Journal of Cellular Biochemistry 64:458–465 (1997)

Influence of Mechanical Activity, Adrenergic Stimulation, and Calcium on the Expression of Myosin Heavy Chains in Cultivated Neonatal Cardiomyocytes

Hans Peter Luther, Simone Hille, Hannelore Haase, and Ingo Morano*
Max Delbrück Centre for Molecular Medicine, Berlin, Germany

Abstract It is generally accepted that mechanical stress of cardiomyocytes increases RNA and protein synthesis of myosin heavy chain (MHC) quantitatively but it is still a matter of debate whether MHC gene expression is also changed qualitatively. We investigated expression of MHC genes of spontaneously contracting neonatal cardiomyocytes experimentally arrested by permanent depolarization [potassium chloride (KCl)] as well as by electromechanical uncoupling [2,3 butanedione monoxime (BDM)]. Relative distribution of MHC mRNA isoforms (α and β) was studied by quantitative polymerase chain reaction. Expression of MHC isoenzymes was the same in contracting (34.5% β-MHC) and arrested (40.5% and 33.0% β-MHC in KCl and BDM, respectively) cardiomyocytes. However, treatment with phenylephrine for the same period increased significantly β-MHC expression to 55%. We conclude that hormonal factors rather than Ca^{2+} or mechanical stress regulate qualitatively MHC gene expression. J. Cell. Biochem. 64:458–465. © 1997 Wiley-Liss, Inc.

Key words: myosin heavy chain; gene expression; neonatal rat heart culture; contraction; 2,3 butanedione monoxime

Two different genes coding for β-MHC and α-MHC are located in tandem 4 kb apart on chromosome 14, and are expressed in the ventricle of rats [Mahdavi et al., 1984]. The products associate to form αα-homodimers, αβ-heterodimers, and ββ-homodimers. Expression of both MHC genes are regulated on a transcriptional level by interaction with enhancer and silencer elements in the promoter region of the genes: Thyroxine increases transcriptional activity of the α-MHC gene while suppressing transcription of the β-MHC gene [Morkin et al., 1989; Mahdavi et al., 1989], a mechanisms which may be involved in the upregulation of α-MHC during postnatal development [Mahdavi et al., 1989]. Testosterone as well enhances the expression of α-MHC mRNA [Morano et al., 1990]. Elevated levels of cAMP selectively promote α-MHC gene transcription having no effect on the β-MHC gene [Gupta et al., 1991]. Peptide growth factors (GF), e.g., basic and acidic fibroblast GF, which interact with tyrosine kinase receptors [Huang and Huang, 1986], and transforming GF-β1 (TGF-β1) interacting with two types of serine/threonine kinase receptors [Wrana et al., 1994], increased β-MHC and decreased α-MHC gene expression [Parker et al., 1990]. In addition, posttranscriptional mechanisms exist, e.g., expression of naturally occurring antisense-mRNA in the skeletal [Heywood, 1986] and cardiac muscle [Boheler et al., 1992].

Enhanced mechanical stress of cardiomyocytes both in vivo by hemodynamic overload [Kira et al., 1984] and in culture by stretching [Mann et al., 1989] or spontaneous contraction [McDermott et al., 1985] increased quantitatively mRNA and protein synthesis rate leading to hypertrophy of the cardiomyocyte. The "mechano-transcriptional coupling" (MTC) process comprises both quantitative as well as qualitative changes of gene expression in favour of genes typical for the fetal state, e.g., the atrial natriuretic hormone [Lattion et al., 1986] and skeletal α-actin [Komuro et al., 1991]. Part of the MTC processes may reside in the stretch-induced autocrine release of angiotensin II (Ang II) from cardiomyocytes [Sadoshima et al., 1993] which may increase both transcription and translation by activation of the "extracellular signal-regulated kinase" (ESRK) system: Ang II

*Correspondence to: Dr. Ingo Morano, Max Delbrück Centre for Molecular Medicine, Robert-Rössle-Str. 10, D-13122 Berlin-Buch, Germany.

Received 16 July 1996; Accepted 18 September 1996

© 1997 Wiley-Liss, Inc.

activates protein kinase C (PKC) through G-protein coupled AT-1 receptor [Sechi et al., 1992] and then induces the sequential activation of the mitogen-activated protein kinase (MAK) pathway (Raf-1, MEKK, MAPKK, MAPK, $p90^{rsk}$) [Yamazaki et al., 1995a,b]. Both activated MAPK and $p90^{rsk}$ translocate to the nucleus and phosphorylated transcription factors which in turn increase the transcription rate of fetal genes [see Davis, 1993 for review]. In addition, Ang II specific activation of $p70^{S6K}$ [Takan et al., 1996] phosphorylates S6 protein in 40 S ribosome [Erikson, 1991] thus increasing translation efficieny [Flotow and Thomas, 1992]. Furthermore, endothelin-1 (ET-1) is released from cultivated cardiomyocytes upon Ang II stimulation [Ito et al., 1993]. By interaction with ET_A receptors, ET-1 induces hypertrophy of cultured cardiomyocytes [Ito et al., 1991; Shubeita et al., 1990] representing an autocrine/paracrine system to control cardiac cell growth. This seems to be mediated via ET-1 induced activation of Raf-1 kinase and MAP kinases [Yamazaki et al., 1996] amplifying the Ang II effect on the Ras/Raf pathway. Besides ESRK, the "stress-activated protein kinase" (SAPK) system is activated by mechanical activity of cardiomyocytes independent from Ang II secretion [Komuro et al., 1996].

In vivo, additional autocrine/paracrine mechanisms may exist which support stress-induced cell growth. Thus, Ang II stimulates the autocrine production of TGF-β1 in adult cardiac fibroblasts [Lee et al., 1995], a potent growth promoting factor in the heart [Roberts et al., 1992].

Similar to mechanical stress, α-adrenergic stimulation promote cardiac hypertrophy [Simpson et al., 1982] and evoked reexpression of fetal genes in cardiomyocytes [Waspe et al., 1990] by G-protein dependent activation of the Ras/Raf pathway.

The responsiveness of the MHC genes to mechanical stress is variable and it is not clear whether the stress per se or hormonal changes contribute to MHC isoenzyme changes in cardiomyocytes. In vivo, cardiac hypertrophy caused by hemodynamic overload was associated with the expression of the fetal MHC-isoform, namely the β-MHC [Izumo et al., 1987; Mercadier et al., 1981]. However, cardiac hypertrophy due to physical activity increased α-MHC [Rupp, 1981]. Conflicting observations exist concerning the effects of contractile activity on MHC isoenzyme expression in cell culture. Qi and coworkers [1994] reported an increased β-MHC mRNA and protein expression of neonatal cardiomyocytes upon mechanical activity while the α-MHC appeared to decrease in response to contractile activity. The same group demonstrated recently a selective upregulation of α-MHC transcription in transient expression experiments [Ojamaa et al., 1996].

In order to elucidate these conflicting observations and to contribute to the understanding of mechanical stress of cardiomyocytes on MHC gene expression, we compared MHC mRNA of spontaneously contracting with experimentally arrested neonatal cardiomyocytes.

This experimental set-up will also provide data concerning the role of free Ca^{2+} on MHC expression. Increased intracellular Ca^{2+} activates Ca^{2+}/calmodulin kinase which phosphorylates the transcription factor CREB which binds to a Ca^{2+}/cAMP regulatory element (CRE) in the promoter region of target genes [Sheng et al., 1990]. In fact, responsiveness of the α-MHC gene to the Ca/cAMP signalling pathway has been mapped to a M-CAT/E-box hybrid element [Gupta et al., 1994].

In a first set of experiments, cardiomyocytes were depolarized with KCl. This treatment arrests contractile activity by abolishing electrical ativity of the plasma membrane and the normal Ca^{2+} fluctuations associated with systole and diastole. In addition, cardiomyocytes were treated with 2,3 butanedione monoxime (BDM) as electromechanical uncoupler. BDM suppresses mechanical activity by stabilzing the cross-bridges in a state with low actin-affinity [Fryer et al., 1988; Horiuti et al., 1988]. However, electrical activity and the systolic/diastolic fluctuations remain about normal [Li et al., 1985]. Since we found the same MHC mRNA expression in contracting and arrested cardiomyocytes we suggest, that mechanical stress or the systolic/diastolic Ca^{2+} fluctuations have only minor effects on qualitative MHC expression in cultivated cardiomyocytes.

MATERIAL AND METHODS

Neonatal Rat Heart Cell Culture

Isolation and cultivation of neonatal heart cells were performed as previously described [Wallukat and Wollenberger, 1987]. Briefly, single cells were dissociated from the minced ventricles of 1- to 2-day-old Wistar rats with a 0.25% solution of crude trypsin and cultured as

monolayers with a density of 800 cells/mm^2 in Halle SM 20-I medium equilibrated with humidified air. The medium contained 10% heat-inactivated BCS (bovine calf serum) and 2 μmol/L fluorodeoxyuridine, the latter to prevent proliferation of non-muscle cells. BDM (20 mmol/L) and KCl (50 mmol/L) were dissolved in the medium and added at day 2 of cultivation. At day 5 the cells were harvested. In another series of experiments cardiomyocytes were incubated in the presence of the α_1-adrenergic agonist phenylephrine (100 μmol/L) for 72 h.

For measurement of chronotropy 10 different synchronously contracting cell clusters per flask were counted for 15 s. This procedure was repeated twice in different cell cultures for each group.

RNA Preparation

Total RNA was prepared according to the protocol of Chomczynski and Sacchi [1987]. In brief, cultivated cardiomyocytes (≈6 * 10^6/flask) were dissolved in 800 μL of 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol, and homogenized. Eighty microliters of 2 mol/L sodium acetate, pH 4, 800 μL water-saturated phenol (Roth), and 160 μL chloroform/isoamyl alcohol (25:1) were added. After centrifugation at 12,000*g* for 20 min at 4°C, the aqueous phase was transferred into a fresh tube, 800 μl isopropanol was added, and the sample was centrifuged at 5,000*g* for 10 min. The pellet was washed with 75% ethanol and the RNA dissolved in 20 μL water. The concentration was determined by UV absorption. The ratio of optical density at 260 nm and optical density at 280 nm was between 1.8 and 2.0 in all cases.

Reverse Transcription

One microgram of total RNA was used for reverse transcription into cDNA. RNA was denaturated for 5 min at 70°C and then reverse-transcribed by incubation for 60 min at 42°C in the presence of 0.5 mmol/L dNTP, 0.01 mmol/L dithiothreitol, 25 pmol of each reverse primer, 1U MMLV superscript reverse transcriptase (BRL), in 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, and 3 mmol/L $MgCl_2$. The reaction was stopped by heating at 70°C for 10 min.

Quantitative Polymerase Chain Reaction

Relative α- and β-mRNA isoforms were studied by the polymerase chain reaction (PCR). Complementary strands of α- and β-MHC were synthesized on the cDNA by adding four specific oligonucleotides with sequences as follows: CAGAAAATGCACGATGAGGA (α-MHC) and GGCC TGAATGAAGAGTAGAT (β-MHC) as forward and GCATTCATATTTATTGTGGG (α-MHC) and GTGTTTCTGCCT AAGGTGCT as reverse primer (β-MHC) [c.f., Morano et al., 1990]. The position in sequence (between 216 and 292 for α-MHC, between 128 and 226 for β-MHC of the sequence (1) of both isogenes was choosen at the 3′ untranslated region. This led to the amplification of two distinct double-stranded cDNA products specific for the α- (77 bp) and β-MHC (99 bp) in the same test tube. cDNA products were amplified using 1.5 units TAQ-DNA polymerase (BRL) in an assay mix containing 1 μmol/L of the respective oligonucleotide primers, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L $MgCl_2$, and 0.2 mmol/L dNTP. The mixture with a final volume of 50 μl was overlaid with mineral oil and amplified in a thermal cycler (Biometra). Denaturation was carried out at 94°C (1 min), followed by an annealing step 58°C (1 min) and an extension step at 72°C (1.5 min). Products were separated by 8% polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide, photographed (Polaroid), and evaluated by film densitometry. The relative amount of the isoforms was expressed as percentage of the total sum of peak areas.

To evaluate number of PCR cycles with sufficient but still exponential amplification PCR-products were amplified in the presence of [α-^{32}P]dCTP (0.5 μCi). The appropriate PCR bands were excised under UV light, and their ^{32}P contain were determined by liquid scintillation counting. Radioactivity of the PCR products was evaluated after 10, 15, 20, 25, and 30 cycles and expressed as counts per min (cpm). The logarithm of the cpm and the optical density was plotted vs. cycle number and analyzed by linear regression.

DNA Sequencing

DNA sequencing of both strands was carried out on an ABI 373 DNA sequencing system (Perkin-Elmer), using ABI PRISM Dye Terminator Cycle Sequencing Kit. 10 to 20 ng of agarose

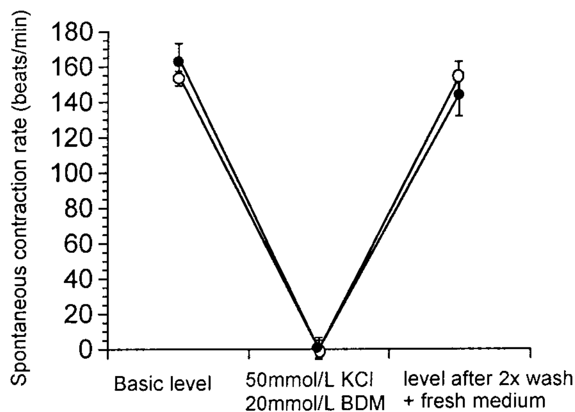

Fig. 1. Chronotropic effect of BDM (○) and KCl (●) on the spontaneous beating rate of cultivated neonatal cardiomyocytes. Washing the cultures and supplying with fresh medium restored spontaneous contraction in both groups. The effect was checked in separated flasks. Values are means ± SD of seven to ten selected cells or synchronously contracting cell clusters counted for 15 s. The procedure was repeated twice in different cultures to yield results representing a total of up to 30 cells for each sample.

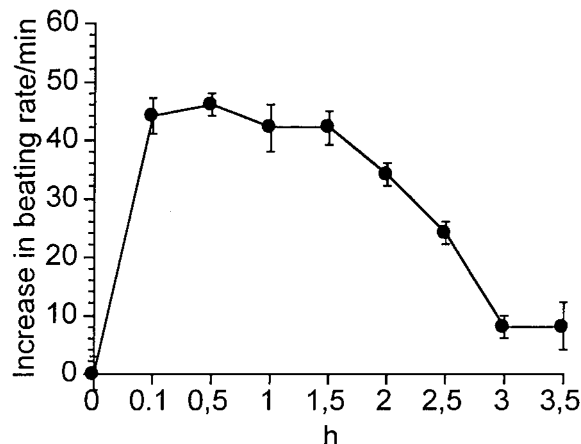

Fig. 2. Time-dependency of the phenylephrine-induced positive chrontropic effect. Desensitization with significant lower values occured 2.5 h after addition of phenylephrine (100 µmol/L).

gel purified PCR fragment and 3,2 pmol primer were used in a total volume of 20 µL. The sequencing reactions were performed on an GeneAmp PCR System 2400 (Perkin Elmer) and run on a 5,5% gel (32 cm "well to read" distance) at 32 watts for 11 h, theoretically yielding about 450 bases.

Statistics

Values are expressed as means ± SD. Student's *t*-test was performed for significance analysis.

RESULTS

Neonatal cardiomyocytes revealed spontaneous contraction with a beating rate of 160 ± 12 beats/min at day 5 of cultivation. Addition of KCl (50 mmol/L) or BDM (20 mmol/L) at day 2 of cultivation caused an immediate complete cessation of spontaneous contraction. The effects of both agents on spontaneous contraction were complete reversible (Fig. 1). Washing three times with phosphate buffered saline and incubation with fresh medium after 3 days restored spontaneous contraction within seconds in cardiomyocytes treated with KCl or minutes in BDM-treated cardiomyocytes. The beating rates after KCl (144 ± 12 beats/min) or BDM (136 ± 16 beats/min) treatment were not significantly different compared to controls at day 5 of cultivation.

The α_1-agonist phenylephrine showed dose-dependent stimulation of spontaneous beating frequency. Desensitization of this effect occurred within 3 h (Fig. 2).

We analyzed both α- and β-MHC mRNA in the same test tube by semi-quantitative RT-PCR. Oligonucleotide primers were chosen from the non-coding regions producing two different cDNAs specific for α-MHC (77 bp) and β-MHC (99 bp). Analysis of both cDNA products revealed the expected sequences (not shown).

Prerequisites for this type of MHC mRNA quantification are: 1) exponential product amplification, 2) identical amplification efficiencies, and 3) evaluation must be performed within the exponential amplification rate [c.f., Morano et al., 1990]. To check our quantification system for these requirements, we amplified cDNA in the presence of ^{32}P-labeled dCTP. The cDNA products were separated by PAGE, evaluated by liquid scintillation counting, and plotted as logarithm of counts per min vs. cycle number. In our PCR system, the ratios between product amplification of MHC mRNA and the number of cycles were identical for both α- and β-MHC cDNAs and could be described by the formula $P = m * 1.33^c$ (an increase of cDNA products by a factor of 1.33 after each cycle). This amplification rate could be observed up to 25 cycles (Fig. 3). Since all requirements for semi-quantitative RT-PCR were fulfilled, this system was applied for MHC mRNA analysis. For detection of MHC mRNA 16 PCR cycles were performed to yield a product signal in the PAGE after fluorescence staining. This is well

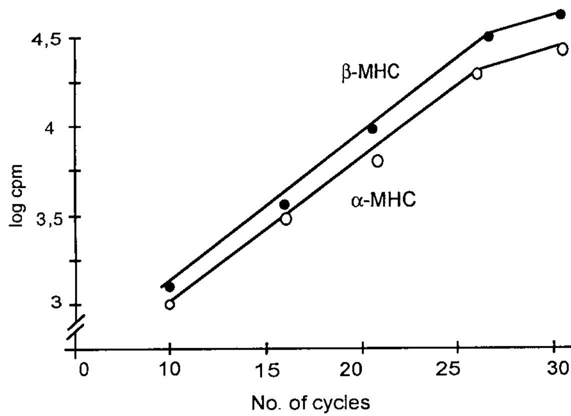

Fig. 3. Evaluation of the amplification efficiencies of PCR product generation from both α- and β-MHC cDNA obtained by reverse transcription. Semilogarithmic plot of the PCR products (expressed as cpm) following the simultaneous amplification of both cDNAs vs. PCR cycle number. ○ α-MHC mRNA, ● β-MHC mRNA. RT-PCRs were performed with 1 μg of total RNA preparation in the presence of [α-^{32}P]dCTP (0.5 μCi). Aliquots of PCR mix were taken after 10, 15, 20, 25, and 30 cycles and separated by 8% polyacrylamide gel electrophoresis. The appropriate PCR bands were excised under UV light, and their ^{32}P contents were determined by liquid scintillation counting.

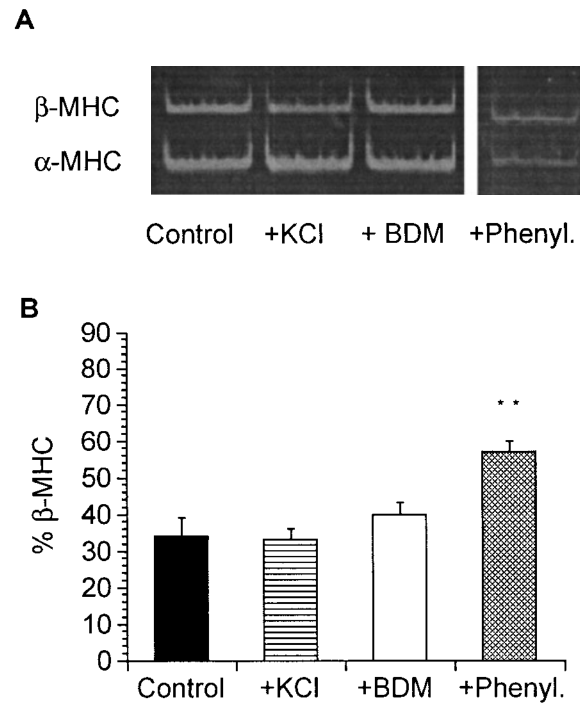

Fig. 4. Levels of MHC isoform mRNAs in cultivated neonatal cardiomyocytes. Data were obtained by densitrometric scanning of PCR products from b-MHC mRNA with a product size of 99 bp and of 77 bp for a-MHC mRNA. **A:** Photograph of PCR products from neonatal cardiomyocytes of control group, cardiomyocytes incubated with BDM (20 mmol/L), KCl (50 mmol/L), and phenylephrine (100 μmol/L) each for 72 h. **B:** Comparison of relative β-MHC mRNA (% of total MHC mRNA) levels. Each data point is based on cells from four primary cell cultures. Values are means ± SD $^*P < 0.05$, Student's *t*-test.

within the exponential phase of product amplification.

During cultivation, spontaneously beating neonatal cardiomyocytes changed their MHC mRNA expression in favour of the α-MHC: The relative level of β-MHC mRNA decreased from 52.5 ± 6% at day 0 to 34.5 ± 11% (control level) at day 5 of cultivation (not shown).

We could not find significant changes of this cultivation-dependent down-regulation of the β-MHC isoform upon arresting the cardiomyocytes with either BDM or KCl for 3 days. MHC pattern of mRNA in the cardiomyocytes treated with BDM from day 2 to day 5 was 33 ± 5.6% β-MHC, i.e., not different from the control value (Fig. 4). Arresting the cells with KCl for the same time period revealed a small increase of the β-MHC mRNA level to 40.5 ± 5%, which was, however not statistically significant different from spontaneously beating cells (Fig. 4).

Cardiomyocytes treated with phenylephrine expressed more β-MHC mRNA and decreased α-MHC mRNA, respectively. The level of 55 ± 5% β-MHC was significantly ($P < 0.05$) higher compared to all other (control, BDM-, and KCl-treated) groups.

DISCUSSION

Increased mechanical stress and contractile activity is thought to act as a trigger for cardiac hypertrophy associated with increased MHC protein and mRNA transcription [McDermott et al., 1985]. Besides these quantitative changes, qualitative changes of gene expression were associated with mechanical overload, in general, upregulation of gene products typical for the embryonic state, e.g., ANF [Lattion et al., 1986] and skeletal α-actin [Komuro et al., 1991]. However, it is not yet clear whether the associated qualitative changes of MHC gene transcription are due to different mechanical demands and/or to changes in the hormonal state of the heart.

The aim of our study was to investigate whether mechanical stress induces qualitative changes of MHC gene expression. We used neonatal cardiomyocytes since mRNA of both β-MHC (fetal form) and α-MHC (adult form) are coexpressed in this developmental state. We compared MHC mRNA expression of normal spontaneously beating neonatal cardiomyocytes in culture with experimentally arrested

cells. Those studies are of additional interest in the light of conflicting results recently published on the effects of mechanical activity of neonatal cardiomyocytes on MHC gene expression: α-MHC mRNA appeared to be decreased in response to contractile activity of neonatal cardiomyocytes [Qi et al., 1994]. In transient expression experiments, however, contractile activity selectively activated the α-MHC gene, an effect which could be localized within −80 to −40 base pairs of the transcription start site by deletion analysis of the α-MHC promoter [Ojamma et al., 1996].

During cultivation of beating neonatal cardiomyocytes, MHC isoform expression shifted in favour of the α-MHC isoenzyme. This change of gene expression was dependent on the presence of serum in the cultivation medium since cardiomyocytes in serum-free medium maintained their initial high β-MHC expression [Luther et al., in press].

To compare the effects of mechanical activity with α-adrenergic effects, we incubated cardiomyocytes with the α_1-adrenergic agonist phenylephrine. α_1-Adrenergic stimulation was believed to share some common signal transducing pathways with mechanical stress, including G-protein coupled activation of the β-protein kinase C [Kariya et al., 1991] and activation of the ESRK system: α1-adrenergic agonists induced hypertrophy with increased cell size and protein content [Simpson et al., 1982] and reexpression of fetal genes in cardiomyocytes similar to mechanical stress [Waspe et al., 1990] probably by sharing conserved response elements in the promoter region of genes typical for the fetal state [Karns et al., 1995; Kariya et al., 1994]. We found an increased β-MHC expression in cardiomyocytes treated with phenylephrine for 3 days which is in accordance with a previous report [Waspe et al., 1990]. The induction of β-MHC was not dependent on the positive chronotropic effect of phenylephrine, since this effect desensitized rapidly and was absent after 3 h of stimulation.

Permanent depolarization by KCl abolishes electrical activity of the sarcolemma, the systolic/diastolic fluctuations of intracellular free Ca^{2+} and, therefore arrests contraction. This intervention did not change qualitative MHC mRNA expression of neonatal cardiomyocytes, suggesting that mechanical activity per se does not alter MHC pattern.

In another experimental approach, we arrested the cells using the electromechanical uncoupler BDM which stabilizes the cross-bridges in a state with low actin affinity inhibiting force generation [Fryer et al., 1988; Horiuti et al., 1988]. BDM at the concentration used in our study abolished mechanical activity of the contractile apparatus completely without significantly changing electrical activity of the plasma membrane [Li et al., 1985]. The cardiomyocytes, therefore, revealed about normal fluctuations of systolic/diastolic free intracellular Ca^{2+} but do not beat. This experimental model allows some suggestions concerning both the role of mechanical activity as well as free intracellular Ca^{2+} on gene expression in the cardiomyocyte. Similar to the KCl arrested cells, a 3 day treatment of cardiomyocytes with BDM did not change normal MHC gene expression. We cannot exclude the possibility that arresting the cardiomyocytes for longer time-periods would induce qualitative changes of MHC gene expression. However, the experimental time period was long enough to detect changes of MHC expression as demonstrated with the 3 day treatment of the cells with the α1-agonist phenylephrine.

Furthermore, intracellular free Ca^{2+} cannot play a significant role as messenger for qualitative regulation of MHC genes. Mechanically arrested cells revealed the same MHC gene transcription with or without intracellular systolic/diastolic free Ca^{2+} fluctuations. This suggests that activation of the Ca/cAMP signalling pathway by Ca^{2+} alone is not sufficient for changing MHC gene transcription.

From our results we suggest that, in contrast to α-adrenergic stimulation, mechanical activity and Ca^{2+} may be weak, if any stimuli for qualitative changes of MHC gene expression in cardiomyocytes. MTC and α-adrenergic stimulation, therefore may comprise different signal transducing mechanisms although they share some common pathways.

Qualitative modulation of MHC gene in vivo, therefore may not directly resides on changes of mechanical stress of the cardiomyocytes. Additional autocrine/paracrine mechanisms exist acting in the MTC process. Thus, the stretch-induced Ang II release from cardiomyocytes which induces TGF-β1 production and release from fibroblast [Lee et al., 1995], promotes β-MHC expression [Parker et al., 1990]. Furthermore, changes in the cAMP/PKA system

may be important in the chronically overloaded hypertrophied heart, since adenylyl cyclase activity is low most likely due to overexpression of the inhibitory G-protein [Böhm et al., 1992]. Suppression of the intracellular second messenger cAMP in the overloaded hypertrophied heart may be responsible for the down-regulation of the α-MHC gene transcription since it could be shown that cAMP selectively promoted α-MHC gene transcription having no effect on the β-MHC gene [Gupta et al., 1991]. Cardiac hypertrophy caused by physical training increased α-MHC gene transcription [Rupp, 1981] probably by the same signal transducing mechanism: the elevated sympathetical drive increased adenylyl cyclase activity via β-adrenergic receptor stimulation and thus increased intracellular cAMP and α-MHC gene transcription.

In summary, mechanical stress increases quantitatively gene expression by numerous "early" and "late" responses and changes qualitatively expression of some genes, excluding the MHC genes.

REFERENCES

Boheler KR, Chassagne C, Wisnewsky C, Martin X, Schwartz K (1992): Cardiac expression of alpha- and beta myosin heavy chains and sarcomeric alpha-actins are regulated through transcriptional mechanisms. J Biol Chem 267:12979–12985.

Böhm M, Gierschick P, Knorr A, Larisch K, Weismann K, Erdmann E (1992): Role of enhanced Giα expression in the regulation of myocardial adenylate cyclase activity and force of contraction in spontaneous hypertensive cardiomyopathy in rats. J Hyperten 10:1115–1128.

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Davis RJ (1993): The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268:14553–14556.

Erikson RL (1991): Structure, expression, and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. J Biol Chem 266:6007–6010.

Flotow H, Thomas G (1992): Substrate recognition determinants of the mitogen-activated 70K S6 kinase from rat liver. J Biol Chem 267:3074–3078.

Fryer MW, Neering IR, Stephenson DG (1988): Effects of 2,3-butanedione monoxime on the contractile activation properties of fast- and slow-twitch rat muscle fibres. J Physiol 407:53–55.

Gupta MP, Gupta M, Stewart A, Zak R (1991): Activation of alpha-myosin heavy chain gene expression by cAMP in cultured fetal rat heart myocytes. Biochem Biophys Res Commun 174:1196–1203.

Gupta MP, Gupta M, Zak R (1994): An E-box/M-CAT hybrid motif and cognate binding protein(s) regulate the basal muscle-specific and cAMP-inducible expression of the rat cardiac alpha-myosin chain gene. J Biol Chem 269:29577–29687.

Heywood SM (1986): tcRNA as a naturally occurring antisense RNA in eukaryotes. Nucl Acid Res 14:6771–6772.

Horiuti K, Higuchi H, Umazume Y, Konishi M, Okazaki O, Kurihara S (1988): Mechanisms of action of 2,3-butanedione 2-monoxime on contraction of frog skeletal muscle fibres. J Mus Res Cell Mot 9:156–164.

Huang SS, Huang JS (1986): Association of bovine brain-derived growth factor receptors with protein tyrosine kinase activity. J Biol Chem 261:9568–9571.

Ito H, Hirata Y, Adachi S, Tanaka M, Tsujino M, Koike A, Nogami A, Marumo F, Hiroe M (1993): Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes. J Clin Invest 92:398–403.

Ito H, Hirata Y, Hiroe M, Tsujino M, Adachi S, Takamoto T, Nitta M, Taniguchi K, Marumo F (1991): Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in dultured neonatal rat cardiomyocytes. Circ Res 69:209–215.

Izumo S, Lompré AM, Matsuoka R, Koren G, Schwartz K, Nadal-Ginard B, Mahdavi V (1987): Myosin heavy chain messenger RNA and protein isoform transition during cardiac hypertrophy. J Clin Invest 79:970–977.

Kariya K, Karns LR, Simpson PC (1991): Expression of a constitutively activated mutant of the β-isozyme of protein kinase C in cardiac myocyte stimulates the promoter of the β-myosin heavy chain isogene. J Biol Chem 266: 10023–10026.

Kariya K, Karns LR, Simpson PC (1994): An enhancer core element mediates stimulation of the rat beta-myosin heavy chain promoter by an alpha 1-adrenergic agonist and activated beta-protein kinase C in hypertrophy of cardiac myocytes. J Biol Chem 269:3775–3782.

Karns LR, Kariya K, Simpson PC (1995): M-CAT, CArG, and Spq elements are required for alpha 1-adrenergic induction of the skeletal alpha-actin promoter during cardiac myocyte hypertrophy. Transcriptional enhancer factor-1 and protein kinase C as conserved transducers of the fetal program ion cardic growth. J Biol Chem 270:410–417.

Kira Y, Kochel PJ, Gordon EE, Morgan HE (1984): Aortic perfusion as a determinant of cardiac protein synthesis. Am J Physiol 246:C247–C258.

Komuro I, Katoh Y, Kaida T, Shibazaki Y, Kurabayashi M, Takaku F, Yazaki Y (1991): Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiomyocytes. J Biol Chem 266:1265–1268.

Komuro I, Kudo S, Yamazaki T, Zou Y, Shiojima I, Yazaki Y (1996): Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. FASEB J 10:631–636.

Lattion AL, Michel JB, Arnauld E, Corvol P, Sourbier F (1986): Myocardial recruitment during ANF mRNA increase with volume overload in the rat. Am J Physiol 251:H890–H896.

Lee AA, Dillmann WH, McCulloch AD, Vikllarreal FJ (1995): Angiotensin II stimulates the autocrine production of transforming growth factor-β1 in adult rat cardiac fibroblasts. J Mol Cell Cardiol 27:2347–2357.

Li T, Sperelakis N, TenEick RE, Solaro JR (1985): Effect of diacetyl monoxime on cardiac excitation-contraction coupling. J Pharmacol Exp Ther 232:688–693.

Luther HP, Morwinski R, Wallukat G, Haase H, Morano I: Expression of sense and naturally occurring antisense mRNA of myosin heavy chain in rat heart tissue and cultivated cardiomyocytes. J Mol Cell Cardiol, in press.

Mahdavi V, Chambers A, Nadal-Ginard B (1984): Cardiac α- and β-myosin heavy chain genes are organized in tandem. Proc Natl Acad Sci USA 81:2626–2630.

Mahdavi V, Koren G, Michand S, Pinset C, Izumo S (1989): Identification of the sequences for the tissue-specific and hormonal regulation of the cardiac myosin heavy chain gene. In Kedes LH, Sockdale FE (eds): "Cellular and Molecular Biology of Muscle Development." New York: Alan R. Liss Inc.

Mann DL, Kent RL, Cooper G (1989): Load regulation of the properties of adult feline cardiocytes growth induction by cellular deformation. Circ Res 64:1079–1090.

McDermott P, Daood M, Klein I (1985): Contraction regulates myosin synthesis and myosin content of cultured heart cell. Am J Physiol 18:H763–H769.

Mercadier JJ, Lompré AM, Wisnewsky C, Samuel JL, Bercovici J, Swynghedauw B, Schwartz K (1981): Myosin isoenzymic changes in several models of rat cardiac hypertrophy. Circ Res 49:525–531.

Morano I, Gerstner J, Rüegg JC, Ganten U, Ganten D, Vosberg HP (1990): Regulation of myosin heavy chain expression in the hearts of hypertensive rats by testosterone. Circ Res 66:1585–1590.

Morkin E, Bahl JJ, Markham BE (1989): Control of cardiac myosin heavy chain gene expression by thyroid hormone. In Kedes LH, Sockdale FE (eds): "Cellular and Molecular Biology of Muscle Development." New York: Alan R. Liss Inc.

Ojamaa K, Samaral AM, Klein I (1996): Identification of a contractile-responsive element in the cardiac α-myosin heavy chain gene. J Biol Chem 52:31276–31281.

Parker TG, Packer SE, Schneider MD (1990): Peptide growth factor can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. J Clin Invest 85:507–514.

Qi M, Ojamaa K, Eleftheriades EG, Klein I, Samarel AM (1994): Regulation of rat ventricular myosin heavy chain expression by serum and contractile activity. Am J Physiol 36:C520–528.

Roberts AB, Roche NS, Winokur TS, Burmester JK, Sporn MB (1992): Role of transforming growth factor-β in maintenance of function of cultured neonatal cardiac myocytes. J Clin Invest 90:2056–2062.

Rupp H (1981): The adaptive changes in the isoenzyme pattern of myosin from hypertrophied rat myocardium as a result of pressure overload and physical training. Basic Res Cardiol 76:79–88.

Sadoshima J, Xu Y, Slayter HS, Izumo S (1993): Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell 75:977–984.

Sechi LA, Griffin CA, Grady EF, Kalinyak JE, Schambelan M (1992): Characterization of angiotensin II receptor subtypes in rat heart. Circ Res 71:1482–1489.

Sheng M, McFadden G, Greenberg ME (1990): Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron 4:571–582.

Shubeita HE, McDonough M, Harris AN, Knowlton KU, Glembotski CC, Brown JH, Chien KR (1990): Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. J Biol Chem 265:20555–20562.

Simpson PC, McGrath A, Savion S (1982): Myocyte hypertrophy in neonatal rat heart cultures and ist regulation by serum and by catecholamines. Circ Res 51:787–801.

Takan H, Komuro I, Zou Y, Kudoh S, Yamazaki T, Yazaki Y (1996): Activation of p70 S6 protein kinase is necessary for angiotensin II-induced hypertrophy in neonatal rat cardiac myocytes. FEBS Lett 379:255–259.

Wallukat G, Wollenberger A (1987): Effects of the serum gamma globulin fraction of patients with allergic asthma and dilated cardiomyopathy on chronotropic b-adrenoceptor function in cultured rat heart myocytes. Biomed Biochem Acta 78:S634–S639.

Waspe LE, Ordahl CP, Simpson PC (1990): The cardiac β-myosin heavy chain isogene is induced selectively in α_1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes. J Clin Invest 85:1206–1214.

Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J (1994): Mechanism of activation of the TGF-β1 receptor. Nature 370:341–347.

Yamazaki T, Komuo I, Kudoh S, Zou Y, Shiojima, Hiroi Y, Mizuno T, Maemura K, Kurihara H, Aikawa R, Takano H, Yazaki Y (1996): Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. J Biol Chem 271:3221–3228.

Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Mizuno T, Takano H, Hiroi Y, Ueki K, Tobe K, Kadowaki T, Nagai R, Yazaki Y (1995a): Mechanical stress activates protein kinase cascade of phosphorylation in neonatal rat cardiac myocytes. J Clin Invest 96:438–446.

Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Mizuno T, Takano H, Hiroi Y, Ueki K, Tobe K, Kadowaki T, Nagai R, Yazaki Y (1995b): Angiotensin II partly mediates mechanical stress-induced cardiac hypertrophy. Circ Res 77:258–265.