More severe cellular phenotype in human idiopathic dilated cardiomyopathy compared to ischemic heart disease

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Abstract Activation of the \( \beta \)-adrenergic receptor (\( \beta \)AR) pathway is the main mechanism of the heart to increase cardiac output via protein kinase A (PKA)-mediated phosphorylation of cellular target proteins, and perturbations therein may contribute to cardiac dysfunction in heart failure. In the present study a comprehensive analysis was made of mediators of the \( \beta \)AR pathway, myofilament properties and cardiac structure in patients with idiopathic (IDCM; \( n = 13 \)) and ischemic (ISHD; \( n = 10 \)) cardiomyopathy in comparison to non-failing hearts (donor; \( n = 10 \)) for the following parameters: \( \beta \)AR density, \( \beta \)-coupled receptor kinases 2 and 5, stimulatory and inhibitory G-proteins, phosphorylation of myofilament targets of PKA, protein phosphatase 1, phospholamban, SERCA2a and single myocyte contractility. All parameters exhibited the expected alterations of heart failure, but for most of them the extent of alteration was greater in IDCM than in ISHD. Histological analysis also revealed higher collagen in IDCM compared to ISHD. Alterations in the \( \beta \)AR pathway are more pronounced in IDCM than in ISHD and may reflect sequential changes in cellular protein composition and function. Our data indicate that cellular dysfunction is more severe in IDCM than in ISHD.

Keywords \( \beta \)-Adrenergic receptor · Protein phosphorylation · Myofilament function · Cardiomyocyte · Collagen

Introduction

During increased cardiac stress stimulation of the \( \beta \)-adrenergic receptors (\( \beta \)AR) exerts a positive inotropic and lusitropic effect on the heart, via activation of protein kinase A (PKA)-mediated phosphorylation of Ca\(^{2+}\)-handling and contractile proteins (Bers 2002; Brodde and Michel. 1999; Kranias et al. 1985; Kentish et al. 2001; Zhang et al. 1995). The positive inotropic effect largely originates from increased cytosolic [Ca\(^{2+}\)] during the systolic phase of the cardiac cycle. The positive lusitropic effect results from increased cytosolic Ca\(^{2+}\) re-uptake into the sarcoplasmic reticulum, increased Ca\(^{2+}\) removal out of the cardiomyocyte, and desensitization of the myofilaments for Ca\(^{2+}\). In patients with heart failure (HF) the sympathetic nervous system is chronically activated to maintain perfusion of vital organs via peripheral vasoconstriction and via an increase in heart rate and in myocardial contractility. Although aimed at maintaining cardiac pump...
function, chronic neurohumoral stimulation is detrimental for cardiac function and results in uncoupling and down-regulation of mediators of the βAR pathway (Brodde and Michel 1999; Packer 1995). Abnormalities in this pathway have been implicated as important determinants of diminished function of the failing human heart. The adverse effects of neurohumoral overstimulation is illustrated by the negative correlation between noradrenaline plasma levels and prognosis of the patients (Cohn et al. 1984), and by the improvement of symptoms and prolonged survival of patients treated with β-blockers (Bohm and Maack 2000).

In line with reduced β-adrenergic signaling, previous studies in end-stage HF patients with idiopathic cardiomyopathy showed reduced PKA-mediated phosphorylation of downstream myofilament target proteins, cardiac myosin binding protein C (cMyBP-C) and troponin I (cTnI) (El-Armouche et al. 2007; van der Velden et al. 2003; Messer et al. 2007, 2009; Copeland et al. 2010), which coincided with increased myofilament Ca2+-sensitivity (pCa50). In patients with diastolic heart failure (i.e., HF with preserved ejection fraction, HFPEF) hyper-phosphorylation of titin has been associated with increased HF with preserved ejection fraction, HFPEF) hypofunction, chronic neurohumoral stimulation is detrimental to cardiac function.

Insight in the modifications of the β-adrenergic pathway and coincident changes in cardiomyocyte function is needed to develop targeted therapy in HF patients. In a recent study we reported diverse changes at the myocardial ultrastructural level with prominent cardiomyocyte hypertrophy in HFPEF patients and low myofibrillar density in HF patients with reduced EF (HFREF) (van Heerebeek et al. 2006). Moreover, Fpassive was higher in HFPEF than in HFREF. These cellular differences may alter responsiveness to current HF treatment, which includes β-blocker therapy. Likewise, patients with idiopathic (IDCM) and ischemic (ISHD) cardiomyopathy may show diverse cellular changes.

Although many studies investigated mediators of the βAR signaling pathway and sarcomeric function in human heart failure, a direct comparative investigation of both in samples from IDCM and ISHD patients is lacking. Within the present study a systematic analysis was performed from the βAR to sarcomeric protein composition and function in IDCM and ISHD patients, and compared to non-failing donor myocardium (donor). Radioligand binding studies were performed to determine βAR density. Protein analysis included down-stream components of the adrenergic signalling cascade, the phospho-proteome of the myofilaments, and SERCA2a and phospholamban levels. Myofilament function was determined in permeabilized single cardiomyocytes. Histological analysis included cardiomyocyte diameter, collagen volume fraction and myofibrillar density.

**Methods**

**Human ventricular tissue**

Left ventricular (LV) tissue samples were obtained during heart transplantation surgery from end-stage HF patients (NYHA III-IV) with IDCM (n = 13; 10 males, 3 females; mean age 51 ± 2 years) or ISHD (n = 10; 7 males, 3 females; mean age 51 ± 2 years)(Table 1). Patients were classified to idiopathic dilated cardiomyopathy when the heart showed LV dilatation and LV systolic dysfunction with no identifiable cause and in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease sufficient to cause the observed myocardial abnormality (Elliott et al. 2008). In contrast, patients with ischemic heart disease had a history of myocardial infarction and explanted hearts showed severe coronary artery disease upon pathologic inspection. Medication included angiotension-converting-enzyme inhibitors, angiotensin II receptor antagonists, diuretics, β-blockers, digoxin and anti-arrhythmic agents and did not differ between the IDCM and ISHD group. Tissue from 10 donor hearts (Table 1; 8 males, 2 females; mean age 38 ± 6 years) served as reference for non-failing myocardium. The tissue was collected in cardioplegic solution and stored in liquid nitrogen. Samples were obtained after informed consent and with approval of the local Ethical Committee (St Vincent’s Hospital Human Research Ethics Committee, Sydney, Australia: File number: H03/118; Title: Molecular Analysis of Human Heart Failure). The investigation conforms with the principles outlined in the Declaration of Helsinki (1997).

**Protein analysis**

**Radioligand binding**

Tissue samples (~200 mg wet weight) were thawed in ice-cold 1 mM KHCO3 solution, minced with scissors and then homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) for 10 s at maximum speed followed by twice 20 s at 2/3 of maximum speed. The homogenates were centrifuged for 15 min at 250 × g and 4°C. The supernatant was filtered through medical gauze and centrifuged for 20 min at 50,000 × g at 4°C. The sediment was resuspended in binding buffer (10 mM Tris, 154 mM NaCl, pH 7.4). Protein content was measured by the method of Bradford using bovine IgG as standard. Radioligand binding was performed as described earlier (Niclauss et al. 2006) using a 90 min incubation at 37°C with [125I]Iodoceyanopindolol (ICYP; specific activity 2200 Ci/mmol, Perkin Elmer, Zaventem, Belgium) in a...
total volume of 250 μl. Non-specific binding was defined as binding in the presence of 100 μM isoproterenol (Sigma-Aldrich). All experiments were performed in duplicates in 96 well plates, and incubations were terminated by rapid vacuum filtration over Whatman GF/C using a Filtermate harvester (Perkin Elmer). Each filter was washed with approximately 10 ml of buffer. Radioactivity adherent to the filters was quantified in a Topcount NXT (Perkin Elmer) using Microsint O scintillator (Perkin Elmer). To determine the relative amount of β₁AR and β₂AR, membranes were incubated with ICYP (100 pM) in the presence or absence of eight concentrations (range $10^{-10}$ to $10^{-3}$ M) of the highly selective β₁AR antagonist CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy) phenoxy) ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulfonate).

**Components of the β-adrenergic receptor pathway**

Protein expression levels of G-coupled receptor kinases (GRK2 and GRK5), G-proteins (Gₛ and Gᵢ) and protein phosphatase 1 (PP-1) were analyzed by one-dimensional 15% SDS-polyacrylamide gel electrophoresis (1D-PAGE) and subsequent Western blotting. Samples were applied in

Table 1 Patient/heart characteristics

|   | Sex | Age | cMyBP-C-P ProQ | cTnI-P ProQ | cTnI-P Phos-tag | pC₅₀ |
|---|-----|-----|----------------|-------------|----------------|------|
| ISHD1 | Male | 47 | 0.27 | 0.20 | 0.14 | 5.69 |
| ISHD2 | Male | 50 | 0.36 | 0.41 | 0.54 | 5.60 |
| ISHD3 | Female | 43 | 0.42 | 0.50 | – | – |
| ISHD4 | Male | 45 | 0.21 | 0.32 | – | – |
| ISHD5 | Female | 54 | 0.46 | 0.67 | 0.79 | 5.41 |
| ISHD6 | Male | 55 | 0.67 | 2.20 | – | 5.70 |
| ISHD7 | Male | 54 | 0.40 | 1.25 | 1.70 | 5.50 |
| ISHD8 | Male | 54 | 0.31 | 0.33 | 0.44 | 5.73 |
| ISHD9 | Male | 50 | 0.33 | 0.21 | 1.18 | 5.45 |
| ISHD10 | Male | 61 | 0.20 | 0.13 | 0.74 | 5.56 |
| IDCM1 | Male | 56 | 0.30 | 0.07 | 0 | 5.65 |
| IDCM2 | Male | 27 | 0.32 | 0.11 | 0 | 5.65 |
| IDCM3 | Male | 53 | 0.31 | 0 | – | 5.65 |
| IDCM4 | Male | 53 | 0.44 | 0.22 | 0.38 | 5.59 |
| IDCM5 | Male | 53 | 0.29 | 0.64 | – | – |
| IDCM6 | Male | 62 | 0.25 | 0.16 | 0.47 | – |
| IDCM7 | Male | 53 | 0.18 | 0.38 | 0.46 | – |
| IDCM8 | Male | 44 | 0.50 | 0.33 | 1.18 | – |
| IDCM9 | Female | 50 | 0.28 | 0.16 | 0.65 | 5.65 |
| IDCM10 | Male | 53 | 0.25 | 0.08 | 0.46 | 5.52 |
| IDCM11 | Female | 52 | 0.18 | 0.17 | 0.51 | 5.68 |
| IDCM12 | Male | 58 | 0.19 | 0.12 | 0.49 | 5.71 |
| IDCM13 | Female | 45 | 0.32 | 0.10 | – | – |
| DONOR1 | Male | 39 | 0.62 | 1.96 | 1.75 | 5.65 |
| DONOR2 | Male | 56 | 0.69 | 2.09 | 1.94 | 5.48 |
| DONOR3 | Male | 21 | 0.60 | 4.54 | 1.59 | 5.48 |
| DONOR4 | Female | 37 | 0.61 | 1.11 | 1.57 | 5.44 |
| DONOR5 | Male | 23 | 0.64 | 0.94 | 1.74 | 5.52 |
| DONOR6 | Male | 19 | 0.75 | 1.96 | 1.97 | 5.51 |
| DONOR7 | Female | 53 | 0.73 | 1.12 | 1.92 | – |
| DONOR8 | Male | 13 | 0.75 | 1.75 | – | 5.43 |
| DONOR9 | Male | 65 | 0.49 | 1.09 | 0.98 | 5.63 |
| DONOR10 | Male | 51 | 0.57 | 1.56 | – | – |

Age and sex of all patients and donors with the corresponding mean value for cMyBP-C and cTnI phosphorylation determined on ProQ Diamond stained gels (values in arbitrary units), for cTnI phosphorylation assessed by Phos-tag (in mol per mol cTnI) and for Ca²⁺-sensitivity of force.
concentrations which were within the linear range of detection: 20 μg for GRK2, GRK5, Gs and PP-1, and 10 μg for Gs. Blots were pre-incubated with 0.5% milk powder in TTBS (TWEEN-tris-buffered-saline: 10 mM Tris–HCl pH 7.6, 75 mM NaCl, 0.1% Tween) for 1 h at room temperature. The blots were incubated overnight at 4°C with primary rabbit polyclonal antibodies (Santa Cruz) against GRK2 (dilution 1:1000; sc-562), GRK5 (dilution 1:1000; sc-565), Gs (Gsolf; dilution 1:1000; sc-3783), Gi (dilution 1:1000; Gi-1 sc-262, Gi-2 sc-7276, Gi-3 sc-262) or primary mouse polyclonal antibody against PP-1 (dilution 1:50; sc-7482, Santa Cruz). Specificity of the antibodies has been shown in previous studies (Vinge et al. 2001; Cho and Kehrl 2007) and all antibodies revealed one protein band in our Western blot analysis indicative for their specificity. Primary antibody binding was visualized using a secondary horseradish peroxidase-labeled goat-anti-rabbit/mouse antibody (dilution 1:2000; DakoCytomation) and enhanced chemiluminescence (ECL plus Western blotting detection, Amersham Biosciences). All signals were normalized to actin (dilution 1:1000; clone KJ43A; Sigma) stained on the same blots.

Myofilament protein phosphorylation

Myofilament protein phosphorylation was determined using Pro-Q Diamond Phosphoprotein Stain as described previously (Zaremba et al. 2007). To preserve the endogenous phosphorylation status, frozen biopsies were homogenized in liquid nitrogen and re-suspended in 1 ml cold 10% trichloroacetic acid solution (TCA; dissolved in acetone containing 0.1% (w/v) dithiothreitol (DTT)). TCA-treated tissue pellets were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT. Tissue samples were separated on gradient gels (Criterion tris–HCl 4–15% gel, BioRad) and proteins were stained for one hour with Pro-Q Diamond Phosphoprotein Stain. Fixation, washing and de-staining were performed according to the manufacturer’s guidelines (Molecular Probes). To assess protein content subsequently gels were stained overnight with SYPRO Ruby stain (Molecular Probes). Myofilament protein phosphorylation status of isolated cardiomyocytes was expressed relative to protein expression of cMyBP-C to correct for differences in sample loading. Staining was visualized using the LAS-3000 Image Reader and signals were analyzed with AIDA.

Cardiac troponin I phosphorylation at PKA sites Ser 23/24 was detected with a primary rabbit polyclonal antibody (dilution 1:500; Cell signaling) in Western blotting. In addition, the recently developed Phos-tag™ acrylamide (FMS Laboratory; Hiroshima University, Japan) (Kinoshita et al. 2006) was used to visualize phosphorylated cTnI species using alkoxide-bridged dinuclear metal (Mn²⁺) complex as phosphate-binding tag (Phos-tag) molecule. Mn²⁺-Phos-tag molecules preferentially capture phosphomonoester dianions bound to Ser, Thr and Tyr residues. Non-phosphorylated and phosphorylated cTnI species were separated in 1D-PAGE with polyacrylamide-bound Mn²⁺-Phos-tag and transferred to Western blots. Phosphorylated cTnI species in the gel are visualized as slower migration bands compared to the corresponding dephosphorylated cTnI form (Kooij et al. 2010; Messer et al. 2009).

SERCA2a and phospholamban

SERCA2a protein levels were determined immunochemically by dot-blot analysis, as described before, with minor modifications (Muller et al. 1991). Briefly, homogenized tissue samples (typically 0.5 μg total protein) were spotted in triplo onto a nitrocellulose membrane. The blot was then incubated with a 1:2500 dilution of a polyclonal antiserum to SERCA2a and subsequently with 125I-labeled anti-rabbit immunoglobulin G (0.05 mg/ml, specific activity 7 mCi/mg). To detect phospholamban, blots were first incubated with a 1:2500 dilution of a monoclonal anti mouse antibody (Affinity Bioreagents) and subsequently with 125I-labeled anti-mouse immunoglobulin G. Blots were exposed to Phosphor Imager screens, which were then scanned and spots were quantified using ImageQuant software (Molecular Dynamics). All protein values for ISHD and IDCM samples were normalized to the average value observed in donor, which was set to 1.

Force measurements in single cardiomyocytes

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously (Kooij et al. 2010; van der Velden et al. 2003). Tissue samples were defrosted in relaxing solution (in mmol/l: free Mg, 1; KCl, 145; EGTA, 2; MgATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 min in relaxing solution supplemented with 0.5% Triton X-100 to remove all membrane structures. Subsequently, cells were washed twice in relaxing solution, after which single cardiomyocytes were attached with silicone adhesive between a force transducer and a motor. Sarcomere length of isolated cardiomyocytes was adjusted to 2.2 μm. The pCa (−log [Ca²⁺]) ranged from 9.0 (relaxation solution) to 4.5 (maximal activation). All force values were normalized for cardiomyocyte cross-sectional area. Exposure to a series of solutions with intermediate pCa values (pCa 6.0–5.0) yielded the baseline force-pCa relation. On transfer of the cardiomyocyte from relaxing to activating solution, isometric force started to develop. Once a steady state force level was reached, the cell was shortened within
1 ms to 80% of its original length to determine the baseline of the force transducer. The distance between the baseline and the steady force level is the total force ($F_{\text{total}}$). After 20 ms, the cell was restretched and returned to the relaxing solution, in which a second slack test, of 10 s duration was performed to determine passive force ($F_{\text{passive}}$). Active force was obtained by subtracting passive force from the total force, i.e., $F_{\text{active}} = F_{\text{total}} - F_{\text{passive}}$.

Quantitative histomorphometry

Histomorphologic analysis of tissues was performed on elastica-von-Giesson and hematoxylin-eosin-stained 4 μm thick sections of tissue fixed in 5% formalin (Borbély et al. 2005). Images of these sections were acquired using a projection microscope (×50). Subsequent image analysis, using SlidebookTM 4.0 software (3I, Denver, Co), was performed to determine cardiomyocyte diameter and the extent of interstitial fibrosis, which was expressed as collagen volume fraction. Areas of reparative and perivascular fibrosis were excluded. Cardiomyocyte diameter was determined perpendicularly to the outer contour of the cell membrane at nucleus level in 15 representative myocytes of the cardiac tissue section, and collagen volume fraction was calculated as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas averaged over 4–6 representative fields of the cardiac tissue section. Data were averaged per heart sample and averaged to obtain the mean of the group. For analysis of cardiomyocyte diameter and collagen volume fraction 9 ISHD, 7 IDCM and 5 donor hearts were included.

Analysis of myofibrillar density was performed in 8 ISHD, 6 IDCM and 5 donor hearts. Tissues samples were fixed in 2% (v/v) gluteraldehyde for 30 min and 1.5% (w/v) osmium tetroxide for 10 min dehydrated with acetone and embedded in Epon812. Ultrathin sections were collected on 300-mesh Formavar-coated Nickel-grids. The sections were contrasted with uranyl acetate and lead citrate and were examined in a Jeol-1200EX electron microscope. Quantitative analysis was performed with the above mentioned automated image analyzer. Cardiomyocyte myofibrillar density was calculated from the sum of the myofibrillar areas related to total cellular area in 4–6 representative myocytes per sample. Data were averaged per heart.

Data analysis

Competition binding data were analyzed by fitting mono- and biphasic sigmoidal functions to the experimental data; a biphasic fit was accepted only if it resulted in a significant improvement as judged by an F-test. Resulting IC$_{50}$ values for the high and low affinity component of inhibition were converted to $K_i$ values based upon the Cheng–Prusoff equation (Cheng and Prusoff 1973) and assuming a $K_d$ value of ICYP of 72 pM ($n = 4$). Due to limited tissue availability, receptor density ($B_{\text{max}}$) was estimated based upon the specific binding (SB) of the single radioligand concentration ($L$) in the competition experiments relative to its $K_d$ using the equation $B_{\text{max}} = \text{SB} / (1 - L / K_d + 1))$. The statistical significance of inter-group differences was assessed by one-way ANOVA followed by Dunnett’s post-hoc tests.

Values are given as mean ± SEM of n observations. The mean data values of protein and histological analysis and force measurements in ISHD, IDCM and donor samples were compared using one-way ANOVA and Bonferroni-post tests. $P < 0.05$ was considered significant. All statistical analysis was performed with Prism (version 4.01, Graphpad Software, San Diego, CA, USA).

Results

Components of the β-adrenergic receptor pathway

In all cases except one competition by the highly β$_1$-selective antagonist CGP 20712A resulted in biphasic curves (Fig. 1a). The −log $K_i$ values for high and low affinity sites, which identify β$_1$AR and β$_2$AR, respectively, were not significantly different between IDCM (8 hearts; 8.21 ± 0.19 and 5.59 ± 0.09) and ISHD (8 hearts; 8.17 ± 0.09 and 5.62 ± 0.10) myocardium, and did not differ from the values observed in donor (7 hearts; 8.55 ± 0.12 and 5.74 ± 0.11). Single point estimates of total βAR density, revealed significantly reduced βAR density in IDCM compared to donor, while the value observed in ISHD was intermediate between IDCM and donor (Fig. 1b). The percentage of β$_1$ARs did not differ between IDCM (50 ± 4%) and ISHD (47 ± 9%) samples and was comparable to the value observed in donor (52 ± 6%).

No difference was found in expression of GRK2 between IDCM and ISHD, while GRK5 was significantly higher in ISHD than in IDCM myocardium (Table 2). Compared to donor GRK2 was significantly lower in both failing groups, while GRK5 was significantly higher in the ISHD group. $G_s$ expression did not differ between ISHD and IDCM, though was significantly lower in both failing groups compared to donor (Fig. 1c; Table 2). In addition to $G_s$, we have analyzed three isoforms of inhibitory G-protein: $G_{\alpha_1}$, $G_{\alpha_2}$ and $G_{\alpha_3}$. The heterotrimeric G-proteins are composed of α, β and γ subunits, of which the α-subunit confers specificity to the G-protein (Wang and Dhalla 2000). $G_{\alpha_1}$ expression was significantly higher in IDCM than in ISHD and donor (Fig. 1d), while $G_{\alpha_2}$ was significantly higher in both failing groups compared to donor.
Fig. 1  a Competition curve of radioligand binding as a function of CFP 20712A.  b βAR density was significantly lower in IDCM compared to donor, but the receptor distribution was the same.  c Western blot examples of βAR pathway proteins in IDCM, ISHD and donor. Expression of Gs was significantly lower in failing compared to donor, but did not differ between IDCM and ISHD.  d The expression level of G_{ia-3} was significantly increased in IDCM compared to donor, while ISHD did not differ from donor. *P < 0.05 in Bonferroni-post tests, IDCM versus ISHD; #P < 0.05, in Bonferroni-post tests, failing versus donor. Donor values obtained in Western blot analyses were set to 1.

Myofilament and calcium handling proteins

ProQ Diamond phosphostaining was used to detect phosphorylation of myofilament proteins. Figure 2a shows ProQ Diamond-stained cardiac samples from ISHD, donor (Table 2). The G_{ia-3} expression level was not altered in patient groups (data are summarized in Table 2).

ProQ Diamond stained gel shows lower endogenous phosphorylation of the PKA-target proteins cMyBP-C and cTnI in failing compared to donor. Noteworthy, cTnI phosphorylation was significantly higher in ISHD compared to IDCM (Fig. 2c). In addition, phosphorylation of cMyBP-C was higher in ISHD compared to IDCM, though not significantly (P = 0.07). When the failing and donor were combined, a monovariate linear regression analysis revealed significant correlations between the ProQ Diamond-assessed phosphorylation status of cMyBP-C and cTnI (P < 0.0001; R^2 = 0.75) (Fig. 2d).

Higher PKA-mediated phosphorylation of cTnI (at Ser23 and Ser24, i.e., PKA sites) in ISHD compared to IDCM was confirmed in Western blotting (Fig. 3). Consistent with ProQ Diamond analysis of cTnI phosphorylation, the Western blots revealed significantly lower cTnI phosphorylation at PKA-sites in failing compared to donor.

Analysis of cTnI species separated by Phos-tag polyacrylamide gels revealed three bands upon staining of Western blots with a specific anti-cTnI antibody (8I-7) in donor, while only two bands were observed in the failing sample shown in Fig. 4a. The lower band was recognized by an antibody directed against cTnI unphosphorylated at PKA sites (22B11). The upper band was recognized by the phosphorylation-specific antibody which recognizes the PKA-sites Ser 23 and Ser 24. The second band on the blot is the mono-phosphorylated form of cTnI (Messer et al. 2009). Phos-tag analysis of samples from all groups are

### Table 2  Protein analysis

|          | ISHD (n = 9) | IDCM (n = 13) | Donor (n = 8) |
|----------|-------------|---------------|---------------|
| GRK2     | 0.72 ± 0.07* | 0.62 ± 0.13*  | 1.00 ± 0.07   |
| GRK5     | 1.92 ± 0.23* | 1.33 ± 0.19*  | 1.00 ± 0.20   |
| Gs       | 0.39 ± 0.10* | 0.45 ± 0.13*  | 1.00 ± 0.30   |
| Giz-1    | 1.00 ± 0.20  | 3.36 ± 0.91*  | 1.00 ± 0.30   |
| Giz-2    | 2.17 ± 0.40* | 3.18 ± 0.80*  | 1.00 ± 0.10   |
| Giz-3    | 0.81 ± 0.18  | 1.19 ± 0.30   | 1.00 ± 0.30   |
| MLC-2P   | 1.60 ± 0.22* | 0.89 ± 0.16*  | 1.00 ± 0.07   |
| PP-1     | 1.51 ± 0.19  | 2.45 ± 0.35*  | 1.00 ± 0.40   |
| Phospholamban | 1.43 ± 0.14* | 1.55 ± 0.08*  | 1.00 ± 0.04   |
| SERCA2a  | 0.99 ± 0.07  | 0.72 ± 0.06*  | 1.00 ± 0.07   |

All signals in IDCM and ISHD samples were normalized to the averaged value observed in donor, which was set to 1. GRK G-coupled receptor kinase, G_{s} G-stimulatory, G_{i} G-inhibitory, MLC-2P phosphorylated myosin light chain 2, PP-1 protein phosphatase 1, a.u. arbitrary units, n number of heart samples. *P < 0.05 in Bonferroni-post tests, IDCM versus ISHD; #P < 0.05, in Bonferroni-post tests, failing versus donor.
The distribution of the cTnI forms was significantly different among the three groups (Fig. 4c). The unphosphorylated form was highest in IDCM, while the bisphosphorylated form was highest in donor. A significant correlation was observed between phosphorylated cTnI on Phos-tag and cTnI phosphorylation analyzed by ProQ Diamond (Fig. 4d; \( P < 0.0001; R^2 = 0.75 \)).

ProQ Diamond analysis also revealed higher phosphorylation of myosin light chain 2 (MLC-2) in ISHD compared to both IDCM and donor (Table 2), while there were no significant differences in phosphorylation of the other myofilament proteins, desmin and troponin T (data not shown). The difference in MLC-2 phosphorylation may be in part explained by differences in PP-1 expression. Western blot analysis of PP-1 revealed significantly higher PP-1 expression in IDCM compared to ISHD and donor (Table 2).

Western blot analysis showed increased phospholamban protein levels in IDCM and ISHD compared to donor and a significantly lower SERCA2a level in IDCM compared to both ISHD and donor (Table 2). The ratio of phospholamban over SERCA2a significantly differed among groups and was highest in IDCM.
Higher Ca\(^{2+}\)-sensitivity in IDC\textsuperscript{2}M than in ISHD

Force measurements were performed in single cardiomyocytes isolated from 8 IDC\textsuperscript{2}M hearts (42 cardiomyocytes), 8 ISHD hearts (27 cardiomyocytes) and 8 donor hearts (41 cardiomyocytes). Cross-sectional area of the cardiomyocytes determined at a sarcomere length of 2.2 \(\mu\)m was significantly higher in IDC\textsuperscript{2}M (556 ± 44 \(\mu\)m\(^2\)) and ISHD (690 ± 75 \(\mu\)m\(^2\)) compared to donor (339 ± 26 \(\mu\)m\(^2\)) (\(P < 0.05\) in one-way ANOVA).

Maximal active tension (\(F_{\text{active}}\)) did not differ (Fig. 5a), while passive tension (\(F_{\text{passive}}\)) was significantly lower in IDC\textsuperscript{2}M and ISHD compared to donor (Fig. 5b).

Noteworthy, myofilament pCa\textsubscript{50} was significantly lower in ISHD compared to IDC\textsuperscript{2}M (Fig. 5c, d). A significantly higher pCa\textsubscript{50} was found in failing heart compared to donor (Fig. 5c, d). Moreover, the steepness of the sigmoidal force-pCa relationship (\(n_{\text{Hill}}\)) was significantly higher in ISHD (4.05 ± 0.12) compared to IDC\textsuperscript{2}M (3.58 ± 0.10) and donor (3.70 ± 0.14). A monovariate linear regression analysis revealed a significant correlation between pCa\textsubscript{50} and phosphorylated cMyBP-C (\(P < 0.05\); \(R^2 = 0.24\)) (Fig. 5e), while no significant correlation was found between pCa\textsubscript{50} and ProQ Diamond-analyzed phosphorylated cTnI (\(P = 0.09\); \(R^2 = 0.12\)). Troponin I phosphorylation determined by Phos-tag analysis did significantly
correlate with pCa_{50} (P \leq 0.01; R^2 = 0.43) (Fig. 5f). The individual data per patient/donor for phosphorylation of the PKA target proteins, cMyBP-C and cTnI, and myofilament Ca^{2+}-sensitivity are summarized in Table 1.

Discussion
In the present study, diverse changes were found in the βAR pathway in IDCM and ISHD patients. The results indicate that there are differences in the cellular structure and function between both patient groups. Compared to ISHD, IDCM was characterized by a more severe receptor βAR down-regulation and increased G_i expression, increased PP-1 expression, an enhanced reduction in protein phosphorylation, increased myofilament pCa_{50}, reduced SERCA2a expression and higher collagen volume fraction. The data indicate that cellular dysfunction is more severe in IDCM than in ISHD patients.

Changes in components of the β-adrenergic pathway
In the present study, total βAR density was significantly reduced in IDCM compared to donor with intermediate
values in ISHD. This down-regulation may have been caused by reduced mRNA levels (Hadcock and Malbon 1998). In addition, \( \beta \)AR may be degraded upon internalization of receptors, which is preceded by the process of receptor uncoupling (Wang and Dhalla 2000). The regulating step in the latter process is receptor phosphorylation, either by second-messenger activated kinases such as PKA, or by GRKs, which only phosphorylate agonist occupied receptors (Fig. 7). Both GRK2 and GRK5 are determinants of \( \beta \)AR responsiveness of the heart, as they disrupt the interaction between receptors and the heterotrimeric G proteins (Wang and Dhalla 2000). This process of receptor uncoupling is thought to be an important contributor to \( \beta \)AR desensitization and down-regulation due to increased sympathetic tone in cardiac disease. In failing human tissue, Ungerer et al. (1993, 1994) showed increased GRK2 mRNA and activity in both ISHD and IDCM. In contrast, in the present study we observed lower GRK2 protein expression in both failing groups compared to donor, while GRK5 protein expression was significantly higher in ISHD compared to IDCM and donor.

The discrepant observation in GRK5 between ISHD and IDCM may reflect a difference in severity of disease, as Leineweber et al. (2005) observed an increase in GRK activity in right atrial tissue from patients at NYHA I and II, while in more severe stages (NYHA III and IV) GRK activity was similar as observed in controls. Interestingly, \( \beta \)AR density did not differ in NYHA I and II and was
significantly reduced compared to controls in NYHA III and IV. These data are suggestive for a transient increase in GRK activity during the course of heart failure, and indicate that increases in GRK precede receptor down-regulation in the human heart. Accordingly, the high GRK5 expression and the lower reduction in βAR density in ISHD compared to IDCM observed in the present study may reflect a less severe disease stage in ISHD, despite similar NYHA classification. The latter is further supported by the significantly higher expression of inhibitor G-protein in IDCM compared to ISHD.

More severe cellular phenotype in IDCM compared to ISHD

Analysis of sarcomeric protein phosphorylation revealed significant differences in the βAR target proteins cMyBP-C and cTnI. Consistent with previous studies (El-Armouche et al. 2007; van der Velden et al. 2003; Messer et al. 2007, 2009; Copeland et al. 2010) phosphorylation of cMyBP-C and cTnI was significantly lower in both failing groups compared to donor, which has been explained by reduced PKA-mediated phosphorylation due to βAR desensitization and down-regulation in cardiac disease (Wolff et al. 1996; van der Velden et al. 2003). Noteworthy, cMyBP-C and cTnI phosphorylation was lower in IDCM compared to ISHD, which fits the larger reduction in βAR density and larger increase in G_{i-1} expression observed in IDCM compared to ISHD. The higher myofilament pCa_{50} in IDCM compared to ISHD may be well explained by the significantly lower (PKA-mediated) myofilament protein phosphorylation observed in IDCM.

Interestingly, analysis of cTnI species using Phos-tag polyacrylamide gels showed that the difference in cTnI phosphorylation between IDCM and donor can be largely explained by a difference in the bis-phosphorylated form of cTnI (Fig. 4e). It is tempting to speculate that a difference in cTnI phosphorylation at PKA sites Ser 23/24 underlies the differences in pCa_{50} among groups. Although a significant correlation was found between myofilament pCa_{50} and phosphorylation status of cMyBP-C (Fig. 5e), the correlation with ProQ Diamond assessed cTnI phosphorylation did not reach statistical significance (P = 0.09). Noteworthy, cTnI phosphorylation determined with the Phos-tag assay did significantly correlate with myofilament Ca^{2+}-sensitivity (Fig. 5f), indicating that Phos-tag analysis is a very accurate method to determine cTnI phosphorylation.

The correlation between cMyBP-C phosphorylation and pCa_{50} was stronger when cMyBP-C phosphorylation was combined with ProQ-Diamond assessed cTnI phosphorylation (P < 0.05; R^2 = 0.27; not shown). In a recent study in human myocardium we showed that myofilibrillar targets of PKA, cMyBP-C and/or titin, play an important role in modulating the effect of cTnI phosphorylation on pCa_{50} in human myocardium (Kooij et al. 2010). Exchange of endogenous (mostly unphosphorylated) cTnI with PKA-phosphorylated troponin complex did not reduce pCa_{50} in failing cardiomyocytes, in which endogenous cMyBP-C phosphorylation is low. Accordingly, our present data are compatible with a modulatory role for cMyBP-C in force development at submaximal calcium concentrations in human myocardium.

In addition to the PKA targets, cTnI and cMyBP-C, phosphorylation of MLC-2 was significantly lower in IDCM than in ISHD. Dephosphorylation of MLC-2 occurs via PP-1, which was significantly higher in IDCM compared to ISHD. Although increased PP-1 expression may explain the relatively low MLC-2 phosphorylation in IDCM, it cannot explain the significantly lower MLC-2 phosphorylation in donor compared to ISHD, as PP-1 expression tended to be even lower in donor compared to ISHD. Other kinases/phosphatases most likely underlie the differences in MLC-2 phosphorylation observed between ISHD and donor myocardium and warrant future research.

Moreover, upon activation of βAR, PP-1 activity is depressed by PKA via phosphorylation of protein inhibitor 1 (I1) (Neumann et al. 1991). Thus, apart from increased PP-1 expression, perturbations in βAR signaling will increase activity of PP-1, which may explain the lower MLC-2 phosphorylation in IDCM compared to
Increased activity and mRNA level of PP-1 has been reported previously in human IDCM (Neumann et al. 1997). Increased activity of PP-1 in a transgenic mouse model resulted in decreased myocardial contractility and dilated cardiomyopathy underscoring a detrimental role for PP-1 in heart failure (Carr et al. 2002). Increased PP-1 will also impair Ca$^{2+}$-handling by dephosphorylation of phospholamban. The resulting reduction in SR Ca$^{2+}$ loading will be enhanced further by the reduction in SERCA2a expression in IDCM. Although several studies did not find differences in SERCA2a and PLB at mRNA levels between ISHD and IDCM (Arai et al. 1993; Flesch et al. 1996), our study showed reduced SERCA2a protein expression in IDCM, and increased phospholamban expression in both failing groups. Higher PP-1 expression and lower SERCA2a expression are indicative for a more severe cellular phenotype in patients with IDCM.

In line with previous studies (Anversa et al. 1986; Parodi et al. 1993; Weber 1989) we observed three structural alterations in end-stage heart failure: increased cardiomyocyte diameter (Fig. 6c), increased collagen volume fraction (Fig. 6d) and decreased myofibrillar density (Fig. 6e). While no difference was found in cardiomyocyte diameter and myofibrillar density between IDCM and ISHD, collagen volume fraction was significantly higher in IDCM compared to ISHD, which is in line with a previous study by Parodi et al. (1993).

Conclusions

Comparison of myocardium from patients with ISHD and IDCM end-stage HF revealed more severe cellular perturbations in patients with IDCM which are summarized in Fig. 7. Our data support previous studies in which transient changes were observed in the $\beta$AR pathway. Increased GRK expression may be a primary event and possibly precedes the reduction in $\beta$AR density, and the changes in expression of proteins involved in calcium handling and PP1. Noteworthy, Leineweber et al. (2005) showed that GRK5 was significantly lower in patients treated with $\beta$-blockers only in the relatively early stages of heart failure (i.e., NYHA I and II), in which GRK5 was higher compared to the end-stage of HF. Hence, the response to current treatment may depend on cause of cardiac disease, and may be less in patients with IDCM.

Conflict of Interest None.

References

Anversa P, Ricci R, Olivetti G (1986) Quantitative structural analysis of the myocardium during physiologic growth and induced cardiac hypertrophy: a review. J Am Coll Cardiol 36(7):1140–1149
Arai M, Alpert NR, Mac Lennan DH, Barton P, Petiasamy M (1993) Alterations in sarcoplasmic reticulum gene expression in human heart failure a possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. Circ Res 72(2):463–469
Bers DM (2002) Cardiac excitation-contraction coupling. Nature 415(6868):198–205
Bohm M, Maack C (2000) Treatment of heart failure with beta-blockers. Bas Res Cardiol 95:I15–I24
Borbély A, van der Velden J, Papp Z, Bronzwaer JG, Edes I, Stienen GJM, Paulus WJ (2005) Cardiomyocyte stiffness in diastolic heart failure. Circulation 111(6):774–781
Borbély A, Falcao-Pires I, van Heerbeeck L, Hamdani N, Edes I, Gavina C, Leite-Moreira AF, Bronzwaer JG, Papp Z, van der Velden J, Stienen GJM, Paulus WJ (2009) Hypophosphorylation of the Stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. Circ Res 104(6):780–786
Broodde OE, Michel MC (1999) Adrenergic and muscarinic receptors in the human heart. Pharmacol Rev 51(4):651–690
Carr AN, Schmidt AG, Suzuki Y, del Monte F, Sato Y, Lanner C, Breeden K, Jing SL, Allen PB, Greengard P, Yatani A, Hoit BD, Grupp IL, Hajjar RJ, DePaoli-Roach AA, Kranias EG (2002) Type 1 phosphatase a negative regulator of cardiac function. Mol Cell Biol 22(12):4124–4135
Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant ($K_i$) and the concentration of an inhibitor, which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. Biochem Pharmacol 22(23):3099–3108
Cho H, Kehrl JH (2007) Localization of Gi alpha proteins in the centrosomes and at the midbody: implication for their role in cell division. J Cell Biol 178(2):245–255
Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T (1984) Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med 311(13):819–823
Copeland O, Sadayappan S, Messer AE, Stienen GJM, van der Velden J, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart muscle. J Mol Cell Cardiol 49:1003–1011
El-Armouche A, Pohlmann L, Schlossarek S, Starbatty J, Yeh YH, Nattel S, Dobrev D, Eschenhagen T, Carrier L (2007) Decreased phosphorylation levels of cardiac myosin-binding protein-C in human and experimental heart failure. J Mol Cell Cardiol 43(2):223–229
Elliot P, Andersson B, Arbustini E, Bilinska Z, Cecchi F, Charron P, Dabour O, Kuhl U, Maisch B, McKenna WJ, Monserrat L, Panukweit S, Rapezzi C, Seferovic P, Tavazzi L, Keren A (2008) Classification of the cardiomyopathies: a position statement from the European society of cardiology working group on myocardial and pericardial diseases. Eur Heart J 29:270–276
Flesch M, Schwinger RH, Schnabel P, Schiffer F, van Gelder I, Bavendiek U, Südkamp M, Kuhn-Renfier G, Böhm M (1996) Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and phospholamban mRNA and protein levels in end-stage heart failure due to ischemic or dilated cardiomyopathy. J Mol Med 74(6):321–332
Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Koishi E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757
Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, dos Remedios C, van der Velden J, Stienen GJM, van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, van Heerebeek L, Borbély A, Niessen HW, Bronzwaer JG, van der Meulen JP, Messer AE, Jacques AM, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart disease. Circulation 113(16):1966–1973
Leineweber K, Rohe P, Beilfuss A, Wolf C, Sporkmann H, Bruck H, Hadcock JR, Malbon CC, Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Kintsh JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88(10):1095–1065
Kinoshata E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757
Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, dos Remedios C, van der Velden J, Stienen GJM, van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, van Heerebeek L, Borbély A, Niessen HW, Bronzwaer JG, van der Meulen JP, Messer AE, Jacques AM, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart disease. Circulation 113(16):1966–1973
Leineweber K, Rohe P, Beilfuss A, Wolf C, Sporkmann H, Bruck H, Hadcock JR, Malbon CC, Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Kintsh JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88(10):1095–1065
Kinoshata E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757
Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, dos Remedios C, van der Velden J, Stienen GJM, van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, van Heerebeek L, Borbély A, Niessen HW, Bronzwaer JG, van der Meulen JP, Messer AE, Jacques AM, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart disease. Circulation 113(16):1966–1973
Leineweber K, Rohe P, Beilfuss A, Wolf C, Sporkmann H, Bruck H, Hadcock JR, Malbon CC, Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Kintsh JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88(10):1095–1065
Kinoshata E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757
Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, dos Remedios C, van der Velden J, Stienen GJM, van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, van Heerebeek L, Borbély A, Niessen HW, Bronzwaer JG, van der Meulen JP, Messer AE, Jacques AM, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart disease. Circulation 113(16):1966–1973
Leineweber K, Rohe P, Beilfuss A, Wolf C, Sporkmann H, Bruck H, Hadcock JR, Malbon CC, Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Kintsh JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88(10):1095–1065
Kinoshata E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757
Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, dos Remedios C, van der Velden J, Stienen GJM, van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, van Heerebeek L, Borbély A, Niessen HW, Bronzwaer JG, van der Meulen JP, Messer AE, Jacques AM, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart disease. Circulation 113(16):1966–1973
Leineweber K, Rohe P, Beilfuss A, Wolf C, Sporkmann H, Bruck H, Hadcock JR, Malbon CC, Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Kintsh JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88(10):1095–1065
Kinoshata E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757
Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, dos Remedios C, van der Velden J, Stienen GJM, van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, van Heerebeek L, Borbély A, Niessen HW, Bronzwaer JG, van der Meulen JP, Messer AE, Jacques AM, Marston SB (2010) Analysis of cardiac myosin binding protein C in human heart disease. Circulation 113(16):1966–1973
Leineweber K, Rohe P, Beilfuss A, Wolf C, Sporkmann H, Bruck H, Hadcock JR, Malbon CC, Hadcock JR, Malbon CC (1998) Down-regulation of \(\beta\)-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proc Natl Acad Sci USA 85(14):5021–5025
Kintsh JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88(10):1095–1065
Kinoshata E, Kinosita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5(4):749–757