Biochimica et Biophysica Acta, 483 (1977) 294—302

BBA 68189

CYCLIC AMP-DEPENDENT PROTEIN KINASE PHOSPHORYLATION OF CARDIAC ($Na^+ + K^+$)-ATPases

EFFECT ON CALCIUM BINDING

KENICHI KANIIKE *, BARRY J.R. PITTS and ARNOLD SCHWARTZ **

Department of Cell Biophysics, Baylor College of Medicine, and the Fondren-Brown Cardiovascular Research and Training Center of The Methodist Hospital, Houston, Texas 77030 (U.S.A.)

(Received February 28th, 1977)

Summary

1. Calcium binding to ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparations from beef and pig heart preparations of varying degrees of purity was measured.

2. Binding was inhibited by Mg^{2+}, Na^+ and K^+. Inhibition by Na^+ and K^+ appeared to be due to an ionic strength effect.

3. Four classes of binding sites were identified with K_d values for calcium of about 0.03, 1, 15 and 200 μM.

4. Cyclic AMP-dependent phosphorylation of the enzyme by protein kinase (ATP : protamine *O*-phosphotransferase, EC 2.7.1.70) had no effect on ($Na^+ + K^+$)-ATPase activity.

5. Phosphorylation also had no effect on either K_d or B_{max} for calcium binding at any of the four sites whether measured in the presence or absence of NaCl or KCl.

6. It is concluded that previous reports of an effect of phosphorylation on calcium binding to a ($Na^+ + K^+$)-ATPase preparation may have been due to the presence of membrane material not directly associated with ($Na^+ + K^+$)-ATPase.

Introduction

Interest in cyclic AMP-dependent protein kinases (ATP : protamine *O*-phosphotransferase, EC 2.7.1.70) as modulators of muscle cell function began with

* Present address: Kinki University School of Medicine, Sayama-cho, Minamikawachi-gun, Osaka, 589 Japan.

** Present address and address for reprints: The University of Cincinnati College of Medicine, Department of Cell Biophysics, 231 Bethesda Avenue, Cincinnati, OH 45267, U.S.A.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

the discovery of a cyclic AMP-induced phosphorylation, and simultaneous activation of rabbit skeletal muscle phosphorylase kinase, first described in 1968 by Walsh et al. [1].

Since that time, interest in the possible regulatory function of protein kinases has increased significantly, particularly with the discovery that there appears to be a close connection between carbohydrate metabolism, protein kinases and cellular calcium [1—3]. Furthermore, it is now apparent that cyclic AMP-dependent and -independent protein kinases may participate in cellular control of the phosphorylation of a wide variety of substrates, including troponin [3,4,19,20] and sacroplasmic reticulum [5,6]. It is understandable, therefore, that attention should be paid to the possible alteration of calcium binding to cardiac muscle sarcolemma resulting from cyclic AMP-dependent, protein kinase-catalyzed phosphorylation [7] which may cause significant changes in calcium influx or efflux from the cell and hence regulate contraction or relaxation [22].

The $(Na^+ + K^+)$-ATPase enzyme (ATP phosphohydrolase, EC 3.6.1.3) system, which is thought to reside in the cell membrane, not only governs the movements of sodium and potassium, but also may regulate calcium movements [21]. It is possible that phosphorylation of a specific protein associated with the $(Na^+ + K^+)$-ATPase may change the affinity of sites for calcium. Recently, Wollenberger and his colleagues [7—10] reported that a $(Na^+ + K^+)$-ATPase enzyme preparation isolated from pig myocardium by a procedure developed in this laboratory [11] was phosphorylated by an endogenous cyclic AMP-dependent protein kinase. Phosphorylation of a 24 000 dalton protein in the enzyme preparation caused a 4-fold increase in calcium affinity at two high affinity calcium binding sites. Although we have been able to demonstrate both phosphorylation [12,18] and calcium binding [22] with a variety of $(Na^+ + K^+)$-ATPase preparations, we have been unable to find any effect of phosphorylation on calcium binding.

Methods

Enzyme preparation. Three different types of $(Na^+ + K^+)$-ATPase preparations were employed in this study and are designated "NaI-treated enzyme", "glycerol enzyme" and "citrate enzyme". These three fractions, which were of differing degrees of purity and specific activity, were prepared from beef and pig hearts as described by Pitts and Schwartz [14]. The procedure used to obtain the NaI-treated enzyme is a modification of the method of Matsui and Schwartz [11] and is essentially the same as that used by Wollenberger and his colleagues [7—10] in their studies. The technique used to obtain the glycerol enzyme was slightly modified from our earlier method [13].

Measurement of ^{32}P incorporation. The procedure of Kuo et al. [15], as modified by Dowd and Schwartz [12], was employed to measure phosphorylation. The enzyme preparation (1 mg/ml) was incubated for 15 min at 30° C in 50 mM potassium phosphate (pH 6.5) containing 0.2 mM [γ-^{32}P]ATP ($0.8 \cdot 10^6$—$3 \cdot 10^6$ cpm/tube), 10 mM $MgCl_2$, 10 mM KF, 2.5 mM theophylline, 0.3 mM EGTA and, where indicated, protein kinase (0.1 mg/ml Sigma) and cyclic AMP ($5 \cdot 10^{-6}$ M). The reaction was terminated by the addition of trichloro-

acetic acid. The phosphorylated protein was recovered by centrifugation, treated with hydroxylamine, washed and counted as previously described [12].

Phosphorylation of enzyme for Ca^{2+} binding studies. Modification of the above phosphorylation procedure was necessary for Ca^{2+} binding studies since it was important to avoid denaturation of the enzyme and to exclude substances which might interfere with Ca^{2+} binding. The enzyme preparation (1 mg/ml) was incubated at 30° C for 15 min in a medium containing 10 mM $MgCl_2$, 0.3 mM EGTA, 40 mM Tris · HCl buffer, (pH 7.4), 0.2 mM ATP and where indicated, cyclic AMP ($5 \cdot 10^{-6}$ M) and protein kinase (0.1 mg/ml). A control experiment contained everything except ATP, cyclic AMP and protein kinase. After incubation, the reaction was terminated by the addition of 10 mM EDTA and cooled in ice. The suspension was then centrifuged for 15 min at 105 000 × *g*. The pellet was suspended in 5 ml of 40 mM Tris · HCl (pH 7.4) containing 1 mM EDTA, and recentrifuged as above. The pellets were resuspended in a small amount of the same buffer (without EDTA) and employed in the Ca^{2+} binding experiments. In some experiments, 100 mM KCl or 10 mM NaCl were included in both the incubation medium and washing buffer.

Measurement of calcium binding. Phosphorylated or non-phosphorylated enzyme preparations (0.2 mg) were incubated at 0° C for 2—10 min in a medium (1 ml) containing various concentrations of ^{45}Ca and 40 mM Tris · HCl (pH 7.4). After incubation, the suspension was centrifuged at 105 000 × *g* for 15 min and the supernatant was removed. The inside of the tube was wiped with tissue and the pellet was dissolved in 0.2 M NaOH (0.4 ml). This was transferred to a scintillation vial containing 10 ml of BBS-3 fluoralloy TLA (Beckman) dissolved in toluene and counted in a Packard Tricarb liquid scintillation counter. Correction was made for the amount of ^{45}Ca trapped in the pellet by the inclusion of controls containing a 100-fold excess of unlabeled $CaCl_2$ [22].

In order to measure Ca^{2+} binding at low Ca^{2+} concentrations, 0.1 mM EGTA was included in the binding medium and the free Ca^{2+} concentration was calculated using the dissociation constant of Portzell et al. [16].

Protein was determined by the method of Lowry et al. [17].

Results

Phosphorylation of $(Na^+ + K^+)$-ATPase preparations by cyclic AMP-dependent protein kinase

Four different types of preparations with varying specific activities were used. The beef heart NaI-treated preparation had a specific activity (μmol/mg per h) of 42, the pig heart NaI-treated preparation, 56, the beef heart glycerol preparation was about 185, and the citrate preparation about 250 (Table I). All preparations were phosphorylated by cyclic AMP-dependent protein kinase, but the extent of phosphorylation did not correlate with $(Na^+ + K^+)$-ATPase activity (Table I). For example, the beef heart NaI-treated enzyme had the lowest specific activity, but gave the highest level of phosphorylation. The citrate enzyme was the most active, but was phosphorylated to the least extent. Since the central aim of these experiments was to study the effect of phosphorylation on Ca^{2+} binding to $(Na^+ + K^+)$-ATPase, the citrate enzyme, which was only phosphorylated to a small degree, was omitted from the Ca^{2+} binding experi-

TABLE I

EFFECT OF CYCLIC AMP AND PROTEIN KINASE ON PHOSPHORYLATION OF $(Na^+ + K^+)$-ATPase PREPARATIONS OF VARYING DEGREES OF PURITY

Phosphorylation was carried out as described in Methods. Protein kinase (0.1 mg/ml) or cyclic AMP ($5 \cdot 10^{-6}$ M) or both were added as indicated below.

Type of preparation	Source	$(Na^+ + K^+)$-ATPase activity (μmol P_i/mg per h)	^{32}P incorporated (pmol/mg) Additions			
			None	cyclic AMP	Protein kinase	Protein kinase + cyclic AMP
NaI-treated	Beef heart	42	284	436	476	1070
NaI-treated	Beef heart	45	262	333	336	1040
NaI-treated	Pig heart	56	131	146	n.d.	405
Glycerol enzyme	Beef heart	188	345	301	305	873
Glycerol enzyme	Beef heart	183	100	90	162	369
Glycerol enzyme		157	132	133	192	663
Citrate enzyme	Beef heart	250	40	85	6	42
Citrate enzyme	Beef heart	252	91	54	265	383

n.d., not determined.

ments. Electrophoresis profiles showed that this preparation sometimes lacked the small molecular weight (12 000) substrate for phosphorylation [18]. Both the NaI-treated and glycerol enzymes showed a 3—4-fold stimulation of phosphorylation by cyclic AMP plus exogenous protein kinase. The NaI-treated enzyme was stimulated by cyclic AMP alone, but the glycerol enzyme required both cyclic AMP and protein kinase indicating that the NaI-treated enzyme probably contained an endogenous protein kinase as described by Krause et al. [10]. Phosphorylation had no effect on $(Na^+ + K^+)$-ATPase activity (Table II).

The purified $(Na^+ + K^+)$-ATPase contains two polypeptides with apparent molecular weights (by gel electrophoresis) of 95 000 and 55 000 [21], but neither is phosphorylated by protein kinase [18]. We have previously reported phosphorylation of the larger polypeptide [12]. but the extent of phosphorylation was less than 0.03 mol per mol indicating that the substrate protein may

TABLE II

LACK OF EFFECT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE PHOSPHORYLATION ON $(Na^+ + K^+)$-ATPase ACTIVITY OF A BEEF HEART GLYCEROL ENZYME PREPARATION

Phosphorylation was measured as described in Methods. $(Na^+ + K^+)$-ATPase activity of the same enzyme preparation was measured after phosphorylation by the procedure given in Methods for use in Ca^{2+} binding studies. In the control, ATP (or [^{32}P]ATP) protein kinase and cyclic AMP were omitted from the incubation medium.

Additions to incubation medium	^{32}P incorporation (pmol/mg)	$(Na^+ + K^+)$-ATPase activity (μmol/mg per h)
Control	0	178
+ATP	233	171
+ATP, protein kinase and cyclic AMP	702	183

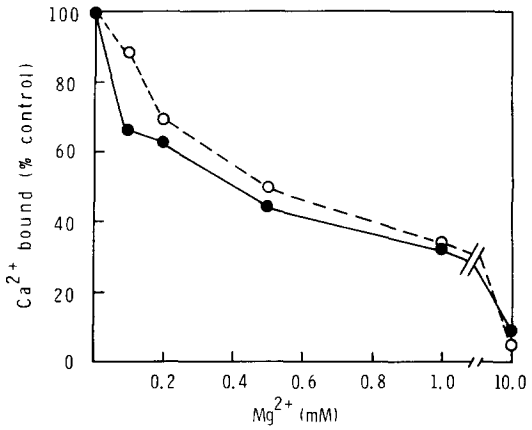

Fig. 1. Inhibition of Ca^{2+} binding to $(Na^+ + K^+)$-ATPase by magnesium. ^{45}Ca binding to beef heart glycerol enzyme was measured as described in Methods with either 10 μM (●) or 100 μM (○) ^{45}Ca and the indicated concentrations of magnesium.

have been a contaminant. The NaI-treated enzyme contains many other proteins besides the two subunits and contains two substrates for protein kinase with molecular weights of approx. 16 000 and 12 000 [18]. The glycerol enzyme is approx. 10% pure [14] as judged by gel electrophoresis and contains only the lower molecular weight substrate protein (unpublished data).

Effects of various ligands and inhibitors on calcium binding to $(Na^+ + K^+)$-ATPase

Mg^{2+} (0.1—10 mM) competitively inhibited Ca^{2+} binding to $(Na^+ + K^+)$-ATPase (Fig. 1). NaCl and KCl also inhibited Ca^{2+} binding and the inhibition also appeared to be competitive (data not shown). However, there was no difference in the extent of inhibition by NaCl or KCl in the presence of high or low Ca^{2+} concentrations. It appeared that the effects of Na^+ and K^+ on Ca^{2+} binding were non-specific and were probably due to ionic strength. Phosphorylation of $(Na^+ + K^+)$-ATPase had no effect on Ca^{2+} binding in the presence of NaCl or KCl concentration ranging from 4 to 40 mM (Figs. 2a and 2b).

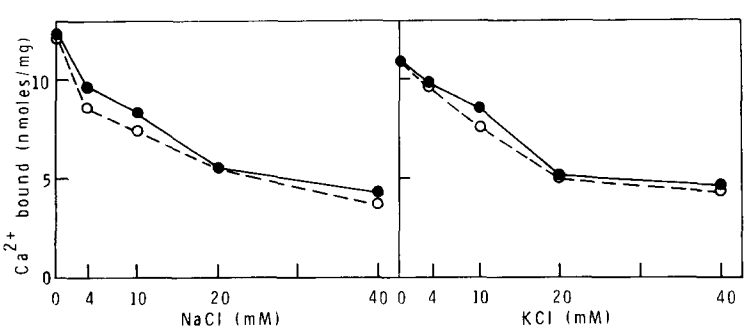

Fig. 2. Inhibition of Ca^{2+} binding to $(Na^+ + K^+)$-ATPase by NaCl and KCl. ^{45}Ca binding to NaI-treated enzyme was measured as described in Methods in the presence of 100 μM ^{45}Ca. ○, control enzymes; ●, phosphorylated enzyme. a, shows the effect of varying NaCl and b, shows the effect of varying KCl.

TABLE III

EFFECT OF TEMPERATURE AND pH ON Ca^{2+} BINDING TO $(Na^+ + K^+)$-ATPase (BEEF HEART GLYCEROL ENZYME)

Ca^{2+} binding was measured as described in Methods with either 0.2 or 100 μM ^{45}Ca at 0 or 20°C. Binding at pH 7.4 was carried out in 40 mM Tris · HCl and at pH 6.8 in 40 mM Tris/maleate.

Ca^{2+} (μM)	pH	nmol ^{45}Ca bound/mg protein	
		0°C	20°C
100	7.4	59.5	53.5
100	6.8	36.7	36.8
0.2	6.8	0.76	0.73

Effect of temperature and pH

The same amount of Ca^{2+} bound to the enzyme at 0 and 20°C, whether measured at 0.2 or 100 μM Ca^{2+} or at pH 6.8 or 7.4 (Table III).

Effect of cyclic AMP-dependent protein kinase phosphorylation on Ca^{2+} binding to $(Na^+ + K^+)$-ATPase

Scatchard analysis of Ca^{2+} binding to a NaI-treated enzyme (Fig. 3) revealed two types of Ca^{2+} binding sites: one with low affinity (site I) possessing a K_d for Ca^{2+} of 0.2 mM and a maximum number of binding sites (B_{max}) of 35 nmol/mg, and the other with high affinity (site II) with a K_d of 7 μM and a B_{max} of 3.7 nmol/mg. In the presence of 100 mM KCl, Ca^{2+} binding was depressed. That is, the K_d of the low affinity site increased to 0.73 mM and the number

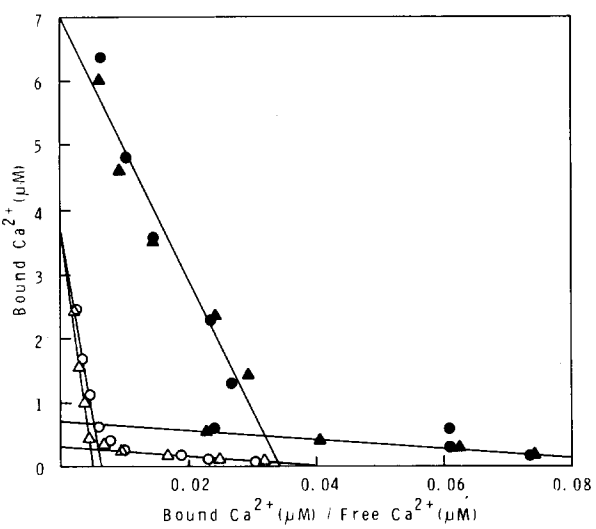

Fig. 3. Scatchard plot of Ca^{2+} binding to $(Na^+ + K^+)$-ATPase at high Ca^{2+} concentrations in the presence or absence of cyclic AMP and protein kinase. ^{45}Ca binding to beef heart NaI-treated enzyme was measured as described in Methods at ^{45}Ca concentrations ranging from 2.5 μM to 1 mM in the presence (open symbols) or absence (closed symbols) of 100 mM KCl. ○,●, enzyme phosphorylated with cyclic AMP and protein kinase; or △,▲, control enzyme.

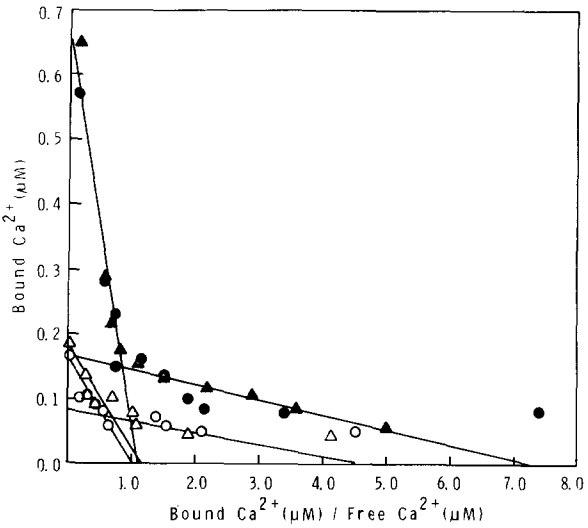

Fig. 4. Scatchard plot of Ca^{2+} binding to $(Na^+ + K^+)$-ATPase at low calcium concentrations in the presence or absence of cyclic AMP and protein kinase. ^{45}Ca binding to beef heart NaI-treated enzyme was measured as described in Methods at calculated free ^{45}Ca concentrations ranging from 11 nM to 3.95 μM in the presence (open symbols) or absence (closed symbols) of 100 mM KCl. ○,●, enzyme phosphorylated with cyclic AMP and protein kinase or △,▲, not phosphorylated. The binding medium contained 0.1 mM EGTA and varying amounts of ^{45}Ca. The concentration of free ^{45}Ca was calculated by the Method of Portzell et al. [16].

of binding sites decreased to 19 nmol/mg protein. KCl had no effect on the K_d for the high affinity sites, but decreased B_{max} to 1.5 nmol/mg. Phosphorylation of the enzyme by cyclic AMP and protein kinase had no effect either on K_d or B_{max} for Ca^{2+} binding. Three types of enzyme preparation (NaI-treated and glycerol enzymes from beef heart and NaI-treated enzyme from pig heart) were then used to investigate the possible effects of phosphorylation on Ca^{2+} binding to preparations of differing degrees of purity in the presence or absence of KCl (0.1 M) or NaCl (0.01 M). We could find no significant differences either in K_d or in B_{max} (data not shown). These experiments were carried out in the presence of a relatively high concentration of Ca^{2+}. When low concentrations of Ca^{2+} were used, Scatchard analysis again revealed two sites, one with low affinity (site III) and the other with high affinity (site IV) as shown in Fig. 4. For site III, the K_d was 0.6 μM and B_{max} was 3.4 nmol/mg and for site IV, K_d was 23 nM and B_{max} was 0.85 nmol/mg. Again, KCl significantly reduced B_{max} for both sites, and again, phosphorylation had no effect. Similar experiments were carried out with the three preparations of differing degrees of purity, and again, no effect of phosphorylation on Ca^{2+} binding parameters was observed with any preparation (data not shown). The nature of the four binding sites for Ca^{2+} observed in these preparations has not yet been clarified, but previous studies in this laboratory of Ca^{2+} binding to a purified $(Na^+ + K^+)$-ATPase preparation from sheep kidney indicated that Ca^{2+} binds to phospholipids associated with the enzyme [22]. The possibility that some Ca^{2+} binds to protein in these preparations cannot, however, be ruled out.

Discussion

Wollenberger and his colleagues [7—10] first reported that an NaI-treated (Na^+ + K^+)-ATPase preparation isolated from pig heart by the procedure of Matsui and Schwartz [11] was phosphorylated by cyclic AMP-dependent protein kinase. They further reported that their preparation was able to bind calcium and identified two classes of calcium binding sites (sites I and II) and found that cyclic AMP-dependent phosphorylation of the enzyme by an endogenous protein kinase increased the affinity of the enzyme for calcium at both sites.

We have also identified two classes of calcium binding sites in our beef heart preparations, but could find no effect of phosphorylation on either K_d or B_{max} for calcium binding. The maximum amount of phosphorylation reported by Wollenberger and his colleagues (123 pmol/mg protein) was lower than that usually obtained by us. Consequently, the absence of an effect on calcium binding cannot have been due to failure to phosphorylate the enzyme. We have also used pig heart (data not shown) as a source of the enzyme because we were unable to reproduce the findings of Wollenberger and his colleagues using the beef heart enzyme, but were similarly unable to find an effect of cyclic AMP and protein kinase on calcium binding.

At free Ca^{2+} concentrations below 10^{-6} M our data are in good agreement with those of Will et al. [7]. The K_d for our low affinity site (site III) was 0.6—1.2 μM compared to 0.26 and 1.1 μM for the native and phosphorylated enzymes of Will et al. [7] (their site II). For the high affinity site (site IV), the K_d was 0.02—0.048 μM compared to 0.023 and 0.06 μM for the native and phosphorylated enzymes of Will et al. [7].

In view of the close agreement of the K_d values for the calcium binding sites, it is curious that we did not observe the large changes in these values when the enzyme was phosphorylated. A possible explanation for this is that the specific activity of our pig heart preparation was 56 μmol/mg per h while that employed by Will et al. [7] was 18 μmol/mg per h. Their preparation was therefore about one-third as pure as ours and may have contained proteins not found in our preparations. The substrate for phosphorylation in their preparation had a molecular weight of 24 000 [10] while even our crudest preparations contained two substrate proteins, with molecular weights of 11 700 and 16 600 [18]. While measurements of molecular weight by sodium dodecyl sulfate gel electrophoresis are inaccurate for proteins below 20 000, it seems unlikely that their protein kinase substrate with a molecular weight of 24 000 was present in our preparations. It is of interest that Katz et al. [5] observed phosphorylation of a 24 000 dalton component of cardiac sarcoplasmic reticulum (which they named phospholamban) which stimulated calcium uptake by the sarcoplasmic reticulum. We have been able to duplicate these results [6]. This leads us to speculate that the preparation used by Will et al. [7] may have contained "phospholamban" and this may have caused the changes in K_d for calcium binding (possibly due to the presence of calcium binding components of sarcoplasmic reticulum in their preparation). Wollenberger et al. [9] in a recent publication, pointed out that ". . . .doubt is raised about the purity of cardiac sarcoplasmic reticulum and sarcolemmal preparations obtainable by present methods. . . .". The present study supports this caveat and demon-

strates clearly that, although a variety of membrane preparations containing ($Na^+ + K^+$)-ATPase activity can be phosphorylated by protein kinase and that this phosphorylation is indeed markedly stimulated by cyclic AMP, no known function or characteristic of these preparations, such as calcium binding or ($Na^+ + K^+$)-ATPase activity, is necessarily altered.

Acknowledgements

This study was supported in part by National Institutes of Health Grant No. HL 7906-13 and Contract NO1 HV52998 and by the Section of Myocardial Biology of the National Heart and Blood Vessel Research and Demonstration Center, Baylor College of Medicine, a grant-supported research project of the National Heart, Lung and Blood Institute, National Institutes of Health, Grant No. 17269-02.

References

1 Walsh, D.A. and Krebs, E.G. (1973) The Enzymes, Vol. 8, pp. 518—555, Academic Press, New York
2 Hofmann, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975) J. Biol. Chem. 250, 7795—7801
3 Krebs, K.C., Stull, J.T., England, P.J., Huang, T.S., Brostrom, C.O. and Vandenheede, J.R. (1973) Protein Phosphorylation in Control Mechanisms, Vol. 5, pp. 31—45, Academic Press, New York
4 Reddy, Y.S., Ballard, D., Giri, N.Y. and Schwartz, A. (1973) J. Mol. Cell. Cardiol. 5, 461—471
5 Katz, A.M., Tada, M. and Kirchberger, M.A. (1975) in Control of Calcium Transport in the Myocardium by the Cyclic Pump-Protein Kinase System (Drummond, G.I., Greengard, P. and Robinson, G.A., eds.), Vol. 5, pp. 453—490, Raven Press, New York
6 Schwartz, A., Entman, M.L., Kaniike, K., Lane, L.K., Van Winkle, W.B. and Bornet, E.P. (1976) Biochim. Biophys. Acta 426, 57—72
7 Will, H., Schirpke, B. and Wollenberger, A. (1973) Acta Biol. Med. Ger. 31, 45—52
8 Krause, E.G., Will, H. and Wollenberger, A. (1975) in Advances in Cyclic Nucleotide Research (Drummond, G.E., Greengard, P. and Robinson, G.A., eds.), Vol. 5, pp. 473—490, Raven Press, New York
9 Wollenberger, A., Will, H. and Krause, E.G. (1975) in Recent Advances in Studies on Cardiac Structure and Metabolism (Fleckenstein, S. and Dhalla, N.S., eds.), Vol. 5, pp. 81—93, University Park Press, Baltimore
10 Krause, E.G., Will, H., Pelouch, V. and Wollenberger, A. (1973) Acta Biol. Med. Ger. 31, 37—43
11 Matsui, H. and Schwartz, A. (1966) Biochim. Biophys. Acta 128, 380—390
12 Dowd, F. and Schwartz, A. (1975) J. Mol. Cell. Cardiol. 7, 483—497
13 Pitts, B.J.R., Lane, L.K. and Schwartz, A. (1973) Biochem. Biophys. Res. Commun. 53, 1060—1066
14 Pitts, B.J.R. and Schwartz, A. (1975) Biochim. Biophys. Acta 401, 184—195
15 Kuo, J.F., Krueger, B.K., Sanes, J.R. and Greengard, P. (1970) Biochim. Biophys. Acta 212, 79—91
16 Portzell, H., Caldwell, P.C. and Ruegg, J.C. (1964) Biochim. Biophys. Acta 79, 581—591
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Dowd, F.J., Pitts, B.J.R. and Schwartz, A. (1976) Arch. Biochem. Biophys. 173, 321—331
19 Ezrailson, E.G., Potter, J.D., Michael, L. and Schwartz, A. (1977) J. Mol. Cell Cardiol., in the press
20 England, P.J. (1975) FEBS Lett. 50, 57—60
21 Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3—134
22 Gervais, A., Lane, L.K., Anner, B.A., Lindenmayer, G.E. and Schwartz, A. (1976) Circ. Res. 40, 8—10