CALCIUM AND CYCLIC AMP AS POSSIBLE MEDIATORS OF NEUROHORMONE ACTION IN THE HINDGUT OF THE COCKROACH, LEUCOPHAEA MADERAE

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Abstract—Adenosine 3',5'-monophosphate (cyclic AMP) (10⁻⁴ g/ml) often caused a gradual increase in spontaneous contractile activity of the hindgut of the cockroach, Leucophaea maderae, and on rare occasions it would evoke a hormone-like response. However, aminophylline (2.5 × 10⁻⁴ g/ml) was capable of mimicking the neurohormone, and a concentration of 2.5 × 10⁻⁴ g/ml potentiated the contractile response evoked by the neurohormone: these responses were blocked by either the presence of 1 mM manganous ion or in a high potassium solution (162 mM). Propranolol (10⁻⁴ g/ml) and dopamine (10⁻⁴ g/ml) suppressed both spontaneous contractile events and neurohormone action. Dopamine (5 × 10⁻⁴ g/ml) also blocked action potential generation as did propranolol at 10⁻⁴ g/ml.

These results lead us to suppose that cyclic AMP might serve as a mediator of neurohormone action by increasing calcium transport across the surface membrane of muscle fibres. Caffeine (2.5 × 10⁻⁴ g/ml), like aminophylline, caused a hormone-like response in normal hindguts. Even when the visceral muscles of the hindgut were depolarized in 162 mM potassium solution (without calcium), caffeine was still capable of inducing a phasic response. However, the addition of 2 mM calcium to such potassium-depolarized preparations caused a gradual increase in muscle tonus and substantially potentiated the response to caffeine.

Such findings clearly implicate calcium as the mediator of excitation-contraction coupling in visceral muscle. While the interactions between the neurohormone, cyclic AMP, and calcium seem to be largely associated with the surface membrane and action potential generation.

INTRODUCTION

Some insect neurohormones seem to regulate the homoeostasis of physiological systems on a day-to-day basis. The regulation of excretion by Malpighian tubules in Rhodnius prolixus (Maddrell, 1963); the conversion of glycogen to trehalose in the fat body of the American cockroach, Periplaneta americana (Steele, 1963); and the control of visceral muscle contractions in many insects (Davies, 1964) are examples of this type of hormonal mediation. Likewise, recent experiments have shown that the membrane of visceral muscle fibres is the prime site of action for the neurohormone that regulates motility of the hindgut of the cockroach, Leucophaea maderae (F.) (Holman and Cook, 1972; Cook and Holman, 1975). Indeed, these results suggested the presence of a loosely bound source of calcium at the surface of muscle membranes that in some way interacts with the neurohormone to change muscle excitability. A possible explanation for this interaction was suggested by the obvious rôle that both calcium ion and adenosine 3',5'-monophosphate (cyclic AMP) often perform in mediating the hormonal effects of many cellular processes (Rasmussen, 1970). Consequently, a series of experiments was conducted to determine whether cyclic AMP was implicated in hormonal action in this instance.

MATERIALS AND METHODS

The preparation of the deganglionated hindgut of the cockroach and the procedures used to record motility were described in detail elsewhere (Holman and Cook, 1970). Bioelectric events were recorded extracellularly as described by Cook and Reincke (1973). The normal saline solution had the following composition (in mM): Na 154, K 2.7, Ca 1.8, Cl 160, glucose 22. The pH was adjusted to 6.8. The 162 mM KCl saline solution used to depolarize the membrane was made up by simply replacing K for Na and other cations present in normal saline solution. In calcium-free saline solutions, the Ca in normal saline solution was omitted.
Fig. 1. Potentiation of the hindgut response to the neurohormone by aminophylline. (A) Myographic record of the contractile response to 1 activity unit of the neurohormone (arrow). (B) Weak response to aminophylline (2.5 x 10^{-6} g/ml) (arrow) after a rinse in fresh saline solution. (C) Reaction of the hindgut to the simultaneous addition (arrow) of the neurohormone (1 activity unit) and aminophylline (2.5 x 10^{-4} g/ml). Horizontal time mark, 20 sec; vertical calibration, 2 mm displacement.

The hindgut stimulating neurohormone (HSN) used in the study was obtained from whole body extracts of cockroaches (HOLMAN and COOK, unpublished data and contained 10 activity units per μl (an activity unit was defined as the minimum amount of neurohormone necessary to elicit a threshold response, an increase in frequency or amplitude of contraction, of the isolated cockroach hindgut in a 4 ml chamber). Each activity unit of the whole body extract represented 150 ng of residue.

RESULTS

In discussing the criteria for establishing the presence of cyclic AMP as a second messenger in a physiological system, SUTHERLAND (1971) stated that it should be possible to increase the magnitude of the physiological response by administering the hormone together with a phosphodiesterase inhibitor such as aminophylline. Our experiments with the cockroach hindgut showed a synergistic relationship between the neurohormone and aminophylline. These substances were first tested separately and then together on the isolated hindgut (after each of these steps, the preparation was rinsed several times in fresh saline solution). At a threshold concentration of one activity unit, the HSN caused an increase in the amplitude and frequency of contractions of the hindgut (Fig. 1A). When this same hindgut was then treated with aminophylline (2.5 x 10^{-6} g/ml), a barely perceptible increase in contractile activity was observed (Fig. 1B). Then, when HSN and aminophylline were added simultaneously, both the amplitude and duration of contractions showed a substantial increase (Fig. 1C), about twice that obtained from each substance alone. At concentrations of 250 μg/ml, aminophylline caused an increase in amplitude, frequency, and tonus of contractions quite similar to that caused by the neurohormone (Figs. 2A, 3A).

The calcium ion seemed to be just as important in the action of aminophylline as it was to the action of the neurohormone. When hindguts were exposed to 1 mM manganous ion, spontaneous contractions ceased (Fig. 2B); then after 5 min, aminophylline failed to evoke a response though caffeine did cause a phasic contraction (Fig. 2C). These results suggest that the manganous ion disrupts the function of calcium at the level of the surface membrane through competitive inhibition, but that sufficient intracellular calcium is present to ensure mechanical activation of the muscle with caffeine. Confirmation of the surface membrane site of action for aminophylline was obtained from experiments performed in high potassium solutions. If hindgut muscles were depolarized in 162 mM solution for 12 min, only a slight elevation was observed in the baseline of the myograph when aminophylline (2.5 x 10^{-4} g/ml) was added to the muscle chamber (Fig. 3A). At the same conditions, comparable amounts of caffeine produced a distinct phasic response (Fig. 3A). Thus aminophylline acts at the surface membrane of muscle fibres just like the neurohormone (COOK and HOLMAN, 1975). The slight rise in the baseline in Fig. 3A after the addition of aminophylline probably signifies a small release of intracellularly bound calcium by the drug.
Since propranolol has been reported as one of the more effective inhibitors of adenyl cyclase in several smooth muscle preparations (Robison et al., 1970; Triner et al., 1971), we decided to try it on the cockroach hindgut. At a concentration of $1 \times 10^{-4}$ g/ml propranolol caused a brief initial increase in contractile activity and an elevated tonus (Fig. 3B). However, after a 7 min exposure to the drug the hindgut showed little spontaneous activity, and two successive exposures to HSN (2 activity units each) failed to evoke a response (Fig. 3B). We have no direct evidence that this effect of propranolol is linked to adenyl cyclase inhibition in the hindgut, but the results do not contradict our supposition that cyclic AMP may have a role in mediating hormone action.

Dopamine was also found to be an antagonist for the peptide as is shown in Fig. 4A; the addition of $10^{-4}$ g/ml caused a marked suppression of myogenic activity in the isolated hindgut. The subsequent application of the hindgut-stimulating peptide (25 activity units) to this hindgut produced the characteristic response, but it was attenuated. Fig. 4A shows the results obtained after the same deganglionated hindgut was rinsed in saline solution: the first arrow marks the point at which the same amount of peptide was added to the muscle bath (25 activity units). There was a 2.5-fold increase in the contractile response. Then dopamine ($10^{-4}$ g/ml) was added to the preparation (second arrow), and this addition caused a pronounced drop in tonus but no change in the frequency and amplitude of contractions. The suppression of contractile activity by dopamine and propranolol (Figs. 4A, 3B) seems to be explained by the fact that both amines block spontaneous action potentials (Figs. 4B, 5C). These results and the suppression of the neurohormone response by both propranolol and dopamine suggest that the membrane site of action for these two amines is similar if not identical. Catecholamines generally stimulate the production of cyclic AMP in cellular processes, but some decreases in this nucleotide have been reported to result from exposure to a catecholamine (Weiss, 1970). Such an exception may explain our results.
The ability to exogenous cyclic AMP to mimic the physiological effect of a hormone was mentioned by SUTHERLAND (1971) as another essential fact in establishing cyclic AMP as a mediator in a specific physiological mechanism. Although it was occasionally possible to evoke a hormone-like response from the hindgut with the sodium salt of cyclic AMP (Fig. 5A), it never gave consistent results, and the dibutyl derivative of cyclic AMP proved less effective. Nevertheless, in the many experiments where cyclic AMP failed to give a hormone-like response, gradual increase in spontaneous activity was often observed. Such inconclusive results are not surprising considering the facts that (1) most cells are relatively impermeable to phosphorylated compounds and (2) cyclic AMP is particularly sensitive to hydrolysis.

Aminophylline had a noticeable effect on action potential generation in addition to its obvious influence on contractile events. In several experiments, both the frequency and amplitude of spontaneously occurring action potentials were increased 1 to 2 min after the addition of the drug \(10^{-4}\) g/ml. Fig. 5B illustrates the sequence of events in such an experiment. Immediately after the addition of aminophylline, spontaneous action potentials stopped but within 30 sec they had reappeared and at an increased rate (Fig. 5B). The initial drop in the amplitude of potentials showed a return to normal levels at 1.5 min. Four min after the application of the drug (Fig. 5B) the frequency of action potentials had increased 70 per cent over the normal rate and the amplitude of most potentials was 30 per cent higher and had larger prepotentials.

Aminophylline could also reinitiate action potentials at sites on the rectum that had become silent after prior treatment with propranolol (Fig. 5D). In the present circumstances, it is not possible to be certain about the site of action for either of these drugs. Nevertheless, our results can still fit the conceptual framework of the cyclic AMP system if one assumes that aminophylline inhibited the membrane phosphodiesterase responsible for the degradation of cyclic AMP and that propranolol inhibits the receptor site for adenyl cyclase. In these circumstances the accumulation of intramembrane cyclic AMP would presumably reactivate calcium transport across the membrane. This in turn would cause the reappearance of action potentials in spite of the inhibition of the receptor site of adenylic cyclase by propranolol.

Caffeine, like aminophylline, evoked a hormone-like response from the isolated hindgut. In fact, successive additions of the drug caused progressively larger contractile responses (Fig. 6A). However, 12 min after the hindgut muscles were depolarized by a solution containing 158 mM KCl and 2 mM CaCl₂, the same successive exposures to the drug evoked smaller and smaller contractile responses (Fig. 6A).

Although caffeine acts as a potent inhibitor of phosphodiesterases, the drug can also release calcium from binding sites in the sarcoplasmic reticulum; the result is muscular contraction (ISAACSON and SANDOW, 1967). The fact that caffeine activates depolarized visceral muscle (Fig. 6) indicates that the drug may have similar effects on the cockroach hindgut. More convincing evidence was obtained from an investigation of the interaction of calcium and caffeine. After hindgut preparations were exposed to calcium-free KCl solutions for 10 min, caffeine evoked only a small response (Fig. 6B); and the addition of 2 mM CaCl₂ to the muscle chamber several minutes later caused a gradual increase in tonus that eventually levelled off and became steady (Fig. 6B). At this time, caffeine \((2.5 \times 10^{-4}\) g/ml) was again added to the preparation; the result was a large phasic contraction (Fig. 6B). Subsequent additions of the same amount of caffeine in these circumstances again produced progressively smaller responses as noted above (Fig. 6A). Such results seem to indicate that the rapid recovery of the intracellular stores of calcium depends on active transport across the surface membrane. Nevertheless, a simple diffusion of calcium across the membrane may account for partial restoration of these intracellular stores. Indeed, the gradual rise in tonus of depolarized visceral muscle after the addition of 2 mM CaCl₂ (Fig. 6B) suggests a process of diffusion. Moreover,
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Fig. 5. Mechanical and electrical responses of the isolated hindgut to cyclic AMP, aminophylline, and propranolol. (A) Contractile response of the hindgut to exogenous cyclic AMP (10^{-6} g/ml) (arrow). (B) Spontaneous action potentials recorded from the circular muscles of the rectum with a suction electrode. The preparation was suspended in a 400 μl chamber. (B) Same recording site 40 sec after the addition of aminophylline (10^{-4} g/ml). (D) Same recording site 4 min after treatment. (C) Action potentials recorded from the circular muscles of another untreated preparation. (C) Gradual disappearance of potentials at the same recording site 1.5 min after the addition of propranolol (10^{-4} g/ml). (D) Silent recording site on circular muscles of another rectum 12 min after treatment with propranolol (10^{-4} g/ml). (D) Initiation of action potentials at the same site 1 min after the addition of aminophylline (10^{-4} g/ml). Time mark for (A), (C-1,-4), and (D-1,-4), 20 sec; (B,-4, 2 sec; vertical displacement for (A), 2 mm. Voltage calibration in (B) is the same for all other records, 0.2 mV.

the ten-fold increase in the contractile response of such muscles to caffeine in the presence of extracellular calcium (Fig. 6B4) indicates an intracellular sequestering of calcium. Attempts to obtain a tonic response from depolarized muscle with caffeine as reported for insect skeletal muscle by HUDDART (1968) were unsuccessful. Even at a concentration of 8 mM the contractions remained phasic.

**DISCUSSION**

The dependence of muscular contraction on calcium has been known for many years (RINGER, 1883) but only recently have the details of the excitation-contraction sequence been defined (SANDOW, 1965). Calcium performs a central role in this process in vertebrate muscle and the findings of AIDLEY (1965) and HUDDART (1968) indicate that the same is true for insect skeletal muscle. Indeed, the view that calcium acts as an intracellular transmitter for excitation-contraction coupling in insect visceral muscle is supported by the following observations on hindgut preparations depolarized in high potassium solutions: (1) Calcium evoked contractures from such preparations. (2) The response of such hindguts to caffeine was calcium dependent. (3) The rapid sequential addition of caffeine evoked progressively smaller contractions indicating a depletion of calcium from its intracellular storage sites. Moreover, the presence or absence of calcium in the external medium did not alter this response. Thus caffeine was acting directly on such intracellular constituents as the sarcoplasmic reticulum. (4) The contractile response of depolarized visceral muscle to calcium and caffeine were qualitatively different. The response to caffeine was rapid and phasic and could be superimposed on the effect of calcium (Fig. 6B4) suggesting that the two agents have different sites of action. The slow tonic response of depolarized visceral muscle to the addition of calcium suggests a change in surface membrane permeability but the rapid response to caffeine implies a largely intracellular action. Finally (5), the calcium dependence of hindgut potassium contractures (COOK and HOLMAN, 1975) emphasizes the importance of this
Fig. 6. The action of caffeine on hindgut contractile activity and its dependence on calcium. (A1) Normal spontaneous activity. (A2) Three successive additions of caffeine (2.5 × 10⁻⁴ g/ml, arrows) evoked progressively larger responses. (A3) Potassium contracture initiated by exposure to 158 mM KCl solution containing normal levels of calcium. (A4) Three additions of caffeine (2.5 × 10⁻⁴ g/ml, arrows) in quick succession caused a progressive reduction in the phasic responses. (B1) Potassium contracture occurring in another hindgut after exposure to a 162 mM KCl solution without calcium. (B2) Hindgut response to caffeine (2.5 × 10⁻⁴ g/ml, arrow) 10 min after the addition of isotonic potassium solution. (B3) Tonic response caused by the addition of 2 mM CaCl₂ (arrow) to the muscle chamber. (B4) An augmented phasic contracture superimposed on the tonic response to calcium after the addition of caffeine (2.5 × 10⁻⁴ g/ml, arrow). Horizontal time mark, 20 sec; vertical displacement, 2 mm.

In discussing calcium and cellular communication, RASMUSSEN (1970) cited evidence supporting the idea that the cell membrane can exist in a resting calcium-associated state or in an active calcium dissociated state and that the transition between states is signalled by the generation of an action potential. Moreover, he believed that the magnitude of this potential change depends on the rate of calcium dissociation from its binding sites on the membrane. This hypothesis seems to explain the effects that calcium has on both the electrical and mechanical events accompanying visceral muscle contraction in the cockroach hindgut. Once preparations of the hindgut were placed in a calcium-free medium, the amplitude of action potentials was markedly reduced; and in the presence of 2 mM manganese ion, action potentials were completely abolished (COOK and HOLMAN, 1973). Such results clearly implicate calcium as a current-carrying ion in action potential electogenesis.

Although hindgut contractile activity often persisted in a calcium-free medium for as long as 1 hr, the addition of the chelating agent EGTA (0.5 mM) brought all activity to an abrupt end. Nevertheless, the addition of caffeine could still release sufficient intracellular calcium to cause a substantial mechanical response (COOK and HOLMAN, 1975). These results seemed to support the assumption that calcium exists in a loosely bound form on the surface membrane of muscle fibres. Such an assumption is not without precedent. HAGIWARA and TAKAHASHI (1967), employing the same supposition, have recently shown that the overshoot of calcium spikes in barnacle muscle fibre membranes was determined by the surface density of calcium ions while the threshold membrane potential for spike initiation depended on the total density of divalent cations.

Fig. 7 shows how the surface membrane might interact with calcium at the molecular level. In the resting state, calcium ions in the external medium (stippled circle) are in equilibrium with ions loosely bound to the globular proteins of the membrane. Once the transition to the active state begins, the calcium is dissociated from this loose binding site and moves inward toward the intra-
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Fig. 7. A schematic three-dimensional view of the surface membrane of a visceral muscle fibre illustrating how such a lipid-globular protein mosaic might mediate the interactions of calcium, cyclic AMP, and the neurohormone during the active transport of calcium.

cellular spaces. As the membrane continues in the active state, additional calcium from the external medium is carried inward. Perhaps this continued inward flux of calcium is enhanced by the opening of additional ion channels through the agency of the neurohormone. In any case, after the intracellular calcium reaches a certain level in the muscle fibres, the calcium-dissociated phase ends and the surface membranes return to the resting state.

Calcium dissociation of the surface membrane seems to be closely associated with neurohormone action in the cockroach hindgut (Cook and Holman, 1975) and the results of the present study suggest a possible mechanism of interaction. The functional sequence for this proposed interaction (shown in Fig. 7) can be summarized as follows: (1) The HSN activates adenyl cyclase by combining with a receptor site on the membrane surface. As adenyl cyclase utilizes ATP, calcium is released from its bound sites on the membrane, and cyclic AMP is formed. (2) The increased levels of cyclic AMP within the membrane accelerate the inward flow of calcium by activating a protein kinase system. At this point, a maximum membrane depolarization is attained. (3) The inward flow of calcium is stopped by a reduction of the intramembrane pool of cyclic AMP. Thus the membrane returns to the resting state by (a) activation of a specific phosphodiesterase and/or by (b) inhibition of adenyl cyclase from excessive intracellular calcium.

Since ATP is a good chelator of calcium, it may serve as a binding site for this ion on the surface membrane in addition to the globular proteins. Such an arrangement might allow two channels for calcium dissociation to exist in close proximity within the membrane (1) via adenyl cyclase, and (2) via a specific ATPase. Perhaps with normal contractile activity, only a single channel for calcium transport is open (i.e. a specific ATPase) but once the neurohormone is present, both channels are available. Such a sequence might explain why the HSN will occasionally cause a sudden increase in the amplitude of spontaneously occurring action potentials at a recording site (Cook and Holman, 1975).

Because such calcium-dependent events as action potential generation, spontaneous hindgut motility, and neurohormone action were either suppressed or abolished by propranolol and dopamine, one might reason that these amines inhibit adenyl cyclase and that aminophylline inhibits a specific membrane phosphodiesterase. However, the exact site of action for these drugs is uncertain, and calcium transport could be altered by some other mechanism. Nevertheless, our hypothesis concerning the involvement of cyclic AMP in membrane calcium dissociation has been further strengthened by the recent demonstration of an adenyl cyclase in homogenates of the cockroach hindgut (Nelson and Marks, 1975). Moreover, the neurohormone can reverse the calcium inhibition in the basal rate of this enzyme. But this adenyl cyclase was found in the supernatant fraction of homogenates and not in the particulate fraction where it is generally found. Thus any direct association of the enzyme with the surface membrane of muscle fibres is uncertain. Also, verification of our proposed mechanism must await a quantitative determination of cyclic AMP during calcium dissociation.

*Note added in proof*—Recent experiments with intracellular electrodes have shown that the surface membranes of rectal muscles were depolarized by the neurohormone. When 5 activity units of HSN were added to the perfusion chamber a depolarization of 10 mV was occasionally recorded prior to muscular contraction.

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