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Differential functional expression of human myocardial G protein receptor kinases in left ventricular cardiac diseases

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Abstract

The relationship between myocardial G protein receptor kinase (GRK) expression and β-adrenoceptor signalling in human left heart diseases has not been fully elucidated yet. In this study, we characterized and compared the GRK2–7 expression in patients with left ventricular volume overload disorders and dilated cardiomyopathic hearts, and evaluated the relationship of this expression with alterations in myocardial β-adrenoceptor signalling in volume overload, in order to test the notion that GRK functional expression is influenced in a disease-specific and selective fashion. We established that GRK2, GRK3, and GRK5 are well expressed, while GRK4, GRK6, and GRK7 are only scarcely detectable in the healthy human heart. Compared to control hearts ($n=8$), GRK2 mRNA expression was elevated by 71% ($P<0.005$) in the left ventricle, 110% ($P<0.05$) in the right ventricle, 130% ($P<0.05$) in the left atrium, and 1300% ($P<0.005$) in the right atrium (RA) of the dilated cardiomyopathy hearts ($n=6$). In the volume overload group ($n=10$), it was increased by approximately 40% ($P<0.05$) in the left ventricle, 38% in the right ventricle, 81% ($P<0.05$) in the left atrium, and 850% ($P<0.005$) in the right atrium. On the other hand, GRK5 was significantly elevated only in the left ventricle by 68% ($P<0.05$) in the dilated cardiomyopathy hearts and by 48% ($P<0.01$) in volume overload patients, while in contrast, GRK3 remained unchanged in dilated cardiomyopathy, but was slightly elevated by 36% ($P=0.05$) in the right ventricle of the volume overload patients. The alterations in GRK expression were accompanied with a decrease in myocardial β_1-adrenoceptor mRNA in all four chambers, and these trends in gene expression were paralleled with those of their immunodetectable protein levels. Furthermore, these changes were in association with a decrease in downstream receptor-stimulated, adenylyl cyclase-mediated functional expression and an increase in ventricular protein kinase A activity. The results point to differences in which myocardial GRKs are regulated in cardiac disease, whereby changes in GRK2 expression may be related to the global effects of the disease on myocardial adrenoceptor function and those in GRK5 may be localized to the ventricles, depending on the nature of the myocardial load.

Keywords: G protein-coupled receptor kinase; Adenylyl cyclase; β-Adrenoceptor; Protein kinase; Human myocardial tissue; Hemodynamic load; Left ventricular volume overload

1. Introduction

In experimental heart failure, the downregulation of myocardial β-adrenoceptors is partly due to the receptor phosphorylation in their agonist-bound state mediated by their selective guanine nucleotide-binding (G) protein receptor kinases (GRKs) 2/3 in conjunction with their cognate proteins, the β-arrestins (Benovic et al., 1986, 1989; Bouvier et al., 1989; Ferguson et al., 1996a,b,c, 1998a,b). The GRK2/3 as well as cardiac-specific GRK5 are implicated in the control of cardiac function, both under normal conditions and in disease (Benovic et al., 1989; Ferguson, 2001; Ferguson et al., 1996a, 1998a; Inglese et al., 1993; Lefkowitz et al., 1990, 1992). In animal study models, the expression of these receptor kinases appears to be regulated in peculiar fashions in different cardiac diseases. Thus, for example, in the porcine heart, treatment with a β-adrenoceptor antagonist has been associated with a reduction in left ventricular GRK2/3 activity (Ping et al., 1995), while in the progression of pacing-induced congestive heart failure, an

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increase was observed only in GRK5, but not GRK2 (Ping et al., 1997). Some studies in transgenic mouse hearts overexpressing the GRK5 suggested that its upregulation might be partially responsible for alterations in myocardial function in chronic heart failure (Chen et al., 2001). Besides, recently, GRK2 has been linked primarily with the regulation of endothelial function, while GRK3 and GRK5 were associated with cardiac myocyte function (Vinge et al., 2001), pointing to the dependence of their regulation on the type of prevailing cardiac disorder, and to their different roles in circulatory function and disease.

At present, only limited information is available in the literature regarding the myocardial GRK distribution and regulation in various human cardiac diseases. The currently available evidence for their different roles in cardiac disease has been derived primarily from experimental and transgenic animal models associating a downregulation of β-adrenoceptor signalling with an elevation in their function and expression in, for example, cardiomyopathy-induced heart failure (Koch et al., 1995; Pippig et al., 1993; Rockman et al., 1998a,b), or reversal of disease manifestation with altered expression of the adrenoceptor signalling components, such as the β-arrestins (Rockman et al., 1996). In humans, a study by Ungerer et al. (1993) showed an association of elevated left ventricular GRK2 mRNA and activity with a reduction in β_1-adrenoceptor function in failing hearts from patients with both dilated cardiomyopathy and ischaemic heart disease. Recently, we reported an association between the increase in lymphocyte GRK2, GRK3, and GRK5 with an attenuation in the β-adrenoceptor signalling levels in patients with left ventricular volume overload (Dzimiri et al., 2002). This led us to suggest that cardiac diseases such as volume overload, in which heart failure per se is not necessarily the primary indicator of the severity of the disease, may influence the functional expression of these GRKs in fashions that differ from dilated cardiomyopathy. Besides, in dilated cardiomyopathy and ischaemic heart disease, downregulation of the β_1-adrenoceptor is associated with defective downstream signalling as reflected by a global myocardial reduction in receptor-mediated adenylyl cyclase activities (Bohm et al., 1992; Bristow and Feldman, 1992; Brodde et al., 1998; Fu et al., 1992). It is still not known whether the left ventricular overload influences myocardial β-adrenoceptor functional expression, and whether or not such effects are localized to the left ventricle or manifest globally throughout the myocardium. The objective of this study was, therefore, to characterize the expression of GRKs in the normal human heart, and to evaluate its possible relationship with the level of the β_1-adrenoceptor and its downstream signalling in volume overload. We accomplished this by determining, at both mRNA and protein levels, the association between the myocardial expression of the GRK2 to GRK7 and that of the β_1-adrenoceptor, as well as its downstream signalling, as denoted by the levels of the various adenylyl cyclase-mediated activities, and the second messenger-dependent protein kinase A (PKA) function in volume overload, using healthy donor hearts as controls. We further compared the changes in gene expression in volume overload with that in dilated cardiomyopathy hearts, in order to evaluate the possibility that the GRK expression may be influenced in a disease-specific fashion.

2. Materials and methods

2.1. Study patients and materials

In all, 10 patients (mean age 35.7 ± 4.9 years) admitted for surgical correction of stenotic and regurgitant heart valvular lesions at our institution were selected for the study as the volume overload group, using the same exclusion criteria described previously (Dzimiri et al., 2002). Two of the patients were female and eight were male. In brief, the diagnosis of the disease was established by the patients' history, as well as the echocardiographic appearance of the valve and confirmed at surgery. Five of the patients presented with aortic regurgitation, three with mitral regurgitation, and two had mixed aortic and mitral regurgitation. These patients did not have any active rheumatic carditis, and had not taken β-adrenoceptor antagonists at least within the last 4 months prior to—or inotropes at—the time of surgery. Two of the patients were over 45 years of age. These were studied by coronary angiography to exclude the presence of significant coronary artery disease. Transthoracic two-dimensional colour echocardiography was used to evaluate the type of valvular pathology and ventricular dysfunction. The hemodynamic data of the patients were recorded as clinical routine. The various arterial pressures were determined by sphygmomanometry or direct cannulation, and cardiac output was determined by the thermodilution method. Systemic vascular resistance, pulmonary vascular resistance, and cardiac index were derived from the above hemodynamic parameters.

The second study group consisted of dilated cardiomyopathy hearts excised from six patients (four males, two females, mean age 33.4 ± 5.3 years) with end-stage heart failure who were undergoing heart transplantation at our institution. These patients were on antifailure medication including digoxin, diuretics, and/or angiotensin-converting enzyme inhibitors prior to explantation. Hearts explanted from patients on inotropic support, such as dobutamine and dopamine, were excluded from the study. As controls, we used eight healthy donor (34.9 ± 3.9 years) hearts excised from individuals who had died of traffic accidents with no history of cardiac disease, and had not been previously exposed to β-adrenoceptor agonists or antagonists. These hearts had originally been intended for cardiac transplantation, but failed to get suitable matching recipients. Full informed consent was obtained from all patients or family members before participating in the study. The study was performed in accordance with the rules and regulations laid down by the Hospital's Ethics Committee.

2.2. G protein receptor kinase (GRK) and β-adrenoceptor gene expression

For the gene expression experiments, similar biopsies were obtained from all three groups: dilated cardiomyopathic hearts, volume overload hearts, and healthy donor hearts. In volume overload patients, myocardial biopsies were obtained at the time of surgery. The right atrial biopsy was obtained as surgical waste, while the procurement of biopsies from other chambers is known to cause no untoward effects on the patient. For the dilated cardiomyopathy and control hearts, the biopsies were obtained at the time of donation, and frozen at − 80 °C if not used immediately. Total RNA was extracted by pulverizing the biopsies in liquid nitrogen, followed by homogenization in Tris reagent for simultaneous isolation of total RNA, DNA, and proteins (Molecular Research Center, Cincinnati, OH, USA) according to Chomczynski (1993). The RNA was precipitated from the aqueous layer by adding isopropanol, washed with ethanol, and solubilized according to the instruction of the manufacturer. The RNA was reverse-transcribed to cDNA at 65 °C with AMV reverse transcriptase (Promega, Madison, WI, USA) and amplified by polymerase chain reaction (PCR) using the Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). The upstream and downstream primer pairs used for amplifying the respective genes are given in Table 1. We first evaluated the suitability of using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Its coamplification permits standardization of the baseline on which the levels of the different GRK amplicons could be comparatively analyzed in the myocardial tissue. We established that amplification of the PCR products for 30 cycles at annealing temperatures of 55– 57 °C was accomplishable within the exponential range of the products. The amplicons were quantified by densitometric imaging technique using the Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD, USA).

2.3. Immunodetectable GRK and β_1-adrenoceptor expression

The tissue biopsies were homogenized and the proteins were precipitated with isopropanol from the organic phase of the cardiac homogenate prepared using the Tri reagent, as described above. Samples of 20-μg protein were boiled for 5 min in 1:1 ratio with Laemmli sample buffer, applied to 5% stacking/12% resolving SDS polyacrylamide gels, and electrophoresed at 100 V for 2 h on the Biorad minigel system (Biorad Laboratories, Richmond, CA, USA). Two gels were run simultaneously for each set of samples, one of which was subjected to Coomassie blue staining and another to Western blotting. The proteins were then transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), presoaked in ethanol using the Biorad miniprotean transfer cell for 5 h at 80 V. The membranes were first washed, blocked with enhanced chemiluminescence (ECL) blocking agent, and incubated with the rabbit anti-GRK primary antibodies overnight at 4 °C. They were then washed, incubated with antirabbit or antimouse IgG conjugated with horseradish peroxidase, treated with Western blotting detection reagents, and immediately exposed to ECL Hyperfilm (Amersham Biosciences, Buckinghamshire, UK). The detected bands were quantified by densitometric imaging technique with GS-800 Calibrated Densitometer (Bio-Rad Laboratories). The GRK2, GRK4, GRK5, and GRK6 antibodies recognize a domain in the carboxyl (C)-terminal region of the human origin, while the GRK3 antibody recognizes the C-terminal region of the bovine origin corresponding to the sequence of the human GRK3.

2.4. Adenylyl cyclase activity

Adenylyl cyclase activity was determined in cardiomyocyte membrane protein suspended in buffer containing 5 mmol/l Tris HCl (pH 7.5), 250 mmol/l sucrose, 1 mmol/l EGTA, and 10 mmol/l $MgCl_2$, as described previously (Dzimiri et al., 2002). In brief, the cardiac tissue was homogenized using a Stedfast Stirrer (Fisher Scientific, Pittsburgh, PA, USA), and the homogenate was centrifuged at $200 \times g$ to remove cellular debris. The supernatant was then centrifuged at $10{,}000 \times g$, and the resultant pellet was washed twice with the same buffer, snap-frozen in liquid nitrogen, and stored at − 70 °C or used immediately. The different adenylyl cyclase activities were assessed by its ability to catalyze the conversion of [α^{32}P]ATP to [^{32}P]cAMP under the given reaction conditions. About 15–20 μg of protein was suspended in an assay buffer

Table 1
List of the downstream and upstream primer pair sequences used in the study

	Downstream 5′ primer	Upstream 3′ primer
GAPDH	GCTTTTAACTCTGGTAAAGTGG	TCACGCCACAGTTTCCCGGAGG
β_1-AR	CTCACCAACCTCTTCATCATG	GAAACGGCGCTCGCAGCTGTC
GRK2	AGCGATAAGTTCACACGGTT	TGCCACCGCTCCGAGATGGTGA
GRK3	AATGAAGCTGTACCTCAGGTG	CTGGGGTATGGAAGGCATAGG
GRK4	TATGAAGTTGCCGATGATGAG	GCCCAGGTTGTAAATGTGAA
GRK5	CCCCGAAGTCCTGAACAACCA	AACCCCCTCCCCGCTAAAATG
GRK6	TTCCGAGAGTTCTGTGCCACGA	TCATAGGCGTAGGCCAAGCTC
GRK7	GAAACCAGAGCAACGCTTAGG	TAGGACTTTGCGTAATACACA

β_1-AR, β_1-adrenoceptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRK, G protein-coupled receptor kinase.

containing 25 mmol/l Hepes (pH 7.5), 5 mmol/l $MgCl_2$, 20 mmol/l creatine phosphate, 200 μg of creatine kinase, 1 mmol/l cAMP, and 100 μmol/l [α^{32}P]ATP (specific activity 30 Ci/mmol; Amersham International, Buckinghamshire, UK) in a final volume of 50 μl and incubated at 37 °C for 20 min. The reaction was terminated by adding 10 μl of stop solution containing 30 mmol/l each of EDTA, ATP, cAMP, and AMP (pH 7.5). Three microliters of the assay mixture was chromatographed in 0.25 M LiCl on 0.1 mm of cellulose MN300 polyethyleneimine impregnated with a fluorescent indicator. The cAMP and ATP spots were cut from the chromatogram and radioactivity was counted in 10 ml of Optifluor scintillation fluid (Packard, Meridian, CT, USA) on a Beckman beta counter.

2.5. Cyclic AMP-dependent protein kinase A activity

The cAMP-dependent PKA activity was assayed by the [^{32}P]phosphorylation of a synthetic substrate Kemptide (GIBCO-BRL/Sigma, St. Louis, MO) using its pseudosubstrate inhibitor peptide as a control for specificity. The tissues were homogenized in extraction buffer containing 5 mmol/l EDTA and 50 mmol/l Tris (pH 7.5) in a precooled dounce homogenizer on ice. The homogenate was centrifuged at 200 × *g* for 10 min to remove cellular debris, and the supernatant snap-frozen in liquid nitrogen and stored at − 70 °C. Twenty micrograms of the protein was incubated for 15 min at ambient temperature with 50 μmol/l Kemptide and 0.25 mg/ml BSA in a buffer containing 50 mmol/l Tris (pH 7.5) and 10 mmol/l $MgCl_2$ to determine the kinase activity under various reaction conditions. The cAMP-stimulated activity was determined in the presence of 10 μmol/l cAMP. Ten microliters of 100 μM [γ^{32}P]ATP ($1–2 \times 10^5$ dpm/nmol) was added to the assay mixture and further incubated at 30 °C for 5 min; 20 μl was withdrawn and spotted on phosphocellulose discs. The discs were washed three times in 2 M NaCl, then four times with 2 M NaCl in 1% phosphoric acid, followed by two washes with H_2O to remove unbound radioactivity. The radioactivity was counted in 10 ml of Optifluor scintillation fluid on a Beckman beta scintillation counter. The extent of PKA-mediated peptide phosphorylation was determined as the average of duplicate experiments. The protein concentrations for all assays were determined according to Bradford (1976) using Biorad standard reagents.

2.6. Materials

The upstream and downstream primer pairs used for amplifying the respective genes were designed in our laboratory and synthesized in the core facility of our institution. The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and all other reagents were of chemical quality grade.

2.7. Statistical analysis

The data were analyzed using analysis of variance (ANOVA) and Student's *t* test, and are presented as mean ± S.E.M. $P < 0.05$ was employed to indicate statistical significance.

3. Results

3.1. G protein receptor kinase and β_1-adrenoceptor gene expression

We first established the distribution of myocardial GRKs in the normal human chambers. Three of the six GRKs studied, (GRK2, GRK3, and GRK5) were well expressed in all four chambers (Figs. 1–3), while GRK4, GRK6, and GRK7 were only partially detectable at mRNA level in the left ventricles of the healthy human heart. However, while

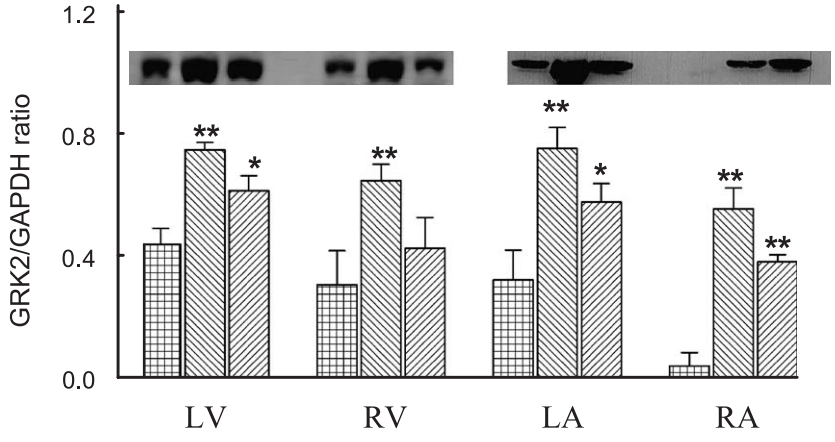

Fig. 1. Assessment of GRK2 expression in myocardial biopsies of 6 dilated cardiomyopathic hearts (rising left bar) and 10 left ventricular volume overload patients (rising right bar), compared with 8 control donor hearts (horizontal cross hatch). The values are given as mean ± S.E.M. of the densitometric unit ratios for the GRK2 mRNA to that of the GAPDH as an internal control. Insert depicts a typical immunodetectable protein profile of GRK2 in the corresponding groups. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium. *$P < 0.05$, **$P < 0.005$ vs. control.

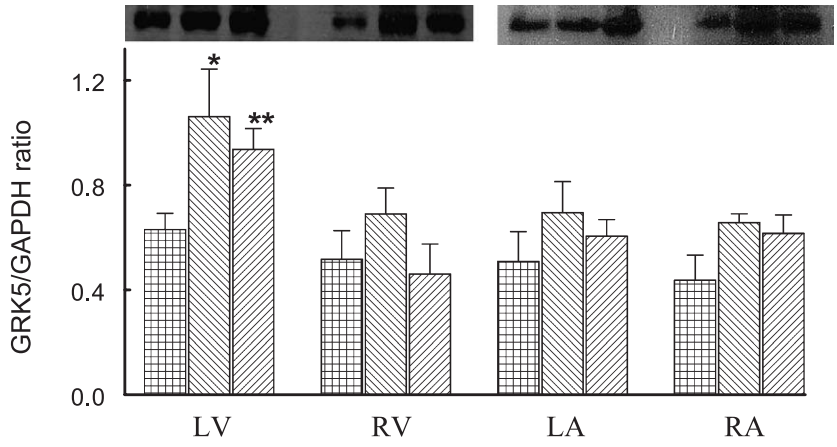

Fig. 2. Assessment of GRK5 expression in myocardial biopsies of 6 dilated cardiomyopathic hearts (rising left bar) and 10 left ventricular volume overload patients (rising right bar) compared with 8 heart donors (horizontal cross-hatch). The values are given as mean ± S.E.M. of the densitometric unit ratios for the GRK5 mRNA to that of the GAPDH as an internal control. Insert depicts a typical immunodetectable protein profile of GRK5 in the corresponding groups. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium. *$P<0.05$, **$P<0.01$ vs. control.

GRK3 and GRK5 were uniformly distributed in the four chambers, GRK2 appeared to be predominantly localized in the left heart side, and was partially undetectable in the right atrial chambers.

Following the establishment of myocardial GRK mRNA distribution, we proceeded to compare the influence of dilated cardiomyopathy and volume overload on the three well-expressed GRKs (GRK2, GRK3, and GRK5) in all four chambers. Compared to healthy donor hearts ($n=8$), in the cardiomyopathic hearts ($n=6$), GRK2 mRNA was elevated by 71% ($P<0.005$) in the left ventricle, 110% ($P<0.05$) in the right ventricle, 130% ($P<0.005$) in the left atrium, and 1300% ($P<0.005$) in right atrium (RA) (Fig. 1). In the volume overload patients ($n=10$), the GRK2 mRNA was increased by 40% ($P<0.05$) in the left ventricle, 38% in the right ventricle, 81% ($P<0.05$) in the left atrium, and 850% ($P<0.005$) in right atrium. It is noteworthy that the apparently great increase in GRK2 expression in the atria, particularly the right atrium, is primarily due to its partial lack of expression in normal hearts, since its levels in either disease type were not significantly different from those in the other three chambers.

The GRK5 mRNA was increased by 68% ($P<0.05$) in the left ventricle, 33% in the right ventricle, 35% in the left atrium, and 50% in the right atrium in the dilated cardiomyopathic hearts. In the volume overload patients, it was also significantly elevated by 48% ($P<0.01$) in the left ventricle, but appeared to remain uninfluenced in the other three chambers (Fig. 2). Thus, unlike the GRK2 mRNA expression, significant alteration in GRK5 expression was observed primarily in the left ventricle of both study groups. In contrast to GRK2 and GRK5, GRK3 mRNA was not altered at all in the dilated cardiomyopathy heart biopsies, and was slightly elevated by 36% ($P=0.05$) only in the right ventricle of the volume overload patients (Fig. 3). Hence, put together, while the GRK2 mRNA expression

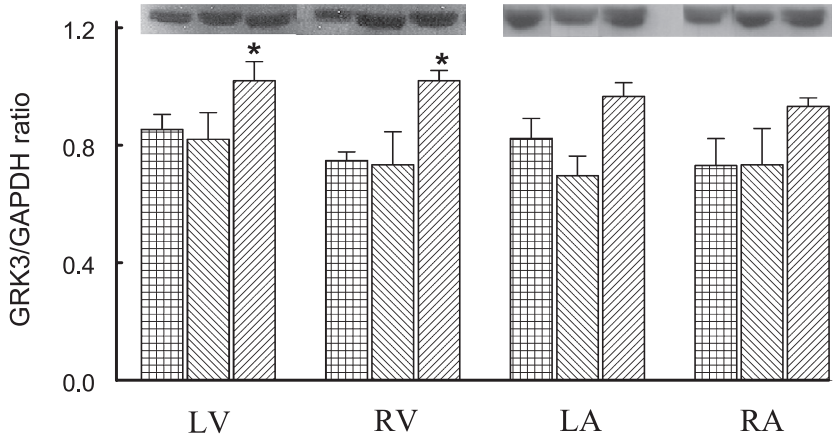

Fig. 3. Assessment of GRK3 expression in biopsies of 6 dilated cardiomyopathic hearts (rising left bar) and 10 left ventricular volume overload patients (rising right bar) compared with 8 heart donors (horizontal cross hatch). The values are given as mean ± S.E.M. of the densitometric unit ratios for the GRK3 mRNA to that of the GAPDH as an internal control. Insert depicts a typical immunodetectable protein profile of GRK3 in the corresponding groups. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium. *$P<0.05$ vs. control.

differed between the two types of cardiac diseases, that of GRK5 was similar, and GRK3 was slightly influenced in the volume overload patients, but remained unaffected in the dilated cardiomyopathic chambers. On the other hand, no delineable trends or alterations were observed in the expression of the GRK4, GRK6, and GRK7 mRNA in both disease groups (results not shown).

In order to assess the relationship between GRK expression and AR functional expression in the two types of diseases, we determined the levels of β_1-adrenoceptor mRNA in the same biopsies employed to study the GRK expression. In the dilated cardiomyopathic hearts, the β_1-adrenoceptor mRNA was reduced by 60% ($P<0.005$) in the left ventricle, 73% ($P<0.005$) in the right ventricle, 58% ($P<0.01$) in the left atrium, and 63% ($P<0.01$) in the right atrium (Fig. 4). In the volume overload hearts, on the other hand, the β_1-adrenoceptor mRNA was reduced by 44% ($P<0.005$) in the left ventricle, 35% ($P<0.05$) in the right ventricle, 39% ($P<0.01$) in the left atrium, and 44% ($P<0.05$) in the right atrium. Put together, these results show a global reduction in β_1-adrenoceptor mRNA in both diseases, which is more significant in dilated cardiomyopathy than in volume overload, and hence inversely matches more closely the expression pattern of the elevation in GRK2 than that of the GRK5 mRNA.

3.2. Immunodetectable GRK and β_1-adrenoceptor expression

The potential functional consequence of the alterations in GRK and β_1-adrenoceptor mRNA expression was assessed by determining the immunodetectable protein profiles of these genes in the same myocardial biopsies from the four chambers. The protein expression is given as inserts to the respective figures. In general, the trends in the immunodetectable protein levels of both GRKs and β_1-adrenoceptors reflect the changes in their gene expression. Thus, the increase in the immunodetectable GRK2 protein expression in dilated cardiomyopathy was observed in all four chambers, while in volume overload patients, it was greatest in the left ventricle (insert, Fig. 1). Furthermore, the increase in GRK5 expression was most evident in the left ventricle, as was its level of gene expression (insert, Fig. 2). On the other hand, like the mRNA expression, GRK3 protein was not significantly altered in either the dilated cardiomyopathic heart biopsies or volume overload patients (insert, Fig. 3), while the attenuation in the β_1-adrenoceptor mRNA expression was similarly marked by a global reduction in the myocardial protein expression (insert, Fig. 4).

3.3. Adenylyl cyclase activity

The downstream signalling consequences of altered myocardial β_1-adrenoceptor and GRK expression can be assessed by determining various adenylyl cyclase-mediated activities. In the present study, we evaluated the implications of these changes in gene expression in volume overload by determining the basal, β_1-adrenoceptor-stimulated, forskolin (FSK)-mediated, and nonreceptor (G protein)-mediated adenylyl cyclase catalytic activities. The basal activity of the enzyme in control hearts ($n=6$) was 195.8 ± 31.8 pmol phosphate/min/mg protein in the left ventricle, 225.3 ± 39.3 pmol in the right ventricle, 200.7 ± 42.9 pmol in the left atrium, and 132.7 ± 18.6 pmol in the right atrium. In the volume overload group ($n=6$), this activity was 167.4 ± 33.2 pmol phosphate/min/mg protein in the left ventricle, 197.2 ± 26.4 pmol in the right ventricle, 182.6 ± 43.5 pmol in the left atrium, and 171.3 ± 38.9 pmol in the right atrium, suggesting a slight depression, particularly in the ventricles, as compared to control hearts (Fig. 5). We first evaluated the possibility that an elevation in GRK-mediated β_1-adrenoceptor phosphorylation would lead to blunted receptor-mediated adenylyl cyclase activity, employing different isoproterenol (IPN)

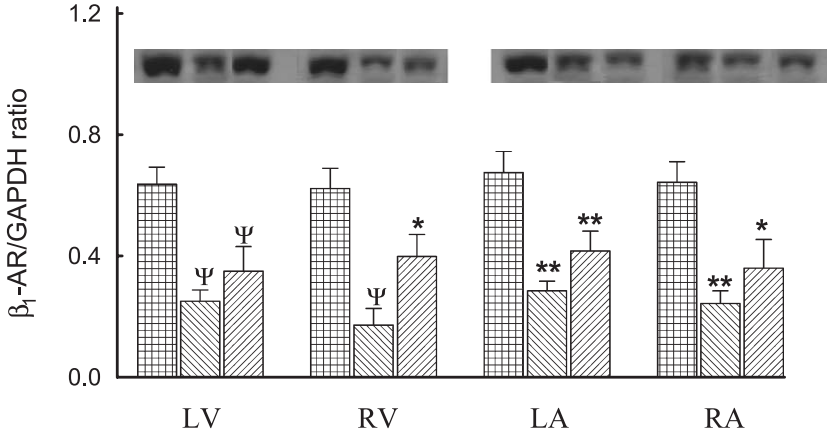

Fig. 4. Assessment of β_1-adrenoceptor (β_1-AR) expression in 6 dilated cardiomyopathic hearts (rising left bar) and 10 left ventricular volume overload patients (rising right bar) compared with 8 heart donors (horizontal cross hatch). The values are given as mean ± S.E.M. of the densitometric unit ratios for the β_1-adrenoceptor mRNA to that of the GAPDH as an internal control. Insert depicts a typical immunodetectable protein profile of the β_1-adrenoceptor in the corresponding groups. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium. $^{*}P<0.05$, $^{**}P<0.01$, $^{\Psi}P<0.005$ vs. control.

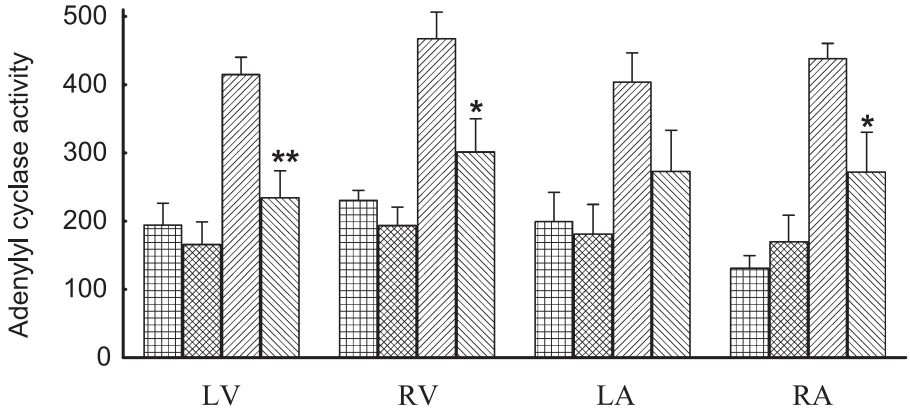

Fig. 5. Stimulation of adenylyl cyclase basal activity by 1.0 μM IPN in the presence of 100 μM guanosine triphosphate in patients with left ventricular volume overload ($n=6$) compared to normal donor hearts ($n=6$). The enzyme activity is given in picomoles of phosphate per milligram of protein per minute, and values are given as mean ± S.E.M. Horizontal cross-hatch represents adenylyl cyclase basal activity in control; cross-hatch represents basal activity in patients; rising right hatch represents stimulated activity in controls; rising left hatch represents stimulated activity in patients. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium. *$P<0.05$, **$P<0.01$ vs. control.

concentrations in the presence or absence of guanosine triphosphate (GTP). Under our study conditions, the adenylyl cyclase basal activity in both control hearts ($n=6$) and volume overload patients ($n=6$) was elevated only slightly by GTP (100 μM) or isoproterenol (100 μM) alone, but increased significantly ($P<0.05$) in the presence of both agents (Fig. 5). However, compared to the healthy controls, the isoproterenol/GTP-stimulated enzyme activity was approximately 57–71% lower in the four chambers of the volume overload group (Fig. 5).

We were further interested in establishing how the adenylyl cyclase-mediated signalling pathway(s) bypassing the β-adrenoceptor/stimulatory G protein-dependent system, or mediated by other G proteins such as the inhibitory G protein, may be influenced under these disease conditions. In this study, the adenylyl cyclase basal activity was elevated in the presence of 3 mM sodium fluoride (NaF) in the control group, but was blunted in the patient group. In the left ventricle, for example, the NaF-stimulated activity was increased by 68% ($P<0.05$) in the control group, and by 43% in the volume overload group. The adenylyl cyclase catalytic function, as estimated by the extent of its stimulation in the presence of 5 mM manganese chloride ($MnCl_2$), was elevated by approximately 94% ($P<0.05$) in the control group. This activity was reduced by about 67% ($P<0.05$) in the left ventricle of the volume overload patients compared to the control population. Generally similar trends were observed in the other three chambers. Furthermore, FSK-stimulated enzyme activity exhibited a concentration-dependent increase in the adenylyl cyclase basal activity in the tested range of 10 nM–1.0 μM, which was remarkably less in the volume overload group than in all four chambers of the controls. At 1.0 μM, for example, this signified an overall slight depression of the FSK-

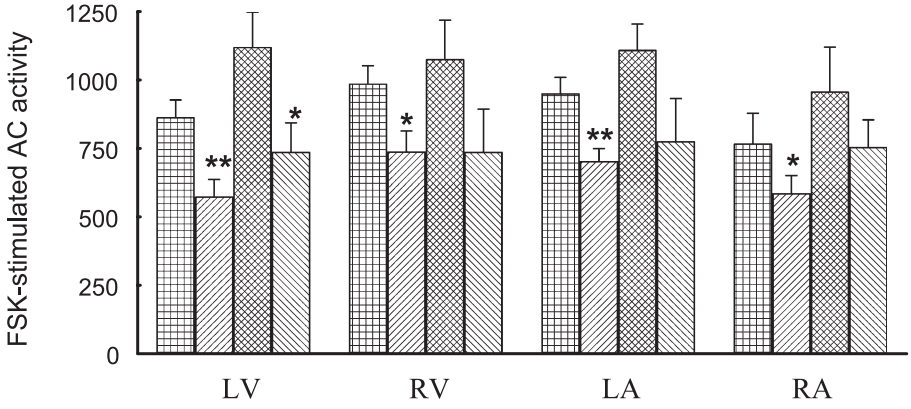

Fig. 6. Stimulation of adenylyl cyclase (AC) basal activity by 1.0 μM FSK in the presence/absence of 100 μM Gpp(NH)p in patients with left ventricular volume overload ($n=6$) compared to normal donor hearts ($n=6$). The enzyme activity is given in picomoles of phosphate per milligram of protein per minute, and values are given as mean ± S.E.M. Horizontal cross-hatch represents FSK-stimulated activity in control; rising right hatch represents FSK-stimulated activity in patients; cross-hatch represents FSK/Gpp(NH)p-stimulated activity in controls; rising left hatch represents FSK/Gpp(NH)p-stimulated activity in patients. LV, left ventricle; RV, right ventricle, LA, left atrium; RA, right atrium. *$P<0.05$, **$P<0.01$ vs. control.

stimulated activity by approximately 25–37% in the volume overload patients ($n=6$) compared to the controls (Fig. 6). While coincubation of the membrane proteins with FSK and guanylimidodiphosphate [Gpp(NH)p] resulted in a further slight increase in adenylyl cyclase activity in all four chambers, the levels of the enzyme activity in the patient population were approximately 41–52% lower than in control hearts (Fig. 6). Thus, put together, these results point to intact G protein-mediated and catalytic functions of the adenylyl cyclase in both groups, and an attenuation of the β_1-adrenoceptor-mediated enzyme activity in the patient population.

3.4. Protein kinase A activity

Alterations in signalling events mediated via second messenger-dependent protein kinases, particularly the serine/threonine PKA and PKC, are associated with heterologous desensitization of the β_1-adrenoceptor pathway. We therefore evaluated the functional expression of PKA, in order to assess the consequences of the above-observed changes downstream of the adenylyl cyclase signaling in our volume overload population, and compared it with that in dilated cardiomyopathy. In the controls ($n=8$), the cAMP-stimulated PKA activity was 12.5 ± 3.7 pmol/mg protein/min in the left ventricle, 10.3 ± 2.9 in the right ventricle, 15.6 ± 5.2 in the left atrium, and 21.3 ± 3.8 in the right atrium (Table 2). This activity was slightly elevated to 18.4 ± 4.4 in the left ventricle, but remained unchanged at 12.5 ± 7.5 in the right ventricle and decreased slightly to 6.2 ± 5.2 in the left atrium and 11.7 ± 6.6 in the right atrium in the volume overload patient group ($n=6$). Interestingly, in the dilated cardiomyopathy group ($n=6$), the PKA activity increased significantly to 60.5 ± 18.4 ($P<0.05$) in the left ventricle and 76.6 ± 17.7 ($P<0.01$) in the right ventricle, but decreased slightly to 10.3 ± 1.8 in the left atrium and 14.7 ± 2.0 in the right atrium. The increase in ventricular PKA activity in dilated cardiomyopathy was significantly higher ($P<0.05$) than that in the volume overload patients, suggesting a greater influence of dilated cardiomyopathy than the latter on the PKA activity.

Table 2
Assessment of myocardial cAMP-stimulated PKA activity in myocardial biopsies of six dilated cardiomyopathic hearts and six left ventricular volume overload patients compared with eight control donor hearts

	Controls	DCM	VOL
Left ventricle	12.5 ± 3.7	60.5 ± 18.4*	18.4 ± 4.4
Right ventricle	10.3 ± 2.9	76.6 ± 17.7**	12.5 ± 7.5
Left atrium	15.6 ± 5.2	10.3 ± 1.8	6.2 ± 5.2
Right atrium	21.3 ± 3.8	14.7 ± 2.0	11.7 ± 6.6

The activity is given as picomoles per milligram of protein per minute, and values are given as mean ± S.E.M.
DCM, dilated cardiomyopathy; VOL, left ventricular volume overload.
* $P<0.05$.
** $P<0.01$ vs. control.

4. Discussion

The primary objective of the present study was to establish the pattern of myocardial GRK expression and its possible implications for the regulation of adrenoceptor signalling in human left ventricular overload disorders. Our results demonstrate that the GRK2, GRK3, and GRK5 are well expressed, while GRK4, GRK6, and GRK7 are scarcely detectable at mRNA level in the human myocardial chambers. In the healthy individuals, the expression of the three GRKs (GRK2, GRK3, and GRK5) was greatest in the left ventricle, with GRK2 being only partially evident in the right atrial chambers. In the volume overload patients, on the other hand, although the expression of both GRK2 and GRK5 was evident in all four chambers, the elevation in GRK2 was manifest globally, while that of GRK5 appeared to be localized primarily in the left ventricle. The fact that GRK2 was globally distributed in the myocardial chambers of both patient groups, yet partly undetectable in the atria of the normal individuals, is particularly noteworthy. Among others, the global nature of its expression in disease seems to imply that changes in the cardiac signalling events in disease may be accountable for by inducing its expression. However, further studies are warranted to verify this notion.

The changes in the GRK mRNA and protein levels were accompanied by a global reduction in myocardial β_1-adrenoceptor expression as well as receptor-mediated adenylyl cyclase activity. To our knowledge, this study is the first tangible evidence demonstrating a direct association between a decrease in the cardiac β_1-adrenoceptor and an increase in GRK expression in human diseases, particularly the left ventricular overload disorders. Thus, while the basal cyclase activity of the enzyme was only slightly reduced, the β_1-adrenoceptor-stimulated, G_s protein-coupled adenylyl cyclase function was significantly blunted, pointing to a compromised downstream signalling of this pathway in these diseases. Since the trends in gene expression were paralleled by similar alterations in the immunodetectable protein levels, it can be inferred that the changes in the activities of the adrenoceptor downstream signalling components are results of a quantitative increase in the proteins triggering these modifications. The reduction in myocardial β_1-adrenoceptor expression is in agreement with our previous findings of a similar attenuation in myocardial β-adrenoceptor numbers and binding activity (Dzimiri et al., 1996), as well as an association between increased lymphocyte GRK2 and reduced β_2-adrenoceptor expression in left ventricular overload disease (Dzimiri et al., 2002). In the present study, it appears that the global nature of the increase in myocardial GRK2 expression correlates more closely with the decrease in myocardial β_1-adrenoceptor and its agonist-stimulated adenylyl cyclase functional expression than the increase in GRK5. This points to a greater role for the GRK2 in the alterations in β_1-adrenoceptor signalling in cardiac muscle disease. Furthermore, transgenic animal and cell culture studies have also shown that over-

expression of GRK2 leads to increased phosphorylation and desensitization of agonist-activated adrenoceptors (Diviani et al., 1996, 1997; Eckhart et al., 2002; Ferguson et al., 1995). Put together, our present findings of elevated cardiac GRK levels in left ventricular disease seem to point to their altered expression as a primary trigger of the deficiency in the β_1-adrenoceptor–G protein–adenylyl cyclase circuit, resulting in the depression of the downstream signalling of this pathway.

In fact, our present finding of an increase in GRK2 is in contrast to a previous report of a decrease in the porcine model of congestive heart failure, in association with the desensitization of the β-adrenoceptor signalling pathway (Choi et al., 1997; Ungerer et al., 1993). Therefore, we were also interested in verifying whether the pattern of their expression in volume overload differs from that in predominantly human heart muscle diseases, such as dilated cardiomyopathy. Our results showed that dilated cardiomyopathy is associated with an even greater increase in GRK expression than in volume overload. Interestingly, unlike the increase in GRK expression in the volume overload patients, which was manifest primarily in the left ventricle, in dilated cardiomyopathy, the elevation was observed in all four chambers. Furthermore, in contrast to the differential elevation in GRK2, the increase in GKR5 was more pronounced on the left side in both diseases, while GRK3 expression appeared to display no significant changes. Thus, although GRK2 and GRK5 profiles were at variance in the volume overload group, they exhibited similar patterns in dilated cardiomyopathy hearts. This demonstrates distinctly different patterns of expression by the three GRKs, possibly pointing to selectivity in the influence of heart disease on GRK expression in the human myocardium. The finding of differential alterations in the GRK expression in these two disorders further suggests functional specificity in different cardiac diseases. In a recent study, we established a de novo expression in GRK5 mRNA in lymphocytes, which may be unique to the volume overload disease (Dzimiri et al., 2002). It appears, therefore, that volume overload influences the functional expression of the myocardial GRK complexes in a peculiar fashion, which is markedly different from heart failure resulting from cardiac muscle disease, such as dilated cardiomyopathy. Other previous studies also showed that, in contrast to heart failure in which predominantly the myocardial β_1-adrenoceptors are desensitized and/or downregulated, in volume overload or heart valvular diseases in general, both β_1- and β_2-adrenoceptors are attenuated to the same extent (Brodde et al., 1989; Dzimiri et al., 1996).

Our functional studies also indicated that the fluoride, FSK-stimulated enzyme activity both in the absence and presence of Gpp(NH)p as well as the Mn^{2+}-dependent activity were depressed in the left ventricle of the volume overload patients, suggesting that an attenuation in the β-adrenoceptor signalling events downstream of cAMP synthesis may be augmented by changes directly affecting the function of the catalytic unit of the enzyme. A reduction in the Mn^{2+}-mediated adenylyl cyclase function has also been reported in some animal study models of ischaemic heart disease (Warner et al., 1992) and pressure overload hypertrophy (Holmer et al., 1996), in conjunction with decreased receptor-mediated enzyme activity. However, other studies have failed to establish a change in Mn^{2+}-activated adenylyl cyclase in failing human ventricular myocardium (Hershberger et al., 1991), as well as experimental animal model of heart failure (Fan et al., 1987) and hypertension (Umemura et al., 1985). Put together, these findings point to differences in the modes by which the adenylyl cyclase catalytic function may be influenced in cardiac disease, which may provide a mechanistic basis for differentiating between the signalling pathways and events underlying the manifestation and course of various cardiac disorders, such as hypertension, ischaemic heart disease, and left ventricular overload dysfunction. Interestingly, in the present study, despite a decrease in adenylyl cyclase-mediated cAMP synthesis, the PKA activity appeared to be elevated in the ventricles of both volume overload and dilated cardiomyopathy groups, possibly suggesting an association with signalling events related to ventricular dysfunction and linking it with the pattern in GRK5 expression in cardiac disease. This increase in PKA activity points to the probability that some other factors, unrelated to cAMP levels, may be involved in its stimulation under these conditions. Hence, the influence of the overload is likely to involve both adenylyl cyclase-stimulated cAMP-dependent and other PKA-dependent pathways. Furthermore, since in the cardiomyocytes the β-adrenoceptor can be phosphorylated by both GRKs and cAMP-dependent PKA, this scenario indicates that β_1-adrenoceptor is potentially subject to elevated phosphorylation by both types of kinases in these patients. Rapacciuolo et al. (2003) recently suggested that GRKs and PKA mediate receptor endocytosis through different pathways, and that the pathway selected for β_1-adrenoceptor internalization is primarily determined by the kinase that phosphorylates the receptor. Hence, the events regulating the adrenoceptor functional expression in various cardiac diseases may depend on the predominating changes influencing the signalling components. In the present study, it seems that changes in β-adrenoceptor downstream signaling in left ventricular overload may occur primarily at receptor–G protein coupling level, possibly as a maladaptive response to the overload in an endeavor to meet the demands of the load on the left ventricle.

Our previous observation of a de novo expression in GRK5 mRNA in lymphocytes in the volume overload disease led us to suggest that this might be an adaptive mechanism directly emanating from modifications in the volume-regulating factors due to the overload. Furthermore, the observation—that, in volume overload, the GRK5 expression in particular was greatest primarily in the left ventricle—seems to indicate the probability of a chamber-specific hemodynamic influence on the functional expression of the receptor kinases. Put together, the present findings provide supportive evidence for the notion that

GRK expression may be disease-specific and possibly dependent on their functional role in normal physiology. Several lines of evidence also suggest strongly that increased hemodynamic load might indeed constitute a major contributing factor to the increase in GRK expression, and therefore the attenuation in β-adrenoceptor signalling in volume overload disease. Since volume overload differs from dilated cardiomyopathy in both localization and determinant variables for the severity of the disease, it can be inferred that the changes in GRK5 are probably dependent primarily on the left ventricular hemodynamic load, while those in GRK2 may be directly related to alterations in the cardiac myocyte structural components, such as in dilated cardiomyopathy, leading to heart failure. In addition, the observations of an elevated PKA activity in the left ventricle in the presence of a decrease in cAMP production as well as dissociation of regulation of the adenylyl cyclase catalytic function from the receptor-mediated enzyme activity may also point to selectivity between event-associated adaptational signaling in left ventricular volume overload from those related to other cardiac disorders, such as ischaemic heart disease. It may also signify an increase in PKA-dependent, adrenoceptor-independent downstream signalling resulting from the left ventricular overload. Further studies should clarify these points more explicitly.

In summary, it appears that, in general, the GRK2, GRK3, and GRK5 are expressed differently in the human myocardial chambers and influenced in different fashions by various cardiac diseases. The differences in expression may signify different physiological roles in cardiac function and underlying regulatory mechanisms for the functional expression of the three GRKs in cardiac disease. Thereby, the elevation in GRK2 appears to be distributed throughout the myocardial chambers, suggesting an equally global influence of dilated cardiomyopathy-related cardiac failure on its functional expression, while that of GRK5 is probably localized mainly in the left ventricle, pointing to an association with the more or less localized effects of left ventricular overload disease. Altogether, our results point to a functional role of changes in GRK expression and adrenoceptor signalling in left ventricular overload disorders.

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