Guanosine 3’: 5’-cyclic monophosphate-dependent pathway alterations in ventricular cardiomyocytes of spontaneously

British Journal of Pharmacology (2001) 134, 596–602

www.nature.com/bjp

Guanosine 3′:5′-cyclic monophosphate-dependent pathway alterations in ventricular cardiomyocytes of spontaneously hypertensive rats

[1]Luca Mazzetti, [1]Carlo Ruocco, [1]Lisa Giovannelli, [1]Mario Ciuffi, [1]Sergio Franchi-Micheli, [2]Fabio Marra, [1]Lucilla Zilletti & *,[1]Paola Failli

[1]Department of Pharmacology, Viale Pieraccini, 6, University of Florence, 50139 Florence, Italy and [2]Department of Internal Medicine, Viale Morgagni, 85, University of Florence, 50139 Florence, Italy

1 We investigated the effect of the NO-donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) on cardiomyocytes isolated from control normotensive Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats.

2 Ventricular cardiomyocytes were isolated from SHR and WKY hearts and imaging analysis of fura-2-loaded cells was performed in order to evaluate calcium transient in electrical field paced (0.5 Hz) cells.

3 In WKY cardiomyocytes, 1–200 μM SNAP dose-dependently increased cyclic GMP content. In basal conditions, cyclic GMP content of SHR cardiomyocytes was significantly higher than in WKY, but SNAP failed to further increase cyclic GMP over the basal level.

4 In control conditions, the $\Delta F/F$ and decay time of the calcium transient were similar in both strains. In WKY cardiomyocytes, SNAP (1–100 μM) reduced the decay time. In SHR cardiomyocytes, SNAP was ineffective. Dibutyryl cyclic GMP (10^{-6}–10^{-8} M), a membrane permeable cyclic GMP analogue, behaved similarly to SNAP.

5 In WKY and SHR cardiomyocytes, 10^{-8} M isoprenaline similarly increased $\Delta F/F$ and decreased the decay time. SNAP and dibutyryl cyclic GMP prevented the effect of isoprenaline in WKY, whereas both molecules were ineffective in SHR cardiomyocytes. In WKY, SNAP effects were blocked by pretreating cells with the cGK inhibitor KT-5823.

6 Western blotting analysis of cGK type I showed that the enzyme was expressed in WKY isolated cardiomyocytes, but absent in four out of five SHR preparations.

7 We concluded that the low expression of cGKI may determine the lack of NO/cyclic GMP-dependent regulation on calcium transient in SHR cardiomyocytes. This alteration may contribute to the development of heart hypertrophy in hypertensive status.
British Journal of Pharmacology (2001) **134,** 596–602

Keywords: Normotensive Wistar Kyoto rats; spontaneously hypertensive rats; cardiomyocytes; guanosine 3′:5′-cyclic monophosphate-dependent protein kinase I; isoprenaline; SNAP; fura-2 fluorescence

Abbreviations: AcPen, N-acetyl-DL-penicillamine; cGKI, guanosine 3′:5′-cyclic monophosphate-dependent protein kinase I; cyclic GMP, guanosine 3′:5′-cyclic monophosphate; dibutyryl cyclic GMP, N^2,2′-O-Dibutyryl guanosine 3′:5′-cyclic monophosphate; eNOS, endothelial nitric oxide synthase (type III); $\Delta F/F$, ratio between fluorescence modification at the systolic peak and basal diastolic fluorescence; IBMX, isobutylmethylxanthine; L-NMMA, L-N^G-monomethylarginine; SHR, spontaneously hypertensive rats; SNAP, S-nitroso-N-acetyl-DL-penicillamine; WKY, Wistar Kyoto rats

Introduction

Nitric oxide (both endogenous produced and exogenous applied as NO-donor molecules) can regulate the heart rate and contractility (for recent review see Balligand, 1999; Paulus & Shah, 1999). In particular nitric oxide can exert either a positive or a negative inotropic effect according to dose (Kojda *et al*., 1996; Mohan *et al*., 1996) and inhibits the β-adrenergic-mediated positive inotropic effect (Balligand *et al*., 1993). Several NO effects are mimicked by guanosine 3′:5′-cyclic monophosphate (cyclic GMP) analogues (Shah *et al*., 1994 ; Mohan *et al*., 1996), blocked by cyclic GMP pathway interfering agents (Kojda *et al*., 1997) and an increase in cyclic GMP after NO stimulation is documented in heart preparations (Mery *et al*., 1993; Kaye *et al*., 1996), thus suggesting that soluble guanylyl cyclase activation is mainly involved in NO actions. Cardiomyocytes express the constitutive type III nitric oxide synthase (eNOS, NOS III – Balligand *et al*., 1995). In the rat, the enzyme is activated by muscarinic cholinergic agonists (Balligand *et al*., 1993; 1995) and by a high frequency rate (Kaye *et al*., 1996). The exact mechanism(s) by which NO regulates cardiomyocyte functions is still controversial. Cyclic GMP can inhibit type 3 phosphodiesterase (PDE3)

*Author for correspondence at: Department of Pharmacology, Viale Pieraccini, 6, 50139 Florence, Italy;
E-mail: Failli@server1.pharm.unifi.it

and activates type 2 phosphodiesterase (PDE2), thus regulating the cyclic adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content in an opposite direction and therefore influencing the β-adrenergic-mediated positive inotropic effect (Hove-Madsen *et al*., 1996). Several phosphodiesterase subtypes are involved in the regulation of L-type calcium current in rat ventricular cardiomyocytes (Verde *et al*., 1999) and can therefore strongly influence the β-adrenergic-mediated positive inotropic effect. Moreover, cyclic GMP can decrease L-type calcium current stimulated by cyclic AMP through the activation of a cyclic GMP-dependent protein kinase (cGK) in rat ventricular cardiomyocytes (Mery *et al*., 1991). Other cyclic GMP-dependent mechanisms can not be excluded (Balligand, 1999).

On the other hand, NOSIII-deficient mice (NOS3-KO) do not show cardiac abnormalities, and muscarinic agonists can still regulate β-adrenergic-mediated inotropic effect and inhibit L-type calcium current, indicating at least in this genetically engineered experimental model a minor role of NO in the anti-adrenergic muscarinic action (Vandecasteele *et al*., 1999).

However, NO fails to antagonize the β-adrenoceptor mediated positive inotropic effect in left ventricular strips isolated from 12–16-week-old spontaneously hypertensive rats (SHR), while in WKY left ventricular strips the increase in contractile force induced by isoprenaline is prevented by S-nitroso-N-acetyl-DL-pencillamine (SNAP) (Kotchi Kotchi *et al*., 1998). Whereas in WKY ventricular strips 10^{-5} M SNAP shifts the isoprenaline dose-response curve to the right, it is ineffective in SHR.

This loss of NO regulatory effect in SHR ventricular strips could be dependent on the decreased activity of cGK measured in homogenized ventricular and atrial tissue as a decrease in cyclic GMP-dependent phosphorylated proteins (Kuo *et al*., 1976). A decreased concentration of cGK is also described in stroke-prone SHR and renovascular hypertensive rats (Goldblatt II) cardiac tissue extracts by Ecker *et al*. (1989). Using a radioimmunoassay and enzyme-linked immunosorbent assay methodology, the authors demonstrate a half concentration of cGK in stroke-prone SHR total heart extract. However, these authors demonstrate a similar high expression of cGK in coronary smooth muscle cells of both strains by immunohistochemical and therefore they conclude that the low content of cGK is mainly due to a low index of vascularization in stroke-prone SHR hearts since cGK expression is very low in cardiomyocytes. This cGK decrease is selectively present only in the heart, but not in aorta and brain microvessels.

In microcoronary endothelial cells isolated from 12-week-old SHR, we demonstrated that the intracellular calcium is not regulated by endogenous and exogenous applied NO (Failli *et al*., 2000). We reasoned that a similar calcium deregulation may be responsible for the loss of NO-mediated prevention of β-adrenergic effect in SHR cardiomyocytes.

Therefore the aim of our study was to evaluate the effect of exogenous applied NO and cyclic GMP on the calcium transient in paced ventricular cardiomyocytes isolated from SHR heart and to compare these data with those obtained in WKY cardiomyocytes. Preliminary data of this research were presented at the XIIIth International Congress of Pharmacology (Failli *et al*., 1998).

Methods

Isolation of ventricular cardiomyocytes and microcoronary endothelial cells

This investigation conforms to the rules for the care and use of laboratory animals of the European Community.

Cardiomyocytes were isolated from 12–15-week-old WKY and age-matched SHR (Charles River, Italy) as described (Failli *et al*., 1992). Briefly, hearts were mounted in Langendorff's apparatus, washed for 5 min with nominally calcium-free Krebs-Heinselet solution (K-H, mM composition): NaCl 110, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $MgCl_2$ 1.2, D(+)glucose 11, 10 taurine, and then perfused for 20 min with KH containing 0.1% type I collagenase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), 0.1% fraction V, fatty acid free bovine serum albumin (BSA, Roche Molecular Biochemicals, Milan, Italy). Ventricles, including the septum, were cut in small pieces and fragments were dispersed in K-H, 100 μM $CaCl_2$ and 1% BSA. After centrifugation ($25\times g$ for 3 min), the pellet containing heavy cells was resuspended in 10 ml of K-H solution, 100 μM $CaCl_2$ and cardiomyocytes were purified using a discontinuous BSA gradient (4%). Microcoronary endothelial cells (MCECs) were obtained from the supernatant, purified as described and used at the first culture passage (Failli *et al*., 2000). Solutions were gassed with 95% O_2: 5% CO_2. All the perfusion steps were performed at 37°C, whereas heart disgregation and cardiomyocyte purification were conducted at room temperature (20–25°C). Only suspensions containing more than 70% of rod-shaped cardiomyocytes were used for further investigations. Contamination from other cell types were evaluated as being less than 7%.

Cyclic GMP determination

Cardiomyocytes (5×10^5 cells ml^{-1}) were preincubated (10 min) with K-H containing 100 μM isobutylmethylxanthine (IBMX, Sigma Chemical Co.); cells were then incubated for 5 min at 37°C in either control conditions or with SNAP (final concentration 200, 100, 10 and 1 μM). Reaction was stopped by cooling and centrifugation ($500\times g$ for 5 min); the supernatant was discharged and cyclic GMP was twice extracted with ice-cold 65% ethanol. Hydroalcoholic supernatant was dried under N_2 stream (Lin *et al*., 1995) and used for the determination of cyclic GMP using a commercial [^{3}H]-cyclic GMP RIA Kit (Amersham International).

Western blot analysis of type I cGK

Cardiomyocytes and confluent MCECs were lysed in RIPA buffer (mM composition): Tris-HCl 20, ph 7.4, NaCl 150, EDTA 5, Na_3VO_4 1, phenyl methyl sulphonyl fluoride 1; nonidet P-40 1%, aprotinin 0.05% [w/v]. Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate (Bradford, 1976). Western blot analysis was carried out essentially as described elsewhere (Marra *et al*., 1997). Proteins (75 μg) were separated by 10% SDS–PAGE according to Laemmli (1970) and electroblotted on a polyvinyliden-difluoride membrane. The membranes were

blocked overnight at 4°C with 2% BSA in 0.1% PBS–Tween, and then sequentially incubated at room temperature with the primary rabbit polyclonal antibody for cGK typeI (cGKI) and horseradish peroxidase-conjugated secondary antibodies. Detection was carried out using chemiluminescence according to the manufacturer's protocol (Amersham, Arlington Heights, IL, U.S.A.).

Immunocytochemistry of cGKI in MCECs MCECs were grown until confluence on tissue culture chamber slides (Lab-tek, Nunc Inc., Naperville, Il, U.S.A.) and fixed in 10% formaldehyde solution. The slides were incubated overnight at RT with the primary polyclonal rabbit antibody for cGKI (used at a 1 : 600 dilution in PBS containing 0.1% Triton-X and 0.5% BSA). On the following day, they were washed and incubated for 1 h at RT with the anti-rabbit secondary antibody Cy3 (Sigma) diluted 1 : 500 in PBS–0.5% BSA and 0.1% Triton-X. Analysis was performed by means of a Nikon Labophot-2 epifluorescence microscope. Negative control for the immunostaining was obtained by omission of the primary antibody. No fluorescence was detected in absence of the primary antibody.

Imaging analysis of fura-2 fluorescence and cytosolic intracellular calcium

Isolated cardiomyocytes were resuspended at a density of 500,000 cells ml^{-1} in 500 μM $CaCl_2$ K-H solution and loaded with 4 μM fura-2AM (Grynkiewicz *et al*., 1985) for 45 min at room temperature. Cells were washed and resuspended in K-H 1.5 mM $CaCl_2$. Fura-2 loaded cardiomyocytes were sedimented into a perfusion chamber supporting two platinum wires (distance between electrodes: 10 mm) connected with an electrostimulator (Harvard Apparatus, South Natick, Massachusetts, U.S.A.) and placed on the stage of an inverted fluorescence microscopy (Nikon Diaphot, Tokyo, Japan) equipped with a 75 W UV–Xenon lamp. Cells were paced by electrical field stimulation at 0.5 Hz, 100 mV peak voltage. Imaging analysis of fura-2 specific fluorescence was performed. Fluorescence images obtained at the single excitation wavelength of 380 nm, emission 510 nm (Ishide *et al*., 1990; Failli *et al*., 1997) were collected with an intensified charge coupled device (CCD) video camera ISIS-M extended camera (Photonic Science, U.K.) at video rate (25 Hz), digitalized by an analogical to digital converter (256×256 pixels $\times 8$ bits) and dynamically analysed on-line using an image analysis, Microsoft Windows based software (Autolab®, RCS, Florence, Italy). Fluorescence of fura-2 was converted in a grey scale recognizing 256 levels. Experiments were performed at $32° \pm 1$°C. The excitation wavelength of 380 nm for fura-2 was chosen in order to evaluate fura-2 fluorescence in an optimal linear range during electrical field stimulation. All cells found in an optical field (using $20\times$ magnification objective) were analysed. The following parameters of the calcium transient induced by electrical-field stimulation were evaluated: the ratio between the fluorescence maximal variation induced by electrical-field stimulation and the diastolic fluorescence ($\Delta F/F$) and the decay time (see later, mathematical method). Fura-2 loading of WKY and SHR cardiomyocytes was homogeneous and the fluorescence intensity of whole cells was similar in both strains. In control experiments after recording the calcium transient in basal condition for at least 3 min, SNAP, dibutyryl guanosine 3′:5′-cyclic monophosphate (cyclic dibutyryl GMP), N-acetyl-DL-penicillamine (AcPen) or the solvent were administered and followed by 10^{-8} M isoprenaline after 6 min. Electrical pacing was followed for at least 6 min after isoprenaline. Cardiomyocytes were incubated 10 min with KT-5823 before performing experiments.

Intracellular calcium quantification performed with the ratiometric method (Failli *et al*., 1992) showed that the intracellular calcium concentration in unstimulated cardiomyocytes was not significantly different in either strain (126 ± 7.1 nM, $c = 12$, $n = 5$ and 135 ± 4.1 nM, $c = 11$, $n = 4$ in WKY and SHR respectively).

Data were exported as ASCII file format and elaborated for graphic presentation using MicroCal Origin® (2.8 version).

Materials L-NMMA, isoprenaline, (d,l)propranolol, cyclic dibutyryl GMP and AcPen were purchased from Sigma Chemical Co. (Milan, Italy). SNAP was purchased from Tocris Cookson Ltd. (Bristol, U.K.), KT-5823 an inhibitor of cyclic GMP-dependent protein kinase (Kase *et al*., 1987) by Calbiochem (Milan, Italy), and fura-2AM from Molecular Probes (Eugene, Oregon, U.S.A.). All other reagents were of analytical grade. The cGKI polyclonal antibody was a kind gift of Prof Franz Hofmann.

Mathematical and statistical methods

For each experimental protocol, 2–4 cardiomyocytes isolated from at least three different hearts were analysed. The number of cells analysed is reported as c and the number of different preparations used as n. Values are presented as means ± s.e.mean of analysed cells (c). In order to fit calcium transient decay with a mono-exponential mathematical function, data were subtracted from the reference value of 256 gray levels. The decay time of calcium transient was calculated according to the followed equation:

$$Y = y_0 + A \cdot e^{(-x \cdot \tau)} \quad (1)$$

According to the fitting function, the 'tau' (τ) parameter represented the time necessary for $[Ca^{2+}]i$ to reach 36.79% of the maximal value. The constant 'tau' of the monoexponential fitting was therefore reported as the decay time value (ms). At least six calcium transients for each different treatment were analysed and averaged.

Statistical comparisons between data groups were performed using Student's t-test (paired or independent as indicated); dose-response curves were also analysed by one-way ANOVA. A P value of <0.05 was considered significant.

Results

Effect of SNAP on cyclic GMP content in cardiomyocytes

As shown in Figure 1, SNAP (1–200 μM) dose-dependently increased cyclic GMP content in WKY cardiomyocytes ($F = 3.128$, $P < 0.05$, one-way ANOVA, $n = 5$). In SHR cardiomyocytes, cyclic GMP content in basal conditions

was significantly higher than in WKY, but SNAP failed to further increase cyclic GMP over the basal level at all doses tested. Indeed, at all SNAP doses tested no difference from basal level was observed (Figure 1).

Electrical pacing in ventricular cardiomyocytes

In control conditions electrical field stimulation in both WKY and SHR cardiomyocytes induced a typical calcium transient as observed by the change in the fura-2 fluorescence (Figure 2). The ΔF/F and decay time were similar in both strains (Figure 3). Isoprenaline (10^{-8} M) increased the ΔF/F by about +4.5% in WKY ($c=64$, $n=21$) and by +4.0% in SHR ($c=58$, $n=18$) and decreased the decay time (−17.6%, −36.2±2.84 ms in WKY and −15.5%, −30.3±1.98 ms in SHR respectively), its effect being comparable in both WKY and SHR cardiomyocytes (Figure 3). This effect was observed shortly (60–120 s) after addition of isoprenaline, and still present after 10 min in the continuous presence of the drug. In both strains, 10^{-6} M (d,l)propranolol prevented isoprenaline effects.

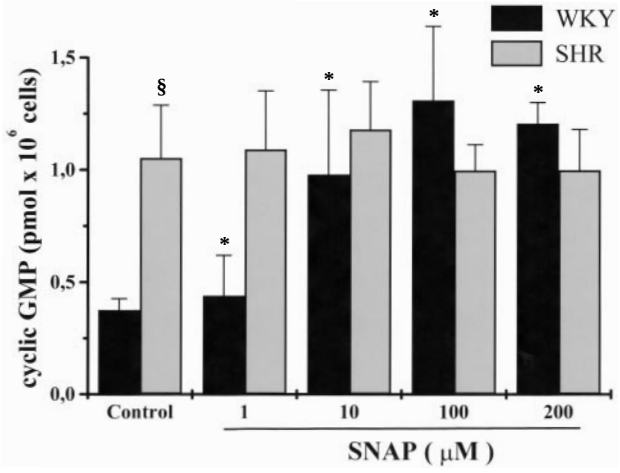

Figure 1 Cyclic GMP content of WKY and SHR ventricular cardiomyocytes after preincubation with buffer (control) or in the presence of 1–200 μM S-nitroso-N-acetylpenicillamine (SNAP). Values are the mean(±s.e.mean) of five independent experiments. *$P<0.05$ one-way ANOVA; §$P<0.001$ *vs* control, WKY.

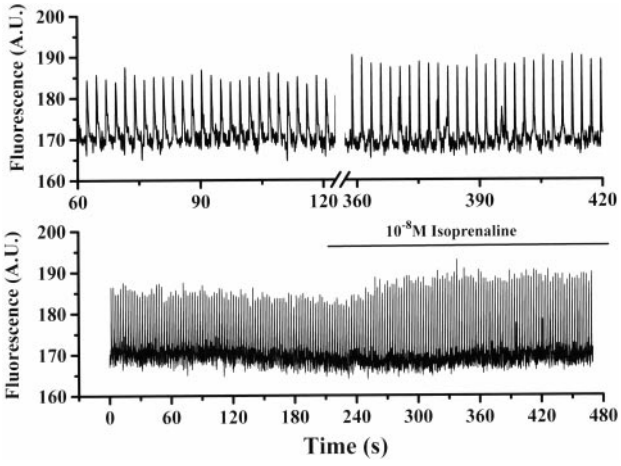

Figure 2 Typical time-course of fura-2 fluorescence in an electrical-field stimulated WKY cardiomyocyte paced at 0.5 Hz. Fura-2 fluorescence was obtained at 380 nm excitation wavelength, emission 510 nm. For graphical presentation values were subtracted from a reference value of 256 gray levels. Isoprenaline (10^{-8} M) was administered as indicated and maintained till the end of the experiment. The upper panel shows a magnification of calcium transients in control conditions (before axis break) and after isoprenaline.

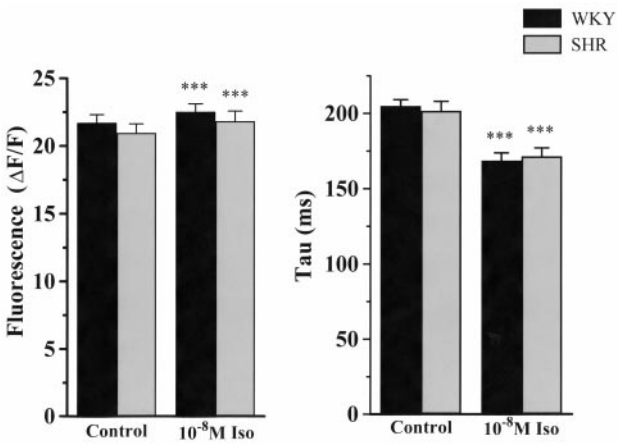

Figure 3 Effect of 10^{-8} M isoprenaline on ΔF/F and decay time in WKY ($c=64$, $n=24$) and SHR ($c=55$, $n=16$) ventricular cardiomyocytes. ***$P<0.001$ *vs* control, Student's *t*-test, paired data.

Effect of SNAP and dibutyryl cyclic GMP on fura-2 fluorescence

Effect of SNAP and dibutyryl cyclic GMP in basal conditions Then, we analysed the effect of SNAP (1–200 μM) on fura-2 fluorescence in unstimulated and in electrical-field stimulated cells. No significant changes in fura-2 fluorescence were observed when SNAP was administered to unpaced cardiomyocytes in both strains. However, in paced WKY cardiomyocytes, 200 μM SNAP induced a modest (not significant) increase in decay time, whereas in the range 100–1 μM SNAP significantly reduced it (Table 1). These effects were measured after 300–480 s of SNAP. The amplitude of calcium transient was not modified. In SHR cardiomyocytes, SNAP slightly decreased the decay time at the higher dose tested, but was ineffective in the 100–1 μM range. Dibutyryl cyclic GMP, a membrane permeable cyclic GMP analogue, behaved similarly to SNAP. Indeed, dubutyryl cyclic GMP in the dose range 10^{-6}–10^{-8} M reduced the decay time in WKY, while it was practically ineffective in SHR (Table 2).

Effect of isoprenaline on SNAP- and dibutyryl cyclic GMP-treated cells Thereafter we tested the effect of 10^{-8} M isoprenaline in SNAP-treated myocytes. In WKY cardiomyocytes, SNAP (administered 6 min before 10^{-8} M isoprenaline) prevented the effect of isoprenaline. The increase in ΔF/F was totally blocked by SNAP, while the decrease in decay time was dose-dependently reduced, 10^{-5} M SNAP being the more effective dose (Figure 4, $F=8.73$, $P<0.01$, one-way ANOVA). Administration of 200 μM AcPen in control conditions did not influence either the ΔF/F or the decay time, and was also ineffective in preventing the increase

in ΔF/F and the decrease in decay time induced by 10^{-8} M isoprenaline. Indeed, the ΔF/F increase induced by isoprenaline in AcPen-treated cardiomyocytes was 4.8±0.82% ($c=18$, $n=6$) of basal value, a value similar to that obtained in control conditions. Also the decrease in the decay time was 38±4.48 ms ($c=18$, $n=6$) in 200 μM N-AcPen treated cells; this value was not significantly different from that obtained in control conditions. Moreover, in WKY cardiomyocytes dibutyryl cyclic GMP in the 10^{-6}–10^{-8} M dose range blocked the increase in ΔF/F induced by isoprenaline and dose-dependently counteracted the decrease in decay time ($F=4.78$, $P<0.01$ one-way ANOVA, Figure 5). Neither the ΔF/F, nor the decay time were significantly modified by either SNAP or dibutyryl cyclic GMP in SHR cardiomyocytes (Figures 4 and 5).

Table 1 Effect of SNAP (200-1 μM) on the decay time of isolated cardiomyocytes from WKY and SHR

	WKY	*SHR*
	Δ *decay time* (ms)	
SNAP (μM)		
200	+9.3±4.75	−13.0±9.12
100	−18.9±4.85**	−11.2±5.53
10	−19.5±7.17**	−9.7±8.86
1	−8.2±8.16	−4.2±8.31

**$P<0.01$ *vs* control, Student's *t*-test paired data. Data are reported as differences from control values (before the administration of drugs) and represent the mean (±s.e.mean) of at least 11 cells obtained in four separate experiments.

Table 2 Effect of dibutyryl cyclic GMP (10^{-6}–10^{-8} M) on the decay time of isolated cardiomyocytes from WKY and SHR

	WKY	*SHR*
	Δ *decay time* (ms)	
Dibutyryl cyclic GMP (M)		
10^{-6}	−18.8±8.75*	−12.6±8.68
10^{-7}	−18.9±4.85*	−5.3±5.79
10^{-8}	−19.5±7.17*	−9.3±7.48

*$P<0.05$ *vs* control, Student's *t*-test paired data. Data are reported as differences from control values (before the administration of drugs) and represent the mean (±s.e.mean) of at least 10 cells obtained in four separate experiments.

Effect of KT-5823 In WKY cardiomyocytes, we tested the influence of the cyclic GMP-dependent protein kinase inhibitor KT-5823 on SNAP effects. The preincubation with 1 μM KT-5823 did not modify the ΔF/F and decay time as compared to controls. The effect of 10^{-8} M isoprenaline was fully maintained; the increase in ΔF/F was 4.3±0.81%, while decrease in the decay was −32.5±4.58 ms ($c=8$, $n=3$). These values were not significantly different to those obtained in control cells. However, after KT-5823 preincubation, 100 μM SNAP was ineffective in reducing the decay time and no longer prevented isoprenaline effects. After 10^{-8} M isoprenaline the increase in ΔF/F was +3.9±0.63% and the decrease in decay time was −28.7±3.52 ms ($c=7$, $n=3$), an effect similar to that induced by isoprenaline in cells pretreated only with KT-5823.

cGKI expression in isolated cardiomyocytes and microcoronary endothelial cells

Western blotting analysis of GK I showed that the enzyme highly expressed in WKY isolated cardiomyocytes, but was totally absent in four out of five SHR preparations, while in one preparation it was expressed (Figure 6).

On the other hand, in isolated microcoronary endothelial cells obtained from the same animal as cardiomyocytes, cGKI was similarly expressed as determined by Western analysis and immunocytochemistry (not shown).

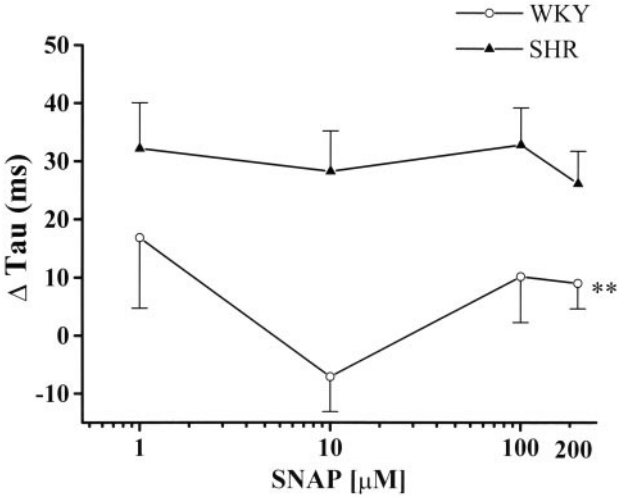

Figure 4 Effect of 1–200 μM S-nitroso-N-acetylpenicillamine (SNAP) on isoprenaline-induced decrease in decay time in WKY and SHR ventricular cardiomyocytes. Each point is the mean (±s.e.mean) of at least 15 cells obtained in at least five separate experiments. Values are presented as Δ decay time (decay time SNAP minus decay time 10^{-8} M isoprenaline administered 6 min after SNAP). For more details, see text. **$P<0.01$, one-way ANOVA.

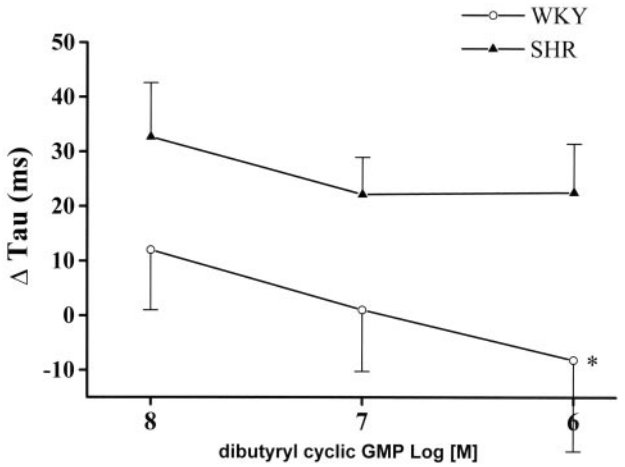

Figure 5 Effect of 10^{-6}–10^{-8} M dibutyryl guanosine 3′:5′ cyclic monophosphate (dibutyryl cyclic GMP) on isoprenaline-induced decrease in decay time in WKY and SHR ventricular cardiomyocytes. Each point is the mean (±s.e.mean) of at least 12 cells obtained in at least four separate experiments. Values are presented as Δ decay time (decay time dibutyryl cyclic GMP minus decay time 10^{-8} M isoprenaline administered 6 min after dibutyryl cyclic GMP). For more details, see text. *$P<0.05$, one-way ANOVA.

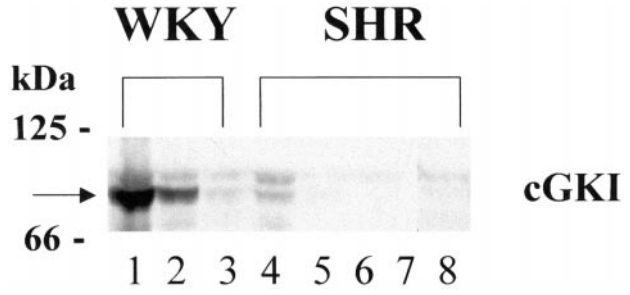

Figure 6 Western blot analysis of cGKI in WKY and SHR ventricular cardiomyocytes. The arrow indicates the cGKI band.

Discussion

Our data show that in WKY cardiomyocytes, the NO-donor SNAP dose-dependently increases cyclic GMP content, the highest effective dose being 100 μM. Therefore, the soluble guanylyl cyclase (sGC) is present and active in WKY cardiomyocytes. A different behaviour is present in SHR cardiomyocytes. In control conditions cyclic GMP content is higher in SHR cardiomyocytes than in WKY. However, SNAP is unable to further increase cyclic GMP content in SHR cardiomyocytes, suggesting a low or absent responsiveness of the enzyme. We have also described a reduced SNAP responsiveness of sGC in MCECs isolated from SHR (Failli *et al*., 2000), indicating that this alteration is common in different cell types of SHR hearts. According to the result obtained by Ruetten *et al*. (1999) the protein expression of sGC (α and β subunits) is slightly increased in young SHR hearts, whereas it is decreased in SHR aortic rings. This slight increase in sGC protein expression might justify the high basal cyclic GMP content in SHR cardiomyocytes, although we can hypothesise alternative explanation of our data.

SNAP administration to electrical stimulated WKY cardiomyocytes can induce in the dose interval of 1–100 μM a decrease in decay time, whereas at a higher dose of 200 μM the decay time is slightly increased. Since the effect of 100 μM SNAP is absent after blocking cGKI with KT5823 and the cyclic GMP analogue dibutyryl cyclic GMP in the range 10^{-6}–10^{-8} M reduces the decay time, the reduction in decay time induced by SNAP is probably mediated by cyclic GMP throughout the activation in cGKI. 200 and 100 μM SNAP are equally effective in increasing cyclic GMP content and therefore other pathways may influence the slight increase in decay time observed with 200 μM SNAP.

In the range 1–200 μM SNAP linearly prevents the decrease in decay time and the increase in $\Delta F/F$ induced by isoprenaline. AcPen, the NO-lacking molecular skeleton of SNAP, is ineffective. Moreover, 100 μM SNAP no longer counteracts the isoprenaline effect in the presence of a cGKI block. Therefore, it is likely that SNAP activity is mediated by a NO pathway that, increasing the cyclic GMP content, activates the cGKI. The membrane permeable cyclic GMP dibutyryl cyclic GMP (a membrane permeable cyclic GMP analogous) behaves as SNAP in preventing the isoprenaline effect, further supporting an involvement of cyclic GMP/cGKI pathway in SNAP effectiveness. These results are in line with earlier observations on NO antagonism of the β-adrenergic inotropism (Balligand *et al*., 1993; also reviewed in Balligand, 1999). Our data support a main role of cGKI in SNAP effects, since SNAP is ineffective in the presence of a cGKI block, thus suggesting that cyclic GMP direct influence on β-adrenergic inotropism is negligible in our condition.

On the other hand, in SHR cardiomyocytes SNAP is unable to modulate calcium transient or to increase cyclic GMP. A similar lack of NO effectiveness is observed also with dibutyryl cyclic GMP. SNAP does not counteract the decrease in decay time and the increase in $\Delta F/F$ induced by isoprenaline. These results may explain the lack of β-adrenergic inotropism modulation in SHR heart preparation reported by Kotchi Kotchi *et al*. (1998). Since in WKY the SNAP effect is mainly mediated by cGKI activation, we analysed the expression of cGKI in cardiomyocytes of both strains and found low or absent expression of cGKI in SHR cardiomyocytes. This alteration is specific for SHR cardiomyocytes as compared to MCECs, since in SHR MCECs, the cGKI expression is similar to that observed in WKY. It should be noted that the cGK activity is lower in SHR ventricular and atrial homogenates than in WKY (Kuo *et al*., 1976). Our data are only partially in agreement with those reported by Ecker *et al*. (1989). On the other hand, we detected the cGKI expression in cardiomyocytes by Western blot while Ecker *et al*. (1989) analysed it immunohistochemically. However, we can not exclude alterations of other cGKI-independent NO targets that can influence the ineffectiveness of NO/cyclic GMP pathway in SHR cardiomyocytes. Although in NOS3-KO mice, β-adrenergic-mediated positive inotropic effects are still antagonized by muscarinic agents (Vandecasteele *et al*., 1999), these data do not rule out a big NO influence in cardiomyocytes isolated from naïve animals, since alternative pathways may develop in KO mice.

cGKI-deficient mice are hypertensive and show an impaired NO/cyclic GMP mediated relaxation of smooth muscles (Pfeifer *et al*., 1998). Moreover, the cGK gene expression is down regulated in cultured rat VSMCs after exposure to hypertrophic/hyperproliferative agents such as platelet-derived growth factor, angiotensin II, transforming growth factor-β, or tumour necrosis factor-α (Tamura *et al*., 1996), indicating that this enzyme may play a role under hypertrophic/hyperproliferative conditions. The low sCG responsiveness to NO and the low expression of cGKI may determine the lack of NO/cyclic GMP-dependent regulation on calcium transient in SHR cardiomyocytes and can therefore influence the cardiac hypertrophic status of SHR.

This work was supported by grants from the University of Florence. We would like to thank Prof Franz Hofmann and Dr Aldo Ammendola for reading the manuscript and kind suggestions, Ms Mary Forrest for her revision of the English and Paolo Ceccatelli and Mauro Beni for their technical assistance.

References

BALLIGAND, J.-L. (1999). Regulation of cardiac β-adrenergic response by nitric oxide. *Cardiovasc. Res.,* **43,** 607 – 620.

BALLIGAND, J.L., KELLY, R.A., MARSDEN, P.A., SMITH, T.W. & MICHEL, T. (1993). Control of cardiac muscle cell function by and endogenous nitric oxide signaling system. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 347 – 351.

BALLIGAND, J.L., KOBZIK, L., HAN, X., KAYE, D.M., BELHASSEN, L., O'HARA, D.S., KELLY, R.A., SMITH, T.W. & MICHEL, T. (1995). Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (typeIII) nitric oxide synthase in cardiac myocytes. *J. Biol. Chem.,* **270,** 14582 – 14586.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 – 254.

ECKER, T., GÖBEL, C., HULLIN, R., RETTIG, R., SEITZ, G. & HOFMANN, F. (1989). Decreased cardiac concentration of cGMP-kinase in hypertensive animals. An index for cardiac vascularization? *Circ. Res.,* **65,** 1361 – 1369.

FAILLI, P., FAZZINI, A., FRANCONI, F., STENDARDI, I. & GIOTTI, A. (1992). Taurine antagonizes the increase in intracellular calcium induced by α-adrenergic stimulation in freshly isolated guinea-pig cardiomyocytes. *J. Mol. Cell Cardiol.,* **24,** 1253 – 1265.

FAILLI, P., FAZZINI, A., RUOCCO, C., MAZZETTI, L., CECCHI, E., GIOVANNELLI, L., MARRA, F., MILANI, S. & GIOTTI, A. (2000). Lack of nitric oxide- and guanosine 3′:5′-cyclic monophosphate-dependent regulation of α-thrombin-induced calcium transient in endothelial cells of spontaneously hypertensive rat hearts. *Br. J. Pharmacol.,* **130,** 1468 – 1476.

FAILLI, P., RUOCCO, C., FAZZINI, A. & GIOTTI, A. (1997). Calcium waves in unstimulated left ventricular cardiomyocytes isolated from aged spontaneously hypertensive rats. *Biochem. Biophys. Res. Commun.,* **237,** 103 – 106.

FAILLI, P., RUOCCO, C., MAZZETTI, L., ZILLETTI, L. & GIOTTI, A. (1998). Effect of S-nitroso-N-acetylpenicillamine (SNAP) on intracellular calcium dynamic in cardiac cells isolated from normotensive and spontaneously hypertensive rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **358,** R304.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440 – 3450.

HOVE-MADSEN, L., MERY, P.F., JUREVICIUS, J., SKEBERDIS, A.V. & FISCHMEISTER, R. (1996). Regulation of myocardial calcium channels by cyclic AMP metabolism. *Basic Res. Cardiol.,* **91 (S2),** 1 – 8.

ISHIDE, N., URAYAMA, T., INOUE, K., KOMARU, T. & TAKISHIMA, T. (1990). Propagation and collision characteristics of calcium waves in rat myocytes. *Am. J. Physiol.,* **259,** H940 – H950.

KASE, H., IWAHASHI, K., NAKANISHI, S., MATSUDA, Y., YAMADA, K., TAKAHASHI, M., MURAKATA, C., SATO, A. & KANEKO, M. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.,* **142,** 436 – 440.

KAYE, D.M., WIVIOTT, S.D., BALLIGAND, J.L., SIMMONS, W.W., SMITH, T.W. & KELLY, R.A. (1996). Frequency-dependent activation of a constitutive nitric oxide synthase and regulation of contractile function in adult ventricular myocytes. *Circ. Res.,* **78,** 217 – 224.

KOJDA, G., KOTTENBERG, K., NIX, P., SCHLUTER, K.D., PIPER, H.M. & NOACK, E. (1996). Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular muscle. *Circ. Res.,* **78,** 91 – 101.

KOJDA, G., KOTTENBERG, K. & NOACK, E. (1997). Inhibition of nitric oxide synthase and soluble guanylate cyclase induces cardiodepressive effects in normal rat hearts. *Eur. J. Pharmacol.,* **334,** 181 – 190.

KOTCHI KOTCHI, E., WEISSELBERG, T., RÖHNERT, P., PREISS, M., HEINROTH-HOFFMANN, I., OSTEN, B. & BRODDE, O.E. (1998). Nitric oxide inhibits isoprenaline-induced positive inotropic effects in normal, but not in hypertrophied rat heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **357,** 579 – 583.

KUO, J.F., DAVIES, G.W. & TSE, J. (1976). Depressed cardiac cyclic GMP-dependent protein kinase in spontaneously hypertensive rats and its further depression by guanethidine. *Nature,* **261,** 335 – 336.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature,* **348,** 699 – 704.

LIN, X., HÄNZE, J., HEESE, F., SODMANN, R. & LANG, R.E. (1995). Gene expression of natriuretic peptide receptors in myocardial cells. *Circ. Res.,* **77,** 750 – 758.

MARRA, F., GENTILINI, A., PINZANI, M., GHOSH-CHOUDHURY, G., PAROLA, M., HERBST, H., DIANZANI, M.U., LAFFI, G., ABBOUD, H.E. & GENTILINI, P. (1997). Phosphatidylinositol 3-kinase is required for platelet-derived growth factors actions on hepatic stellate cells. *Gastroenterology,* **112,** 1297 – 1306.

MERY, P.F., LOHMANN, S.M., WALTER, U. & FISCHMEISTER, R. (1991). Ca2+ current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 1197 – 1201.

MERY, P.F., PAVOINE, C., BELHASSEN, L., PECKER, F. & FISCHMEISTER, R. (1993). Nitric oxide regulates cardiac Ca2+ current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J. Biol. Chem.,* **268,** 26286 – 26295.

MOHAN, P., BRUTSAERT, D.L., PAULUS, W.J. & SYS, S.U. (1996). Myocardial contractile response to nitric oxide and cGMP. *Circulation,* **93,** 1223 – 1229.

PAULUS, W.J. & SHAH, A.M. (1999). NO and cardiac diastolic function. *Cardiovasc. Res.,* **43,** 595 – 606.

PFEIFER, A., KLATT, P., MASSBERG, S., NY, L., SAUSBIER, M., HIRNEISS, C., WANG, G.X., KORTH, M., ASSODI, A., ANDERSSON, K.E., KROMBACH, F., MAYERHOFER, A., RUTH, P., FASSLER, R. & HOFMANN, F. (1998). Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J.,* **17,** 3045 – 3051.

RUETTEN, H., ZABEL, U., LINZ, W. & SCHMIDT, H.H. (1999). Downregulation of soluble guanylyl cyclase in young and aging spontaneously hypertensive rats. *Circ. Res.,* **85,** 534 – 541.

SHAH, A.M., SPURGEON, H.A., SOLLOTT, S.J., TALO, A. & LAKATTA, E.G. (1994). 8-Bromo-cGMP reduces the myofilament response to Ca2+ in intact cardiac myocytes. *Circ. Res.,* **74,** 970 – 978.

TAMURA, N., ITOH, H., OGAWA, Y., NAKAGAWA, O., HARADA, M., CHUN, T.H., SUGA, S., YOSHIMASA, T. & NAKAO, K. (1996). cDNA cloning and gene expression of human type Ialpha cGMP-dependent protein kinase. *Hypertension,* **27,** 552 – 557.

VANDECASTEELE, G., ESCHELHAGEN, T., SCHOLZ, H., STEIN, B., VERDE, I. & FISCHMEISTER, R. (1999). Muscarinic and beta-adrenergic regulation of heart rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase. *Nat. Med.,* **5,** 331 – 334.

VERDE, I., VANDECASTEELE, G., LEZOUALC'H, F. & FISCHMEISTER, R. (1999). Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca2+ current in rat ventricular myocytes. *Br. J. Pharmacol.,* **127,** 65 – 74.

(Received April 14, 2001
Revised May 21, 2001
Accepted July 3, 2001)