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cGMP compartmentation in rat cardiac myocytes

**Liliana R. V. Castro, MS^{1,2,3}, Ignacio Verde, PhD³, Dermot M. F. Cooper, PhD⁴
& Rodolphe Fischmeister, PhD^{1,2}**

¹INSERM U-769, Châtenay-Malabry, F-92296 France;

²Université Paris-Sud, Faculté de Pharmacie, IFR-141, Châtenay-Malabry, F-92296 France;

³Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6201-001
Covilhã, Portugal

⁴Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge
CB2 1PD, United Kingdom

Correspondence to:

Rodolphe FISCHMEISTER

INSERM U-769

Faculté de Pharmacie

5, Rue J.-B. Clément

F-92296 Châtenay-Malabry Cedex

France

Tel. +33-1-46 83 57 57

Fax +33-1-46 83 54 75

E-mail: fisch@vjf.inserm.fr

Background – Cyclic GMP is the common second messenger for the cardiovascular effects of nitric oxide (NO) and natriuretic peptides, such as ANP or BNP, which activate, respectively, the soluble and particulate form of guanylyl cyclase. Yet, 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 CNG channel α subunit and recording of the associated cGMP-gated current (I_{CNG}). ANP (10 nM) or BNP (10 nM) induced a clear activation of I_{CNG} while NO-donors (SNAP, SNP, DEANO, SIN-1, spermine NO, all at 100 μM) had little effect. The I_{CNG} current was strongly potentiated by non-selective PDE inhibition with IBMX (100 μM) and by the PDE2 inhibitors EHNA (10 μM) and Bay 60-7550 (50 nM). Surprisingly, sildenafil, a PDE5 inhibitor, produced a dose-dependent increase of I_{CNG} activated by NO-donors but had no effect (at 100 nM) on the current elicited by ANP.

Conclusions – These results indicate that, in rat cardiomyocytes: i) the ‘particulate’ cGMP pool is readily accessible at the plasma membrane, while the ‘soluble’ pool is not; ii) 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.

Key words: cGMP ■ nitric oxide ■ natriuretic peptides ■ phosphodiesterases ■ sildenafil

Introduction

Cyclic GMP (cGMP) is an 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,^{1,2} while chronic elevation prevents and reverses cardiac hypertrophy.³⁻⁵ cGMP synthesis is controlled by two 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 (CNP);⁶⁻⁸ and a soluble guanylyl cyclase (sGC) present in the cytosol and activated by nitric oxide (NO).^{8,9}

Although NO and natriuretic peptides use cGMP as a common second messenger, there are many instances in which activation of pGC and sGC lead to different functional effects.¹⁰⁻¹⁴ 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 cAMP, a cGMP closely related counterpart.¹⁵ For example, work from this laboratory has shown that cAMP compartmentation occurs during β -adrenergic stimulation of adult cardiac myocytes¹⁶ and is responsible for a local activation of cardiac L-type Ca^{2+} channels.^{17,18} Such a compartmentation involves activation of subsarcolemmal cyclic nucleotide phosphodiesterase (PDE) by cAMP-dependent protein kinase (PKA),¹⁶ most likely through the formation of multimolecular signaling complexes involving PKA, PKA anchoring proteins (AKAPs) and PDE4 isoforms.¹⁹⁻²¹

Despite its crucial role in smooth muscle relaxation, the subcellular spatiotemporal organization of cGMP signaling has been relatively unexplored. AKAP-like proteins have been identified for targeting cGMP-dependent protein kinase (PKG) to protein substrates,^{22,23} but their characterization remains limited. FRET-based cGMP fluorescent probes have been

developed²⁴ but their availability as plasmid constructs currently restricts their use to cultured cells only.²⁵

Previous work from this laboratory has examined the response of cardiac L-type 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.²⁶ 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) α -subunit of the rat olfactory cyclic nucleotide-gated channel (CNGA2) as a real-time sensor for subsarcolemmal cGMP.²⁷ This channel binds cGMP with a >10-fold higher affinity than cAMP.²⁷ 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 phosphodiesterases 2 and 5 isoforms as key elements in this phenomenon.

Methods

Detailed methods are included in the online-only Data Supplement to this article, which is available at <http://www.circulationaha.org>.

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 h of culture. These results revealed the selective expression of recombinant WT CNGA2 channels at the sarcolemmal structures in Ad-CNGA2 infected cells (see Data Supplement).

Functional Expression of CNGA2 Channels in ARVMs

The CNGA2 current (I_{CNG}) was recorded 24 h after cell isolation, in Ca^{2+} - and Mg^{2+} -free external solution. The individual I_{CNG} traces in Fig. 1A show that application of the membrane permeant cGMP analogue Sp-8 (100 μM) induced a time-independent inward current at -50 mV in Ad-CNGA2 cells, but not in non infected myocytes. This current displayed other characteristic features of CNG currents, such as Mg^{2+} block (Fig. 1B) and a linear current-voltage relationship crossing at 0 mV (Fig. 1C). Such current was not detected in non infected ARVMs and was thus attributed to the functional expression of WT CNGA2. Figure 1D summarizes the effect of SP-8 on I_{CNG} density (dI_{CNG}). In non infected myocytes, application of Sp-8 (100 μM) did not change dI_{CNG} ($+0.1 \pm 0.1$ 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 μM) increased dI_{CNG} to a value which was not statistically different from 100 μM (22.9 ± 0.7 pA/pF, $n=4$) indicating that the current generated by 100 μM Sp-8 corresponded to the maximal amplitude of I_{CNG} that the infected cell can generate. Thus, the response of I_{CNG} to drug application was subsequently normalized to the response obtained at 100 μM Sp-8 (see Data Supplement for details).

cGMP Signals Elicited by Activation of Soluble Guanylyl Cyclase

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 μM concentration: SNAP, SIN-1, DEANO and SPNO. Figure 2A shows a typical experiment in which SPNO and SNAP were tested. While SPNO produced a small increase in I_{CNG} , SNAP had no effect. On average (Fig. 2B), all four NO-donors produced only a small, although

significant increase in I_{CNG} , which ranged from 5 to 8% of the maximal response obtained with Sp-8 (100 μM). Unlike the NO-donors, a direct activator of sGC, HMR1766 (HMR, 10 μM),²⁸ produced no significant effect on I_{CNG} (Fig. 2B). To examine whether the overall small increase in subsarcolemmal cGMP upon activation of sGC was due to a limited production of cGMP or to a rapid hydrolysis of the nucleotide by phosphodiesterases (PDEs), we re-examined the effect of the NO-donors and HMR in the presence of isobutyl methylxanthine (IBMX, 100 μM), a broad spectrum PDE inhibitor. Figure 2A shows that the effects of SPNO and SNAP on I_{CNG} were strongly enhanced by IBMX. On average (Fig. 2B), while IBMX had no effect *per se* on I_{CNG} , it increased ~10-fold the effects of the four NO-donors and of HMR to values ranging from 63 to 73% of the maximal response induced by Sp-8 (100 μM). These results indicate that PDE activity prevents cGMP produced by sGC to reach the sarcolemmal membrane.

cGMP Signals Elicited by Activation of Particulate Guanylyl Cyclase

Because particulate guanylyl cyclase (pGC) produces cGMP right at the sarcolemmal membranes, where CNG channels are expressed, we anticipated that the effect of natriuretic peptides on I_{CNG} might be more prominent than those of NO-donors. Figure 3 shows this was indeed the case. In the typical experiment illustrated in Fig. 3A, ANP (10 nM) or BNP (10 nM) produced clear and reversible increases in I_{CNG} . On average, ANP and BNP similarly increased I_{CNG} to ~25% of the Sp-8 response (Fig. 3B). The effect of ANP on I_{CNG} could be further increased by raising the concentration of the peptide, with a half-maximal effect seen between 10 and 30 nM, and a maximal stimulation to ~80% of the Sp-8 response seen at 100 nM concentration (Fig. 3C). BNP (10 nM) had no additional effect on I_{CNG} when ANP was already present at maximal concentration (100 nM), indicating that the two peptides likely share a common receptor (Fig. 3D). More interestingly, SNAP (100 μM) produced no

additional effect on I_{CNG} stimulated by ANP either, even though the concentration of ANP used in this case was not maximal (10 nM, Fig. 3D). This strongly suggests that cGMP rises in two different compartments upon activation of sGC and pGC.

To evaluate the role of PDEs in the effects of ANP and BNP on I_{CNG} , the natriuretic peptides were tested again in the presence of IBMX (100 μM). Figure 3A shows that the PDE inhibitor strongly increased the response of I_{CNG} to both ANP and BNP (10 nM) indicating that PDEs limit the accumulation of cGMP at the plasma membrane. However, although the final amplitude of I_{CNG} was similar with ANP, BNP and the NO-donors when IBMX was present (60-70% 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 as compared to pGC.

Cross-Activation of WT CNGA2 by cAMP?

Cyclic GMP elevation can affect cAMP metabolism, in particular through inhibition of cGMP-inhibited PDE3 and activation of cGMP-stimulated PDE2.²⁹ Thus, an obvious question was whether subsarcolemmal cAMP changes contaminated the response of I_{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 towards cAMP than cGMP.^{16,27} We found that neither SNAP (100 μM , n=3), nor HMR (10 nM, n=3), nor ANP (10 nM, n=3), nor BNP (10 nM, n=3), alone or in presence of 100 μM 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 analog L-858051 (100 μM) strongly increased the current (n=6, data not shown). Thus, the I_{CNG} changes measured with

the WT CNGA2 channel upon 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 results above 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 which are involved. Four major PDE isoforms bind and hydrolyze intracellular cGMP in cardiomyocytes: PDE1 which is activated by Ca^{2+} -calmodulin; PDE2 which is activated by cGMP; PDE3 which hydrolyzes preferentially cAMP and is inhibited by cGMP; PDE5 which is highly specific for cGMP. Selective inhibitors exist only for the three latter PDE isoforms and were used here: EHNA and Bay 60-7550 for PDE2, cilostamide for PDE3 and sildenafil (Viagra) for PDE5. None of the PDE inhibitors tested had any effect *per se* on basal I_{CNG} (Fig. 4A & B, and data not shown for cilostamide). Figure 4A shows a typical experiment where PDE2 and PDE5 inhibitors were tested during sGC activation with SNAP (100 μM). As shown before (Fig. 2B), SNAP alone induced a slight increase in I_{CNG} . However, addition of EHNA (10 μM) or sildenafil (Sil, 100 nM) in the presence of SNAP considerably amplified the effect of the NO-donor. On average (Fig. 4B), EHNA in the presence of SNAP increased ~4-fold I_{CNG} (from 5 to 22% of the maximal Sp-8 response). Bay 60-7550 (BAY, 50 nM), another more potent PDE2 inhibitor which, unlike EHNA, does not inhibit adenosine deaminase,³⁰ produced a similar effect. Sildenafil produced a dose-dependent effect on I_{CNG} in the presence of SNAP (Fig. 4C), with a maximal response observed at 100 nM which was almost 2-fold larger than that of EHNA or BAY (from 5 to 39% of the Sp-8 response). When both EHNA and Sil were present, I_{CNG} increased further (Fig. 4A), to ~60% of the maximal Sp-8 response (Fig. 4B). Interestingly, the effect of EHNA+Sil was identical to the effect of IBMX (Fig. 4A & B), suggesting that no other PDE

subtype is involved. This was confirmed in separate experiments where PDE3 inhibition by cilostamide (1 μ M) had no effect on I_{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 upon 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 where PDE2 and PDE5 inhibitors were tested during pGC activation with ANP (10 nM). As shown before (Fig. 3), ANP induced a clear increase in I_{CNG} . However, in this case, addition of Sil (100 nM) on top of ANP failed to increase I_{CNG} while PDE2 inhibition with EHNA (10 μ M) considerably increased I_{CNG} . On average (Fig. 5B), EHNA in the presence of ANP increased ~2-fold I_{CNG} (from 26 to 55% of the maximal Sp-8 response), while Sil had no effect. When both PDE inhibitors were present, the stimulation of I_{CNG} was not different from the effect of EHNA alone nor from the effect of IBMX (100 μ M, Fig. 5A & B) suggesting that no other PDE was involved. This was confirmed in separate experiments where PDE3 inhibition by cilostamide (1 μ M) had no effect on I_{CNG} 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.^{27,31,32} Here, 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 spatio-temporal distribution of intracellular cGMP upon activation of its two main routes of synthesis. When particulate guanylyl cyclase (pGC) is activated by the natriuretic peptides ANP and BNP, cGMP steadily raises beneath the membrane; on the contrary, when soluble guanylyl cyclase (sGC) is activated by NO-donors or HMR1766, a direct activator,²⁸ 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 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 Ca²⁺ efflux, while activation of sGC and not pGC is involved in the stimulation of Ca²⁺ sequestration into the intracellular Ca²⁺ stores.¹⁰ In pig airway smooth muscle cells, stimulation of pGC causes relaxation exclusively by decreasing intracellular Ca²⁺ concentration, whereas stimulation of sGC decreases both Ca²⁺ concentration and Ca²⁺ sensitivity of the myofilaments.¹¹ In human endothelial cells from umbilical vein (HUVEC), activation of sGC induces relaxation in a more efficient manner than does activation of pGC.¹⁴ Differences between pGC and sGC activation have also been reported in cardiac preparations. For instance, in frog ventricular myocytes, sGC activation causes a pronounced inhibition of the L-type Ca²⁺ current ($I_{Ca,L}$) upon stimulation by intracellular cAMP³³ while pGC activation has little effect.³⁴ In rabbit atria, activation of pGC caused a larger cAMP accumulation (via PDE3 inhibition), cGMP efflux and ANP release

than activation of sGC.¹² In mouse ventricular myocytes, pGC activation caused a decrease in intracellular Ca²⁺ transient while sGC activation had little effect.¹³

Given the separate sources of cGMP within the cell, *i.e.* membrane vs. cytosol, it was tempting to speculate in the above studies that the functional differences between pGC and sGC activators arose from a functional compartmentation of cGMP. However, since 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, this 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 co-workers^{27,31} 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 Data Supplement) that this channel is not normally expressed in native rat cardiomyocytes,³⁵ but becomes functionally expressed 24h after infection of the myocytes with the Ad-CNGA2 construct developed by Fagan *et al.*³² A similar method was used recently to follow cAMP changes in the same preparation,¹⁶ using mutants of the CNGA2 channel with a higher sensitivity towards cAMP *vs.* cGMP.

The WT-CNGA2 channel responds to cGMP changes with a threshold at 0.1-0.5 μM concentration, a $K_{1/2}$ of 1.4 μM and a maximal amplitude obtained at 5-10 μM cGMP.²⁷ Using these parameters and the amplitude of the CNG current measured at the end of each experiment after application of a saturating concentration (100 μM) of the cGMP analog 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 below 0.5 μM when sGC was maximally activated (with any of the four NO-donors tested, all used at 100 μM concentration), while it reached 2.3 μM upon maximal activation of pGC (with 300

nM ANP). This 5-fold difference was not due to a lower activity of sGC *vs.* pGC, because, on the contrary, the level of cGMP rose proportionally higher during sGC *vs.* pGC activation upon PDE inhibition with IBMX (compare Fig. 2B & 3B). Thus, we conclude that intracellular cGMP is highly compartmentalized within adult rat cardiomyocytes, and that homogenous distribution is prevented by PDE activity.

At least four different PDE isoforms account for the hydrolysis of cGMP in heart tissue:³⁶ PDE1 which is activated by Ca²⁺-calmodulin and hydrolyzes equally well cAMP and cGMP, 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;³⁷ PDE3 which has a similar affinity for cAMP and cGMP, but a higher V_{max} for the former, making it a cGMP-inhibited cAMP-PDE; PDE5 which is highly specific for cGMP and is also activated by cGMP acting both on GAF domains and via phosphorylation by PKG.³⁸ A fifth isoform (PDE9A), highly specific of cGMP, has been shown to be expressed at the mRNA level in human³⁹ but not mouse heart.⁴⁰ All PDE isoforms but PDE9A^{39,40} are inhibited by IBMX and a number of drugs have been developed as selective inhibitors of PDE2, 3 and 5. In this study, we used EHNA⁴¹ and Bay 60-7550³⁰ to evaluate the contribution of PDE2, cilostamide for PDE3, and sildenafil (Viagra) for PDE5.³⁸ PDE1, which is essentially expressed in a non-myocyte fraction of cardiac tissue,⁴² unlikely plays a major role under our experimental conditions, since Ca²⁺ ions were omitted both from 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,⁴³ and this is achieved by a marked reduction in cAMP level due to cGMP activation of PDE2.⁴³ In frog ventricular^{26,33,41} and human atrial myocytes,^{44,45} NO-donors or intracellular cGMP induce

activation of PDE2 causing a decrease in cAMP and an inhibition of $I_{Ca,L}$.²⁹ 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 myocytes⁴⁶ 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.^{38,47} In spite of the detection of its mRNA in heart,⁴⁸ the presence and role of a functional PDE5 protein in cardiomyocytes has been controversial.^{36,49} However, recent evidence strongly support the presence of a functional PDE5 in cardiac myocytes.² 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 dog⁵⁰ and mouse ventricle.²⁵ Second, PDE5 inhibition using sildenafil (Viagra) decreases the β -adrenergic-stimulation of cardiac systolic and diastolic function in dog,⁵⁰ mouse,²⁵ and human⁵¹ as well as the β -stimulation of $I_{Ca,L}$ in guinea pig ventricular myocytes.⁵² Finally, chronic exposure to sildenafil was found to prevent and reverse cardiac hypertrophy in mouse hearts exposed to sustained pressure overload.⁵ 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 PDE5,³⁸ 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 Ca^{2+} channels.⁵³ Interestingly, sildenafil and other new PDE5 inhibitors are contraindicated in men who use nitrate medications,⁵⁴ because the co-administration 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 co-administration 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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Conflict of Interest

Rodolphe Fischmeister has served as expert witness for Pfizer.

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Figure Legends

Figure 1. Functional expression of CNGA2 channels in ARVMs. A, individual current traces of I_{CNG} obtained during 200 ms voltage pulse at -50 mV in infected (Ad-CNGA2) or non infected (NI) myocytes. Each trace was recorded in the presence of control solution supplemented (solid lines) or not (dotted lines) with the cGMP analog Sp-8 (100 μM). B, time course of I_{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 μM). The Sp-8 activated current was blocked in a reversible manner by the presence of Mg^{2+} (10 mM). C, current-voltage relationships of I_{CNG} obtained with control solution (■) in the presence of 100 μM Sp-8 alone (▲) or with 10 mM Mg^{2+} (●). D, density of I_{CNG} obtained in Ad-CNGA2 or NI myocytes challenged with Sp-8 (10 μM or 100 μM). ***, $p < 0.005$ vs. NI cells.

Figure 2. cGMP signals elicited by activation of soluble guanylyl cyclase (sGC). Typical experiment (A) and summary (B) of the effects of sGC activators on I_{CNG} in Ad-CNGA2 myocytes. I_{CNG} was measured as in Fig. 1. Specific activation of sGC was achieved by a direct agonist HMR1766 (HMR, 10 μM) or several NO-donors (SNAP, SIN-1, DEANO, SPNO, all at 100 μM concentration) and non selective PDE inhibition by IBMX (100 μM). At the end of each experiment the cell was challenged with Sp-8 (100 μM) as an internal control for WT-CNGA2 channel expression. ***, $p < 0.005$ vs. IBMX; #, $p < 0.05$ vs. basal; ###, $p < 0.005$ vs. basal.

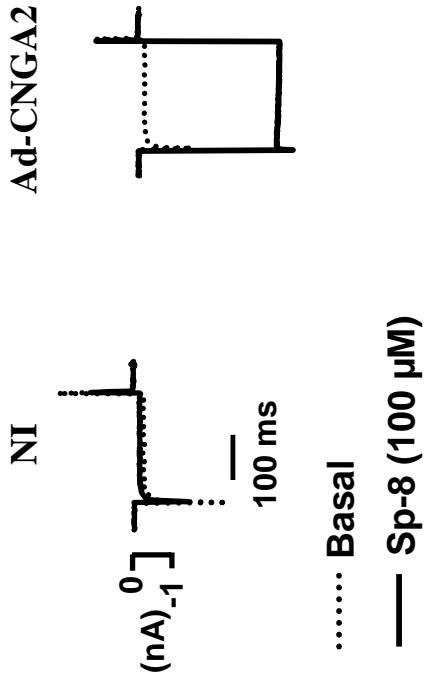
Figure 3. cGMP signals elicited by activation of particulate guanylyl cyclase (pGC). Typical experiment (A) and summary (B) of the effects of pGC activators on I_{CNG} in Ad-CNGA2

myocytes. Specific activation of pGC was achieved by ANP (10 nM) or BNP (10 nM) and non selective PDE inhibition by IBMX (100 μ M). The effect of Sp-8 (100 μ M) serves as an internal control for WT-CNGA2 channel expression. C, Concentration-dependent effects of ANP in Ad-CNGA2 myocytes. D, Non additive effect of both SNAP (100 μ M) or BNP (10 nM) in ANP activated I_{CNG} . *, $p < 0.05$ vs. IBMX; ###, $p < 0.005$ vs. basal; ns, non significant.

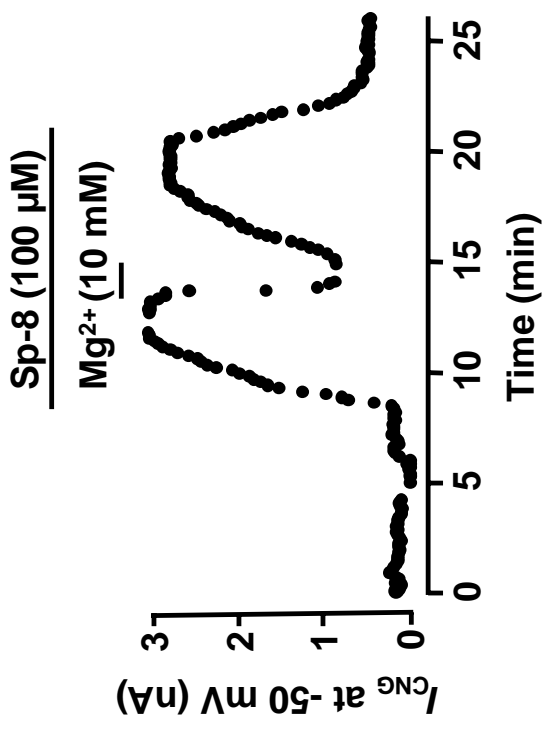
Figure 4. Role of PDE2 and PDE5 in cGMP signals generated by sGC. Typical experiment (A) and summary (B) of the effects of SNAP (100 μ M) alone or in the presence of PDE inhibitors: EHNA (10 μ M), sildenafil (Sil, 100 nM), Bay 60-750 (BAY, 50 nM), IBMX (100 μ M). The effect of Sp-8 (100 μ M) serves as an internal control for WT-CNGA2 channel expression. C, concentration-dependent effects of Sil in Ad-CNGA2 myocytes in the presence of SNAP. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.005$ vs. SNAP; ###, $p < 0.005$ vs. basal I_{CNG} .

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 nM) alone or in the presence of PDE inhibitors: EHNA (10 μ M), sildenafil (Sil, 100 nM), IBMX (100 μ M). The effect of Sp-8 (100 μ M) serves as an internal control for WT-CNGA2 channel expression. ***, $p < 0.005$ vs. SNAP; ###, $p < 0.005$ vs. basal; ns, non significant.

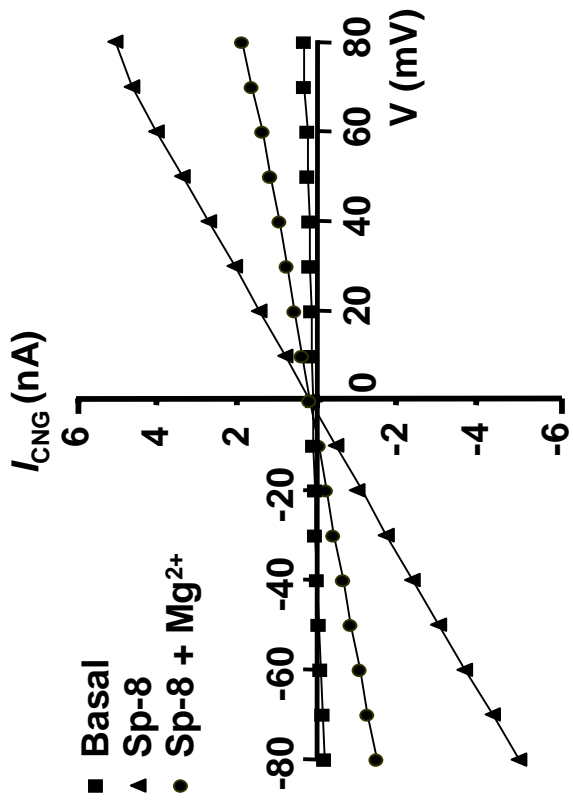
A



B



C



D

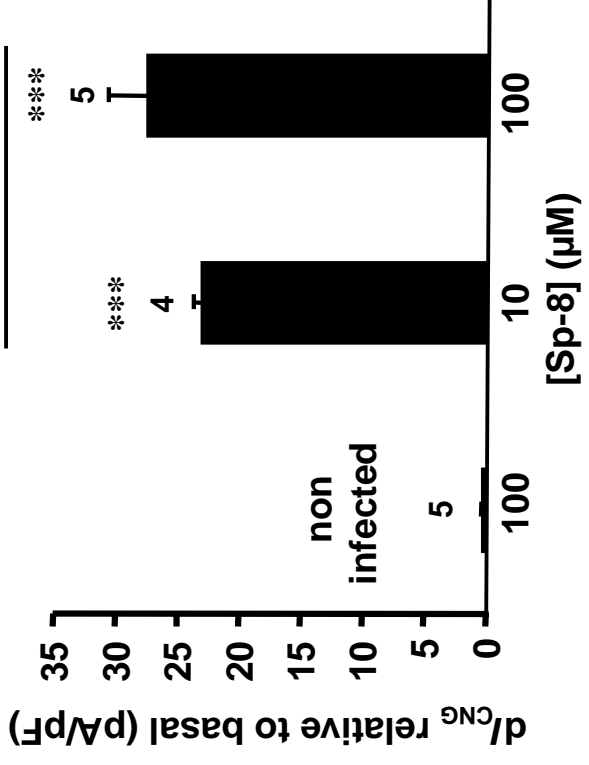


Figure 1

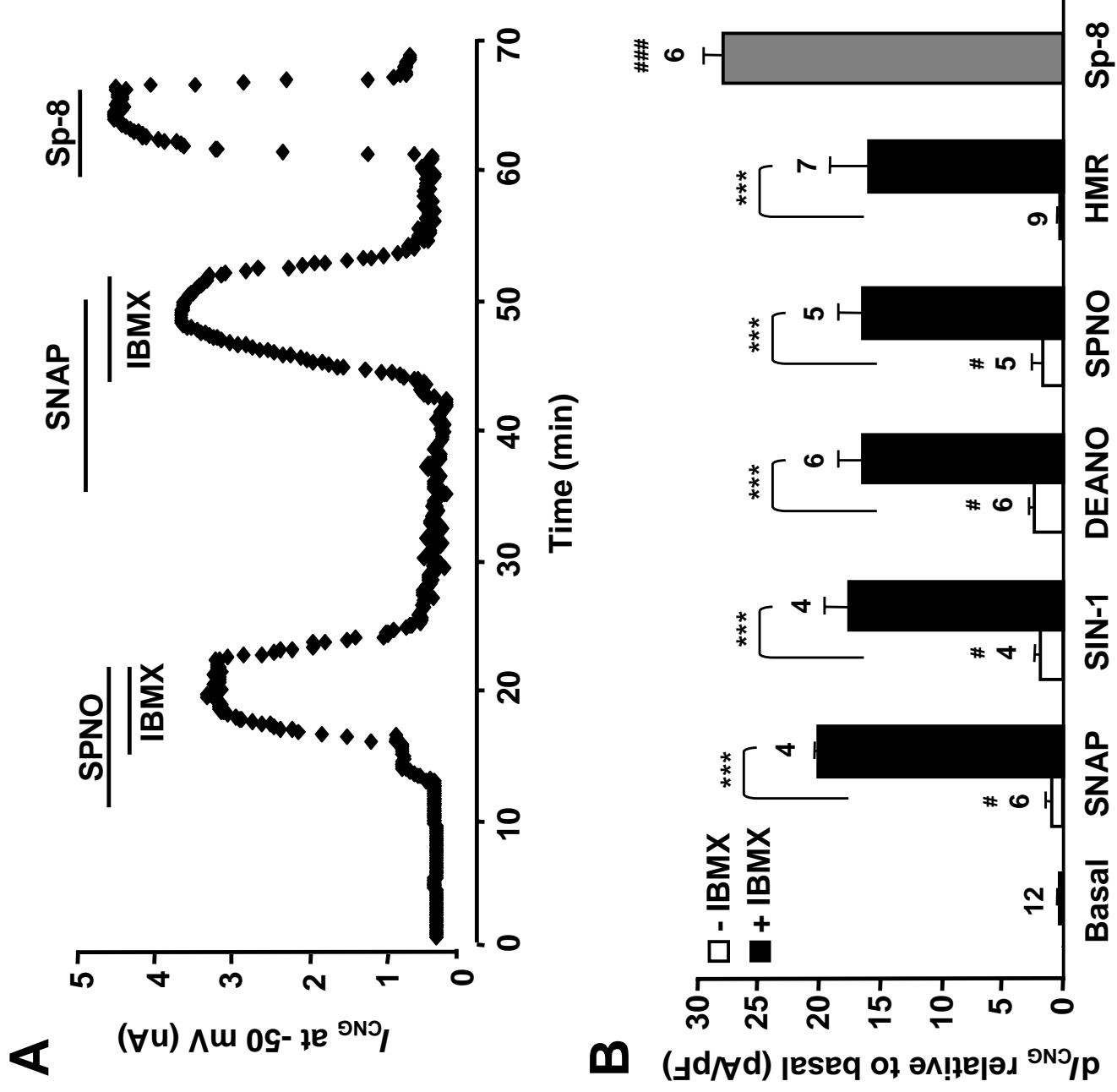


Figure 2

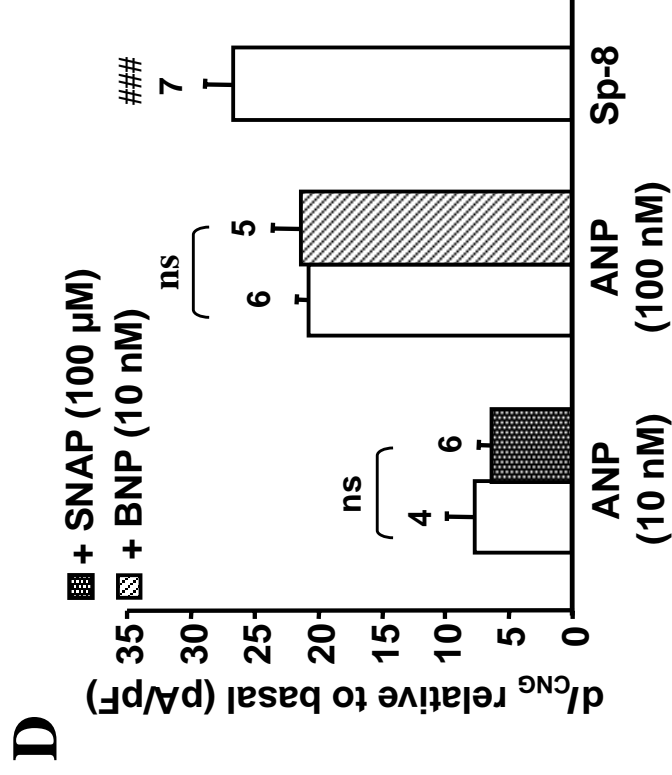
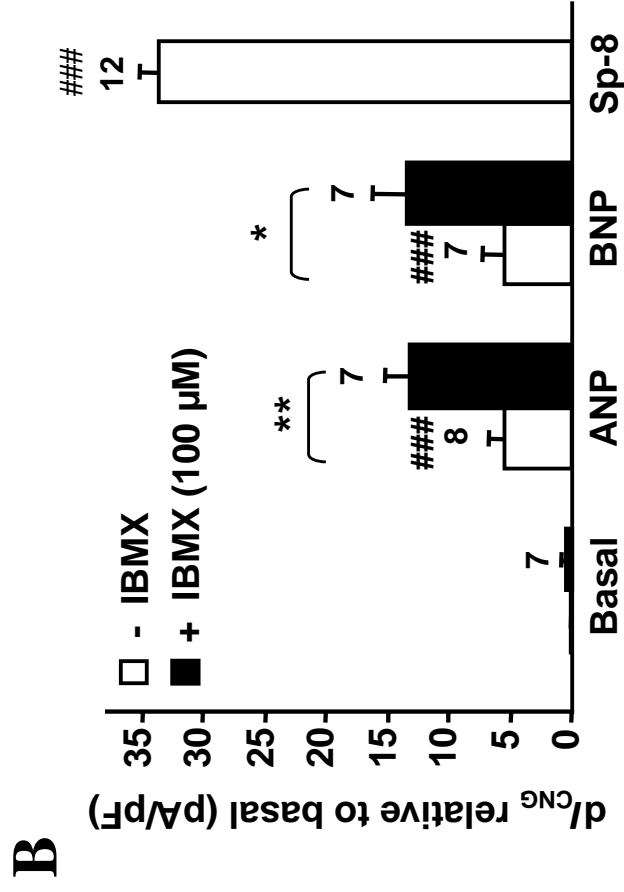
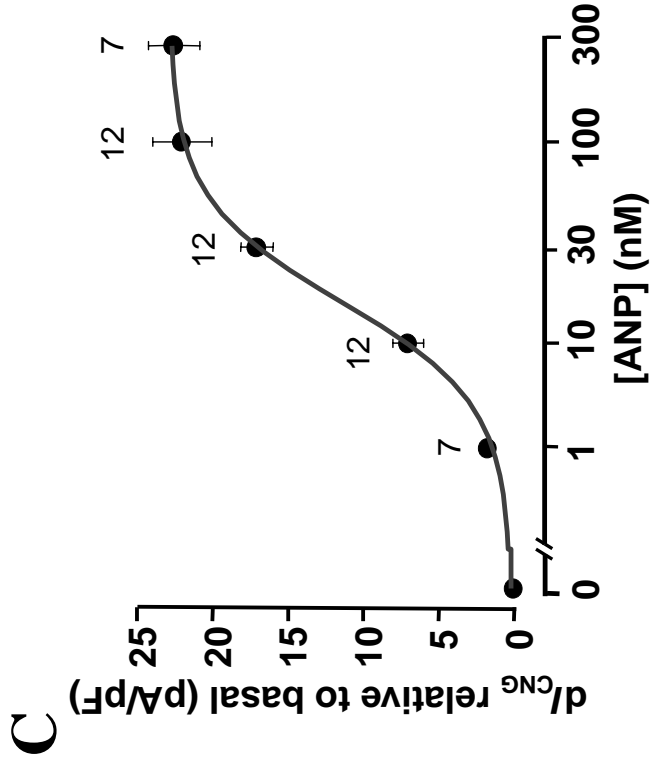
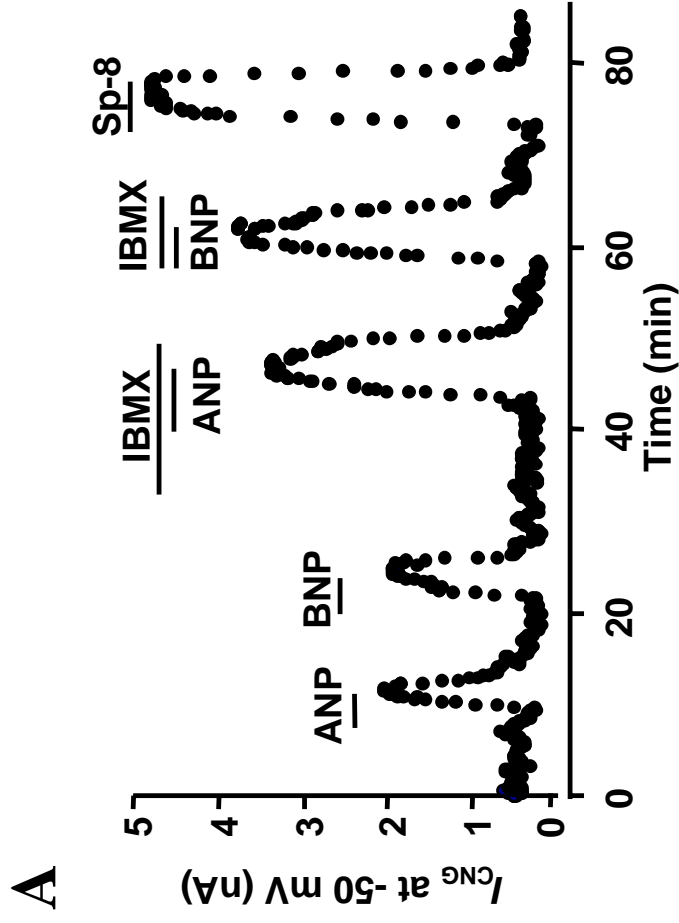


Figure 3

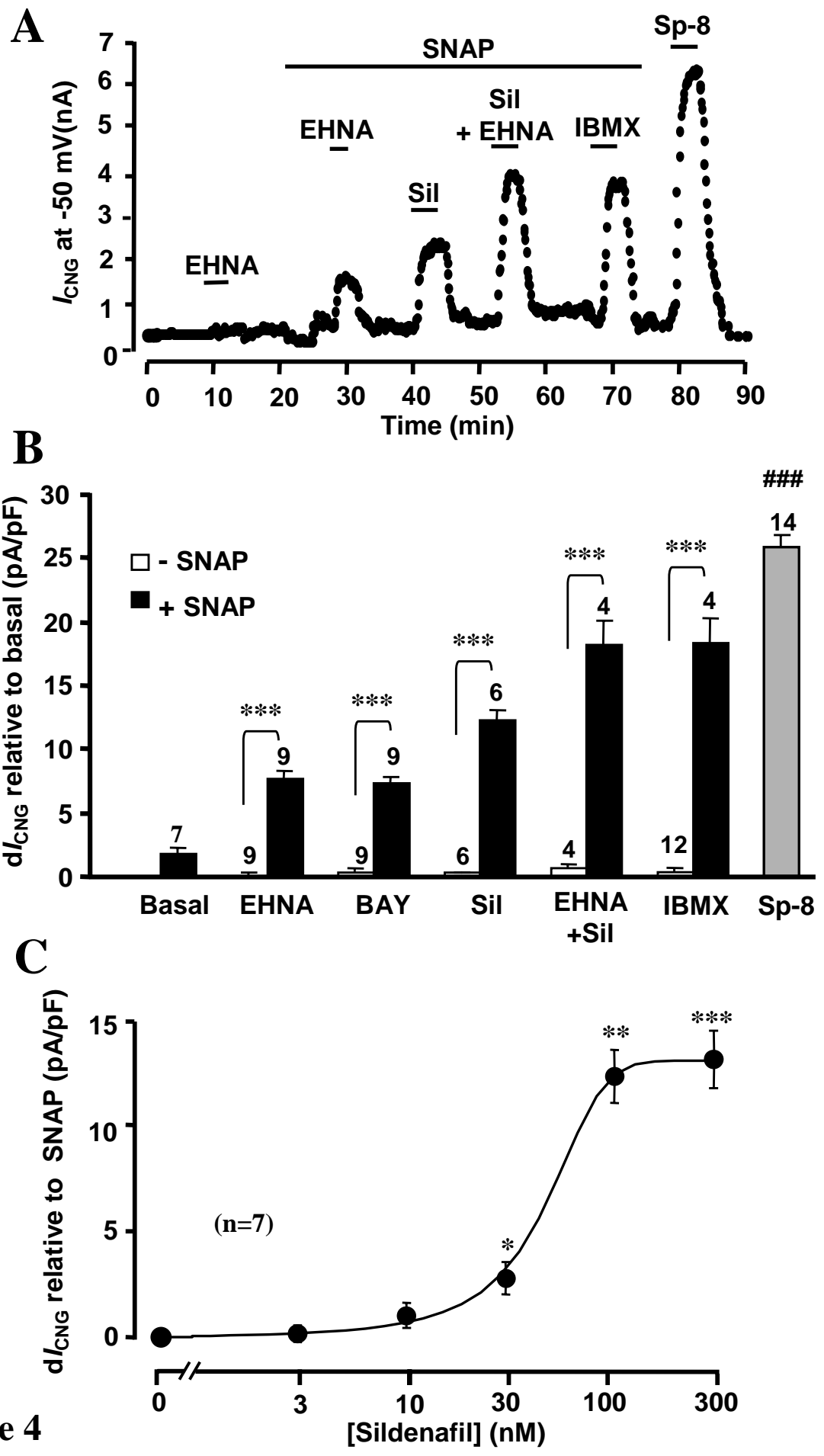


Figure 4

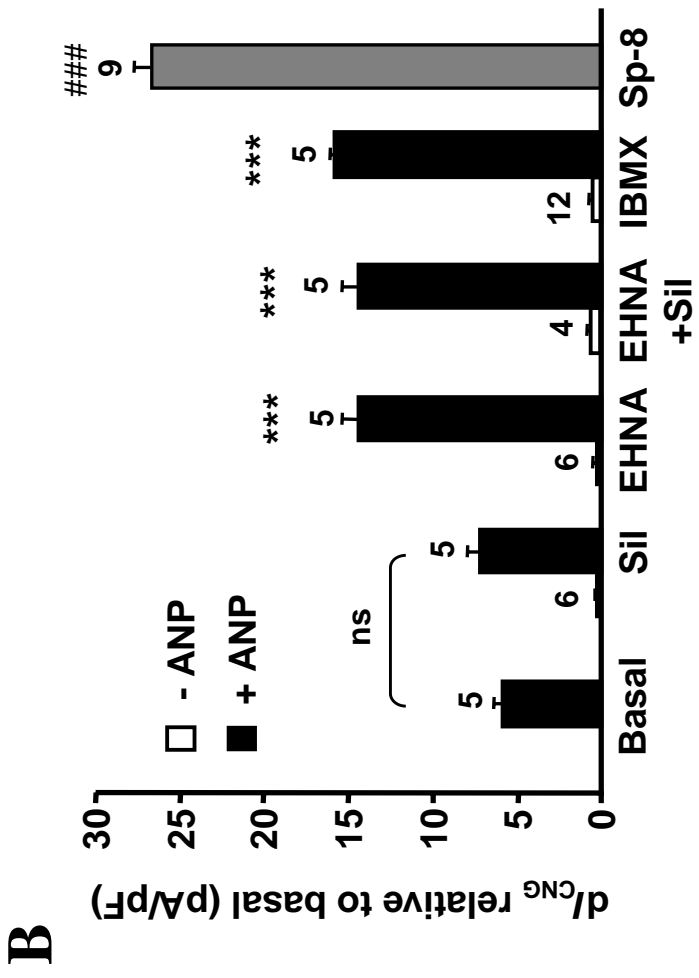
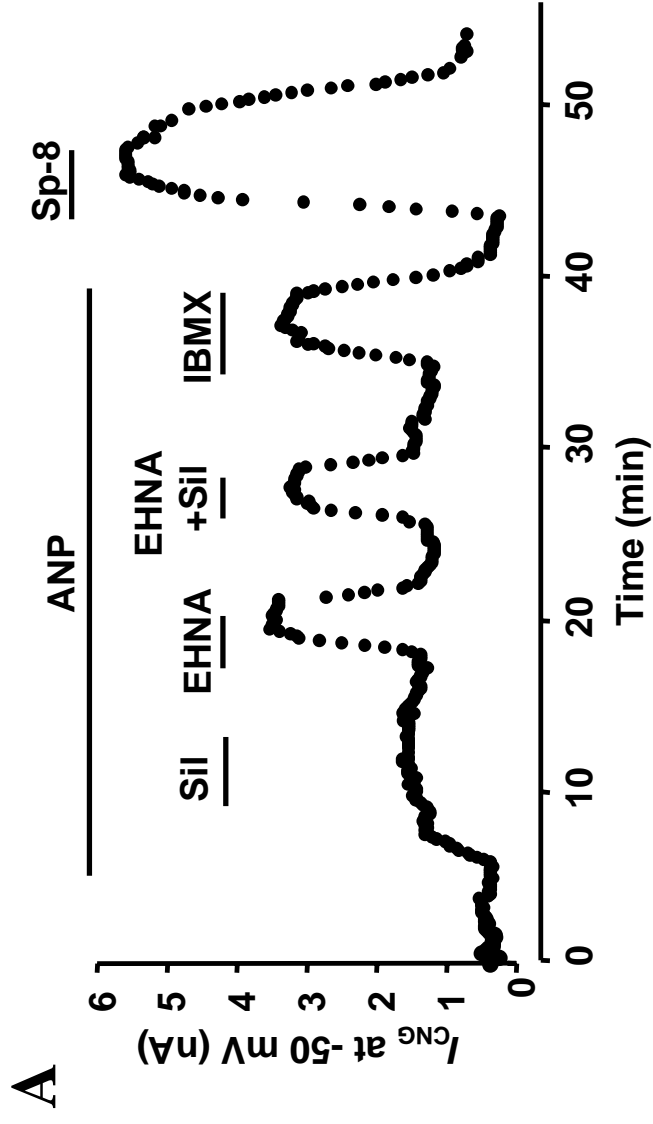


Figure 5

DATA SUPPLEMENT TO MANUSCRIPT: CIRCULATIONAHA/2005/599241/R1**Methods**

Our investigations conform with the European Community guiding principles in the care and use of animals (86/609/CEE), *CE Off J* n° L358, 18 December 1986), the use of ethics committee (CREEA Ile- de-France Sud) guidelines and the French decree n°87-848 of October 19, 1987 (*J of 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 (n°7475, May 27, 1997).

Isolation, Culture and Infection of Adult Rat Ventricular Myocytes

Adult rat ventricular myocytes (ARVMs) were obtained by retrograde perfusion from hearts of male Wistar rats (160-180 g) as previously described.¹ Freshly isolated cells were suspended in minimal essential medium (MEM: M4780; Sigma) containing 1.2 mM Ca²⁺, 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, 2h) at a density of 10⁴ cells per dish. The cells were left to adhere for 1h in a 95% O₂, 5% CO₂ incubator at 37°C. The medium was replaced by 200 µl of FBS-free MEM containing or not the WT CNGA2 encoding adenovirus (Ad-CNGA2) or the double mutant E583M/C460W CNGA2 encoding adenovirus. Adenovirus was used at a multiplicity of infection (MOI) of 3000 plaque forming units (pfu) per cell. After 2h, the same volume of FBS-free medium without adenovirus was added, and the cells were placed overnight in an incubator.

Immunocytochemistry

Cardiomyocytes attached onto coverslips were fixed, permeabilized and incubated with a mouse monoclonal antibody against CNGA2 as previously described.² This antibody was a generous gift from Drs F. Mueller and B. Kaupp (Juelich, Germany). Cells were revealed with Alexa fluor 488 goat antibody, and mounted in Mowiol antifadent mounting medium (France Biochem, Meudon, France) and examined under a laser scanning confocal microscope (Zeiss LSM 510). Optical section series were obtained with a Plan Apochromat ×63 objective (NA 1.4 oil immersion). The fluorescence was observed with a LP 505-nm emission filter under 488-nm laser illumination.

Electrophysiological Experiments

The whole cell configuration of the patch-clamp technique was used to record the CNG current (I_{CNG}). The cells were maintained at 0 mV holding potential and routinely hyperpolarized every 8 s to -50 mV test potential during 200 ms. Current-voltage relationships were obtained by varying the test potential amplitude to values ranging from -50 to +50 mV. The 0 mV holding potential was chosen because it corresponds to the reversal potential of I_{CNG} under our experimental conditions (Fig. 1C of main article). Indeed, I_{CNG} was recorded in the absence of divalent cations in the extracellular solution (see below) allowing monovalent cations to flow through the channels in a non specific manner. Currents were not compensated for capacitance and leak currents. All experiments were done at room temperature (21-27°C), and the temperature did not vary by more than 1°C in a given experiment.

Solutions and Drugs

Control zero $\text{Ca}^{2+}/\text{Mg}^{2+}$ extracellular Cs^+ -Ringer solution contained (in mM): HEPES 10, NaCl 107.1, CsCl 20, NaHCO_3 4, NaH_2PO_4 0.8, D-glucose 5, sodium pyruvate 5, adjusted to

pH 7.4 with NaOH. This solution was supplemented with nifedipine (1 μ M) to block non specific cation current through L-type Ca^{2+} channels. Control and drug-containing solutions were applied to the exterior of the cell, by placing the cell at the opening of a 250 μ m (inner diameter) capillary tube. Patch electrodes (0.7-1.2 M Ω) were made of soft glass (Drummond, Broomall, PA, USA) and filled with control internal solution containing (in mM): HEPES 10, CsCl 118, EGTA 5, MgCl_2 4, sodium phosphocreatine 5, Na_2ATP 3.1, Na_2GTP 0.42, CaCl_2 0.0062 (pCa 8.5), adjusted to pH 7.3 with CsOH. 2-(N,N6Diethylamino)-diazonolate-2-oxide sodium salt (DEANO) and SpermineNONOate (SPNO) were from Alexis Corp. (La Jolla, CA); S-nitroso-N-acetylpenicillamine (SNAP) was from Tocris-Cookson (Bristol, UK); HMR1766 was a generous gift from Dr. Ursula Schindler, Aventis Pharma Deutschland GmbH (Frankfurt am Main, Germany); 3-morpholinopyridone (SIN1) was a generous gift from Dr J. Winicki (Hoechst Houdé Laboratories, Paris La Défense, France); 8-(4-chlorophenylthio) guanosine- 3', 5'- cyclic monophosphorothioate, Sp- isomer (Sp-8) was from Biolog L.S.I. (Bremen, Germany); L-858051 (L-85, a hydrosoluble analogue of forskolin) and cilostamide were from France Biochem; Sildenafil was a generous gift from Dr Claire Lugnier (Strasbourg, France); 2-(3,4-dimethoxybenzyl)-7-[(1R)-1-[(1R)-1-hydroxyethyl]-4-phenylbutyl]-5-methylimidazo[5,1-f][1,2,4]triazin-4(3H)-one (Bay 60-7550, a PDE2 inhibitor) was a generous gift from Bayer Healthcare AG (Wuppertal, Germany); all other drugs were from Sigma-Aldrich (Saint Quentin Fallavier, France).

Data Analysis

I_{CNG} amplitude is time-independent and was measured at the end of the 200 ms pulse. In a total of 93 cells, mean membrane capacitance (C_m) was 152.6 ± 2.8 pF. I_{CNG} density (dI_{CNG}) was calculated for each experiment as the ratio of current amplitude to 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'= $(I_{\text{CNG}} - \text{'basal current'})/C_m$. When applicable, the response of I_{CNG} was also normalized to the maximal current amplitude obtained on the same cell with 100 μM Sp-8 (a membrane permeant cGMP analog) following the relation: 'normalized response'= $(I_{\text{CNG}} - \text{'basal current'})/(I_{\text{Sp-8}} - \text{'basal current'})$, where $I_{\text{Sp-8}}$ is the current obtained at 100 μM Sp-8. The concentration-response curve (CRC) for the effects of ANP on I_{CNG} was fitted to the Hill equation: $dI_{\text{CNG}} = E_{\text{max}} / (1 + (EC_{50}/[\text{ANP}])^n)$, where EC_{50} is the ANP concentration required to produce half-maximal stimulation, E_{max} is the maximal effect, and n the Hill coefficient. All data are expressed as mean \pm S.E.M.. When appropriate, the Student's t test was used for statistical evaluation of I_{CNG} variation induced by the different drugs and a p value of <0.05 was considered statistically significant.

Results

Subsarcolemmal Localization of Recombinant CNGA2 Channels in ARVMs

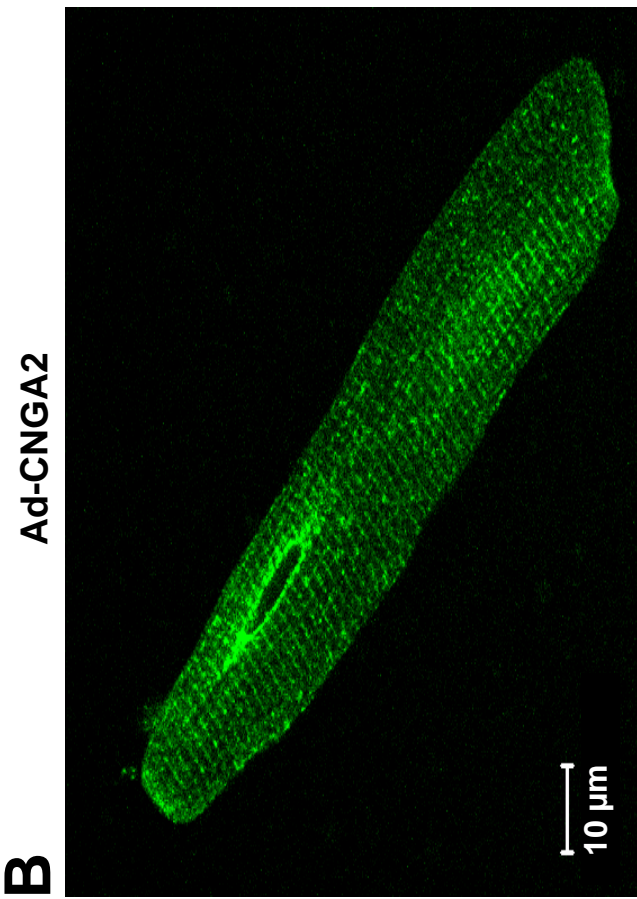
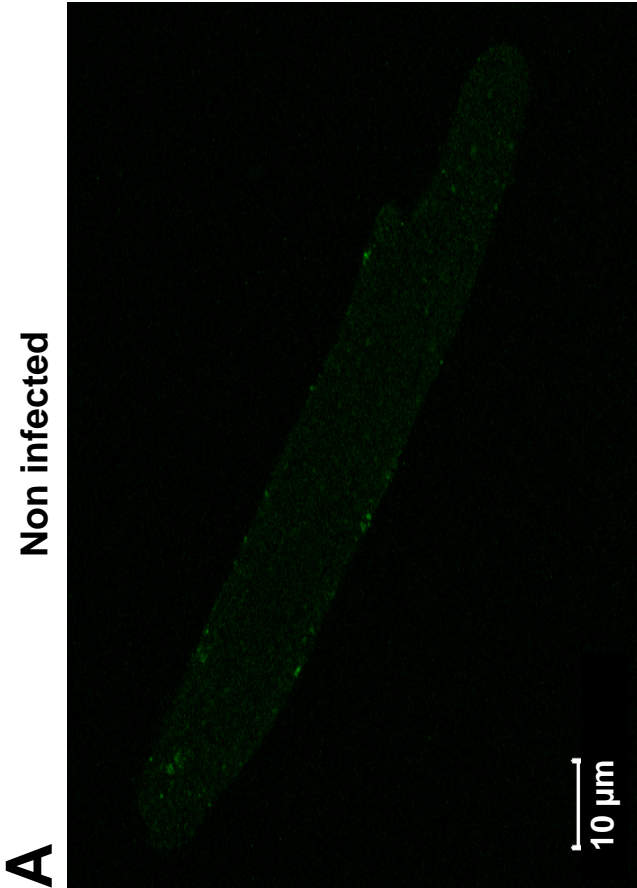
CNGA2 expression was investigated by immunofluorescence in ARVMs after 24 h of culture. Native or Ad-CNGA2 myocytes were labeled with primary antibody against CNGA2 and visualized with fluorophore conjugated secondary antibody (Alexa 488). Supplemental Fig. 1 shows confocal images of representative cells in Ad-CNGA2-infected or non infected cardiac myocytes. Infected cells elicited a strong fluorescent signal, mainly around the nucleus and in transversal T-tubules. Such a pattern was not observed in non-infected cells or when the primary antibody against CNGA2 was omitted. These results revealed the selective expression of recombinant WT CNGA2 channels at the sarcolemmal structures in Ad-CNGA2 infected cells.

Supplementary Figure Legends

Supplementary Figure 1. Immunocytochemical detection of the recombinant WT-CNGA2 channels in ARVMs. Myocytes were either infected (Ad-CNGA2, B) or not (A) with an adenovirus encoding the wild type (WT) α -subunit of rat olfactory CNG channels and labeled with anti-CNGA2 antibody after 24h of culture. Images were produced using a laser scanning confocal microscope. Each image represents one optical slice of a stack of images collected every 0.5 μ m.

Supplementary References

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Supplemental Figure 1