PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 19–24 (1982)

Characterization of the Phosphoprotein Profile in Spontaneously Beating Cultured Rat Heart Cells (41389)

IRWIN KLEIN, JOHN S. LEVEY, AND MONICA GONDEK

Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, and the Veterans Administration Hospital, Pittsburgh, Pennsylvania 15240

Abstract. Culture of neonatal rat hearts yields a fairly homogeneous population of synchronous and spontaneously beating myocardial cells. Incubation of the culture with [^{32}P]-orthophosphate followed by polyacrylamide gel electrophoresis and autoradiography yields a reproducible profile of both high- and low-molecular-weight phosphoproteins. This phosphoprotein profile is clearly distinct from that observed with mesenchymal cells, the major contaminant of the heart cell cultures. By comparison with purified standard proteins, and by copurification with neonatal rat myosin, we have identified one of these phosphoproteins as the 20,000-dalton myosin light chain. The demonstration of phosphoproteins in intact contracting myocardial cells provides a system to study the complex interaction of the phosphoproteins involved in the control of cardiac contractility.

There are multiple mechanisms by which cardiac contractility is controlled (1, 2). Cardiac inotropic and chronotropic agents such as isoproterenol, glucagon, histamine, and nonepinephrine have been shown to bind to specific cardiac membrane receptors and increase the activity of adenylate cyclase (3, 4). The resulting rise in cellular levels of cyclic AMP then activates a protein kinase which in turn mediates the phosphorylation of specific intercellular proteins (5, 6). A number of phosphoproteins have previously been characterized in heart muscle, and their relation to changes in contractile activity have been suggested (7–9). In order to better understand the capacity for cell regulation through alterations in protein phosphorylation, we have examined the phosphoprotein profile in a relatively homogenous population of contracting cardiac cells in culture. This system yields a reproducible pattern of phosphoproteins and allows for a correlation of changes in contractile events with changes in protein phosphorylation.

Materials and Methods. Eagles modification of minimal essential media (MEM), essential and nonessential amino acids, supplemented vitamins, penicillin, and streptomycin were obtained from Grand Island Biological Company. Calf serum and trypsin were from Flow Laboratories. Acrylamide, sodium dodecyl sulfate, and Coomassie blue were from Bio-Rad, and [^{32}P]orthophosphoric acid from New England Nuclear. Plastic petri dishes and culture flasks were from Falcon and Corning, and colchicine was from Sigma. Zivic-Miller strain of Sprague–Dawley rats were used throughout.

Heart cells were obtained by trypsin treatment of 2- to 4-day-old rats using the technique as described by Harary and Farley (10) with minor modifications. Hearts were aseptically removed from 12 to 20 neonatal rats, and the blood was rinsed clean in Hank's balanced salt solution without calcium or magnesium. The ventricular tissue was finely minced and then incubated at 37° with gentle stirring in Hank's salt solution with 0.125% trypsin. The resulting cell suspension was collected without removing the minced tissue fragments and centrifuged at 600 *g* for 10 min. The supernatant was removed and the cell pellet resuspended in MEM supplemented with complete amino acids *l*-glutamine 200 m*M*, 10% heat-inactivated calf serum, vitamins, penicillin, streptomycin, and additional calcium chloride to yield a final concentration of 4 m*M*. This mixed cell population was then incubated in a 120-cm^2 Fal-

0037-9727/82/050019-06$01.00/0

con flask. After 90 min the nonadherent cells (predominantly myocardial cells) (10, 11) were gently removed and replated in 60-cm Falcon dishes that had been previously coated with gelatin. The flasks, containing predominantly mesenchymal tissue elements, were then overlaid with culture media (supplemented MEM, 1.8 m*M* calcium) and maintained for periods of up to 14 days.

The culture dishes containing cardiac cells were incubated at 37° in an atmosphere of 5% CO_2/95% room air and after 24 to 48 hr the media changed to supplemented MEM with a final calcium concentration of 1.8 m*M*. Cells were observed daily utilizing an inverted stage, phase-contrast Nikon microscope, with the stage warmed by a curtain of air to between 35 and 40°. Within 24 hr of plating of the heart cells, spontaneous contractile activity was observed. Rates of contraction were assessed by manual counting for two successive 15-sec periods and the average rate calculated. Duplicate counts did not vary by more than 10%. In experiments where it was employed colchicine 1×10^{-6} *M* was added after baseline heart rates were determined and just prior to the addition of [^{32}P]orthophosphoric acid.

All cultures used for the identification of phosphoproteins were incubated for 48 to 96 hr and had spontaneous synchronous heart rates of between 90 and 120 beats/min. Visual observation of the percentage of heart cells, using either the criteria of cell morphology (11, 12) or the presence of contractile activity, demonstrated that between 60 and 90% of the cells in culture were myocardial cells.

Phosphate incorporation into cultured cell proteins was performed as previously described by Klein *et al.* (13). [^{32}P]Orthophosphate (250 μCi/culture dish) was added to MEM prepared without serum. Actinomycin D at 2.5 μg/ml was added to inhibit the synthesis of ^{32}P containing RNA and this improved the clarity of the autoradiographs. After 1 hr of incubation in this media, the cells were scraped from the dishes into 0.5–1.0 ml of phosphate-buffered saline (pH 7.40) by means of a rubber policeman. Subcellular fractionation of ^{32}P-labeled heart cells was obtained as previously described (13) after Dounce homogenization. In addition to the whole cell homogenate, a crude nuclear fraction (1000 *g* pellet), plasma membrane (12,000 *g* pellet), mixed microsomal, mitochondrial (100,000 *g* pellet), and soluble cell fraction was obtained.

Aliquots of the ^{32}P-labeled myocardial cells containing between 20 and 40 μg of protein were applied to a vertical slab gel electrophoresis system to allow for identification of individual protein subunits containing ^{32}P. Samples for electrophoresis were prepared by heating at 100° in 2% SDS, 0.1 *M* dithiotreitol, 10% glycerol, and 0.01% bromphenol blue. Sodium dodecyl sulfate gel electrophoresis employing either 5 or 11% acrylamide resolving gels overlayered with 3 or 5% acrylamide stacking gels, respectively, were used as described (13). Purified molecular weight standards were routinely used for comparison. Two-dimensional gel electrophoresis using isoelectric focusing in the first dimension (pH 5 to 7) and 11% SDS–gel electrophoresis in the second stage was performed as described by O'Farrell (14). Myosin was purified from adult and 2-day-old rats by the technique described by Shiverick *et al.* (15). Since there were insufficient amounts of cardiac cell protein to directly purify heart cell myosin, [^{32}P]orthophosphate-labeled heart cells were copurified with chromotographically pure cardiac myosin. After addition of the purified myosin to the heart cells, the mixture was homogenized in high-salt buffer and then centrifuged at 45,000 g for 30 min. The supernatant was then diluted with 20 vol of water, allowed to sit for 24 hr, and the partially purified myosin was collected by centrifugation at 12,000 *g* for 10 min. An aliquot of the precipitate containing 25 μg protein was applied to an 11% polyacrylamide gel as described above.

To analyze radioactively labeled proteins, the gels were dried and exposed to Kodak RP "X-omat" film for varying lengths of time. Specific protein bands were identified by scanning the photographic

negatives on a E-C Model 910 densitometer. Subsequent to the scanning of the autoradiograph from 11% gels, the relative amount of ^{32}P radioactivity in each band was determined by cutting out the individual peaks traced by the densitometer and weighing them (13). This allowed for comparison of the degree of [^{32}P]phosphate incorporation into specific molecular weight regions from different samples with identical amounts of cell protein applied to a single acrylamide gel. The validity of this method was established by demonstrating a linear relationship between the measured peak volume and the amounts of protein applied (range 10 to 60 μg protein).

Results. *Phosphoprotein profile of cultured myocardial cells.* Figures 1 and 2 are graphic reproductions of the phosphoprotein profile obtained from [^{32}P]orthophosphate-treated myocardial cells in culture. These figures are derived from the densitometer tracings recorded from the autoradiographs corresponding to 5 and 11% acrylamide gels, respectively. As can be seen, there are a number of fairly discrete peaks in each of these tracings. By comparison with known molecular weight standards, Fig. 1 demonstrates phosphoproteins that comigrate with an apparent molecular weight of 200,000, a broad peak in the molecular weight range of 130,000–140,000, a peak at 116,000 and two additional broad peaks at approximately 105,000 and 94,000. This latter peak has the greatest relative amount of radioactivity and comigrates with the enzyme standard phosphorylase *b*. In Fig. 2, there are a minimum of seven discrete phosphoprotein peaks in the region between the molecular weight standards of 43,000 to 14,000. A major phosphoprotein in this region has identical electrophoretic mobility with the purified 20,000-mol wt myosin light chain (MLC_2). In contrast to the 5% acrylamide gels (Fig. 1), the 11% gels yield a much clearer separation of the phosphoproteins.

Further support for the identification of the 20,000-mol wt phosphoprotein as a myosin light chain comes from three additional lines of investigation. First this phosphoprotein shows identity with the purified MLC_2 from cardiac muscle when subject to two-dimensional electrophoresis. Second, when a ^{32}P-labeled cell extract was added to a previously purified preparation of rat cardiac myosin and further partially purified as described the resulting product demonstrated one major phosphoprotein (Fig. 3). This phosphoprotein exhibited identical mobility to the Coomassie blue staining band of the 20,000 MLC_2. No significant radioactivity could be identified in the 200,000 (myosin heavy chain) or 26,000-mol wt regions (myosin light $chain_1$). Third,

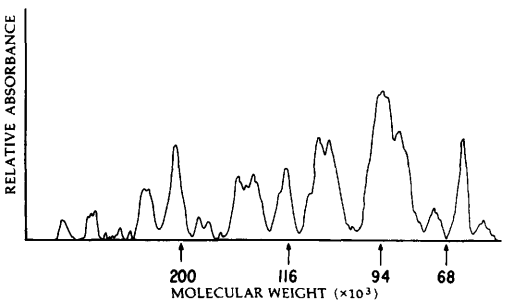

FIG. 1. High-molecular-weight phosphoprotein profile. This is a densitometer tracing from the autoradiograph obtained after electrophoresis of ^{32}P-labeled rat heart cells on 5% acrylamide gels. Standard proteins are myosin 200,000, β-galactosidase 116,000, phosphorylase *b* 94,000, and bovine serum albumin 68,000.

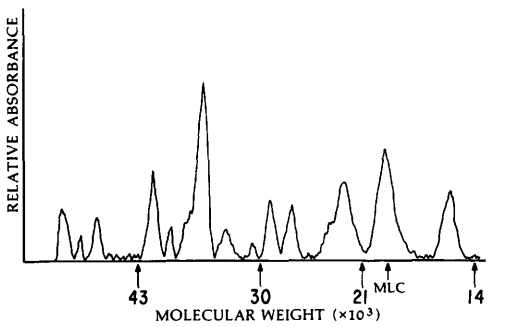

FIG. 2. Low-molecular-weight phosphoprotein profile. This is a densitometer tracing of an autoradiograph obtained from an 11% acrylamide gel. Standard proteins are ovalbumin 43,000, carbonic anhydrase 30,000, soybean trypsin inhibitor 21,000, myosin light $chain_2$ 20,000, and lysozyme 14,300.

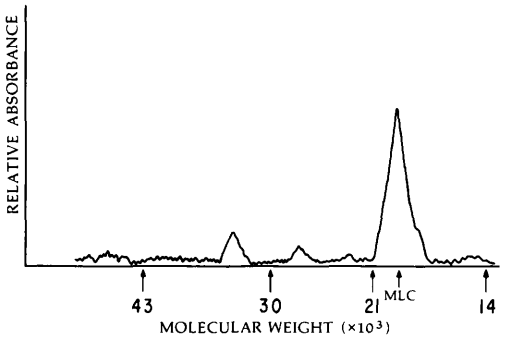

FIG. 3. Partial purification of cardiac cell myosin. After incubating cultured cells with [^{32}P]orthophosphoric acid, the cell extract was partially purified with unlabeled myosin as described under Methods. This is a densitometer tracing of an autoradiograph obtained after electrophoresis of 25 μg of purified protein. Compared to Fig. 2, standard proteins including MLC_2 are the same.

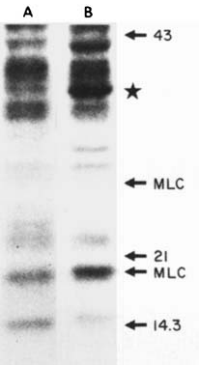

FIG. 4. Comparison of the autoradiographs obtained from cultures enriched in nonmuscle cell (A), and myocardial cells (B) after incubation with [^{32}P]orthophosphate and employing 11% acrylamide gels. Standard proteins are the same as Fig. 2. The two purified myosin light chains are indicated as MLC. The * indicates a major low-molecular-weight phosphoprotein in heart cells. Each lane contains 40 μg of cell protein.

when subcellular fractionation was performed the 20,000-mol wt phosphoprotein could be identified only in the whole cell homogenate and the 100,000 *g* soluble cell supernatant.

In two separate experiments colchicine, 1×10^{-6} *M*, but not lumicolchicine, 1×10^{-6} *M* (16), increased the spontaneous heart rate 70% ($P < 0.01$) and the quantitative incorporation of ^{32}P into the MLC_2 by 35% ($P < 0.05$) when compared to control cardiac cell cultures (Table I).

Phosphoprotein profile of mesenchymal cells. The major contaminant of the heart cultures were mesenchymal (fibroblast cells). To determine which, if any, of the major cardiac phosphoproteins were due to nonmuscle cell proteins, a fibroblast-enriched culture was incubated with [^{32}P]-orthophosphate and treated identically to that described for the heart cells. Equal amounts of cell protein were then electrophoresed simultaneously with ^{32}P-labeled heart cells. Figure 4 illustrates the phosphoprotein profile of a mesenchymal cell culture (lane A) in contrast with a cardiac cell culture (lane B) from an 11% acrylamide gel. It can be seen that these two autoradiographs show quite dissimilar phosphoprotein profiles. Most strikingly, the major cardiac band at approximately 37,000 (Fig. 4*) is virtually absent from the mesenchymal cell pattern. In addition, quantiation of the peaks in the region (not identical mobility) of the MLC_2 and the region of approximately 42,000 reveal that the nonmuscle cells contain less than 30% of the activity recorded for the heart muscle cells.

TABLE I. EFFECT OF COLCHICINE ON ^{32}P INCORPORATION INTO CELL PROTEINS

	[^{32}P]Phosphoprotein activity[b] (percentage of control)	
Protein peak[a]	Colchicine	Lumicolchicine
37,000 mol wt	96 ± 6	103 ±4
20,000 mol wt (MLC_2)	135 ± 8	93 ± 12

[a] Refers to the two major protein peaks as seen in Figs. 2 and 4.

[b] Data are mean ± SEM and are expressed as a percentage of ^{32}P incorporation into protein peaks of control cultures.

Discussion. Myocardial cells can be maintained in tissue culture and provide a relatively homogenous system to study factors which alter cardiac intropy and chronotropy (10–12). The present report demonstrates the presence of a family of both high- and low-molecular weight phosphoproteins from cultured heart cells. Most

prior studies with cardiac tissue have examined either individual proteins phosphorylated *in situ* or utilized those proteins as substrate for *in vitro* phosphorylation (7–9). In contrast, the present report describes the spectrum of phosphoproteins in intact, spontaneously contracting myocardial cells.

One of these phosphoproteins has an identical electrophoretic mobility when examined with both single and two-dimensional gel electrophoresis to the purified 20,000-mol wt myosin light $chain_2$. In addition, this phosphoprotein was identified in the soluble cell supernatant after cell fractionation and copurified with myosin from rat heart. Similar to the findings of Stull *et al.*, we could not identify [^{32}P]phosphate incorporation into the region of the slower migrating of the two myosin light chains (Fig. 3, molecular weight approximately 26,000 MLC_1). This later observation suggests that there was neither *in vitro* protein phosphylation occurring at 3° during myosin purification nor nonspecific exchange of ^{32}P label between cardiac proteins. These separate lines of evidence suggest that a major phosphoprotein in cultured rat heart cells is the myosin light $chain_2$.

Recent reports have suggested the presence of additional phosphoproteins in muscle (17). These include myosin light chain phosphatase (approximately 70,000) (18), and the troponin subunits TN-T (37,000) and TN-I (28,000) (7). While similar electrophoretic mobilities do not prove identity, we can tentatively identify regions of ^{32}P labeling corresponding to these previously reported phosphoproteins.

Cultures of heart cells are contaminated by nonmuscle mesenchymal cells, and fibroblasts contain contractile proteins that are substrate for phosphorylation (19). However, it is unlikely that these cells contribute significantly to the profile we observed since: (i) they are a small proportion of the cells in our cultures; (ii) the lack of correspondence between many of the phosphoprotein peaks seen in the muscle and nonmuscle cell cultures; and (iii) the quantitative decrease in protein phosphorylation observed in cultures of nonmuscle cells. It is possible that the decrease in ^{32}P-protein labeling of the mesenchymal cells was due to a decreased incorporation of the nuclide into ATP compared to heart cells. In either case, the current data support the fact that the phosphoprotein profile we observed is primarily attributable to the myocardial cells.

Cultured cells serve as a model system for studying the effect of various physiologic and pharmacologic agents upon cellular functions. Many drugs can alter the rate and force of contraction of heart cells. Colchicine has been previously reported to increase the spontaneous rate of contraction of cultured heart cells (16, 20). In the present study this increase in chronotropy was associated with a significant increase in ^{32}P incorporation into the 20,000-mol wt myosin light chain. In addition, we have recently reported that trifluoperazine, a drug that binds to calmodulin, both reversibly inhibits the contraction of cultured heart cells, as well as decreases the ^{32}P content of MLC_2 (21). A knowledge of the different phosphoproteins in myocardial cells thus allows for further investigation into the action of cardioactive agents at the molecular level.

The authors thank Ms. Susan Daley for preparation of the figures and Miss Chris Wuchina for secretarial assistance. Dr. Klein is a Research Associate of the Veterans Administration. This work was supported in part by HRSF Grant W83.

1. Perry SV. The regulation of contractile activity in muscle. Biochem Soc Trans 7:593–619, 1979.
2. Entman ML. The role of cyclic AMP in the modulation of cardiac contractility. Advan Cyclic Nucleotide Res 4:163–193, 1974.
3. Klein I, Levey GS. Activation of myocardial adenyl cyclase by histamine in guinea pig, cat and human heart. J Clin Invest 50:1012–1015, 1971.
4. Klein I, Fletcher M, Levey GS. Evidence for a dissociable glucagon binding site in a solubilized preparation of myocardial adenylate cyclase. J Biol Chem 284:5552–5554, 1973.
5. Adelstein RS. Phosphorylation of muscle contractile proteins. Fed Proc 39:1544–1546, 1980.
6. Corbin JD, Keely SL. Characterization and regulation of heart adenosine 3′:5′ monophosphate-

dependent protein kinase isoenzymes. J Biol Chem **252**:910–918, 1972.
7. Stull JT, Manning DR, High CW, Blumenthal DK. Phosphylation of contractile proteins in heart and skeletal muscle. Fed Proc **39**:1552–1558, 1980.
8. England PJ. Studies on the phosphylation of the inhibitory subunit of troponin during modification of contraction in the perfused rat heart. Biochem J **160**:295–304, 1976.
9. Kirchberger MA, Tada M, Katz AM. Adenosine 3′:5′ morophosphate-dependent protein kinase-catalyzed dependent phosphylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J Biol Chem **249**:6166–6173, 1974.
10. Harary I, Farley B. *In vitro* studies of single isolated beating heart cells. Science **131**:1674–1675, 1960.
11. Blondel B, Roijen I, Cheneval JP. Heart cells in culture a simple method for increasing the proportion of myoblasts. Experentia **27**:356–358, 1971.
12. Norwood CR, Castaneda AR, Norwood WI. Heterogeneity of rat cardiac cells of defined origin in single cell culture. J Mol Cell Cardiol **12**:201–211, 1980.
13. Klein I, Willingham M, Pastan I. A high molecular weight phosphoprotein in cultured fibroblasts that associates with polymerized tubulin. Exp Cell Res **114**:229–238, 1978.
14. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem **25**:4007, 1975.
15. Shiverick KT, Thomas LL, Alpert NR. Purification of cardiac myosin application to hypertrophied myocardium. Biochem Biophys Acta **393**:124–133, 1975.
16. Klein I, Gondek M. Colchicine stimulates the rate of spontaneous contraction in cultured heart cells. Clin Res **29**:215A, 1981.
17. Conti MA, Adelstein RS. Phosphylation by cyclic adenosine 3′:5′-monophosphate-dependent protein kinase regulates myosin light chain kinase. Fed Proc **39**:1569–1573, 1980.
18. Morgan M, Perry SV, Ottaway J. Myosin light chain phosphatase. Biochem J **157**:687–697, 1976.
19. Pastan I, Willingham M, Yamada K, Davies P, Klein I. Biochemical basis of the morphologic phenotype of transformed cells. In: Brinkley BR, Porter KR, eds. International Cell Biology. New York, Rockefeller Press, Vol 1:p161, 1977.
20. Nath K, Shay J, Bollon AP. Relationship between dibutyryl cyclic AMP and microtubule organization in contracting heart muscle cells. Proc Nat Acad Sci USA **75**:319, 1978.
21. Klein I. Colchicine stimulates the rate of spontaneous contraction in cultured heart cells. Circulation **64**:IV–164, 1981.

Received September 11, 1981. P.S.E.B.M. 1982, Vol. 170.