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MODE OF STIMULATION BY ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE OF THE SODIUM EFFLUX IN BARNACLE MUSCLE FIBRES

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SUMMARY

1. Giant fibres of the barnacle *Balanus nubilus* have been used as a preparation for studying the mode of action of cAMP on sodium transport.

2. It is shown that a concentration of cAMP as low as 10^{-6} M, when micro-injected, causes a sharp rise in the radio-Na efflux. Ouabain fails to reverse the cAMP effect.

3. The magnitude of the response of the Na efflux to cAMP is markedly reduced by pre-injecting 100 or 500 mM-EGTA solutions or by omitting Ca^{2+} from the bathing medium. Both together fail to bring about a greater reduction in the response.

4. The response to cAMP is greatly reduced by pre-injecting the protein inhibitor of Walsh and practically abolished by pre-injecting 500 mM-EGTA and soaking in Ca-free artificial sea water, ASW.

5. The Ca^{2+}-independent component of the Na efflux which is also stimulated by cAMP is shown to involve Na for H exchange. The magnitude of this exchange is governed by external pH.

6. The Na efflux into Ca^{2+}-free, Li^{+}-ASW is shown to be markedly stimulated by injecting cAMP, an effect which is enhanced by reducing external pH.

7. The Na efflux at 0° C is stimulated by injecting cAMP. This is shown to be related to activation of the protein kinase by cAMP and to depend on the presence of external Ca^{2+}.

8. (i) Ethacrynic acid when injected reduces the ouabain-insensitive Na efflux into HEPES–Ca^{2+}-free ASW at pH 6·3. These same fibres show a marked response to cAMP.

(ii) The ouabain-insensitive Na efflux into HCO_3^-, Ca^{2+}-free ASW from fibres pre-treated with ethacrynic acid fails to respond to external acidification. This is interpreted as indicating that ethacrynic acid inactivates

the CO_2-sensitive adenyl cyclase system. These same fibres when injected with cAMP show a marked response.

(iii) Stimulation of the ouabain-insensitive Na efflux into HCO_3^-, Ca^{2+}-free ASW by external acidification is reversed by injecting ethacrynic acid. These fibres when injected with cAMP show a reduced response.

9. It is concluded that: (i) stimulation of the Na efflux by injected cAMP is mainly due to activation of cAMP-dependent protein kinase;

(ii) the underlying exchange mechanism consists of Na:Ca and Na:H exchange. Interaction of Ca^{2+} with a phosphorylated membrane, thereby modifying permeability remains as a real possibility;

(iii) the site of action of CO_2 and ethacrynic acid is the adenyl cyclase system.

10. The implications of activation of the adenyl cyclase system by CO_2 and Na:H exchange are briefly touched upon.

INTRODUCTION

In an earlier paper it was shown that Na efflux in barnacle muscle fibres is stimulated by the injection of adenosine 3′:5′-cyclic monophosphate (cAMP), and that the observed response is enhanced by prior application of ouabain (Bittar, Hift, Huddart & Tong, 1974*b*). This paper raised two basic questions: firstly, is the stimulatory response wholly or partly the result of a fall in myoplasmic pCa, and is its magnitude dependent on external Ca^{2+}, Na^+ or H^+ ions. Secondly, is the stimulatory response partly or wholly attributable to activation of a cAMP-dependent protein kinase, and hence to increased phosphorylation of the fibre membrane? The following communication represents an attempt to answer these questions, and to find out whether the ouabain-insensitive Na efflux at 0° C is similarly stimulated by injecting cAMP.

A preliminary account of some of this work has already appeared (Bittar, Chambers & Schultz, 1974*a*).

METHODS

Materials. Specimens of *Balanus nubilus* and *Balanus aquila* were obtained from Cleve Vandersluys, Friday Harbor, Washington, and maintained in a 150-gallon Instant Ocean aquarium containing artificial sea water, ASW. The temperature of the aquarium water was kept in the region of 12° C.

Dissection and cannulation. Single fibres were dissected from the rostral and lateral depressor muscle bundles and cannulated in the same way as *Maia* fibres (Caldwell & Walster, 1963). The fibres used were 3–5 cm in length and 1–2 mm in width. A 50–80 mg weight was attached to the tendon, thereby keeping the fibre in a vertical position when suspended in ASW.

The micro-injector. The micro-injector used was similar in design to that devised by Hodgkin & Keynes (1956) as modified by Caldwell & Walster (1963). The volume of fluid discharged per centimetre excursion of the micromanipulator was about

0·1 μl. Since the fibres used in this work had an intrafibre fluid volume of 25–50 μl., the dilution factor owing to the sarcoplasm may be taken as 250–500.

Solutions. The experiments were carried out with ASW or with Li^+-ASW having the following composition (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10; and pH 7·8; or LiCl, 475; $CaCl_2$, 10; $MgCl_2$, 10; $KHCO_3$, 10; and pH 7·8. Solutions with varying concentrations of Ca^{2+} were prepared by raising or reducing NaCl in osmotically equivalent amounts. In those experiments where the buffer *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) was used instead of HCO_3^- the pH of the solution was adjusted as necessary by adding HCl or NaOH. The pH of the solutions was measured with a Beckman pH meter and standard glass electrode.

Agents. Adenosine 3′:5′-monophosphoric acid (cAMP), ouabain, HEPES, ethylene glycol-bis-(β-amino ethyl ether)*N*,*N'*-tetra-acetic acid (EGTA) and iodoacetamide were obtained from Sigma Chemical Company, St Louis, Missouri. Ethacrynic acid was obtained from Merck, Sharp and Dohm, West Point, Pennsylvania. The protein inhibitor was obtained as a gift from Dr Donal Walsh of the Department of Biological Chemistry, University of California at Davis.

Radioactivity measurements. ^{22}NaCl in aqueous solution was obtained from Amersham-Searle Corporation, Arlington Heights, Illinois or New England Nuclear, Boston, Massachusetts. The solution was dried, and then re-dissolved in distilled water so that volumes of *ca.* 0·1 μl. gave at least 75,000 c.p.m., an activity sufficiently high for work of this type. The procedures used for collecting the effluent and for counting its activity as well as the activity remaining in the fibre at the end of each experiment were those described by Bittar (1966) and Bittar, Caldwell & Lowe (1967). A well-type scintillation counter fitted with a solid phosphor and connected to a scaler, was used for counting. Except where stated otherwise, all experiments were performed at room temperature, 22–25° C. In those experiments done at *ca.* 0° C, the fibres used were cooled down in a special microbeaker surrounded by ice.

The results given in this paper are means ± S.E. of mean and significance levels were computed by using Student's *t* test. Estimates of the size of the observed effects on Na efflux were computed on the basis of the rate constant plot, as mentioned previously (Bittar *et al.* 1974*b*).

RESULTS

Stimulation by cAMP of Na efflux

Earlier work showed that the Na efflux is stimulated by micro-injection of 5×10^{-2} M-cAMP. Since the concentration of cAMP in skeletal muscle is in the region of 10^{-7} M (Robinson, Butcher & Sutherland, 1971), it seemed important to know whether a much smaller concentration of cAMP would stimulate the Na efflux. Fig. 1 shows that micro-injection of 10^{-6} M-cAMP caused a prompt rise in the Na efflux. The magnitude of this stimulatory effect averaged 55·57 ± 30·08 % (n = 6). Taking into account dilution of the injected cAMP by the myoplasm, one can safely deduce that the observed stimulation was caused by cAMP when its myoplasmic concentration was 10^{-7} to 10^{-8} M. This estimate is not very different from the reported K_m value for cAMP in skeletal muscle (Beavo, Bechtel & Krebs, 1974).

In order to obtain a clearer view of this sensitivity to cAMP, experiments

were designed in which the effect of graded concentrations of cAMP was investigated by using unpoisoned and ouabain-poisoned fibres. The results obtained are summarized in Fig. 2. These experiments, though done with fibres from the same barnacle specimen, show that 10^{-5} to 10^{-4} M-cAMP failed to produce an appreciable rise in the Na efflux. The reason for this is not clear, particularly since repetition of this type of experiment led to identical results. Fig. 2 also indicates that ouabain-poisoned fibres are not always more sensitive to cAMP than unpoisoned fibres. It will be remembered that this was previously not the case when 5×10^{-2} M-cAMP or GMP was injected (Bittar *et al.* 1974*b*).

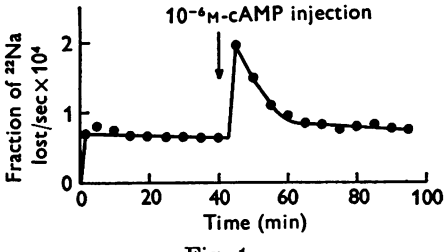

Fig. 1

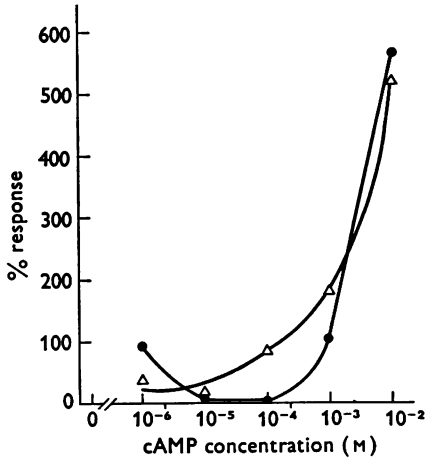

Fig. 2

Fig. 1. Effect on the Na efflux of internal application of 10^{-4} M-cAMP.

Fig. 2. Dose–response curve for the effect of graded concentrations of cAMP on the Na efflux in unpoisoned, ●, and 10^{-4} M ouabain-poisoned, △, fibres. Each point represents the mean of data from six fibres.

The finding that cAMP stimulates the ouabain-insensitive Na efflux raised the possibility that membrane Na-pump Na^+-K^+ ATPase activity can be reduced by cAMP. Hence experiments were done in which 10^{-4} M ouabain was applied externally following stimulation of the Na efflux by micro-injection of 3×10^{-2} M-cAMP. The results show a complete lack of effect with ouabain ($n = 4$), indicating that a reduction in the Na^+-K^+ ATPase takes place. This not only confirmed the see-saw theory (Bittar & Tong, 1975), but also provided justification for the method we have adopted in estimating the size of the stimulation of the ouabain-insensitive Na efflux caused by cAMP, namely to take into account the rate constant for Na efflux observed before the application of cAMP rather than that immediately before the application of ouabain.

Lack of effect of 5′-AMP

An essential preliminary was to rule out the possibility that stimulation of the Na efflux by cAMP was actually caused by 5′-AMP following degradation of cAMP by the phosphodiesterase system. Three fibres injected with 5×10^{-2} M-5′-AMP failed to display a response, but when subsequently injected with 5×10^{-2} M-cAMP, they displayed an average stimulation of $208{\cdot}2 \pm 66{\cdot}8\,\%$.

Stimulation by cAMP of Na efflux in EGTA-pre-treated fibres

It is well established that micro-injection of $CaCl_2$ into barnacle muscle fibres causes stimulation of the Na efflux (Bittar, Chen, Danielson, Hartmann & Tong, 1972) and that the ouabain-insensitive Na efflux is the component stimulated by a fall in myoplasmic pCa (Danielson, Bittar, Chen & Tong, 1971). It is also well established that the stimulation of the Na efflux caused by external caffeine depends on the presence of external Ca^{2+}, whereas the stimulation of the ouabain-insensitive Na efflux caused by external caffeine depends only in part on external Ca^{2+}. It would thus appear that under these conditions myoplasmic pCa is a factor governing the response of the Na efflux.

Since a myoplasmic pCa of 6·0 is enough to produce half-maximal activation of the phosphorylase kinase (Heilmeyer, Meyer, Haschke & Fischer, 1970), and since cAMP is able to mobilize mitochondrial Ca^{2+} (Borle, 1974), it became of special interest to control pCa by means of EGTA and see whether cAMP is still able to stimulate the Na efflux. Experiments were therefore done with EGTA by micro-injecting 1, 100 and 500 mM solutions of EGTA (pH 7·0) almost throughout the entire length of the fibres tested. This was necessary because experience had disclosed that longitudinal diffusion of EGTA in these fibres is rather slow. The fibres responded to internal application of 3×10^{-2} M-cAMP by showing a rise in the Na efflux of the order of $187{\cdot}8 \pm 27{\cdot}8\,\%$ ($n = 11$), in 1 mM-EGTA (controls: $255{\cdot}7 \pm 44{\cdot}2\,\%$, $n = 11$, $P < 0{\cdot}3$); $118{\cdot}1 \pm 22{\cdot}9\,\%$, $n = 11$, in 100 mM-EGTA (controls: $302{\cdot}4 \pm 44{\cdot}6\,\%$, $n = 9$, $P < 0{\cdot}01$); and $105{\cdot}0 \pm 28{\cdot}4$, $n = 6$ in 500 mM-EGTA (controls: $212{\cdot}6 \pm 24{\cdot}1$, $n = 6$, $P < 0{\cdot}05$). A typical experiment of micro-injection of 500 mM-EGTA before 3×10^{-2} M-cAMP is shown in Fig. 3. It is clear therefore that there is a close connexion between the magnitude of the response of the Na efflux to cAMP and myoplasmic pCa.

Dependence of the cAMP effect on external Ca^{2+}

Measurements of the Na efflux were first made into Ca^{2+}-free ASW before and after the micro-injection of 3×10^{-2} M-cAMP. Calcium was then

restored to the bathing medium, and the fibres re-injected with 3×10^{-2} M-cAMP. Such an experiment is shown in Fig. 4*A*, where it can be seen that both Ca^{2+} restoration and re-injection with cAMP led to further stimulation of the Na efflux. Estimates indicate that the first injection of cAMP

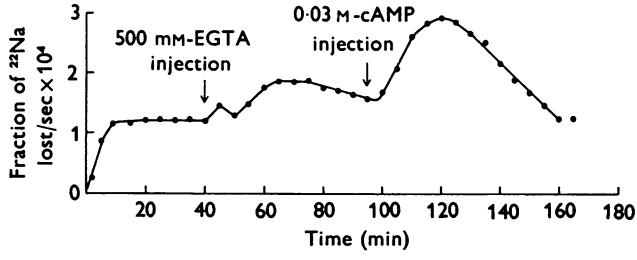

Fig. 3. Effect on the Na efflux of internal application of 3×10^{-2} M-cAMP following 500 mM-EGTA.

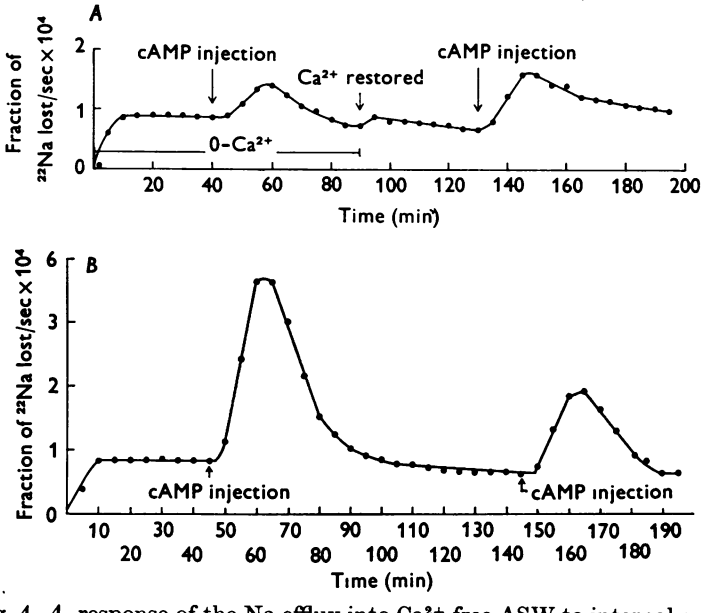

Fig. 4. *A*, response of the Na efflux into Ca^{2+}-free ASW to internal application of 3×10^{-2} M-cAMP, restoration of external Ca^{2+} and re-injection of cAMP. *B*, control experiment in which 3×10^{-2} M-cAMP was injected twice in succession.

caused an average stimulation of $128{\cdot}4 \pm 8{\cdot}6\,\%$ ($n = 15$). This is to be compared with a value of $348{\cdot}0 \pm 62{\cdot}3\,\%$ ($n = 8$) obtained with controls ($P < 0{\cdot}001$) (Fig. 4*B*). Calcium restoration caused a rise in the Na efflux in the order of $71{\cdot}6 \pm 22{\cdot}0\,\%$ ($n = 6$), while re-injection with cAMP caused

a $50{\cdot}5 \pm 12{\cdot}3\%$ rise ($n = 10$). Re-injection of control fibres with cAMP caused $91{\cdot}4 \pm 22{\cdot}6\%$ ($n = 7$) stimulation, a value which is not very different from that obtained with the test fibres ($P < 0{\cdot}2$).

In the next group of experiments, fibres bathed in Ca^{2+}-free ASW were injected with 500 mM-EGTA followed by 3×10^{-2} M-cAMP. The results show that cAMP caused an average stimulation of $120{\cdot}0 \pm 8{\cdot}0\%$ ($n = 5$). This is to be compared with a value of $135{\cdot}0 \pm 8{\cdot}3\%$ ($n = 4$) obtained with controls where EGTA was not injected ($P < 0{\cdot}5$). It is thus all too obvious that the effect of cAMP on the Na efflux is dependent on external Ca^{2+} as found in the preceding experiments. Failure to see a greater reduction in the size of the response in EGTA-treated fibres was not an unexpected situation. For it will be recalled that fibres bathed in Ca^{2+}-free ASW show rupture of mitochondria and mitoplast formation (Bittar *et al.* 1974*b*). The loss of mitochondrial Ca^{2+} before micro-injection of cAMP, however, provides a tentative explanation which should not be pressed too far.

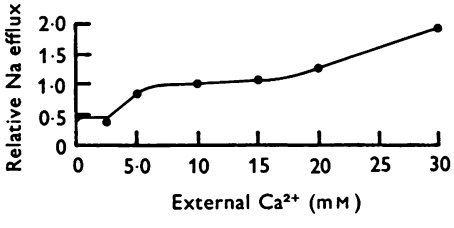

Fig. 5. Behaviour of the Na efflux towards internal application of 3×10^{-2} M-cAMP with variation of the external Ca^{2+} concentration. Ordinate is the maximal rate constant for the Na efflux observed following internal application of 3×10^{-2} M-cAMP relative to that measured in 10 mM-Ca^{2+} ASW. Each point represents the mean of data obtained from the following number of experiments, n: Ca^{2+}-free ASW, 3; 2·5 mM-Ca^{2+} ASW, 2; 5·0 mM-Ca^{2+} ASW, 5; 7·5 mM-Ca^{2+} ASW, 2; 10 mM-Ca^{2+} ASW, 8; 15·0 mM-Ca^{2+} ASW, 6; 20·0 mM-Ca^{2+} ASW, 6; and 30·0 mM-Ca^{2+} ASW, 4.

To gain a better understanding of the Ca^{2+}-dependent action of cAMP, experiments were done with varying concentrations of external Ca^{2+}. The response of the Na efflux to micro-injection of 3×10^{-2} M-cAMP, as shown in Fig. 5, is sigmoidal. The latter part of the curve suggests the existence of a second threshold of activation of Na:Ca exchange in the presence of a high external Ca^{2+} concentration. The shape of the curve resembles that obtained with caffeine (Bittar *et al.* 1974*b*).

Response to cAMP of fibres pre-injected with the protein inhibitor of Walsh

Evidence has come to light that the activity of the catalytic subunit of the c-AMP-dependent protein kinase present in skeletal muscle is blocked by a heat-stable protein whose molecular weight is 26 000 daltons (Ashby & Walsh, 1972, 1973). This protein inhibitor occurs in a wide variety of tissues in a concentration sufficient to almost completely inactivate the free catalytic subunit (Beavo *et al.* 1974). The following two reactions describe the dissociation of the protein kinase by cAMP into two subunits and the inactivation of the free catalytic subunit by the inhibitor.

$$\underset{\substack{\text{Inactive}\\\text{protein}\\\text{kinase}}}{RC} + \text{cAMP} \rightleftharpoons \underset{\substack{\text{Catalytic}\\\text{subunit}}}{C} + R\text{cAMP} \quad (1)$$

$$C + \underset{\substack{\text{Protein}\\\text{inhibitor}}}{I} \rightleftharpoons CI. \quad (2)$$

This information based on biochemical studies carried out with cell-free systems raised the question whether micro-injection of the protein inhibitor before cAMP would lead to a reduced response. The protein inhibitor (from rabbit skeletal muscle) supplied by Dr Donal Walsh of the University of California at Davis, was in a solvent containing 0·3 M-K_2HPO_4 and 0·001 M-EDTA. Control experiments were therefore done by micro-injecting solvent before cAMP. The solvent when injected led to a brief and slight rise in the Na efflux, presumably caused by the presence of EDTA. This is thought to be so because injection of 1 mM-EDTA or EGTA is known to modify the Na efflux in a similar way (E. E. Bittar and R. Schultz, unpublished). The results obtained by micro-injection of 3×10^{-2} M-cAMP following the solvent show an average stimulation of $378{\cdot}9 \pm 40{\cdot}6\,\%$ ($n = 13$). In sharp contrast, fibres injected with the inhibitor, followed by 3×10^{-2} M-cAMP show an average stimulation of $80{\cdot}5 \pm 13{\cdot}4\,\%$ ($n = 16$), $P < 0{\cdot}001$. These results are illustrated in Fig. 6*A* and *B*. It is thus evident that the theory advanced by Ashby & Walsh (1972, 1973) holds for barnacle fibres and that the bulk of the stimulation caused by cAMP can be accounted for by a phosphorylation, probably of the fibre membrane.

To answer the question whether phosphorylation of the membrane is associated with increased Na:Ca exchange, experiments were carried out in which 500 mM-EGTA-treated fibres were bathed in Ca^{2+}-free ASW, micro-injected with the inhibitor and then with 3×10^{-2} M-cAMP. As shown in Fig. 7, the fibre was barely responsive to cAMP when Na:Ca exchange was suppressed. Estimates of the magnitude of this response

indicate a value of $57{\cdot}7 \pm 19{\cdot}1\,\%$ ($n = 8$). This is to be compared with values of $120{\cdot}0 \pm 8{\cdot}0\,\%$ ($n = 5$) and $85{\cdot}9 \pm 9{\cdot}56\,\%$ ($n = 4$) obtained with 500 mM-EGTA pre-treated fibres, bathed in Ca^{2+}-free ASW, following injection of 3×10^{-2} M-cAMP. The difference is not significant. Such results can only mean that there is a close relationship between membrane phosphorylation and activation of Na:Ca exchange.

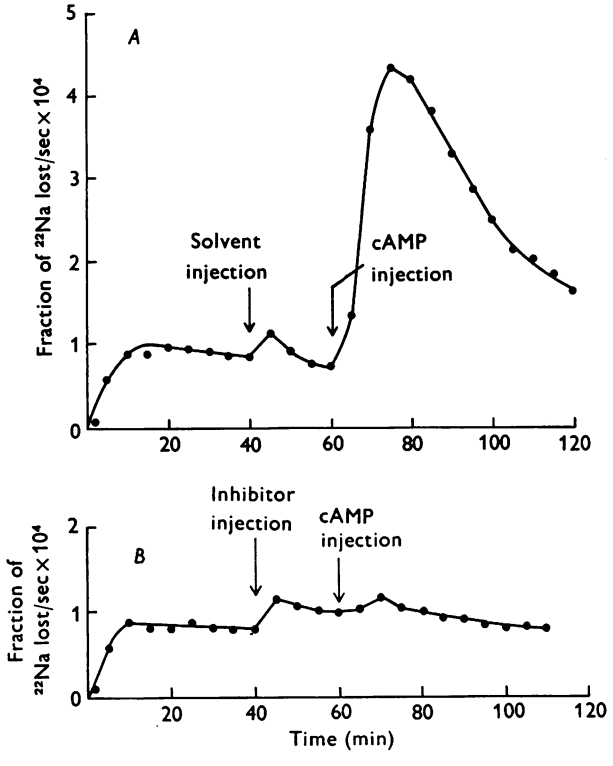

Fig. 6. *A*, effects on the Na efflux of internal application of solvent (H_2O containing 300 mM-K_2HPO_4, 1 mM-EDTA at pH 7·0) followed by 3×10^{-2} M-cAMP. *B*, effects on the Na efflux of internal application of the protein inhibitor of Walsh (in solvent) followed by 3×10^{-2} M-cAMP. In both types of experiments, *A* and *B*, solvent and inhibitor were injected throughout the length of the fibre.

Dependence of the response to cAMP on external pH

The finding that a fraction of the Na efflux stimulated by cAMP is independent of external Ca^{2+} led to the question whether phosphorylation of the membrane is also attended by increased Na:H exchange. Experiments were therefore designed in which the fibres used were bathed in ASW containing HEPES as buffer rather than bicarbonate. The results

obtained indicate that micro-injection of 3×10^{-2} M-cAMP in the presence of an external pH of 6·3 resulted in an average stimulation of $164{\cdot}8 \pm 12{\cdot}8\%$ ($n = 4$). Controls, by contrast, showed a stimulation of $44{\cdot}0 \pm 16{\cdot}4\%$ ($n = 4$) in the presence of an external pH of 7·8 ($P < 0{\cdot}01$). The poor response of control fibres to cAMP could have been related to replacement of bicarbonate with HEPES. That this is the case, and that CO_2 and not HCO_3^- is the species augmenting the response to cAMP are problems which will be dealt with later. Since the present results gave support to

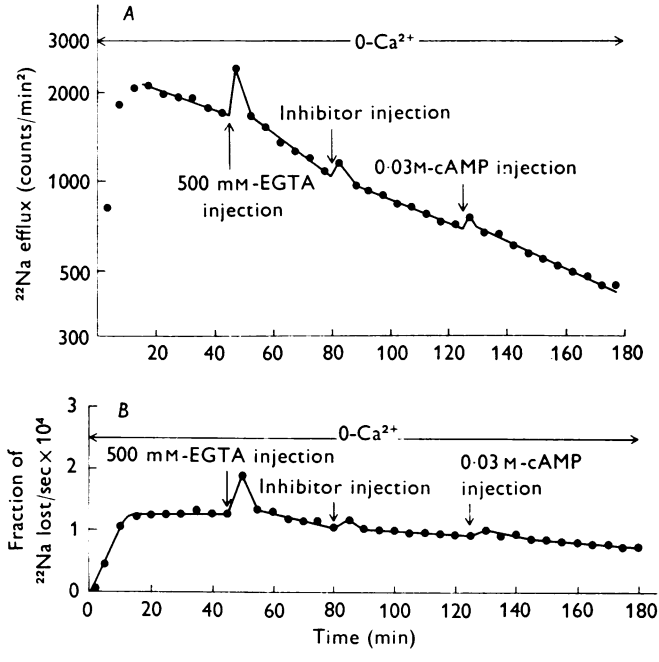

Fig. 7. Effects on the Na efflux into Ca^{2+}-free ASW of internal application of 500 mM-EGTA, followed by inhibitor and 3×10^{-2} M-cAMP. *A*, efflux plot; *B*, rate constant plot.

the view that the magnitude of the response of the Na efflux is partly governed by external pH, more experiments of this type were done along two different lines. In the first series, the Na efflux into 3 mM-HEPES–Ca^{2+}-free ASW at pH 7·8 and 6·3 was measured before and after micro-injection of 3×10^{-2} M-cAMP. The results show that only at the lower pH was there a stimulation and this averaged $155{\cdot}3 \pm 4{\cdot}05\%$ ($n = 2$). These results are illustrated in Fig. 8*A*. Keeping in mind then that when the external pH is 7·8, the response of the Na efflux to cAMP depends on the presence of external Ca^{2+}, and that at external pH 6·3, it is independent of external Ca^{2+}, the question asked was whether another buffer such as histidine

would lead to similar results. Hence parallel experiments with 3 mM histidine-Ca^{2+}-free ASW at pH 7·8 and 6·3 were done. The results were identical to those obtained with HEPES-ASW. To see how dependent the response of the Na efflux to cAMP is on external pH, experiments were designed in which the external H^+ ion concentration was varied over a wide range, and in which Ca^{2+} in the bathing medium was not omitted. As shown in Fig. 8*B*, the external pH-dependency curve is characterized by a steep inflexion as an external pH of 6·0 is approached.

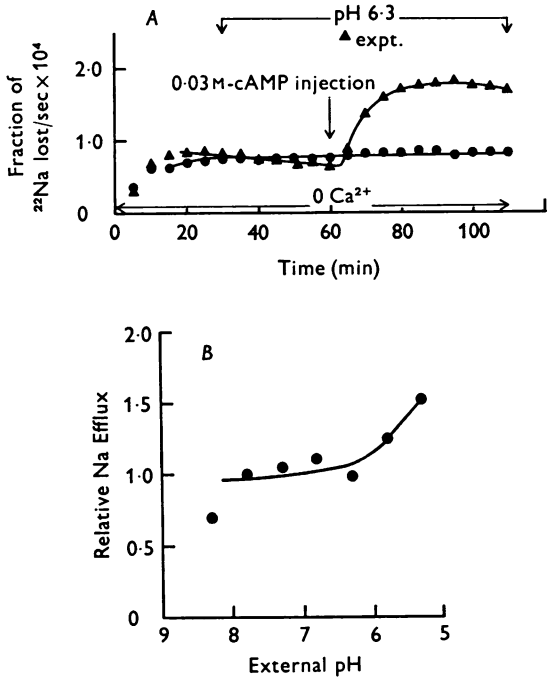

Fig. 8. *A*, ▲, effect on the Na efflux into 3 mM-HEPES–Ca^{2+}-free ASW at pH 6·3 of internal application of 3×10^{-2} M-cAMP; ●, lack of effect on the Na efflux into 3 mM-HEPES–Ca^{2+}-free ASW at pH 7·8 of internal application of 3×10^{-2} M-cAMP. *B*, behaviour of the Na efflux towards internal application of 3×10^{-2} M-cAMP with variation of external pH (using 3 mM-HEPES-ASW as bathing medium). Ordinate is the maximal rate constant for Na efflux relative to that measured in ASW at pH 7·8. Each point represents the mean of data from the following number of experiments, *n*: pH 8·3, 4; pH 7·8, 5; pH 7·3, 4; pH 6·8, 4; pH 6·3, 4; pH 5·8, 4; and pH 5·3, 3. In this particular experiment the fibres used were isolated from the same barnacle specimen.

Responses to cAMP of the efflux into Li⁺-ASW

It now became important to find out whether the response to cAMP at an external pH of about 6·0 depends on external Na^+. The results showed that complete replacement of external Na^+ by Li^+ in the presence of Ca^{2+} failed to stop the Na efflux from responding to the internal application of 3×10^{-2} M-cAMP. The average stimulation was in the order of $216{\cdot}6 \pm 33{\cdot}7\%$ ($n = 4$), which is to be compared with a value of $139{\cdot}0 \pm 33{\cdot}0\%$

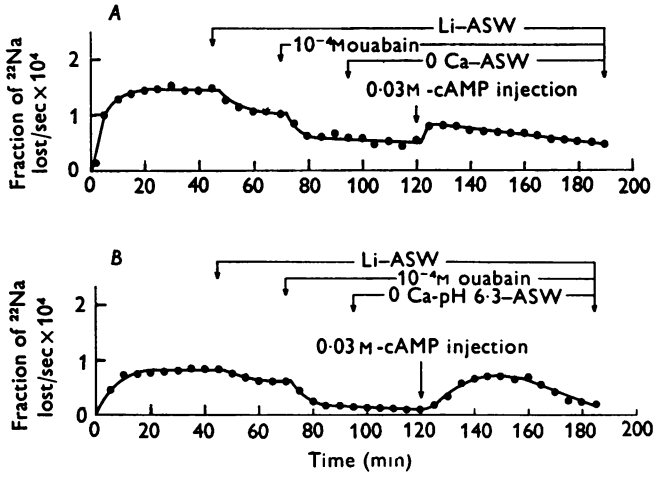

Fig. 9. *A*, effect on the ouabain-insensitive Na efflux into 3 mM-HEPES Ca^{2+}-free, Li^+-ASW at pH 7·8 of internal application of 3×10^{-2} M-cAMP. *B*, effect on the ouabain-insensitive Na efflux into 3 mM-HEPES–Ca^{2+}-free Li^+-ASW at pH 6·3 of internal application of 3×10^{-2} M-cAMP.

($n = 4$) obtained with controls ($P < 0{\cdot}1$). The question then asked was: how dependent on external Ca^{2+} is the ouabain-insensitive Na efflux into Li^+–ASW in its response to cAMP? Fig. 9*A* shows that internal application of 3×10^{-2} M-cAMP into a fibre pretreated with 10^{-4} M ouabain caused a rise in the Na efflux into Ca^{2+}-free-Li^+-ASW. The magnitude of the stimulation averaged $97{\cdot}0 \pm 17{\cdot}6\%$ ($n = 5$). It thus seems clear that the stimulation caused by cAMP is completely independent of external Na^+, and that Li^+, if anything, is inert.

Having established the fact that the ouabain-insensitive Na^+ efflux stimulated by cAMP is independent of external Na^+, and that a fraction of the stimulated efflux is independent of external Ca^{2+}, it seemed natural to enquire about the nature of this fraction and whether it consists of Na^+:H^+ exchange. Hence a similar series of experiments was done except that this time the bathing medium used contained HEPES as buffer and

not bicarbonate, and the external pH was adjusted to 6·3. As illustrated in Fig. 9*B*, internal application of 3×10^{-2} M-cAMP caused a marked stimulation of the Na efflux into Ca^{2+}-free–Li^{+}-ASW. The average effect was in the order of $274{\cdot}4 \pm 106{\cdot}7\,\%$ ($n = 6$), a result which lends support to the view that cAMP stimulates $Na^{+}:H^{+}$ exchange.

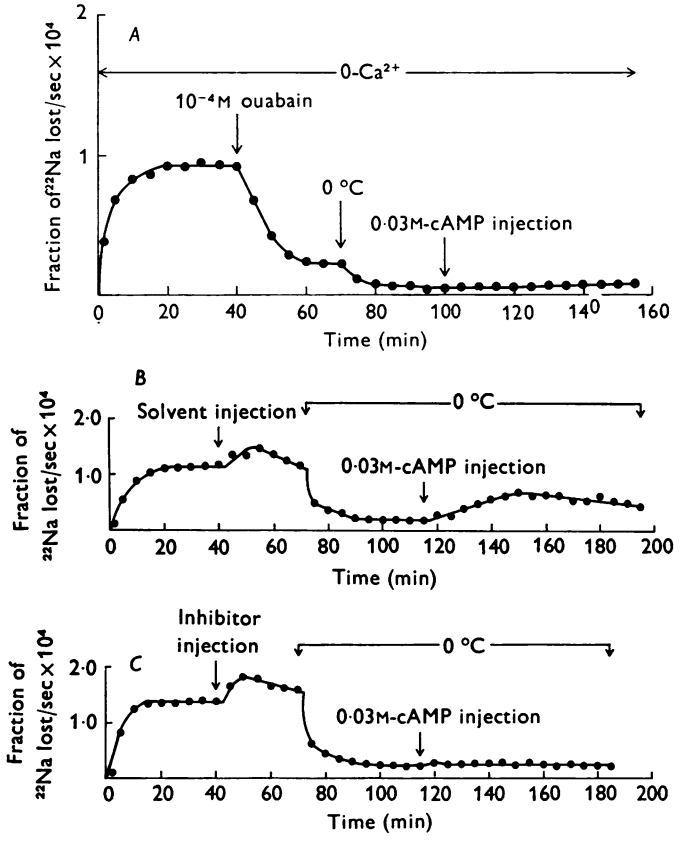

Fig. 10. *A*, absence of stimulation following internal application of 3×10^{-2} M-cAMP in a fibre bathed in Ca^{2+}-free ASW containing 10^{-4} M ouabain and cooled to 0° C. *B*, stimulation of the Na efflux by internal application of 3×10^{-2} M-cAMP in a fibre pre-treated with solvent and cooled to 0° C in 10 mM-Ca^{2+} ASW. *C*, absence of an effect following internal application of 3×10^{-2} M-cAMP in a fibre pre-treated with inhibitor and cooled to 0° C in 10 mM-Ca^{2+} ASW.

Experiments with cAMP at an environmental temperature of about 0° C

The dependence on external Ca^{2+} of the action of cAMP on Na efflux at 0° C was investigated both in unpoisoned and ouabain-poisoned fibres. It was found that internal application of 3×10^{-2} M-cAMP at about 0° C

(in contrast to 22–25° C) consistently failed to stimulate the Na efflux into Ca^{2+}-free ASW ($n = 5$ in each group of experiments). A typical experiment with ouabain is shown in Fig. 10*A*. This makes it possible to decide whether the wholly Ca^{2+}-dependent stimulatory response to cAMP is likely to be related to activation by cAMP of the cAMP-dependent protein kinase. To answer this question, four fibres were injected with the protein inhibitor and cooled to 0° C followed by injecting 3×10^{-2} M-cAMP. As shown in Fig. 10*C*, cAMP failed to stimulate the Na efflux into 10 mM-Ca^{2+}-ASW. By contrast, companion controls injected with solvent ($n = 2$) showed a response at 0° C (Fig. 10*B*). Thus there emerges a new aspect of cell control at 0° C which is of physiological interest. This is that it is likely that Na:Ca exchange which is stimulated by cAMP is dependent on a mechanism which involves phosphorylation of the membrane.

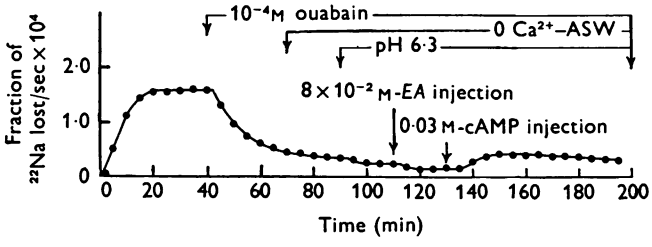

Fig. 11. Response of the Na efflux into 3 mM-HEPES-Ca^{2+}-free ASW to internal application of 3×10^{-2} M-cAMP following external application of 10^{-4} M ouabain, reduction in external pH from 7·8 to 6·3 and then internal application of 8×10^{-2} M ethacrynic acid, *EA*.

Experiments with ethacrynic acid

Ethacrynic acid was chosen because it acts not only as an inhibitor of glycolysis (Gordon & Hartog, 1969; Klahr, Yates & Bourgoignie, 1971) but also of the adenyl cyclase system (Ebel, 1974). Knowing from earlier work that external or internal application of ethacrynic acid inactivates the CO_2-sensitive Na efflux in barnacle fibres (Danielson, Bittar, Chen & Tong, 1972*a*, *b*), it seemed natural to wonder what would happen in the presence of a reduced external pH to the ouabain-insensitive Na efflux following the micro-injection of ethacrynic acid. To distinguish between an effect involving activation of the adenyl cyclase system by CO_2 and possibly an effect involving prior protonation of the bathing medium, experiments with HEPES as buffer and not bicarbonate, were done. The results obtained with 3 mM-HEPES–Ca^{2+}-free ASW at pH 6·3 show that the ouabain-insensitive Na efflux was reduced by internal application of 8×10^{-2} M ethacrynic acid. The average effect was of the order of $34{\cdot}68 \pm 5{\cdot}94$ % ($n = 4$). These fibres when injected with 3×10^{-2} M-cAMP exhibited a stimulation of the remaining efflux of the order of $235{\cdot}24 \pm 38{\cdot}04$ %, as

illustrated in Fig. 11. Controls in which pre-treatment with ethacrynic acid was omitted, showed stimulation in the order of $184{\cdot}12 \pm 46{\cdot}12\%$ ($n = 4$, $P > 0{\cdot}05$). It thus emerges that in the absence of a raised P_{CO_2} the stimulation of Na:H exchange as the result of phosphorylation of the fibre membrane is not augmented by a reaction involving an acceleration of glycolysis. The observed fall in the ouabain-insensitive Na efflux into Ca^{2+}-free ASW at pH 6·3 caused by ethacrynic acid is precisely what one would expect if the action of the agent is internal pH-dependent. Evidence in support of this argument comes from recent experiments based on the 2-^{14}C–DMO technique which show that the internal pH of barnacle fibres is 7·1, and that it falls whenever the pH of HCO_3-free ASW is reduced (E. E. Bittar, B. G. Danielson and W. Lin, unpublished).

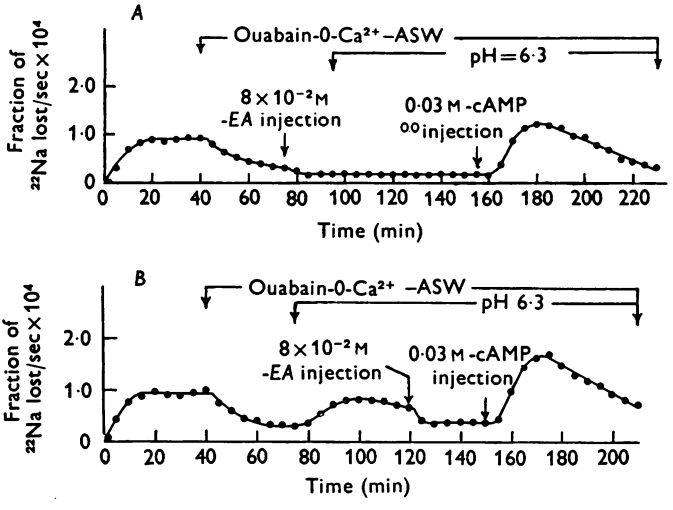

Fig. 12. *A*, effects on the Na efflux into Ca^{2+}-free ASW of external application of 10^{-4} M ouabain followed by internal application of 8×10^{-2} M ethacrynic acid, *EA*, reduction in external pH from 7·8 to 6·3, and then 3×10^{-2} M-cAMP. *B*, effects on the Na efflux into Ca^{2+}-free ASW of external application of 10^{-4} M ouabain followed by reducing the external pH from 7·8 to 6·3, internal application of 8×10^{-2} M ethacrynic acid, and then 3×10^{-2} M-cAMP.

In the next group of experiments, the Ca^{2+}-free ASW used contained HCO_3^- as buffer. As illustrated in Fig. 12*A*, micro-injection of 8×10^{-2} M ethacrynic acid before external acidification led to complete abolition of the response of the Na efflux to a raised P_{CO_2}. It can also be seen that subsequent micro-injection of 3×10^{-2} M-cAMP caused a large rise in the ouabain- and ethacrynic acid-insensitive Na efflux the magnitude of which averaged $457{\cdot}57 \pm 35{\cdot}79\%$ ($n = 11$). This type of experiment served as

the control. Hence it became possible to find out whether the adenyl cyclase system plays a role in the stimulatory response of the Na efflux following activation of the cyclic AMP-dependent protein kinase by cAMP. As shown in Fig. 12*B*, stimulation of the ouabain-insensitive Na efflux into Ca^{2+}-free ASW brought about by suddenly reducing the external pH from 7·8 to 6·3 was promptly reversed by micro-injecting 8×10^{-2} M ethacrynic acid. In this case, micro-injection of 3×10^{-2} M-cAMP led to a rise in the Na efflux averaging $279{\cdot}13 \pm 55{\cdot}99\%$ in magnitude ($n = 11$). This is a value appreciably less than that observed in the preceding control experiments ($P < 0{\cdot}02$). Two explanations of these results suggest themselves. One is that cAMP-dependent protein kinase is rate-limiting. This is quite reasonable, since the concentration of the native enzyme in skeletal muscle is about 10^{-7} M, i.e. the same as the concentration of cAMP (Beavo, Bechtel & Krebs, 1975). The other is that activation of the adenyl cyclase system by CO_2 reduces the myoplasmic ATPMg concentration sufficiently to reduce the rate of cAMP release from the *R*cAMP complex. Evidence assigning such a role to ATPMg derives from the recent work of Beavo *et al.* (1975). This argument, however, is weak for two reasons: in the first place, it overlooks the possible role of phosphagen in the re-phosphorylation of ADP; and secondly, it also overlooks the fact that ATPMg interferes with the binding of cAMP to protein kinase in skeletal muscle (Haddox, Newton, Hartle & Goldberg, 1972). It is, nonetheless, possible that the explanation is threefold: (i) the concentration of protein kinase is rate-limiting; (ii) ADP is re-phosphorylated by ArP, but (iii) the equilibrium reaction involving the activation and reconstitution of protein kinase remains poised towards *R*cAMP formation.

DISCUSSION

The picture which emerges is a straightforward one. In response to the micro-injection of cAMP, the Na efflux rises mainly because of activation of a cAMP-dependent protein kinase. The two key findings leading to this conclusion are: firstly, the protein inhibitor is able to abolish most of the stimulatory effect caused by cAMP; secondly, barnacle muscle fibres possess a cAMP-dependent protein kinase (D. Walsh, private communication). This kinase, according to Walsh, is half-maximally activated by 2×10^{-8} M-cAMP, a concentration not very different from that which is found to bring about stimulation of the Na efflux in the order of 55·0%. One is therefore justified in arguing that the myoplasmic cAMP concentration in barnacle fibres is considerably less than 10^{-7} M. This is not surprising for two reasons: the values cited in the literature represent total cellular content; and the myoplasmic cAMP concentration is determined not only

by its rate of formation from $(ATP\text{-}Mg)^{-2}$ by adenyl cyclase and its rate of degradation by phosphodiesterase (a system present in barnacle fibres: F. Siegel, private communication) but also by its degree of binding and rate of loss from the fibre. Interest in the possibility of measuring the myoplasmic cAMP has been aroused as the result of the availability of the protein inhibitor without EDTA. The inhibitor when injected causes a prompt fall in the Na efflux, an effect which is reversed by injecting graded concentrations of cAMP (E. E. Bittar and R. Schultz, unpublished). This is precisely what one would expect if activation by cAMP of the protein kinase is a mechanism responsible for maintaining the resting ouabain-insensitive Na efflux.

The view that stimulation of the Na: Ca exchange is the consequence of phosphorylation of the fibre membrane is strengthened by the results obtained at 0° C. The mechanism underlying stimulation of Na: Ca exchange at 0° C is connected with activation of the cAMP-dependent protein kinase. This follows because the stimulation caused by cAMP is completely blocked by the protein inhibitor. Another salient feature of the results obtained with unchilled and chilled fibres is that they fail to contract when injected with cAMP. It is possible to explain this by supposing that cAMP stimulates the sarcoplasmic reticulum Ca^{2+}-pump (see Entman, 1974) or the plasmalemma Ca^{2+}-pump. Evidence favouring the latter idea is provided by the work of Cheng & Chen (1975) showing that the stimulatory response of the ^{45}Ca efflux to cAMP in barnacle fibres is independent of external Ca^{2+}. Thus a question of obvious interest is whether cAMP causes the release of mitochondrial Ca^{2+}. Information on this point is lacking, but one useful way of approaching this problem would be either to use aequorin or to use cAMP analogues which either cause, or do not cause, release of Ca^{2+} from isolated mitochondria.

It is a fact of some importance that cAMP stimulates Na: H exchange and that external pH governs the magnitude of the response of the Na efflux to cAMP. Similar findings have been reported by Bittar & Tallitsch (1976) in connexion with the action of aldosterone on the Na efflux in barnacle fibres. The agreement is not a coincidence but on the contrary significant since the action of aldosterone on the ouabain-insensitive Na efflux is accounted for in terms of increased adenyl cyclase activity. Thus, one reason for arguing here that the CO_2-sensitive component of the Na efflux and the ouabain-insensitive Na efflux are one, and the same, is that ethacrynic acid is found to abolish that part of the delayed stimulation caused by aldosterone which is unaffected by ouabain (Bittar & Tallitsch, 1976). The impact of these conclusions is fairly clear. It is not hard to see, for example, that in systems such as the kidney medulla where a raised P_{CO_2} does exist, it is quite likely that the *Einzeleffekt*, the single

effect of the active transport of Na or Cl out of the ascending limb of Henle's loop, which is known to be abolished by ethacrynic acid, is due to activation by CO_2 of an ion transport system. The other possibility which seems unmistakable is that stimulation of Na:H exchange by cAMP in barnacle fibres may well provide a partial yet necessary clue to the problem of how ion transport acts as the pace-maker of cell metabolism in the context of changes in cytoplasmic cAMP concentration and acid–base balance.

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REFERENCES

ASHBY, C. D. & WALSH, D. A. (1972). Characterization of the interaction of a protein inhibitor with adenosine 3′,5′-monophosphate-dependent protein kinases. 1. Interaction with the catalytic subunit of the protein kinase. *J. biol. Chem.* **247**, 6637–6642.

ASHBY, C. D. & WALSH, D. A. (1973). Characterization of the interaction of a protein inhibitor with adenosine 3′,5′-monophosphate-dependent protein kinases. II. Mechanism of action with the holoenzyme. *J. biol. Chem.* **248**, 1255–1261.

BEAVO, J. A., BECHTEL, P. & KREBS, E. G. (1974). Activation of protein kinase by physiological concentrations of cyclic-AMP. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3580–3583.

BEAVO, J. A., BECHTEL, P. J. & KREBS, E. G. (1975). Mechanisms of control for cAMP-dependent protein kinase from skeletal muscle. *Adv. cyclic nucleotide Res.* **5**, 241-251.

BITTAR, E. E. (1966). Effect of inhibitors and uncouplers on the Na pump of the *Maia* muscle fibre. *J. Physiol.* **187**, 81–103.

BITTAR, E. E., CALDWELL, P. C. & LOWE, A. G. (1967). The efflux of sodium from single crab muscle fibres. *J. mar. biol. Ass. U.K.* **47**, 709–721.

BITTAR, E. E., CHAMBERS, G. & SCHULTZ, R. (1974*a*). On the mode of action of cyclic nucleotides on Na efflux in single barnacle muscle fibres. *Fedn Proc.* **33** (5), 1359.

BITTAR, E. E., CHEN, S., DANIELSON, B. G., HARTMANN, H. A. & TONG, E. Y. (1972). An investigation of sodium transport in barnacle muscle fibres by means of the micro-injection technique. *J. Physiol.* **221**, 389–414.

BITTAR, E. E., HIFT, H., HUDDART, H. & TONG, E. Y. (1974*b*). The effect of caffeine on sodium transport, membrane potential, mechanical tension and ultrastructure in barnacle muscle fibres. *J. Physiol.* **242**, 1–34.

BITTAR, E. E. & TALLITSCH, R. B. (1976). Mode of stimulation by aldosterone of the sodium efflux in barnacle muscle fibres: effects of ouabain, ethacrynic acid, diphenylhydantoin, $[ATP\text{-}Mg]^{-2}$, adenine translocase inhibitors, pyruvate and oxythiamine. *J. Physiol.* **255**, 29–56.

BITTAR, E. E. & TONG, E. T. (1975). Sensitivity of the sodium efflux in barnacle muscle fibres to the micro-injection of ATP. *Life Sci. Oxford* **16**, 289–296.

BORLE, A. G. (1974). Cyclic AMP stimulation of calcium efflux from kidney, liver and heart mitochondria. *J. membrane Biol.* **16**, 221–236.

CALDWELL, P. C. & WALSTER, G. E. (1963). Studies on the micro-injection of various substances into crab muscle fibres. *J. Physiol.* **169**, 353–372.

CHENG, S. C. & CHEN, S. S. (1975). Stimulation by cyclic nucleotides of calcium efflux in barnacle muscle fibres. *Life Sci. Oxford* **16**, 1711–1716.

DANIELSON, B. G., BITTAR, E. E., CHEN, S. & TONG, E. (1971). The influence of low pH, high K and microinjected $CaCl_2$ on the ouabain-insensitive component of sodium efflux in barnacle muscle fibres. *Life Sci. Oxford* **10**, 833–839.

DANIELSON, B. G., BITTAR, E. E., CHEN, S. & TONG, E. (1972*a*). On the mode of action of ethacrynic acid, using the barnacle muscle fibre as a model. *Life Sci. Oxford* **11**, 13–21.

DANIELSON, B. G., BITTAR, E. E., CHEN, S. & TONG, E. (1972*b*). Inhibition of the CO_2-sensitive Na efflux in barnacle muscle fibres by microinjection of ethacrynic acid. *Experientia* **28**, 1304.

EBEL, H. (1974). Effects of diuretics on renal Na-K-ATPase and adenyl cyclase. *Naunyn-Schmiedebergs Arch. Pharmacol.* **281**, 301–314.

ENTMAN, M. L. (1974). The role of cyclic AMP in the modulation of cardiac contractility. *Adv. cyclic nucleotide Res.* **4**, 163–193.

GORDON, E. E. & HARTOG, M. DE (1969). The relationship between cell membrane potassium ion transport and glycolysis. *J. gen. Physiol.* **54**, 650–663.

HADDOX, M. K., NEWTON, N. E., HARTLE, D. K. & GOLDBERG, N. D. (1972). ATP (Mg^2.) induced inhibition of cyclic AMP reactivity with a skeletal muscle protein kinase. *Biochem. biophys. Res. Commun.* **47**, 653–661.

HEILMEYER, L. M. G., MEYER, F., HASCHKE, R. H. & FISCHER, E. H. (1970). Control of phosphorylase activity in a muscle glycogen particle. II. Activation by calcium. *J. biol. Chem.* **245**, 6649–6656.

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid giant axons by means of a microsyringe. *J. Physiol.* **131**, 592–616.

KLAHR, S., YATES, J. & BOURGOIGNIE, J. (1971). Inhibition of glycolysis by ethacrynic acid and furosemide. *Am. J. Physiol.* **221**, 1038–1043.

ROBISON, G. A., BUTCHER, R. W. & SUTHERLAND, E. W. (1971). *Cyclic AMP*, p. 30. New York: Academic Press.