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THE EFFECT OF CYCLIC NUCLEOTIDES AND PROTEIN PHOSPHORYLATION ON CALCIUM PERMEABILITY AND BINDING IN THE SARCOPLASMIC RETICULUM

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Summary

In the absence of cyclic nucleotides heart microsomes have two classes of calcium binding sites with binding constants of 0.69 and 0.071 μM^{-1} and capacities of 2.2 and 9.7 nmol/mg protein, respectively. Neither cyclic AMP nor monobutyryl cyclic AMP affect binding but cyclic GMP and monobutyryl cyclic GMP cause the complete loss of the high affinity calcium binding sites. Cyclic GMP (but not monobutyryl cyclic GMP) also causes a decrease in the binding constant of the low affinity binding sites. AMP, GMP and Tris-butyrate do not affect calcium binding. The effects of the cyclic nucleotides are direct and not mediated by protein phosphorylation.

Phosphorylation of microsomal proteins increases the binding constant but not the capacity of the high affinity calcium binding sites. The capacity and also, perhaps, binding constant of the low affinity sites is also increased by phosphorylation.

In addition to their effects on calcium binding the cyclic nucleotides also affect the movements of calcium into and out of the microsomes. The effects are again direct and not mediated by protein phosphorylation. Cyclic GMP decreases the rate of Ca^{2+} efflux from preloaded cardiac microsomes and also appers to decrease the rate of uptake of Ca^{2+} by cardiac microsomes though this effect is less clear cut than the action on efflux. The cyclic nucleotide has a half maximal effect at a concentration of 100 μM. By contrast cyclic AMP increases the rate of influx of Ca^{2+} into heart microsomes and the rate of efflux of Ca^{2+} from preloaded preparations. The effect is, however, rather slight. It is suggested that the most obvious interpretation of these results is that cyclic GMP decreases the Ca^{2+} permeability of the cardiac microsomal membrane while cyclic AMP increases the permeability.

In contrast to the results found with membrane preparations from certain other tissues phosphorylation of cardiac microsomal proteins does not appear to alter Ca^{2+} efflux or influx out of, or into, cardiac microsomal preparations. It is thus concluded that phosphorylation of cardiac microsomal proteins does not affect the Ca^{2+} permeability of the microsomal membrane.

Introduction

It is well established that membrane fragments from a variety of tissues including heart [1—3], brain [4—6], liver [2], fat cells [7] and gastric mucosa [8] contain a bound protein kinase which catalyses the phosphorylation of endogenous proteins in a reaction which is stimulated by cyclic AMP [9]. The function of this reaction is uncertain but several studies have shown that phosphorylation of proteins in cardiac microsomes [10—12], cardiac plasma membranes [13,14] and slow-, but not fast-, contracting skeletal muscle microsomes [15,16] stimulates active calcium transport by activating the Ca^{2+}-ATPase.

It is well known that cyclic AMP has a positive inotropic effect on heart muscle, probably mediating the action of noradrenalin on the tissue [17]. It is, however, difficult to reconcile a positive inotropic effect (which must, presumably, be caused by an increase in the amount of free Ca^{2+} in the heart muscle cell) with a stimulation of Ca^{2+} uptake into the sarcoplasmic reticulum. It has recently been found, however, that, in the case of erythrocytes, cyclic AMP interacts directly with the cell membrane and increases calcium permeability [18,19]. By contrast it has been observed that phosphorylation of proteins in the synaptic membrane [20], the erythrocyte membrane [18,19] and the retinal rod outer segment disc membrane [21] lowers the permeability of the membranes to Ca^{2+}.

In addition to the effect of cyclic AMP on heart muscle discussed above it is well known that cyclic GMP has a negative inotropic effect on the tissue and may mediate, at least at part, the action of acetylcholine [22]. It has been suggested that cyclic GMP may act by decreasing the entry of Ca^{2+} into the heart cell [23].

In view of the various observations discussed above experiments were carried out to determine the effects of cyclic nucleotides and the phosphorylation of membrane proteins on the binding of calcium to heart microsomes and on the movement of calcium into and out of such preparations.

Methods

Preparation of heart microsomes. Heart microsomes were prepared by an adaption of the method of Harigaya and Schwartz [24]. Rats were killed by decapitation and 20 g of heart muscle taken and ground in liquid nitrogen with a pestle and mortar. The frozen powder was then homogenised in 100 ml 10 mM $NaHCO_3$/1 mM $MgCl_2$ (pH 7.0) and centrifuged at 8700 $\times g$ for 10 min. These, and all subsequent steps, were carried out at 0° C. The supernatant was taken and again centrifuged at 8700 $\times g$ for 20 min. The second supernatant was then centrifuged at 37 500 $\times g$ for 30 min. The pellet was suspended in

40 ml of 20 mM Tris-maleate buffer (pH 6.8) containing 0.6 M KCl and 1 mM $MgCl_2$ and centrifuged at 37 500 × *g* for 30 min. The pellet was then suspended in 40 ml of 0.32 M sucrose/10 mM Tris-HCl (pH 7.0) and again centrifuged at 37 500 × *g* for 10 min. The pellet was finally suspended in a small volume of 0.32 M sucrose/10 mM Tris-HCl (pH 7.4) at a concentration of approx. 10 mg of protein/ml. Preparations were kept at 0—4° C and used within 24 h.

In the original method of Harigaya and Schwartz [24] the tissue was broken up by a Polytron homogeniser while we ground the hearts in liquid nitrogen. We could however find no difference between the ATP-stimulated calcium transport of microsomes prepared by either method (uptake measured in the presence of 100 mM KCl/5 mM $MgCl_2$/20 mM Tris-maleate (pH 6.8)/5 mM Tris-oxalate/10 μM $^{45}CaCl_2$ in the presence or absence of 2 mM ATP). In addition we could find no great difference between the lactate dehydrogenase [32], cytochrome oxidase [33] and rotenone-insensitive NADPH cytochrome reductase [34] activities of the preparations. Indeed preparations from frozen tissue tended to have lower lactate dehydrogenase and higher NADP cytochrome reductase activities than those from fresh tissue indicating a lower contamination with cytoplasm and a higher concentration of endoplasmic reticulum. Typical values for lactate dehydrogenase, cytochrome oxidase and rotenone-insensitive NADPH cytochrome reductase activities of preparations from fresh and frozen tissues were 223, 43, 10 and 140, 51, 17 nmol/mg protein per min, respectively. There was no difference between the yields of the two methods of preparation which both gave approx. 1.5 mg microsomal protein/g heart tissue.

Determination of protein phosphorylation. Protein phosphorylation was determined as previously described [25]. Reaction were carried out in a volume of 0.5 ml at 37° C in the presence of 1 mM $MgCl_2$/1 mM [γ-^{32}P]ATP (specific radioactivity approx. $1 \cdot 10^7$ cpm/μmol) and 50 mM Tris-HCl (pH 7.4) using approx. 200 μg of microsomal protein. Reactions were stopped at the stated times by addition of 2 ml ice-cold 15% trichloroacetic acid. The precipitated protein was then washed and extracted with 10% trichloroacetic acid, 1 M phosphoric acid, 0.1 M NaOH and ethanol/diethyl ether (1 : 1, v/v) exactly as previously described [25] and the amount of ^{32}P bound to the protein determined by measuring the Cerenkov radiation.

Determination of calcium binding. Samples of heart microsomes were suspended at a concentration of approx. 1 mg protein/ml in 0.32 M sucrose/10 mM Tris-HCl (pH 7.4) and a small quantity of a solution of $^{45}CaCl_2$ (specific radioactivity approx. $1 \cdot 10^7$ cpm/μmol) added to give the stated final concentration. The samples were kept in ice and aliquots taken at stated times. These aliquots were filtered through moistened Millipore filters (cellulose ester; 0.45 μm pore size). The filters were washed twice with 4-ml lots of 180 mM choline chloride, the entire washing procedure being complete within 30 s. The filters were then placed in scintillation vials, dried at 80° C, 5 ml of scintillation fluid added, and the amount of bound radioactivity measured. Samples of the microsomal suspensions were saved for protein determinations [26].

Determination of calcium efflux. Samples of heart microsomes were suspended at a concentration of approx. 1 mg protein/ml in 0.32 M sucrose/10 mM Tris-HCl (pH 7.4) containing 10 μM $^{45}CaCl_2$ (specific radioactivity

approx. $1 \cdot 10^7$ cpm/μmol). The suspension was left in ice for 45 min and 0.1-ml samples taken and filtered as described above to determine the initial level of bound $^{45}Ca^{2+}$ in the loaded microsomes. Aliquots (2 ml) were then centrifuged at 45 000 $\times g$ for 15 min. The pellets were suspended in 2-ml lots of ice-cold 0.32 M sucrose/10 mM Tris-HCl (pH 7.4) with stated additions and 0.1-ml aliquots filtered at stated times to determine the amount of bound ^{45}Ca as described above. The time between adding the 0.32 M sucrose to the ^{45}Ca-loaded pellets and filtering the first 0.1 ml sample was always exactly 2 min.

Results

The effect of cyclic nucleotides on the binding of calcium to heart microsomal preparations

Samples of heart microsomes were incubated for 45 min with the stated concentrations of $^{45}CaCl_2$ in the presence or absence of various cyclic nucleotides as shown in Fig. 1A. It may be seen that cyclic AMP (100 μM) has no effect on binding (monobutyryl cyclic AMP similarly has no effect but this data has not been included in Fig. 1 for the sake of clarity). Cyclic GMP (100 μM), however, decreases binding though monobutyryl cyclic GMP (100 μM) only decreases binding at low concentrations of Ca^{2+}. A Scatchard analysis [27] of the results (Fig. 1B) shows that in the absence of cyclic nucleotides there are two classes of calcium binding sites with binding constants of 0.69 ± 0.16 and 0.071 ± 0.008 μM^{-1} and capacities of 2.2 ± 0.5 and 9.7 ± 1.2 nmol/mg protein (means ± S.D.), respectively. In the presence of cyclic AMP (100 μM) the equivalent values of these constants are 0.47 ± 0.13 and 0.071 ± 0.003 μM^{-1} and 2.7 ± 0.9 and 10.1 ± 0.9 nmol/mg protein (means ± S.D.), respectively, not significantly different from the values found in the absence of cyclic AMP. In the presence of 100 μM cyclic GMP, however, the high affinity binding sites are completely lost. The binding constant of the low affinity sites is also decreased from 0.071 ± 0.008 to 0.048 ± 0.004 μM^{-1} (means ± S.D.) though the number of these sites, is not significantly altered (capacities in the absence and presence of cyclic GMP 9.7 ± 1.2 and 10.0 ± 0.9 nmol/mg protein (means ± S.D.), respectively). Monobutyryl cyclic GMP similarly removes the high affinity binding sites but unlike cyclic GMP has little effect on the low affinity sites. The binding constants of these sites in the presence and absence of monobutyryl cyclic GMP are 0.068 ± 0.01 and 0.071 ± 0.008 μM^{-1} and the capacities 9.0 ± 2 and 9.7 ± 1.2 nmol/mg protein (means ± S.D.), respectively.

Neither GMP, AMP nor Tris-butyrate (all at a concentration of 100 μM) have any effect on calcium binding over the concentration range shown in Fig. 1 (data not given).

The effect of cyclic nucleotides on the rates of efflux and influx of Ca^{2+} out of and into heart microsomal preparations

Samples of heart microsomes were loaded with ^{45}Ca as described in Methods then incubated in the absence of Ca^{2+} in the presence or absence of various cyclic nucleotides as described in Fig. 2. Monobutyryl cyclic GMP (100 μM) causes a decreased rate of loss of ^{45}Ca from the preparation and cyclic GMP (100 μM) causes a similar, though rather slight, effect. Cyclic AMP (100 μM)

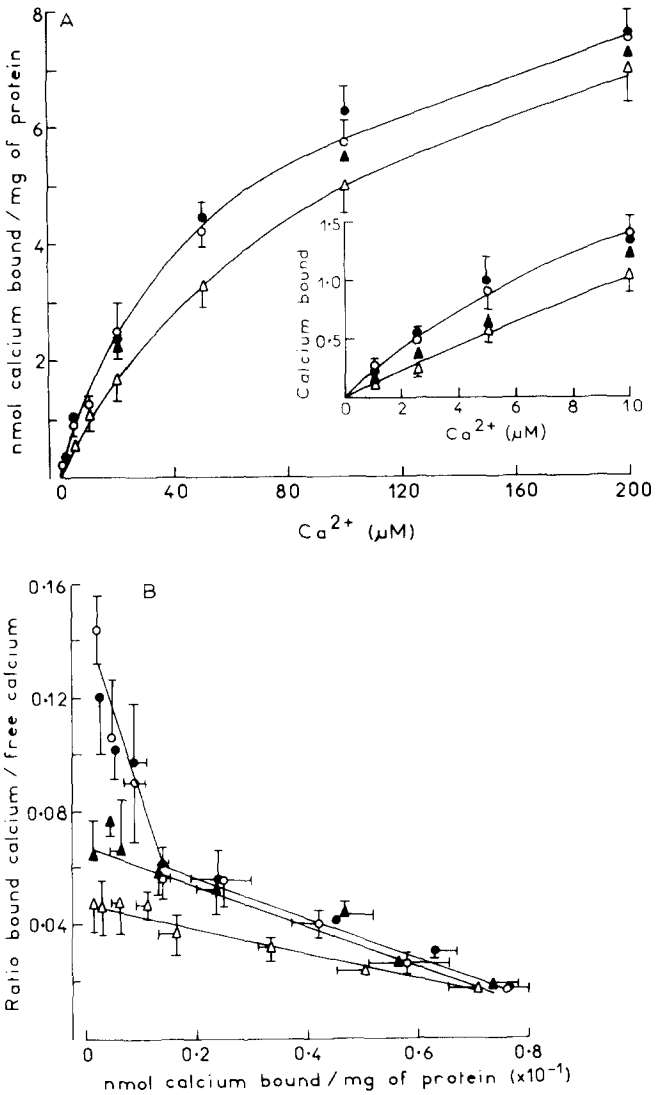


Fig. 1. The effect of cyclic nucleotides on the binding of calcium to heart microsomal fragments. Samples of cardiac microsomal preparations were incubated for 45 min in ice with various concentrations of $^{45}CaCl_2$ and the amount of bound calcium determined as described in the text. Binding was either determined in the absence of cyclic nucleotides (○) or in the presence of 100 μM cyclic AMP (●), 100 μM cyclic GMP (△) or 100 μM monobutyryl cyclic GMP (▲). Results are shown as means ± S.D. and are taken from eight observations with four separate microsomal preparations. In B the data of A are shown as a Scatchard plot.

causes a slight increase in the rate of loss of ^{45}Ca but monobutyryl cyclic AMP (100 μM) has no effect (data not shown). Neither AMP, GMP nor Tris-butyrate (all at a concentration of 100 μM) have any significant effect on efflux (data not shown).

The effect of the cyclic nucleotides on calcium efflux cannot be due to an action on calcium binding. Cyclic GMP and monobutyryl cyclic GMP decrease

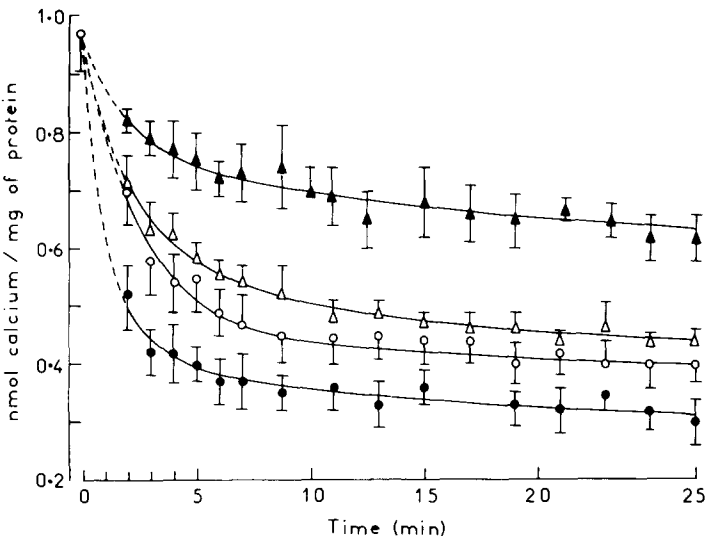


Fig. 2. The effect of cyclic nucleotides on the efflux of Ca^{2+} from preloaded heart microsomal preparations. Samples of heart microsomes were loaded with $^{45}CaCl_2$, incubated in the absence of Ca^{2+} and samples taken at various times to determine the amount of bound calcium as described in the text. Efflux was either measured in the absence of cyclic nucleotides (○) or in the presence of 100 μM cyclic AMP (●), 100 μM cyclic GMP (△) or 100 μM monobutyryl cyclic GMP (▲). Results are shown as means ± S.D. and are taken from five observations with five separate microsomal preparations.

calcium binding (Fig. 1) and it would be anticipated that this would cause an increase, rather than a decrease, in calcium efflux. Cyclic AMP on the other hand has no effect on calcium binding (Fig. 1).

One possible explanation of the above results on efflux could be that monobutyryl cyclic GMP and cyclic GMP decrease the calcium permeability of heart microsomal membranes while cyclic AMP slightly increases the permeability. If this were the case then it would be anticipated that cyclic GMP would decrease, and cyclic AMP increase, the rate of passive uptake of Ca^{2+} into preparations of heart microsomes. It may be seen from Fig. 3 that this is, in fact, the case. Care must, however, be taken in interpreting the results of this experiment. While the situation appears clear in the case of cyclic AMP which does not effect equilirbium calcium binding (Fig. 1) the situation is not so simple in the case of cyclic GMP and monobutyryl cyclic GMP since both compounds decrease calcium binding (Fig. 1), and effect which would naturally cause an apparent decrease in Ca^{2+} uptake. The fact that cyclic GMP is more effective than monobutyryl cyclic GMP in inhibiting calcium binding may account for the fact that while monobutyryl cyclic GMP has a greater effect than cyclic GMP on calcium efflux the reverse is the case with calcium influx. Although part of the effect of cyclic GMP and monobutyryl cyclic GMP on the rate of calcium influx is undoubtedly due to their action on calcium binding it would still seem that these compounds may inhibit uptake as such.

AMP, GMP and Tris-butyrate (all at a concentration of 100 μM) have no effect on calcium uptake (data not shown).

All the above experiments were carried out with rather high concentrations

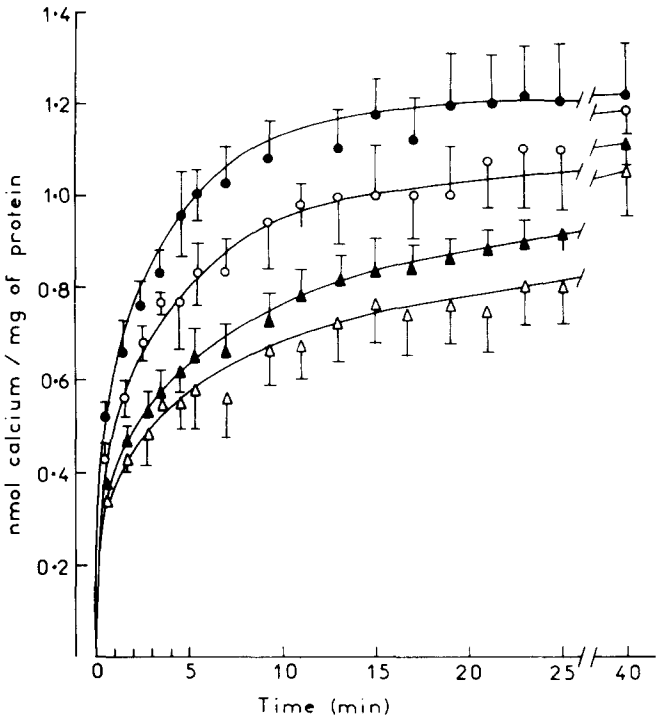


Fig. 3. The effect of cyclic nucleotides on the rate of Ca^{2+} uptake into preparation of heart microsomes. Samples of cardiac microsomes were incubated with 10 μM $^{45}CaCl_2$ and samples taken at various times to determine the amount of bound calcium as described in the text. Experiments were carried out either in the absence of cyclic nucleotides (○) or in the presence of 100 μM cyclic AMP (●), 100 μM cyclic GMP (△) or 100 μM monobutyryl cyclic GMP (▲). Results are shown as means ± S.D. and are taken from four observations with four separate microsomal preparations.

of cyclic nucleotides (100 μM). An experiment was thus carried out to determine the effect of cyclic GMP concentration on the uptake of Ca^{2+}. Samples of heart microsomes were incubated at 0° C for 7 min with 10 μM $^{45}CaCl_2$ and various concentrations of cyclic GMP. The amount of calcium taken up into the microsomes was determined as described above. It may be seen from Fig. 4 that cyclic GMP has a half maximal effect at a concentration of 100 μM and a maximal effect at a concentration of 200 μM.

The effect of protein phosphorylation on the binding of calcium to preparations of heart microsomes

Preliminary experiments demonstrated that incubation of heart microsomal fragments with 1 mM ATP for 10 min at 37° C saturated all the phosphate acceptor sites, approx. 70 pmol phosphate being incorporated/mg protein. In order to examine the effect of protein phosphorylation on calcium binding samples of phosphorylated and non-phosphorylated heart microsomes were prepared.

Three samples of microsomes were taken and treated under the following conditions: (1) suspended at a concentration of about 0.5 mg protein/mg in in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 4 mM Tris-ATP and incubated at

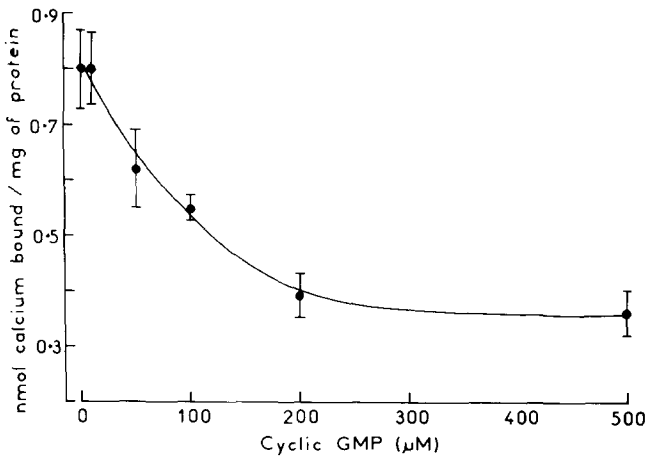
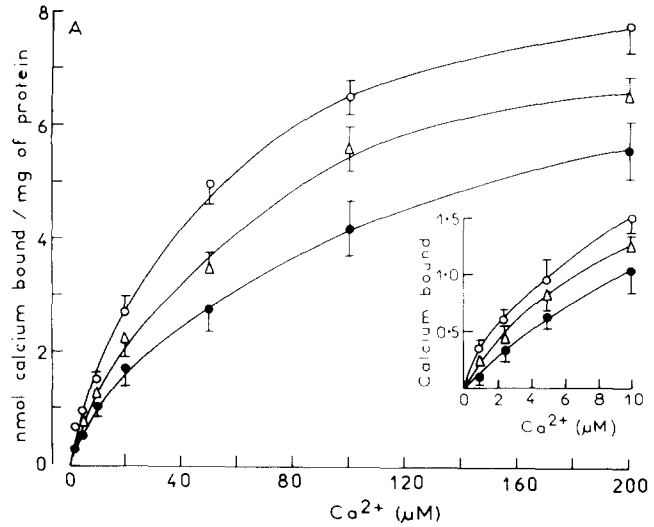


Fig. 4. The effect of cyclic GMP concentrations on calcium binding to heart microsomal preparation. Samples of heart microsomes were incubated for 7 min with 10 μM $^{45}CaCl_2$ in the presence of various concentrations of cyclic GMP and the amount of bound calcium determined as described in the text. Results are shown as means ± S.D. and are taken from four observations, with two separate membrane preparations.

37°C for 10 min (phosphorylated samples); (2) incubated under the same conditions as (1) but in the absence of ATP (non-phosphorylated samples); (3) suspended under the same conditions as (1) but kept at 0°C instead of being incubated at 37°C (control samples). After treatment the samples were centrifuged at 50 000 × *g* for 10 min and the pellets washed once by resuspension in and centrifugation from 0.32 M sucrose/10 mM Tris-HCl. The pellets were finally suspended at a concentration of approx. 1 mg protein/ml in 0.32 M sucrose/10 mM Tris-HCl (pH 7.4). In some experiments the ATP used for the initial treatment of the microsomes was labelled in the γ position with ^{32}P and the amount of protein-bound radioactive phosphate determined after the final resuspension of the treated samples in 0.32 M sucrose. It was found that phosphorylated and control samples had incorporated 68 ± 5 and 15 ± 4 (means ± S.D. of four observations) pmol phosphate/mg protein, respectively. It was also found that there was no detectable loss of protein-bound phosphate during the washing of the phosphorylated samples with 0.32 M sucrose. This procedure was, however, carried out as rapidly as possible using ice-cold solutions.

Aliquots of the treated microsomes were taken, incubated with various concentrations of $^{45}CaCl_2$ for 45 min, and the binding of ^{45}Ca determined as described in Methods. The results of this experiment are shown in Fig. 5A from which it may be seen that phosphorylation of heart microsomes increases calcium binding. Control samples show more binding than non-phosphorylated samples possibly because some phosphorylation has occurred under the control conditions or possibly because the non-phosphorylated samples became dephosphorylated during incubation. Scatchard analysis of the results (Fig. 5B) indicates the phosphorylation increases the binding constant of the high affinity sites without having much effect on the number of these sites (the binding constants of the high affinity sites of phosphorylated, control and non-



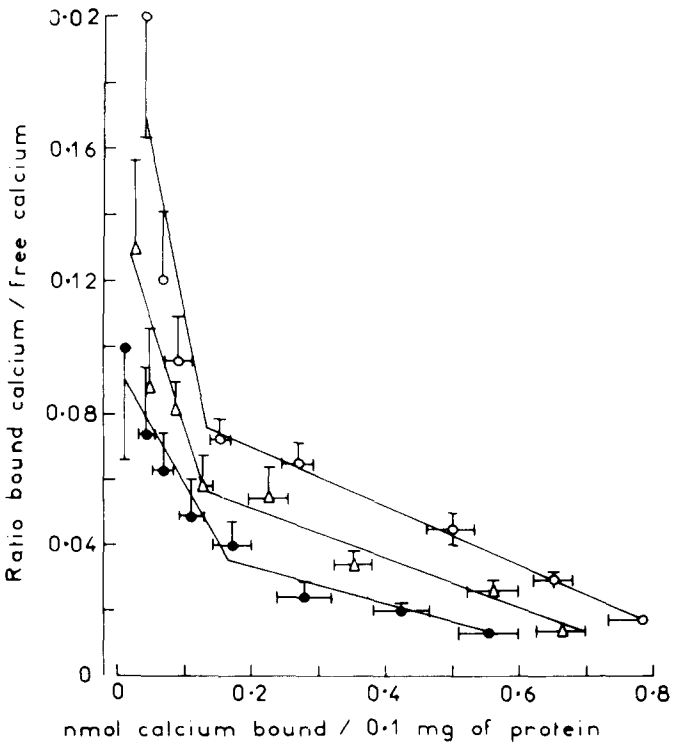


Fig. 5. The effect of protein phosphorylation on the binding of calcium to heart microsomal preparations. Phosphorylated (○), non-phosphorylated (●) and control (△) samples of cardiac microsomes were prepared as described in the text and incubated for 45 min with various concentrations of $^{45}CaCl_2$. The amount of bound calcium was then determined as described in the text. Results are shown as means ± S.D. and are taken from eight observations with four separate preparations of heart microsomes. In B the data of A are presented as a Scatchard plot.

phosphorylated samples are 0.98 ± 0.1, 0.6 ± 0.04 and 0.35 ± 0.09 μM^{-1}, respectively. The corresponding capacities are 2.08 ± 0.7, 2.13 ± 0.7 and 2.6 ± 0.7 nmol/mg protein (all results are shown as means ± S.D.). By contrast phosphorylation increases the number of low affinity binding sites and may possibly also increase their affinity. (The capacities of the low affinity binding sites of phosphorylated, control and non-phosphorylated samples are 9.7 ± 0.37, 8.4 ± 0.8 and 7.3 ± 2 nmol/mg protein. The corresponding affinities are 0.09 ± 0.003, 0.008 ± 0.01 and 0.063 ± 0.1 μM^{-1} (all results are shown as means ± S.D.).

Effect of protein phosphorylation on Ca^{2+} efflux and influx into and out of heart microsomes

Samples of heart microsomes were treated as described in the previous section to obtain either phosphorylated, control or non-phosphorylated samples. These preparations were then loaded with $^{45}Ca^{2+}$ as described in Methods (no detectable loss of protein-bound phosphate occurred during the loading procedure). The samples were then incubated in the absence of Ca^{2+} as is shown in Fig. 6. The interpretation of this experiment is made difficult because phosphorylated preparations bind more calcium than non-phosphorylated samples. The starting level of bound calcium thus differs between the two samples. Nevertheless it seems reasonably clear that, if this factor is taken into account, there is no real difference between the rate of loss of calcium from phosphorylated and non-phosphorylated heart microsomes. Presuming that Ca^{2+} actually entered inside the microsomes during the loading procedure (as is indicated by the effect of cyclic nucleotides on Ca^{2+} efflux and influx

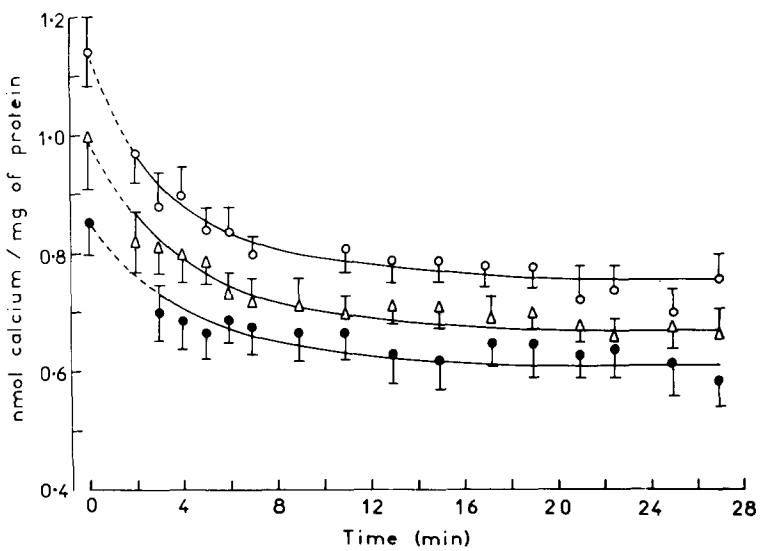


Fig. 6. The effect of protein phosphorylation on the efflux of calcium from preloaded heart microsomes. Samples of phosphorylated (○), non-phosphorylated (●) and control (△) microsomes were prepared, loaded with $^{45}CaCl_2$ and incubated in the absence of Ca^{2+}. Samples were taken at various times to determine the amount of bound calcium as described in the text. Results are shown as means ± S.D. and are taken from four observations with four separate membrane preparations.

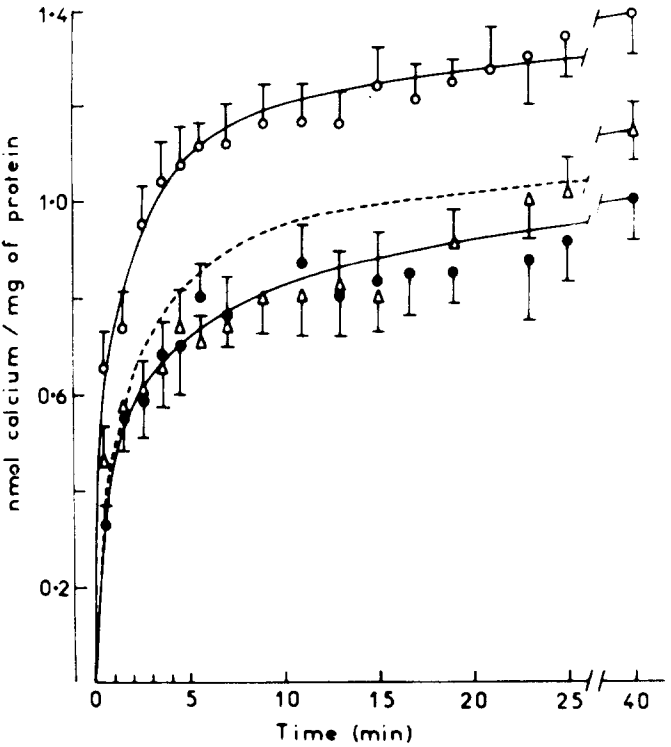


Fig. 7. The effect of protein phosphorylation on the influx of Ca^{2+} into heart microsomal preparations. Phosphorylated (○), non-phosphorylated (●) and control (△) samples of cardiac microsomes were prepared, incubated with 10 μM $^{45}CaCl_2$ and samples taken at various times to determine the amount of bound calcium as described in the text. Results are shown as means ± S.D. and are taken from four observations with four separate preparations of heart microsomes. The broken line represents uptake into phosphorylated heart microsomes when allowance is made for the fact that such preparations bind more calcium than control samples (N.B. the differences between the amounts of calcium bound by phosphorylated and control samples after 40 min incubation (that is when the system is at equilibrium) is subtracted from all the figures obtained for calcium binding to phosphorylated samples at the various times measured).

described above) this indicates that, in contrast to the situation with synaptosome membranes [20], retinal rod outer segment disc membranes [21] and erythrocyte plasma membranes [18,19], phosphorylation of proteins in cardiac microsomal membranes does not alter Ca^{2+} permeability. In order to check this conclusion the rate of uptake of calcium into phosphorylated and non-phosphorylated heart microsomes was measured as shown in Fig. 7. Since phosphorylation increases the amount of calcium binding there is obviously more calcium uptake into phosphorylated than into non-phosphorylated samples. If, however, allowance is made for the effect of phosphorylation on calcium binding (by subtracting the difference between the amount of calcium bound by phosphorylated and control samples at equilibrium (that is after incubation for 40 min) from all the figures for calcium binding to phosphorylated samples at various times) it may be seen that phosphorylation has no effect on Ca^{2+} uptake into heart microsomes.

Discussion

The results described above show that cyclic GMP specifically blocks the high affinity calcium binding sites in heart microsomes. In addition this cyclic nucleotide also inhibits the efflux of Ca^{2+} from preloaded cardiac microsomes. The compound may also decrease the rate of passive influx of Ca^{2+} into the preparation. A rather high concentration of cyclic GMP is needed to cause these effects (a concentration of 100 μM is needed to give half maximal effect) but it must be remembered that heart microsomal preparations contain an active phosphodiesterase which may, perhaps, account for this point. By contrast cyclic AMP has no effect on calcium binding and, unlike cyclic GMP increases, rather than decreases, the rate of efflux of Ca^{2+} from preloaded heart microsomes increases, rather than decreases, the rate of passive uptake of Ca^{2+} into heart microsomes.

It has been argued before [20,21] that when a compound decreases both the rate of efflux of Ca^{2+} out of a vesicular preparation and the rate of influx of Ca^{2+} into such a preparation it is difficult to explain the results unless one assumes that Ca^{2+} enters into an internal compartment of the preparation and that the compound in question reduces the Ca^{2+} permeability of the bounding membrane. We would thus like to suggest that cyclic GMP decreases the Ca^{2+} permeability of the microsomal membrane while cyclic AMP slightly increases the permeability.

It must be emphasised that the above effects of cyclic nucleotides were observed under condition were no protein phosphorylation could take place (N.B. no phosphate donor was present). The effects must thus have been caused by a direct action of the cyclic nucleotides not mediated by protein phosphorylation.

In contrast to the results obtained with synaptosomal membranes and retinal rod outer segment disc membranes it is of considerable interest that phosphorylation of proteins in heart microsomal preparations increases the binding of calcium without causing a detectable additional effect on the rate of Ca^{2+} influx into heart microsomal preparations or on the rate of Ca^{2+} efflux from preloaded microsomal preparation. It can thus be concluded that phosphorylation of proteins in cardiac microsomal membranes, in contrast to phosphorylation of proteins in synaptosome membranes [20] or in rod outer segment disc membranes [21], does not alter the permeability of the membranes to Ca^{2+}. It may be noted that phosphorylation of proteins in liver plasma membranes also increases calcium binding [28].

The physiological implications of the results described above are of some interest. It is well known that the amount of bound calcium can regulate the permeability of a membrane to Na^+ or K^+ [29] and it is interesting to postulate that changes in the concentration of cyclic GMP and in the state of phosphorylation of membrane proteins could, by altering the amount of calcium bound to the heart cell membrane, regulate Na^+ and/or K^+ permeability and thus control the membrane potential and excitability of the myocardial cell [30].

The observation that cyclic GMP decreases the efflux of Ca^{2+} from cardiac microsomes may help to explain the fact that cyclic GMP decreases the force of

contraction of heart muscle [22]. In view of our conclusion that this compound may act by decreasing the permeability of the microsomal membrane to Ca^{2+} it is interesting to note that Nawrath [23] observed that cyclic GMP decreases the influx of Ca^{2+} into the heart cell, presumably by decreasing the calcium permeability of the plasma membrane.

Although the effect of cyclic AMP on Ca^{2+} movement in heart microsomes is rather slight the fact that this nucleotide increases the efflux of Ca^{2+} may explain the fact that cyclic AMP increases the force of contraction of heart muscle [17]. The increased level of cyclic AMP would then stimulate phosphorylation of proteins in the sarcoplasmic reticulum which would cause an increase in the active uptake of Ca^{2+} lowering the cytoplasmic level of Ca^{2+} causing relaxation. This sort of process would help to explain the fact that the positive inotropic effect of catecholamines is characterised by an increase in the rate of rise of tension, a decrease in the duration of systole and an increase in the rate of relaxation [31].

It is apparent that cyclic nucleotides and protein phosphorylation are intimately involved in the control of Ca^{2+} binding and movement in membrane preparations from a variety of tissues. It is obvious that this will repay much further study.

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