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# $\beta_2$-Adrenergic Signaling in Human Heart: Shift from the Cyclic AMP to the Arachidonic Acid Pathway

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## ABSTRACT

We have recently established that enhancement of intracellular calcium cycling and contraction in response to $\beta_2$-adrenergic receptor ($\beta_2$-AR) stimulation exclusively relies on the activation of the cytosolic phospholipase $A_2$ ($cPLA_2$) and arachidonic acid production, via a pertussis toxin-sensitive G protein (possibly Gi), in embryonic chick cardiomyocytes. We aimed to investigate the relevance of the $\beta_2$-AR/Gi/$cPLA_2$ pathway in the human myocardium. In left ventricular biopsies obtained from explanted hearts, $\beta_2$-AR stimulation exerted either an inhibition of $cPLA_2$ that was insensitive to pertussis toxin (PTX) treatment, or an activation of $cPLA_2$, sensitive to PTX treatment. In right atrial appendages from patients who were undergoing open heart surgery, we demonstrated that $\beta_2$-AR-induced activation of $cPLA_2$ was favored in situations of altered $\beta_1$-AR and/or $\beta_2$-AR/adenylyl cyclase (AC) stimulations. Alterations were characterized by an increase in $EC_{50}$ value of norepinephrine and a decrease in the maximal AC activation in response to zinterol, respectively. Quantitative reverse transcription-polymerase chain reaction analyses highlighted a positive correlation between the expression of AC5 and AC6 mRNAs in human cardiac atria, which suggested that functional alterations in AC responses were unlikely to be related to changes in the AC5/AC6 mRNA ratio. In addition, the shift from the cyclic AMP to the arachidonic acid pathway was not supported at the transcriptional level by opposite regulation of AC and $cPLA_2$ mRNAs expression. This study gives the first evidence of the recruitment of $cPLA_2$ by $\beta_2$-ARs in the human heart and suggests that the Gi/$cPLA_2$ pathway could substitute for a deficient Gs/AC pathway in mediating $\beta_2$-AR responses.

The sympathetic nervous system plays a crucial role in the regulation of cardiac function, and β-adrenergic signaling defects are central features of human heart failure (Brodde and Michel, 1999). The β-adrenergic signaling system seems to be a promising target for therapeutic intervention to treat this increasingly common clinical problem that is only partially mitigated by current therapy.

In healthy human heart, activation of $\beta_1$- and $\beta_2$-ARs by catecholamines induces positive inotropic, chronotropic, and lusitropic responses (for reviews, see Brodde and Michel, 1999; Steinberg, 1999). Both $\beta_1$- and $\beta_2$-ARs stimulate the classic Gs/adenylyl cyclases (AC)/cAMP/protein kinase A (PKA) cascade. Among all identified receptor systems expressed in the human heart, the β-AR/Gs/AC pathway is the most powerful physiological mechanism to accurately augment cardiac contractility (for review, see Brodde et al., 1995a). It is noteworthy that, in healthy human myocardium, despite the predominance of $\beta_1$-ARs (around 70% $\beta_1$-ARs versus 30% $\beta_2$-ARs), the functional responses mediated by $\beta_1$- and $\beta_2$-ARs are not necessarily different (Kaumann and Lemoine, 1987), because of the more effective coupling of $\beta_2$-ARs to AC (Levy et al., 1993; Brodde and Michel, 1999).

In failing or aging human heart, the β-AR/Gs/AC pathway exhibits two marked alterations: 1) a selective decrease in $\beta_1$-ARs number, and 2) an impairment of the coupling of both $\beta_1$-ARs and $\beta_2$-ARs to Gs and AC. Both alterations may

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**ABBREVIATIONS:** β-AR, β-adrenergic receptor; AC, adenylyl cyclase; PKA, protein kinase A; $cPLA_2$, cytosolic phospholipase $A_2$; AA, arachidonic acid; ICM, ischemic cardiomyopathy; DCM, dilated cardiomyopathy; PTX, pertussis toxin; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); nt, nucleotide(s); NE, norepinephrine; CGP 20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol.

account for the reduced physiological responses to β-AR stimulation (for review, see Brodde and Michel, 1999). Interestingly, the number of $\beta_2$-ARs is preserved. In fact, the essential role of $\beta_2$-ARs in pathological hearts, inferred from previous studies (Bristow et al., 1986), has been substantiated by the recent finding that the genetic variability of $\beta_2$-ARs is one determinant of clinical outcome in the setting of myocardial dysfunction. Thus, $\beta_2$-AR polymorphisms that exhibit abnormal receptor-effector coupling or pattern of desensitization modify the prognosis of patients with congestive heart failure (Liggett et al., 1998) and the exercise capacity in patients with heart failure (Wagoner et al., 2000).

The exclusive role of cAMP as the messenger of $\beta_1$-AR-mediated contractile responses is uncontested. Regarding $\beta_2$-AR-mediated effects, most of the experimental evidence to date indicates a dominant role of the Gs/AC pathway. However, some authors have argued that $\beta_2$-ARs may also signal through a cAMP-independent inotropic mechanism, especially in humans (Altschuld et al., 1995). Interestingly, in contrast to $\beta_1$-ARs that only interact with Gs, $\beta_2$-ARs may also couple to Gi (Communal et al., 1999; Kilts et al., 2000).

Our group has recently identified the cytosolic phospholipase $A_2$ ($cPLA_2$) as a new signaling pathway of $\beta_2$-AR in heart. In embryonic chick ventricular cardiomyocytes, $\beta_2$-AR stimulation triggers positive inotropic and lusitropic responses, associated with an enhancement of intracellular calcium cycling, that are independent of cAMP production and exclusively mediated by arachidonic acid (AA) (Pavoine et al., 1999). AA production relies on a p38 and p42/44 mitogen-activated protein kinase-dependent activation of the $cPLA_2$, via a pertussis toxin-sensitive G protein (possibly Gi) (Magne et al., 2001).

Thus, one can speculate that, in human, physiopathological situations associated with a defective β-AR/AC pathway could promote the activation of the $cPLA_2$ pathway by $\beta_2$-AR agonists, as a compensatory mechanism. The aim of the present study was to investigate the effect of $\beta_2$-AR stimulation on $cPLA_2$ activity in human myocardium. We observe a PTX-sensitive activation of $cPLA_2$ by $\beta_2$-AR agonists that prevails in situations in which β-AR stimulation of AC is altered.

## Materials and Methods

**Materials.** Zinterol was supplied by Bristol-Myers Squibb Co. (Stamford, CT). Bromoenol lactone and arachidonic acid were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Nucleotides, (±)-isoproterenol, CGP20712 A, ICI 118551, and bovine serum albumin were obtained from Sigma Chimie (Saint Quentin Fallavier, France). Silicic acid and *n*-heptane were from Merck (Chelles, France). Isopropyl alcohol was purchased from Carlo-Erba (Val de Reuil, France). [L-3-Phosphatidylcholine,1-stearoyl-2-[1-$^{14}$C]arachidonyl (53 mCi/mmol), [α-$^{32}$P]ATP (30 Ci/mmol), and [8-$^{3}$H]cAMP (38–50 Ci/mmol) were from Amersham Biosciences Inc. (Les Ulis, France). Moloney murine leukemia virus reverse transcriptase was from Invitrogen (Cergy Pontoise, France) and oligo(dT)$_{12–18}$ was from Pharmacia Biotech (Saclay, France)

## Materials and Methods

### Patients

With the approval of local ethics committees, left ventricular samples were obtained from explanted hearts from patients with end-stage ischemic or dilated cardiomyopathies (ICM or DCM) and right atrial appendages were obtained from patients undergoing open heart surgery for coronary bypasses or valve replacement. All patients had given written informed consent before surgical procedures were performed. Ventricular and auricular samples were freed of fatty and connective tissues and immediately frozen in liquid nitrogen. Patient characteristics are reported in Tables 2 and 3. Anesthesia was induced and maintained with midazolam, propofol, and sufentanil. Pancuronium was used as neuromuscular blocker. Controlled ventilation was performed with an inspired oxygen fraction of 50%.

### Preparation of Cardiac Fractions

Frozen specimens were ground in liquid nitrogen, using a mortar, and homogenized using an Ultraturrax (T25; Janke-Kunkel, Staufen, Germany) in 160 mM ice-cold Tris-HCl, pH 8.5, supplemented with 4 mM EDTA, 250 mM sucrose, and 4 mM phenylmethylsulfonyl fluoride. The homogenates were filtered on two layers of cheesecloth and centrifuged at 1,000*g* for 10 min at 4°C.

### Adenylyl Cyclase Assay

The 1,000*g* pellet fractions were resuspended in 50 mM HEPES, pH 7.4, and AC activity was immediately measured as described previously (Pavoine et al., 1999). Results were obtained from triplicate determinations.

### Cytosolic Phospholipase $A_2$ Assay

The 1,000*g* supernatant fractions were incubated with 50 μM GTP, with or without $\beta_2$-adrenergic agonist (zinterol alone or in the presence of ICI 118551, a $\beta_2$-adrenergic antagonist, or isoproterenol in the presence of CGP20712A, a $\beta_1$-adrenergic antagonist). After 20 min at 37°C, samples were centrifuged at 20,900*g* for 30 min at 4°C. The 20,900*g* pellet and supernatant fractions were referred to as membrane and cytosol fractions, respectively. The $cPLA_2$ activity was assayed in the 20,900*g* fractions, by measuring the release of [$^{14}$C]AA from the *sn*-2 position of 1-stearoyl-2-[1-$^{14}$C]arachidonyl phosphatidylcholine, as described previously (Magne et al., 2001). Briefly, the reaction was carried out in a final volume of 250 μl, in a buffer containing 40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 50 mM NaF, 200 μM $Na_3VO_4$, 10 mM $Na_4P_2O_7$, 1 mg/ml fatty acid-free bovine serum albumin, inhibitors of $Ca^{2+}$-independent $PLA_2$ (10 μM bromoenol lactone) and secreted $PLA_2$ (2 mM dithiothreitol) isoforms and [$^{14}$C]phosphatidylcholine (2 μM, $10^6$ cpm) substrate vesicles. After 30 min at 37°C, the reaction was stopped by adding 800 μl of the Dole's reagent and 0.1 mg of unlabeled arachidonic acid. The upper phase was then mixed with silicic acid, collected after centrifugation, and counted for radioactivity. Results were obtained from triplicate (Fig. 4) or quadruplicate (Figs. 1 and 2) determinations.

### Treatment of Cardiac Homogenates with Pertussis Toxin (PTX)

Before use, PTX was activated by incubation with 20 mM dithiothreitol for 30 min at 37°C. Cardiac tissues, homogenized as described above, were divided into two equal fractions and incubated either with 10 μg/ml pertussis toxin or with vehicle, in a final volume of 1 ml containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM ATP, 3 mM dithiothreitol, 0.1 mM GTP, and 10 mM NAD, as described previously (Lotersztajn et al., 1987). PTX-treated fractions as well as vehicle-treated fractions were subjected to a 30-min incubation at 30°C, before centrifugation at 1,000*g* for 10 min at 4°C. The 1,000*g* supernatant fraction was used for the assay of $\beta_2$-AR-induced modifications of the $cPLA_2$ activity, as described above. In parallel, the efficiency of PTX treatment was checked in the 1,000*g* pellet fraction by the blockade of Gi-mediated acetylcholine inhibition of isoproterenol effect on AC activity (not shown), as described previously (Pavoine et al.,1999).

### Quantitative RT-PCR: AC5, AC6, cPLA$_2$, and GAPDH mRNA Expression Levels

Total RNA was extracted from right atrial appendages, using acid guanidinium thiocyanate (Chomczynski and Sacchi, 1987) and first-strand cDNAs (RT) were synthesized from 1 to 10 $\mu$g of total RNA using Moloney murine leukemia virus reverse transcriptase (16 U/$\mu$l) and Oligo-(dT)$_{12-18}$ (4 $\mu$M). Quantitative PCR was performed using the Light Cycler technology (Roche Diagnostics, Mannheim, Germany) according to the supplier conditions. Quantification of amplified products was determined using standard curves made from PCR of eight dilutions (from 10 to $10^8$ copies) of the relevant plasmids. AC5 and AC6 plasmids were obtained by TA cloning of PCR products: a 473-bp fragment for the AC5 plasmid (nt 2970 to 3442 of the rat AC5 cDNA) and a 590-bp fragment for the AC6 plasmid (nt 1615 to 2204 of the rat AC6 cDNA). The cPLA$_2$ plasmid was from the IMAGE clone library (1226314) and contained the 640-bp fragment corresponding to nt 2168 to 2808 of the human cPLA$_2$ cDNA.

The sizes of the PCR-amplified products were 305, 453, 195, and 165 bp for AC5, AC6, cPLA$_2$, and GAPDH, respectively (Table 1). The relative value for each amplified product was evaluated using the Light Cycler analysis software. PCR reactions were normalized to GAPDH expression to control variations in RNA extraction and RT reaction.

### Statistics

**Univariate and Bivariate Analyses.** Clinical characteristics of the patients were compared with independent tests (nonparametric Mann-Whitney *U* test) for continuous variables. Nonparametric Spearman tests of correlation were calculated to evaluate the association between AC and cPLA$_2$ regulations. Full dose-response curves were compared by analysis of variance for repeated measures. Results from Fig. 1 were analyzed by unpaired Student's *t* test. Each test of significance was two-tailed. Results were expressed as mean ± S.E.M. (Figs. 1, 2, and 3), and in each analysis, $p < 0.05$ was considered statistically significant.

## Results

**$\beta_2$-AR Induced Redistribution of cPLA$_2$ in Human Left Ventricle.** cPLA$_2$ activation relies on its translocation from the cytosol to membranes, where its phospholipid substrate is located (Magne et al., 2001). In contrast, redistribution of the cPLA$_2$ toward the cytosol is regarded as cPLA$_2$

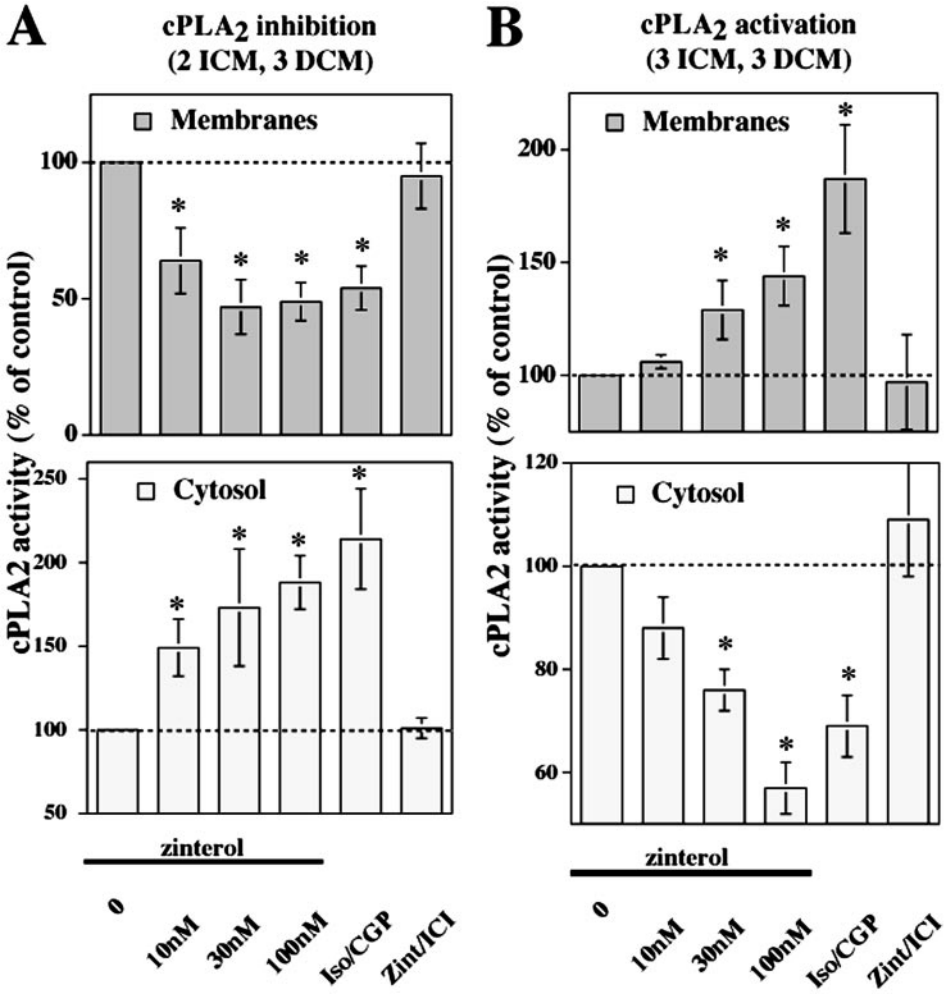


**Fig. 1.** $\beta_2$-AR stimulation elicits a dose-dependent cPLA$_2$ inhibition (A) or activation (B) in human left ventricular tissues: left ventricular tissues were obtained from explanted hearts of patients with DCM or ICM undergoing transplant heart surgery (Table 2). cPLA$_2$ activity was measured in the membrane and cytosol fractions, after incubation with varying concentrations of zinterol or with 1 $\mu$M isoproterenol added with 300 nM CGP 20712A, a $\beta_1$-AR antagonist, or with 1 $\mu$M isoproterenol added with 100 nM ICI 118551, a $\beta_2$-AR antagonist. Results from A and B are expressed in percentage of control (100% corresponded to 4,280 and 6,164 total dpm in supernatants and 4,959 and 5,151 total dpm in pellets in A and B fractions, respectively; and total proteins were 4,262 and 4,232 $\mu$g in supernatants and 780 and 903 $\mu$g in pellets in A and B fractions, respectively). Results are the mean ± S.E.M. of five or six different experiments performed in quadruplicates. *, statistical difference compared with the control.

inhibition. We studied $\beta_2$-AR-induced redistribution of $cPLA_2$ in left ventricular biopsies derived from explanted hearts of patients with DCM ($n = 6$) or with ICM ($n = 5$), undergoing transplant heart surgery (see characteristics of patients in Table 2). In each biopsy, $cPLA_2$ activity in the membranes and the cytosolic fractions was assayed after treatment with either a $\beta_2$-AR agonist or vehicle. Activity measured in vehicle-treated fractions was referred to as 100% of $cPLA_2$ activity. Two opposite patterns of $cPLA_2$ redistribution were detected in response to $\beta_2$-AR stimulation.

In 5 of 11 left ventricular fractions (two ICM and three DCM), zinterol, a specific, partial $\beta_2$-AR agonist (Bristow et al., 1989; Kaumann et al., 1996), evoked a dose-dependent decrease in $cPLA_2$ activity in the membranes that correlated with a dose-dependent increase in $cPLA_2$ activity in the cytosol (Fig. 1A). This illustrated a redistribution of $cPLA_2$ apart from its membranous substrate, toward the cytosol, considered as $cPLA_2$ inhibition (Fig. 1A). Another $\beta_2$-AR stimulus, 1 $\mu$M isoproterenol associated with CGP20712A, a selective $\beta_1$-AR antagonist, reproduced the effect of zinterol, whereas preincubation for 10 min with 100 nM of the selective $\beta_2$-AR antagonist ICI 118551 blocked zinterol effect on $cPLA_2$ activity (Fig. 1A). Conversely, in six other left ventricular fractions (three ICM and three DCM), $\beta_2$-adrenergic stimuli, zinterol, or isoproterenol added with CGP20712A produced a dose-dependent increase in $cPLA_2$ activity in the membranes, associated with a dose-dependent decrease in $cPLA_2$ in the cytosol, illustrating a redistribution of $cPLA_2$ from the cytosol, toward the membranes, that was indicative of $cPLA_2$ activation (Fig. 1B). Zinterol induced-$cPLA_2$ activation was no more observed after preincubation for 10 min with 100 nM ICI 118551 (Fig. 1B). Together, these data highlighted two alternative profiles of action of $\beta_2$-adrenergic agonists on $cPLA_2$ redistribution: redistribution toward the membranes, indicative of $cPLA_2$ activation; or redistribution toward the cytosol, regarded as $cPLA_2$ inhibition.

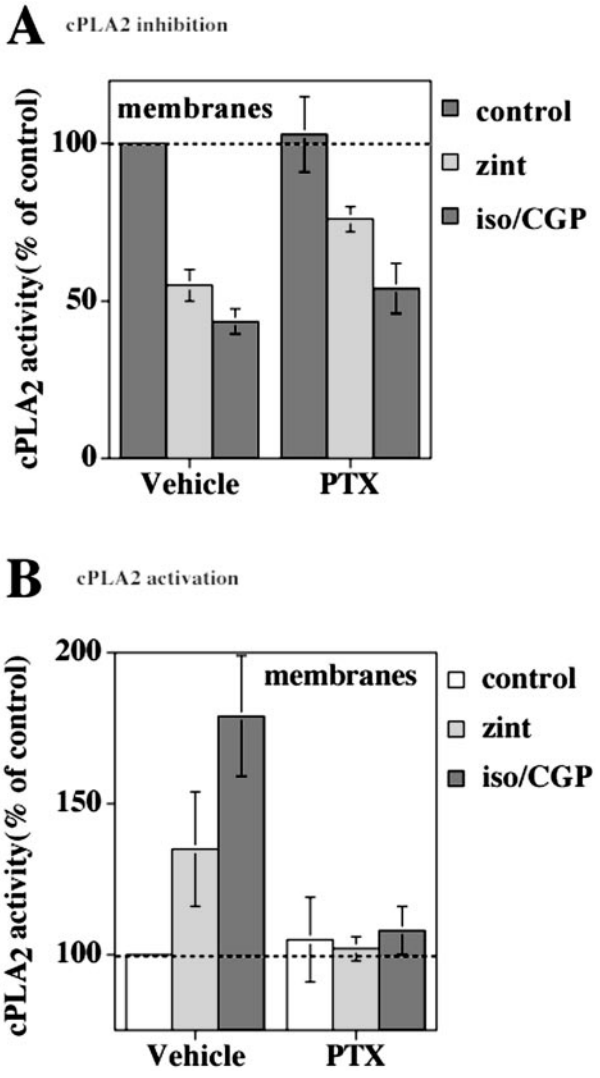


**Fig. 2.** Consequence of a PTX-treatment on $\beta_2$-AR-induced $cPLA_2$ inhibition or activation. Left ventricular tissues were obtained from explanted hearts of patients with ICMs undergoing transplant heart surgery (Table 2). Cardiac homogenates were divided into two equal fractions and were incubated, either in the absence (vehicle) or in the presence (PTX) of 10 $\mu$g/ml PTX for 30 min at 37°C. Cardiac fractions were subsequently incubated with or without 30 nM zinterol or 1 $\mu$M isoproterenol added with 300 nM CGP 20712A, a $\beta_1$-AR antagonist, and $cPLA_2$ activity was measured in the membranes, as described under *Materials and Methods*. Results from A and B are expressed in percentage of control and are the mean ± S.E.M. of quadruplicate determinations.

**Mechanism of $\beta_2$-AR Induced $cPLA_2$ Activation or Inhibition.** Because a PTX-sensitive G protein has been previously involved in $\beta_2$-AR induced $cPLA_2$ activation, in embryonic chick heart cells, we examined consequences of a PTX treatment in two left ventricular biopsies obtained from explanted hearts of patients with ICM undergoing transplant heart surgery (see characteristics in Table 2). $cPLA_2$ activity was measured in membranes from biopsies that displayed typical $cPLA_2$ inhibition or activation profiles, respectively, in response to $\beta_2$-AR stimulation (Fig. 2). It has to be noted that PTX-treated fractions were compared with vehicle-treated fractions submitted to equal preincubation procedure. PTX treatment did not affect $\beta_2$-AR-induced inhibition of $cPLA_2$ (Fig. 2A). In contrast, PTX treatment impaired $\beta_2$-AR-induced activation of $cPLA_2$ (Fig. 2B). This result confirmed that $\beta_2$-AR-induced activation of $cPLA_2$ resulted from $\beta_2$-AR coupling to a Gi/o-like protein. It clearly argued for divergent signaling pathways leading to either $\beta_2$-AR-induced activation or inhibition of $cPLA_2$.

***β*-AR Stimulation of AC Activity in Human Right Atrial Appendages: Relation with $\beta_2$-AR-Induced Redistribution of the $cPLA_2$.** Because the AC pathway is considered the dominant pathway of both $\beta_1$- and $\beta_2$-adrenergic agonists in heart, our next experiments aimed to compare zinterol-induced $cPLA_2$ redistribution with $\beta_1$- and $\beta_2$-AR-induced AC activation. The study was performed on 33 right atrial appendages obtained from patients undergoing open heart surgery for coronary artery bypass or valve replacement (aortic or mitral) (the characteristics of patients are listed in Table 3). Most of the subjects (27 of 33) were without advanced heart failure, and their left ventricular ejection fraction was above 40%.

Because of material limitation, we measured the change in $cPLA_2$ activity in the cytosol fraction only, in response to optimal 30 nM zinterol concentration. As described previously, we assayed $cPLA_2$ activity in the cytosolic fraction after treatment either with zinterol or vehicle. $cPLA_2$ activity in the cytosol from fractions treated with vehicle was referred to as 100% $cPLA_2$ activity. Biopsies that displayed a zinterol-induced decrease in $cPLA_2$ activity in the cytosolic fraction

(<100% control) were assigned to the cPLA$_2$ activation group. Conversely, biopsies in which zinterol induced an increase in cPLA$_2$ activity in the cytosolic fraction (≥100% control) were classified in the cPLA$_2$ inhibition group. Zinterol induced cPLA$_2$ activation in 18 of the 33 biopsies (mean cPLA$_2$ activity detected in the cytosol was 70 ± 5% of control activity) and evoked cPLA$_2$ inhibition in the remaining 15 biopsies (mean cPLA$_2$ activity in the cytosol was 141 ± 10% of control activity).

In the 33 biopsies, AC activity was measured, in parallel, in response to increasing doses of either norepinephrine (NE), a β-AR physiological agonist that is around 20-fold more selective for human $\beta_1$ versus $\beta_2$-AR (Bristow, 2000), or the selective $\beta_2$-AR agonist zinterol. The mean full dose-response curves to NE and zinterol of AC activity measured in the 18 biopsies displaying activation of cPLA$_2$ in response to zinterol proved to be statistically different from those assayed in the 15 biopsies displaying inhibition of cPLA$_2$ in response to zinterol (Fig. 3, A and B). Eadie-Hofstee's plots were derived from these curves, to determine the mean agonist concentrations for half-maximal effects ($EC_{50}$) and the mean maximal AC stimulations ($V_{max}$). As shown in Fig. 3A and inset in Fig. 4A, the cPLA$_2$ activation group displayed an $EC_{50}$ value of NE statistically higher than the cPLA$_2$ inhibition group (8 ± 1.3 μM in the cPLA$_2$ activation group versus 2 ± 0.5 μM in the cPLA$_2$ inhibition group; $p = 0.0006$). The $V_{max}$ in response to NE was similar in both groups (102 ± 10% over basal, in the cPLA$_2$ activation group versus 120 ± 17% over basal in the cPLA$_2$ inhibition group). In contrast, as shown in Fig. 3B and inset in Fig. 4B, the $V_{max}$ in response to zinterol was lower in the cPLA$_2$ activation group than in the cPLA$_2$ inhibition group (44 ± 7% over basal in the cPLA$_2$ activation group versus 84 ± 10% over basal in the cPLA$_2$ inhibition group; $p = 0.0017$), whereas both groups displayed similar $EC_{50}$ values of zinterol (26 ± 6 nM in the cPLA$_2$ activation group versus 23 ± 7 nM in the cPLA$_2$ inhibition group). Individual data from the 33 biopsies were plotted in Fig. 4. Figure 4A evidenced a statistically significant linear correlation (Spearman $r = -0.61$; $p = 0.0002$) between the $EC_{50}$ value of NE for activation of AC- and the zinterol-induced changes in cPLA$_2$ activity detected in the cytosol; high $EC_{50}$ values, which denoted low sensitivity of AC toward NE, were related to zinterol-induced decreases in cPLA$_2$ activities in the cytosolic fraction, compared with 100% control level, indicative of cPLA$_2$ activation (Fig. 4A, inset). Figure 4B further showed a statistical significant linear correlation (Spearman $r = 0.55$; $p = 0.0008$) between maximal activation of AC ($V_{max}$) by zinterol and the change in cPLA$_2$ activity detected in the cytosol in response to 30 nM zinterol: low $V_{max}$ of AC in response to zinterol was associated with zinterol-induced increases in cPLA$_2$ activities in the cytosolic fraction, compared with 100% control level and thus with cPLA$_2$ activation (Fig. 4B, inset). Together, these results argued for a link between cPLA$_2$ activation by zinterol and a defect in the activation of AC by β-AR, characterized by a decrease in $V_{max}$ in response to zinterol and an increase in $EC_{50}$ value of NE. Heterogeneity of β-AR responses in atrial biopsies argued for an early evolution of $\beta_2$-AR coupling from an inhibitory profile toward an activatory profile of cPLA$_2$, related to the degree of alteration of $\beta_1$-AR and/or $\beta_2$-AR coupling to AC, and initiated before symptomatic failure develops.

It has to be noted that analyses of the data with respect to age, gender, diagnoses (left ventricular hypertrophy and hypertension), or medications (β-blocker) did not reveal any statistical significant modification in AC responses to NE and zinterol, apart from a maximal AC stimulation by zinterol that was statistically higher in the nonhypertensive patients compared with the hypertensive patients (77 ± 10%, $n = 18$, compared with 50 ± 8%, $n = 15$, respectively, with $p = 0.04$). However, a slight alteration in AC responses could be associated with left ventricular hypertrophy, and biopsies from patients treated with β-blockers displayed a tendency to improved AC responses (not shown). In terms of age, the lack of statistical difference was not surprising because patients belong to the old stratum of age (65 mean age value) according to published age-related studies (Brodde et al., 1995b; Davies et al., 1996). We did not detect any statistical significant interference between β-AR coupling to AC and treatment of patients with the cyclooxygenase inhibitor aspirin. However, no definitive conclusion could be drawn because of the restricted number of patients in this group (5 of 33).

**Quantitative RT-PCR Analysis of AC5 and AC6 mRNAs: Correlation with cPLA$_2$ mRNA Expression.** We quantified the mRNA expression of the two main cardiac AC isoforms, AC5 and AC6, by quantitative RT-PCR technique using a Light Cycler (Roche Diagnostics), in right atrial appendages obtained from a second cohort of 32 patients undergoing open heart surgery, without advanced heart failure (see characteristics in Table 3). A mean 3-fold higher expression of the AC5 mRNA was observed, compared with AC6 mRNA (Fig. 5). Our data established a significant

TABLE 1

Primers used in quantitative RT-PCR

Probes specific for human AC5 and AC6 were synthesized by RT-PCR using human heart mRNA and oligonucleotides specific for each isoform. The sequence of the human AC5 amplified product has 93% identity with the rat AC5 cDNA sequence, 61.6% identity with the rat AC6 cDNA sequence, and 62.7% with the human AC6 cDNA sequence. The sequence of the human AC6 amplified product has 90.3% identity with the rat AC6 cDNA sequence and 72.7% identity with the rat AC5 cDNA sequence. In all cases the annealing temperature was 60°C and the number of PCR cycles was 40.

| Sequence | GenBank Accession Number | Primer | Oligonucleotide Sequence | Position | Expected Size Product |
|---|---|---|---|---|---|
| | | | | | *bp* |
| AC5 | M96159 | Forward | 5′-CCCTGGTGTTCCTCTCGGCTTTTG-3′ | 2971–2994 | 305 |
| | | Reverse | 5′-CACGTAGATGAGCTCGATGGCCAGC-3′ | 3275–3251 | |
| AC6 | AF250226 | Forward | 5′-GTGACCCTGGCCAACCACATGG-3′ | 2189–2209 | 453 |
| | | Reverse | 5′-CAGAAACCGGCGCACATGGTC-3′ | 2641–2621 | |
| cPLA$_2$ | IM72393 | Forward | 5′-GATGTGATAAAAGAAGCCATGGTTGAAAGC-3′ | 2241–2270 | 195 |
| | | Reverse | 5′-GTTGTCATGGGATTGCAAACTGCCTC-3′ | 2436–2411 | |
| GAPDH | AF261085 | Forward | 5′-GGATTTGGTCGTATTGGGCGC-3′ | 131–150 | 165 |
| | | Reverse | 5′-GTTCTCAGCCTTGACGGTGC-3′ | 295–276 | |

positive correlation between the expression of both mRNA subtypes (Spearman $r = 0.59$; $p < 0.0004$; Fig. 5), which suggested a parallel transcriptional regulation of AC5 and AC6 mRNAs. Thus, the alteration in AC responses to β-AR stimulation described above was unlikely to be related to changes in the AC5/AC6 mRNA ratio.

Quantitative analysis of $cPLA_2$ mRNA expression revealed a mean 7 ± 1 copies of $cPLA_2$ mRNA/10,000 copies of GAPDH mRNA and no correlation with the expression of AC(5 + 6) mRNAs (not shown). These results suggested that $cPLA_2$ recruitment by $\beta_2$-AR stimulation, favored in case of defective β-AR/AC coupling, did not originate from opposite transcriptional regulations of AC versus $cPLA_2$ mRNAs.

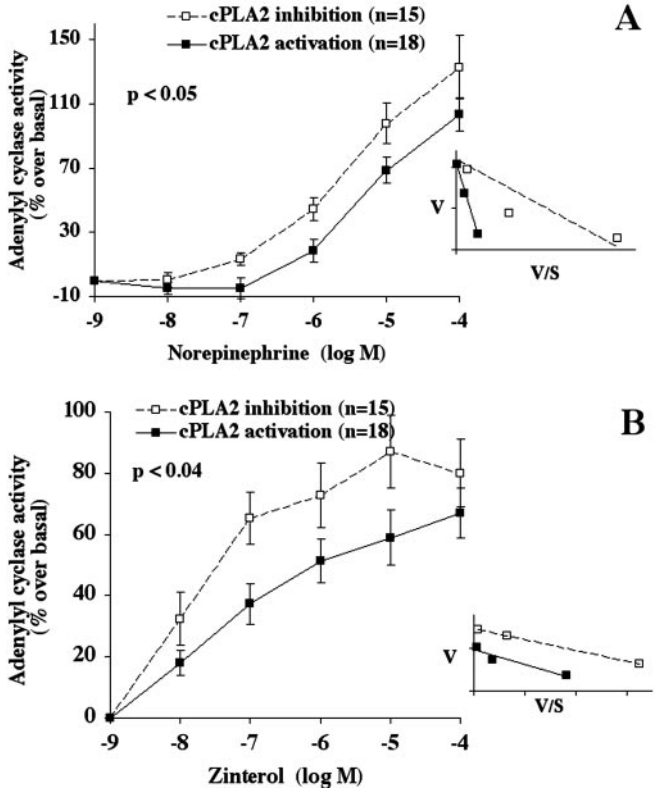


**Fig. 3.** β-AR-induced AC activation in the $cPLA_2$ activation versus the $cPLA_2$ inhibition group of patients. Right atrial appendages were obtained from 33 patients undergoing open heart surgery (Table 3). Classification in the $cPLA_2$ activation or the $cPLA_2$ inhibition groups was performed by dosage of the $cPLA_2$ activity detected in the cytosol in response to 30 nM zinterol, compared with control (referred to as 100%), as described under *Results*. Dose-response-curves of AC activation to either NE (A) or zinterol (B) in each group of biopsies ($cPLA_2$ activation and $cPLA_2$ inhibition) were compared and analyzed according to the Eadie-Hofstee method (insets). AC experiments in each atrial appendage were performed in triplicate and expressed as percentage over basal AC activity. Results are the mean ± S.E.M. of the number of individual experiments summarized under brackets. $p < 0.04$ (A) and $p < 0.05$ (B).

## Discussion

Our study provides the first evidence for the regulation of the $cPLA_2$ pathway by $\beta_2$-ARs in human heart. It demonstrates that the regulation of $cPLA_2$ by $\beta_2$-ARs depends on the status of the β-AR/AC coupling. An alteration of the AC response to β-AR stimulation promotes activation of the $cPLA_2$ pathway. In contrast, efficient β-AR-induced AC activation is linked to $cPLA_2$ inhibition. Consistent with this observation, we previously demonstrated that cAMP, through PKA activation, exerts a negative constraint on the $cPLA_2$ pathway in embryonic chick heart cells (Pavoine et al., 1999). Furthermore, inhibition of the $cPLA_2$ activity upon PKA-mediated phosphorylation has been reported in smooth muscle cells (Murthy and Makhlouf, 1998).

AC stimulation in response to either $\beta_1$-AR or $\beta_2$-AR agonists is altered with aging (White and Leenen, 1994) and in the course of heart failure (Brodde and Michel, 1999). Whereas $\beta_1$-AR subsensitivity of AC is caused by selective $\beta_1$-AR down-regulation, $\beta_2$-AR subsensitivity of AC mainly relies on the partial uncoupling of $\beta_2$-AR to subsequent biochemical events in the $\beta_2$-AR pathway (Bristow et al., 1989). It is noteworthy that our study in right atrial appendages highlights a marked variability of the AC response to β-AR stimulation, despite the fact that the cohort of patients was devoid of advanced heart failure. This is consistent with the observation that β-AR signaling alteration is an early phenomenon that precedes symptomatic heart failure (Schotten et al., 2000). Interestingly, our results suggest that defects in cAMP production can occur without apparent impact on the cardiac function. This questions the exclusive role of cAMP as the contractile messenger of β-AR agonists and the setting up of compensatory mechanism(s). In this context, our results clearly demonstrate a recruitment of $cPLA_2$ upon $\beta_2$-AR stimulation not only in left ventricles from explanted hearts but also in right atrial appendages obtained from patients without advanced heart failure. In human atrial and ventricular tissues, $\beta_2$- as well as $\beta_1$-AR stimuli are known to trigger positive inotropic and lusitropic responses, with an increasing role of $\beta_2$-AR with ageing and failure (Brodde and Michel, 1999). Thus, our results argue for a potential role of $cPLA_2$ in mediating $\beta_2$-AR responses in the atria as well as in the ventricle, even in the absence of symptomatic heart failure. Interestingly, $\beta_2$-AR-induced $cPLA_2$ activation correlates with increased $EC_{50}$ value of NE for AC stimulation, which probably reflects selective "high-affinity" down-regulation of $\beta_1$-AR, as suggested previously (Bristow et al., 1989). The

TABLE 2
Studies in left ventricular tissues

| | Redistribution Study (Fig. 1) | PTX Study (Fig. 2) |
|---|---|---|
| Number of patients | 11 | 2 |
| Mean Age ± S.D. (years) | 56 ± 2 (34–65) | 63–52 |
| Gender | 10 M, 1 F | 2 M |
| LVEF (%) ± S.D. | 20 ± 2 | 20 −20 |
| Indication | 6 DCM/5 ICM | 2 ICM |
| ß-blocker treatment | 4/11 | 1/2 |
| Aspirin treatment | 0/11 | 0/2 |
| Zinterol effect on $cPLA_2$ activity | 6, activation/ 5, inhibition | 1, activation/1, inhibition |

M, male; F, female; DCM dilated cardiomyopathy; ICM, ischemic cardiomyopathy; LVEF, left ventricular ejection fraction.

absence of decrease in $V_{max}$ upon NE stimulation probably relies on the limited selectivity of NE for $\beta_1$-AR, and the binding of high doses of NE to $\beta_2$-AR more efficiently coupled to AC than $\beta_1$-AR. $\beta_2$-AR-induced cPLA$_2$ activation is also correlated with a decrease in the $V_{max}$ value of zinterol AC activation without change in $EC_{50}$ value. Such modifications are consistent with a partial uncoupling of $\beta_2$-AR from the AC pathway, without $\beta_2$-AR down-regulation.

Specific properties distinguish the two subtypes of $\beta$-AR, $\beta_1$ and $\beta_2$. The role of $\beta_1$-ARs in the regulation of cardiac contractility is predominant in the healthy heart. $\beta_1$-ARs only interact with Gs and modulate cardiac contractility exclusively through a cAMP-dependent mechanism. In contrast, $\beta_2$-ARs become major modulators of clinical outcome in the setting of myocardial dysfunction, as attested by studies on $\beta_2$-AR polymorphisms. $\beta_2$-ARs variants that exhibit abnormal receptor-effector coupling or pattern of desensitization, modify the prognosis of patients with heart failure (Liggett et al., 1998; Steinberg, 1999; Wagoner et al., 2000). Noteworthy, in human myocardium, $\beta_2$-ARs can couple to Gs, but also to Gi (Kilts et al., 2000) and the Gs/Gi ratio decreases in the failing or aged hearts. Interestingly, our results suggest that $\beta_2$-ARs activate cPLA$_2$ in human heart through a PTX-sensitive pathway. This raises the possibility that the $\beta_2$-AR/PTX-sensitive/cPLA$_2$ pathway could be a functional substitute for a defective or missing $\beta$-AR/Gs/AC pathway. Functional studies performed on human biopsies are currently under investigation to support this hypothesis. Note-

TABLE 3
Studies in right atrial appendages

| | Biochemical Study (Figs. 3 and 4) | RT-PCR study (Fig. 5) |
|---|---|---|
| Number of patients | 33 | 32 |
| Mean Age ± S.D. (years) | 65 ± 14 (21–89) | 65 ± 10 (37–80) |
| Gender | 16 M/17 F | 22 M/10 F |
| LVEF <40% | 6/33 | 4/32 |
| Indication | 6 MVR/15 AVR/12 CAB | 9 AVR/23 CAB |
| $\beta$-blocker treatment | 15/33 | 20/32 |
| Aspirin treatment | 5/33 | 6/32 |
| Zinterol effect on cPLA$_2$ activity | 18, activation/15, inhibition | N.D. |

M, male; F, female; LVEF, left ventricular ejection fraction; MVR, mitral valve replacement; AVR, aortic valve replacement; CAB, coronary artery bypass; N.D., not determined.

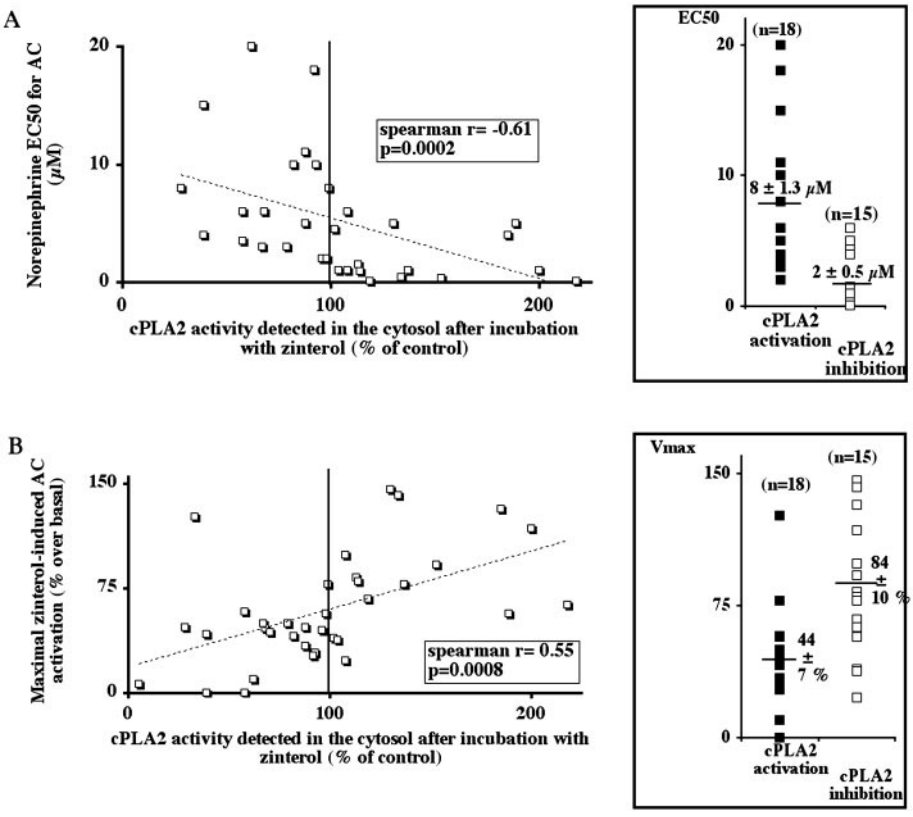


**Fig. 4.** Relationship between $\beta$-AR-induced AC activation and cytosolic cPLA$_2$ activity. Right atrial appendages were obtained from 33 patients undergoing open heart surgery (Table 3). Measurement of cPLA$_2$ activity in response to 30 nM zinterol was performed in the cytosol fraction and compared with the 100% control cPLA$_2$ activity, as described under *Results*. $EC_{50}$ value of NE for AC activation as well as maximal zinterol-induced AC stimulation was determined in the same biopsies. Individual data of $EC_{50}$ value of NE (A) and zinterol-induced maximal AC activation (B) are represented as a function of changes in zinterol-induced cytosolic cPLA$_2$ activity. Spearman $r = -0.61$; $p = 0.0002$ (A) and $r = 0.55$; $p = 0.0008$ (B) and are also represented by group of patients (activation or inhibition) in insets.

worthy, we showed that, in embryonic chick heart cells, the $\beta_2$-AR-positive effects on intracellular calcium cycling and cell contraction did exclusively rely on the production of AA via a pertussis toxin sensitive-$cPLA_2$ pathway (Pavoine et al., 1999). In addition, preliminary results in rats indicate that a $\beta_2$-AR/PTX-sensitive/$cPLA_2$ pathway supports contraction in cardiomyocytes isolated from a model of cardiac failure (our unpublished data).

However, the noxious nature of the signaling triggered by $cPLA_2$ could be questioned in heart because the promotion of $cPLA_2$-initiated pathways might hasten rather than limit the progression toward heart failure. In fact, the model of $cPLA_2$-deficient mice revealed the essential role of $cPLA_2$ in inflammation, asthma, neurodegenerative diseases, and bleomycin-induced pulmonary fibrosis. In addition, $cPLA_2$ has now been identified as an attractive therapeutical approach in the design of new anti-inflammatory drugs (Bonventre et al., 1997; Uozumi et al., 1997; Nagase et al., 2000, 2002).

Our study provides the first quantitative RT-PCR analysis of the expression of AC5, AC6, and $cPLA_2$ mRNAs in human heart. We show that, in human heart, the alteration in the AC response to $\beta$-AR stimulation is unrelated to changes in the AC5/AC6 mRNA ratio. The parallel regulation of AC5 and AC6 mRNA levels in human myocardium establishes a clear disparity with studies performed in rat and dog hearts where opposite and/or dissociated evolution of steady-state AC5 and AC6 mRNA levels have been reported previously (Ishikawa et al., 1994; Espinasse et al., 1999). Quantification of the $cPLA_2$ mRNA in human cardiac tissue demonstrates a regulation of expression unrelated to the expression of mRNA isoforms of AC.

In conclusion, this study highlights the recruitment of $cPLA_2$ by $\beta_2$-AR agonists in the human myocardium, under conditions of altered $\beta$-AR-induced AC signaling. An important unanswered question concerns the protective or the deleterious nature of the signaling triggered by $cPLA_2$, with regards to the potential development of cardiac dysfunctions.

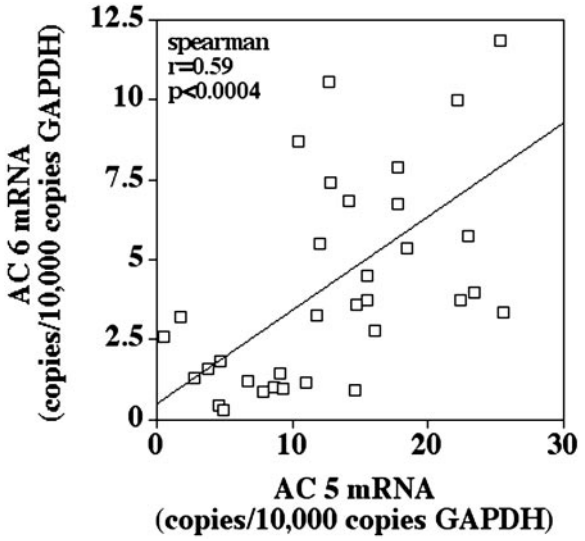


**Fig. 5.** Quantitative RT-PCR analyses of mRNAs coding for AC5 and AC6. Right atrial appendages were obtained from a second cohort of 32 patients undergoing open heart surgery (Table 3). Quantitative RT-PCR was performed as described under *Materials and Methods*. Correlation between AC6 and AC5 mRNAs (Spearman $r = 0.59$; $p < 0.0004$).

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**References**

Altschuld RA, Starling RC, Hamlin RL, Billman GE, Hensley J, Castillo L, Fertel RH, Hohl CM, Robitaille PM, Jones LR, et al. (1995) Response of failing canine and human heart cells to beta 2-adrenergic stimulation. *Circulation* **92:**1612–1618.

Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, and Sapirstein A (1997) Reduced fertility and postischemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature (Lond)* **390:**622–625.

Bristow MR (2000) What type of beta-blocker should be used to treat chronic heart failure (Editorial)? *Circulation* **102:**484–486.

Bristow MR, Ginsburg R, Umans V, Fowler M, Minobe W, Rasmussen R, Zera P, Menlove R, Shah P, Jamieson S, et al. (1986) Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. *Circ Res* **59:**297–309.

Bristow MR, Hershberger RE, Port JD, Minobe W, and Rasmussen R (1989) $\beta$1- and $\beta$2-adrenergic receptor-mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium. *Mol Pharmacol* **35:**295–303.

Brodde OE and Michel MC (1999) Adrenergic and muscarinic receptors in the human heart. *Pharmacol Rev* **51:**651–690.

Brodde OE, Michel MC, and Zerkowski HR (1995a) Signal transduction mechanisms controlling cardiac contractility and their alterations in chronic heart failure. *Cardiovasc Res* **30:**570–584.

Brodde OE, Zerkowski HR, Schranz D, Broede-Sitz A, Michel-Reher M, Schafer-Beisenbusch E, Piotrowski JA, and Oelert H (1995b) Age-dependent changes in the beta-adrenoceptor-G-protein(s)-adenylyl cyclase system in human right atrium. *J Cardiovasc Pharmacol* **26:**20–26.

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

Communal C, Singh K, Sawyer DB, and Colucci WS (1999) Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein. *Circulation* **100:**2210–2212.

Davies CH, Ferrara N, and Harding SE (1996) Beta-adrenoceptor function changes with age of subject in myocytes from non-failing human ventricle. *Cardiovasc Res* **31:**152–156.

Espinasse I, Iourgenko V, Richer C, Heimburger M, Defer N, Bourin MC, Samson F, Pussard E, Giudicelli JF, Michel JB, et al. (1999) Decreased type VI adenylyl cyclase mRNA concentration and $Mg^{2+}$-dependent adenylyl cyclase activities and unchanged type V adenylyl cyclase mRNA concentration and $Mn^{2+}$-dependent adenylyl cyclase activities in the left ventricle of rats with myocardial infarction and longstanding heart failure. *Cardiovasc Res* **42:**87–98.

Ishikawa Y, Sorota S, Kiuchi K, Shannon RP, Komamura K, Katsushika S, Vatner DE, Vatner SF, and Homcy CJ (1994) Downregulation of adenylylcyclase types V and VI mRNA levels in pacing-induced heart failure in dogs. *J Clin Investig* **93:**2224–2229.

Kaumann AJ and Lemoine H (1987) Beta 2-adrenoceptor-mediated positive inotropic effect of adrenaline in human ventricular myocardium. Quantitative discrepancies with binding and adenylate cyclase stimulation. *Naunyn-Schmiedeberg's Arch Pharmacol* **335:**403–411.

Kaumann AJ, Sanders L, Lynham JA, Bartel S, Kuschel M, Karczewski P, and Krause EG (1996) Beta 2-adrenoceptor activation by zinterol causes protein phosphorylation, contractile effects and relaxant effects through a cAMP pathway in human atrium. *Mol Cell Biochem* **163–164:**113–123.

Kilts JD, Gerhardt MA, Richardson MD, Sreeram G, Mackensen GB, Grocott HP, White WD, Davis RD, Newman MF, Reves JG, et al. (2000) $Beta_2$-adrenergic and several other G protein-coupled receptors in human atrial membranes activate both $G_s$ and $G_i$. *Circ Res* **87:**705–709.

Levy FO, Zhu X, Kaumann AJ, and Birnbaumer L (1993) Efficacy of beta 1-adrenergic receptors is lower than that of beta 2-adrenergic receptors. *Proc Natl Acad Sci USA* **90:**10798–10802.

Liggett SB, Wagoner LE, Craft LL, Hornung RW, Hoit BD, McIntosh TC, and Walsh RA (1998) The Ile164 beta2-adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. *J Clin Investig* **102:**1534–1539.

Lotersztajn S, Pavoine C, Mallat A, Stengel D, Insel PA, and Pecker F (1987) Cholera toxin blocks glucagon-mediated inhibition of the liver plasma membrane ($Ca^{2+}$-$Mg^{2+}$)-ATPase. *J Biol Chem* **262:**3114–3117.

Magne S, Couchie D, Pecker F, and Pavoine C (2001) Beta 2-adrenergic receptor agonists increase intracellular free $Ca^{2+}$ concentration cycling in ventricular cardiomyocytes through p38 and p42/44 MAPK-mediated cytosolic phospholipase A2 activation. *J Biol Chem* **276:**39539–39548.

Murthy KS and Makhlouf GM (1998) Differential regulation of phospholipase A2 (PLA2)-dependent $Ca^{2+}$ signaling in smooth muscle by cAMP- and cGMP-dependent protein kinases. Inhibitory phosphorylation of PLA2 by cyclic nucleotide-dependent protein kinases. *J Biol Chem* **273:**34519–34526.

Nagase T, Uozumi N, Ishii S, Kita Y, Yamamoto H, Ohga E, Ouchi Y, and Shimizu T (2002) A pivotal role of cytosolic phospholipase $A_2$ in bleomycin-induced pulmonary fibrosis. *Nat Med* **8:**480–484.

Nagase T, Uozumi N, Ishii S, Kume K, Izumi T, Ouchi Y, and Shimizu T (2000) Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A2. *Nat Immunol* **1:**42–46.

Pavoine C, Magne S, Sauvadet A, and Pecker F (1999) Evidence for a beta2-adrenergic/arachidonic acid pathway in ventricular cardiomyocytes. Regulation by the $\beta$1-adrenergic/camp pathway. *J Biol Chem* **274:**628–637.

Schotten U, Filzmaier K, Borghardt B, Kulka S, Schoendube F, Schumacher C and Hanrath P (2000) Changes of beta-adrenergic signaling in compensated human cardiac hypertrophy depend on the underlying disease. *Am J Physiol Heart Circ Physiol* **278:**H2076–H2083.

Steinberg SF (1999) The molecular basis for distinct beta-adrenergic receptor subtype actions in cardiomyocytes. *Circ Res* **85:**1101–1111.

Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, Komagata Y, Maki K, Ikuta K, Ouchi Y, et al. (1997) Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature (Lond)* **390:**618–622.

Wagoner LE, Craft LL, Singh B, Suresh DP, Zengel PW, McGuire N, Abraham WT, Chenier TC, Dorn GW 2nd, and Liggett SB (2000) Polymorphisms of the beta(2)-adrenergic receptor determine exercise capacity in patients with heart failure. *Circ Res* **86:**834–840.

White M and Leenen FH (1994) Aging and cardiovascular responsiveness to beta-agonist in humans: role of changes in beta-receptor responses versus baroreflex activity. *Clin Pharmacol Ther* **56:**543–553.

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