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Research Article

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Trifluoperazine Inhibits the Contraction of Cultured Rat Cardiac Cells and the Phosphorylation of Myosin Light Chain

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ABSTRACT Cultured, spontaneously beating heart cells were used to study the role of calmodulin in regulating cardiac contraction. Trifluoperazine (TFP), an antipsychotic drug that binds to calmodulin, reversibly inhibited myocardial contraction. This effect occurred over a TFP concentration range of 5–100 μ M with half maximal activity at \sim 15 μ M TFP. When the phosphoprotein profile of TFP-treated cells was compared with control cultures, there was a significant decrease in 32 P content of the 20,000-D myosin light chain. As measured by two-dimensional gel electrophoresis, the fraction of phosphorylated myosin light chain decreased from 0.31 ± 0.06 in control to 0.16 ± 0.05 in TFP-treated cells ($P < 0.05$). This inhibition of protein phosphorylation was relatively selective, as two other phosphoproteins (\sim 41,000 and 36,000 D) were unaffected, and a third protein (\sim 28,000 D) showed an increase in 32 P activity. In contrast, the cessation of spontaneous beating by 50 mM KCl did not reproduce these changes. This inhibition of contraction mediated by TFP associated with alterations in the phosphorylation of certain cardiac phosphoproteins suggests a role for calmodulin and for the myosin light chain in the regulation of cardiac cell contraction.

INTRODUCTION

Phosphorylation of the myosin light chain (MLC)¹ is an important regulatory step in the control of smooth muscle and platelet contraction. It has previously been

shown that phosphorylation of this protein is mediated by a MLC kinase that requires the presence of both calcium and the calcium binding protein, calmodulin (1–3). Thus, increases in the contractile state of smooth muscle are associated with demonstrable increases in the phosphate content of the 20,000-D MLC (4). The role of MLC phosphorylation in the control of cardiac contractility is less well established (2, 3, 5). There are reports of increased myosin ATPase activity in association with increased protein phosphorylation (6), whereas other studies show no effect of increased light chain phosphate content on the interaction of cardiac myosin and actin (3).

Trifluoperazine (TFP) is an antipsychotic drug that is capable of inhibiting the intracellular effects of calmodulin. This property accounts for the inhibition of vascular smooth muscle contraction and MLC phosphorylation that is seen after drug treatment (4, 7, 8). In order to better understand the role of calmodulin (9) and of MLC phosphorylation in the regulation of cardiac muscle, we have examined the effect of TFP on cultured heart cells. This report demonstrates that micromolar concentrations of TFP are capable of reversibly inhibiting the contraction of neonatal rat heart cells in culture. This change in contractility is associated with a decrease in the phosphorylation of the 20,000-D MLC, and an increase in the phosphorylation of a 28,000-D protein.

METHODS

Materials. Eagle's modification of minimal essential media (MEM), essential and nonessential amino acids, supplemental vitamins, penicillin, and streptomycin were obtained from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY); calf serum and trypsin, from Flow Laboratories, Inc. (Rockville, MD); acrylamide, sodium dodecyl sulfate (SDS), Coomassie Blue, and ampholytes (pH 5–7), from Bio-Rad Laboratories (Richmond, CA); and [32 P]orthophosphoric

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¹ Abbreviations used in this paper: MEM, minimum essential medium; MLC, myosin light chain; TFP, trifluoperazine.

acid and [³⁵S]methionine from New England Nuclear (Boston, MA). Plastic petri dishes were obtained from Falcon Labware, Div. of Becton, Dickinson & Co. (Oxnard, CA) and culture flasks from Corning Glass Works (Corning, NY). Zivic-Miller (Pittsburgh, PA) strain of Sprague-Dawley rats were used in all experiments. TFP dihydrochloride was a gift from SmithKline & French Laboratories, SmithKline Corp. (Philadelphia, PA). Colchicine, β -mercaptoethanol, and Nonidet (P-40) were from Sigma Chemical Co. (St. Louis, MO), and 3-isobutyl-1-methylxanthine, from Aldrich Chemical Co., Inc., Milwaukee, WI.

Preparation of heart cells. Heart cells were obtained by trypsinization of 2–4-d-old rat hearts as described by Harary and Farley (10). Hearts were aseptically removed from 12 to 20 neonatal rats, the ventricular tissue finely minced and incubated at 37°C in 0.125% trypsin. The resulting cell suspension was centrifuged at 600 *g* for 10 min and the cell pellet resuspended in MEM supplemented with complete amino acids, 200 mM L-glutamine, 10% heat inactivated calf serum, vitamins, penicillin, streptomycin, and additional calcium chloride to yield a final concentration of 4 mM. This mixed cell population was then incubated in a 120-cm² Falcon flask. After 90 min the nonadherent cells (predominantly myocardial cells) were gently removed and replated at a concentration of $1-2 \times 10^6$ cells/3 ml in 60-cm² Falcon dishes that had been previously coated with gelatin.

The culture dishes were incubated at 37°C in an atmosphere of 5% CO₂:95% room air and after 24 h the media was changed to supplemented MEM with a final calcium concentration of 2.0 mM. Cells were observed daily using an inverted stage, phase-contrast microscope from Nikon Inc., Instrument Div. EPOI (Garden City, NY). Within 24 h of plating of the heart cells, spontaneous contractile activity was observed. Rates of contraction were assessed by manual counting for two successive 15-s periods and the average rate calculated. Cultures were maintained at temperatures between 35 and 40°C. Duplicate counts did not vary by >5%. Visual observation of the percentage of heart cells, using either the criteria of cell morphology or the presence of contractile activity, demonstrated that between 60 and 90% of the cells in cultures were muscle cells (11).

Measurement of phosphoproteins. Phosphate incorporation into cultured cell proteins was performed as previously described (12). [³²P]Orthophosphate (250 μ Ci/culture dish) was added to MEM prepared without serum. After 1 h of incubation in this media, the cells were scraped from the dishes into 0.5–1.0 ml of phosphate-buffered saline (PBS) (pH 7.40). Aliquots of the ³²P-labeled myocardial cells containing between 20 and 50 μ g of protein were applied to a vertical slab gel electrophoresis system to allow for identification of individual protein subunits containing ³²P. SDS slab gel electrophoresis using 11% acrylamide resolving gels overlaid with 5% acrylamide stacking gels, respectively, were used as described (12). Purified molecular weight standards were routinely used for comparison including myosin purified from 2-d-old rat hearts as described by Shiverick et al. (13).

To analyze radioactively labeled proteins, the gels were dried and exposed to Kodak "X-omat" film (Eastman Kodak Co., Rochester, NY) for varying lengths of time. Specific protein bands were identified by their electrophoretic mobility when compared with standard proteins. The amount of ³²P incorporated into individual molecular weight regions of the gel was quantitated by scanning the photographic negatives on an E-C model 910 densitometer, E-C Apparatus Corp., St. Petersburg, FL. Subsequent to the scanning of the autoradiograph, the relative amount of radioactivity in each

band was determined by cutting out the individual peaks traced by the densitometer and weighing them (12). This allowed for comparison of the degree of [³²P]phosphate incorporation into specific molecular weight regions from control and experimental samples when identical amounts of cell protein were applied to a single acrylamide gel. The validity of this method was established by demonstrating a linear relationship of measured densitometer peak volumes from control samples with variable amounts of cell protein applied (range 10–60 μ g protein).

Because nonmuscle as well as myocardial cells contain the contractile proteins actin and myosin, two-dimensional gel electrophoresis using isoelectric focusing in the first dimension (14) was used to identify the specific phosphoproteins from heart muscle. There are recent reports that the phosphorylated and nonphosphorylated subunits of the MLC can be resolved by two-dimensional gel electrophoresis (15, 16). In this series of experiments, heart cells in culture were incubated with [³⁵S]methionine (100 μ Ci/dish) for 16 h. Before harvest the cells were washed with PBS, and scraped into an isoelectric focusing buffer containing 5.0 M urea, 2% Nonidet, 1.6% ampholytes, pH 5–7 and 5% β -mercaptoethanol (14). Approximately 100 μ g of protein was applied to disc isoelectric focusing gels and run overnight at 400 V. These gels were then equilibrated in 2% SDS sample buffer and electrophoresed as described with 5% stacking gel and an 11% resolving gel (15). By comparison with previously purified neonatal rat heart myosin the Coomassie Brilliant Blue staining spots corresponding to the phosphorylated and nonphosphorylated forms of cardiac MLC ($\sim 20,000$ D) were identified. These regions were cut from the gels solubilized with 60% perchloric acid and 30% hydrogen peroxide and counted in a Packard model 300C liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). The phosphorylated fraction of the 20,000-D MLC was calculated as the percentage of ³⁵S activity (disintegrations per minute) in the phosphorylated region compared with the total ³⁵S incorporated into the MLC (phosphorylated and nonphosphorylated forms). All measurements were performed in duplicate and the results expressed as the mean \pm SEM. In addition, cultures of heart cells were labeled with [³²P]orthophosphoric acid for 1 h and the cells were subject to two-dimensional gel electrophoresis as described.

Addition of TFP to cell culture. In order to determine the effects of TFP on cardiac contraction, concentrations of 1–100 μ M were added to the culture media. TFP was dissolved in sterile MEM without serum at concentrations ranging from 0.5 to 5 mM and then added to the cell cultures to yield the final concentration as stated in the text. Before drug addition, two base-line heart rates were determined and then again at 2, 5, 10, and 20 min subsequent to the addition of TFP. After 20 min, the media was washed twice and replaced with fresh MEM with 10% serum. When TFP was added after treatment with 1 μ M colchicine or 10 μ M isobutylmethylxanthine, the latter agents were preincubated with the cells for 30 min.

In experiments where the effect of TFP upon phosphorylation was assayed, the cells were incubated with [³²P]orthophosphoric acid or [³⁵S]methionine as described above. 10 min before harvesting the cells, either TFP at a final concentration of 20 μ M, KCl at a final concentration of 50 mM, or control MEM was added to the media, and contractile activity was measured 5 and 10 min later. Cells were then scraped from the dish, the concentration of cell protein determined, and equal amounts of protein applied to 11% acrylamide gels (11). Quantitation of the amount of ³²P in specific protein bands was performed as described.

Alternatively, cells incubated with [³⁵S]methionine were similarly treated with TFP or control media, scraped, and subject to two-dimensional gel electrophoresis. The fraction of ³⁵S radioactivity recovered in the phosphorylated form of the 20,000-D MLC was calculated as described above and compared between treated and control cultures.

RESULTS

Inhibition of contraction by TFP. Cultures of neonatal rat heart cells demonstrate spontaneous and synchronous rhythmic contractile activity after 48 h in culture. Control cells maintain this rate for periods in excess of 96 h. When TFP was added to the culture media at 10 and 20 μ M, there was a rapid decrease in the rate of contraction (Fig. 1). The effect of various concentrations of TFP was assessed by measuring the percent of inhibition of heart rate at 5 min after exposure to the drug. The effect of TFP was dose-dependent over the concentration range of 1–100 μ M (Fig. 2). Half maximal inhibition occurred at \sim 15 μ M (Fig. 2). When followed for periods > 10 min, all concentrations of TFP > 20 μ M led to complete cessation of contractile activity.

The effect of TFP was partially reversible (Fig. 1). 20 min after exposure to 5, 10, 20, 40, and 100 μ M TFP, the drug was washed from the culture media as described (Table I). Recovery from drug treatment was both time and dose dependent. Fig. 1 demonstrates the return of contractile activity for cultures treated with 10 and 20 μ M TFP. Table I compares the percent return of heart rate for five different TFP dosages. For

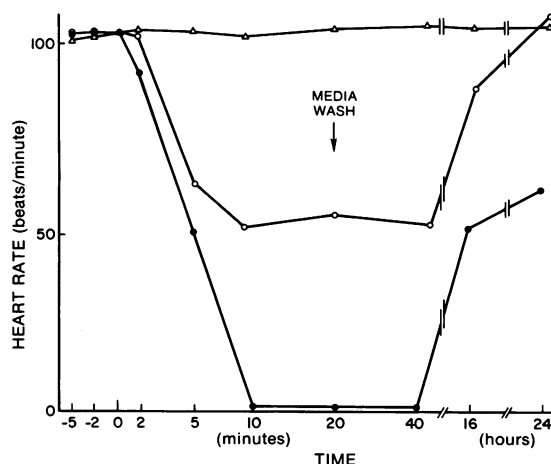


FIGURE 1 Reversibility of the effect of TFP on cardiac contractility. At time zero, TFP at 10 μ M (○) and 20 μ M (●) or diluent (△) was added to spontaneously beating heart cells, and contractility assessed at the times indicated. At 20 min, the TFP was removed by washing and replacing all cultures with fresh MEM. Data are means of duplicate determinations.

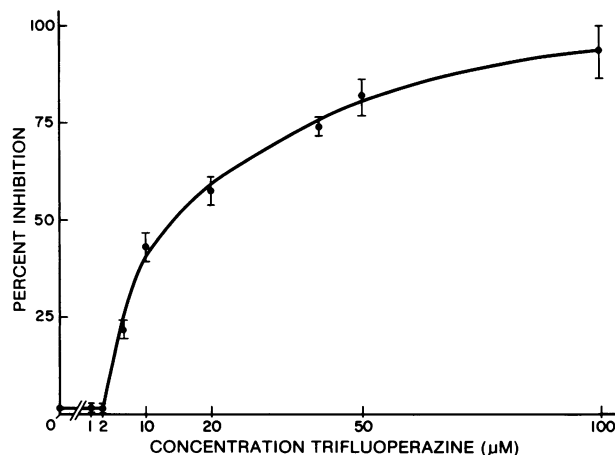


FIGURE 2 Dose-response curve for TFP upon cardiac contractility. TFP was added to spontaneously beating heart cells and after 5 min the heart rate was measured and expressed as a percentage of the base-line activity. Data are means \pm SEM.

TFP at 5 and 10 μ M, recovery of activity was also measured 2 h after drug removal and found to be 112 and 55% of the pretreatment values, respectively. Periods > 24 h after washout of the drug were not examined. In cultures where the drug was not removed, there was no return of contractile activity. Control cultures to which no drug was added (diluent alone) maintained a constant rate of contractility within a 10% variation over the 24 h of observation (Fig. 1).

Pretreatment of heart cell cultures with either isobutylmethylxanthine at 10 μ M or colchicine at 1 μ M, agents that stimulate contractile activity (15), did not overcome the inhibition produced by 10 or 100 μ M TFP.

Effect of TFP on protein phosphorylation. Fig. 3 is the autoradiograph from an 11% polyacrylamide

TABLE I
Recovery of Contractile Activity

TFP concentration μ M	Heart rate (Hours after media wash)	
	16 h	24 h
5	112	108
10	83	106
20	50	64
40	27	52
100	24	41

After the removal of TFP, heart rate was measured and expressed as a percentage of the pretreatment value. Data are means of duplicate determinations. Range of pretreatment heart rate was 90–110 beats/min.

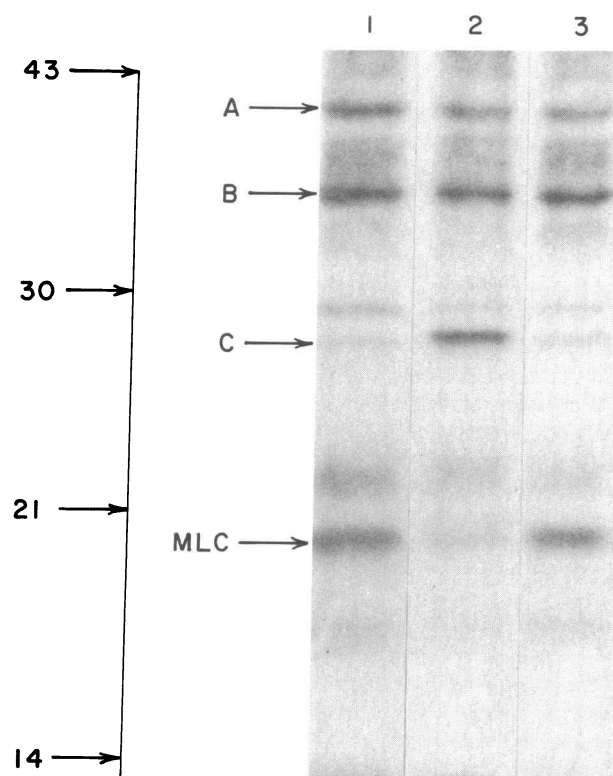


FIGURE 3 Effect of 20 μ M TFP on protein phosphorylation. Cardiac cells were incubated with 32 P, and identical amounts of 32 P-labeled cardiac cells were applied to one-dimensional SDS-polyacrylamide gels and then subject to autoradiography as described. This is a typical experiment in which lane 1 is a control cell population, lane 2 are cells treated with 20 μ M TFP, and lane 3 are cells treated with 50 mM KCl. Bands A, B, and C identify three of the major phosphoprotein regions, and MLC denotes the 20,000-D myosin light chain. Left-hand margin indicates migration of standard reference proteins (ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme 14,000-D).

gel of cultured cardiac cells incubated with [32 P]-orthophosphoric acid as described. Lane 1 is a control cell population that demonstrates a number of discrete phosphoproteins; lane 2 is from cells treated with 20 μ M TFP. Three of the phosphoproteins common to both preparations are labeled as A, B, and C corresponding to subunit molecular weights of \sim 41,000, 36,000, and 28,000. These molecular weight estimates are based upon the mobility of four simultaneously run standards proteins (Fig. 3, left margin). An additional [32 P]phosphate band has identical electrophoretic mobility with a 20,000-D purified rat cardiac MLC. This same 32 P band from cultured heart cells also copurifies with rat heart MLC (15) and, therefore, is designated MLC.

Treatment with 20 μ M TFP for 10 min before harvesting the 32 P-labeled cardiac cells both inhibits cell contraction and alters the phosphoprotein profile. As seen in Fig. 3, lane 2, TFP decreased 32 P labeling in the region of the MLC. In contrast, the activity of band C was enhanced when compared with control cultures. The degree of phosphate incorporation was determined and found to be the same for bands A and B.

The amount of [32 P]phosphate incorporated into each of these four bands was quantitated by the use of a scanning densitometer as previously described (12). This technique was used to compare the amount of [32 P]phosphorus in protein bands of identical electrophoretic mobility from individual experiments. Thus, the degree of protein phosphorylation in Table II is expressed as a percent of the simultaneously run control. Analysis of this data from three separate experiments demonstrates an inhibition of MLC phosphorylation and an increase in phosphorylation of peak C. Peaks A and B were unaffected by TFP treatment.

To determine whether the reduction of MLC phosphorylation was due to treatment with TFP or a function of the associated decrease in heart rate, 50 mM KCl was used to arrest the beating cells. When added to the culture media in a manner identical to that for TFP 10 min before harvesting the 32 P-labeled cells, KCl produced a complete cessation of contractile activity. An equal aliquot of protein from KCl-treated heart cells was applied to 11% acrylamide gels and the degree of phosphate incorporation compared with both control and TFP (20 μ M) cultures (Fig. 3, lane 3). Under these conditions the activity corresponding to peaks A, B, and MLC were unchanged while there was a 34% increase in peak C when compared with controls (Table II).

To more specifically identify that the change in [32 P]phosphate incorporation was due to changes in the phosphate content of the 20,000-D MLC, two-dimensional gel electrophoresis was used. As described above, this was performed with isoelectric focusing in the first

TABLE II
Effect of TFP on Protein Phosphorylation

Peak*	[32 P]Phosphoprotein activity (% control)	
	TFP†	KCl‡
A	88 \pm 6	86 \pm 11
B	96 \pm 4	114 \pm 6
C	225 \pm 46	134 \pm 8
MLC	32 \pm 6	108 \pm 5

* Peaks refer to the autoradiograph bands identified in Fig. 3.

† Data are expressed as mean \pm SD ($n = 6$).

‡ Data are mean \pm SD ($n = 4$).

dimension and SDS-polyacrylamide in the second dimension. When cells that had been incubated with [35 S]methionine were subject to two-dimensional gel electrophoresis, two discrete areas of activity were observed at 20,000-D (Fig. 4, left). These two spots corresponded identically to the Coomassie Blue staining pattern of previously purified rat cardiac myosin. Further proof that these regions represented the phosphorylated (more acidic) and nonphosphorylated forms of the MLC was provided by 32 P-labeling of the cells. When cells that had been incubated with [32 P]orthophosphate were subjected to two-dimensional gel electrophoresis, essentially all of the radioactivity in the 20,000-D region comigrated with the more acidic light chain subunit (Fig. 4, right arrowhead). No 32 P activity could be discerned comigrating with the less acidic 20,000-D subunit and similarly no activity was seen to migrate in the region corresponding to the 26,000-D MLC (Fig. 4).

The ability to identify and separate the phosphorylated and nonphosphorylated forms of the MLC then allowed for direct measurement of the extent of light chain phosphorylation. Cardiac cells that had been incubated with [35 S]methionine for 16 h were scraped and an aliquot of cell protein subject to two-dimensional gel electrophoresis. The Coomassie Blue staining bands corresponding to the previously identified phosphorylated and nonphosphorylated forms of the 20,000-D MLC were cut out of the gel and counted by liquid scintillation spectrometry. Using this technique, the fraction of phosphorylated MLC was calculated to be 0.31 ± 0.06 (mean \pm SEM, $n = 6$) for con-

trol heart cells. Before harvest these cells had a mean heart rate of 118 ± 21 . After exposure to $20 \mu\text{M}$ TFP, there was a prompt decrease in heart rate to 24 ± 14 beats/min and the fraction of phosphorylated MLC decreased to 0.16 ± 0.05 (mean \pm SEM, $n = 4$). This decrease in MLC phosphorylation was significant at $P < 0.05$.

DISCUSSION

There is a complex system of control mechanisms that mediates the interaction of actin and myosin and thereby regulates cardiac contractility (1-3, 17, 18). Although it has been demonstrated that phosphorylation of the MLC is an important regulatory mechanism in smooth muscle, the role of light chain phosphorylation in cardiac muscle has not been defined (3-5, 19). Adelstein (1) and Conti and Adelstein (20) working with platelets and Baron et al. (4) in studies of smooth muscle have shown that the phosphorylation of the light chain is catalyzed by a specific kinase that requires the presence of calcium bound to calmodulin as a cofactor for activity.

TFP binds to the calcium-dependent regulator, calmodulin, and has been used to study some of the many reactions mediated by calcium via calmodulin (7, 8). Sheterline (21) has shown that TFP can distinguish between the actomyosin interaction regulated by phosphorylation of the MLC and that which is under the control of troponin C (not calmodulin dependent). In order to better understand the role of calmodulin and the possible role of myosin light phosphorylation in the control of cardiac contractility, the present study used cultured heart cells treated with varying doses of TFP.

The data demonstrate that TFP can reversibly inhibit the spontaneous contractile activity observed in cultured heart cells. In contrast to previous reports (7, 8), we observed TFP inhibition with concentration of the drug as low as $5 \mu\text{M}$ and the dose-response relationship revealed that half maximal inhibition occurred at $\sim 15 \mu\text{M}$ TFP. The onset of drug effect was rapid and was reversible with removal of the drug. TFP treatment was accompanied by a decrease in ^{32}P activity associated with the 20,000-D protein that we have previously identified as a MLC (15). Drug treatment, however, was selective in its effects on [^{32}P]phosphate incorporation. Two major phosphoproteins were not apparently altered by TFP, whereas a third protein demonstrated an increase in ^{32}P activity. This latter protein is of interest, and although we have not established the identity of this band, the apparent subunit mol wt of 28,000 D is quite similar to that reported for troponin I in cardiac muscle (15, 19, 22).

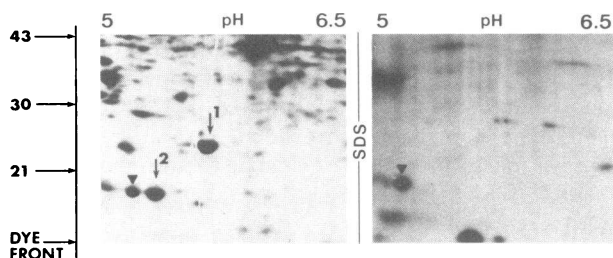


FIGURE 4 Two-dimensional gel electrophoresis of cardiac cell proteins. On the left is an autoradiogram of cells incubated with [35 S]methionine. Arrow 1 indicates the region of migration of purified 26,000-D myosin light chain; arrow 2, the major species of the 20,000-D light chain; and the arrowhead, to the more acidic light chain subunit. These regions correspond exactly to the Coomassie Blue staining of previously purified rat heart myosin. On the right, cells labeled with [32 P]orthophosphate and subject to autoradiography show activity corresponding only to the more acidic subunit of the 20,000-D light chain (arrowhead). The left hand margin is the migration of reference standard proteins ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (21,000-D).

Our observation that arrest of contractile activity with 50 μ M KCl did not produce the same alterations in protein phosphorylation as seen with 20 μ M TFP suggests that the latter changes were not merely due to an alteration in heart rate. The experimental design used makes it quite unlikely that the changes in 32 P-labeling activity were due to differences in 32 P-ATP-specific activity, content of MLC, or of the other phosphoproteins in heart muscle.

To further characterize the effect of TFP on MLC phosphorylation, [35 S]methionine-labeled cell extracts were analyzed by two-dimensional gel electrophoresis. Similar to previous reports (16, 23) this technique allows for the identification and separation of the phosphorylated and nonphosphorylated subunits of the 20,000-D light chain. Thus, by prelabeling cells with [35 S]methionine and then directly counting the activity in the more acidic (phosphorylated) fraction as well as in the nonphosphorylated subunit, a direct measure of the degree of phosphorylation could be made. Under control conditions, 31% of the light chain chromatographed as the acidic form. This is similar to the percentage of phosphorylated cardiac MLC reported by Holroyde et al. (24). After treatment with 20 μ M TFP and an accompanying fall in heart rate this significantly decreased to 16%, confirming the observation made with 32 P-labeled cells.

The finding of an alteration in MLC phosphorylation after treatment of heart cells with TFP suggests a role for the calcium-calmodulin-mediated phosphorylation of MLC in the control of cardiac contractility (9, 19, 25). In association with the decrease in light chain phosphorylation, we observed an increase in the phosphorylation of a 28,000-D muscle protein. This supports the prior suggestion that the phosphorylation of Troponin I may play a role in the regulation of myocardial contractility (2, 18, 26, 27). The present studies do not distinguish whether the effects of TFP are mediated through alterations in the activity of a MLC kinase (1-3) or alternatively by a change in calcium transport by the sarcoplasmic reticulum (9, 16). TFP-mediated changes in contraction allow for the further study of coordinate changes in cardiac phosphoproteins. In the present studies we have used heart rate as a measure of contraction. Many factors can potentially alter heart rate independent of the interaction of actin and myosin. Future studies using direct measurements of heart cell contractility (28) will be required to resolve this question.

Our in vitro observations may explain the in vivo effects of TFP, which has been shown to induce both bradycardia and hypotension when given to psychotic patients under controlled conditions (29). Similar to the present finding, the in vivo effects of TFP on car-

diovascular function were reversible after removal of the drug.

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