cGMP-Phosphodiesterase Activity Is Up-regulated in Response to Pressure Overload of Rat Ventricles

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Although expression of natriuretic peptides in cardiac tissues is up-regulated in response to pressure overload, no significant change in cGMP level in hypertrophied ventricles was observed. Activities of two cyclic nucleotide phosphodiesterase (PDE) isoforms, Ca2+/calmodulin-stimulated PDE (PDE1) and cGMP-stimulated PDE (PDE2), were significantly higher in rat left ventricles 14 days after aortic banding. The absence of significant changes in PDE1A and PDE2A mRNA levels indicated that the two PDE activities were post-transcriptionally up-regulated. These results suggested that the increased cGMP-PDE activity in response to pressure overload plays an important role in neutralizing cGMP action in cardiac tissue.

Key words: cGMP; natriuretic peptide; phosphodiesterase; pressure overload

Hemodynamic overload evokes a series of acute and chronic responses in the heart that are directed toward neutralizing the stresses. The acute responses are focused on making the heart more mechanically efficient and more capable of dealing with the increased workload.1) The chronic changes are typified by a growth response within the cellular elements that make up the cardiac tissue. This is characterized by hypertrophy of nondividing myocardial cells, which leads to an increase in wall thickness and reduces wall stress, and hyperplasia of mesenchymal elements that make up the cardiac interstitium. The increased density of these frameworks that are affected during cardiac hypertrophy serves to abate the increase in wall tension that accompanies hemodynamic overload.

Cyclic GMP is now recognized to participate in regulations of numerous physiological responses including smooth muscle relaxation, retinal photo signal transduction, and intestinal secretion, such as acting as a second messenger of natriuretic peptides (NP) and nitric oxide (NO).2) These responses are mediated through direct actions on ion channels and cGMP-dependent protein kinases. Modulation of some specific cyclic nucleotide phosphodiesterases (PDEs), which contribute to elimination of intracellular cAMP, also indirectly participates in the regulation of physiological responses via cAMP signaling. While cGMP has been considered to play important roles for the control of systemic blood pressure and intravascular volume,3) it is noteworthy that this molecule has been shown to have important growth-suppressing properties in a variety of tissues, including cardiac myocytes4) and cardiac fibroblasts.5,6) These reports indicated that increased expression of NPs in hypertrophic cardiac tissue has protective and compensatory roles against the development of cardiac hypertrophy through intracellular cGMP production.

Physiological functions of cGMP are regulated by a balance of the production/degradation rate. PDEs are also critical determinants of intracellular cyclic nucleotide concentrations through the hydrolysis of cAMP and cGMP. Currently eleven distinct PDE families (PDEs 1–11) have been identified in mammalian tissues based on their amino acid sequence similarity, biochemical characteristics, pharmacological profiles, and sensitivities to inhibitors.7–11) PDE isoforms in the cardiac tissue have been isolated and characterized, showing that four major distinct PDEs coexist in the heart muscle: (a) a Ca2+/calmodulin-stimulated PDE (PDE1), (b) a cGMP-stimulated PDE (PDE2), (c) a cGMP-inhibited PDE (PDE3), and (d) a cAMP-specific PDE (PDE4). However, little is known about the regulation of PDE expression in the development of certain cardiac pathologies. In this study, we characterize PDE activities in pressure-overloaded ventricles of aortic-banded rats, and investigate to find whether PDE activities are altered during the development of cardiac hypertrophy.
Materials and Methods

Materials. Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Bio (Kyoto, Japan). [3H]cAMP, [3H]cGMP, [α-32P]dCTP, [γ-32P]ATP, first strand cDNA synthesis kit, and Hybond-N+ nylon membrane were from Amersham Biosciences (Buckinghamshire, UK). cAMP and cGMP were products of Sigma. CI-930 was synthesized at Discovery Research Laboratory (Tanabe Seiyaku Co., Ltd.). cDNA encoding human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was described previously.12

Animals and surgical procedures. Eight-week-old male Sprague-Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the aorta was exposed through a midline abdominal incision. The abdominal aorta was constricted above the renal arteries, using a 20-gauge needle to establish the diameter of the ligature. Sham-operated control rats underwent an identical procedure except for the ligature. Two weeks after aortic banding, the rats were weighed, and quickly frozen in liquid nitrogen. Left ventricles were divided into the left ventricle, including intraventricular septum, and right ventricular free wall, and they were rinsed with cold saline, weighed, and quickly frozen in liquid nitrogen. Left ventricular hypertrophy was assessed by the left ventricular wet weight-to-body weight ratio. cGMP and cAMP concentrations were measured using enzyme immunoassay kits (Amersham Biosciences). This study was approved by the Animal Research Committee of Tanabe Seiyaku Co., Ltd.

FPLC resolution of PDE activities. Isolated left ventricles from aortic banded and sham-operated rats were homogenized in 3 ml of ice-cold homogenization buffer A (50 mM Bis-Tris (pH 6.5), 15 mM benzamidine, 15 mM MgSO4, 15 mM dithiothreitol, 0.5 μg/ml leupeptin, and 5 mM EDTA). The homogenates were centrifuged at 50,000 g for 30 min at 4°C. The resulting supernatants were filtered through 0.45-μm filter (Millipore), and used for the PDE assay or applied onto a Mono Q FPLC column (Amersham Biosciences; 0.5 cm6×5 cm). Supernatant fractions from left ventricles (500 mg protein in 3 ml) were eluted with a linear gradient of NaCl from 0 mM to 500 mM (in 25 mM Bis-Tris (pH 6.5) and 1 mM EDTA) at a flow rate of 2 ml/min. Fractions (1.6 ml) were collected, of which 15 μl were assayed for PDE activity.

Assay for PDE activity. PDE activity was measured as previously reported.13 Total protein concentration was measured using a DCA Protein assay kit (Bio-Rad) and bovine serum albumin as the standard.

Preparation of cDNA probes for three cGMP-PDEs, PDE1A, PDE2A, and PDE3A. To obtain a cDNA probe for northern blotting analysis of rat PDE1A mRNA, we did PCR using a λZap rat heart cDNA library (Stratagene, La Jolla, CA, USA) as a template. The primer set (5'-TAGAGTTGTTA-CAGCAAGTACAA-3' plus 5'-CCTAATTCAGCT-TCTTTATCC-3') was designed according to the nucleotide sequence of the bovine PDE1A,10 and used for amplifications. Denaturing, annealing, and polymerase reaction were done 30 times at 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, respectively. A cDNA probe for the rat PDE2A was produced by RT-PCR using rat ventricle mRNA as a template. Poly(A)+ RNA was isolated from the ventricle of WKY rats using Isogen (Nippon gene, Toyama, Japan) and mRNA separator kit (Clontech, Palo Alto, CA, USA). After cDNA synthesis using a first strand cDNA synthesis kit (Amersham Biosciences) and the primer 5'-GGACATCCTGGTT-CTGAACCCAGGAC-3' corresponding to the 3' sequence of the rat PDE2A cDNA,15 we did PCR with a primer set (5'-CACCACCTCCGCATCTTCCA-GGAC-3' plus 5'-CCCTCAGGCTGCAGCAGC-3') designed according to the rat PDE2A cDNA sequence. To isolate a cDNA probe for the PDE3A3, we did PCR using the primer set (5'-TATT-TCTGAGCTAACCATG-3' plus 5'-CTGTCAAGATCTGAATCCTG-3') designed according to the nucleotide sequence of the human PDE3A cDNA10 and a λgt11 human fetal lung cDNA library (Clontech) as a template. The cDNA fragments encoding PDE isozymes and GAPDH were labeled with 32P using Random Primer DNA Labeling Kit Ver. 2 (Takara Bio). 32P-labeling of synthetic oligonucleotides (5'-GGCCTGGAGGCTGCACTCACTAC-3' and 5'-GTGATAAGATGAAAGAACAGAGGATCGAG-3'), which are complementary to the nucleotide sequence of the rat atrial natriuretic peptide (ANP), was done using Megalabel (Takara Bio).

RNA analysis by northern blotting hybridization. Poly(A)+ RNAs were isolated from left ventricles (n = 3) as described above. Northern blot hybridization for PDE1A and PDE2 was done in 50% formamide, 5×SSC, 0.5% SDS, 5× Denhardt’s, 100 μg/ml salmon sperm DNA, and the 32P-labeled probe, at 42°C overnight as we previously reported.17 Blots were washed finally in 0.2×SSC and 0.1% SDS at
Table 1. Hemodynamic Parameters and Heart Weight in Aortic Banding and Sham-operated Control Rats 14 Days after Surgery

Values are expressed as means ± SD (n = 5). **P < 0.01, *P < 0.05, versus sham-operated control rats. LVEDP, left ventricular end-diastolic pressure.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sham</th>
<th>Aortic banding</th>
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<tbody>
<tr>
<td>Body weight (BW, g)</td>
<td>371.4 ± 16.0</td>
<td>342.0 ± 14.8</td>
</tr>
<tr>
<td>Left ventricle weight (LVW, mg)</td>
<td>712.7 ± 52.3</td>
<td>854.3 ± 65.1</td>
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<tr>
<td>LVW/BW (mg/g)</td>
<td>1.9 ± 0.1</td>
<td>2.5 ± 0.1**</td>
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<tr>
<td>Mean Blood Pressure (mmHg)</td>
<td>130.8 ± 5.1</td>
<td>212.2 ± 4.5**</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>395.8 ± 21.4</td>
<td>393.4 ± 6.7</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>3.2 ± 0.3</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>cGMP (pmol/mg)</td>
<td>1.5 ± 0.3</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>cAMP (pmol/mg)</td>
<td>476.9 ± 153.8</td>
<td>581.6 ± 65.8</td>
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60°C for 1 h. Northern blot hybridization for PDE3A was done in 6× SSC, 0.5% SDS, 5× Denhardt’s, 100 μg/ml salmon sperm DNA, and the 32P-labeled probe at 55°C overnight, and then blots were washed finally in 1× SSC and 0.1% SDS at 55°C for 20 min.12) Hybridization with 32P-labeled oligonucleotides was done in 6× SSC, 0.5% SDS, 5× Denhardt’s, and 100 μg/ml salmon sperm DNA at 55°C overnight, and then blots were washed with 6× SSC and 0.1% SDS at 55°C for 5 min. All membranes were exposed to x-ray film at −70°C. Values were obtained from a densitometric scan of hybridized signals using MCID (Imaging Research Inc., Ontario, Canada).

Statistical analysis. All values were expressed as mean ± S.D. Statistical significance was measured by an unpaired Student’s t test.

Results

Effects of aortic banding on rats

Animals were killed with an anesthetic overdose 1 day or 14 days after ascending aortic banding. Hemodynamic parameters and heart weight-to-body weight ratio measured in aortic-banded rats and sham-operated age-matched control rats were summarized in Table 1. Body weight was similar in sham-operated rats and banded rats. Mean carotid arterial pressure was significantly higher in aortic-banded rats. The left ventricular weight-to-body weight ratio was greater in aortic-banded rats than in sham-operated rats, indicating that aortic-banded rats developed a left ventricular hypertrophy. The right ventricular weight-to-body weight ratio was not significantly different between the two groups (data not shown).

PDE activities of rat left ventricles

We compared specific PDE activities in left ventricles from aortic banding and sham-operated control rats 1 day and 14 days after surgery. The specific activities of cytosolic cAMP- and cGMP-PDEs were similar in both groups 1 day after surgery, but those of 14 days after surgery were significantly higher in aortic banding rats than in sham-operated rats (Table 2). Cytosolic PDE activities of left ventricles 14 days after surgery were resolved by anion-exchange chromatography (Fig. 1AB). Although the levels of PDE activities differed, two peaks showing cGMP-PDE activity were recognized in the elution profiles of ventricle extracts from both animals. A specific cGMP-PDE activity, which was stimulated by addition of Ca2+ and calmodulin (data not shown), was designated Peak I. The Km values for cGMP of the Peak I fractions from control and hypertrophied ventricles were 5–10 μM in the presence of Ca2+ /calmodulin. The Peak II fraction included both cGMP-PDE and cAMP-PDE activities, and the cAMP-PDE activity was augmented (544%) in the presence of 2 μM of cGMP. Specific cAMP-PDE activity in the Peak III fraction was moderately inhibited by addition of 2 μM of cGMP (−18%) or 10 μM of CI-930 (−29%), which is a known PDE3-specific inhibitor (data not shown). From these observations, we suspected that Peak I, Peak II, and Peak III include a Ca2+/calmodulin-stimulated PDE (PDE1), a cGMP-activated PDE (PDE2), and a cGMP-inhibited PDE (PDE3), respectively.

Preparation of PDE cDNA probes

To clone the rat PDE1A cDNA from a rat heart cDNA library, we did PCR using a set of primers designed according to the nucleotide sequences of the human and bovine PDE1A. The nucleotide sequence of a specifically amplified 576-base pair DNA fragment was shown to be similar to the human and bovine PDE1A.18 Comparison of the deduced amino acid sequences with the human and bovine PDE1A showed that rat PDE1A shares 89 and 90% identities with human and bovine orthologues at amino acid level, respectively (data not shown), suggesting that mammalian PDE1 orthologues are well-conserved. During the course of this study, the rat PDE1A
cDNA sequence (NM_030871), which is completely matched with our sequence, was disclosed. We also obtained DNA fragments of 576 bp and 881 bp corresponding to rat PDE2A and human PDE3A cDNAs, respectively.

Northern blot analysis of rat left ventricles

Northern blot analysis using poly(A)⁺ RNA from the left ventricles 14 days after surgery showed that PDE1A and PDE3A were slightly increased in the mRNA levels compared with age-matched and sham-operated control rats (116 ± 1% and 122 ± 2% (n = 3), respectively), whereas no significant change was observed for PDE2A mRNA between the two groups (108 ± 7%) (Fig. 2). Expression of the ANP mRNA was up-regulated in the left ventricles of aortic banding rats (1560 ± 59%), suggesting its counter-regulation against an increase in arterial pressure. Two major transcripts of rat PDE1A with different sizes suggested the presence of alternative splice variants.

Discussion

In this study, the influence of the development of cardiac hypertrophy or pressure overload of rat ventricles on cGMP hydrolytic activity was investigated. The cytosolic cAMP- and cGMP-PDE activities (per mg protein) in the left ventricle of aortic banding rats were significantly high compared with those of sham-operated rats 14 days after surgery. PDE1 and PDE2 were major cGMP-PDE activities in the tissue, and aortic banding augmented both activities in the tissue.
The PDE1 family is constituted of multiple gene products of three distinct genes, PDE1A, PDE1B, and PDE1C.\textsuperscript{14,18–20} The PDE1A is known to be highly expressed in canine, bovine, and human hearts as a major form in cardiac tissue.\textsuperscript{14,23,26} PDE1C transcripts are also documented to be weakly expressed in the rat heart.\textsuperscript{29} In contrast, PDE1B transcripts are not detectable in rat heart.\textsuperscript{25} The increase in PDE1 activity in the rat ventricles with aortic banding was accompanied by a slight increase in the PDE1A mRNA level. Augmentation of expression of PDE1 isoforms other than PDE1A might participate in up-regulation of the Ca\textsuperscript{2+}/calmodulin-stimulated PDE activity. However, because a high cGMP \( K_m \) value (\( K_m = 5–10 \mu M \)) of the Peak I fraction in the aortic banding rats was not matched with the value of rat PDE1C (\( K_m = 1.1 \mu M \)) reported in Ref. 19, the PDE1C isoform is unlikely to be induced.

Possible explanations for the augmentation are increased \textit{de novo} PDE1A protein synthesis or post-translational modification of PDE1 proteins, that is, a change in the affinity of PDE1 for calmodulin. It has been reported that PDE1A is a substrate for cAMP-dependent protein kinase, and that its hydrolytic activity is lowered by the phosphorylation.\textsuperscript{20,27} The effect of phosphorylation is reversed by a Ca\textsuperscript{2+}-activated phosphatase, calcineurin, through dephosphorylation.\textsuperscript{26,27} Enzymatic activity of calcineurin in the heart is known to be increased in response to pressure-overload hypertrophy in the rats with aortic banding.\textsuperscript{28} In the hypertrophied heart ventricle and cardiac myocytes, it is plausible that calcineurin-mediated dephosphorylation of PDE1A proteins occurs. In addition, the endogenous calmodulin concentration is reported to be elevated by aortic banding in the hypertrophied cardiac muscle,\textsuperscript{29} which might affect the phosphorylation/dephosphorylation state of PDE1 proteins. Thus, PDE1 activities are regulated by cross-talk between Ca\textsuperscript{2+} and cAMP signal transduction. Because treatment with norepinephrine and isoproterenol but not phenylephrine also up-regulated the cGMP hydrolytic activity in primary cardiac myocytes (unpublished observation), this system may be useful for further exploration of the mechanism of augmentation of PDE1 activities.

Northern blot analysis in rat hearts demonstrated that expression levels of transcripts of another cGMP-PDE, PDE2A, were unchanged or rather slightly increased during the course of development of hypertrophy. Since post-translational modification of PDE2A is not known, augmentation of PDE2A activity may be related to the reduction of PDE2A protein degradation. The effect of cGMP on cardiac Ca\textsuperscript{2+} channels through PDE2 activation is controversial. Several reports have shown that the desensitization of the increased Ca\textsuperscript{2+} channel current by cGMP may be mediated through PDE2 activation in frog ventricular and human atrial myocytes,\textsuperscript{30,31} whereas cGMP possibly inhibits the basal and \( \beta \)-adrenergic agonist-stimulated L-type Ca\textsuperscript{2+} channel currents via activation of cGMP-dependent kinase in rabbit ventricular myocytes.\textsuperscript{32} Our results demonstrating the up-regulation of PDE2 activity in pressure-overloaded ventricles is intriguing in the aspect with the inotropic function of the PDE2 activity in cardiac myocytes.

A current study shows the regulation of the cyclic nucleotide action by alteration of the PDE activities, providing new information on the role of the NO or NPs/cGMP signaling pathway in cardiac diseases. Recent reports have shown that NPs in hypertrophic cardiac tissue have protective and compensatory roles against development of cardiac hypertrophy, and that its physiological effects are mediated with production of intracellular cGMP through the guanylyl cyclase-coupled receptor, GC-A.\textsuperscript{33,34} Because up-regulation of cGMP-PDEs was considered to be tightly associated with neutralization of NPs actions in acute and chronic responses in the pressure-overloaded heart, selective inhibition of the cGMP-PDE is a potential pharmacological target for therapeutic agents.

References

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