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MOLECULAR MECHANISM BY WHICH CYCLIC AMP REGULATES MYOCARDIAL CONTRACTILITY

by

Jean Marie Bidlack

Submitted in Partial Fulfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by: Dr. Adil E. Shamoo

Department of Radiation Biology and Biophysics

School of Medicine and Dentistry

The University of Rochester

Rochester, New York

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DISCLAIMER

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VITAE

The author was born on [redacted] in Rochester, New York. She attended the Blessed Sacrament and the Academy of the Sacred Heart schools and graduated from Brighton High School in 1971. That fall she entered Skidmore College in Saratoga Springs, New York. During her senior year, she was awarded the Periclean Scholar Award for the best undergraduate thesis, based on research on programmed cell death done under the direction of Dr. Richard Lockshin in the Physiology Department at the University of Rochester. She also conducted a study on the excitation-contraction coupling in the frog conus arteriosus, the only pathway out of the frog heart. Both projects resulted in publications. In May 1975, she was awarded a Bachelor of Arts degree in Biology-Chemistry.

In the fall of 1975, the author started graduate work in the Department of Radiation Biology and Biophysics at the University of Rochester. She received a Masters of Science degree in Biophysics in 1977. During the last half of 1977, she accompanied her advisor, Dr. Adil E. Shamoo, on a sabbatical leave to the Max-Planck-Institut fur Biophysik in Frankfurt, West Germany. During her work on cyclic AMP-dependent phosphorylation of cardiac sarcoplasmic reticulum, the author was supported by a NIH Biophysics Training Grant.

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ABSTRACT

The addition of cyclic AMP - dependent protein kinase and cyclic AMP to canine cardiac sarcoplasmic reticulum is known to increase Ca^{2+} transport into the sarcoplasmic reticulum. The mechanism of this enhanced transport is investigated here. Cyclic AMP - dependent protein kinase phosphorylates a 6,000 and a 22,000 dalton protein as determined by SDS - polyacrylamide slab gel electrophoresis and autoradiography. Maximal phosphorylation occurs when protein kinase and cyclic AMP are incubated with the microsomes. However, the isolated sarcoplasmic reticulum does contain endogenous adenylate cyclase and protein kinase, which phosphorylate both proteins. Phosphodiesterase completely inhibits phosphorylation. In the presence of a phosphatase inhibitor, the time course of the phosphorylation of the two proteins differs. The 22,000 dalton protein is phosphorylated more rapidly than the 6,000 dalton protein. Once phosphorylated the 22,000 dalton protein is soluble in acidified chloroform:methanol, while the 6,000 dalton protein is not. Prior to phosphorylation, both proteins can be digested by trypsin and cannot be phosphorylated later. When phosphorylated first, both proteins are resistant to digestion by trypsin. Prior to phosphorylation, both proteins are soluble in a low concentration of the detergent, deoxycholate (DOC). After phosphorylation, neither protein can be solubilized by DOC. Phosphorylation appears to cause the proteins to become buried in the membrane.

By employing very low concentrations of DOC (less than 1 μg DOC/mg microsomal protein), purification of the 22,000 dalton protein and the Ca^{2+} + Mg^{2+}-ATPase has been accomplished. After solubilizing the extrinsic membrane proteins including the 22,000 dalton protein by a low concentration of DOC, passage of these proteins through a Sephadex G - 75 column results in the purification of the 22,000 dalton protein. The protein is still specifically phosphorylated by cyclic AMP - dependent protein kinase, incorporating approximately 0.15 moles of phosphate/mole of protein. Approximately 5 moles of phospholipid are bound to 1 mole of purified protein.

The Ca^{2+} + Mg^{2+}-ATPase is purified by first solubilizing the extrinsic proteins with DOC. Then the addition of an increasing amount of DOC to the pellet from the first solubilization results in the solubilization and purification of the Ca^{2+} + Mg^{2+}-ATPase to at least 95% purity. The Ca^{2+} concentration required for half maximal activity of the ATPase is approximately 5.2 μM. The purified ATPase hydrolyzes ATP at the rate of 2.47 μmole Pi/mg/min.

When the purified Ca^{2+} + Mg^{2+}-ATPase is reconstituted into asolectin vesicles, the ATPase actively transports Ca^{2+} into the vesicles, with the transport dependent on the presence of ATP. When the 22,000 dalton protein is reconstituted with the ATPase, no change in the transport of Ca^{2+} is observed during a short time course. However, when phosphate is included inside the vesicles to precipitate entering Ca^{2+}, maximal transport is observed when the ATPase and the phosphorylated 22,000 dalton protein are reconstituted together. The reconstituted ATPase and the nonphosphorylated 22,000 dalton protein do

not transport Ca^{2+} at a greater rate than that observed by the ATPase alone. The phosphorylated 22,000 dalton protein reconstituted without the ATPase, facilitates the transport of Ca^{2+}. The enhanced transport seen when the phosphorylated 22,000 dalton protein and the ATPase are reconstituted together is an additive effect of the two individual processes. The phosphorylated 22,000 dalton protein does not regulate the ATPase's ability to transport Ca^{2+}, but instead is capable of transporting Ca^{2+}, itself. In vesicles without phosphate inside, the initial rate of Ca^{2+} - uptake for the reconstituted 22,000 dalton protein is approximately 8 times greater for the phosphorylated protein than the nonphosphorylated one. Including phosphatases in the uptake medium reduces the uptake of the originally phosphorylated 22,000 dalton protein to that of the nonphosphorylated protein. Ba^{2+}, Sr^{2+}, and Mn^{2+} have virtually no effect on the transport of Ca^{2+} into the vesicles. Zn^{2+} inhibits transport by approximately 30% and Hg^{2+} and ruthinum red inhibit Ca^{2+} transport almost completely.

As a result of these experiments, it appears that the phosphorylated 22,000 dalton protein does not regulate the transport properties of the ATPase. Instead phosphorylation of the 22,000 dalton protein causes it to become buried in the membrane, transporting Ca^{2+} into the sarcoplasmic reticulum and thereby, elevating the Ca^{2+} concentration in the sarcoplasmic reticulum available for release to the myofibrils.

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BACKGROUND

Effects of β-Adrenergic Agonists on Myocardial Contractility

Classification of adrenergic receptors was first suggested by Dale in 1906 (Dale, 1906), who showed that preparations of ergot abolished the motor effects of adrenaline or sympathetic stimulation. Ahlquist subsequently subclassified the adrenergic receptors as alpha and beta receptors (Ahlquist, 1948). The alpha adrenotropic receptors are associated with most of the excitatory functions (vasocontriction, and stimulation of the uterus, ureter and dilator pupillae) and one important inhibitory function (intestinal relaxation). The beta adrenotropic receptors are associated with most of the inhibitory function (vasodilation, and inhibition of the uterine and broncial musculature) and one excitatory function (myocardial stimulation).

The mechanical responses of cardiac and skeletal muscle to β-adrenergic agonists can be summarized as follows: in cardiac muscle, the rate of tension rise and the total tension are increased while the rate of relaxation is accelerated (Rolett, 1974); in slow skeletal muscle, the rate of relaxation is increased (Bowman and Nott, 1969), while in fast skeletal muscle, the relaxation rate is decreased (Marsden and Meadows, 1970).

Since the discovery of adenosine 3':5'-monophosphate (cyclic AMP) during the late 1950s by Sutherland and Rall (1958), there has been continued interest in the possible involvement of cyclic AMP in the adrenergic responses. Beta-adrenergic amines are known to significantly elevate the intracullular level of cyclic AMP in cardiac muscle

(Drummond et al., 1969; Drummond and Hemmings, 1973; Kukovetz et al., 1975; Ones and Øye, 1975; Posner et al., 1965). A second-messenger hypothesis (Sutherland et al., 1968; Epstein et al., 1971) involves a sequence of events connecting the appearance of the β-agonist with the eventual increase in contractility: a) catecholamine diffusion to the vicinity of the cell membrane; b) binding to the β-receptor; c) activation of adenyl cyclase; d) elevation of cyclic AMP concentration; e) diffusion of cyclic AMP to its sites of action: f) modification of some aspect(s) of excitation-contraction coupling; g) enhancement of systolic calcium concentration; and h) greater contractile strength (Tsien, 1977). Drummond et al. (1966) found that the level of cyclic AMP in cardiac cells increased by nine-fold within 1 sec after epinephrine administration.

Attempts have been made to see if cyclic AMP can produce the same physiological responses in cardiac tissues that catecholamines do. Exposure of a perfused heart to cyclic AMP did not increase twitch tension (Rall and West, 1963; Robison et al., 1965). Reasoning that this lack of effectiveness might be due to a slow entry of cyclic AMP into heart cells, other investigators turned to acyl substituted derivatives, such as $N^6,2^1$-O-dibutyryl cyclic AMP (DBcAMP), in the hope that the lipophilic side chains would facilitate movement across the cell membrane (Posternak et al., 1962) or provide temporary protection against degradation by phosphodiesterase (Henion et al., 1967). It is now generally agreed that DBcAMP does give adrenaline-like effects in increasing the strength of contraction (Skeleton et al., 1970;

Kukoveta and Poch, 1970), and in some cases, shortening the duration of the twitch (Meinertz et al., 1975).

Skinned cardiac cells have been used to study the function of cyclic AMP. Skinned cardiac preparations have been produced by EDTA treatment (Winegrad, 1971) or by microdissection (Fabiato and Fabiato, 1973). These approaches allow rapid acceleration of substances like cyclic AMP to the myoplasmic space. They also eliminate steps in excitation-contraction coupling which involve the surface membrane. Fabiato and Fabiato (1975) found that a twitch-like force response was stimulated in the presence of cyclic AMP. The rate of relaxation was also enhanced by cyclic AMP. The skinned cell procedure should also be useful in dissecting subcellular mechanisms for cyclic AMP action.

Another useful procedure for directly introducing cyclic AMP to cardiac cells has been the cut-end procedure (Tsien and Weingart, 1976). This method takes advantage of the fact that cardiac cells are connected by nexuses, low-resistance junctions, which allow small molecules to move from cell to cell. One end of a muscle strip was loaded with cyclic AMP. The cyclic AMP moved longitudinal along the muscle, reaching the "test" part of the muscle in 40-50 min after loading (Tsien, 1977). The inotropic effect seen with epinephrine was also seen by this method using cyclic AMP (Tsien and Weingart, 1976). Thus, from these studies, it can be concluded that cyclic AMP is the active messenger from catecholamines, being capable of producing the same physiological effects that catecholamines do. The catecholamines acting through cyclic AMP appear to regulate Ca^{2+} transport in cardiac cells.

Calcium Transport in Myocardial Cells

Calcium has been shown to play a dominant role in myocardial contractility (Katz, 1970). In mammalian heart, full contractile activity occurs when 50 to 100 nmoles of calcium per gram weight of ventricular tissue are made available to troponin, the calcium receptor protein of the contractile system (Ebasi and Endo, 1968; Katz, 1970; Shigekawa et al., 1976; Solaro et al., 1974). During each cardiac cycle at maximal contractility, this amount of calcium must first bind to and then be removed from the regulatory sites of troponin. Movement of this activator calcium within the myocardial cell is controlled by the sarcoplasmic reticulum (Langer, 1973; Legato and Langer, 1969; Martonosi, 1972; Weber, 1966), a membraneous intracellular structure which surrounds the myofibrils, by the cell surface membrane (Langer, 1973; Reuter, 1973), and possibly by the mitochondria (Horn et al., 1971; Lehninger, 1974; Patriarca and Carafoli, 1968).

It is generally believed that a signal generated by an action potential is transmitted to the interior of the cell, either by propagation of an intracellular message to the heart's sarcoplasmic reticulum or by entry of calcium from the extracellular fluid (or both), leading ultimately to an increase in the calcium concentration around the contractile proteins. This calcium initiates systole by binding to troponin A, one of the constituents of troponin, which has been identified as the calcium receptor of the contractile apparatus (Katz, 1971). Relaxation occurs when the calcium concentration in the myoplasm falls to a level too low to support contraction. The decrease

in the calcium concentration in the myoplasm is believed to be achieved by the resequestering of calcium into the sarcoplasmic reticulum and the movement of calcium out of the cell.

The sarcotubular system in skeletal muscle is highly developed and is capable of releasing large quantities of calcium in response to the depolarization of the sarcolemmal membranes as well as of sequestering this cation from the sarcoplasm during muscle relaxation (MacLennan and Holland, 1975). In cardiac muscle, the sarcoplasmic reticulum is sparse compared to that in skeletal muscle (Langer and Brady, 1974). As a result of the sparsity of sarcoplasmic reticulum, the possibility exists that the limited calcium stores of the cardiac sarcoplasmic reticulum may not fully saturate all the potential sites of actomyosin interactions. Evidence is available which shows rapid calcium fluxes across cardiac sarcolemma and that heart contractility is augmented by increased filling of sarcoplasmic reticulum during the plateau phase of the cardiac action potential (Langer, 1973; Morad and Goldman, 1973; Repke and Katz, 1969; Reuter, 1974; Trautwein, 1973). Cardiac muscle also contains abundant mitochondria, which display *in vitro* active calcium accumulation (Carafoli, 1975). However, to what extent these organelles contribute to the regulation of intracellular calcium in heart still remains an unanswered question.

Isolated cardiac sarcoplasmic reticulum vesicles can accumulate calcium against a concentration gradient in the presence of ATP and magnesium (Fanburg *et al.*, 1964; Hasselbach and Makinose, 1961; Inesi *et al.*, 1964; Katz and Repke, 1967; Weber *et al.*, 1964). Calcium accumulation by cardiac microsomes is coupled to ATP hydrolysis, which

is catalyzed by a membrane-bound Ca^{2+} + Mg^{2+}-ATPase. As in microsomes prepared from fast skeletal muscle (Hasselbach and Makinose, 1961; Weber, 1966), a 2:1 stoichiometric relationship between the amount of calcium taken up and ATP hydrolyzed is found in fresh cardiac microsomes (Tada et al., 1974). ATP hydrolysis by cardiac sarcoplasmic reticulum has been reported to involve a phosphoprotein intermediate (Fanburg and Matsushita, 1973; Namm et al., 1972) and to proceed by a mechanism similar to that of the more completely characterized sarcoplasmic reticulum of fast skeletal muscle (Hasselbach, 1972; Inesi, 1972; Martonosi, 1972). The rates of calcium transport and the concomitant Ca^{2+} -dependent hydrolysis of ATP, however, are considerably lower in cardiac than in skeletal muscle microsomes (Harigaya and Schwartz, 1969; Martonosi, 1972; Weber, 1966). The difference in these rates appears to be due to significant quantitative difference existing between several kinetic properties of these calcium transport ATPases. The concentration of phosphorylation sites in the cardiac microsomes has been found to be about four times lower than that for fast skeletal microsomes (Shigekawa et al., 1976). The turnover rates of these sites, however, did not differ significantly. The slower rate of calcium transport by cardiac microsomes, therefore, reflects primarily a lower density of calcium pumping sites that have a lower affinity for calcium than the corresponding sites in fast skeletal muscle.

Regulation of Calcium Transport in the Sarcoplasmic Reticulum by Cyclic AMP

Cardiac microsomes contain adenylate cyclase activity which responds to β-adrenergic amines and other hormones (Dhalla et al., 1970; Dhalla et al., 1973; Entman et al., 1969; Katz et al., 1974, Sulakhe and Dhalla, 1973). In addition, cyclic AMP has been shown to be specifically localized in the area of the sarcoplasmic reticulum (Ong and Sceiner, 1977). Since the central role of the sarcoplasmic reticulum appears to be the regulation of calcium concentrations in contractile tissues, the mechanism(s) by which calcium fluxes across the sarcoplasmic reticulum are regulated is being investigated in many laboratories.

Cyclic AMP is known to activate soluble protein kinase by inducing dissociation into cyclic AMP-binding regulatory and cyclic AMP-independent catalytic subunits (Krebs, 1972), while the heat stable protein inhibitor (Ashley and Walsh, 1973) binds tightly to the catalytic subunit and blocks the catalytic activity of the purified enzyme. Through studies concerning the interaction between cyclic AMP and sarcoplasmic reticulum, it has become increasingly evident that the phosphorylation of membranes by cyclic AMP-dependent protein kinases may represent one such regulation of the calcium fluxes. When isolated cardiac microsomes were incubated in the presence of protein kinase and cyclic AMP, the initial rates of both Ca^{2+} uptake and ATPase activity were enhanced (Kirchberger et al., 1974; Kirchberger et al.,

1975; Tada et al., 1975; Tada et al., 1974). These findings indicate that cyclic AMP-dependent protein kinase can increase the rate of calcium transport in the sarcoplasmic reticulum.

Tada and Kirchberger then proceeded to see which protein(s) were phosphorylated by cyclic AMP-dependent protein kinase. Microsomes incubated in the presence of cyclic AMP, protein kinase and [γ-^{32}P] ATP were electrophoresed on a sodium dodecyl sulfate polyacrylamide slab gel. A protein component of approximately 22,000 daltons was found to bind most of the ^{32}P label (Tada et al., 1975). This 22,000 dalton phosphoprotein had stability characteristics of a phosphoester bond rather than an acyl phosphate (Tada et al., 1975).

A procedure for the isolation of the 22,000 dalton protein will be presented here. It will also be shown that the 22,000 dalton protein can facilitate transport of Ca^{2+} into reconstituted vesicles. The ability of the 22,000 dalton protein to transport Ca^{2+} is dependent on the phosphorylation of the protein. In addition, another protein with a molecular weight of 6,000 is also being identified as being specifically phosphorylated by cyclic AMP-dependent protein kinase. The further characterization of these phosphoproteins will aid in understanding the molecular mechanism by which cyclic AMP regulates myocardial contractility.

REFERENCES

Ahlquist, R.P. (1948) A study of the adrenotropic receptors. Am. J. Physiol. 153: 586-600

Bowman, W.C. and Nott, M.W. (1969) Actions of sympathomimetic amines and their antagonists on skeletal muscle. Pharmac. Rev. 21: 27-72

Carafoli, E. (1975) The role of mitochondria in contraction-relaxation cycle and other Ca^{2+} -dependent activites of heart cells. In "Recent Advances in Studies on Cardiac Structure and Metabolism". (Fleckenstein, A. and Dhalla, N.S., eds.). Vol. 5, pp. 151-163. Univ. Park Press, Baltimore

Dale, H.H. (1906) On some physiological action of ergot. J. Physiol. 34: 163-206

Drummond, G.I., Duncan, L. and Hertzman, E. (1966) Effect of epinephrine on phosphorylase β kinase in perfused rat hearts. J. Biol. Chem. 241: 5899-5903

Drummond, G.I., Harwood, J.P. and Powell, C.A. (1969) Studies on the activation on phosphorylase in skeletal muscle by contraction and epinephrine. J. Biol. Chem. 244: 4235-4240

Drummond, G.I. and Hemmings, S.J. (1973) Role of adenylate cyclase-cyclic AMP in cardiac actions of adrenergic amines. In "Recent Advances in Studies on Cardiac Structure and Metabolism". (Dhalla, N.S., ed.) Vol. 3, pp. 213-222. Univ Park Press, Baltimore

Ebasi, S. and Endo, M. (1968) Calcium ion and muscular contraction. Prog. Biophys. Mol. Biol. 18: 123-183

Epstein, S.E., Levey, G.S. and Skeleton, C.L. (1971) Adenyl cyclase and cyclic AMP. Circulation 43: 437-450

Fabiato, A. and Fabiato, F. (1973) Activation of skenned cardiac cells: Subcellular effects of cardioactive drugs. Eur. J. Cardiol. 1: 143-155

Fabiato, A. and Fabiato, F. (1975) Relaxing and inotropic effects of cyclic AMP on skinned cardiac cells. Nature (Lond.) 253: 556-558

Fanburg, B., Finkel, R.M. and Martonosi, A. (1964) The role of calcium in the relaxation of cardiac muscle. J. Biol. Chem. 239: 2298-2306

Fanburg, B.L. and Matsushita, S. (1973) Phosphorylated intermediate of ATPase of isolated cardiac sarcoplasmic reticulum. J. Mol. Cell. Cardiol. 5: 111-115

Krebs, E.G. (1972) Protein kinases. Curr. Top. Cell. Regul. 5: 99-133

Langer, G.A. and Brady, A.J. (1974) "The Mammalian Myocardium". Wiley, New York

Legato, M.J. and Langer, G.A. (1969) The subcellular localization of calcium ion in mammalian myocardium. J. Cell Biol. 41: 401-423

Lehninger, A.L. (1974) Ca^{2+} transport by mitochondria and its possible role in the cardiac contraction - relaxation cycle. Circ. Res. 35: Suppl. 3, 83-90

MacLennan, D.H. and Holland, P.C. (1975) Calcium transport in sarcoplasmic reticulum. Ann. Rev. Biophys. Bioeng. 4: 377-404

Marsden, C.D. and Meadows, J.C. (1970) The effect of adrenaline on the contraction of human muscle. J. Physiol. 207: 429-448

Martonosi, A. (1972) Biochemical and clinical aspects of sarcoplasmic reticulum function. In "Current Topics in Membranes and Transport". (Bronner, F. and Kleinzeller, A., eds.). Vol. 3, pp. 83-197 Academic Press, New York

Meinertz, T., Nawrath, H. and Scholz, H. (1975) Relaxant effects of dibutyryl cyclic AMP on mammalina cardiac muscle. J. Cyclic Nucleotide Res. 1: 31-36

Morad, M. and Goldman, Y. (1973) Excitation - contraction coupling in heart muscle: Membrane control of development of tension. Progr. Biophys. Mol. Biol. 27: 257-412

Namm, D.H., Woods, E.L. and Zucker, J.L. (1972) Incorporation of the terminal phosphate of ATP into membranal protein of rabbit cardiac sarcoplasmic reticulum. Circ. Res. 31: 308-316

Ones, J.B. and Øye, I. (1975) Relationship between cyclic AMP metabolism and inotropic response of perfused rat hearts to phenylephrine and other adrenergic amines. In "Recent Advances in Cyclic Nucleotide Research". (Drummond, G.I., Greengard, P. and Robison, G.A., eds.). Vol. 5, pp. 415-433

Posternak, T., Sutherland, E.W. and Henion, W.F. (1962) Derivatives of cyclic 3',5'- adenosine monophosphate. Biochim. Biophys. Acta 65: 558-560

Patriarca, P. and Carafoli, E. (1968) A study of the intracellular transport of calcium in rat heart. J. Cell Physiol. 72: 29-37

Posner, J.B., Stern, R. and Krebs, E.G. (1965) Effects of electrical stimulation and epinephrine on muscle phosphorylase, phosphorylase b kinase and adenosine 3',5', phosphate. J. Biol. Chem. 240: 982-985

Harigaya, S. and Schwartz, A. (1969) Rate of calcium binding and uptake in normal and failing human cardiac muscle. Circ Res. 25: 781-794

Hasselbach, W. (1972) In "Molecular Bioenergetics and Macromolecular Biochemistry". (Weber, H.H., ed.). pp. 149-171. Springer-Verlag, Berlin

Hasselbach, W. and Makinose, M. (1961) Die calciumpumpe der „Erschlaffungsyrana des Muskels und ihre Abhangigbeit von der ATP - spaltung. Biochem. Z. 333: 518-528

Henion, W.F., Sutherland, E.W. and Posternak, T. (1967) Effects of derivatives of adenosine 3',5'-phosphate on liver slices and intact animals. Biochim. Biophys. Acta 148: 106-113

Horn, R.S., Fyhn, A. and Haugaard, N. (1971) Mitochondrial calcium uptake in the prefused contracting heart and the influence of epinephrine on calcium exchange. Biochim. Biophys. Acta 226: 459-466

Inesi, G. (1972) Active transport of calcium ions in sarcoplasmic membranes. Ann. Rev. Biophys. Bioeng. 1: 191-210

Inesi, G., Ebasi, S. and Watanabe, S. (1964) Preparation of vesicular relaxing factor from bovine heart tissue. Am. J. Physiol. 207: 1339-1344

Katz, A.M. (1970) Contractile proteins of the heart. Physiol. Rev. 50: 63-158

Katz, A.M. (1971) Mechanical and biochemical correlates of cardiac contraction (II). Mod. Concepts of Cardiovascular Disease 40: 45-48

Katz, A.M. and Repke, D.I. (1967) Quantitative aspects of dog cardiac microsomal binding and calcium uptake. Circ. Res. 21: 153-162

Kukovetz, W.R. and Poch, G. (1970) Cardiostimulatory effects of cyclic 3',5'- adenosine monophosphate and its acylated derivatives. Naunyn Schmiedebergs Arch. Pharmacol. 266: 236-254

Kukovetz, W.R., Poch, G. and Wurm, A. (1975) Quantitative relations between cyclic AMP and contraction as affected by stimulators of adenylate cyclase and inhibitors of phosphodiesterase. In "Recent Advances in Cyclic Nucleotide Research". (Drummond, G.I., Greengard, P. and Robison, G.A., eds.) Vol. 5, pp. 395-414 Raven Press, New York

Langer, G.A. (1973) Excitation - contraction coupling. Ann. Rev. Physiol. 35: 55-86

Rall, T.W. and West, T.C. (1963) The potentiation of cardiac inotropic responses to norepinephrine by theophylline. J. Pharmacol. Exp. Ther. 139: 269-274

Repke, D.I. and Katz, A.M. (1969) Stabilization of cardiac microsomal calcium uptake by hypertonic sucrose solutions. Biochim. Biophys. Acta 172: 348-350

Reuter, H. (1973) Divalent cations as charge carriers in excitable membranes. Progr. Biophys. Mol. Biol. 26: 1-43

Reuter, H. (1974) Exchange of calcium ions in mammalian myocardium: Mechanism and physiological significance. Circ. Res. 34: 599-605

Robison, G.A., Butcher, R.W., Øye, I., Morgan, H.E. and Sutherland, E.W. (1965) The effects of epinephrine on adenosine 3',5'-monophosphate levels in the isolated perfused rat heart. Mol. Pharmacol. 1: 168-177

Rolett, E. (1974) Adrenergic mechanisms in mammalian myocardium. In "The Mammalian Myocardium". (Langer, G.A. and Brady, A.J., eds.), pp. 219-250. Wiley, New York.

Shigekawa, M., Finegan, J.M. and Katz, A.M. (1976) Calcium transport ATPase of canine cardiac sarcoplasmic reticulum. A comparison with that of rabbit fast skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 251: 6894-6900

Skeleton, C.L., Levey, G.S. and Epstein, S.E. (1970) Positive inotropic effects of dibutyryl cyclic adenosine 3',5'-monophosphate. Circ. Res. 26: 35-43

Solaro, R.J., Wise, R.M., Shiner, J.S. and Briggs, F.N. (1974) Calcium requirements for caridac myofibrillar activation. Circ. Res. 34: 525-530

Sutherland, E.W. and Rall, T.W. (1958) Fractionation and characterization of a cyclic adenine nucleotide formed by tissue particles. J. Biol. Chem. 232: 1077-1090

Sutherland,E.W., Robison, G.A. and Butcher, R.W. (1968) Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). Circulation 37: 279-306

Tada, M., Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974) The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate - dependent protein kinase. J. Biol. Chem. 249: 6174-6180

Trautwein, W. (1973) Membrane currents in cardiac muscle fibers. Physiol. Rev. 53: 793-835

Tsien, R.W. (1977) Cyclic - AMP and contractile activity in heart. Adv. Cyclic Nucleotide Res. 8: 363-420

Tsien, R.W. and Weingart, R. (1976) Inotropic effect of cyclic AMP in calf ventricular muscle studied by a cut end method. J. Physiol. 260: 117-141

Weber, A. (1966) Energized calcium transport and relaxing factors. In "Current Topics in Bioenergetics". (Sanadi, D.R., ed.) Vol. 1, pp. 203-254. Academic Press, New York

Weber, A., Herz, R. and Reiss, I. (1964) Role of calcium in contraction and relaxation of muscle. Fed. Proc. 23: 896-900

Winegrad, S. (1971) Studies of cardiac muscle with a high permeability to calcium produced by treatment with ethylene diaminetetraacetic acid. J. Gen. Physiol. 58: 71-93

CHAPTER 1

ADENOSINE 3':5'-MONOPHOSPHATE DEPENDENT-PHOSPHORYLATION OF A 6,000 AND A 22,000 DALTON PROTEIN FROM CARDIAC SARCOPLASMIC RETICULUM.

SUMMARY

In canine cardiac sarcoplasmic reticulum, adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase specifically phosphorylates two proteins, as seen by sodium dodecyl sulfate (SDS)-slab gel electrophoresis and autoradiography. One protein has a molecular weight ranging between 22,000 and 24,000 daltons and has previously been identified and named phospholamban (Tada et al., 1975). The other protein that the ^{32}P label incorporates into has a molecular weight of approximately 6,000 daltons. Like the 22,000 dalton protein, the 6,000 dalton protein has characteristics of phosphoester bonding. The time-dependent course of phosphorylation shows that initially the ^{32}P label is incorporated more rapidly into the 22,000 dalton protein than the 6,000 dalton protein, with both proteins reaching a steady state level of phosphorylation after 10 minutes of incubation. When both protein kinase and cyclic AMP are eliminated from the incubation medium, both the 22,000 dalton protein and the 6,000 dalton protein are still phosphorylated but only to about a quarter of the activity found when cyclic AMP and protein kinase are included in the incubation mixture. The addition of phosphodiesterase completely eliminates the phosphorylation of both proteins. These results are indicative of an adenylate cyclase and cyclic AMP-depdendent protein kinase system, being directly associated with the cardiac sarcoplasmic reticulum membrane. Treating the microsomes with trypsin prevents subsequent phosphorylation of

either protein. Phosphorylating the microsomes first, then treating with trypsin, renders both the 22,000 and 6,000 dalton proteins resistant to even prolonged trypsin attack. Unphosphorylated, both proteins are solubilized by a very low concentration of deoxycholate (DOC). After phosphorylation the proteins cannot be solubilized by DOC. Phosphorylation appears to greatly alter the physical properties of both these proteins.

Control experiments exclude the possibility that a lipid is being phosphorylated. After phosphorylation, the phosphorylated 22,000 dalton protein is separated from the 6,000 dalton protein by proteolipid extraction. After first treating the microsomes with methanol, the 22,000 dalton protein is soluble in acidified chloroform:methanol, while the 6,000 dalton protein remains insoluble. The finding that both proteins have much different biochemical properties when phosphorylated than not, may be relevant in how they regulate calcium transport in the sarcoplasmic reticulum.

INTRODUCTION

Cyclic AMP[1] is known to be a regulator of myocardial contractility (Rolett, 1974; Tsien, 1977). This regulation is believed to be achieved at least partly by regulating the calcium transport into the sarcoplasmic reticulum (Fabiato and Fabiato, 1975). Tada et al. (1974) and Kirchberger et al. (1972, 1974) have shown that calcium transport into the sarcoplasmic reticulum is stimulated by the presence of cyclic AMP and cyclic AMP-dependent protein kinase. If the sarcoplasmic reticulum transport calcium at a faster rate in the intact myocardium when cyclic AMP is present, the abbreviation of systole seen in the presence of catecholamines may be explained by this transport phenomena. Calcium is removed from troponin at a faster rate in the presence of cyclic AMP than under nonstimulated conditions (Katz et al., 1975; Tada and Kirchberger, 1975). Also, the rate of release of calcium from the sarcoplasmic reticulum is enhanced by the presence of cyclic AMP (Kirchberger and Wong, 1978). The amount of calcium stored in the sarcoplasmic reticulum could add to the amount of calcium available for delivery to the contractile proteins in subsequent contractions (Tada et al., 1978a). The enhanced rate of contractility and the augmentation produced by cyclic AMP can be explained by the enhanced transport of calcium by the sarcoplasmic reticulum. The

[1]The abbreviations used are : cyclic AMP, adenosine 3':5'-monophosphate; protein kinase, adenosine 3':5'-monophosphate-dependent protein kinase; EGTA, ethylene glycol bis (β-amino-ethyl)-N, N'-tetraacetic acid; DOC, deoxycholate; SDS, sodium dodecyl sulfate.

rate of ATP hydrolysis by the Ca^{2+}+Mg^{2+}-ATPase in the sarcoplasmic reticulum is also increased in the presence of cyclic AMP (Tada et al., 1974). However, the amount of phosphorylated intermediate from the ATPase did not vary with cyclic AMP (Tada et al., 1978, 1979).

To elucidate the molecular mechanism by which cyclic AMP enhances myocardial contractility, the phosphorylation of cardiac microsomes by cyclic AMP-dependent protein kinase has been investigated. A 22,000-dalton protein from cardiac sarcoplasmic reticulum has been shown to be phosphorylated specifically by cyclic AMP-dependent protein kinase (Tada et al., 1975). This phosphoprotein has characteristics of phosphoester bonding. The phosphate group is removed by phosphatases (Tada et al., 1975; Tada et al., 1978). In addition, Jones et al. (1979) have recently described the separation and characterization of the sarcoplasmic reticulum and the sarcolemmal membranes. They reported that cyclic AMP-dependent protein kinase stimulates the phosphorylation of a 20,000 and a 7,000 dalton protein in the sarcoplasmic reticulum and proteins of molecular weights, 165,000, 90,000, 56,000, 24,000 and 11,000 in the sarcolemmal membranes. The present communication confirms and extends the findings of Jones et al. (1979) and Bidlack and Shamoo (1979). In addition to the 22,000 dalton protein, another protein with a molecular weight of 6,000 is also specifically phosphorylated by cyclic AMP-dependent protein kinase. This protein has properties similar to the 22,000 dalton protein. Extrinsic before phosphorylation, the phosphorylation of both proteins causes them to become intrinsic, buried in the membrane.

EXPERIMENTAL PROCEDURE

MATERIALS

Bovine heart cyclic AMP-dependent protein kinase, sodium cyclic AMP, sodium cyclic GMP, disodium ATP, hydroxylamine-HCl (grade 1), trypsin and trypsin inhibitor, and all lipids used were purchased from Sigma Chemical Co.

[γ - ^{32}P] ATP, ammonium salt (20 mCi per mmol) was obtained from Amersham/Searle. Deoxycholate was purified by the method of MacLennan (1970).

METHODS

Isolation of Cardiac Microsomes

Cardiac microsomes were prepared from canine heart ventricle according to Harigaya and Schwartz (1969) with the following modifications. Hearts were removed from dogs anesthesized with Nembutal. The hearts were placed in ice-cold saline. Fat and connective tissue were removed and the ventricle was cut in small pieces. The heart muscle was homogenized in 10 mM $NaHCO_3$ in a Waring blendor. The suspension was centrifuged at 8,700 x g for 20 min. The supernatant was strained through 4 layers of cheesecloth and spun at 10,000 x g for 20 min. The supernatant was again strained through cheesecloth, followed by centrifugation at 37,000 x g for 30 min. The pellet was suspended in 20 mM tris-maleate, 0.6 M KCl, pH 6.8. This suspension was centrifuged at 100,000 x g for 20 min to remove the solubilized actomyosin. The pellet from this centrifugation was suspended in 0.25 M sucrose. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Phosphorylation of Sarcoplasmic Reticulum by Cyclic AMP - Dependent Protein Kinase

Cardiac microsomes were phosphorylated at 25° by a method similar to that used by Tada et al. (1975). Microsomes (1.25 mg/ml) were suspended in a reaction mixture consisting of 40 mM histidine buffer (pH 6.8), 0.12 M KCl, 0.5 mM $MgCl_2$, 20 µM [γ- ^{32}P] ATP (1-3 µCi/nmol), 2.5 mM EGTA, 5 mM NaN_3, 25 mM NaF, with and without 1 µM cyclic AMP or 1 µM cyclic GMP and protein kinase (0.125 mg/ml). When phosphorylase kinase or phosphodiesterase were added, they were included at a concentration of 0.325 mg/ml and 0.125 mg/ml, respectively. When Ca^{2+} was added, 391 mM EGTA and 125 mM $CaCl_2$ were used to give a final concentration of 1 µM Ca^{2+}. Phosphorylation was initiated by the addition of the radioactive ATP. The final volume was 125 µl. Unless otherwise stated, the reaction was stopped after 10 min. by the addition of 75 µl of the SDS - solubilizing buffer and incubated at 37° for 1 hr. In order to determine the extent of phosphorylation of endogenous substrates, aliquots containing 50 µg of microsomal protein were subjected to Laemmli SDS - slab gel electrophoresis.

SDS - Slab Gel Electrophoresis

Polyacrylamide slab gel electrophoresis was carried out according to the method of Laemmli and Favre (1978). Slab gels (1.5 x 120 mm) consisted of a 12% acrylamide running gel with a 6% acrylamide stacking gel. The samples to be analyzed by electrophoresis were solubilized in 2% SDS, 62.5 mM tris - HCl (pH 6.8), 1 mM EDTA, 3 mM LiCl, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue (SDS - solubilizing buffer) and incubated at 37^{o} for 1 hr. The solubilized protein was electrophoresed for 2 hrs at 150 V constant voltage. Gels were stained in 0.1% Coomassie blue, 10% acetic acid, 50% methanol overnight and diffusion destained in 10% acetic acid. The slab gels were dried on Whatman No. 5 paper under vacuum and were placed on Kodak NS-2T film for 2-7 days. The resulting autoradiograph revealed those proteins into which ^{32}P had been incorporated. The optical density of the bands on the film was measured with a Beckman densitometer and the areas under the peaks of the optical density tracings were used as a quantitative measure of incorporation of ^{32}P into the SR proteins. Apparent molecular weights were calculated from a graph of relative mobilities versus log of molecular weight. Molecular weight standards used were phosphorylase a (94,600), bovine serum albumin (68,000), catalase (57,500), lactate dehydrogenase (35,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), lysozyme (14,400), cytochrome c (12,600), PTH (9,800) and glucagon (3,400).

Characterization of the Phosphoproteins

Microsomes were phosphorylated as described above. During phosphorylation or after 10 min., the following agents were added directly to the reaction mixture as detailed below.

a. Treatment with Hydroxylamine -
Hydroxylamine and sodium acetate (pH 5.4) were added to the reaction mixture to give a final concentration of 0.8 M and 0.05 M, respectively, in a final volume of 200 µl. After an additional 10 min., 100 µl of the SDS - solublizing buffer was added, and the sample was electrophoresed.

b. Treatment with Protease Inhibitors -
Trypsin inhibitor or toluenesulfonyl fluoride were included in the phosphorylation medium. Trypsin inhibitor was used at a concentration of 0.05 mg/mg SR protein. Toluenesulfonyl fluoride was used at a concentration of 0.5 mM. After 10 min., the reaction was stopped with SDS solubilizing buffer as described above.

c. Proteolipid Extraction -
After phosphorylation, proteolipid extraction was done by a method similar to MacLennan (1974). After

10 min. of phosphorylation, 9 vol of methanol was added to a final concentration of 90% methanol. The sample was incubated for 40 min. at 25°. The suspension was then centrifuged at 1,000 x g for 10 min. The pellet was suspended in 5 vol of chloroform:methanol (2:1). The sample was incubated at 25° for 10 min. and was then centrifuged at 1,000 x g for 10 min. The pellet was suspended in 2 vol of acidified chloroform:methanol (2:1:10 mM HCl). After incubating for 30 min., the sample was centrifuged at 1,000 x g for 10 min. The supernatants from this spin were then treated with 7 vol of ethyl ether. After 30 min., the samples were centrifuged at 1,000 x g for 10 min. The pellets from this spin were resuspended in acidified chloroform:methanol and then reprecipitated by ethyl ether. The washed pellet was finally suspended in acidified chloroform:methanol. The supernatants and pellets from the various fractions were electrophoresed as described above, and subjected to thin layer chromatography as described below.

Thin-Layer Chromatography

The supernatants from the proteolipid extractions were dried under nitrogen and resuspended in the acidified chloroform:methanol. The samples were applied to a thin layer plate consisting of Silica G, and were fractionated with acidified chloroform:methanol (2:1: 10 mM HCl) as the solvent. The spots were stained with iodine vapor. After drying in an oven, a piece of Kodak X-omat X-ray film was laid on top of the plate. After 2-7 days the film was developed to see if any of the spots contained the ^{32}P label.

Trypsin Treatment

Microsomes (1 to 3 mg/ml) were digested with trypsin at 25^{o} in the phosphorylating buffer less cyclic AMP, protein kinase and ATP. The trypsin concentration was varied from 0.1 to 0.5 mg/ml. After 5 min of incubation, trypsin inhibitor was added at a ratio of trypsin to trypsin inhibitor of 1:2. After 5 min cyclic AMP, protein kinase and ATP were added and the phosphorylation was carried out as described above.

In experiments in which phosphorylated microsomes were treated with trypsin, microsomes were phosphorylated in the phosphorylation mixture described above. After 10 min trypsin was added at concentrations varying from 0.1 mg to 0.5 mg/ml. After 5 min the digestion was stopped by the addition of trypsin inhibitor followed by the SDS-solubilizing buffer. As a control, trypsin and trypsin inhibitor were added at the same time. The amount of phosphoproteins formed in these microsomes was determined by slab gel electrophoresis as described above.

DOC Treatment

Cardiac microsomes were phosphorylated as described above in a 1 ml final volume. After 10 min, DOC was added at concentrations varying from 0.01 mg to 1.0 mg DOC/mg protein. The mixture was incubated for 10 min and then centrifuged at 105,000 x g for 30 min. The pellet and the supernatant were suspended in the SDS-solubilizing buffer. Both samples were electrophoresed on a SDS-Laemmli slab gel.

When the microsomes were first treated with DOC and then phosphorylated, the following procedure was employed. Microsomes were suspended in the phosphorylating buffer as described above less the cyclic AMP, protein kinase and radioactive ATP. DOC was added at concentrations varying from 0.01 mg to 1.0 mg DOC/mg protein. After 10 min, cyclic AMP, protein kinase and ATP were added. The mixture was incubated for 10 min after which it was centrifuged at 105,000 x g for 30 min. The pellet was suspended in SDS-solubilizing buffer and the supernatant was diluted with the buffer. Both fractions were electrophoresed to determine whether DOC solubilized the phosphoproteins.

Effect of Cyclic AMP and Protein Kinase on the Phosphorylation of Lipids

Cardiolipin, phosphotidylinositol, phosphotidylserine, phosphotidylcholine, and phosphotidylethanolamine were dried under nitrogen. 1.7 mg of each lipid was used for the experiment. The lipids were incubated in the phosphorylation medium with and without cyclic AMP and protein kinase. After 10 min. the reaction was stopped by the addition of 0.5 ml chloroform:methanol (1:1). Samples were vortexed for 15 sec and centrifuged at 1,000 x g for 10 min. The water phase was removed. Care was taken not to upset the organic phase. Two ml of chloroform:methanol (1:1) was added to the sample plus the addition of 1 ml of water to yield 2 phases. The sample was centrifuged at 1,000 x g for 10 min. 100 µl of the organic phase was counted in a scintillation counter.

RESULTS

Sodium Dodecyl Sulfate - Polyacrylamide Slab Gel Electrophoresis of Cardiac Sarcoplasmic Reticulum

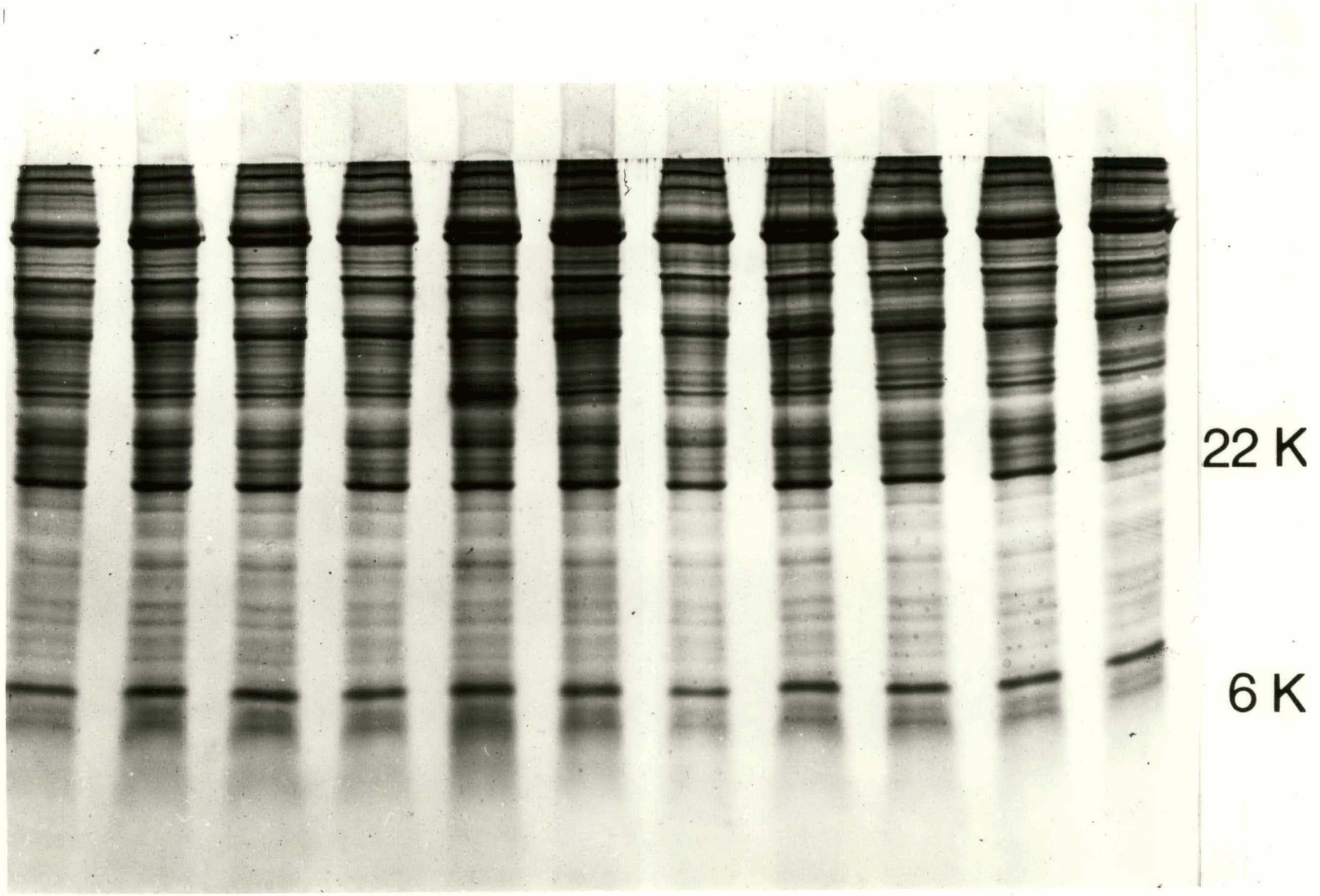
Fig. 1 shows a 12% SDS-polyacrylamide slab gel of cardiac microsomes. The 22,000 dalton protein, phospholamban, identified by Tada et al. (1975), appears as a distinct band on the slab gel. In this gel system, phospholamban runs with a molecular weight of 23,500 when compared to molecular weight standards. For consistency, phospholamban will be referred to as a 22,000 dalton protein in this paper. A 6,000 dalton protein, which is also phosphorylated by cyclic AMP-dependent protein kinase, appears as a diffuse band just below the major low molecular weight protein band. The 6,000 dalton protein appears in Coomassie blue stained gels only when a high concentration of methanol is present in the stain. In addition to these two proteins, the slab gel shows the presence of many proteins and especially higher molecular weight proteins not previously identified by disc gels (Tada et al., 1975).

Phosphorylation of a 6,000 dalton and a 22,000 dalton protein by cyclic AMP-dependent Protein Kinase

Slab gels have the distinction over disc gels in that many different conditions of phosphorylation can be compared directly by doing an autoradiogram of the slab gel. As can be seen in Fig. 2, maximal phosphorylation of the 22,000 dalton and 6,000 dalton protein occur when both cyclic AMP and protein kinase were included

FIGURE 1

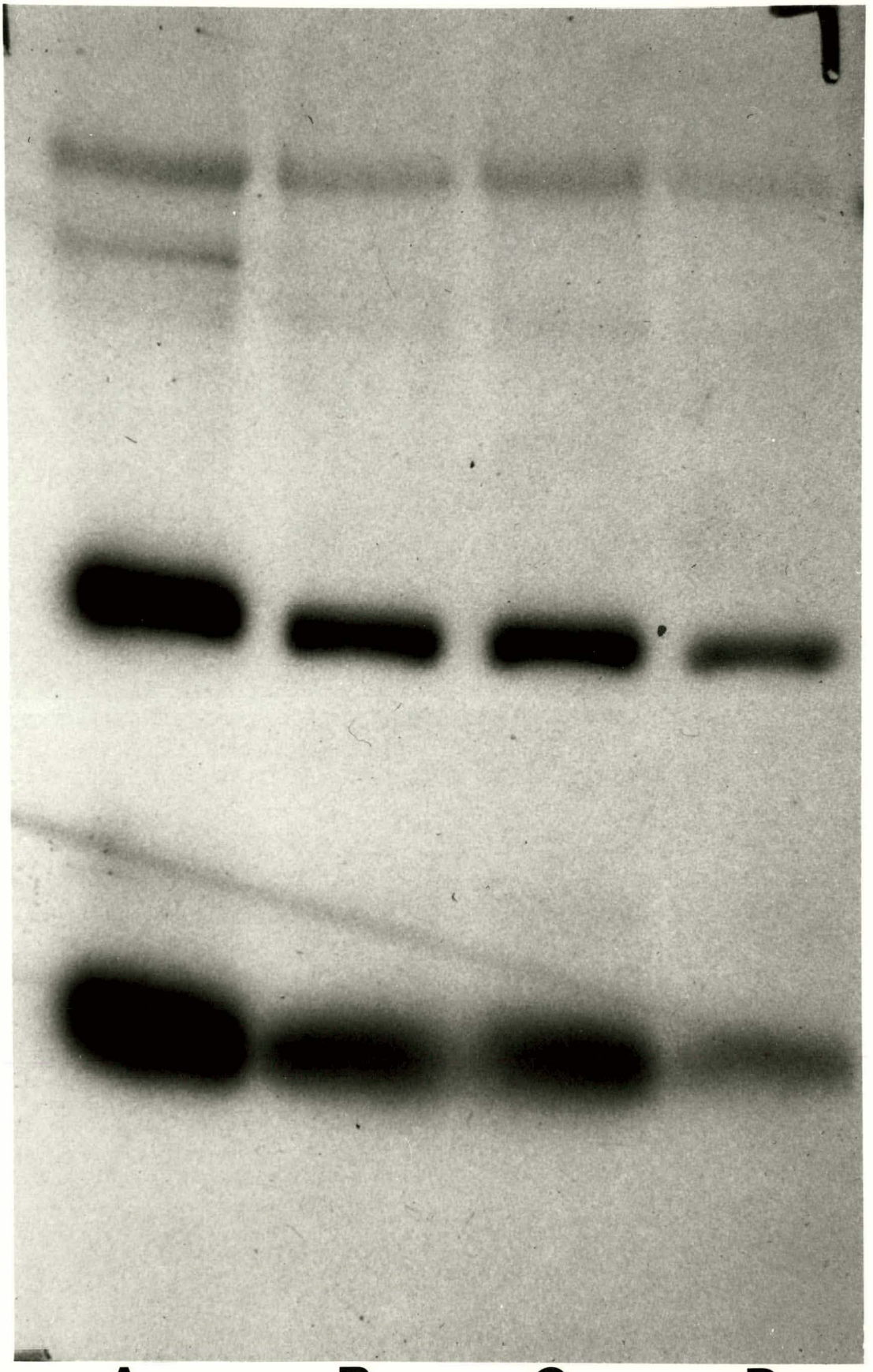
Coomassie blue stained SDS-12% polyacrylamide slab gel of cardiac microsomes. Cardiac microsomes (0.4 mg/ml) were incubated in 40 mM histidine, 0.12 M KCl, 0.5 mM $MgCl_2$, 2.5 mM EGTA, 5 mM NaN_3, 25 mM NaF and 20 μM [γ - ^{32}P] ATP was added to start the reaction. After 10 min, 75 μl of 2x Laemmli solubilizing solution was added. The mixture was incubated at 37^{o} for 1 hr, then 50 μl which contained approximately 50 μg protein was applied to the slab gel. The stacking gel contained 6% acrylamide, the running gel, 12%. Electrophoresis was carried out for 2 hr at 150 V, constant voltage. The gels were stained in 0.1% Coomassie blue, 10% acetic acid, 50% methanol and diffusion destained in 10% acetic acid.


22 K
6 K

79 5255

FIGURE 2

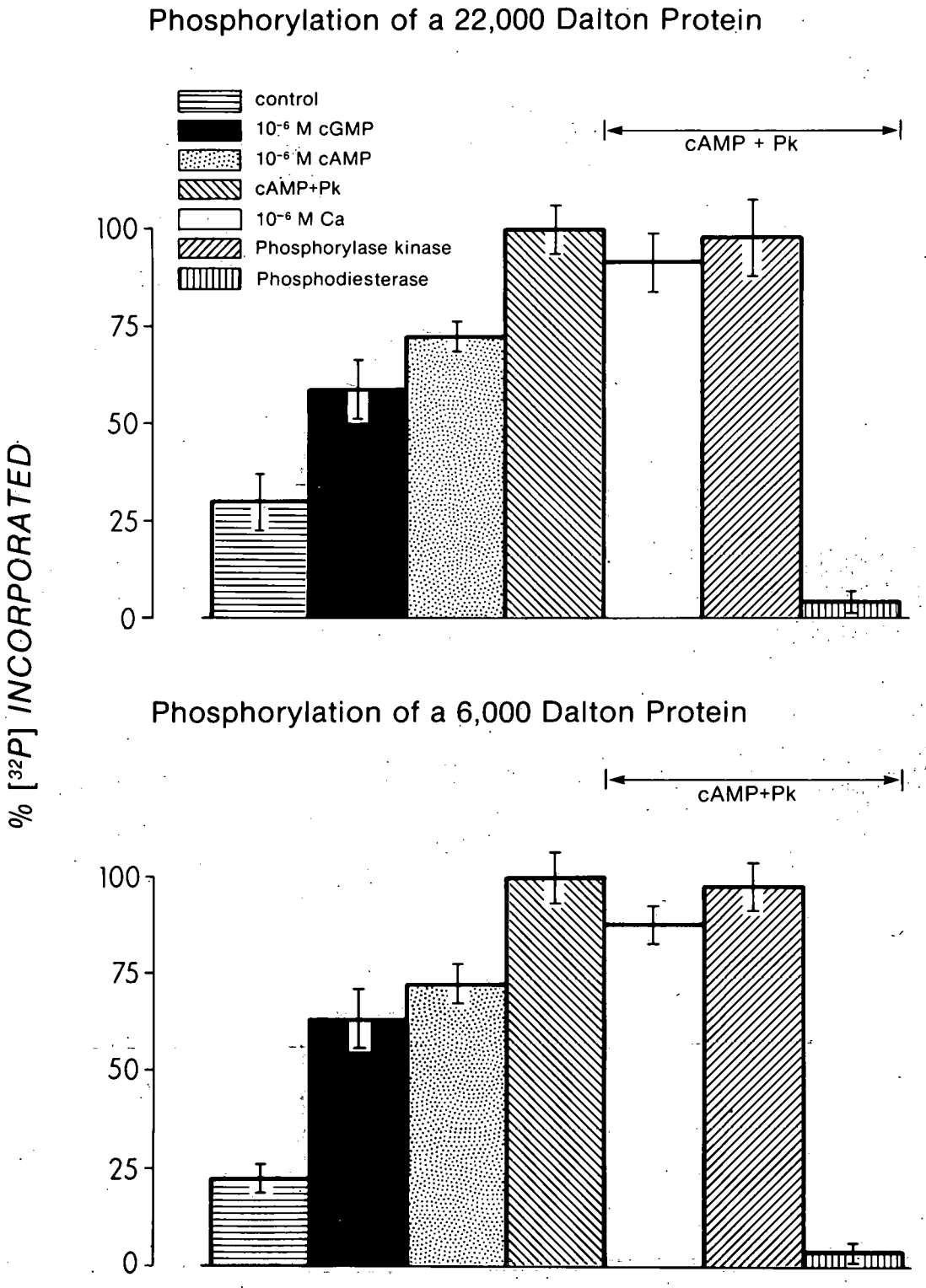
Autoradiogram of polyacrylamide SDS slab gel of cardiac microsomes. The microsomes were phosphorylated as described in "Methods". Electrophoresis was carried out on a 6% acrylamide stacking gel and 12% acrylamide running gel at a constant voltage, 150 V, for 2 hrs. The dried slab gel was exposed to Kodak NS-2T film for 2 days and developed. From left to right, A) Microsomes incubated with cyclic AMP and protein kinase, B) Microsomes incubated with cyclic AMP, C) Microsomes incubated with cyclic GMP, D) Microsomes incubated with no additions.


A
B
C
D

in the incubation medium. The top band is phosphorylase, and the band below it is protein kinase. When exogenous protein kinase was excluded, the 22,000 and 6,000 dalton proteins were still phosphorylated, but to a lesser extent than when it was added. Cyclic GMP did stimulate phosphorylation but not as well as cyclic AMP. When neither cyclic AMP or protein kinase was added, there was a basal level of phosphorylation of both proteins. The phosphorylation of the 22,000 and 6,000 dalton proteins is quantified in Fig. 3. The response of the 22,000 dalton and the 6,000 dalton protein to various conditions of phosphorylation was essentially identical. The basal level of phosphorylation of both proteins was about a quarter of the level attained when cyclic AMP and protein kinase are included in the incubation mixture. Cyclic GMP did stimulate phosphorylation but cyclic AMP has a more pronounced effect on phosphorylation. In the presence of cyclic AMP and protein kinase, the addition of phosphorylase kinase or 1 μM Ca^{2+} had little effect on the phosphorylation of either protein. Higher concentrations of Ca^{2+} did partially inhibit the phosphorylation of both proteins. Phosphodiesterase, which breaks down cyclic nucleotides, totally inhibited the phosphorylation of both proteins. Thus, associated with the isolated sarcoplasmic reticulum, there appears to be an endogenous adenylate cyclase system, capable of producing cyclic AMP, and an endogenous cyclic AMP-dependent protein kinase.

FIGURE 3

Bar graph quantifying the phosphorylation of the 22,000 and 6,000 dalton proteins. The top section represents the phosphorylation of the 22,000 dalton protein and the bottom section the phosphorylation of the 6,000 dalton protein. Quantitation was obtained by scanning autoradiograms on a Beckman densitometer. Maximal phosphorylation was set as 100%.


Phosphorylation of a 22,000 Dalton Protein
control
10⁻⁶ M cGMP
10⁻⁶ M cAMP
cAMP+Pk
10⁻⁶ M Ca
Phosphorylase kinase
Phosphodiesterase
cAMP + Pk
100
75
50
25
0
% [32P] INCORPORATED
Phosphorylation of a 6,000 Dalton Protein
cAMP+Pk
100
75
50
25
0

Time-dependent Course of Phosphorylation of the 22,000 and 6,000 Dalton Proteins

The autoradiograph in Fig. 4 shows the time-dependent phosphorylation of both proteins. At very early time points, the 22,000 dalton protein started to gradually become phosphorylated, while the 6,000 dalton protein did not. Only after about 1 min of incubation did the 6,000 dalton protein start to become phosphorylated. As the 6,000 dalton protein was starting to become phosphorylated, between 1 and 2 min, the phosphorylation of the 22,000 dalton decreased slightly. This result was reproducible, though its significance is not understood.

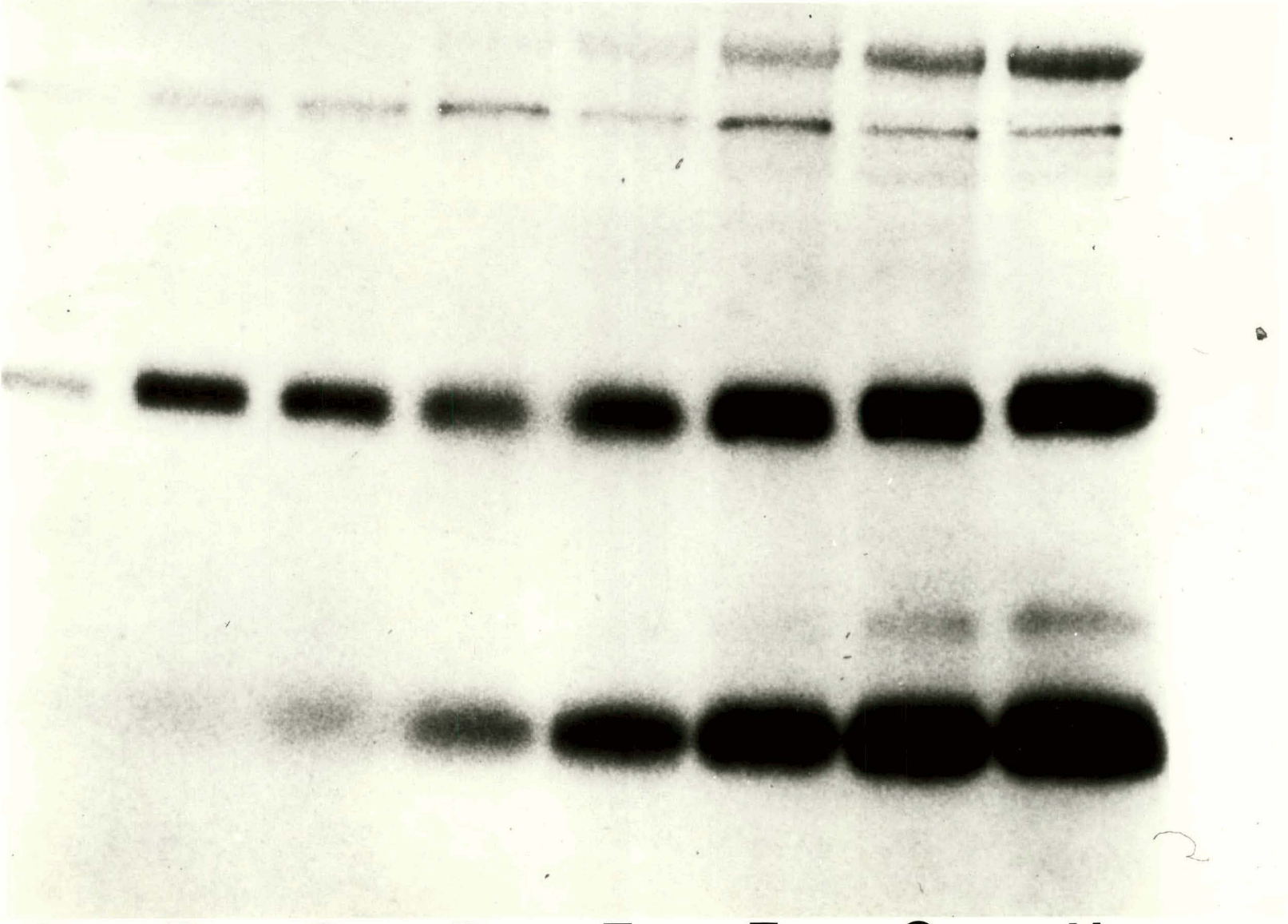
The top band on the autoradiogram is phosphorylase a. The phosphorylation of this protein also increased with time. The phosphorylation of this protein is achieved by cyclic AMP-dependent protein kinase phosphorylating phosphorylase kinase, which in turn phosphorylates phosphorylase a. The band below phosphorylase is protein kinase, whose phosphorylation remains rather constant with time; after 30 min of incubation there is a light band that corresponds to a molecular weight of 11,500 and is believed to be a contaminant of the plasma membrane.

Phosphorylation of Microsomes in the Presence of Protease Inhibitors

Since the phosphorylation of the 6,000 dalton proceeded more slowly than the phosphorylation of the 22,000 dalton protein, the possibility that the 6,000 dalton protein was a breakdown product of the 22,000 dalton protein was investigated. When the cardiac

FIGURE 4

Time-dependent phosphorylation of the 6,000 and 22,000 dalton proteins. The cardiac microsomes were phosphorylated as described in "Methods". The reaction was stopped after the addition of [γ - ^{32}P] ATP at various time points by adding 75 µl of 2x Laemmli solubilizing solution. 50 µg of microsomal protein was electrophoresed in each well. The gels were dried and exposed to Kodak NS-2T film for 2 days. The incubation time of the microsomes with ATP was as follows: A) 5 sec, B) 15 sec, C) 30 sec, D) 1 min, E) 2 min, F) 5 min, G) 10 min, H) 30 min.


A
B
C
D
E
F
G
H

microsomes were phosphorylated, the protease inhibitors, toluenesulfonyl fluoride, bacitracin and trypsin inhibitor, were included in the incubation medium. None of the inhibitors had any effect on the phosphorylation of either protein. While this evidence does not prove that the 6,000 dalton protein is not a breakdown product of the 22,000 dalton protein, it does add supporting evidence that the 22,000 dalton protein is not degraded. Also, the appearance of the 6,000 or 22,000 dalton protein on the Coomassie stained gels does not vary with time.

Treatment of Microsomes with Hydroxylamine

After the microsomes had been incubated in the presence of cyclic AMP and protein kinase for 10 min, hydroxylamine was added to give a final concentration of 0.8 M hydroxylamine in 0.05 M sodium acetate, pH 5.4. After an additional 10 min, SDS-solubilizing buffer was added to the medium. Both the 22,000 dalton protein, as had been previously reported (Tada et al., 1975), and the 6,000 dalton protein were not affected by the addition of hydroxylamine. Like the 22,000 dalton protein, the 6,000 dalton protein also forms a phophoester bond.

Treatment of Microsomes with Trypsin

Similar to the method used by Tada *et al.* (1975), phosphorylated microsomes were subjected to trypsin. First, microsomes were phosphorylated under the standard reaction. After 10 min, various concentrations of trypsin up to 0.5 mg/ml were added to the incubation medium. Table I shows that even at 0.5 mg/ml of trypsin, both the 22,000 dalton and 6,000 dalton proteins remained phosphorylated and nondigested. The addition of sucrose to the mixture had no effect on the phosphorylation.

The bottom half of Table I shows the effect of first treating the microsomes with trypsin for 10 min, followed by the addition of trypsin inhibitor at a ratio of trypsin inhibitor to trypsin of 2:1, followed by phosphorylation. Cyclic AMP, protein kinase and [γ-^{32}P] ATP were then added to the incubation medium. Even with a trypsin concentration of as low as 0.1 mg/ml, the 22,000 and 6,000 dalton proteins were digested and did not become phosphorylated. These data indicate that phosphorylation of both proteins render them resistant to attack by trypsin.

TABLE I

Effect of trypsin on phosphorylated and nonphosphorylated microsomes. In the top half of the table, microsomes were phosphorylated for 10 min as described in "Methods". Trypsin was then added at various concentrations ranging from 0.1 mg/ml to 0.5 mg/ml. After an additional 10 min, the reaction was stopped by adding Laemmli solubilizing buffer. The samples were electrophoresed on a 12% polyacrylamide-SDS-slab gel, autoradiographed, and the amount of radioactivity in each band was compared with the control, which had not been subjected to trypsin. In the bottom half of the table, microsomes were first treated with trypsin for 10 min at 25^{o}, then trypsin inhibitor was added at a trypsin inhibitor to trypsin ratio of 2:1. At this point, cyclic AMP, protein kinase and [γ - ^{32}P]ATP were added. After an additional 10 min, the reaction was stopped with Laemmli solubilizing buffer.

TABLE I

The Effect of Phosphorylation

on the Susceptibility of the 22,000 and 6,000 Dalton Proteins

to Digestion by Trypsin

TREATMENT	% ^{32}P bound to protein	
	22,000 dalton	6,000 dalton
Phosphorylation followed by:		
Control	100	100
Trypsin (0.50 mg/ml)	95	95
Trypsin (0.1 mg/ml), then trypsin inhibitor, followed by:		
Cyclic AMP + Protein Kinase	0	3

Treatment of Microsomes with DOC

The solubility properties of the 22,000 and 6,000 dalton proteins were investigated. Microsomes were first phosphorylated under the standard conditions. After a 10 min incubation period, DOC was added at varying concentrations up to 1.0 mg DOC/mg protein. After centrifugation, the pellet and supernatant were assayed by SDS-slab gel electrophoresis and autoradiography. Table II shows the results. Once phosphorylated, the 22,000 or 6,000 dalton proteins were not solubilized even when 1.0 mg DOC/mg protein was added. However, when DOC was added first to the microsomes, followed by cyclic AMP, protein kinase and ^{32}P-ATP, most of the 22,000 and 6,000 dalton proteins were solubilized in as little as 0.1 mg DOC/mg protein. Greater than 85% of both proteins appeared phosphorylated in the supernatant. The phosphorylation of both proteins appears to render them inaccessible to added DOC.

TABLE II

Effect of DOC on phosphorylated and nonphosphorylated microsomes. In the top half of the table, microsomes were phosphorylated as described in "Methods". After 10 min, DOC was added at concentrations ranging from 0.1-1.0 mg DOC/mg protein. The sample was centrifuged at 100,000 x g for 30 min. Laemmli solubilizing buffer was added to the pellet and supernatant and 50 µg of each was electrophoresed on 12% acrylamide-SDS-slab gel. After autoradiography, the percent of ^{32}P label in each band was determined by scanning the autoradiogram with a Beckman densitometer. In the bottom half of the table, DOC was added first to the microsomes. After 10 min, cyclic AMP, protein kinase and [γ - ^{32}P] ATP was added. After an additional 10 min, the samples were centrifuged at 100,000 x g for 30 min and assayed as described above.

TABLE II

The Effect of Phosphorylation
on the Susceptibility of the 22,000 and 6,000 Dalton Proteins
to Solubilization by DOC

	% ^{32}P bound to protein			
	22,000 dalton		6,000 dalton	
Phosphorylation followed by:	pellet	supernatant	pellet	supernatant
Control	97	3	98	2
DOC (1.0 mg/mg protein)	94	6	96	4
DOC (0.1 mg/mg protein) followed by:				
Cyclic AMP + Protein Kinase	10	90	8	92

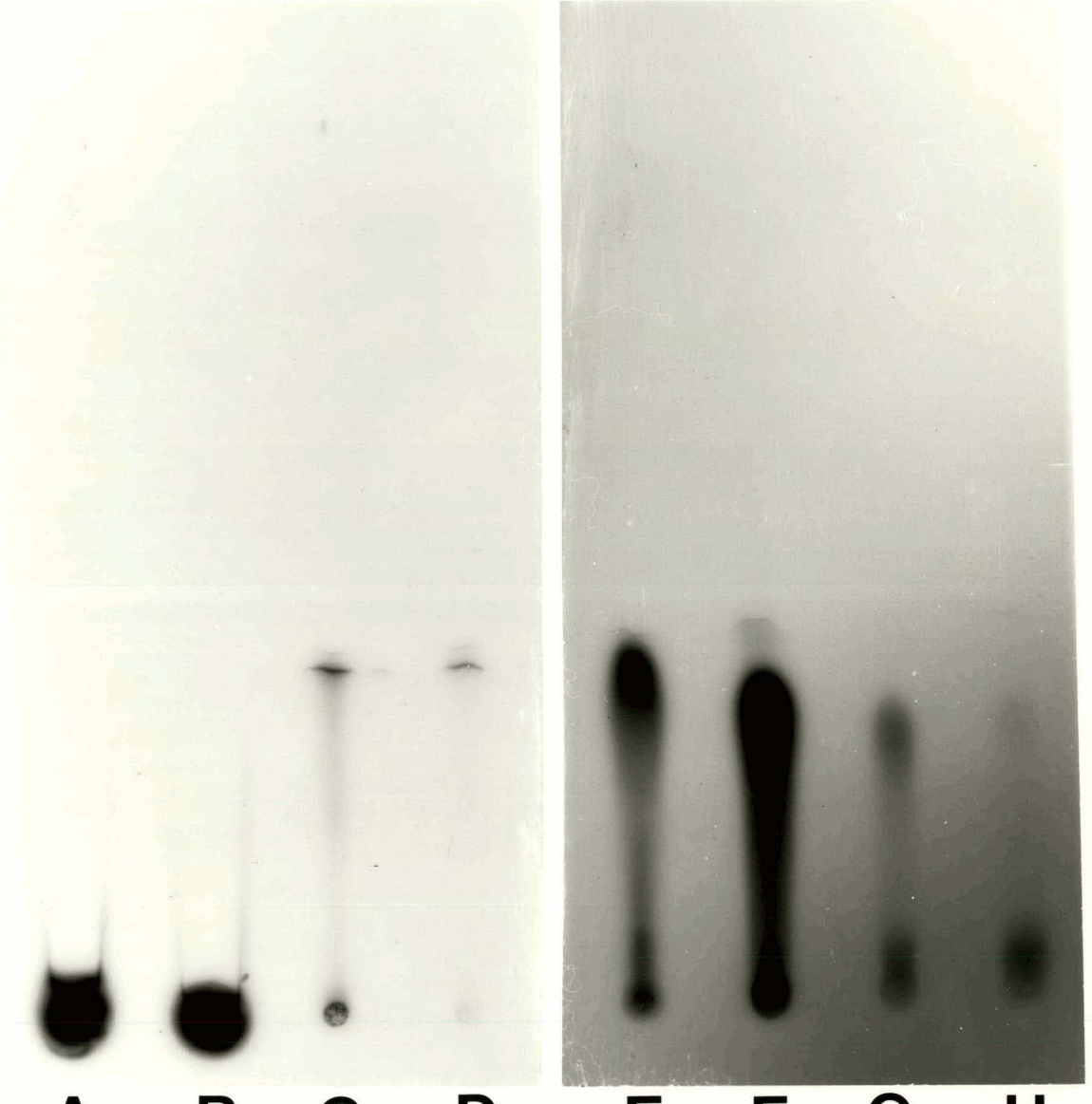
Proteolipid Extraction of Phosphorylated Microsomes

As a means of separating the two phosphorylated protein, the proteolipid extraction procedure of MacLennan (1974) was used. The phosphorylated and nonphosphorylated sarcoplasmic reticulum were extracted with 90% methanol, followed by chlorofrom:methanol (2:1), and finally acidified chloroform:methanol (2:1:10 mM HCl). Proteolipids, proteins with lipids strongly associated with them, are soluble in acidified chloroform:methanol. Ethyl ether can precipitate specifically the proteolipids from the acidified chloroform:methanol phase. Thin layer chromatography can separate lipids and proteolipids. The various fractions were run on Silica G thin layer plates with acidified chloroform:methanol as the solvent. Fig. 5 shows an autoradiogram of the fractions from the proteolipid extraction run on thin layer plated with acidified chloroform:methanol as the solvent. In the methanol supernatant, all counts remained at the origin. These counts represent the ^{32}P-ATP present in the reaction. This finding was generally true for the chloroform:methanol supernatant, with only a few counts moving away from the origin. However, the supernatant from the acidified chloroform:methanol extraction and its subsequent pellet, precipitated with ethyl ether, did show ^{32}P counts at the same place that the proteolipid ran on the thin layer plates.

To rule out the possibility that a lipid might be the component being phosphorylated, all the fractions from the proteolipid extraction were run on thin layer plates with chloroform:methanol:

FIGURE 5

Proteolipid extraction of phosphorylated microsomes. The cardiac microsomes were phosphorylated as described in "Methods". After 10 min, 9 vol of methanol was added to the sample. After 40 min, the sample was centrifuged at 1,000 x g for 10 min. The pellet was suspended in 5 vol chloroform:methanol (2:1) and after 10 min was centrifuged at 1,000 x g for 10 min. The chloroform:methanol pellet was suspended in 2 vol acidified chloroform:methanol (2:1:10 mM HCl). After 10 min, the sample was centrifuged at 1,000 x g for 10 min. To the supernatant was added 7 vol of ethyl ether. After 30 min, the sample was centrifuged at 1,000 x g for 10 min. Each fraction was applied to a Silica G thin layer plate. Acidified chloroform:methanol was the solvent. After the plates were stained with iodine vapor, they were exposed to a sheet of Kodak X-omat film. The film was developed after 5 days. From left to right, A) supernatant from methanol extraction, with cyclic AMP and protein kinase, B) supernatant from methanol extraction, without cyclic AMP and protein kinase, C) supernatant from chloroform:methanol extract, with cyclic AMP and protein kinase, D) supernatant from chloroform:methanol extract, without cyclic AMP and protein kinase, E) supernatant from acidified chloroform:methanol extract, with cyclic AMP and protein kinase, F) supernatant from acidified chloroform:methanol extract, without cyclic AMP and protein kinase, G) pellet from ethyl addition, with cyclic AMP and protein kinase, H) pellet from ethyl ether addition, without cyclic AMP and protein kinase.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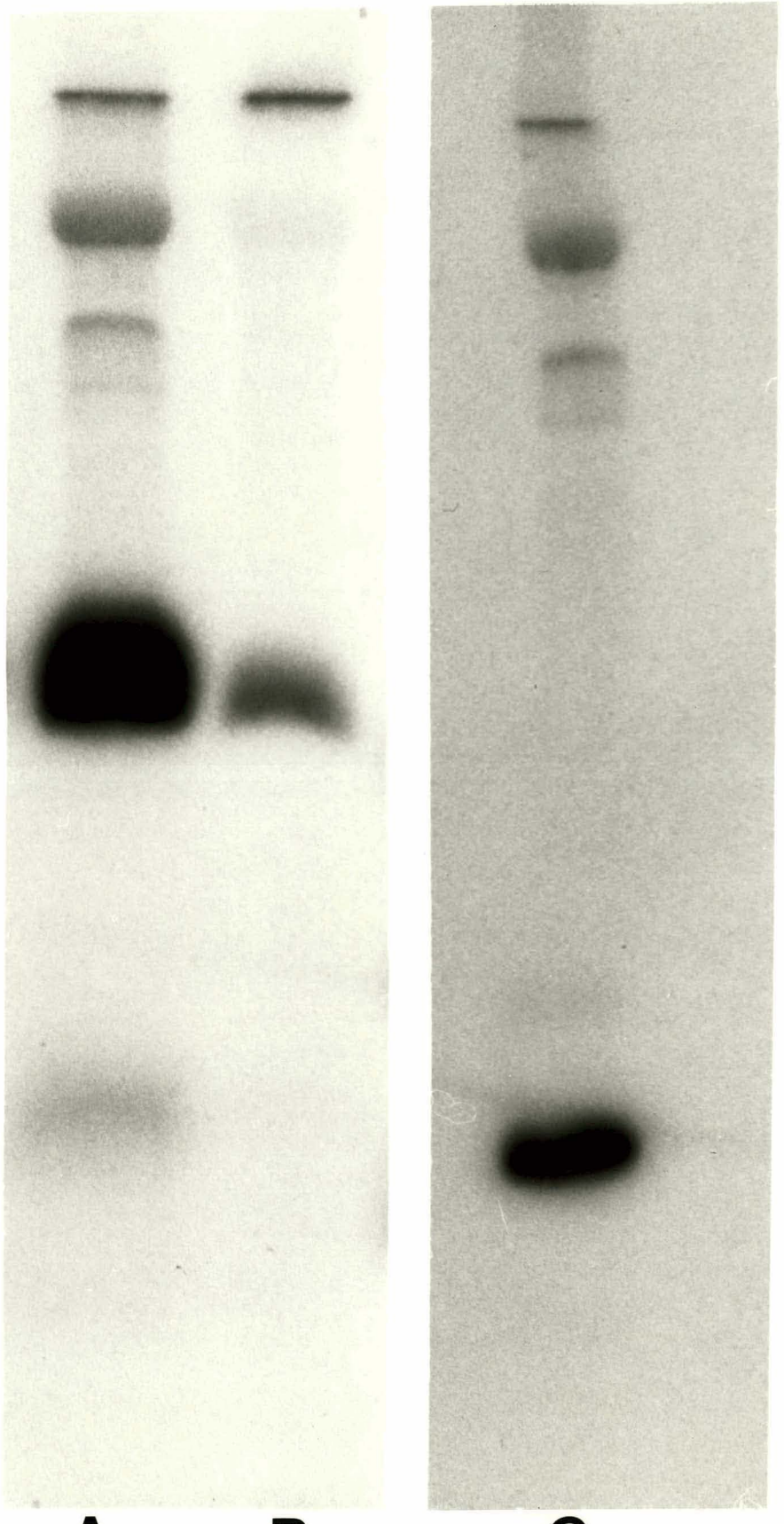
ammonium hydroxide (65:35:5) as the solvent. This solvent system is known to separate lipids but not proteolipids. Using this solvent system, all the ^{32}P counts remained at the origin.

Figure 6 shows an autoradiogram of the proteolipid extraction, run on 12% acrylamide SDS - slab gels. The 22,000 dalton protein appears up in the acidified chloroform:methanol extraction, as does phosphorylase a. The 22,000 dalton protein also appears in the ethyl ether precipitate. The 6,000 dalton protein remains in the pellet, after the addition of acidified chloroform:methanol. The fact that the 22,000 dalton protein is soluble in the acidified chloroform:methanol, and precipitated with ethyl ether, indicates that the 22,000 dalton protein quite probably has lipids associated with it, while the 6,000 dalton protein does not possess these properties.

FIGURE 6

SDS-polyacrylamide slab gel electrophoresis and autoradiography of proteolipid extraction of phosphorylated microsomes. Microsomes were phosphorylated and the proteolipids were extracted as describe in "Methods" and Fig. 5. The extracts and pellets were run on a 12% acrylamide-SDS-slab gel. The gels were dried and autoradiographed. All samples shown were phosphorylated in the presence of cyclic AMP and protein kinase. From left to right, A) supernatant after the addition of acidified chloroform:methanol, B) pellet after the addition of ethyl ether to the acidified chloroform:methanol supernatant, C) pellet after the addition of acidified chloroform:methanol.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C

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Effect of Cyclic AMP and Protein Kinase on the Phosphorylation of Lipids

Even after treating proteins with SDS, lipids are known to be still attached to some proteins. Since it has now been shown that 2 proteins on SDS slab gels appear to be phosphorylated by cyclic AMP-dependent protein kinase and ^{32}P counts do partition into the acidified chloroform:methanol fraction in the proteolipid extraction, a thorough investigation of whether it was a protein or lipid that was being phosphorylated was undertaken. Phosphatidylinositol and cardiolipin were of particular interest because both have a site where phosphate could bind. The lipids were dried down and suspended in the identical phosphorylation mixture used to phosphorylate the sarcoplasmic reticulum. After 10 min the lipids were extracted with chloroform:methanol. Cyclic AMP and protein kinase had no effect on the number of ^{32}P counts extracted into the chloroform:methanol phase. Table III shows the ability of each lipid to extract the ATP into the organic phase. Cardiolipin and phosphatidylinositol extracted the most ^{32}P counts. Phosphatidylserine extracted very few counts.

In addition, phosphorylated microsomes were extracted with chloroform:methanol (2:1). Cyclic AMP and protein kinase had no effect on the number of counts appearing in the organic phase.

TABLE III

1.7 mg of each lipid was dried under nitrogen. The dried lipid was suspended in the phosphorylation buffer as described in "Methods". 20 μM [γ - ^{32}P] ATP was added. After 10 min, the lipids were extracted into chloroform:methanol (2:1). An aliquot was counted in a scintillation counter. The presence of cyclic AMP and protein kinase in the incubation medium had no effect on the results.

TABLE III

Ability of Various Lipids to Extract [γ-^{32}P]ATP into Organic Solvents

Cardiolipin > Phosphatidylinositol > Phosphatidylethanolamine > Phosphatidylserine = Phosphatidylcholine

1.7 mg of each lipid was dried under nitrogen. The dried lipid was suspended in the phosphorylation buffer as described in "Methods". 20μM [-^{32}P]ATP was added. After 10 min, the lipids were extracted into chloroform:methanol (2:1). An aliquot was counted in a scintillation counter. The presence of cyclic AMP and protein kinase in the incubation medium had no effect.

DISCUSSION

Cyclic AMP-dependent protein kinase catalyzes the specific phosphorylation of two proteins from canine cardiac sarcoplasmic reticulum. Like the 22,000 dalton protein, the 6,000 dalton protein is also derived from the sarcoplasmic reticulum, as recently confirmed by Jones et al. (1979). Polyacrylamide slab gel electrophoresis followed by autoradiography have allowed for the direct comparison of the 22,000 and 6,000 dalton proteins under a variety of phosphorylating conditions.

With regard to phosphorylation, both proteins seem to have quite similar properties. In the absence of added, cyclic AMP or protein kinase, both proteins have a basal level of phosphorylation, about a quarter of the level attained when cyclic AMP and protein kinase are included in the incubation medium. This fact demonstrates that there is both endogenous cyclic AMP-dependent protein kinase and adenyl cyclase present in the native sarcoplasmic reticulum. It has been previously suggested that an intrinsic protein kinase is associated with the microsomal membrane (LaRaia and Markin, 1974; Wray et al., 1973). Cyclic GMP does enhance phosphorylation of both proteins slightly. The significance of this finding is not understood. The action of cyclic GMP is often thought to be antagonistic to that of cyclic AMP. The phosphorylation of both proteins by cyclic GMP may be occurring for one of two reasons. Either cyclic GMP too has a role in regulating transport in the sarcoplasmic reticulum by

influencing the phosphorylation of the 22,000 and 6,000 dalton proteins or the specific site for cyclic AMP in the protein kinase moiety is not 100% specific for cyclic AMP, and when cyclic GMP is present, it can partially substitute for cyclic AMP. Maximal phosphorylation occurs when cyclic AMP and protein kinase are both included in the incubation medium (Fig. 3). Phosphodiesterase completely inhibits phosphorylation. This inhibition is accomplished by phosphodiesterase breaking down cyclic nucleotides. The fact that the level of phosphorylation in the presence of phosphodiesterase is significantly lower than the basal levels, gives credence to the idea that there is an adenyl cyclase system in the sarcoplasmic reticulum. Phosphorylase kinase had virtually no effect on the phosphorylation of either the 22,000 or 6,000 dalton proteins (Fig. 3). Schwartz et al. (1976) reported that phosphorylase kinase was capable of both phosphorylating phosphorylase a and stimulating calcium uptake in microsomes from cardiac and skeletal muscles. Including phosphorylase kinase in the incubation medium had no pronounced enhancement on the phosphorylation of the 95,000-dalton protein, phosphorylase a. After long period of incubation, the phosphorylation of phosphorylase did increase but not nearly as dramatically as that found in the 22,000 and 6,000 dalton proteins (Fig. 4). In addition, in skeletal muscle microsomes neither the 22,000 or 6,000 dalton proteins appears phosphorylated; however, phosphorylase a is phosphorylated (Data not shown). In cardiac sarcoplasmic reticulum, only the Ca^{2+}+Mg^{2+}-ATPase, phosphorylase a, protein

kinase and the 22,000 and 6,000 dalton proteins ever appear phosphorylated. In skeletal muscle, particularly in the absence of calcium, many proteins are phosphorylated, but not specifically by cyclic AMP-dependent protein kinase[2].

The time course of phosphorylation of the 22,000 and 6,000 dalton proteins is not identical. The 22,000 dalton protein is phosphorylated at earlier time points than the 6,000 dalton protein (Fig. 4). When the 6,000 dalton protein starts to become phosphorylated, the phosphorylation of the 22,000 dalton protein decreases slightly, and then both proteins continue to be phosphorylated at steady-state levels. After 10 minutes of incubation the phosphorylation of both proteins reaches an equal molar ratio. Added protease inhibitors had no effect on the phosphorylation of either protein. Also, the Coomassie blue stained gel pattern does not change as a function of incubation time. These results strongly suggest that the 6,000 dalton protein is not a degradation product of the 22,000 dalton protein.

The proteins, not the lipids, are the moieties phosphorylated. Since it has now been found that two proteins are phosphorylated by cyclic AMP-dependent protein kinase, the postulate that a lipid was being phosphorylated and that these two proteins merely bind the lipid tightly was raised. The data does not support this contention. While the lipids do vary in their ability to extract phosphate into organic solvents, cyclic AMP has no effect on this property.

[2]Campbell, K.P. and Shamoo, A.E. (1979) submitted to J. Biol. Chem.

One of the most striking findings in this study is that phosphorylation of both the 22,000 and 6,000 dalton proteins causes them to become buried in the membrane. Phosphorylation renders them resistant to attack by trypsin (Table I) and DOC (Table II). Prior to phosphorylation both proteins appear to be extrinsic-solubilized by low concentrations of detergent and digested by trypsin. Phosphorylation appears to cause them to become intrinsic - not solubilized by high concentrations of DOC and resistant to trypsin digestion. The only explanation for this data is that phosphorylation of both proteins causes them to become buried in the membrane and no longer exposed to the external environment. It is very fascinating to find that adding one group, a phosphate, to a protein can change its biochemical characteristics so dramatically. How and if the phosphorylation of these proteins regulates calcium transport must still be discerned. When the proteins are phosphorylated and buried in the membrane they may act directly on the Ca^{2+}+Mg^{2+}-ATPase at this point, or they may facilitate transport directly, possibly by translocating calcium themselves. The isolation of these proteins will greatly aid in our understanding of how they function.

REFERENCES

Bidlack, J.M. and Shamoo, A.E. (1979) Cyclic AMP-dependent phosphorylation of a 6,000 and 22,000 dalton protein in sarcoplasmic reticulum vesicles. Biophysical J. 25: 24a

Fabiato, A. and Fabiato, F. (1975) Relaxing and inotropic effects of cyclic AMP in skinned cardiac cells. Nature 253: 556-558

Harigaya, S. and Schwartz, A. (1969) Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. Membrane vesicles (relaxing system) and mitochondria. Circ. Res. 25: 781-794

Jones, L.R., Besch, Jr., H.R., Fleming, J.W., McConnaughey, M.M. and Watanabe, A.M. (1979) Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. J. Biol. Chem. 254: 530-539

Katz, A.M., Repke, D.I., Upshae, J.E. and Polascik, M.A. (1970) Characterization of dog cardiac-microsomes-use of zonal centrifugation to fractionate fragmented sarcoplasmic reticulum, ($Na^+ + K^+$) - activated ATPase and mitochondrial fragments. Biochim. Biophys. Acta 205: 473-490

Katz, A.M., Tada, M. and Kirchberger, M.A. (1975) Control of calcium transport in the myocardium by the cyclic AMP- protein kinase system. Advan. Cyclic Nucleotide Res. 5: 453-472

Kirchberger, M.A., Tada, M. and Katz, A.M. (1974) Adenosine 3':5'-monophosphate- dependent protein kinase-catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J. Biol. Chem. 249: 6166-6173

Kirchberger, M.A., Tada, M., Repke, D.I. and Katz, A.M. (1972) Cyclic adenosine 3':5' - monophosphate- dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. J. Mol. Cellular Cardiol. 4: 673-680

Kirchberger, M.A. and Wong, D. (1978) Calcium efflux from isolated cardiac sarcoplasmic reticulum. J. Biol. Chem. 253: 6941-6945

Laemmli, U.K. and Favre, M. (1973) Maturation of head of bacteriophage-T4 1. DNA packaging events. J. Mol. Biol. 80: 575-599

LaRaia, P.J. and Morkin, E. (1974) Adenosine 3':5' - monophosphate-dependent membrane phosphorylation. A possible mechanism for the control of microsomal calcium transport in heart muscle. Circ. Res. 35: 298-306

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 246: 2702-2710

MacLennan, D.H. (1970) Purification and properties of an ATPase from sarcoplasmic reticulum. J. Biol. Chem. 245: 4508-4518

MacLennan, D.H. (1974) Isolation of proteins of the sarcoplasmic reticulum. In "Method in Enzymology" (Fleischer, S. and Packer, L., eds.), Vol. 32, pp. 291-302, Academic Press, New York

Rolett, E. (1974) Adrenergic mechanisms in mammalian myocardium. In "The Mammalian Myocardium" (Langer, G.A. and Brady, A.J., eds.), pp. 219-250, Wiley, New York

Schwartz, A., Entman, M.L., Kaniike, K., Lane, L.K., Van Winkle, W.B. and Bornet, E.P. (1976) The rate of calcium uptake into sarcoplasmic reticulum of cardiac muscle and skeletal muscle. Effects of cyclic AMP-dependent protein kinase and phosphorylase b kinase. Biochim. Biophys. Acta 426: 57-72

Tada, M. and Kirchberger, M.A. (1975) Regulation of calcium transport by cyclic AMP. A proposed mechanism for the beta-adrenergic control of myocardial contractility. Acta Cardiologia 30: 231-237

Tada, M., Kirchberger, M.A. and Katz, A.M. (1975) Phosphorylation of a 22,000 - dalton component of the cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 250: 2640-2647

Tada, M., Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974) The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 249: 6174-6180

Tada, M., Ohmori, F., Kinoshita, N. and Abe, H. (1978) Cyclic AMP regulation of active calcium transport across membranes of sarcoplasmic reticulum. Role of the 22,000 - dalton protein phospholamban. Advan. Cyclic Nucleotide Res. 9: 355-369

Tada, M., Ohmori, F., Yamada, M. and Abe, H. (1979) Mechanism of the stimulation of Ca^{2+} - dependent ATPase of cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 254: 319-326

Tada, M., Yamamoto, T. and Tonomura, Y. (1978a) Molecular mechanism of active calcium transport by sarcoplasmic reticulum. Physiol. Rev. 58: 1-79

Tsien, R.W. (1977) Cyclic-AMP and contractile activity in heart. Adv. Cyclic Nucleotide Res. 8: 363-420

Wray, H.L., Gray, R.R. and Olsson, R.A. (1973) Cyclic adenosine 3':5'- monophosphate - stimulated protein kinase and a substrate associated with cardiac sarcoplasmic reticulum. J. Biol. Chem. 248: 1496-1498

CHAPTER 2

PURIFICATION OF TWO PROTEINS FROM CARDIAC SARCOPLASMIC RETICULUM. A 22,000 DALTON PROTEIN, SPECIFICALLY PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE, AND THE Ca^{2+}+ Mg^{2+}-ATPase.

SUMMARY

Very low concentrations of the detergent, deoxycholate (DOC), have been used to isolate two functionally interesting proteins from canine cardiac sarcoplasmic reticulum. These two proteins are the 22,000 dalton protein, specifically phosphorylated by cyclic AMP-dependent protein kinase, and the $Ca^{2+}Mg^{2+}$-ATPase, the major protein of the sarcoplasmic reticulum, responsible for the active transport of calcium. By employing detergents at concentrations of one hundredth to one thousandth lower than normally used, it is possible to selectively solubilize certain proteins completely, while leaving others still embedded in the membrane.

The 22,000 dalton protein is first solubilized in a very low concentration of DOC and then subjected to column chromatography. After molecular weight sieving, the 22,00 dalton protein appears as a purified protein on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The purified protein is still specifically phosphorylated by cyclic AMP-dependent protein kinase. Phospholipids are still bound to the isolated 22,000 dalton protein in the ratio of 5 moles phospholipid to 1 mole protein.

The Ca^{2+}+ Mg^{2+}ATPase is purified by first solubilizing all the extrinsic proteins with a low concentration of DOC. An increasing amount of DOC is then added to yield the purified Ca^{2+} Mg^{2+}-ATPase. This protein is at least 95% pure as determined by

SDS-polyacrylamide gels. Adding additional DOC to the solubilized purified enzyme enhances the enzyme's ability to hydrolyze ATP, approximately 2.5 µmoles Pi/mg/min. The isolation of the 22,000 dalton protein and the Ca^{2+}+ Mg^{2+}-ATPase will aid in understanding how these two proteins function and if they specifically interact with one another.

INTRODUCTION

Purification of a protein is a major step toward understanding the function of the protein. In order to purify most membrane bound proteins, it is first necessary to separate the protein from the lipid membrane. Detergents have been generally used to solubilize proteins (MacLennan, 1970, 1974). This communication reports on the purification of two proteins from canine cardiac sarcoplasmic reticulum. Very low concentrations of detergent were used to selectively solubilize these proteins, greatly aiding in their purification.

In canine cardiac sarcoplasmic reticulum, cyclic AMP-dependent protein kinase[1] specifically phosphorylates two proteins, one with a molecular weight of 6,000 and one with a molecular weight of 22,000 (Bidlack and Shamoo, 1979b, Jones et al., 1979). The 22,000 dalton protein has been identified and studied by others (Tada et al., 1975, 1978, 1979; Kirchberger and Chu, 1976; Kirchberger et al., 1975). It exhibits characteristics of phosphoester bonding (Tada et al., 1975). After phosphorylation, the 22,000 dalton protein is resistant to attack by trypsin (Tada et al., 1975; Bidlack and Shamoo, 1979b). In addition, prior to phosphorylation,

[1]The abbreviations used are: cyclic AMP, adenosine 3':5'-monophosphate-dependent protein kinase; EGTA, ethylene glycol bis (β-amino-ethyl ether)-N, N[1]-tetraacetic acid; DOC, deoxycholate, SDS, sodium dodecyl sulfate.

the 22,000 dalton protein is soluble in a low concentration of deoxycholate. After phosphorylation, the protein cannot be solubilized by deoxycholate. These results intimate that phosphorylation causes the protein to become buried in the membrane, rendering it resistant to agents acting on the exterior of the membrane (Bidlack and Shamoo, 1979b).

The Ca^{2+} + Mg^{2+}-ATPase, a 100,000 dalton protein, is the integral protein of both cardiac and skeletal sarcoplasmic reticulum. It actively transports calcium into the sarcoplasmic reticulum. The ATPase from skeletal rabbit hind muscle was first isolated almost a decade ago by MacLennan (1970). As a result, the skeletal enzyme has been characterized much more extensively than the cardiac enzyme. In both systems, 2 moles of Ca^{2+} are transported for each mole of ATP hydrolyzed (Hasselbach, 1964; Weber, 1966). The Ca^{2+} + Mg^{2+}-ATPase from skeletal and cardiac sarcoplasmic reticulum differ with respect to calcium binding, uptake, K_m for calcium and hydrolytic activity. They have similar K_m values for ATP (Shigekawa et al., 1976). Recently two methods have been published for the isolation of the Ca^{2+} + Mg^{2+}-ATPase from cardiac sarcoplasmic reticulum (Levitsky et al., 1976; Van Winkle et al., 1978). The method presented in this communication has the advantage over these two procedures in that it is a very rapid technique employing very low concentrations of detergent. Moreover, our method also isolates for the first time the 22,000 dalton protein from the cardiac sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURE

MATERIALS

Bovine heart cyclic AMP-dependent protein kinase, sodium cyclic AMP and tris-ATP were purchased from Sigma Chemical Co. [γ-^{32}P]ATP, ammonium salt (20 mCi per mmol) was obtained from Amersham/Searle. Deoxycholate was purified by the method of MacLennan (1970).

METHODS

Isolation of Cardiac Microsomes

Cardiac microsomes were prepared from canine heart ventricle according to Harigaya and Schwartz (1969) with the modifications described in Bidlack and Shamoo (1979b). Briefly, cardiac muscle was homogenized in 10 mM $NaHCO_3$ and centrifuged twice at 8700 x g for 20 min. The supernatant was then centrifuged at 37,000 x g for 30 min. The pellet was suspended in 20 mM tris-maleate, 0.6 M KCl, pH 6.8, and centrifuged at 100,000 x g for 20 min. The pellet, containing microsomes, was suspended in 0.25 M sucrose.

Gel Electrophoresis

Sodium dodecyl sulfate gel electrophoresis was run according to Weber and Osborn (1969). Samples for electrophoresis were suspended in the SDS-sample buffer consisting of 1% SDS, 20 mM sodium phosphate (pH 7.2), 0.1 mM EDTA, 1% β-mercaptoethanol, 50%

glycerol and 0.005% bromophenol blue. The samples were incubated at 37° for 1 hr. An aliquot containing between 50 and 150 µg was applied to the gel. After electorphoresis in the 10% polyacrylamide SDS-gel, the proteins were made visible by staining the gels in 0.25% Coomassie blue for 2 hr at 37° and diffusion destained in 10% acetic acid.

Phosphorylation Assay

Phosphorylation of cardiac microsomes, DOC supernatant and the purified 22,000 dalton protein was accomplished essentially by the method of Kirchberger et al. (1974). The protein fraction (0.25 to 0.50 mg/ml) was suspended in 40 mM histidine (pH 6.8), 0.12 M KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 25 mM NaF buffer. Cyclic AMP was added to a final concentration of 1 µM. Protein kinase was added at the concentration of 0.1 mg per ml. The reaction was started by adding 20 µM [γ-^{32}P]ATP. The final volume was 0.2 ml. After 10 min, the reaction was stopped by adding 2 ml of cold 10% trichloroacetic acid, 0.1 mM KH_2PO_4. 0.2 ml of 0.63% bovine serum albumin was added as a carrier protein. After remaining on ice for 15 min, samples were centrifuged at 2,000 x g for 10 min. The pellets were suspended in 0.2 ml of 0.6 N NaOH. The protein was reprecipitated by the addition of 2 ml of 10% trichloroacetic acid, centrifuged, resuspended in NaOH and washed a couple of times. The pellet was finally suspended in 0.2 ml of 0.6 N NaOH and transferred to scintillation vials containing 10 ml of Instagel (Packard) for counting.

ATPase Activity

Ca^{2+}-dependent ATPase was measured in a manner similar to that used by Tada et al. (1974). Cardiac microsomes or the isolated protein were suspended at a concentration of 30-50 µg/ml in 40 mM histidine (pH 6.8), 0.125 M KCl, 5 mM NaN_3, 5 mM $MgCl_2$ and varying calcium concentrations in the presence of EGTA. The reaction was started by adding 5 mM [γ-^{32}P]ATP (3µCi per µmole). The final volume was 0.4 ml. The Ca^{2+}-independent ATPase activity was measured by including 0.5 mM EGTA instead of calcium-EGTA in the reaction mixture. At various time intervals, the reaction was stopped by adding 0.4 ml of ice-cold 13.33 mM silicotungstic acid in 1.67 M H_2SO_4. 0.3 ml of 10% ammonium molybdate was added. After vortexing, 1 ml of isobutanol:benzene (1:1) was added. The sample was vortexed for 30 sec, followed by centrifugation at 1,000 x g for 10 min. 0.5 ml of the organic phase was counted. To determine the nonspecific binding of phosphate, controls were run by adding the protein to the reaction mixture after the acid had been added. The free Ca^{2+} concentrations were determined by the method of Katz et al. (1970).

Isolation of the 22,000 Dalton Protein

Cardiac microsomes were suspended at a concentration of 7 mg/ml in 0.25 M sucrose. A small aliquot of DOC was added at the ratio of 2 µg DOC/7 mg microsomal protein (0.286 µg DOC/mg protein).

The suspension was incubated on ice for 15 min. After incubation, the suspension was centrifuged at 100,000 x g for 30 min. The supernatant was concentrated on an Amicon PM 10 membrane. The supernatant was then applied to a Sephadex G-75 (fine) column (1.5cm x 1.5m), equilibrated with 10 mM tris (pH 7.8), 0.04% DOC, 0.02% NaN_3. The sample was eluted from the column using the tris-DOC buffer. The absorbance of the sample at 280 nm was read. The 22,000 dalton protein was eluted as a single protein as determined by SDS-polyacrylamide gel elctrophoresis.

Isolation of the Ca^{2+}+ Mg^{2+}-ATPase

As in the isolation of the 22,000 dalton protein, cardiac microsomes were suspended at 7 mg/ml in 0.25 M sucrose. DOC was added at the ratio of 2 µg DOC/7 mg protein. Each 7 mg of protein must be treated in a separate tube. The purification cannot be scaled upwards without losing about 10% in the purification of the ATPase. The mixture was incubated on ice for 15 min, followed by centrifugation at 100,000 x g for 30 min. The pellet was suspended in the original volume of 0.25 M sucrose, 1 ml. Additional DOC was added at the concentration of 2 µg DOC/7 mg of original microsomal protein (i.e. the same amount of DOC as was added initially). The suspension was incubated on ice for 15 min, followed by centrifugation at 100,000 x g for 30 min. The supernatant from this centrifugation contained the Ca^{2+}+ Mg^{2+}-ATPase to at least 95% purity.

Biochemical Assays

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Phospholipid concentration was assayed by determining the phosphorus present by the method of Ames and Dubin (1960).

RESULTS

Purification of the 22,000 Dalton Protein

The first step in the purification of the 22,000 dalton protein was to solubilize it in a low concentration of DOC. The 22,000 dalton protein was completely solubilized by the DOC, with none remaining in the pellet. If the protein is first phosphorylated by cyclic AMP-dependent protein kinase, it cannot be solubilized by DOC (Bidlack and Shamoo, 1979a,b). After solubilizing the 22,000 dalton protein in a very low concentration of DOC, a Sephadex G-75 column was used to purify the protein. The protein elution pattern from the column is shown in Fig. 1. The proteins solubilized by the low concentration of DOC are extrinsic and mainly of high molecular weight eluting in the void volume from the column. SDS-polyacrylamide gels of the various column fractions are shown in Fig. 2. Gel 2A shows the proteins solubilized by the low concentration of DOC. These proteins included the 22,000 dalton protein, mainly higher molecular weight proteins and some of the ATPase. All solubilized proteins with a molecular weight above 60,000 were excluded in the void volume, the major peak from the column. The first fraction separated by the column is shown in the third gel in Fig. 2B. A 50,000 dalton protein was the major band with a few minor proteins also present. The isolated 22,000 dalton protein is seen in the next peak (Gel C, Fig. 2). The column was

FIGURE 1

Elution profile from the Sephadex G-75 column. Cardiac microsomes were suspended at 7 mg/ml in 0.25 M sucrose. 2 μl of 0.1% DOC was added per 7 mg protein. The microsomes were centrifuged at 100,000 x g for 30 minutes. The supernatants were concentrated and approximately 2.7 mg of protein was applied to a Sephadex G-75 (fine) column (1.5 cm x 1.5 m) equilibrated with 10 mM tris (pH 7.8), 0.04% DOC, 0.02% NaN_3. The absorbance of the eluant was monitored at 280 nm. Each fraction was 4.7 ml.

PURIFICATION OF THE 22,000 DALTON PROTEIN
ON A SEPHADEX G-75 COLUMN

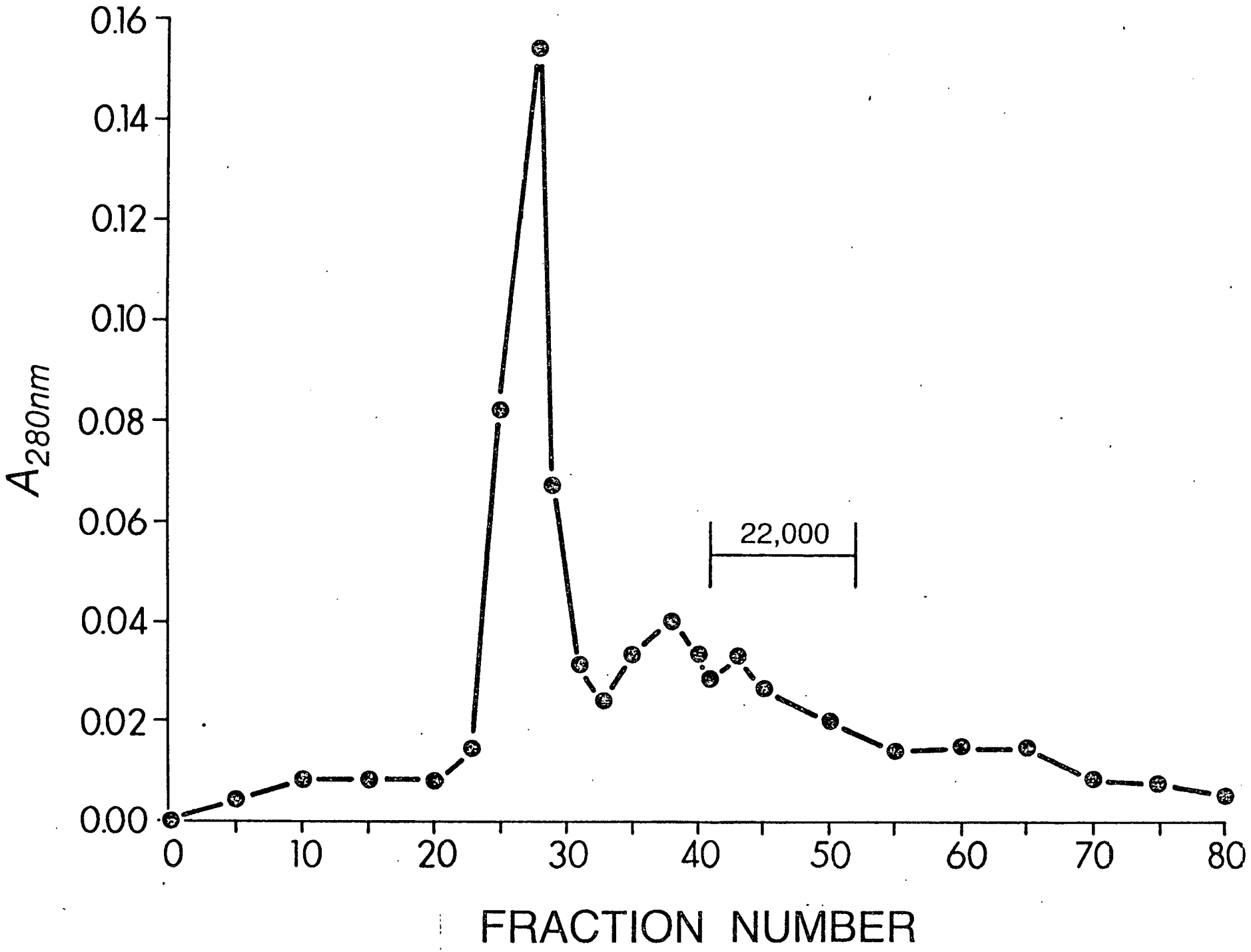
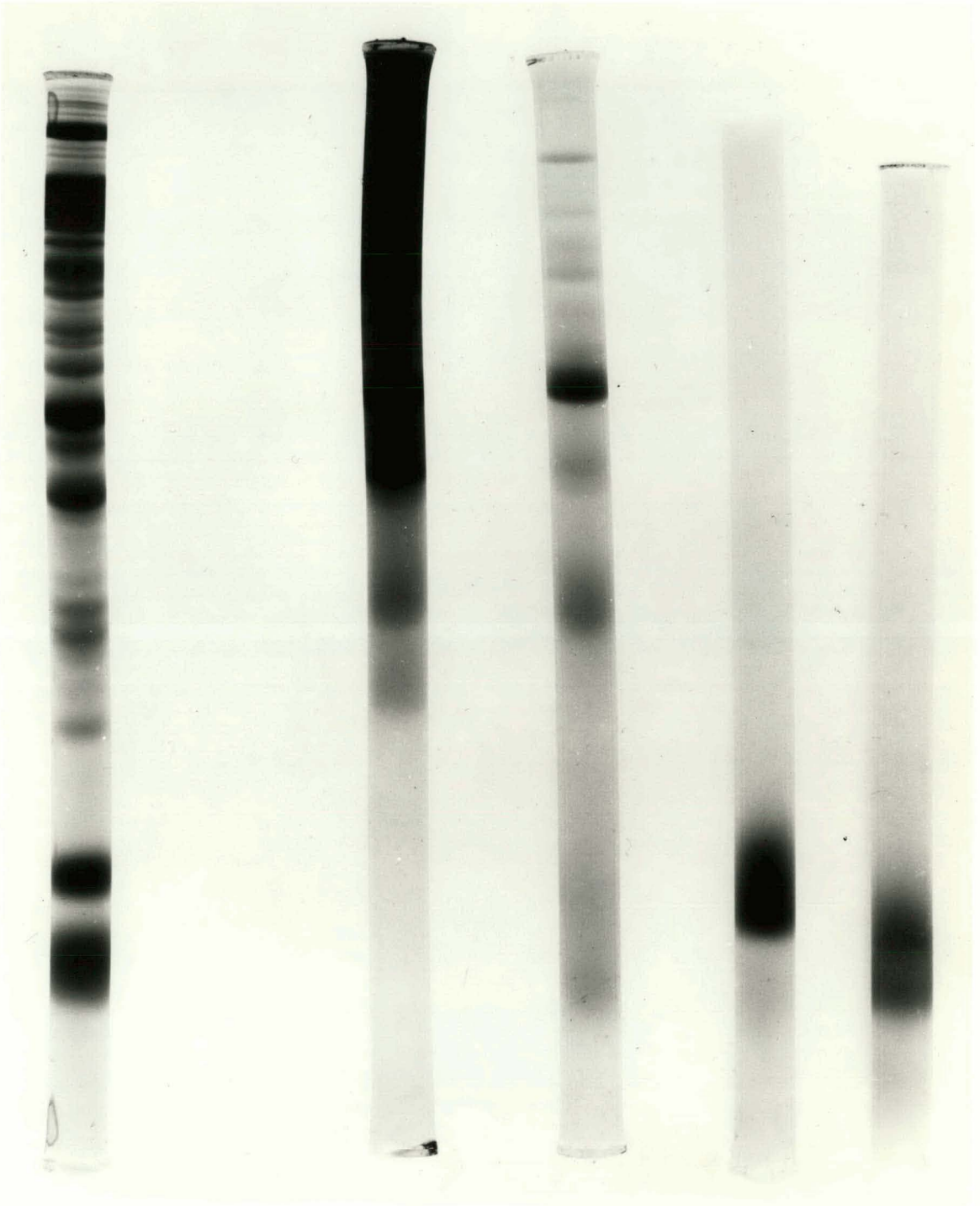


FIGURE 2

Coomassie blue stained gels of the column fractions. Ten percent Weber and Osborn polyacrylamide-SDS-gels were run using of the column fractions shown in Figure 1. The gel on the far left (A), shows the DOC solubilized supernatant that was applied to the column. Gel B shows the proteins in the void volume, fractions 25-30. Gel C shows fractions 31-38. Gel D shows the purified 22,000 dalton protein, fractions 39-51. Gel E shows the fractions 52-61, showing the 18,000 dalton protein with a small amount of the 22,000 dalton protein present.


A
B
C
D
E

capable of separating the 22,000 dalton protein from a 18,000 dalton protein also solubilized by DOC. Fig. 2D shows the next successive fractions. They contain mainly the 18,000 dalton protein with only a small amount of the 22,000 dalton protein eluting in this fraction.

The yield of the 22,000 dalton protein at each step of the purification is shown in Table I. The DOC solubiliztion step results in a yield of 0.37 mg of protein in the supernatant per 7 mg of sarcoplasmic reticulum. Usually about 2.7 mg of the solubilized proteins were applied to the Sephadex G-75 column. This results in a yield of about 150 µg of pure 22,000 dalton protein. About 100 µg of pure 22,000 dalton protein can be obtained from 34 mg of sarcoplasmic reticulum.

Phosphorylation of the Purified 22,000 Dalton Protein

The isolated 22,000 dalton protein was specifically phosphorylated by cyclic AMP-dependent protein kinase as is shown in Table II. Cardiac microsomes incorporated approximately 1.35 nmole ^{32}P per mg protein. This data is consistent with that reported by Kirchberger et al. (1974). The supernatant from the first DOC solubilization incorporated only about a quarter of the phosphate that the microsomal fraction did. The isolated 22,000 dalton protein incorporated 6.83 nmoles phosphate per mg protein, or 0.15 moles of phosphate per mole of protein. This degree of incorporation is considerably lower than the idealistic, 1 mole of phosphate per mole of protein, but is in agreement with the finding

TABLE I

Percent protein yield during isolation. The percent protein at each step of the purification was calculated by taking the cardiac microsomes as 100%. The percent of 22,000 dalton protein at each step was determined by scanning Coomassie blue stained 10% Weber and Osborn polyacrylamide-SDS-gels at 550 nm.

TABLE I

Percent Protein Obtained in Each Isolation Step

Isolation Step	Protein Yield, %	22,000 Dalton Protein, %
Cardiac microsomes	100.0	5.1
DOC supernatant	5.3	10.8
Isolated protein	0.3	100.0

TABLE II

Cyclic AMP-dependent phosphorylation of the sarcoplasmic reticulum, DOC supernatant and the purified 22,000 dalton protein. Each fraction was incubated in the phosphorylating buffer, including [γ-^{32}P] ATP in a final volume of 0.2 ml. After 10 minutes, the phosphorylation was stopped by the addition of 2 ml of 10% trichloroacetic acid (TCA) 0.1 mM KH_2PO_4. 0.2 ml of 0.63% BSA was added as a carrier protein. After incubating on ice for 15 minutes, the sample was centrifuged at 2,000 x g for 10 minutes. The pellet was solubilized by 0.6 N NaOH, reprecipitated with 10% TCA, 0.1 mM KH_2PO_4 and washed two times. Finally, the pellet was solubilized in NaOH, transferred to 10 ml Instagel (Packard) and the radioactivity was counted. The data represents the mean $\pm$ S.E. for five experiments on the cardiac microsomes, and three determinations on two different preparations for the DOC supernatant and the purified 22,000 dalton protein.

TABLE II

Cyclic AMP-dependent Phosphorylation

Protein Fraction	nmoles ^{32}P/mg protein	moles ^{32}P/mole protein
Cardiac microsomes	1.35 ± 0.16	
First DOC supernatant	0.31 ± 0.08	
Purified 22,000	6.83 ± 0.31	0.150 ± 0.027

for some other isolated proteins (Becker-Ursic and Davies, 1976; Hebert, J. et al. 1977). It must be remembered that in the microsomal fraction, two major proteins are being phosphorylated, the 22,000 and 6,000 dalton proteins (Bidlack and Shamoo, 1979a, b; Jones et al., 1979).

Phospholipid Content of the 22,000 Dalton Protein

Phospholipid content remained bound to the 22,000 dalton protein after isolation as shown in Table III. The fact that lipid is still bound to the 22,000 dalton protein, is consistent with the finding that the 22,000 dalton protein can be solubilized by acidified chloroform:methanol (Bidlack and Shamoo, 1979b).

Isolation of the Ca^{2+} + Mg^{2+}-ATPase

The purification of the cardiac Ca^{2+} + Mg^{2+}-ATPase was accomplished by selective solubilization of the membrane proteins by DOC. Figure 3 shows the gel pattern of the purification procedure. The sarcoplasmic reticulum is made up of about 30% Ca^{2+} + Mg^{2+}-ATPase. The first treatment with DOC selectively solubilized the extrinsic proteins including the 22,000 dalton protein and some of the ATPase. Adding an additional small amount

TABLE III

Phospholipid content of the sarcoplasmic reticulum, DOC supernatant, 22,000 dalton protein, and Ca^{2+}+ Mg^{2+}-ATPase. The phosphorus present in each sample was determined by the method of Ames and Dubin (1960). The experimental results ar presented as the mean ± S.E. for 5 experiments.

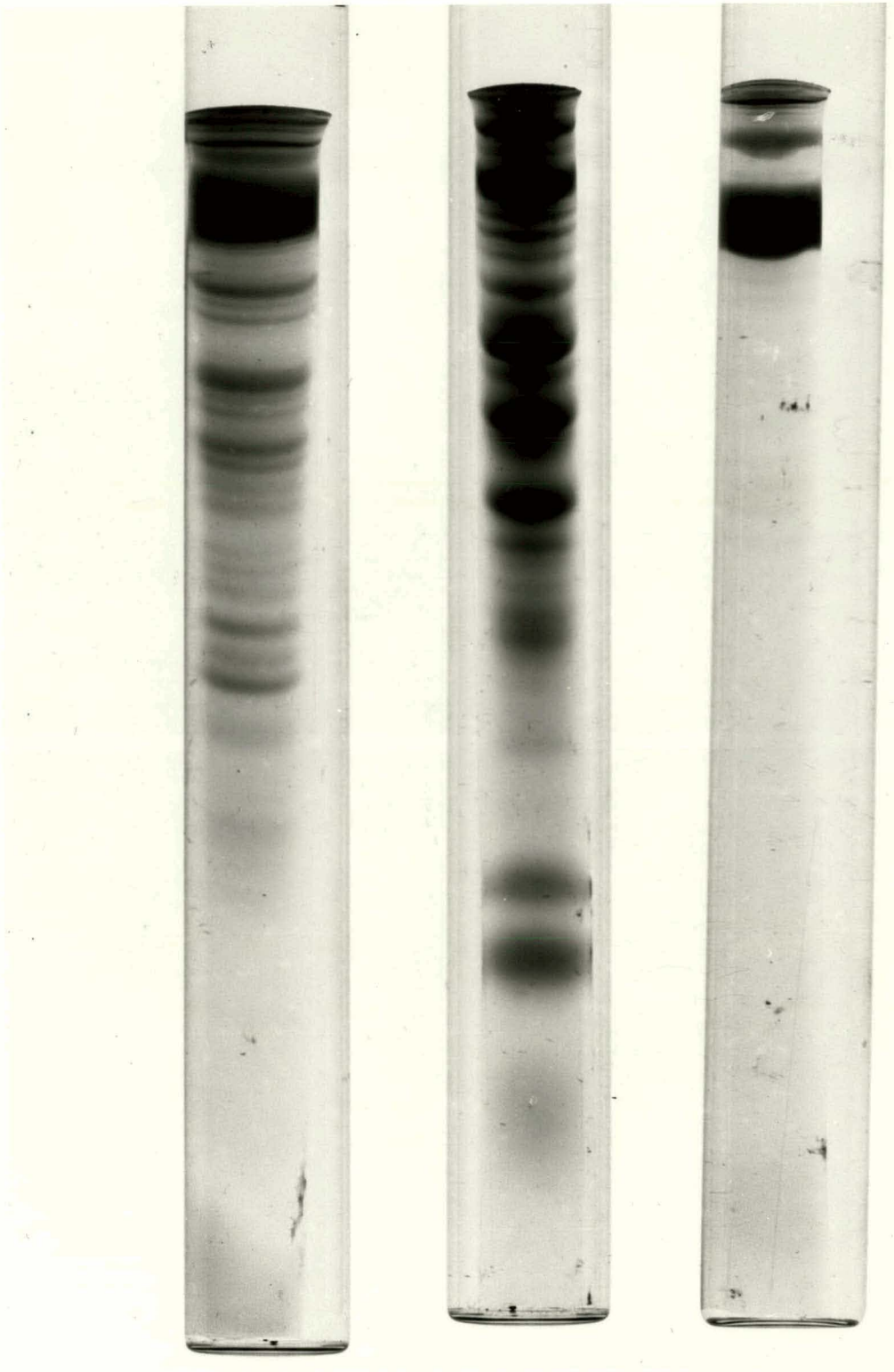
TABLE III

Phospholipid Content

Protein Fraction	Lipid Content	
	µg phospholipid phosphorus/mg protein	mole phospholipid phosphorus/mole protein
Cardiac Microsomes	20.5 ± 0.8	
First DOC supernatant	5.1 ± 0.4	
22,000 dalton protein	7.2 ± 0.5	5.1 ± 0.02
$Ca^{2+} + Mg^{2+}$-ATPase	3.6 ± 0.4	11.5 ± 0.03

FIGURE 3

Coomassie blue stained, 10% Weber and Osborn polyacrylamide-SDS-gels of the isolation of the Ca^{2+}+ Mg^{2+}-ATPase . Gel A shows the cardiac microsomes. Gel B shows the first supernatant after treating the microsomes with DOC. Gel C shows the purified ATPase. The band above the 100,000 dalton band represented a dimer of the ATPase.



A B C

79 8452

of DOC to the pellet from the first extraction resulted in the isolation of the ATPase to at least 95% purity, as shown in Fig. 3. This procedure yields 50 $\pm$ 5 µg of purified Ca^{2+} + Mg^{2+}-ATPase per mg of sarcoplasmic reticulum. The isolated enzyme contained no phosphorylase activity (Data not shown). The protein was soluble but had virtually no detergent bound to it because it was isolated by using very low concentrations of detergent.

ATPase Activity

The purified Ca^{2+} + Mg^{2+}-ATPase is nearly 100% Ca^{2+}-dependent as detailed in Table IV. About a quarter of the hydrolytic activity in sarcoplasmic reticulum fraction is Ca^{2+}-independent. Once solubilized it appears that all the hydrolytic activity is Ca^{2+}-dependent. Even in the supernatant from the first solubilization with DOC, all the ATPase activity is Ca^{2+}-dependent. The purified enzyme exhibited activity in the range of 1.2 µmoles Pi/mg protein/min. By adding additional DOC, the hydrolytic activity of the enzyme was doubled. DOC was also found to stimulate the ATPase activity of the Ca^{2+} + Mg^{2+}-ATPase isolated from skeletal muscle (MacLennan, 1970).

Figure 4 shows the ATPase activity with varying calcium concentrations, a concentration of 5.2 $\pm$ 0.3 µM Ca^{2+} was needed to achieve 50% activation of the ATPase.

TABLE IV

The ATPase hydrolytic activity of cardiac microsomes, DOC supernatant and purified Ca^{2+} + Mg^{2+}-ATPase. Protein samples were suspended at a concentration of 30-50 µg/ml in 40 mM histidine (pH 6.8), 0.125 M KCl, 5 mM NaN_3, 5 mM $MgCl_2$, and 50 M calcium-EGTA buffer. The reaction was started by adding 5 mM [γ-^{32}P] ATP (3 µCi per µmole). The final volume was 0.4 ml. The Ca^{2+}-independent ATPase activity was measured by including 0.5 mM EGTA instead of calcium-EGTA in the reaction mixture. The reaction was stopped by adding 0.4 ml of ice-cold 13.33 mM saltiungstic acid in 1.67 M H_2SO_4. 0.3 ml of 10% ammonium molybdate was added. After vortexing, 1 ml of isobutanol:benzene (1:1) was added. The sample was vortexed for 30 seconds, followed by centrifugation at 1,000 x g for 10 minutes. 0.5 ml of the organic phase was counted. ATPase activities represent mean $\pm$ S.E. for five preparations.

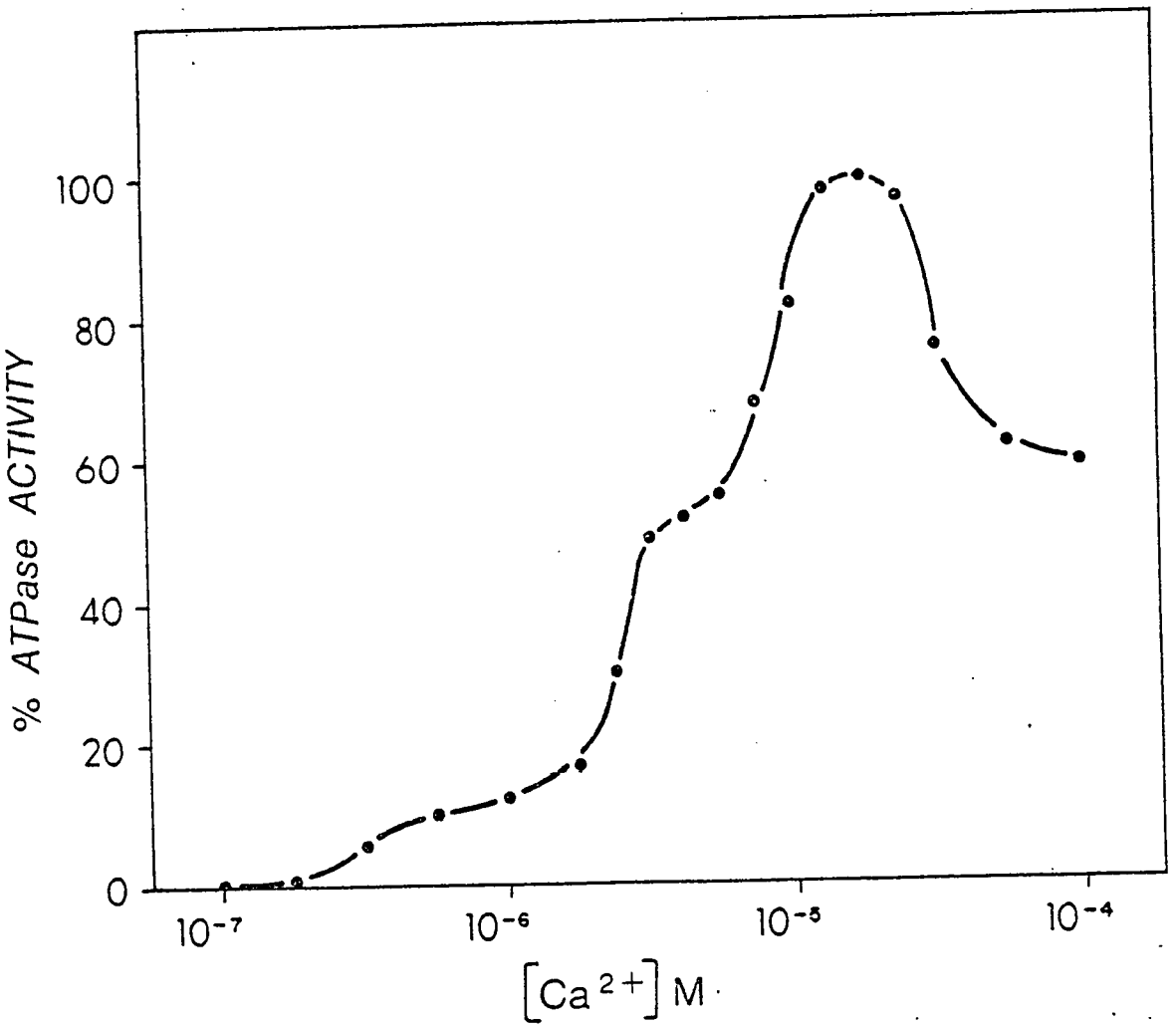
TABLE IV

ATPase Activity

Protein Fraction	Mg^{2+}-ATPase	Ca^{2+}-dependent ATPase
	µmoles Pi/mg/min	µmoles Pi/mg/min
Cardiac microsomes	0.18 ± 0.03	0.24 ± 0.06
First DOC supernatant	0.02 ± 0.03	0.73 ± 0.10
Purified Ca^{2}+Mg^{2+}-ATPase	0.01 ± 0.02	1.27 ± 0.15
Purified Ca^{2+}+ Mg^{2+}-ATPase + 0.1% DOC	0.01 ± 0.03	2.47 ± 0.31

FIGURE 4

ATPase activity with varying calcium concentrations. The purified protein was suspended at 50 μg/ml in 40 mM histidine, pH 6,8, 0.125 M KCl, 5.0 mM $MgCl_2$ and varying calcium concentrations in the presence of EGTA. The reaction was started by the addition of 5 mM [γ-^{32}P] ATP. After 30 seconds the reaction was terminated as described in "Methods".


% ATPase ACTIVITY
100
80
60
40
20
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[Ca²⁺] M

Phospholipid Content of ATPase

The purifed cardiac ATPase bound 11.5 moles of phospholipid per mole of enzyme (Table III). The Ca^{2+}+ Mg^{2+}-ATPase from skeletal muscle isolated by MacLennan (1970) bound considerably more phospholipid than the cardiac enzyme. However, the ATPase purified by Warren et al. (1974) from skeletal muscle bound about the same amount of phospholipid as the cardiac enzyme. These findings undoubtedly result from the method of isolation. MacLennan (1970) used relatively large concentrations of DOC to solubilize most of the membrane, and then selectively precipitated the ATPase. The concentration of DOC used there, was capable of solubilizing much more lipid, which then could remain with the ATPase upon precipitation. The prcedure presented here and the procedure of Warren et al. (1974) use a low concentration of DOC, resulting in fewer phospholipids and DOC molecules being bound to the ATPase. Lipid or a lipid substitute, detergent, is needed for ATPase activity.

DISCUSSION

Selective solubilization of membrane proteins by detergents is a very powerful tool in protein purification. The 22,000 dalton protein, specifically phosphorylated by cyclic AMP-dependent protein kinase, has a very unique property. When phosphorylated it cannot be solubilized by DOC (Bidlack and Shamoo, 1979b). However, unphosphorylated, the protein is soluble in a low concentration of detergent. This fact was used in the purification of the protein. After selective solubilization, only a Sephadex G-75 column was needed to completely purify the protein. The purified protein was specifically phosphorylated by cyclic AMP-dependent protein kinase.

The isolated 22,000 dalton protein bound 6.83 nmoles of phosphate per mg of protein, compared to the sarcoplasmic reticulum, which bound 1.35 nmoles of phosphate per mg of protein. This is not as great an enhancement as might be anticipated considering that the 22,000 dalton protein accounts for at the most 5% of the total microsomal protein. However, in the sarcoplasmic reticulum, more than one protein is phosphorylated. The 22,000 and 6,000 dalton proteins are the major ones, along with the endogenous protein kinase and phosphorylase a. The isolated protein bound 0.150 moles of phosphate per mole of protein. Specific cyclic AMP-dependent protein kinase phosphorylation of isolated ribosomal proteins has shown that between 0.035-0.069 moles of phosphate were incorporated per mole of protein (Becker-Ursic and Davies, 1976;

Herbert et al., 1976). Glycogen synthetase (Soderling et al., 1970) has been shown to incorporate approximately 1 mole of phosphate per mole of protein, while phosphorylase kinase incorporates 0.4 moles of phosphate per mole of protein (Walsh et al., 1970). Thus, there seems to be a large range of values found for the specific incorporation of a phosphate into a protein by cyclic AMP-dependent protein kinase. It is possible that solubilization of the 22,000 dalton protein results in a structural change of the protein, decreasing its ability to become phosphorylated. The possibility also exists that 0.150 moles of phosphate incorporated into the protein per mole of protein is the maximal level of phosphorylation of this protein. The protein may act in a form other than monomeric, such as a tetramer. Regardless, the 22,000 dalton protein is specifically phosphorylated by cyclic AMP-dependent protein kinase at a high enough specific activity that the function of the phosphrylated 22,000 dalton protein can be elucidated.

When isolated, the 22,000 dalton protein still has phospholipids bound to it. This fact is consistent with the finding that the 22,000 dalton protein was soluble in acidified chloroform:methanol, indicating the possibility that it is a proteolipid (Bidlack and Shamoo, 1979b). Whether these lipids play any direct role in the function of the 22,000 dalton protein is not known. It has been shown that the protein, not the lipid, is the component phosphorylated by cyclic AMP-dependent protein kinase (Bidlack and Shamoo, 1979b).

The Ca^{2+}+ Mg^{2+}-ATPase was isolated by employing successive additions of DOC. This procedure allowed for the very rapid purification of the ATPase. Because of solubilizing the enzyme in a very low concentration of detergent, fewer phospholipids were associated with the enzyme than found in the purifed skeletal ATPase isolated by MacLennan (1970). However, the phospholipid content of the isolated cardiac ATPase is in agreement with the phospholipid content from the isolated skeletal enzyme purified by Warren et al. (1974), which also employed a low concentration of detergent. The addition of DOC to the purified enzyme greatly enhanced its hydrolytic activity. In the skeletal enzyme, it appears that the more lipids associated with the enzyme, the greater its hydrolytic activity (MacLennan, 1970; Warren et al., 1974). Lipids appear responsible for maintaining the conformation state of the ATPase. The K_m value for calcium found for the purified enzyme is in agreement with the value found for cardiac microsomes, and is about 5 times higher than the value for the ATPase from skeletal muscle (Shigekawa et al., 1976; Levitsky et al., 1976).

Using the procedures presented here, the rapid purification of the 22,000 dalton protein and the Ca^{2+}+ Mg^{2+}-ATPase is possible. A good yield of the 22,000 dalton protein (approximately 200 µg) can be obtained from as few as 2 canine hearts, while the ATPase can be isolated from 1 heart. For a short period of time (less than 2 days), the ATPase remains the most stable when stored

on ice. For maintaining activity for as long as a week and a half, the best results were obtained when the enzyme was frozen in the presence of calcium and ATP. With the isolation of both these proteins, it will now be possible to elucidate the function of cyclic AMP-dependent phosphorylation of the 22,000 dalton protein in the regulation of calcium transport.

REFERENCES

Ames, B.N. and Dubin, D.T. (1960) The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235: 769-775

Becker-Ursic, D. and Davies, J. (1976) In vivo and in vitro phosphorylation of ribosomal proteins by protein kinases from saccharomyces cerevisiae. Biochem. 15: 2289-2296

Bidlack, J.M. and Shamoo, A.E. (1979a) Cyclic AMP-dependent phosphorylation of a 6,000 and a 22,000 dalton protein from cardiac sarcoplasmic reticulum vesicles. Biophys. J 25: 24a

Bidlack, J.M. and Shamoo, A.E. (1979b) Adenosine 3':5'-monophosphate dependent-phosphorylation of a 6,000 and a 22,000 dalton protein from cardiac sarcoplasmic reticulum. Submitted to J. Biol. Chem.

Harigaya, S. and Schwartz, A. (1969) Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. Membrane vesicles (relaxing system) and mitochondria. Circ. Res. 25: 781-794

Hasselbach, W. (1964) Relaxing factor and the relaxation of muscle. Progr. Biophys. Chem. 14: 167-222

Hebert, J., Pierre, M. and Loeb, J.E. (1977) Phosphorylation in vitro and in vivo of ribosomal proteins from saccharomyces cerevisiae. Eur. J. Biochem. 72: 167-174

Jones, L.R., Besch, Jr., H.R., Fleming, J.W., McConnaughey, M.M., and Watmbe, A.M. (1979) Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. J. Biol. Chem. 254: 530-539

Katz, A.M., Repke, D.I., Upshaw, J.E. and Polascik, M.A. (1970) Characterization of dog cardiac microsomes. Use of zonal centrifugation to fractionate fragmented sarcoplasmic reticulum, (Na^+K^+)-activated ATPase and mitochondrial fragments. Biochim. Biophys. Acta 205: 473-490

Kemp, B.E., Benjamini, E. and Krebs, E.G. (1976) Synthetic hexapeptide substrates and inhibitors of 3':5'-cyclic AMP-dependent protein kinase. Proc. Nat. Acad. Sci. 73: 1038-1042

Kirchberger, M.A. and Chu, G. (1976) Correlation between protein kinase-mediated stimulation of calcium transport by cardiac sarcoplasmic and phosphorylation of a 22,00 dalton protein Biochem. Biophys. Acta 419: 559-562

REFERENCES (con't)

Kirchberger, M.A., Tada, M. and Katz, A.M. (1974) Adenosine 3':5'-monophosphate-dependent protein kinase-catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J. Biol. Chem. 249: 6166-6173

Kirchberger, M.A., Tada, M. and Katz, A.M. (1975) Phospholamban: a regulatory protein of the cardiac sarcoplasmic reticulum. In "Recent Advances in Studies on Cardiac Structures and Metabolism" (Fleckestein, A. and N.S. Dhalla, eds.) Baltimore, Univ. Park Press Vol. 5 pp. 103-115

Levitsky, D.O., Aliev, M.K., Kuzmin, A.V., Levchenko, T.S., Smirnov, V.N. and Chazor, E.I. (1976) Isolation of calcium pump system and purification of calcium ion-dependent ATPase from heart muscle. Biochim. Biophys. Acta 443: 468-484

Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 246: 2702-2710

MacLennan, D.H. (1970) Purification and properties of an ATPase from sarcoplasmic reticulum. J. Biol. Chem. 245: 4508-4518

MacLennan, D.H, (1974) Isolation of proteins of the sarcoplasmic reticulum. In "Methods in Enzymology" (Fleischer, S. and Packer, L., eds.), Vol. 32, pp. 291-302 Academic Press, New York

Sigekawa, M., Finegan, J.M and Katz, A.M. (1976) Calcium transport ATPase of canine cardiac sarcoplasmic reticulum. A comparison with that of rabbit fast skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 251: 6894-6900

Soderling, T.R., Hickenbottom, J.P., Reimann, E.M., Hunkeler, F.L., Walsh, D.A. and Krebs, E.G. (1970) Inactivation of glycogen synthetase and activation of phosphorlyase kinase by muscle adenosine 3':5'-monophosphate-dependent protein kinases. J. Biol. Chem. 245: 6317-6328

Tada, M., Kirchberger, M.A. and Katz, A.M. (1975) Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 250: 2640-2647

Tada, M., Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974) The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 249: 6174-6180

REFERENCES (con't)

Tada, M., Ohmori, F., Kinoshita, N. and Abe, H., (1978) Cyclic AMP regulation of active calicum transport across membranes of sarcoplasmic reticulum. Role of the 22,000-dalton protein. Advan. Cyclic Nucleotide Res. 9: 355-369

Tada, M., Ohmori, F., Yamada, M. and Abe, H. (1979) Mechanism of the stimulation of Ca^{2+}-dependent ATPase of cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 254: 319-326

Van Winkle, W.B., Pitts, B.J.R. and Entman, M.L. (1978) Rapid purification of canine cardiac sarcoplasmic reticulum Ca^{2+}-ATPase. J. Biol. Chem. 253: 8671-8673

Walsh, D.A., Perkins, J.P., Bropstram, C.O., Ho, E.S. and Krebs, E.G. (1971) Catalysis of the phosphorlyase kinase activation reaction. J. Biol. Chem. 246: 1968-1976

Warren, G.V., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J. (1974) Reconstitution of a calcium pump using defined membrane components. Proc. Nat. Acad. Sci. 71: 622-626

Weber, A. (1966) Energized calcium transport and relaxing factors. Current Topics Bioenergetics 1: 203-254

Weber, K. and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412

CHAPTER 3

RECONSTITUTION OF THE CARDIAC Ca^{2+} + Mg^{2+} -ATPase AND THE 22,000 DALTON PROTEIN PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE, IN LIPID VESICLES.

SUMMARY

The Ca^{2+} + Mg^{2+}-ATPase from cardiac sarcoplasmic reticulum was reconstituted in lecithin vesicles along with the 22,000 dalton protein, phosphorylated specifically by cyclic AMP-dependent protein kinase. The Ca^{2+} + Mg^{2+}-ATPase transports Ca^{2+} in an ATP-dependent manner. In the presence of phosphate inside the reconstituted vesicles, the phosphorylated 22,000 dalton protein transports Ca^{2+} 5 times greater than the nonphosphorylated protein. The phosphorylated 22,000 dalton protein has no direct effect on the transport of Ca^{2+} by the Ca^{2+} + Mg^{2+}-ATPase. Vesicles, reconstituted with the phosphorylated 22,000 dalton protein and the ATPase, transport Ca^{2+} in a manner that is additive of the 2 individual transport processes. Without phosphate present inside the vesicles, the phosphorylated 22,000 dalton protein transports Ca^{2+} at a rate of about one-third greater than the non-phosphorylated protein does. When phosphatases are included in the Ca^{2+} - uptake medium, the originally phosphorylated and nonphosphorylated 22,000 dalton protein transport Ca^{2+} at identical rates after 5 min of incubation. In competition experiments, Ba^{2+}, Mn^{2+} and Sr^{2+} have little effect on the transport of Ca^{2+} by the 22,000 dalton protein, while Zn^{2+} inhibits transport by 30% and Hg^{2+} and ruthinum red inhibit transport almost completely.

INTRODUCTION

Cyclic AMP-dependent protein kinase[1] phosphorylates a 6,000 and a 22,000 dalton protein in cardiac sarcoplasmic reticulum (Bidlack and Shamoo, 1979a, b; Jones et al., 1979). Concomitant with this phosphorylation, calcium transport into the sarcoplasmic reticulum is enhanced (Tada et al., 1974; Will et al., 1976). A decrease in the rate of calcium uptake was observed when phosphatase were added to the phosphorylated microsomes (Kirchberger and Raffo, 1977). Because of the changes in calcium transport as a function of the phosphorylation of the 22,000 dalton protein, the suggestion that the 22,000 dalton protein is a regulator of the Ca^{2+} + Mg^{2+}-ATPase in cardiac sarcoplasmic reticulum has been made (Kirchberger et al., 1975; Tada et al., 1975, 1976, 1978).

Reconstitution of membrane proteins into lipid vesicles is one of the best methods to determine the transport properties of these proteins. The Ca^{2+} + Mg^{2+}-ATPase from skeletal muscle has been shown to pump calcium in an ATP-dependent manner in lipid vesicles (Warren et al., 1974; Jilka et al., 1975; Zimniak and Racker, 1978).

Both the 22,000 dalton protein and the Ca^{2+} + Mg^{2+}-ATPase from cardiac sarcoplasmic reticulum have been purified (Bidlack and Shamoo, 1979c). This communication reports that the 22,000 dalton protein can transport calcium into lipid vesicles, and that this ability to transport calcium is dependent on the phosphorylation of the 22,000 dalton protein.

[1]The abbreviations used are:cyclic AMP, adenosine 3':5'- monophosphate; protein kinase, adenosine 3':5' - monophosphate-dependent protein kinase; DOC, deoxycholate; SDS, sodium dodecyl sulfate; HEPES, N-2-hydroxethylpiperazine-N'-2-ethene sulfonic acid.

EXPERIMENTAL PROCEDURE

MATERIALS

Bovine heart cyclic AMP-dependent protein kinase, alkaline phosphatases, sodium cyclic AMP, tris-ATP and soy bean lectin were purchased from Sigma Chemical Co. ^{45}Ca was obtained from Amersham/Searle. Cation exchange resin, AG 50W-X8, 50-100 mesh was purchased from Biorad. Instagel was obtained from Packard Chemical Co.

METHODS

Purification of the 22,000 Dalton Protein

Cardiac microsomes were prepared from canine heart ventricle according to Harigaya and Schwartz (1969) with the modifications described in Bidlack and Shamoo (1979b). The 22,000 dalton protein was isolated according to the procedure described in Bidlack and Shamoo (1979c).

Purification of the Ca^{2+} + Mg^{2+}-ATPase

The Ca^{2+} + Mg^{2+}-ATPase from cardiac sarcoplasmic reticulum was purified as described by Bidlack and Shamoo (1979c) with the following modifications. The sarcoplasmic reticulum was suspended at 7 mg/ml in 0.25 M sucrose. DOC was added at the ratio of 2 µg DOC/7 mg protein.

After incubating on ice for 15 min, the sample was centrifuged at 100,000 x g for 30 min. The pellet was suspended in 1 mM HEPES, pH 7.5,at the original volume. Additional DOC was added at the concentration of 2 μg DOC/7 mg original microsomal protein. The suspension was incubated on ice for 15 min, followed by centrifugation at 100,000 x g for 30 min. The supernatant from this centrifugation contained the Ca^{2+} + Mg^{2+}-ATPase to at least 95% purity. The buffer HEPES was used to resuspend the pellet of the initial DOC solubilization because sucrose has been shown to interfere with the reconstitution of the Ca^{2+} + Mg^{2+}-ATPase from skeletal muscle into lipid vesicle (Zimniak and Racker, 1979).

Reconstitution of the Ca^{2+} + Mg^{2+}-ATPase and the 22,000 Dalton Protein

The reconstitution procedure is essentially that of Kasahara and Hinkle (1977) with the following modifications. Soy bean asolectin was washed with petroleum ether and lyphilized for 3 hrs. The dried lipid was suspended in 10 mM K-HEPES, pH 7.5, at a concentration of 60 mg/ml. The suspension was flushed with nitrogen and sonicated in a covered test tube for 20-30 min in a bath sonicator at 25°.

When the phosphorylated 22,000 dalton protein was included in the reconstituted vesicles, it was first phosphorylated in the following manner. The 22,000 dalton protein was suspended in a final concentration of 0.125 M KCl, 10 mM tris-HCl, pH 7.5, 20 μM $MgCl_2$, 20 μM tris-ATP, 1 μM

cyclic AMP and protein kinase (0.08 mg/ml). After 10 min, lipid was added to yield a final concentration of 1 μg protein to 20 μg lipid. The nonphosphorylated 22,000 dalton was incubated in the above reaction mixture minus the cyclic AMP.

When the Ca^{2+} + Mg^{2+}-ATPase was reconstituted with the 22,000 dalton protein, the ATPase was added to the reaction mixture after the 22,000 dalton had been phosphorylated. The 22,000 dalton protein and the ATPase were reconstituted at equal molar concentrations. Five micrograms of the ATPase were included for every microgram of the 22,000 dalton protein. Reconstituted ATPase without the 22,000 dalton protein consisted of suspending the protein in 0.125 M KCl, 10 mM tris-HCl, pH 7.5, at a lipid to protein ratio of 20:1. When phosphate was included in the inside of the vesicles, it was added as potassium phosphate at a concentration of 0.4 M. Also when phosphate was present, HEPES was used in place of the chloride ion. Reconstitution was accomplished by freezing the protein-lipid suspension in liquid nitrogen and then letting the vesicles thaw at room temperature. The vesicles were then sonicated in a bath sonicator for 30 sec.

Calcium-Uptake into Vesicles

10 μl of the reconstituted vesicles (5 μg ATPase, 1 μg 22,000) were suspended in 0.24 ml of a mixture consisting of, in final concentration,

0.125 M KCl, 5 mM $MgCl_2$, 5 mM tris-ATP, 10 mM tris-HCl, pH 7.5, and 0.1 mM $^{45}CaCl_2$ to a final specific radioactivity of 50 μCi/μmole. When phosphate was incorporated into the vesicles, HEPES was used in place of the chloride ion. In competition experiments, the competing cation was added at a concentration of 0.1 mM. The uptake was stopped by pipetting 0.2 ml of the suspension through an AG 50W-X8 cation exchange 50-100 mesh, resin column. The resin had been made the tris form by washing with 1 M tris. The pH of the resin was adjusted by washing with water until the pH was approximately 7.5. The resin was suspended in 0.25 M sucrose. Prior to use, the cation exchange column (0.5 x 7 cm) was washed with 3 ml of 0.25 M sucrose, 3.3 mg of serum albumin/ml. Each 0.2 ml of sample was eluted with 3 ml of 0.25 M sucrose. A small volume (0.05-0.20 ml) of the eluant was counted in 10 ml of Instagel. Control vesicles consisted of vesicles without protein.

RESULTS

Reconstitution of the Ca^{2+} + Mg^{2+}-ATPase with the 22,000 Dalton Protein in the Absence of Phosphate

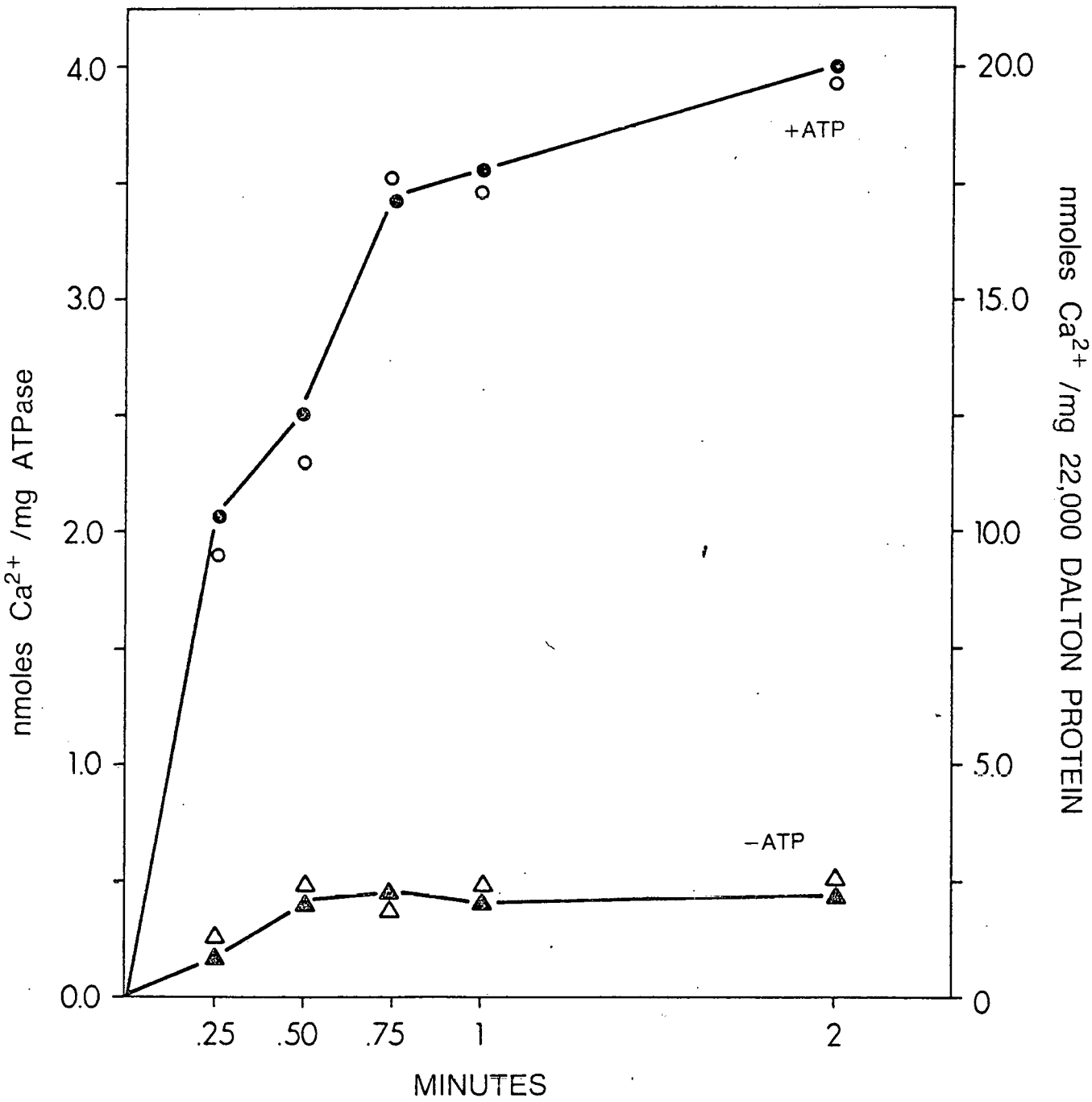
Ca^{2+} + Mg^{2+}-ATPase reconstituted with soy bean asolectin vesicles in the absence of phosphate did transport Ca^{2+}, as is shown in Fig. 1. This transport was dependent on the presence of ATP. As is shown by the open symbols in Fig. 1, the co-reconstitution of the phosphorylated 22,000 dalton protein with the Ca^{2+} + Mg^{2+}-ATPase in an equal molar ratio, had no effect on the transport of Ca^{2+} into the vesicles. Equal molar concentrations of the proteins were used because that is approximately the ratio that they are present in the sarcoplasmic reticulum, with the ATPase accounting for 40-50% of the total protein, while the 22,000 dalton protein accounts for 4-5% of the total protein.

Reconstitution of the Ca^{2+} + Mg^{2+}-ATPase with the 22,000 Dalton Protein in the Presence of Phosphate

When reconstituted lipid vesicles were made in the presence of 0.4 M phosphate, a "sink" was created to trap the transported calcium. As a result of this trapping, the transport of Ca^{2+} into the vesicles is enhanced as is shown in Fig. 2. The greatest transport occurred when the ATPase was reconstituted along with the phosphorylated 22,000 dalton protein (designated 22P). When the ATPase was reconstituted

FIGURE 1

Ca^{2+} - uptake by lipid vesicles in the absence of phosphate reconstituted with the Ca^{2+} + Mg^{2+} -ATPase in the presence and absence of the 22,000 dalton protein, phosphorylated and not. The ATPase was reconstituted in 0.125 M KCl, 10 mM tris-HCl, pH 7.5, and lectin at a lipid to protein ratio of 20:1. When the 22,000 dalton protein was reconstituted with the ATPase, the 22,000 dalton protein was first phosphorylated in a mixture of 0.125 M KCl, 10 mM tris-HCl, pH 7.5, 20 μM $MgCl_2$, 20 μM tris-ATP, 1 μM cyclic AMP and protein kinase (0.08 mg/ml). The nonphosphorylated 22,000 dalton protein was incubated in the above reaction mixture minus the 1 μM cyclic AMP. The 22,000 dalton protein was reconstituted with the ATPase at equal molar ratio. The ratio of lipid to 22,000 dalton protein was 100:1. The uptake buffer consisted of, in final concentration, 0.125 M KCl, 10 mM tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM tris-ATP and 0.1 mM $^{45}CaCl_2$. Uptake was stopped by passing the vesicles through a cation exchange resin column. The open symbols represent uptake from vesicles reconstituted with the ATPase and the phosphorylated 22,000 dalton protein. Closed symbols represent vesicles containing only the ATPase.


4.0
3.0
2.0
1.0
0.0
20.0
15.0
10.0
5.0
0
+ATP
−ATP
.25
.50
.75
1
2
MINUTES
nmoles Ca2+ /mg ATPase
nmoles Ca2+ /mg 22,000 DALTON PROTEIN

with the nonphosphorylated 22,000 dalton protein (designated 22), the Ca^{2+} transport into the vesicles did not significantly differ from the transport observed by the ATPase alone. The phosphorylated 22,000 dalton protein, reconstituted at the same concentration as when the ATPase was present (1 µg 22,000:100 µg lipid), was also capable of transporting Ca^{2+}. The nonphosphorylated 22,000 dalton protein transported Ca^{2+} at about one-fifth the rate that the phosphorylated 22,000 dalton protein did.

While the transport of Ca^{2+} was the greatest when the ATPase and the phosphorylated 22,000 dalton protein were reconstituted together, the phosphorylated 22,000 dalton protein does not have a direct effect on the ATPase. The enhancement observed when the 2 proteins were reconstituted together is an additive effect. Adding the transport of Ca^{2+} observed by the ATPase alone to the transport observed by the phosphorylated 22,000 dalton protein alone yields a transport rate virtually identical to that observed when the ATPase and the phosphorylated 22,000 dalton protein were reconstituted together. These data do indicate that the 22,000 dalton protein can transport Ca^{2+} and that much of this transport is dependent on the phosphorylation of the 22,000 dalton protein.

Ca^{2+} - Uptake by the Reconstituted 22,000 Dalton Protein in the Absence of Phosphate

The ability of the 22,000 dalton protein to transport Ca^{2+} into lipid vesicles when there is free Ca^{2+} inside the vesicles was investigated.

FIGURE 2

Ca^{2+}- uptake by lipid vesicles in the presence of phosphate reconstituted with the Ca^{2+} + Mg^{2+} -ATPase in the presence and absence of the 22,000 dalton protein, phosphorylated and not. Reconstitution was carried out as described in Fig. 1 with the exception that 0.4 M potassium phosphate was included in the reconstituted vesicles and HEPES was used in place of the Cl^- ion. All experiments were done with 5 mM ATP in the uptake medium.

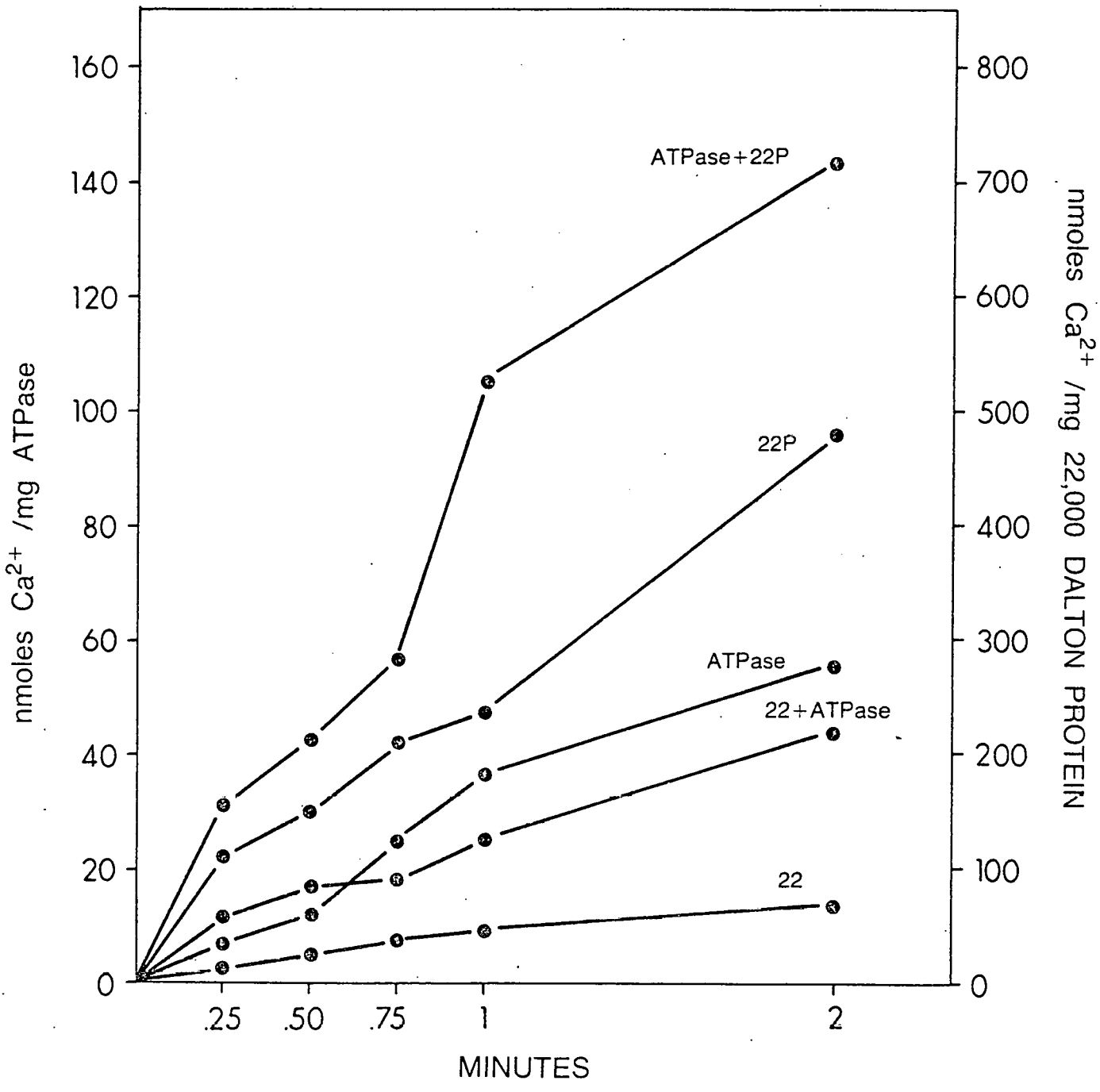
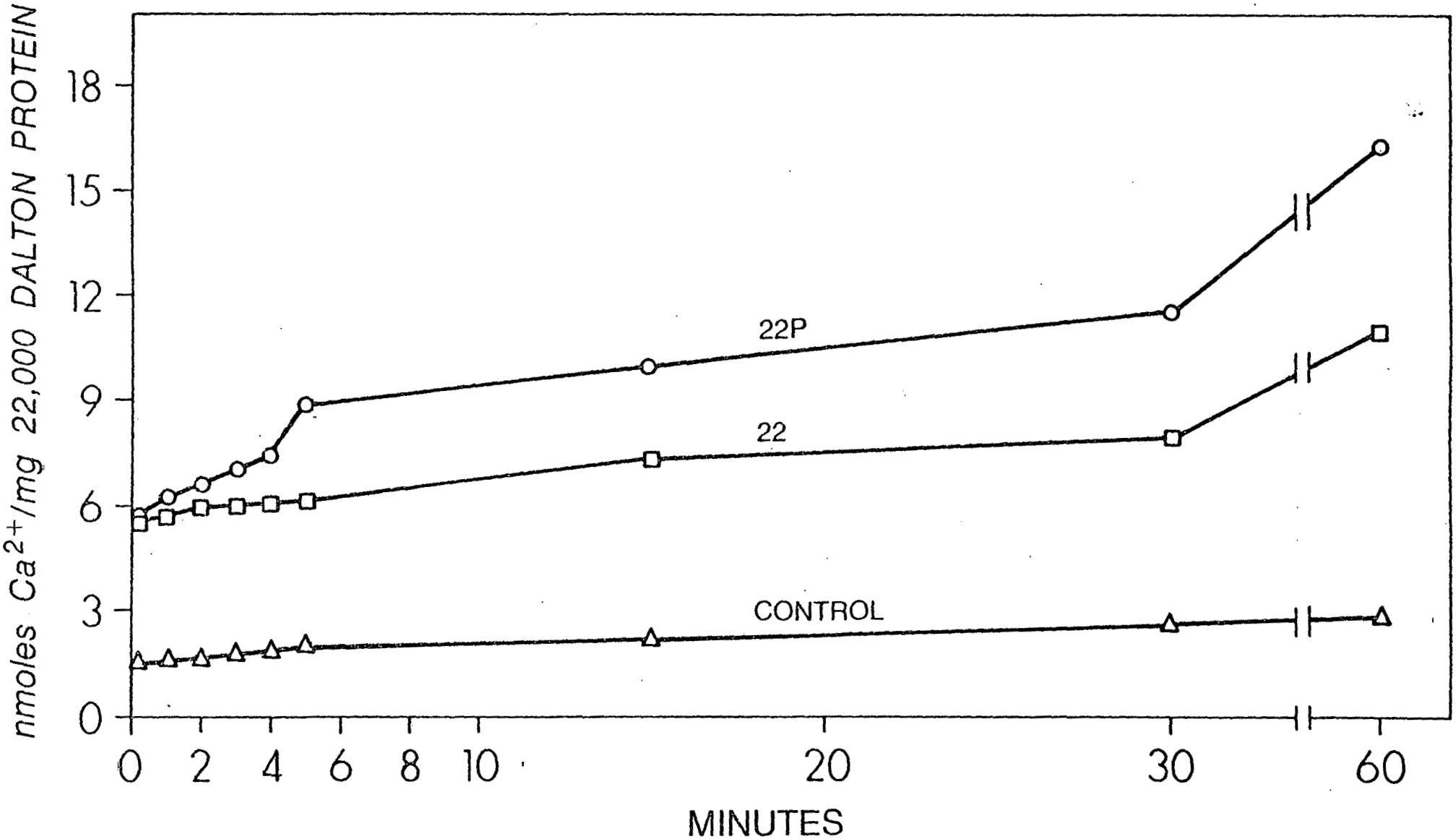

160
140
120
100
80
60
40
20
0
800
700
600
500
400
300
200
100
0
.25
.50
.75
1
2
MINUTES
nmoles Ca2+ /mg ATPase
nmoles Ca2+ /mg 22,000 DALTON PROTEIN
ATPase+22P
22P
ATPase
22+ATPase
22

Fig. 3 shows that the phosphorylated 22,000 dalton protein can transport Ca^{2+} into lipid vesicles better than the nonphosphorylated protein. The time course for uptake without phosphate being present is longer than in its presence. Over a long time course, the nonphosphorylated 22,000 dalton protein transported approximately 70% of the Ca^{2+} transported by the protein when it is phosphorylated. After an initial binding of Ca^{2+} to the vesicles, the initial rate of Ca^{2+} uptake over the first 4 min, was almost 8 times greater for the phosphorylated protein than the nonphosphorylated protein. The slope of the uptake rate for the first 4 min was 0.41, for the phosphorylated protein and 0.06 for the nonphosphorylated protein. Thus, like in the presence of phosphate, the phosphorylated 22,000 dalton protein can transport Ca^{2+} better than the nonphosphorylated protein. The relative enhancement of Ca^{2+} transport by the phosphorylation of the protein did vary depending on whether phosphate was present. In the absence of phosphate, the nonphosphorylated protein transported at a rate of 70% of the phosphorylated protein, while in the presence of phosphate, the transport of Ca^{2+} by the nonphosphorylated protein was only 20% of that observed by the phosphorylated protein.

FIGURE 3

Ca^{2+} - uptake by the 22,000 dalton protein, phosphorylated and not. The 22,000 dalton protein was phosphorylated as described in Fig. 1. The protein was reconstituted at a lipid to protein ratio of 100:1. 10 µl of vesicles were added to 0.24 ml of uptake buffer consisting of 10 mM tris-HCl, pH 7.5, 0.125 M KCl and 0.1 mM $^{45}CaCl_2$. The control vesicles consisted of reconstituted vesicles without protein. The open circles represent the phosphorylated 22,000 dalton protein. The open squares represent the nonphosphorylated 22,000 dalton protein, and the open triangles represent the control vesicles.

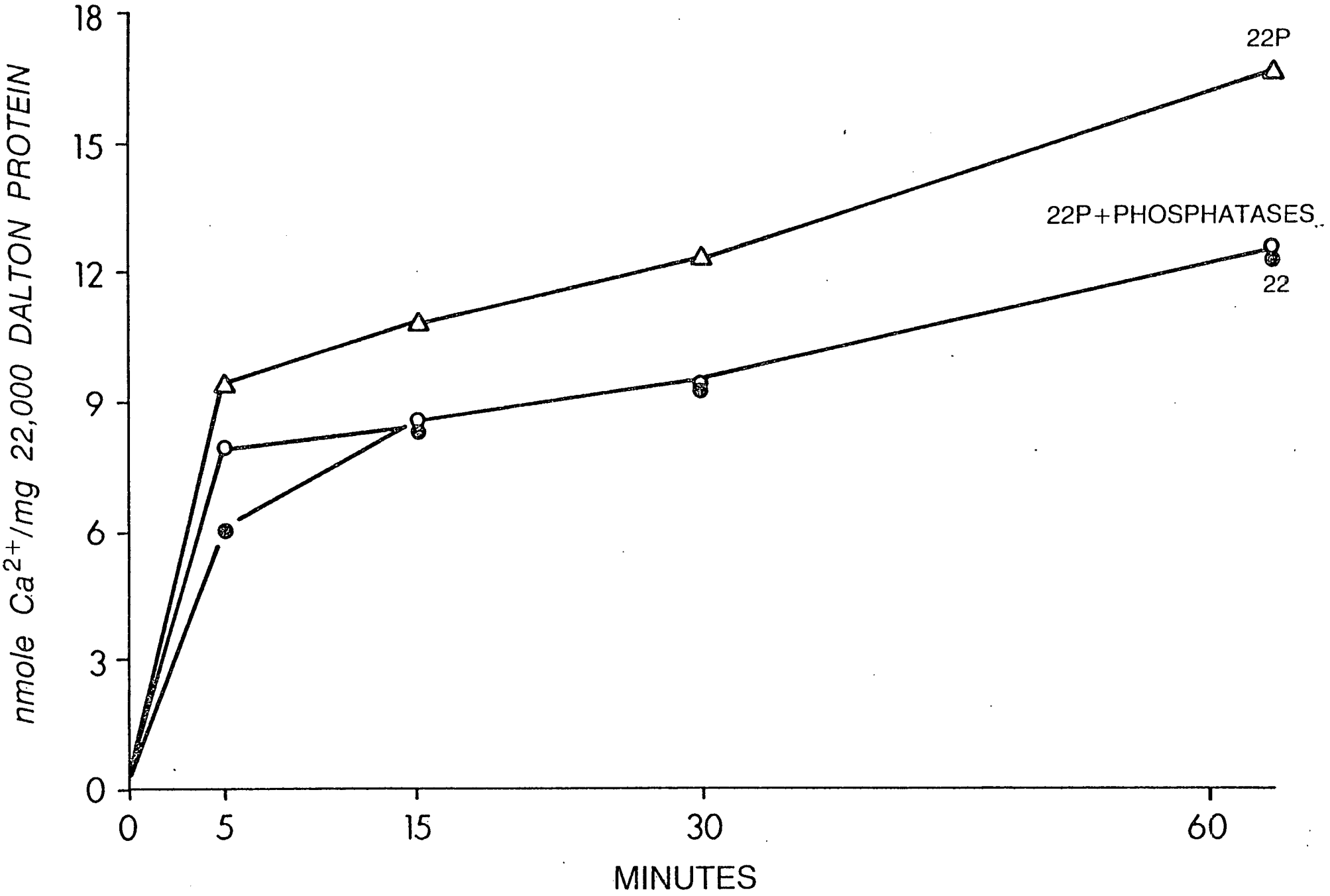

nmoles Ca^{2+}/mg 22,000 DALTON PROTEIN
18
15
12
9
6
3
0
22P
22
CONTROL
0
2
4
6
8
10
20
30
60
MINUTES

Ca^{2+} - Uptake into Vesicles Reconstituted with the 22,000 Dalton Protein in the Presence of Phosphatases

Vesicles with the phosphorylated and nonphosphorylated 22,000 dalton protein were incubated in Ca^{2+} - uptake medium containing alkaline phosphatases (10 µg phosphatases/0.25 ml uptake medium). Fig. 4 shows the effect of phosphatases on Ca^{2+} uptake. After 5 min of incubating in the presence of phosphatase, the Ca^{2+} uptake by the phosphorylated 22,000 dalton protein is reduced significantly in comparison to when there are no phosphatases present. The uptake was still slightly greater than that observed by the nonphosphorylated 22,000 dalton protein in the presence of phosphatases. After 15 min of incubating, there was no difference in the Ca^{2+} - transport by the phosphorylated or nonphosphorylated 22,000 dalton protein in the presence of phosphatase. These data seem to indicate that the phosphoester bond is responsible for the enhanced transport seen by the phosphorylated 22,000 dalton protein. It also appears that both proteins incorporate equally well into the reconstituted vesicles, since once the phosphorylated 22,000 dalton protein loses its phosphoester bond, it transports Ca^{2+} equal to the nonphosphorylated protein.

FIGURE 4

Uptake of Ca^{2+} by the 22,000 dalton protein in the presence of phosphatases. The 22,000 dalton protein was phosphorylated and reconstituted as described in Fig. 3 and the "Methods" section. The uptake medium contained 0.125 M KCl, 10 mM tris-HCl, pH 7.5, and 0.1 mM $^{45}CaCl_2$. 5 µl (10 µg) of alkaline phosphatases were added to 0.235 ml uptake medium. 10 µl of reconstituted vesicles were added to start the uptake. The open triangles represent the phosphorylated 22,000 dalton protein not incubated with phosphatases. The open circles represent the phosphorylated 22,000 dalton protein incubated with phosphatases. The closed circles represent the nonphosphorylated 22,000 dalton protein incubated with phosphatases.


18
15
12
9
6
3
0
nmole Ca^{2+}/mg 22,000 DALTON PROTEIN
22P
22P+PHOSPHATASES
22
0
5
15
30
60
MINUTES

Ca^{2+} - Transport by the 22,000 Dalton Protein in the Presence of Competing Cations

Reconstituted lipid vesicles in the absence of phosphate were incubated for 1 hr in the uptake buffer, containing 0.1 mM $^{45}CaCl_2$ and 0.1 mM of the competing cation. The vesicles were then assayed for Ca^{2+} uptake. Table I summarizes the results. The effect of the competing cation was the same for both the phosphorylated and the nonphosphorylated 22,000 dalton protein. Mn^{2+} and Ba^{2+} had virtually no effect on the transport of Ca^{2+}. Sr^{2+} inhibited transport by approximately 7%, while Zn^{2+} inhibited 30% of the transport. Hg^{2+} was capable of inhibiting 90% of the Ca^{2+} transport, while ruthinum red at a concentration of 10 μM was 99% effective. Mg^{2+} was not tested because all the vesicles were made in the presence of 20 μM Mg^{2+}, an ion necessary for the phosphorylation of the 22,000 dalton protein.

TABLE I

Competitive inhibition by cations of Ca^{2+} transport by the 22,000 dalton protein. The 22,000 dalton protein was phosphorylated and reconstituted as described in "Methods" and Fig. 1. 10 µl of vesicles were transferred to 0.24 ml of an uptake buffer consisting of, in final concentration, 0.125 M KCl, 10 mM tris-HCl, pH 7.5, 0.1 mM $^{45}CaCl_2$ and 0.1 mM of the competing cation. Ruthinum red was added at a concentration of 10 µM. Transport was allowed to proceed for 1 hr before being terminated by passing 0.2 ml of the vesicles in the uptake solution through a cation exchange resin, AG 50W-X8, column equilibrated with 0.25 M sucrose. The phosphorylated 22,000 dalton protein is designated 22,000-P and the nonphosphorylated protein as 22,000. The data represents the mean ± S.E. for three determinations.

TABLE I

Competitive Inhibition by Cation of Ca^{2+} Transport by the 22,000 Dalton Protein

Competing Cation	% Ca^{2+} - transport	
	22,000-P	22,000
None	100 ± 5	71 ± 4
Mn^{2+}	99 ± 6	69 ± 4
Ba^{2+}	98 ± 5	69 ± 5
Sr^{2+}	93 ± 5	62 ± 4
Zn^{2+}	71 ± 4	37 ± 3
Hg^{2+}	10 ± 3	4 ± 2
Ruthinum Red (10 μM)	1 ± 2	1 ± 1

DISCUSSION

Cyclic AMP appears to be a regulator of many transport processes (Tsien, 1977; Nathanson, 1977). Of all transport systems regulated by cyclic AMP, the cardiac system is the best characterized. Both Tada et al. (1974) and Will et al. (1976) have shown that Ca^{2+} transport into the cardiac sarcoplasmic reticulum is enhanced by the presence of cyclic AMP and protein kinase. Concomitant with the enhancement of Ca^{2+} transport, a 22,000 dalton protein was phosphorylated by cyclic AMP-dependent protein kinase. Since the Ca^{2+} + Mg^{2+}-ATPase is the protein actively responsible for transporting Ca^{2+} into the sarcoplasmic reticulum, it has been postulated that the phosphorylated 22,000 dalton protein regulates the ATPase's ability to transport Ca^{2+} (Kirchberger et al., 1975; Tada et al., 1975, 1976, 1978). The experiments detailed in this communication indicate that the phosphorylated 22,000 dalton protein does not act directly on the ATPase, but instead is capable of transporting Ca^{2+} itself.

When both the phosphorylated 22,000 dalton protein and the ATPase were reconstituted in lipid vesicles, Ca^{2+} transport into the vesicles was maximized (Fig. 2). However, this enhanced transport rate is only an additive response of vesicles reconstituted with the phosphorylated 22,000 dalton protein and the ATPase, separately. The transport of Ca^{2+} into all reconstituted vesicles is much greater when phosphate is inside the vesicles. This is a result of the phosphate precipitating the

entering Ca^{2+}, thus maintaining an environment with no free Ca^{2+}.

Over a 2 min time course, in the absence of phosphate, vesicles reconstituted with the ATPase and the phosphorylated 22,000 dalton protein showed no enhanced transport over vesicles reconstituted with the ATPase alone (Fig. 1). As was shown in Fig. 3, the 22,000 dalton protein does transport Ca^{2+} in the absence of phosphate. However, this transport is much smaller than that seen by the ATPase and was thus obscured when both were reconstituted together. When the reconstituted 22,000 dalton portein was first introduced to the uptake medium, there was an initial binding of Ca^{2+}. After this binding, there was an initial uptake of Ca^{2+} lasting for about 5 min. This initial rate was approximately 8 times greater for the phosphorylated 22,000 dalton protein than the nonphosphorylated protein. After 5 min, a gradual increase in Ca^{2+} - uptake was observed, with the nonphosphorylated protein taking up about 70% of the Ca^{2+} that the phosphorylated protein did. Why the vesicles don't reach the same equilibrium is not clear. The volume of the vesicles reconstituted with protein may be larger than those without portein. The possibility also exists that in the presence of Ca^{2+}, vesicles are fusing during the experiments. The initial binding of Ca^{2+} to the vesicles is greater in the presence of protein than in its absence.

From the data presented here, it appears that the phosphorylated 22,000 dalton protein is capable of facilitating Ca^{2+} transport into the sarcoplasmic reticulum. This conclusion is consistent with other biochemical properties reported about the 22,000 dalton protein. When the 22,000 dalton protein is not phosphorylated, it appears to be extrinsic, easily attacked by trypsin and DOC (Bidlack and Shamoo, 1979a,b).

After phosphorylation, the protein is resistant to attack by both trypsin and DOC. Phosphorylation appears to cause the protein to become buried in the membrane, transporting Ca^{2+} across the membrane at this time. When phosphatases were included in the uptake medium, after 5 min, there was no enhanced uptake of Ca^{2+} by vesicles reconstituted originally with the phosphorylated 22,000 dalton protein.

Phosphatases have been shown to be closely associated with isolated cardiac microsomes (Tada et al., 1975a). Repeated washing of microsomes does not remove phosphatase activity (Tada et al., 1975a). Thus, when the 22,000 dalton protein is phosphorylated, there are phosphatases in close proximity, capable of dephosphorylating the protein. In the cardiac cell, the 22,000 dalton protein is undobtedly getting phosphorylated and dephosphorylated fairly rapidly. Adenylate cyclase and endogenous protein kinase have also been shown to be associated with cardiac microsomes (Bidlack and Shamoo, 1979b).

The ATPase hydrolytic activity was reported to increase in the presence of cyclic AMP and protein kinase (Kirchberger et al., 1974). This enhanced increase in inorganic phosphate may be the result of the 22,000 dalton protein being phosphorylated and dephosphorylated, thus generating a new source of Pi. The ATPase activity was not examined in the presence of a phosphatase inhibitor (Kirchberger et al., 1974). The increase in calcium uptake by the sarcoplasmic reticulum in the presence of cyclic AMP and protein kinase (Tada et al.,1974; Will et al., 1976), may well be a direct effect of the phosphorylated 22,000 dalton protein facilitating Ca^{2+} transport. Tada et al. (1979) reported that the phosphorylated intermediate of the ATPase did not vary as a function

of cyclic AMP and protein kinase. But, the rate of dephosphorylation of the ATPase was enhanced in the presence of cyclic AMP and protein kinase (Tada et al., 1979). The level of phosphorylated 22,000 dalton protein at any given time in the presence of phosphatases may be quite low, and thus account for the lack of difference seen in the phosphorylated intermediate in the absence or presence of cyclic AMP and protein kinase. How the rate of dephosphorylation of the ATPase is enhanced in the presence of cyclic AMP and protein kinase is not understood.

The data presented here strongly suggest that the 22,000 dalton protein can facilitate Ca^{2+} transport into the sarcoplasmic reticulum, and that much of this transport is dependent on the phosphorylation of the protein. The dephosphorylation of the protein by phosphatases then seems to complete the cycle. It should also be remembered that in addition to the 22,000 dalton protein, a 6,000 dalton protein is also phosphorylated by cyclic AMP- dependent protein kinase (Bidlack and Shamoo, 1979a,b; Jones et al., 1979). What role this protein plays is not known. It is phosphorylated at a slower rate than the 22,000 dalton protein (Bidlack and Shamoo, 1979b). This may indicate that it is a further back-up mechanism for the transport of Ca^{2+} into the sarcoplasmic reticulum. Phosphorylation appears to also cause it to become buried in the membrane (Bidlack and Shamoo, 1979b). Further characterization of the transport properties of both these proteins will greatly increase our knowledge of the molecular mechanism by which cyclic AMP regulates myocardial contractility, and may well help elucidate how other transport processes are regulated by cyclic AMP.

REFERENCES

Bidlack, J.M. and Shamoo, A.E. (1979a) Cyclic AMP-dependent phosphorylation of a 6,000 and a 22,000 dalton protein from cardiac sarcoplasmic reticulum vesicles. Biophys. J. 25: 24a

Bidlack, J.M. and Shamoo, A.E. (1979b) Adenosine 3':5'-monophosphate dependent-phosphorylation of a 6,000 and a 22,000 dalton protein from cardiac sarcoplasmic reticulum. Submitted to J. Biol. Chem.

Bidlack, J.M. and Shamoo, A.E. (1979c) Purification of two proteins from cardiac sarcoplasmic reticulum. A 22,000 dalton protein, specifically phosphorylated by cyclic AMP-dependent protein kinase, and the Ca^{2+} + Mg^{2+}-ATPase. Submitted to J. Biol. Chem.

Harigaya, S.and Schwartz, A. (1969) Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. Membrane vesicles (relaxing system) and mitochondria. Circ. Res. 25: 781-794

Jilka, R.L., Martonosi, A.N. and Tillack, T.W. (1975) Effect of the purified [Mg^{2+} + Ca^{2+}]-activated ATPase of sarcoplasmic reticulum upon passive Ca^{2+} permeability and ultrastructure of phospholipid vesicles. J. Biol. Chem. 25: 7511-7524

Jones, L.R., Besch, Jr., H.R., Fleming, J. W., McConnaughey, M.M. and Watanabe, A.M. (1979) Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. J. Biol. Chem. 254: 530-539

Kasahara, M. and Hinkle, P.C. (1977) Reconstitution and purification of the D-glucose transporter from human erythrocytes. J. Biol. Chem. 252: 7384-7390

Kirchberger, M.A. and Raffo, A. (1977) Phosphoprotein phosphatase-catalyzed dephosphorylation of the 22,000 dalton phosphoprotein of cardiac sarcoplasmic reticulum. J. Cyclic Nucleotide Res. 3: 45-53

Kirchberger, M.A., Tada, M. and Katz, A.M. (1974) Adenosine 3':5'-monophosphate-dependent protein kinase-catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J. Biol. Chem. 249: 6166-6173

Kirchberger, M.A., Tada, M. and Katz, A.M. (1975) Phospholamban: a regulatory protein of the cardiac sarcoplasmic reticulum. In "Recent Advances in Studies on Cardiac Structure and Metabolism" (Fleckestein, A. and N.S. Dhalla, eds.) Vol. 5 pp. 103-115 Baltimore, Univ. Park Press

Nathanson, J.A. (1977) Cyclic nucleotides and nervous system function. Physiol. Rev. 57: 157-256

Tada, M., Kirchberger, M.A. and Katz, A.M. (1975) Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 250: 2640-2647

Tada, M., Kirchberger, M.A. and Katz, A.M. (1976) Regulation of calcium transport in cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase. In "Recent Advances in Studies on Cardiac Structure and Metabolism" (Roy, P.E. and Dhalla, N.S., eds.) Vol.9 pp. 225-239 Univ. Park Press, Baltimore

Tada, M., Kirchberger, M.A. and Li, H.C. (1975a) Phosphoprotein phosphatase-catalyzed dephosphorylation of the 22,000 dalton phosphoprotein of cardiac sarcoplasmic reticulum. J. Cyclic Nucleotide Res. 1: 329-338

Tada, M. Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974) The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 249: 6174-6180

Tada, M., Ohmori, F., Kinoshita, M. and Abe, H. (1978) Cyclic AMP regulation of active calcium transport across membranes of sarcoplasmic reticulum. Role of the 22,000-dalton protein phospholamban. Advan. Cyclic Nucleotide Res. 9: 355-369

Tada, M., Ohmori, F., Yamada, M. and Abe, H. (1979) Mechanism of the stimulation of Ca^{2+}-dependent ATPase of cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 254: 319-326

Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Reconstitution of a calcium pump using defined membrane components. Proc. Nat. Acad. Sci. 71: 622-626

Will, H., Blanck, J., Smettan, G. and Wollenberger, A. (1976) A quench-flow kinetic investigation of calcium ion accumulation by isolated cardiac sarcoplasmic reticulum. Dependence of initial velocity on free calcium ion concentration and influence of preincubation with a protein kinase, Mg ATP, and cyclic AMP, Biochim. Biophys. Acta 449: 295-303

Zimniak, P. and Racher, E. (1978) Electrogenicity of Ca^{2+} transport catalyzed by the Ca^{2+}-ATPase from sarcoplasmic reticulum. J. Biol. Chem. 253: 4631-4637