Decreased Expression and Activity of cAMP Phosphodiesterases in Cardiac Hypertrophy and Its Impact on β-Adrenergic cAMP Signals

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Rationale: Multiple cyclic nucleotide phosphodiesterases (PDEs) degrade cAMP in cardiomyocytes but the role of PDEs in controlling cAMP signaling during pathological cardiac hypertrophy is poorly defined.

Objective: Evaluate the β-adrenergic regulation of cardiac contractility and characterize the changes in cardiomyocyte cAMP signals and cAMP-PDE expression and activity following cardiac hypertrophy.

Methods and Results: Cardiac hypertrophy was induced in rats by thoracic aortic banding over a time period of 5 weeks and was confirmed by anatomic measurements and echocardiography. Ex vivo myocardial function was evaluated in Langendorff-perfused hearts. Engineered cyclic nucleotide-gated (CNG) channels were expressed in single cardiomyocytes to monitor subsarcolemmal cAMP using whole-cell patch-clamp recordings of the associated CNG current (I_{CNG}). PDE variant activity and protein level were determined in purified cardiomyocytes. Aortic stenosis rats exhibited a 67% increase in heart weight compared to sham-operated animals. The inotropic response to maximal β-adrenergic stimulation was reduced by ≈54% in isolated hypertrophied hearts, along with a ≈32% decrease in subsarcolemmal cAMP levels in hypertrophied myocytes. Total cAMP hydrolytic activity as well as PDE3 and PDE4 activities were reduced in hypertrophied myocytes, because of a reduction of PDE3A, PDE4A, and PDE4B, whereas PDE4D was unchanged. Regulation of β-adrenergic cAMP signals by PDEs was blunted in hypertrophied myocytes, as demonstrated by the diminished effects of IBMX (100 μmol/L) and of both the PDE3 inhibitor cilostamide (1 μmol/L) and the PDE4 inhibitor Ro 201724 (10 μmol/L).

Conclusions: β-Adrenergic desensitization is accompanied by a reduction in cAMP-PDE and an altered modulation of β-adrenergic cAMP signals in cardiac hypertrophy. (*Circ Res*. 2009;105:784-792.)

Key Words: 3′-5′ cyclic nucleotide phosphodiesterase ■ cardiac hypertrophy ■ cAMP ■ β-adrenergic receptors

Stimulation of cardiomyocyte β-adrenergic receptors (β-ARs) by noradrenaline released from the sympathetic nervous system is the most powerful mechanism to increase cardiac output in response to stress or exercise. β-ARs signal primarily through $G_{\alpha s}$ proteins, resulting in the activation of adenylyl cyclases and the elevation in the intracellular concentration of the second messenger cAMP. The positive chronotropic, inotropic, and lusitropic effects of cAMP on cardiomyocytes are primarily mediated by the cAMP-dependent protein kinase (PKA), which phosphorylates and regulates many of the key proteins involved in cardiac excitation-contraction coupling.[1]

β-AR signaling is rapidly terminated through several mechanisms that limit cAMP production, such as reuptake and/or metabolism of noradrenaline, uncoupling and desensitization of β-ARs, and inactivation of $G_{\alpha s}$ signaling on GTP hydrolysis. Intracellular cAMP is degraded by cyclic nucleotide phosphodiesterases (PDEs), thus inactivating PKA. Finally, protein phosphatases reverse the PKA-mediated effects on myocyte contraction by dephosphorylating the PKA downstream targets. PDEs comprise a large group of isoenzymes that are divided into 11 PDE families based on their substrate and inhibitor specificity and sequence homology.[2,3] Of these, PDE3 and PDE4 contribute the majority of the cAMP-hydrolytic activity in cardiomyocytes.[4,5] PDE3 is encoded by 2 genes (PDE3A and PDE3B), with PDE3A being the predominant form expressed in cardiomyocytes.[6] The PDE4 family consists of four genes (PDE4A to D), but only PDE4A, PDE4B, and PDE4D appear to be expressed in rat heart.[7] These multiple PDEs contribute to the generation of

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intracellular cAMP microdomains within cardiomyocytes that are thought to be critical for the specificity of cAMP signaling.[3,5]

Whereas acute stimulation of cardiac output by the sympathetic nervous system is essential for the adaptation of the organism to its environment, chronic activation of the sympathetic nervous system promotes a pathological remodelling of the heart which may ultimately lead to heart failure (HF).[8,9] It is well established that chronically elevated catecholamine levels, which are a hallmark of HF, lead to the desensitization of cardiac β-AR signaling through several mechanisms that limit cAMP production. These include the downregulation of β_1-ARs; the uncoupling of β_2-ARs from $G_{\alpha s}$; an increased activity of β-AR kinases; and an increase in $G_{\alpha i}$ subunits, which promotes the signaling of β-ARs through $G_{\alpha i}$ to inhibit adenylyl cyclase. In canine models of HF, $G_{\alpha s}$ and adenylyl cyclases type V and VI are also decreased.[10]

In comparison, the signaling mechanisms acting downstream of cAMP synthesis have received much less attention. In particular, only a few studies have investigated the potentially critical role of PDEs in controlling cAMP signaling during pathological cardiac hypertrophy (CH). These studies focused on PDE3, reporting a decreased PDE3 expression and/or activity in the failing heart.[11] Such a decrease in PDE3 is thought to have adverse consequences on the heart because it promotes cardiomyocyte apoptosis[12] and exaggerates cardiac dysfunction induced by chronic pressure overload.[13] Although largely ignored until recently, a role of PDE4 in HF is supported by the late onset cardiomyopathy developed in mice with deletion of the PDE4D gene.[14]

Intrigued by these findings, we determined the expression pattern of PDE3 and PDE4 enzymes and investigated the role of these PDEs in the control of β-AR cAMP signals in a rat model of compensated CH. Using cyclic nucleotide-gated (CNG) channels, we provide the first live cell recordings of cAMP in hypertrophied cardiomyocytes and demonstrate that PDE3 and PDE4 regulation of β-AR cAMP signals is blunted in hypertrophy. We report a decreased protein expression of PDE3A, PDE4A, and PDE4B but not of PDE4D in hypertrophied cardiomyocytes compared to sham controls that is paralleled by similar changes in cAMP-PDE activities for these PDE subtypes. These results document profound defects in the 2 main PDEs degrading cAMP in hypertrophied myocytes.

Non-standard Abbreviations and Acronyms

β-AR	β-adrenergic receptor
CH	cardiac hypertrophy
CNG	cyclic nucleotide gated
CRC	concentration–response curve
E_{max}	maximal effect
HF	heart failure
IBMX	isobutylmethylxanthine
I_{CNG}	CNG current
ISO	isoprenaline
LV	left ventricular
LV $+dP/dt_{max}$	maximal positive first derivative of left ventricular pressure
LV $-dP/dt_{max}$	maximal negative first derivative of left ventricular pressure
PDE	phosphodiesterase
PKA	cAMP-dependent protein kinase

Methods

All experiments performed conformed to the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J no. L358, 18 December 1986), the local ethics committee (CREEA Ile-de-France Sud) guidelines, and the French decree no. 87-848 (J Off République Française, 20 October 1987: pp 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture, de la Pêche et de l'Alimentation (no. 92-283, June 27, 2007). Detailed methods are included in the Online Data Supplement at http://circres.ahajournlas.org.

Briefly, CH was induced in rats by thoracic aortic banding over a time period of 5 weeks and was confirmed by anatomic measurements and echocardiography. Ex vivo myocardial function was evaluated in Langendorff perfused hearts. Engineered CNG channels were expressed in single isolated cardiomyocytes to monitor subsarcolemmal cAMP using whole-cell patch-clamp recordings of the associated CNG current (I_{CNG}). PDE variant activity and protein level were determined in purified cardiomyocytes.

Results

Induction and Characterization of Cardiac Hypertrophy in Rats

To generate a model of CH, male Wistar rats were subjected to aortic constriction over a time period of 5 weeks as described in Methods. The development of CH was then assessed through anatomic data and echocardiography (see Table 1). Heart weight of the CH rats was significantly higher (+67%) than that of the sham-operated rats, whereas body weight and tibia length were similar in both groups. The weight of lung, liver, kidney, and right ventricle were also unchanged (data not shown). Telediastolic interventricular septum, telediastolic posterior wall thickness, and left ventricular (LV) end diastolic diameter, as determined by echocardiography were significantly larger in CH rats compared to sham (Table 1). However, the fractional shortening was similar between both groups, indicating a preserved LV function in CH rats. From these data, we can classify this model as compensated CH.[15] Measurement of cell capacitance, perimeter, and surface confirmed that LV myocytes from CH rats exhibited substantial hypertrophy when compared to sham-operated animals (Table 1).

Isolated Heart Function in Sham-Operated and CH Rats

To further characterize our model, myocardial function was evaluated using an isolated Langendorff heart preparation. As shown in Table 2, spontaneous heart rate was similar between sham-operated and CH hearts and was increased to the same extent by a saturating concentration of the β-AR agonist isoprenaline (ISO, 1 μmol/L). Basal LV developed pressure and maximal rate of contraction, measured as the maximal value of LV $+dP/dt_{max}$ (maximal

Table 1. Cardiac Phenotype of Sham-Operated and Hypertrophic Rats

	Sham		CH		
	Parameter Value	n	Parameter Value	n	*P*
Anatomy					
BW (g)	318±6	32	316±7	33	NS
TL (cm)	3.6±0.03	32	3.6±0.04	33	NS
HW (g)	1.29±0.04	32	2.15±0.12	33	<0.001
HW/BW (mg/g)	4.1±0.1	32	6.8±0.3	33	<0.001
HW/TL (mg/cm)	363±13	32	605±37	33	<0.001
Echocardiography					
TD IVS (cm)	0.083±0.002	6	0.140±0.005	6	<0.001
TD PW (cm)	0.090±0.004	6	0.155±0.007	6	<0.001
LVEDD (cm)	0.663±0.016	6	0.54±0.02	6	<0.001
FS (%)	56.2±2.2	6	55.8±2.4	6	NS
Cell parameters					
Cell capacitance (pF)	173±5	84	214±8	64	<0.001
Perimeter (μm)	255±4	63	295±4	60	<0.01
Surface (μm^2)	2368±62	63	3140±84	60	<0.001

All data are expressed as means±SEM. BW indicates body weight; FS, fractional shortening; HW, heart weight; LVEDD, left ventricular end diastolic diameter; n, no. of animals in each group for anatomic and echocardiographic data or no. of cells in each group for cell parameters; TD IVS, telediastolic interventricular septum; TD PW, telediastolic posterior wall; TL, tibia length. Statistically significant differences between CH and sham-operated rats are indicated with a *P* value.

Table 2. Basal and ISO-Stimulated Function of Hearts Isolated From Sham-Operated and CH Rats

	Sham		CH		
	Parameter Value	n	Parameter Value	n	*P*
HR (bpm)					
Baseline	248±7	5	231±17	7	NS
ISO (1 μmol/L)	356±12*	5	344±17*	7	NS
LVDP (mm Hg)					
Baseline	73.5±4.2	5	77.1±7.2	7	NS
ISO (1 μmol/L)	152.0±9.0*	5	87.6±9.3	7	<0.001
LV $+dP/dt_{max}$ (mm Hg · sec^{-1})					
Baseline	1958.6±164.9	5	1846.2±177.3	7	NS
ISO (1 μmol/L)	8428.3±613.5*	5	3757.2±464.8†	7	<0.001
LV $-dP/dt_{max}$ (mm Hg · sec^{-1})					
Baseline	1283.2±72.1	5	1393.6±140.5	7	NS
ISO (1 μmol/L)	5662.6±395.8*	5	2564.6±253.8†	7	<0.001

The hearts were perfused with oxygenated Krebs–Henseleit solution containing 1.25 mmol/L $[Ca^{2+}]_o$ as described in Methods. HR indicates heart rate; LVDP, left ventricular developed pressure. Values are means±SEM. Statistically significant differences within one group are indicated as *$P<0.001$; †$P<0.01$. Statistically significant differences between CH and sham-operated rats are indicated with a *P* value.

positive first derivative of LV pressure), were not different between the 2 groups, nor was the maximal rate of cardiac relaxation, measured as the maximal value of LV $-dP/dt_{max}$ (maximal negative first derivative of LV pressure) (Table 2). ISO (1 μmol/L) increased these 3 parameters by 2- to 4-fold in normal hearts. In contrast, the response to maximal β-AR stimulation was reduced in hypertrophied hearts (Table 2). To further document this difference, concentration–response curves (CRC) to ISO were generated for both groups. Figure 1A shows the CRC obtained for LV $+dP/dt_{max}$ by fitting the experimental data with the Hill function. The 2 resulting curves were statistically different (Fischer test, $P<0.001$), with a decreased maximal effect (E_{max}) of ISO in CH hearts (E_{max} was 8205.0±182.7 mm Hg · sec^{-1} in sham and 3758.7±24.4 mm Hg · sec^{-1} in CH) but identical apparent potency (EC_{50} was 3.4±0.3 nmol/L in sham versus 3.3±0.2 nmol/L in CH). Similar results were obtained when the hearts were electrically paced (at 400 bpm). Thus, in this model of compensated CH, basal cardiac function is preserved but the inotropic reserve in response to β-AR stimulation is reduced.

Subsarcolemmal β-Adrenergic cAMP Signals in Sham-Operated and CH Rat Cardiomyocytes

It is well established that β-adrenergic control of cardiac contractility is associated with changes in the particulate, but not the soluble pool of cellular cAMP.[16,17] To determine whether the attenuated inotropic response to ISO in CH was related to a decrease in plasma membrane cAMP, engineered CNG channels were overexpressed in sham and CH cardiomyocytes and the associated I_{CNG} was recorded as an index of subsarcolemmal cAMP.[18] Figure 1B summarizes the results of initial experiments in which the effect of ISO at 100 nmol/L was tested on sham and CH cardiomyocytes expressing either the low-affinity E583M CNGA2 channel ($K_{1/2\ cAMP}$=10.3 μmol/L) or the high-affinity C460W/E583M CNGA2 channel ($K_{1/2\ cAMP}$=1.4 μmol/L). To correct for cell size, I_{CNG} density was calculated by dividing the current amplitude by the cell capacitance for each experiment. As shown in Figure 1B, E583M CNGA2 detected ISO-induced cAMP signals in cardiomyocytes from sham-operated rats (I_{CNG} density was increased to 4.2±1.5 pA/pF in the presence of 100 nmol/L ISO, n=19) but not in cardiomyocytes from CH rats (I_{CNG} density was 0.7±0.2 pA/pF, n=12). In contrast, using the high-affinity C460W/E583M CNGA2 allowed unambiguous detection of β-AR-dependent cAMP signals in both groups. On average, ISO increased I_{CNG} density to 15.1±1.9 pA/pF (n=28) in sham cardiomyocytes and to 8.3±1.8 pA/pF (n=24) in CH myocytes. These results demonstrate a decrease in the subsarcolemmal concentration of cAMP on β-AR stimulation in hypertrophy. To fully characterize the β-AR response, CRCs to ISO were generated in both groups using C460W/E583M CNGA2 (Figure 1C). Consistent with the measurements of contractility of whole hearts, ISO dose-dependently increased cAMP in sham and CH cardiomyocytes, but maximal stimulation was attenuated in the CH myocytes. Fit of the data to the Hill equation yielded 2 statistically different curves (Fischer test, $P<0.001$)

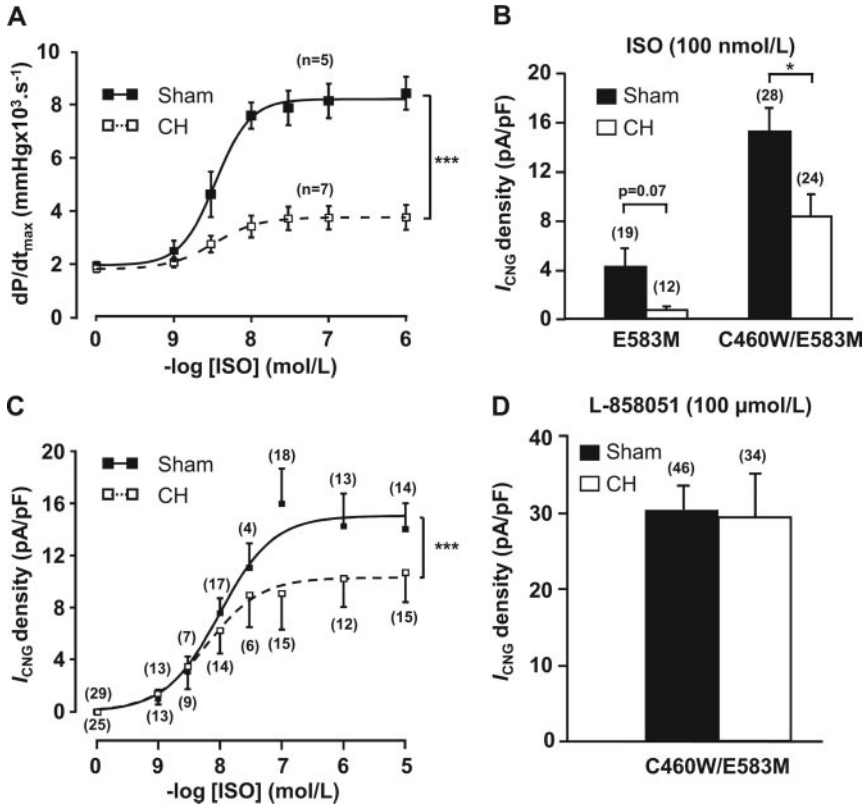

Figure 1. β-AR responses in whole hearts and isolated ventricular myocytes from sham and CH rats. A, CRC to ISO on LV $+dP/dt_{max}$ used as an index of cardiac contractility in 5 normal hearts and 7 hypertrophied hearts. B, Comparison of the effect of ISO (100 nmol/L) on I_{CNG} density in sham (black bars) and hypertrophied (CH) (white bars) cardiomyocytes using the low- and high-affinity CNG channels mutants: E583M CNGA2 and C460W/E583M CNGA2, respectively. I_{CNG} was measured every 8 seconds at −50 mV from a holding potential of 0 mV. The number of cells tested from 2 normal hearts and 3 hypertrophied hearts for E583M CNGA2 and from 18 normal hearts and 19 hypertrophied hearts for E583M/C460W CNGA2 is indicated in brackets above the bars. Statistically significant differences in I_{CNG} density between sham-operated and CH cardiomyocytes are indicated as $^*P<0.05$. C, CRC to ISO on I_{CNG} density in cardiomyocytes from 16 hypertrophied hearts (CH) (dash line) and 13 normal hearts (Sham) (continuous line) using C460W/E583M CNGA2. Cells were first superfused with control external Ringer solution and then exposed to increasing concentrations of ISO. Data represent the means±SEM of the number of cardiomyocytes indicated near each data point. D, Effect of the forskolin analog L-858051 (100 μmol/L) on I_{CNG} density in sham (black bars) and hypertrophied (CH) (white bars) cardiomyocytes using C460W/E583M CNGA2. Data represent the means±SEM of the number of cells (from 23 normal hearts and 21 hypertrophied hearts) indicated near the bars.

with a decreased maximal effect in hypertrophy (E_{max} was 15.1±0.7 pA/pF in sham versus 10.3±0.2 pA/pF in CH cardiomyocytes) but similar apparent potency (EC_{50} was 9.9±2.3 nmol/L in sham versus 6.2±0.7 nmol/L in CH cardiomyocytes). CNGA2 channel expression was the same in sham and CH myocytes as indicated by immunoblot analysis (Online Figure I). In contrast, the response to the forskolin analogue L-858051 (100 μmol/L) was identical in sham and CH cardiomyocytes (30.3±3.0 pA/pF in sham versus 29.4±5.7 pA/pF in CH cardiomyocytes; Figure 1D). Because, at this concentration, L-858051 was shown to saturate the probe,[19] this result only indicates a similar density of functional CNG channels in sham and CH cardiomyocytes. Thus, the ≈32% decrease in maximal ISO response in CH cells reflects a real decrease in the concentration of cAMP at the membrane.

Modulation of β-Adrenergic cAMP Signals by PDEs in Sham and CH Cardiomyocytes

As a first experiment to elucidate the role of PDEs on cAMP signals in sham-operated and CH rat hearts, the effect of the general PDE inhibitor IBMX was tested in cardiomyocytes using the low-affinity E583M CNGA2 channel. We showed previously that 100 μmol/L IBMX had no effect on basal I_{CNG} in cardiomyocytes expressing the E583M CNGA2 channel.[18] As shown in Figure 2, the effects of IBMX in combination with 100 nmol/L ISO are compared. Although IBMX increased the response of I_{CNG} to ISO in both sham (Figure 2A) and CH myocytes (Figure 2B), the response remained on average ≈50% smaller in CH cells (Figure 2C). Because most of the cAMP hydrolytic activity is inhibited at the concentration of IBMX used,[20] this 50% difference between sham and CH cardiomyocytes likely reflects a commensurate reduction in cAMP synthesis. Using the higher-affinity C460W/E583M CNGA2 channel to elicit larger basal responses to ISO, we evaluated the contributions of PDE3 and PDE4 to the β-AR response. Preliminary experiments were performed to check the effect of global PDE inhibition on basal I_{CNG} in rat ventricular myocytes expressing the C460W/E583M CNGA2 channel. In agreement with our previous reports,[5,21] global PDE inhibition with IBMX (100 μmol/L) failed to significantly increase the current in sham (I_{CNG} was 0.9±0.5 pA/pF in the presence of IBMX and 17.3±4.4 pA/pF in the presence of 100 nmol/L ISO; n=3) and CH cardiomyocytes (I_{CNG} was 1.1±0.45 pA/pF in the presence of IBMX and 5.4±0.8 pA/pF the presence of 100 nmol/L ISO; n=6). As shown in Figure 3, the results were quite different on β-AR stimulation. In sham cardiomyocytes, the effect of ISO on I_{CNG} was potentiated ≈2-fold on PDE3 inhibition with cilostamide (1 μmol/L) and 3- to 5-fold on PDE4 inhibition with Ro 201724 (10 μmol/L) (Figure 3A, 3C, and 3D). In CH cardiomyocytes, cilostamide potentiated the ISO response by only ≈40%, whereas Ro 201724 increased the ISO response ≈2-fold (Figure 3B, 3C, and 3D). Consequently, the responses of I_{CNG} to ISO in combination with either PDE inhibitor were significantly reduced in CH versus sham cells ($P<0.01$; Figure 3C and 3D).

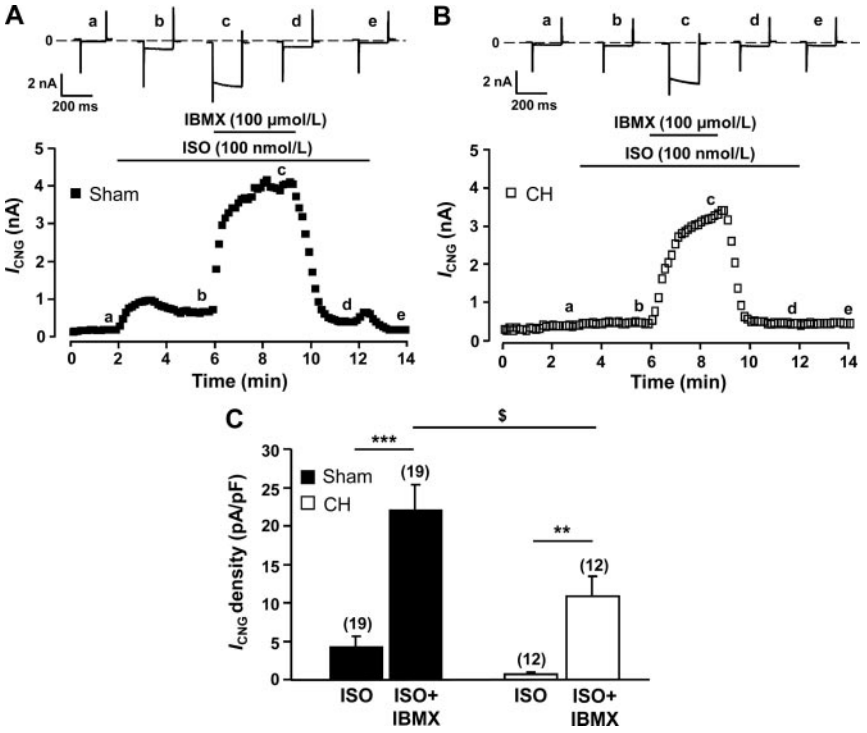

Figure 2. Regulation of β-AR cAMP signals by PDEs in sham and hypertrophied cardiomyocytes. Subsarcolemmal cAMP signals were recorded by measuring I_{CNG} in sham (A) and CH (B) cardiomyocytes expressing E583M CNGA2. Cells were superfused with control external Ringer solution and then challenged with ISO (100 nmol/L) in the absence or presence of IBMX (100 μmol/L) for the periods indicated with horizontal lines. The individual I_{CNG} traces shown on top were recorded at the times indicated by the corresponding letters on the graph below. C, Summary of the responses shown in A and B. Data are expressed as means±SEM. The numbers above the bars represent cardiomyocytes isolated from 2 normal hearts and 3 hypertrophied hearts. Asterisks and dollars signs indicate statistically significant differences (paired *t* test) resulting from the treatment with IBMX or statistically significant differences (unpaired *t* test) between hypertrophied (CH) and sham cardiomyocytes, respectively, and are indicated as: $$P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Expression of PDE3 and PDE4 Isoforms in Sham and CH Cardiomyocytes

The above results suggest that in addition to a decreased β-AR cAMP production, cAMP degradation by PDE3 and PDE4 is also decreased in CH cardiomyocytes. To test this hypothesis further, the expression of PDE3 and PDE4 proteins was measured in cardiomyocytes isolated from sham-operated (n=6) or CH rats (n=4). Equal amounts of proteins prepared from cardiomyocytes isolated from CH or sham-operated animals were separated on SDS-PAGE and PDE3A, PDE4A, PDE4B, and PDE4D proteins were subsequently detected by Western blotting using PDE subtype-selective antibodies. Detergent extracts prepared from brain and heart of PDE knockout mice and wild-type control mice were analyzed on the same blots to control for the specificity of the antibodies. As shown in Figure 4A, all immunoreactive bands detected with PDE4 subtype–selective antibodies in tissues of wild-type mice were not present in the respective PDE4 knockout tissue. This finding confirms the specificity of the antibodies used and indicates that the proteins detected in the rat heart, given their identical migration on SDS-PAGE, are authentic PDEs. Most PDEs are expressed as multiple vari-

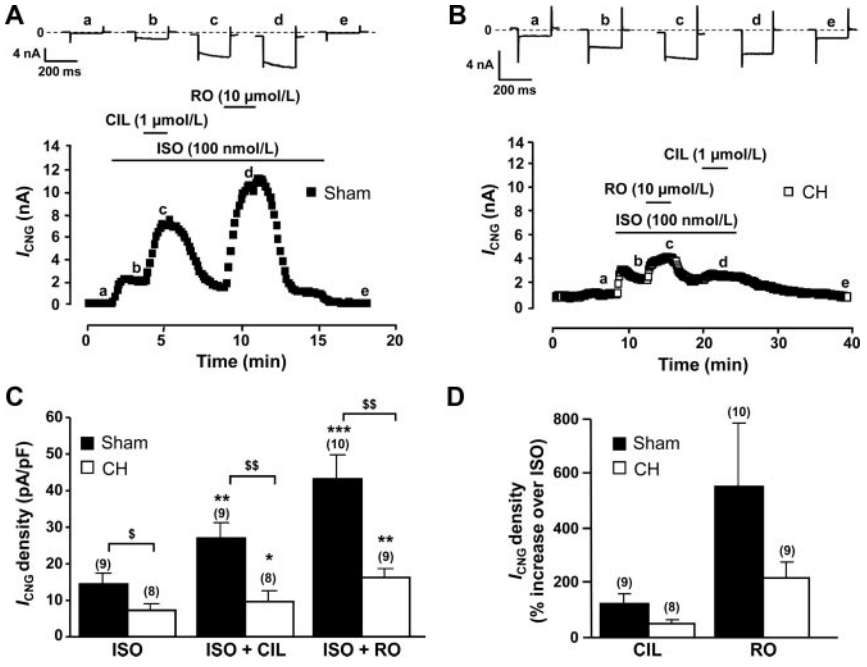

Figure 3. Regulation of β-AR cAMP signals by PDE3 and PDE4 in sham and hypertrophied cardiomyocytes. Subsarcolemmal cAMP signals were recorded by measuring I_{CNG} in sham (A) and CH (B) cardiomyocytes expressing C460W/E583M CNGA2. Cells were superfused with control external Ringer solution and then challenged with different drugs for the periods indicated with horizontal lines. The individual I_{CNG} traces shown on top were recorded at the times indicated by the corresponding letters on the graph below. C and D, Summary of the responses shown in A and B, represented either in absolute current density (C) or in percentage increase over the ISO response (D). All data are expressed as means±SEM. The numbers above the bars represent cardiomyocytes isolated from 2 normal and 5 hypertrophied hearts. Asterisks and dollars signs indicate statistically significant differences (paired *t* test) resulting from the treatment with PDE inhibitors or statistically significant differences (unpaired *t* test) between CH and sham cardiomyocytes, respectively, and are indicated as: $$P<0.05$; $^{**}P<0.01$; $$$P<0.01$; $^{***}P<0.001$.

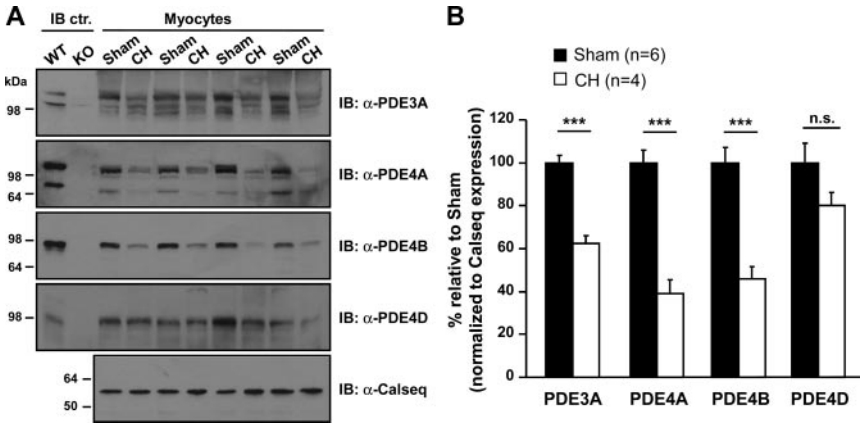

Figure 4. Expression of PDE3 and PDE4 proteins in sham and hypertrophied cardiomyocytes. Equal amounts of proteins from ventricular myocytes isolated from CH (n=4) and sham-operated (n=6) rat hearts were separated on SDS-PAGE and revealed with PDE subtype-specific antibodies. Whole brain (PDE4A, PDE4B, and PDE4B) and total heart extracts (PDE3A) of wild-type (WT) and the respective PDE knockout (KO) mice were loaded as immunoblot controls (IB ctr.). Calsequestrin (Calseq) was used as a loading control. A, Shown are representative blots. B, Quantification of all data obtained in several immunoblots from 6 sham-operated samples and 4 CH samples.

ants through the use of different promoters and alternative splicing, which explains the presence of multiple immunoreactive bands detected in Western blotting for several PDE subtypes. Three immunoreactive bands migrating at approximately 125, 106, and 97 kDa were detected for PDE3A in rat cardiomyocytes. All 3 appeared to be decreased in CH myocytes compared to sham cells. Probing cardiomyocyte extracts with PDE4A-selective antibodies also labeled 3 bands of approximately 105, 95, and 79 kDa. All PDE4A immunoreactive signals were attenuated in CH myocytes compared to sham. A single band migrating at ≈92 kDa was detected in ventricular cardiomyocytes for PDE4B. Its expression was decreased in CH cardiomyocytes. A single band migrating at ≈91 kDa was detected in cardiomyocytes extracts using PDE4D-selective antibodies. Migration of this band corresponds to the migration of PDE4D variants PDE4D3, PDE4D8, and PDE4D9.[22] In contrast to the other PDE subtypes analyzed, the PDE4D immunoreactive signal intensity was not significantly different in CH compared to sham cells (Figure 4B).

cAMP-PDE Activities in Sham and Hypertrophied Cardiomyocytes

To determine whether changes in PDE protein expression are reflected in similar changes of PDE activity, total cAMP-hydrolytic activity in isolated cardiomyocytes from CH and sham-operated rats was measured (Figure 5). Total cAMP-PDE, as well as PDE3 and PDE4 activities, was significantly decreased by 42%, 42%, and 53%, respectively (Figure 5A). To further dissect the contributions of single PDE4 subtypes, total extracts from cardiomyocytes isolated from sham (n=5) and CH (n=4) rats were immunoprecipitated using subtype-specific antibodies against PDE4A, PDE4B, and PDE4D, and the PDE activity recovered in the immunoprecipitation pellets was assayed (Figure 5B). The activity of both PDE4A and PDE4B was significantly reduced in CH cells compared to sham. Conversely, PDE4D activity was not significantly different between the 2 groups.

Discussion

With this study, we provide evidence that cardiac hypertrophy induced by chronic pressure overload in the rat is associated with major changes in the cAMP signaling machinery that controls excitation–contraction coupling. Together with marked β-AR desensitization, we demonstrate a decrease in relative activities and protein densities for PDE isoforms belonging to the PDE3 and PDE4 families, the major cAMP hydrolyzing forms expressed in cardiomyocytes from different species.

In rat cardiomyocytes, PDE3 and PDE4 contribute between 70% and 90% of the total cAMP-hydrolyzing activity.[4,18,21,23] Therefore, a molecular characterization of the various PDE3A and PDE4 isoforms expressed in cardiomyocytes was undertaken. The 3 immunoreactive bands of PDE3A detected here in the rat heart likely represent the counterparts of human PDE3A1, PDE3A2, and PDE3A3 because of their similar migration in SDS-PAGE.[19] Of the 3 immunoreactive species detected for PDE4A, the long 95-kDa form was previously detected in rat heart but not identified.[7] To our knowledge,

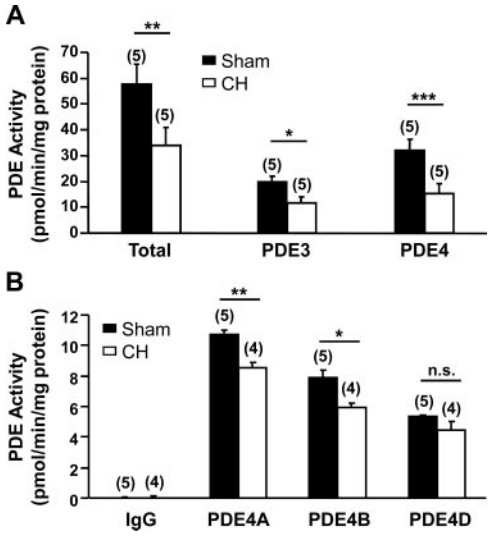

Figure 5. Pattern of cAMP-PDE activities in sham and hypertrophied cardiomyocytes. A, PDE activity in sham and CH cardiomyocytes using 1 μmol/L cAMP as substrate. PDE3 and PDE4 activities are defined as the fraction of the total PDE activity inhibited by cilostamide (1 μmol/L) or rolipram (10 μmol/L), respectively. B, Detergent extracts from sham and CH cardiomyocytes were immunoprecipitated with PDE4 subtype–specific antibodies as described in Methods. The PDE activities recovered in the immunoprecipitation pellets are reported. The number of samples analyzed for each group is shown in brackets above the bars. All data show the means±SEM. Statistically significant differences in PDE activity between CH and sham-operated rats are indicated as: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

the 2 others species migrating at 105 and 79 kDa were not described in cardiomyocytes. The size of the first corresponds to a long form, such as PDE4A5, PDE4A8, PDE4A10, or PDE4A11.[24,25] The short form detected likely corresponds to PDE4A1, which has a mobility similar to the form migrating at ≈75 kDa in rat brain.[24] Only 1 immunoreactive band at ≈92 kDa was detected in cardiomyocytes for PDE4B. This species was previously reported in rat ventricle[24] and likely represents long PDE4B form(s), such as PDE4B1 and/or PDE4B3, for which transcripts were detected in rat heart.[7] In contrast to earlier studies, we could not detect expression of PDE4B2, which is expected to migrate at ≈75 kDa.[4,7] Finally, we detected 1 PDE4D immunoreactive band that exhibited a migration similar to splice variants PDE4D3, PDE4D8, and PDE4D9.[22] These diverse PDE isoforms have been shown to be localized in specific compartments, so as to exert a local control of cAMP signaling; for instance, PDE4D3 associates with the ryanodine receptor complex[14] and with the I_{Ks} potassium channel complex[26]; PDE4D5 associates with β-arrestin near the β_2-AR[27]; and PDE4D8 has been shown to bind to the β_1-AR.[28] In addition, a long isoform of PDE4D was shown recently to coimmunoprecipitate with SERCA2a.[29]

When comparing sham and hypertrophied cardiomyocytes, profound quantitative differences were observed: relative activity and protein expression of PDE3A, PDE4A, and PDE4B were decreased in hypertrophy, whereas that of long PDE4D isoforms remained unchanged. Although our experiments do not exclude the possibility that changes in other PDE families also occur in cardiac hypertrophy, the observed decrease in PDE levels is comparable to the reduction of β_1-ARs[30] and the sarcoplasmic reticulum Ca^{2+} ATPase[31] previously reported in rat heart using a similar model of hypertrophy. Although the mechanisms were not determined, our findings suggest that the genes encoding PDE3A, PDE4A, and PDE4B are not part of the hypertrophic program. A small number of previous studies have investigated the variations of PDE3 and PDE4 during CH with contradictory results.[32] In dogs with CH attributable to aortic valve stenosis, cAMP-PDE activity was reported to be unchanged,[33,34] whereas PDE3 and PDE4 expression and activities were found to be enhanced in Dahl salt–sensitive rats.[35] Our results are consistent with a decreased expression of PDE3A in rodent models of CH induced by chronic infusion of isoprenaline or angiotensin II[12] and with several studies showing a reduction in PDE3 expression and/or activity at the HF stage.[36–39] However, some studies found no difference in PDE3 activity during HF.[34,40] Concerning PDE4, a constant mRNA level for PDE4D was reported in dog with HF,[37] which is consistent with what we observe in rats with CH. However, the PDE4D3 isoform associated to RyR2 was found decreased in human HF.[20] This difference might be attributable to the different species or to the fact that we measured the total cellular PDE4D pool. Therefore, a decrease in PDE4D3 could be compensated by an increase in another splice variant such as PDE4D8 or PDE4D9, which are also expressed in heart.[26] It should be noted that in this study, protein expression and activity measurements were performed using highly purified cardiomyocytes, thus excluding possible variations attributable to other cell types present in the heart.

Despite an important decrease in the 2 main PDEs involved in cAMP hydrolysis, there was a marked impairment of contractile responsiveness to isoprenaline in hypertrophied hearts (Figure 1A) that correlated with reduced subsarcolemmal cAMP levels on β-AR stimulation in hypertrophied cells (Figure 1B and 1C). These results indicate that the PDE diminution was unable to compensate for β-AR desensitization. They are in agreement with numerous studies showing a loss of the β-AR inotropic reserve,[41–44] a decreased β-AR density,[30,41,45] a decreased ISO-stimulated cAMP formation,[46,47] and a decreased β-AR stimulation of the L-type Ca^{2+} current[48] in rodent models of CH induced by pressure overload. In contrast, the response to the forskolin analog L-858051 at 100 μmol/L was unchanged (Figure 1D). We showed previously that this concentration of L-858051 saturates the CNG channels.[19] Thus, this result indicates that the same density of functional channels was expressed in normal and hypertrophied cells.

In conclusion, our study provides strong biochemical and functional evidence for a decreased cAMP-hydrolytic reserve during cardiac hypertrophy and identifies PDE3A, PDE4A, and PDE4B as being specifically altered. PDE downregulation in CH might be regarded as an initial adaptive process because it partly compensates for the deficit in cAMP synthesis. However, such PDE remodeling may be maladaptive in the long term, because of a loss of cAMP compartmentation.[3,18] This in turn may cause unrestricted diffusion of cAMP and chronic activation of cAMP effectors, such as PKA and the exchange factor for Rap1, Epac, both of which were shown to induce pathological cardiac hypertrophy.[49–51]

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Disclosures

None.

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Decreased Expression and Activity of cAMP Phosphodiesterases in Cardiac Hypertrophy and Its Impact on β-Adrenergic cAMP Signals

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Materials and Methods

Reagents

Isoprenaline (ISO) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO, USA). Cilostamide was obtained from Tocris Bioscience (Ellisville, MI, USA). L-858051 (L-85; a hydrosoluble forskolin analogue) was from Calbiochem (San Diego, CA, USA). Ro 201724 (Ro) was kindly provided by Hoffman-La-Roche (Basel, Switzerland). The antibody against calsequestrin was obtained from Affinity BioReagents (Golden, CO, USA). PDE3A was detected in Western blotting using a rabbit polyclonal anti-PDE3A antibody kindly provided by Dr. Chen Yan (Columbia University, NY, USA). The PAN-selective antibodies against PDE4A (AC55), PDE4B (K118) and PDE4D (M3S1) that were used for immunoprecipitation (IP) experiments have been described previously.[1] PDE4A and PDE4B were detected in Western blotting using rabbit polyclonal antibodies generated against the specific C-termini of these PDEs. Mouse monoclonal antibodies against PDE4D and CNGA2 were kindly provided by ICOS Corporation (Bothell, WA, USA) and by Dr. Frank Mueller (Forschungszentrum Jülich, Jülich, Germany), respectively.

Induction of Cardiac Hypertrophy in Rats

77 male Wistar rats were used in this study. Left ventricular hypertrophy was induced at three weeks of age (body weight<60g). The animals were anesthetized (pentobarbital 60 mg/kg) and a stainless steel hemoclip of 0.6 mm ID was placed on the ascending aorta via thoracic incision.[2] Age-matched control animals (sham-operated) underwent the same procedure without placement of the clip. 5 weeks after surgery, 6 rats from each group were anesthetized with isofluorane and subjected to echocardiography as described previously.[3] All other rats were sacrificed 5 weeks after surgery and body, heart, lung, liver and kidney were

weighed. Tibia length was measured using a vernier caliper (Table 1). Rats that exhibited signs of congestive HF (i.e. ascites, serous cavity effusion, or necrotic lungs, kidney or liver) were excluded from the study. The hearts from all other animals were either digested to obtain individual myocytes or used for Langendorff heart studies, as described in the next sections. All procedures conform to the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off J* n°L358, 18 December 1986), the local ethics committee (CREEA Ile-de-France Sud) guidelines and the French decree n°87/748 of October 19, 1987 (*J Off République Française*, 20 October 1987, pp. 12245-12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (nº7475, May 27, 1997).

***Ex vivo* Physiology**

Rats were anesthetized by intraperitoneal injection of pentobarbital (150 mg/kg). The heart was quickly removed and placed in oxygenated Krebs-Henseleit solution (95% O_2 and 5 % CO_2 , pH 7.35) containing (in mmol/L): NaCl 113, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25, glucose 11, Na pyruvate 5, and mannitol 1.1. The aorta was cannulated and perfused by the Langendorff method with oxygenated Krebs-Henseleit solution at constant pressure (75 mmHg, temperature 37±0.2°C). A latex water-filled balloon was inserted into the left ventricular chamber and connected to a pressure transducer (Statham gauge Ohmeda, Bilthoven, Holland) for continuous measurement of heart contractility. Heart rate, left ventricular developed pressure (LVDP) and the first derivatives of LV pressure (LV $+dP/dt_{max}$ and LV $-dP/dt_{max}$) were measured online using a dedicated software (Emka technologies data analyzer, Paris, France). For each heart, the experiment started with a progressive increase of the latex balloon inserted inside the left ventricle to generate a ventricular volume-developed pressure relationship. When the maximal developed

pressure was reached, ten minutes of equilibration in isovolumic working conditions were imposed before infusing increasing concentrations of isoprenaline (from 1 nmol/L to 1 μmol/L).

Isolation of Adult Rat Ventricular Myocytes

Hearts were mounted on a Langendorff apparatus and perfused through the coronaries with collagenase A (Roche, Meylan, France) as described previously.[4] Digestion time was 50 min for hearts from sham-operated animals and varied between 90 and 120 min for hypertrophic hearts. At the end of the enzymatic digest, the left ventricles were excised, chopped finely, and agitated manually to dissociate individual myocytes. A portion of the freshly isolated cells was infected with adenoviruses encoding CNGA2 channels for measurement of intracellular cAMP signals as described below. The remaining cells were frozen in liquid nitrogen and subsequently used to determine PDE expression patterns by Western blotting or PDE activity assay.

Preparation of Protein Extracts from Cardiomyocytes

Left ventricular myocytes isolated from sham-operated and cardiac hypertrophy (CH) rats were homogenized in ice-cold buffer containing (in mmol/L): NaCl 150, Hepes 20 (pH 7.4), EDTA 2, and supplemented with 10% Glycerol, 0.5% NP40, 1 μmol/L microcystin-LR, and Complete Protease Inhibitor Tablets from Roche Diagnostics (Basel, Switzerland). Cell lysates were centrifuged at 14,000 g and 4°C for 20 min, and supernatants were either used directly for PDE assay or Western blotting or first subjected to IPs as described below.

To determine the expression of CNG channels, adult rat ventricular myocytes were homogenized 24 h after infection with C460W/E583M CNGA2 adenoviruses in ice cold buffer containing (in mmol/L): NaCl 150, Tris HCl (pH 7.5) 20, EGTA 5, $NaVO_3$ 1, sodium

fluoride 20, glycerophosphate 20, and supplemented with 1% Triton-X 100, 0.1% Tween 20, and a protease inhibitor cocktail from Calbiochem (LaJolla, CA, USA). Cell lysates were centrifuged at 10,000 g and 4°C for 10 min and the resulting supernatants were used to measure channel expression by Western blotting.

Immunoprecipitation of PDE4 Subtypes from Protein Extracts

For IPs, the respective PDE4 subtype-specific antibodies were coupled to 25 µL of Protein G-Sepharose beads, which were then incubated with protein extracts from adult rat ventricular myocytes (each 1 mg protein) for 2 h at 4°C. The beads were pelleted, washed three times with cell lysis buffer, and the resulting IP pellets were subjected to PDE activity assays as described below.

PDE Assay

Cyclic AMP-PDE activity was measured according to the method of Thompson and Appleman as described in detail previously.[5] In brief, samples were assayed in a reaction mixture of 200 µL containing 40 mmol/L Tris-HCl (pH 8.0), 1 mmol/L $MgCl_2$, 1.4 mmol/L β-mercaptoethanol, 1 µmol/L cAMP, 0.75 mg/mL bovine serum albumin, and 0.1 µCi of [^{3}H]cAMP for 30 min at 33°C. The reaction was terminated by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5'-AMP was then hydrolyzed by incubation of the assay mixture with 50 µg of *Crotalus atrox* snake venom for 20 min at 33°C, and the resulting adenosine was separated by anion exchange chromatography using 1 mL of AG1-X8 resin (BioRad, Hercules, CA, USA) and quantitated by scintillation counting. PDE3 and PDE4 activities were defined as the fraction of cAMP-PDE activity inhibited by 1 µmol/L cilostamide or 10 µmol/L rolipram, respectively.

Single Cell Measurement of cAMP Signals

To measure real-time cAMP signals, cardiac myocytes were infected with adenoviruses encoding for two mutants of the cyclic-nucleotide gated CNGA2 channel, E583M CNGA2 and C460W/E583M CNGA2. The CNGA2 channel carrying the E583M mutation exhibits a lower affinity for cAMP ($K_{1/2\ cAMP}$=10.3 µmol/L) compared to the channel carrying the C460W/E583M double mutation ($K_{1/2\ cAMP}$=1.4 µmol/L).[6] Freshly isolated adult rat ventricular myocytes were cultured in Minimum Essential Medium (M 4780; Sigma, St. Louis, MO) containing 1.2 mM Ca^{2+}, 2.5% fetal bovine

serum (FBS, Invitrogen, Cergy-Pontoise, France), 1% penicillin-streptomycin and 2% HEPES (pH 7.6) and plated on laminin-coated culture dishes (10 µg/ml laminin, 2 h) at a density of 10^4 cells per dish. The cells were left to adhere for 1 h in a 95% O_2, 5% CO_2 atmosphere at 37 °C, before the medium was replaced with 300 µl FBS-free MEM containing the respective adenovirus at a multiplicity of infection of 600 pfu/per cell. 24 h after virus infection, cell membrane capacitance and I_{CNG} were measured using the patch-clamp technique in the whole-cell configuration as previously described.[7] Cell membrane capacitance was determined as the current elicited by a voltage ramp at 1V/s between -90 mV and -70 mV. I_{CNG} was measured at -50 mV in 200 ms steps from a holding potential of 0 mV. Extracellular solution contained (in mmol/L): NaCl 107.1, CsCl 20, $NaHCO_3$ 4, NaH_2PO_4 0.8, D-Glucose 5, sodium pyruvate 5, HEPES 10, nifedipine 0.001, and was adjusted to pH 7.4 with NaOH. Patch electrodes (0.5-1 MΩ) were filled with intracellular solution containing (in mmol/L): CsCl 118, EGTA 5, $MgCl_2$ 4, sodium phosphocreatine 5, Na_2ATP 3.1, Na_2GTP 0.42, $CaCl_2$ 0.062 (pCa 8.5), HEPES 10, and was adjusted to pH 7.3 with CsOH. All experiments were done at room temperature (19-25°C), and the temperature did not vary by more than 1°C in a given experiment.

Data Analysis

Cellular hypertrophy was evaluated by measuring cell surface and cell perimeter using Image J (Wayne Rasband, National Institutes of Health, USA) as well as cell membrane capacitance. I_{CNG} amplitude was measured at the end of the 200 ms pulse at -50 mV. I_{CNG} density (dI_{CNG}) was calculated for each experiment as the ratio of current amplitude to cell capacitance (C_m). In each experimental condition, the response of dI_{CNG} to a drug was expressed relative to the 'basal current' obtained in control extracellular solution following the relation: 'response'=(I_{CNG}-'basal current')/C_m. All data are expressed as mean ± S.E.M. Paired Student's *t*-test was used for statistical evaluation within the same group. When two groups were compared, unpaired Student *t*-test was used. Concentration-response curves (CRC) of ISO on I_{CNG} and LV $+dP/dt_{max}$, were generated by fitting the data to the Hill equation, $dI_{CNG}=E_{max}/(1+(EC_{50}/[ISO])^n)$ or LV $+dP/dt_{max}=E_{max}/(1+(EC_{50}/[ISO])^n)$, where E_{max} is the maximal effect, EC_{50} is the ISO concentration required to produce half of E_{max}, and n is the Hill coefficient. The CRC obtained in both groups were compared using a Fisher test; and a difference was considered statistically significant when p was <0.05.

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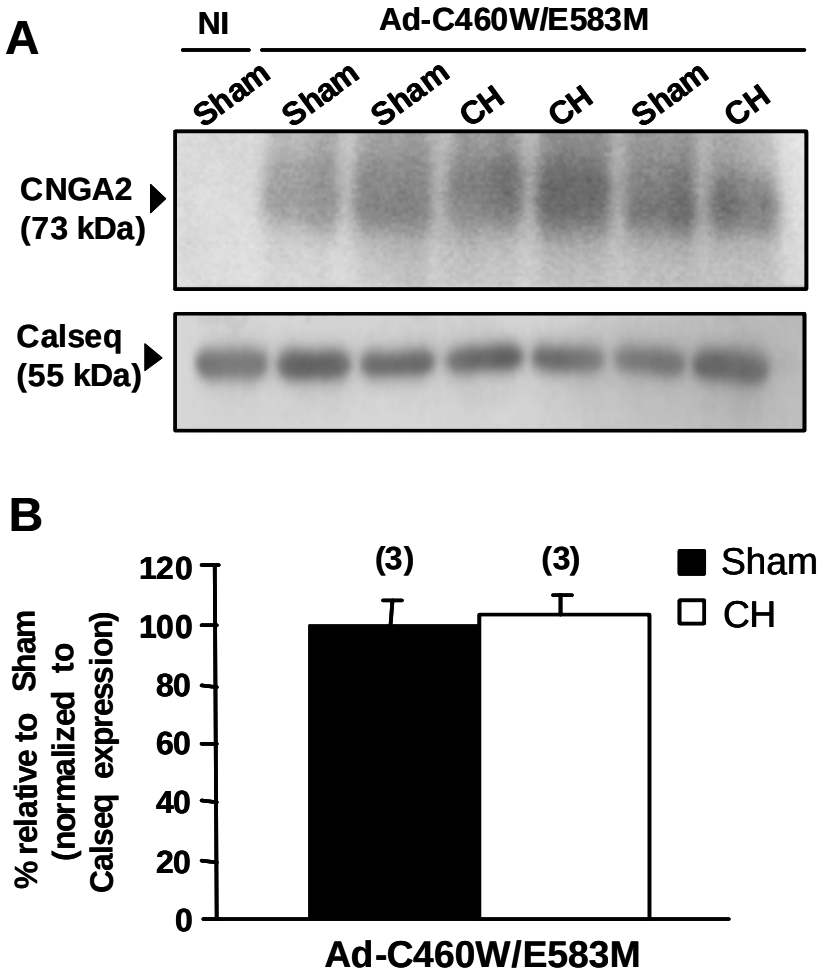

Online Figure 1. Expression of C460W/E583M CNGA2 in sham and CH adult rat ventricular myocytes. (A) Equal amounts of protein from sham and CH cardiomyocytes infected with adenovirus encoding C460W/E583M CNGA2 or from non-infected (NI) sham cells were separated by SDS/PAGE and channel expression was subsequently detected by Western blotting. Expression of Calsequestrin (Calseq) was determined as a loading control. The intensity of the CNGA2 immunoblot signal for all samples is quantified in (B). Expression of C460W/E583M CNGA2 is normalized to the expression level of Calseq in each sample.