

## Cyclic Guanosine Monophosphate Compartmentation in Rat Cardiac Myocytes

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**Background**—Cyclic guanosine monophosphate (cGMP) is the common second messenger for the cardiovascular effects of nitric oxide (NO) and natriuretic peptides, such as atrial or brain natriuretic peptide, which activate the soluble and particulate forms of guanylyl cyclase, respectively. However, natriuretic peptides and NO donors exert different effects on cardiac and vascular smooth muscle function. We therefore tested whether these differences are due to an intracellular compartmentation of cGMP and evaluated the role of phosphodiesterase (PDE) subtypes in this process.

**Methods and Results**—Subsarcolemmal cGMP signals were monitored in adult rat cardiomyocytes by expression of the rat olfactory cyclic nucleotide-gated (CNG) channel  $\alpha$ -subunit and recording of the associated cGMP-gated current ( $I_{\text{CNG}}$ ). Atrial natriuretic peptide (10 nmol/L) or brain natriuretic peptide (10 nmol/L) induced a clear activation of  $I_{\text{CNG}}$ , whereas NO donors (*S*-nitroso-*N*-acetyl-penicillamine, diethylamine NONOate, 3-morpholinonyldimethylamine, and spermine NO, all at 100  $\mu$ mol/L) had little effect. The  $I_{\text{CNG}}$  current was strongly potentiated by nonselective PDE inhibition with isobutyl methylxanthine (100  $\mu$ mol/L) and by the PDE2 inhibitors erythro-9-(2-hydroxy-3-nonyl)adenine (10  $\mu$ mol/L) and Bay 60-7550 (50 nmol/L). Surprisingly, sildenafil, a PDE5 inhibitor, produced a dose-dependent increase of  $I_{\text{CNG}}$  activated by NO donors but had no effect (at 100 nmol/L) on the current elicited by atrial natriuretic peptide.

**Conclusions**—These results indicate that in rat cardiomyocytes (1) the particulate cGMP pool is readily accessible at the plasma membrane, whereas the soluble pool is not; and (2) PDE5 controls the soluble but not the particulate pool, whereas the latter is under the exclusive control of PDE2. Differential spatiotemporal distributions of cGMP may therefore contribute to the specific effects of natriuretic peptides and NO donors on cardiac function. (*Circulation*. 2006; 113:2221-2228.)

**Key Words:** cyclic GMP ■ natriuretic peptides ■ nitric oxide ■ phosphodiesterases ■ sildenafil

Cyclic guanosine monophosphate (cGMP) is a ubiquitous intracellular second messenger in the cardiovascular system. In the heart, acute elevation of cGMP concentration usually exerts negative metabolic as well as inotropic effects,<sup>1,2</sup> whereas chronic elevation prevents and reverses cardiac hypertrophy.<sup>3-5</sup> cGMP synthesis is controlled by 2 types of guanylyl cyclases (GC) that differ in their cellular location and activation by specific ligands: a particulate GC (pGC) present at the plasma membrane, which is activated by natriuretic peptides such as atrial (ANP), brain (BNP), and C-type natriuretic peptide<sup>6-8</sup>; and a soluble guanylyl cyclase (sGC) present in the cytosol and activated by nitric oxide (NO).<sup>8,9</sup>

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Although NO and natriuretic peptides use cGMP as a common second messenger, there are many instances in which activation of pGC and sGC leads to different func-

tional effects.<sup>10-14</sup> One explanation for these divergent effects is that cGMP rises in specific subcellular locations, regulating different targets in different parts of the cell. Such a notion has been extensively substantiated in the case of cyclic adenosine monophosphate (cAMP), a closely related cGMP counterpart.<sup>15</sup> For example, work from this laboratory has shown that cAMP compartmentation occurs during  $\beta$ -adrenergic stimulation of adult cardiac myocytes<sup>16</sup> and is responsible for local activation of cardiac L-type  $\text{Ca}^{2+}$  channels.<sup>17,18</sup> Such a compartmentation involves activation of subsarcolemmal cyclic nucleotide phosphodiesterase (PDE) by cAMP-dependent protein kinase (PKA),<sup>16</sup> most likely through the formation of multimolecular signaling complexes involving PKA, PKA anchoring proteins, and PDE4 isoforms.<sup>19-21</sup>

Despite its crucial role in smooth muscle relaxation, the subcellular spatiotemporal organization of cGMP signaling has been relatively unexplored. PKA-anchoring protein-like

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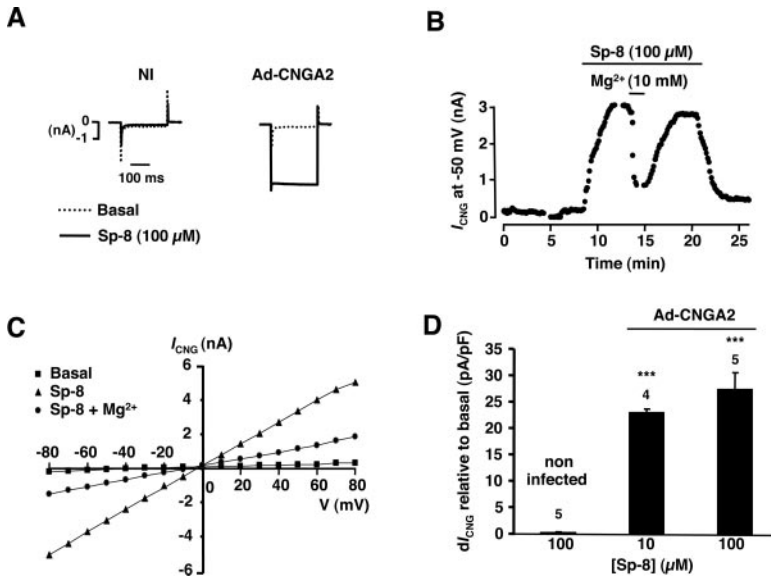
The online-only Data Supplement can be found at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.599241/DC1>.

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**Figure 1.** Functional expression of CNGA2 channels in ARVMs. **A**, Individual current tracings of  $I_{\text{CNG}}$  obtained during 200-ms voltage pulse at  $-50$  mV in infected (Ad-CNGA2) or noninfected (NI) myocytes. Each tracing was recorded in the presence of control solution supplemented (solid lines) or not (dotted lines) with the cGMP analogue Sp-8 ( $100 \mu\text{mol/L}$ ). **B**, Time course of  $I_{\text{CNG}}$  amplitude at  $-50$  mV in an Ad-CNGA2 cell. The cell was initially superfused with control solution and then exposed to Sp-8 ( $100 \mu\text{mol/L}$ ). The Sp-8-activated current was blocked in a reversible manner by the presence of  $\text{Mg}^{2+}$  ( $10 \text{ mmol/L}$ ). **C**, Current-voltage relationships of  $I_{\text{CNG}}$  obtained with control solution (■) in the presence of  $100 \mu\text{mol/L}$  Sp-8 alone (▲) or with  $10 \text{ mmol/L}$   $\text{Mg}^{2+}$  (●). **D**, Density of  $I_{\text{CNG}}$  obtained in Ad-CNGA2 or noninfected myocytes challenged with Sp-8 ( $10 \mu\text{mol/L}$  or  $100 \mu\text{mol/L}$ ).  $***P < 0.005$  vs noninfected cells.

proteins have been identified for targeting cGMP-dependent protein kinase (PKG) to protein substrates,<sup>22,23</sup> but their characterization remains limited. Fluorescence resonance energy transfer-based cGMP fluorescent probes have been developed,<sup>24</sup> but their availability as plasmid constructs currently restricts their use to cultured cells only.<sup>25</sup>

Previous work from this laboratory has examined the response of cardiac L-type  $\text{Ca}^{2+}$  channels to a local application of NO donors and demonstrated a rather limited spread of intracellular cGMP from its site of production to the remote part of the cell not exposed to NO, where sGC was inactive.<sup>26</sup> In this study our aim was to characterize and compare in real time the changes in subsarcolemmal cGMP concentration in response to activators of sGC and pGC. For that purpose, we used the wild-type (WT)  $\alpha$ -subunit of the rat olfactory cyclic nucleotide-gated channel (CNGA2) as a real-time sensor for subsarcolemmal cGMP.<sup>27</sup> This channel binds cGMP with a  $>10$ -fold higher affinity than cAMP.<sup>27</sup> Here we describe experiments performed on adult rat ventricular myocytes (ARVMs) infected with an adenovirus encoding the WT CNGA2 (Ad-CNGA2). Using this model, we provide evidence for cGMP compartmentation and identify PDE2 and PDE5 isoforms as key elements in this phenomenon.

## Methods

Detailed methods are included in the online-only Data Supplement to this article.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

## Results

### Subsarcolemmal Localization of Recombinant CNGA2 Channels in ARVMs

In a first series of experiments, CNGA2 expression was investigated by immunofluorescence in ARVMs after 24 hours of culture. These results revealed the selective expression of recombinant WT-CNGA2 channels at the sarcolem-

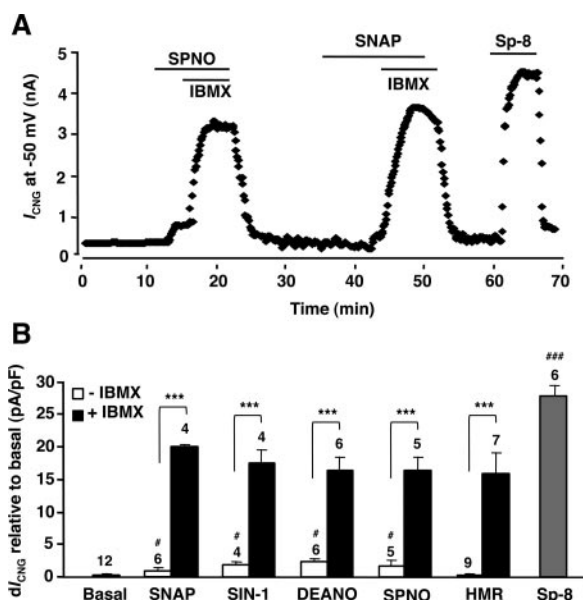
mal structures in Ad-CNGA2-infected cells (see online-only Data Supplement).

### Functional Expression of CNGA2 Channels in ARVMs

The CNGA2 current ( $I_{\text{CNG}}$ ) was recorded 24 hours after cell isolation in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free external solution. The individual  $I_{\text{CNG}}$  tracings in Figure 1A show that application of the membrane-permeant cGMP analogue Sp-8 ( $100 \mu\text{mol/L}$ ) induced a time-independent inward current at  $-50$  mV in Ad-CNGA2 cells but not in noninfected myocytes. This current displayed other characteristic features of cyclic nucleotide-gated (CNG) currents, such as  $\text{Mg}^{2+}$  block (Figure 1B) and a linear current-voltage relationship crossing at 0 mV (Figure 1C). Such current was not detected in noninfected ARVMs and was thus attributed to the functional expression of WT CNGA2. Figure 1D summarizes the effect of Sp-8 on  $I_{\text{CNG}}$  density. In noninfected myocytes, application of Sp-8 ( $100 \mu\text{mol/L}$ ) did not change  $I_{\text{CNG}}$  density ( $0.1 \pm 0.1$  pA/pF;  $n=5$ ), but it induced a large increase in Ad-CNGA2 cells ( $26.7 \pm 2.2$  pA/pF;  $n=7$ ). Application of a 10-fold lower concentration of Sp-8 ( $10 \mu\text{mol/L}$ ) increased  $I_{\text{CNG}}$  density to a value that was not statistically different from  $100 \mu\text{mol/L}$  ( $22.9 \pm 0.7$  pA/pF;  $n=4$ ), indicating that the current generated by  $100 \mu\text{mol/L}$  Sp-8 corresponded to the maximal amplitude of  $I_{\text{CNG}}$  that the infected cell can generate. Thus, the response of  $I_{\text{CNG}}$  to drug application was subsequently normalized to the response obtained at  $100 \mu\text{mol/L}$  Sp-8 (see online-only Data Supplement for details).

### cGMP Signals Elicited by Activation of sGC

We next investigated whether cGMP production by activation of sGC produced a detectable response of CNGA2 channels. Four different NO donors were tested, all at a  $100\text{-}\mu\text{mol/L}$  concentration: *S*-nitroso-*N*-acetyl-penicillamine (SNAP), 3-morpholinopyridone, diethylamine NONOate, and spermine NO (SPNO). Figure 2A shows a typical experiment in which SPNO and SNAP were tested. SPNO produced a small increase in  $I_{\text{CNG}}$ , whereas SNAP had no effect. On



**Figure 2.** cGMP signals elicited by activation of sGC. Typical experiment (A) and summary (B) of the effects of sGC activators on  $I_{\text{CNG}}$  in Ad-CNGA2 myocytes.  $I_{\text{CNG}}$  was measured as in Figure 1. Specific activation of sGC was achieved by a direct agonist HMR (10  $\mu\text{mol/L}$ ) or several NO donors (SNAP, SIN-1, DEANO, SPNO, all at 100  $\mu\text{mol/L}$  concentration) and nonselective PDE inhibition by IBMX (100  $\mu\text{mol/L}$ ). At the end of each experiment the cell was challenged with Sp-8 (100  $\mu\text{mol/L}$ ) as an internal control for WT-CNGA2 channel expression.  $***P < 0.005$  vs IBMX;  $\#P < 0.05$  vs basal;  $###P < 0.005$  vs basal.

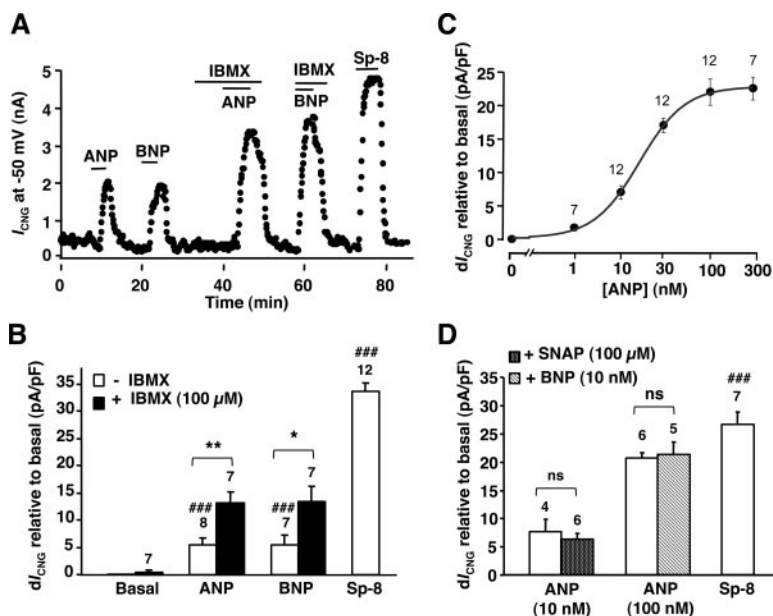
average (Figure 2B), all 4 NO donors produced only a small, although significant increase in  $I_{\text{CNG}}$ , which ranged from 5% to 8% of the maximal response obtained with Sp-8 (100  $\mu\text{mol/L}$ ). Unlike the NO donors, a direct activator of sGC, HMR1766 (HMR) (10  $\mu\text{mol/L}$ ),<sup>28</sup> produced no significant effect on  $I_{\text{CNG}}$  (Figure 2B). To examine whether the overall small increase in subsarcolemmal cGMP on activation of sGC was due to a limited production of cGMP or to a rapid

hydrolysis of the nucleotide by PDEs, we reexamined the effect of the NO donors and HMR in the presence of isobutyl methylxanthine (IBMX) (100  $\mu\text{mol/L}$ ), a broad-spectrum PDE inhibitor. Figure 2A shows that the effects of SPNO and SNAP on  $I_{\text{CNG}}$  were strongly enhanced by IBMX. On average (Figure 2B), whereas IBMX had no effect per se on  $I_{\text{CNG}}$ , it increased  $\approx 10$ -fold the effects of the 4 NO donors and of HMR to values ranging from 63% to 73% of the maximal response induced by Sp-8 (100  $\mu\text{mol/L}$ ). These results indicate that PDE activity prevents cGMP produced by sGC to reach the sarcolemmal membrane.

### cGMP Signals Elicited by Activation of pGC

Because pGC produces cGMP right at the sarcolemmal membrane, where CNG channels are expressed, we anticipated that the effect of natriuretic peptides on  $I_{\text{CNG}}$  might be more prominent than those of NO donors. Figure 3 shows this was indeed the case. In the typical experiment illustrated in Figure 3A, ANP (10 nmol/L) or BNP (10 nmol/L) produced clear and reversible increases in  $I_{\text{CNG}}$ . On average, ANP and BNP similarly increased  $I_{\text{CNG}}$  to  $\approx 25\%$  of the Sp-8 response (Figure 3B). The effect of ANP on  $I_{\text{CNG}}$  could be further increased by raising the concentration of the peptide, with a half-maximal effect seen between 10 and 30 nmol/L, and a maximal stimulation to  $\approx 80\%$  of the Sp-8 response seen at 100 nmol/L concentration (Figure 3C). BNP (10 nmol/L) had no additional effect on  $I_{\text{CNG}}$  when ANP was already present at maximal concentration (100 nmol/L), indicating that the 2 peptides likely share a common receptor (Figure 3D). More interestingly, SNAP (100  $\mu\text{mol/L}$ ) produced no additional effect on  $I_{\text{CNG}}$  stimulated by ANP, even though the concentration of ANP used in this case was not maximal (10 nmol/L; Figure 3D). This strongly suggests that cGMP rises in 2 different compartments on activation of sGC and pGC.

To evaluate the role of PDEs in the effects of ANP and BNP on  $I_{\text{CNG}}$ , the natriuretic peptides were tested again in the presence of IBMX (100  $\mu\text{mol/L}$ ). Figure 3A shows that the



**Figure 3.** cGMP signals elicited by activation of pGC. Typical experiment (A) and summary (B) of the effects of pGC activators on  $I_{\text{CNG}}$  in Ad-CNGA2 myocytes. Specific activation of pGC was achieved by ANP (10 nmol/L) or BNP (10 nmol/L) and nonselective PDE inhibition by IBMX (100  $\mu\text{mol/L}$ ). The effect of Sp-8 (100  $\mu\text{mol/L}$ ) serves as an internal control for WT-CNGA2 channel expression. C, Concentration-dependent effects of ANP in Ad-CNGA2 myocytes. D, Nonadditive effect of SNAP (100  $\mu\text{mol/L}$ ) or BNP (10 nmol/L) in ANP-activated  $I_{\text{CNG}}$ .  $*P < 0.05$  vs IBMX;  $###P < 0.005$  vs basal.

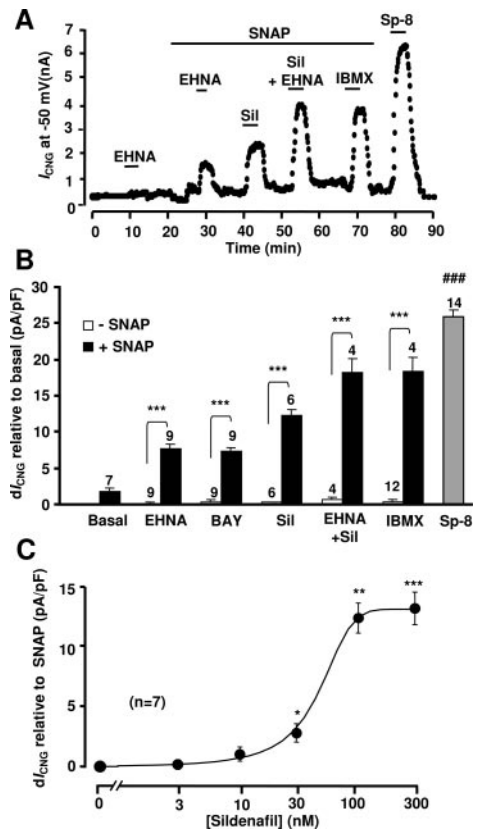
PDE inhibitor strongly increased the response of  $I_{\text{CNG}}$  to both ANP and BNP (10 nmol/L), indicating that PDEs limit the accumulation of cGMP at the plasma membrane. However, although the final amplitude of  $I_{\text{CNG}}$  was similar with ANP, BNP, and the NO donors when IBMX was present (60% to 70% of the Sp-8 response), the relative effect of IBMX was 5-fold larger when used on top of sGC activation than on top of pGC activation. This may indicate a stronger dependence on PDE activity of cGMP produced by sGC compared with pGC.

### Cross-Activation of WT CNGA2 by cAMP?

cGMP elevation can affect cAMP metabolism, in particular through inhibition of cGMP-inhibited PDE3 and activation of cGMP-stimulated PDE2.<sup>29</sup> Thus, an obvious question was whether subsarcolemmal cAMP changes contaminated the response of  $I_{\text{CNG}}$  to activators of sGC or pGC. To address this question, we infected ARVMs with an adenovirus encoding a double mutant of the CNGA2 channel (C460W/E583M-CNGA2), which possesses a 10-fold higher sensitivity toward cAMP than cGMP.<sup>16,27</sup> We found that neither SNAP (100  $\mu\text{mol/L}$ ;  $n=3$ ), nor HMR (10 nmol/L;  $n=3$ ), nor ANP (10 nmol/L;  $n=3$ ), nor BNP (10 nmol/L;  $n=3$ ), alone or in the presence of 100  $\mu\text{mol/L}$  IBMX, produced any significant increase in the CNG current measured with the C460W/E583M-CNGA2 channels, although, as a positive control, a direct stimulation of adenylyl cyclase with the hydrosoluble forskolin analogue L-858051 (100  $\mu\text{mol/L}$ ) strongly increased the current ( $n=6$ ; data not shown). Thus, the  $I_{\text{CNG}}$  changes measured with the WT-CNGA2 channel on manipulations of sGC or pGC pathways indeed reflect subsarcolemmal cGMP changes and not cAMP changes.

### Role of PDE Subtypes in cGMP Signals Generated by sGC

The aforementioned results indicate that PDE activity determines the intracellular distribution of cGMP in rat cardiomyocytes. In the following experiments, our aim was to identify the PDE subtypes that are involved. Four major PDE isoforms bind and hydrolyze intracellular cGMP in cardiomyocytes: PDE1, which is activated by  $\text{Ca}^{2+}$ -calmodulin; PDE2, which is activated by cGMP; PDE3, which hydrolyzes preferentially cAMP and is inhibited by cGMP; and PDE5, which is highly specific for cGMP. Selective inhibitors exist only for the 3 latter PDE isoforms and were used here: erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and Bay 60-7550 for PDE2, cilostamide for PDE3, and sildenafil (Viagra, Pfizer) for PDE5. None of the PDE inhibitors tested had any effect per se on basal  $I_{\text{CNG}}$  (Figure 4A and 4B, and data not shown for cilostamide). Figure 4A shows a typical experiment in which PDE2 and PDE5 inhibitors were tested during sGC activation with SNAP (100  $\mu\text{mol/L}$ ). As shown before (Figure 2B), SNAP alone induced a slight increase in  $I_{\text{CNG}}$ . However, addition of EHNA (10  $\mu\text{mol/L}$ ) or sildenafil (100 nmol/L) in the presence of SNAP amplified the effect of the NO donor considerably. On average (Figure 4B), EHNA in the presence of SNAP increased  $I_{\text{CNG}}$   $\approx$ 4-fold (from 5% to 22% of the maximal Sp-8 response). Bay 60-7550 (50 nmol/L), another more potent PDE2 inhibitor that, unlike EHNA, does not inhibit adenosine deaminase,<sup>30</sup> produced a

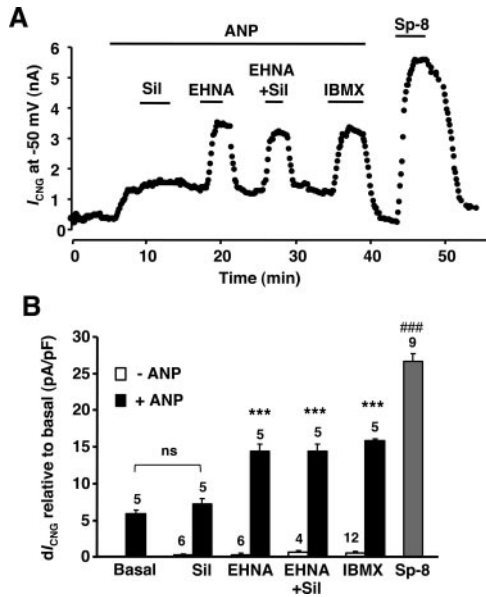


**Figure 4.** Role of PDE2 and PDE5 in cGMP signals generated by sGC. Typical experiment (A) and summary (B) of the effects of SNAP (100  $\mu\text{mol/L}$ ) alone or in the presence of PDE inhibitors: EHNA (10  $\mu\text{mol/L}$ ), sildenafil (Sil) (100 nmol/L), Bay 60-7550 (BAY) (50 nmol/L), and IBMX (100  $\mu\text{mol/L}$ ). The effect of Sp-8 (100  $\mu\text{mol/L}$ ) serves as an internal control for WT-CNGA2 channel expression. C, Concentration-dependent effects of sildenafil in Ad-CNGA2 myocytes in the presence of SNAP. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$  vs SNAP; ### $P<0.005$  vs basal  $I_{\text{CNG}}$ .

similar effect. Sildenafil produced a dose-dependent effect on  $I_{\text{CNG}}$  in the presence of SNAP (Figure 4C), with a maximal response observed at 100 nmol/L that was almost 2-fold larger than that of EHNA or Bay 60-7550 (from 5% to 39% of the Sp-8 response). When both EHNA and sildenafil were present,  $I_{\text{CNG}}$  increased further (Figure 4A), to  $\approx$ 60% of the maximal Sp-8 response (Figure 4B). Interestingly, the effect of EHNA plus sildenafil was identical to the effect of IBMX (Figure 4A and 4B), suggesting that no other PDE subtype is involved. This was confirmed in separate experiments in which PDE3 inhibition by cilostamide (1  $\mu\text{mol/L}$ ) had no effect on  $I_{\text{CNG}}$  in the presence of SNAP ( $n=5$ ; data not shown). Thus, PDE5 and to a lesser extent PDE2 contribute to limit the spread of cGMP on sGC activation.

### Role of PDE Subtypes in cGMP Signals Generated by pGC

Using the same strategy, we examined the contribution of PDE2 and PDE5 to the control of the cGMP pool generated by activation of pGC. Figure 5A shows a typical experiment in which PDE2 and PDE5 inhibitors were tested during pGC activation with ANP (10 nmol/L). As shown before (Figure 3), ANP induced a clear increase in  $I_{\text{CNG}}$ . However, in this



**Figure 5.** Role of PDE2 and PDE5 in cGMP signals generated by pGC. Typical experiment (A) and summary (B) of the effects of ANP (10 nmol/L) alone or in the presence of PDE inhibitors: EHNA (10 μmol/L), sildenafil (Sil) (100 nmol/L), and IBMX (100 μmol/L). The effect of Sp-8 (100 μmol/L) serves as an internal control for WT-CNGA2 channel expression. \*\*\**P*<0.005 vs NAP; ###*P*<0.005 vs basal.

case, addition of sildenafil (100 nmol/L) on top of ANP failed to increase *I*<sub>CNG</sub>, whereas PDE2 inhibition with EHNA (10 μmol/L) increased *I*<sub>CNG</sub> considerably. On average (Figure 5B), EHNA in the presence of ANP increased ≈2-fold *I*<sub>CNG</sub> (from 26% to 55% of the maximal Sp-8 response), whereas sildenafil had no effect. When both PDE inhibitors were present, the stimulation of *I*<sub>CNG</sub> was not different from the effect of EHNA alone or from the effect of IBMX (100 μmol/L; Figure 5A and 5B), suggesting that no other PDE was involved. This was confirmed in separate experiments in which PDE3 inhibition by cilostamide (1 μmol/L) had no effect on *I*<sub>CNG</sub> in the presence of ANP (n=5; data not shown). Thus, unlike when cGMP is produced by sGC, PDE5 is not involved in the hydrolysis of cGMP produced by pGC, which is entirely being hydrolyzed by PDE2.

### Discussion

The use of recombinant CNG channels as cyclic nucleotide biosensors was developed in a series of elegant studies in model cell lines for the measurement of intracellular cAMP.<sup>27,31,32</sup> In the present study we have applied this methodology to differentiated adult cardiomyocytes in primary culture to measure in real time the changes in subsarcolemmal cGMP. Our study reveals major differences in the spatiotemporal distribution of intracellular cGMP on activation of its 2 main routes of synthesis. When pGC is activated by the natriuretic peptides ANP and BNP, cGMP rises steadily beneath the membrane; on the contrary, when sGC is activated by NO donors or HMR, a direct activator,<sup>28</sup> subsarcolemmal cGMP is barely affected. This difference indicates that intracellular cGMP is not uniformly distributed within the cell but is instead compartmentalized in separate, slowly

equilibrating pools. We found that cyclic nucleotide PDEs play a key role in this compartmentation, with different PDE subtypes controlling the pGC (particulate) and sGC (soluble) cGMP pools. Indeed, PDE5 appears to control the soluble but not the particulate pool, whereas the latter is under the exclusive control of PDE2.

Several studies have shown differential effects of cGMP produced by sGC and pGC on various cell functions. For instance, in a human epithelial cell line, activation of pGC and not sGC is coupled to the inhibition of Ca<sup>2+</sup> efflux, whereas activation of sGC and not pGC is involved in the stimulation of Ca<sup>2+</sup> sequestration into the intracellular Ca<sup>2+</sup> stores.<sup>10</sup> In pig airway smooth muscle cells, stimulation of pGC causes relaxation exclusively by decreasing intracellular Ca<sup>2+</sup> concentration, whereas stimulation of sGC decreases both Ca<sup>2+</sup> concentration and Ca<sup>2+</sup> sensitivity of the myofilaments.<sup>11</sup> In human endothelial cells from umbilical vein, activation of sGC induces relaxation in a more efficient manner than does activation of pGC.<sup>14</sup> Differences between pGC and sGC activation have also been reported in cardiac preparations. For instance, in frog ventricular myocytes, sGC activation caused a pronounced inhibition of the L-type Ca<sup>2+</sup> current (*I*<sub>Ca,L</sub>) on stimulation by intracellular cAMP,<sup>33</sup> whereas pGC activation had little effect.<sup>34</sup> In rabbit atria, activation of pGC caused a larger cAMP accumulation (via PDE3 inhibition), cGMP efflux, and ANP release than activation of sGC.<sup>12</sup> In mouse ventricular myocytes, pGC activation caused a decrease in intracellular Ca<sup>2+</sup> transient, whereas sGC activation had little effect.<sup>13</sup>

Given the separate sources of cGMP within the cell, ie, membrane versus cytosol, it was tempting to speculate in the aforementioned studies that the functional differences between pGC and sGC activators arose from a functional compartmentation of cGMP. However, because NO donors were used in these studies to activate sGC and NO and reactive nitrogen species may affect many other processes besides sGC, validation of that hypothesis required a more direct assessment of the cGMP compartmentation. To our knowledge, the present study provides the first direct evidence for intracellular cGMP compartmentation in intact adult cardiac myocytes. We took advantage of the method developed by Rich and coworkers<sup>27,31</sup> in HEK293 cells to follow in real time cGMP changes beneath the sarcolemmal membrane using the WT-CNGA2 channel as a readout. We confirmed by immunocytochemistry (see online-only Data Supplement) that this channel is not normally expressed in native rat cardiomyocytes<sup>35</sup> but becomes functionally expressed 24 hours after infection of the myocytes with the Ad-CNGA2 construct developed by Fagan et al.<sup>32</sup> A similar method was used recently to follow cAMP changes in the same preparation,<sup>16</sup> with the use of mutants of the CNGA2 channel with a higher sensitivity toward cAMP versus cGMP.

The WT-CNGA2 channel responds to cGMP changes with a threshold at 0.1 to 0.5 μmol/L concentration, a *K*<sub>1/2</sub> of 1.4 μmol/L, and a maximal amplitude obtained at 5 to 10 μmol/L cGMP.<sup>27</sup> With the use of these parameters and the amplitude of the CNG current measured at the end of each experiment after application of a saturating concentration (100 μmol/L) of the cGMP analogue Sp-8, it was possible to give a rough

estimate of the subsarcolemmal cGMP concentration reached in each of the experimental conditions tested here. We found that cGMP level was  $<0.5 \mu\text{mol/L}$  when sGC was maximally activated (with any of the 4 NO donors tested, all used at  $100 \mu\text{mol/L}$  concentration), whereas it reached  $2.3 \mu\text{mol/L}$  on maximal activation of pGC (with  $300 \text{ nmol/L}$  ANP). This 5-fold difference was not due to a lower activity of sGC versus pGC because, on the contrary, the level of cGMP rose proportionally higher during sGC versus pGC activation on PDE inhibition with IBMX (compare Figures 2B and 3B). Thus, we conclude that intracellular cGMP is highly compartmentalized within adult rat cardiomyocytes and that homogeneous distribution is prevented by PDE activity.

At least 4 different PDE isoforms account for the hydrolysis of cGMP in heart tissue<sup>36</sup>: PDE1, which is activated by  $\text{Ca}^{2+}$ -calmodulin and hydrolyzes cAMP and cGMP equally well, at least in heart; PDE2, which hydrolyzes either cAMP or cGMP and is stimulated by cGMP binding to amino terminal allosteric regulatory sites known as GAF domains<sup>37</sup>; PDE3, which has a similar affinity for cAMP and cGMP but a higher  $V_{\text{max}}$  for the former, making it a cGMP-inhibited cAMP-PDE; and PDE5, which is highly specific for cGMP and is also activated by cGMP acting both on GAF domains and via phosphorylation by PKG.<sup>38</sup> A fifth isoform (PDE9A), highly specific for cGMP, has been shown to be expressed at the mRNA level in human<sup>39</sup> but not mouse heart.<sup>40</sup> All PDE isoforms but PDE9A<sup>39,40</sup> are inhibited by IBMX, and a number of drugs have been developed as selective inhibitors of PDE2, PDE3, and PDE5. In this study we used EHNA<sup>41</sup> and Bay 60-7550<sup>30</sup> to evaluate the contribution of PDE2, cilostamide for PDE3, and sildenafil (Viagra) for PDE5.<sup>38</sup> PDE1, which is essentially expressed in a nonmyocyte fraction of cardiac tissue,<sup>42</sup> is unlikely to play a major role under our experimental conditions because  $\text{Ca}^{2+}$  ions were omitted from both extracellular and pipette solutions.

Our results demonstrate that both particulate and soluble pools of cGMP are controlled by PDE2. Until now, PDE2 has been shown to be essentially involved in the control of intracellular cAMP concentration. For instance, in primary bovine glomerulosa cells, PDE2 is the main enzyme by which ANP inhibits aldosterone secretion,<sup>43</sup> and this is achieved by a marked reduction in cAMP level due to cGMP activation of PDE2.<sup>43</sup> In frog ventricular<sup>26,33,41</sup> and human atrial myocytes,<sup>44,45</sup> NO donors or intracellular cGMP induces activation of PDE2, causing a decrease in cAMP and an inhibition of  $I_{\text{Ca,L}}$ .<sup>29</sup> Our study demonstrates that PDE2 is also an important component of the cGMP catabolism in cardiomyocytes. Its preferential location in the membrane fraction of cardiac myocytes<sup>46</sup> likely accounts for the unique role of PDE2 in the control of the particulate pool of cGMP.

PDE5 is highly expressed in vascular smooth muscle, and its inhibition is a primary target for the treatment of erectile dysfunction and pulmonary hypertension.<sup>38,47</sup> Despite the detection of its mRNA in heart,<sup>48</sup> the presence and role of a functional PDE5 protein in cardiomyocytes have been controversial.<sup>36,49</sup> However, recent evidence strongly supports the presence of a functional PDE5 in cardiac myocytes.<sup>2</sup> First, PDE5 protein has been detected by immunocytochemistry and found to be located both in the cytosol and at Z bands in

normal cardiomyocytes from dog<sup>50</sup> and mouse ventricle.<sup>25</sup> Second, PDE5 inhibition with the use of sildenafil (Viagra) decreases the  $\beta$ -adrenergic stimulation of cardiac systolic and diastolic function in dog,<sup>50</sup> mouse,<sup>25</sup> and human<sup>51</sup> as well as the  $\beta$ -stimulation of  $I_{\text{Ca,L}}$  in guinea pig ventricular myocytes.<sup>52</sup> Finally, chronic exposure to sildenafil was found to prevent and reverse cardiac hypertrophy in mouse hearts exposed to sustained pressure overload.<sup>5</sup> With our study, we confirm the presence of functional PDE5 in ARVMs. In addition, we demonstrate that this PDE subtype specifically controls the soluble pool of cGMP but not the particulate pool. This could be either because PDE5 is more closely compartmentalized with sGC than pGC or because PKG, which activates PDE5,<sup>38</sup> is compartmentalized with sGC but not pGC. Co-administration of sildenafil and an NO donor but not ANP produced a large rise of cGMP at the membrane, favoring the inhibition via PKG of L-type  $\text{Ca}^{2+}$  channels.<sup>53</sup> Interestingly, sildenafil and other new PDE5 inhibitors are contraindicated in men who use nitrate medications<sup>54</sup> because the coadministration of these agents may cause cGMP to accumulate, resulting in marked and unpredictable decreases in blood pressure, accompanied by symptoms of hypotension. Inasmuch as our results in ARVMs apply to vascular smooth muscle, this could be due to a specific role of PDE5 in controlling the soluble pool of cGMP. Under such circumstances, pGC and PDE5 might control different pools of cGMP, and one may speculate that coadministration of PDE5 inhibitors and natriuretic peptides instead of nitrates might lead to less adverse effects. Further studies will be required to test this hypothesis.

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### Disclosures

Dr Fischmeister has served as an expert witness for Pfizer. The other authors report no conflicts.

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### CLINICAL PERSPECTIVE

Cyclic guanosine monophosphate (cGMP) is the common second messenger for the cardiovascular effects of nitric oxide (NO) and natriuretic peptides, such as atrial or brain natriuretic peptide, which activate the soluble and particulate forms of guanylyl cyclase, respectively. However, natriuretic peptides and NO donors exert different effects on cardiac and vascular smooth muscle function. In this study we demonstrate that these differences are due to different intracellular compartmentation of cGMP. We used the rat olfactory cyclic nucleotide-gated (CNG) channel as a reporter gene to measure subsarcolemmal cGMP concentration in adult rat ventricular myocytes. We found that when particulate guanylyl cyclase is activated by atrial natriuretic peptide and brain natriuretic peptide, cGMP steadily rises beneath the membrane; on the contrary, when soluble guanylyl cyclase is activated by NO donors, subsarcolemmal cGMP is barely affected. This difference indicates that intracellular cGMP is not uniformly distributed within the cell but is instead compartmentalized in separate, slowly equilibrating pools. We found that cyclic nucleotide phosphodiesterases (PDEs) play a key role in this compartmentation, with different PDE subtypes controlling the particulate and soluble cGMP pools. In particular, when sildenafil (Viagra) was used to inhibit PDE5, the effect of NO donors on subsarcolemmal cGMP was strongly increased, whereas that of atrial natriuretic peptide was not affected. Our results indicate that PDE5 controls the soluble but not the particulate pool, whereas the latter is under the exclusive control of the cGMP-stimulated PDE2. Inasmuch as our results in cardiac myocytes apply to vascular smooth muscle, they may help to explain why sildenafil and other PDE5 inhibitors are contraindicated in men who use nitrate medications.