pacemaker neurones. A feasible mechanism would be the displacement of regulatory divalent cations (i.e. Ca^{2+} by H^+ ions) that seem to be responsible for the initiation of bursting pacemaker activity in Ca^{2+}-inhibited snail neurones (Barker & Gainer, 1975*b*). As temperature elevation reduces the regulatory effectiveness of Ca^{2+} in snail and *Aplysia* neurones (see Barker & Gainer, 1975*b*), the heat liberated from the splitting of ATP during the substrate cycle may actually facilitate the displacement of Ca^{2+} by H^+. Such a proton-controlled gating mechanism could equally operate for the two components of the inward current as the time course for the opening and inactivation of both the Na^+ and Ca^{2+} channels in molluscan neurones appear closely similar (Standen, 1975).

All the spike patterns recorded under the influence of the various metabolic effectors can be predicted by a quantitative model in which a metabolically controlled activation of Na^+ (and Ca^{2+}) conductance determines the spike frequency and the amplitude of the after-spike hyperpolarization (Both *et al.* 1976). When the inward current conductance is already high (e.g. at low regulatory Ca^{2+} levels in the medium), the model predicts correctly the short burst recorded at 0·1 mM-Ca^{2+}, in which only the first spike develops the normal amplitude, while the height of the subsequent four impulses is progressively reduced (Fig. $2i_4$). The operation of the substrate cycle does not appear to depend on external Ca^{2+}. This is also shown by the unimpaired slow-wave activity in TTX-treated neurones immersed in Ca^{2+}-free media (Barker & Gainer, 1975*a*). The model thus incorporates the assumption that in addition to their regulatory role Ca^{2+} ions that contribute to the inward current increase the spike amplitude without directly affecting spike frequency. The periodic variation of the outward current K^+ conductance, which in terms of the model is responsible for the burst termination and changes in interburst interval, may reflect two major control mechanisms. For example, the oscillatory reduction of the near-membrane ATP level may activate the K^+ conductance, as ATP addition to mammalian tissue cultures

has been shown to inhibit markedly the K^+ efflux in a ouabain-insensitive manner (Trams, 1974). The accumulation of Ca^{2+} ions on the internal side of the membrane as a consequence of the inward current flow of Ca^{2+} associated with the bursting activity would additionally activate the K^+ current conductance, as shown by Meech (cf. Meech & Standen, 1975).

Although oscillatory fluctuations of ATP level and H^+ release could also arise by the operation of a Na^+/K^+ pump, the latter does not appear to be intimately coupled to rhythmic spike activity. The slow membrane potential oscillations which underlie the spike activity in *Aplysia* neurones are not abolished by ouabain (Chen *et al.* 1973). Further, 0·4 mM ouabain actually increased the spike activity for 30 min periods in R_3–R_8 neurones and for as long as 2 h in unidentified *Aplysia* neurones. Under these conditions, there was a 15 mV depolarization of the resting membrane potential suggesting an effective inhibition of the Na^+/K^+ pump. At any rate, by using a metabolically controlled substrate cycle instead of some modulations in Na^+/K^+ pump activity, Nature would have achieved a much more sensitive control of endogeneous pacemaker activity.

Modulation of the rhythmic discharge pattern through additional metabolic reactions and synaptic stimulation. As an example of the immediate effect of neurogenic amino acids on the neuronal metabolic state the action of L-glutamate on these isolated pacemaker neurones should be described (see Chaplain & Krämer, 1976). Irrespective of whether 0·75 mM-Na-glutamate was present in the medium or whether 0·5 mM-K-glutamate was injected intracellularly, oscillations in the neuronal rhythmic activity appeared, with phases of depressed discharge frequency alternating with periods of very high pacemaker activity (Fig. 2*l*). An analysis of the neuronal concentrations of some of the PFK modifiers listed in Fig. 1 revealed that the activators AMP and F-1,6-P_2 were high under conditions of glutamate-promoted rhythmic activity. In contrast, when the neuronal activity was inhibited in the presence of 7·5 mM glutamate, the neuronal levels of ATP and citrate, known to slow the substrate cycling rate, actually increased. The rise in intraneuronal citrate is very likely caused by the glutamate-specific inhibition of the brain citrate lyase (Szutowicz, Stepien & Angielski, 1974). As other well-known gluconeogenic amino acids such as L-serine, L-glutamine and L-alanine equally affected the discharge rate, the intraneuronal supply of gluconeogenic substrates may exert a regulatory effect, consistent with the membrane potential changes induced specifically by gluconeogenic substrates in perfused rat liver (Dambach & Friedman, 1974).

In this context it is of interest that the enzymes involved in the initial reversal of the glycolytic sequence, and hence the channelling of gluconeogenic equivalents to the PFK–FDPase system, are equally under reciprocal control in these *Aplysia* neurones. Here the two ATP-consuming enzyme reactions – *pyruvate carboxylase* catalysing the formation of oxalacetate from pyruvate and HCO_3^- and *PEP carboxykinase* catalysing the conversion of oxaloacetate to phosphoenol pyruvate (PEP) – are important. PEP itself can be recycled into the glycolytic pathway under ATP formation by *pyruvate kinase*, the only one of the three enzymes bound to the cellular membrane. Comparable to the enzymes from other sources (Scrutton & Utter, 1968; McClure & Lardy, 1971; Cannata & Flombaum, 1974), pyruvate carboxylase becomes increasingly inhibited by Ca^{2+} ions above 10^{-6} M, while the PEP carboxykinase is

under rigid control by ADP. In this way an increase in intraneuronal Ca^{2+} level, as part of the inward current flow during spike activity (Meech & Standen, 1975), as well as increased ADP concentrations accompanying high substrate cycle rates would turn-off the provision of substrates for the PFK-FDPase cycle. As this would shut-off the immediate oscillatory driving for the inward currents across the membrane, this constitutes a way of terminating the bursts, a metabolic equivalent to the Ca^{2+}-induced post-tetanic hyperpolarization (cf. Meech & Standen, 1975). In contrast, the key glycolytic enzyme pyruvate kinase was inhibited by ATP and cyclic AMP (compare also Ljungström, Hjelmquist & Engström, 1974), thus ensuring that PEP is channelled back to the F-6-P level under conditions of high bursting activity. A major stimulus for the PFK–FDPase substrate cycle and its substrate supply is provided by NH_4^+ ions which activate the PFK and the pyruvate carboxylase. Double-labelling studies have shown that NH_4^+ ions are produced in the oxidation of gluconeogenic amino acids. In contrast, Li^+ ions slowed the circadian rhythms by inhibiting the pyruvate carboxylase.

Needless to say, the interplay between the various metabolic reactions can easily give rise to oscillating pacemaker activity and circadian rhythms, particularly as the concentrations of substrates and modifiers for the PFK and FDPase, such as ATP, F-6-P, F-1,6-P_2 and 3-PGA have themselves been shown to undergo oscillatory fluctuations in other systems (Betz, 1968; Hess & Boiteux, 1968).

As Cedar, Kandel & Schwartz (1972) have demonstrated that there is an increased synthesis of cyclic AMP in response to synaptic stimulation of *Aplysia* pacemaker neurones, a close coupling between normal neuronal inputs and the metabolically entrained rhythms is suggested at the level of these regulatory enzymes.

The effect of PFK modifiers on memory facilitation and information storage

Information storage in Aplysia *pacemaker neurones.* Isolated *Aplysia* neurones can recognize, store and reproduce time intervals; the interspike and interburst frequency can be locked with the rhythm of an imposed depolarizing current stimulus, with the new rhythmic periodicity outlasting the stimulation period (von Baumgarten, 1970). As similar effects had previously been observed in intact ganglia for pacemaker neurones subjected to regularly spaced synaptic inputs (Perkel *et al.* 1964) and in the EEG after rhythmic stimulation (Livanov & Poliakov, 1945), a hypothesis has been suggested according to which learning takes place by stimulus-induced re-self-excitation of rhythmically active neurones (von Baumgarten, 1970). According to this hypothesis, information is stored in pacemaker neurones around the time interval at which an external stimulus is entrained on a pre-existing rhythm.

Attempts were made to study the biochemical consequence of resetting the spike activity of *Aplysia* neurones. Stimulus-induced shortening or lengthening of the spontaneous impulse discharges was accompanied by the redistribution between two kinetically different and immunologically distinct forms of PFK and pyruvate kinase as revealed by cross-reacting *Helix* antibodies in a sensitive radioimmunoassay (Chaplain & von Baumgarten, 1975). Further evidence for some biochemical changes was obtained by testing the effects of the metabolic modifiers of the substrate cycle. Whereas the stimulus-induced resetting of spike activity outlasted the conditioning current stimulation by only 18 periods, an entrained faster discharge pattern could be

stabilized by the addition of F-1,6-P_2 to the medium, while a change in interburst interval was stabilized by citrate. For neurones in which spike activity had been reset by F-1,6-P_2 (see Fig. 2*a*) facilitation in the conditioning effect of the threshold-reaching stimulus (compare von Baumgarten, 1970) could be observed.

Metabolic nature of rhythmic activity in vertebrate microneurones. As evolution of memory and learning throughout the animal kingdom has been paralleled by a steep rise in the number of microneurones (the so-called granule cells), these elements might be responsible for memory storage (Altman, 1967). In localized regions of microneurones in the cerebral and cerebellar cortex (Bremer, 1958), the hippocampus (Green, Maxwell & Petsche, 1961) and the bulbus olfactorius (cf. von Baumgarten, 1975) extracellular recordings revealed spontaneous activity, appearing predominantly in the form of slow waves. Using slices of granule cell layers from the bulbus olfactorius of the rabbit, it was shown with intracellular recordings that spontaneous rhythmic activity exhibited a comparable pattern to that established by *Aplysia* pacemaker neurones (see Fig. 2, a_2, k_2 and m_2), which was also of metabolic origin (Chaplain, 1977). The most potent modifiers in this preparation were F-1,6-P_2 and the gluconeogenic substrate pyruvate. Both metabolites accelerated the potential waves, but when F-1,6-P_2 was administered in addition to pyruvate, the amplitude of the waves increased as well (compare Fig. 2, m_2+m_3). Different granule cells displayed characteristic rhythmic potential changes with impulse discharges intermingled (Chaplain, 1977). Slow endogeneous rhythms could also be recorded from frontal, cortical and hippocampal microneurones and neurones of the nucleus suprachiasmaticus.

Memory facilitation in rats and honey-bees. As frontal cortical neurones are thought to be the site of short-term memory (Pribram & Tubbs, 1967) rats were administered PFK modifiers through implanted push–pull electrodes. The rats were trained with a weak electrical shock in a one-trial inhibitory avoidance task (10 s drinking followed by a footshock). Avoidance learning was measured as the latency to drink from a water-bottle after 24 h on a low-liquid diet. Immediate post-trial injections of F-1,6-P_2 or AMP resulted in marked lengthening of the retention latency to 130–280 s compared with the 10–30 s in the controls receiving only artificial cerebrospinal fluid. In this respect it would certainly be tempting to explain the observation that norepinephrine actually promotes memory consolidation in avoidance learning (McGaugh *et al.* 1975) in terms of its known action on neuronal glycolytic substrate levels (Leonhard, 1975).

An even better subject for studying the effects of metabolic modifiers on complex learning tasks is the honey-bee. In discrimination experiments, honey-bees had to make a choice between shape and colour in their flying approaches before receiving sugar water as a reward (Beckmann & Chaplain, 1976). Two effects have been noted. Ingestion of F-1,6-P_2 (80 mM) during a 45 min pre-learning period led to a very much faster memorization of the correct feeding place and colour pattern. Feeding the F-1,6-P_2 solution selectively at the end of ten learning trials ensured that the bees favoured the experienced colour–shape combination (in our case a black square strongly disfavoured in the initial spontaneous choice because of its unlikeness to flowers) for as long as 3–4 days without the need for any new incentives in the meantime. In contrast, the controls fed only on glucose lost all interest in the black square after 3 h once no sugar water was available any longer. As changes in general activity

or harvesting motivation could be excluded and other metabolites such as AMP proved ineffective or in the case of citrate plus 3-PGA even reduced the performance, F-1,6-P_2 appears to induce a characteristic effect both on memory facilitation and consolidation.

Changes in a circadian rhythm induced by F-1,6-P_2. Ample evidence in favour of a circadian involvement in learning and the rhythmic organization of animal behaviour exists in the literature (for Review see Rusak & Zucker, 1975). The unimpaired persistence of circadian rhythms in isolated *Aplysia* pacemakers (Strumwasser, 1967) and the metabolic nature of circadian rhythms in other systems (Aschoff, 1965) suggests the close relation to the metabolic control described for the *Aplysia* pacemaker neurones. A classical circadian rhythm is the time sense of the honey-bee, which remains unaltered in presence of a range of drugs, under narcosis or stress. Honey-bees can be trained on three successive days to visit a feeding place at a specific time of day. After their visit on the third day the bees were placed in a small box containing either glucose solution or glucose supplemented with 80 mM F-1,6-P_2 and released on the following morning. The control bees which ingested only glucose returned at their entrained 24 h interval on the 4th day, while bees fed on F-1,6-P_2 appeared 1 h earlier, with minor appearance peaks at earlier hours of the day (Beckmann & Chaplain, 1976). In terms of the concept of 'learning by stimulus-induced re-self-excitation' F-1,6-P_2 as an activator of neuronal discharges would simply reset the neuronal trigger for temporal coding in case of the learned 24 h circadian rhythm.

Metabolic control of neurones governing cardiovascular activity respiration and body temperature

From an evolutionary point of view the neural control of circulation in molluscs is a particularly well suited system to study modulation of heart beat by the CNS. In *Aplysia* the cardiac output is controlled from the abdominal ganglion by two excitatory (RB_{HE} and LD_{HE}) and two inhibitory motoneurones (LD_{H11} and LD_{H12}) and three vasoconstrictor neurones (Koester *et al.* 1974). A pacemaker neurone has been identified, the L_{10} cell, which induces increases in heart rate and a decrease in vasomotor tone. Using this semi-intact preparation of *Aplysia* introduced by Kandel and his collaborators, it becomes possible to monitor heart rate and blood pressure under conditions where different neurones of the abdominal ganglion are impaled by microelectrodes. Injection of 1 mM F-1,6-P_2 or 1 mM F-6-P into the soma of the L_{10} pacemaker elicited high-frequency bursts of spikes lasting for 15–20 min. Under these conditions the normal 12–14 heart beats/min increased initially to 19–22 beats/min and thereafter settled at 17 beats/min. At the same time the spike activity of the RB_{HE} motoneurones increased to about 39 imp./min. Following injections of 1·5 mM ATP into the L_{10} neurone both the rhythmic activity and the heart beat were converted within 10–14 min to low-frequency tonic discharges; the L_{10} even became silent after 30–40 min. Again the rhythmic activity of the RB_{HE} followed that of the L_{10} pacemaker. Recordings from the inhibitory motoneurones LD_{HE} revealed that their spike activity changed in an inverse pattern with the metabolically induced discharges of the L_{10} neurone.

In view of the observed high permeability of the neuronal membrane for various metabolites and effectors of intermediary metabolism, an attempt was made to affect

pacemaker activity governing certain rhythmic body functions in laboratory animals.

Extending the original studies of Bousquet *et al.* (1975) elliptically shaped neurones, firing with a spontaneous frequency of 3–3·5 imp./min, were localized within the ventrolateral part of the cat nucleus paragigantocellularis (Schläfke & Chaplain, 1977). When the ventral medullary surface was superfused with F-1,6-P_2 plus F-6-P, the discharge rate was raised to 14–20 imp./min, an effect which was accompanied by a reduction in arterial pressure by 24–30 mmHg. Superfusion with citrate plus 3-PGA in turn led to a depression in neuronal activity to about 1·5 imp./min, together with a 15 mmHg increase in arterial blood pressure. The induced changes in rhythmic activity of the blood-pressure regulating neurones were, after a short time-lag, reflected in altered plasma renin activity, suggesting the likely site in the CNS responsible for the observed circadian rhythms of renin (Modlinger, 1976).

Within the hypothalamic nucleus supraopticus of the cat, small-sized neurones were discovered which discharged in synchrony with the heart beat. Microelectrophoretic application of PFK modifiers resulted in major increases both in neuronal activity and heart rate. In contrast, metabolic modifiers failed to affect the activity of cardiovascular follower neurones in the rostromedial area of the medulla oblongata (characterized by short spike bursts lagging 50–100 msec behind the QRS complex of the electrocardiogram) nor under these conditions the heart rate (Böhmer, Chaplain & Fallert, 1976).

The inspiratory and expiratory phases of respiration are under immediate control by rhythmic neurones located in the bulbus reticularis. Probable pacemakers have been identified by their uninterrupted generation of long-lasting spike trains under conditions of low CO_2 levels after partial hyperventilation, which inactivates the feedback inhibitory loop responsible for periodic modulation of the respiratory network (cf. Cohen, 1974). Following superfusion or microintophoretic application the spike density of inspiratory pacemakers is increased by F-1,6-P_2, ATP and low concentrations of 3-PGA and becomes reduced in presence of 10 mM citrate. Expiratory pacemakers are actually activated by 10 mM citrate but become inhibited by ATP (Chaplain, Dinse & Fallert, 1976; Böhmer *et al.* 1976). These reciprocal relations could be explained if, as a result of evolutionary pressure, the neuronal PFK was no longer inhibited by the FDPase activator ATP in inspiratory neurones, while in expiratory neurones the inhibitory effectiveness of the FDPase activator citrate for PFK was strongly reduced. As expected, unspecific reticular follower neurones were not affected by metabolite administration (Böhmer *et al.* 1976). There appears to exist an intricate coupling relation between pacemakers controlling blood pressure and respiration. Changes in arterial blood pressure induced by the metabolic effectors on rhythmic neurones in the nucleus paragigantocellularis are mediated through activity changes in the cervical sympathetic nerve, with high neuronal firing rates being associated with reductions in both sympathetic discharges and blood pressure and vice versa. However, the actual pattern of the sympathetic discharges could be made to follow the inspiratory-related discharge pattern by microiontophoretic application of 3-PGA, F-6-P, F-1,6-P_2 or ADP to the so-called primary pacemakers of the respiratory system, the EI_α neurones (compare Cohen & Goodman, 1970).

In the pre-optic area of the hypothalamus warm-sensitive and cold-sensitive neurones can be identified which are involved in the control of body temperature

(Eisenman, 1972; Jahns & Werner, 1974). Similar temperature-sensitivity has been noticed among isolated *Aplysia* pacemakers. For example, with the cold-sensitive R_6 and R_{14} neurones both the spike frequency and the loss of ^3H from [3-^3H, U-^{14}C]F-6-P increased by 300–400% as the temperature was reduced from 19 to 14 °C. As R_6 and R_{14} neurones are equally activated by F-1,6-P_2 and AMP (see Fig. 2) and by the gluconeogenic substrates pyruvate and serine, these metabolites were applied through stereotaxically implanted push–pull cannulae to the pre-optic area of the anterior hypothalamus of unanaesthetized rabbits. Effector-induced increases of 0·8–1·5 °C in the rectal temperature could be observed after a lag period of about 6–11 min. The increase in temperature was accompanied by shivering, vasoconstriction and pilo-erection, with the colon temperature remaining at the higher level for 20 min. These effects could not be duplicated with glucose or the non-gluconeogenic amino acid lysine. Thus, hypothalamic thermoregulation seems to represent yet another instance in which rhythmic command elements are controlled in their operation by the PFK–FDPase substrate cycle. This hypothesis would also explain the earlier finding that endotoxin injection into the anterior hypothalamus of monkeys induces a hyperthermic state (Myers, Rudy & Yaksh, 1971) as endotoxin is known to promote substrate cycling (Williamson, Refino & La-Noue 1972). The same effect may explain the shivering of pigs under light halothane narcosis (Sinz *et al.* 1973) as halothane is known to increase F-6-P recycling in hyperthermic pigs (Clark *et al.* 1973*b*).

The high permeability of the neuronal membrane to metabolites modifying the pacemaker activity of CNS neurones suggests a logical reason for the existence of a highly selective blood–brain barrier, as otherwise the control of reference values for essential rhythmic body functions would be impossible.

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