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Effects of C-type natriuretic peptide on rat cardiac contractility

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1 Natriuretic peptide receptors have been found in different heart preparations. However, the role of natriuretic peptides in the regulation of cardiac contractility remains largely elusive and was, therefore, studied here.

2 The rate of relaxation of electrically stimulated, isolated rat papillary muscles was enhanced ($114.4 \pm 1.4\%$, $P<0.01$) after addition of C-type natriuretic peptide (CNP; 1 μM). Time to peak tension decreased in parallel (88 ± 3 and 75 ± 2 msec before and 5 min after addition of CNP, respectively, $P<0.01$). On the other hand, the rate of contraction slowly decreased when CNP was added to the papillary muscles. These results show that CNP displays a positive lusitropic effect associated with a negative inotropic effect. The effects of CNP were mimicked by 8-bromo-guanosine 3′,5′ cyclic monophosphate.

3 Addition of CNP to isolated adult rat cardiomyocytes, induced a 25 fold increase in guanosine 3′,5′ cyclic monophosphate (cGMP) levels and stimulated the phosphorylation of phospholamban and troponin I, two proteins involved in the regulation of cardiac contractility. The levels of adenosine 3′,5′ cyclic monophosphate (cAMP) were not affected by the addition of CNP to the myocytes. The CNP-dependent phospholamban phosphorylation was accompanied by the activation of the sarcoplasmic reticulum Ca^{2+}-ATPase.

4 In summary, CNP exerts a positive lusitropic effect, in rat papillary muscles. The putative mechanism involved in the lusitropism induced by this peptide, a cGMP-dependent enhancement of the rate of relaxation with a slowly developing negative inotropic effect, seems different to that described for catecholamines.

Keywords: Natriuretic peptides; inotropism; lusitropism; guanosine 3′,5′ cyclic monophosphate (cGMP); cardiomyocytes

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Ca^{2+}-ATPase, sarcoplasmic reticulum Ca^{2+} pump; cAMP, adenosine 3′,5′ cyclic monophosphate; cGMP, guanosine 3′,5′ cyclic monophosphate; CNP, C-type natriuretic peptide; DMEM, Dulbecco's Modified Eagle Medium; DT, developed tension; EC_{50}, half-maximal transport activity; L-NAME, L-N^G-nitroarginine methyl ester; NPR-A and NPR-B, natriuretic peptide receptor type A and type B; PLN, Phospholamban; (−T), maximal rate of relaxation; (+T), maximal rate of tension development; TnI, troponin I

Introduction

The natriuretic peptide family consists of three different molecules: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Three types of natriuretic peptide receptors (NPRs) have been described: NPR-A preferentially binds ANP and BNP while NPR-B displays a higher affinity towards CNP (Anand-Srivastava & Trachte, 1993; Espiner *et al*., 1995, for reviews). Both receptors contain a guanylate cyclase moiety, which is activated upon binding of the ligands. In contrast, NPR-C (or clearance receptor) lacks the guanylate cyclase domain and cannot discriminate between the different ligands (Anand-Srivastava & Trachte, 1993; Espiner *et al*., 1995). The vascular and renal effects of natriuretic peptides are well documented and usually attributed to an increase in the intracellular levels of guanosine 3′,5′ cyclic monophosphate (cGMP) (Anand-Srivastava & Trachte, 1993; Espiner *et al*., 1995). In the rat and human heart, the three natriuretic receptor mRNAs were found (Nunez *et al*., 1992). Recently, Beaulieu *et al*. (1996) and Hirose *et al*. (1998) reported, respectively, direct chronotropic and dromotropic effects of CNP on anaesthetized dogs. While some studies show that ANP decreases contractility in isolated cardiomyocytes (Neyses & Vetter, 1989; McCall & Fried, 1990), the role of natriuretic peptides in the regulation of cardiac contractility has not been clearly established. Endothelial cells are capable of synthesizing and secreting CNP suggesting that this peptide could act on adjacent cardiomyocytes as a paracrine agent to regulate contractility (Suga *et al*., 1992; 1993). The aim of the present study was, therefore, to determine whether CNP could influence the contractile state of an isolated heart muscle preparation.

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Methods

Papillary muscles experiments

The research complied with national legislation and with the company policy on the care of use of animals and with related codes of practice. All animal procedures conformed to the guide for the animal research and welfare (European Federation of Pharmaceutical Industries Associations).

Male Sprague Dawley rats (250–350 g) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Left ventricular papillary muscles were quickly removed from hearts and vertically disposed in a chamber containing 20 ml of Krebs-Ringer solution containing (mM): NaCl, 118; KCl, 4.7; $MgSO_4$-$7H_2O$, 1.2; KH_2PO_4, 1.1; $NaHCO_3$ 24; $CaCl_2$-$6H_2O$, 2.5 and glucose, 5. The bathing solution was bubbled with a gas mixture of $95\%O_2-5\%CO_2$, resulting in a pH of 7.4 and the temperature was maintained at 29°C. The lower muscular

end was fixed in a platinum clip. The upper end of the muscle was tied to an isometric force transducer (UC2). Cross-sectional area of muscles were <1.2 mm^2. A passive tension (preload) of 1 g was applied to the muscles in the resting state. Muscles were stimulated at a frequency of 0.2 Hz by means of 2 platinum electrodes positioned parallel to the muscle and delivering 5 msec rectangular pulses at a voltage slightly above threshold. The tension generated was recorded with a multi-channel chart recorder (Gould TA5000) equipped with pressure processor preamplifiers (Gould). To determine maximal tension and rates of tension rise and fall, contraction twitches were digitized and analysed on line using LS-Lapp1 software (Buxco). CNP (Peninsula Laboratories Inc., U.S.A.), 8-Br-cGMP (Sigma, St Louis, U.S.A.) and L-N^G-nitroarginine methyl ester (L-NAME, Sigma, St Louis, U.S.A.) dissolved in water were administered by adding 10 μl directly into the tissue bath.

To damage endocardial endothelium, papillary muscles were exposed to a 0.5% Triton X-100 (Sigma, St Louis, U.S.A.) Krebs-Ringer solution, for 1 s, as described by Brutsaert *et al.* (1988).

Preparation of isolated cardiomyocytes

Isolated cardiomyocytes were prepared as described (Piper *et al.*, 1982). Determination of viability was based on the cell morphology: only rod-shaped myocytes were considered. Routinely, $5-7 \times 10^6$ cells were obtained with about 70% rod-shaped myocytes. After isolation, cardiomyocytes were suspended to yield 5×10^5 cells ml^{-1} in Dulbecco's Modified Eagle Medium (DMEM) and kept for 1 h at 37°C before use.

Determination of cyclic nucleotide contents

Cardiomyocytes (10^5 in 200 μl DMEM) were incubated at 37°C with the indicated CNP or ANP (Sigma, St Louis, U.S.A.) concentrations. After a 5 min stimulation, 400 μl ethanol at -20°C was added to the cell suspension. The cell lysate was shaken for 1 h at 4°C, then centrifuged 10 min at 14,000 $\times$ g. The supernatant was evaporated to dryness using a Speed Vac (Savant). cGMP and adenosine 3′,5′ cyclic monophosphate (cAMP) were measured by scintillation proximity immunoassay (SPA-Amersham, U.K.).

Phosphorylation state of cardiomyocyte proteins

Cardiomyocytes were incubated at 37°C in phosphate-free DMEM for 1 h in the presence of 1–2 mCi [^{33}P] orthophosphate (0.5 mCi/10^6 cells ml^{-1}). The myocytes were sedimented and rinsed three times with complete DMEM. Cells (10^5) were then treated with the indicated CNP or isoprenaline hydrochloride (Sigma, St Louis, U.S.A.) concentrations. After a 5 min stimulation, cells were lysed in SDS-gel sample buffer (Tris-HCl 50 mM, pH 6.9, glycerol 2.5%, SDS 1%, β-mercaptoethanol 5%, 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride 100 μg ml^{-1}, leupeptin 1 μg ml^{-1}, aprotinin 1 μg ml^{-1}, pepstatin 1 μg ml^{-1}, okadaic acid 1 μM). Cells were then shaken for 1 h at 4°C and stored at -80°C before electrophoresis. Solubilized myocyte proteins were subjected to SDS–PAGE (12.5% acrylamide). The dried gels were exposed to a phosphor screen for 1–2 days. The bands of interest were quantified using a STORM (Molecular Dynamics). Phospholamban (PLN) has been identified based on its pentameric structure that is disrupted upon boiling in sample buffer.

Measurement of the sarcoplasmic reticulum Ca^{2+} pump activity

Isolated cells were incubated at 37°C in DMEM in 5 min in the presence of 1 μM CNP or 0.1 μM isoprenaline or in the absence of any added reagent (control). After stimulation, cells were centrifuged (1 min at 30 $\times$ g). All the following operations were carried out at 4°C. Myocytes were disrupted by alternating three cycles of 15 strokes with a Dounce homogenizer and 15 strokes with a motorized Potter-Elvehjem in lysis buffer ($NaHCO_3$ 10 mM, pH 7.4, dithiothreitol 1 mM, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride 100 μg ml^{-1}, leupeptin 1 μg ml^{-1}, aprotinin 1 μg ml^{-1}, pepstatin 1 μg ml^{-1}, okadaic acid 1 μM). The homogenates were centrifuged 15 min at 5000 $\times$ g. The determination of oxalate-facilitated, ATP-dependent, $^{45}Ca^{2+}$ uptake was immediately performed on the supernatants using multiscreen 96 well microplates (Millipore MAHV 0.45 μm). Briefly, 10 μg of each homogenate (1 mg protein/ml) were incubated for 10 min at 37°C in a buffer containing (mM): MOPS, 30, pH 6.9; KCl, 100; $MgCl_2$ 5; NaN_3, 5; ouabain, 1; ATP, 5; and oxalate, 5. The reaction was initiated by the addition of EGTA-buffered $^{45}Ca^{2+}$. The final reaction volume was 125 μl. The rate of $^{45}Ca^{2+}$ uptake was linear for at least 20 min. In the experiment described in Figure 4, the microplates were transferred to a vacuum manifold (Millipore) after 10 min at 37°C. Filters were rapidly washed twice with a free-calcium buffer (MOPS 30 mM, pH 6.9, KCl 100 mM). After dying, the radioactivity on the filters was determined by liquid scintillation counting. Free Ca^{2+} concentrations were calculated using the computer program of Fabiato & Fabiato, 1979. In parallel wells, and under the same stimulation conditions, the phosphorylation of PLN and TnI was assessed as indicated above.

Data and statistical analysis

Results are expressed as mean $\pm$ s.e.mean. Statistical analysis was performed by use of Student's t-test or one-way ANOVA where appropriate. Statistical significance was set at $P<0.05$. Data from Figures 2, 4A, C where treated with a sigmoid fit analysis using Origin software (version 3.5). Geometric EC_{50} were estimated with 95% confidence intervals. Data from Figure 5 were fitted by the Hill equation using Origin software (version 3.5).

Results

Effects of natriuretic peptides on the contractile function of rat papillary muscles

Addition of 1 μM CNP to electrically-stimulated, isolated rat papillary muscles induced an immediate enhancement of the maximal rate of relaxation ($-$T) (Figure 1). After 5 min, the $-$T value reached 114.4 $\pm$ 1.4% of the baseline ($P<0.01$), subsequently declined and remained stable after 20 min at 105.9 $\pm$ 2.5% over the control values (non-significant, NS). The maximal rate of tension development (+T) increased to a lower extent than the relaxation rate (105.0 $\pm$ 0.5% after 5 min, $P<0.01$). However, +T decreased progressively to become slower than the baseline 10 min after the addition of CNP. In addition, the natriuretic peptide induced a decrease in the developed tension (DT) (negative inotropic effect) which was already detectable 10 min after addition of the drug.

The +T/$-$T ratio is considered to be a useful index of the lusitropic effect of a drug which modifies DT, +T and $-$T

(Grassi de Gende *et al*., 1977). The +T/−T ratio immediately decreased after addition of the natriuretic peptide and attained, after 15 min, 89.6±1.2% of the control ($P<0.01$) reflecting a positive lusitropic effect of the peptide. Although −T and +T evolved differently, the lusitropic effect was sustained even 60 min after the addition of CNP as illustrated by a decrease in the +T/−T ratio of 14.6±2.1% ($P<0.01$). Time to peak tension, another parameter used to characterize relaxation (Mattiazzi *et al*., 1986), also decreased upon treatment with CNP (88±3 and 75±2 msec, before and 5 min after addition of CNP, respectively, $P<0.01$). Inset A in Figure 1 shows superimposed twitches to illustrate that, in papillary muscles treated for 5 min with CNP, only the relaxation phase of the twitch was altered, whereas the developed force remained unchanged. The same representation is used to depict the combined positive lusitropism and negative inotropism observed after a more prolonged treatment with CNP (Figure 1, inset B). The effects of CNP on the different contractile parameters were dose-related. The increase in −T is illustrated in Figure 2, half-maximal effect was obtained at 0.18±0.04 μM of CNP. Similar results were obtained for +T and DT (data not shown). In contrast, ANP did not produce any effect on any contractile parameter at concentrations up to 10 μM (not shown).

Treatment of papillary muscles with 50 μM of the cell permeable analogue of cGMP, 8-Br-cGMP, enhanced the rate of relaxation by 118.7±0.5% of the baseline (Figure 3). The increase in −T was detectable after a lag time of 5 min, probably reflecting the time required to accumulate active intracellular concentrations of 8-Br-cGMP. Similar results were found when the kinetics of the effect of noradrenaline and a cell-permeable analogue of cAMP were compared on cat papillary muscles (Skelton *et al*., 1970). As for CNP, 8-Br-cGMP also produced a slowly developing decrease in +T and in DT. Both parameters continued to decline throughout the experiment whereas the −T value reached its maximum at about 20 min. The +T/−T ratio decreased up to 75.3±0.7% of the baseline, 45 min after treatment with the cGMP analogue (Figure 3). These results show that 8-Br-cGMP, albeit with a different kinetics, mimics the effect of CNP on the different contractile parameters. Taken together, all the above mentioned results suggest that the effect of CNP on the contractile state of papillary muscles could be mediated by a cGMP-dependent mechanism.

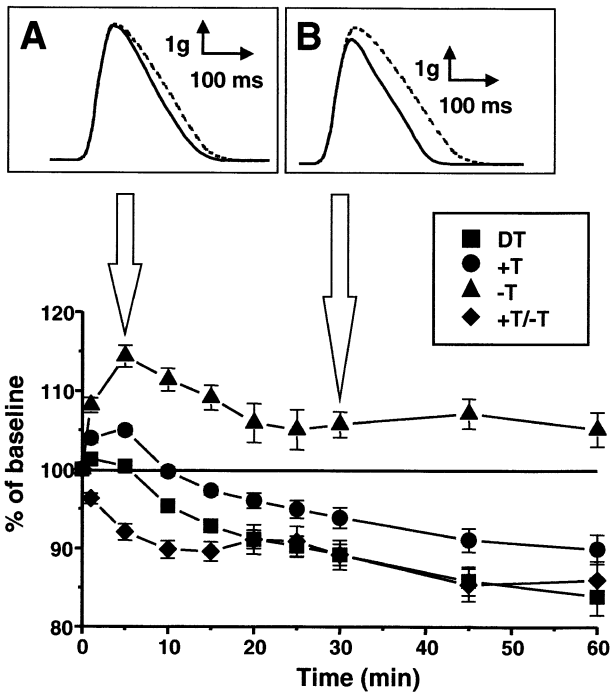

Figure 1 Time course of the effect of C-type natriuretic peptide (CNP; 1 μM) on developed tension (DT), maximal velocity of tension development (+T), maximal velocity of relaxation (−T) and the +T/−T ratio of isolated papillary muscles from rat left ventricles. Insets A and B: Superimposed traces representing sample twitches before treatment (dash lines) and after addition of CNP (solid lines) from the time point indicated by the arrows. For the DT curve, CNP *vs* baseline NS for all determinations up to 10 min and $P<0.01$ after 10 min; for the +T curve, CNP *vs* baseline $P<0.01$ up to 5 min and NS after 5 min; for the −T curve, CNP *vs* baseline $P<0.01$ up to 15 min and NS after that time; for the +T/−T ratio curve, CNP *vs* baseline $P<0.01$ up to 30 min and $P<0.05$ after that time. All the data represent the mean±s.e.mean of results from 23 papillary muscles isolated from 15 rats.

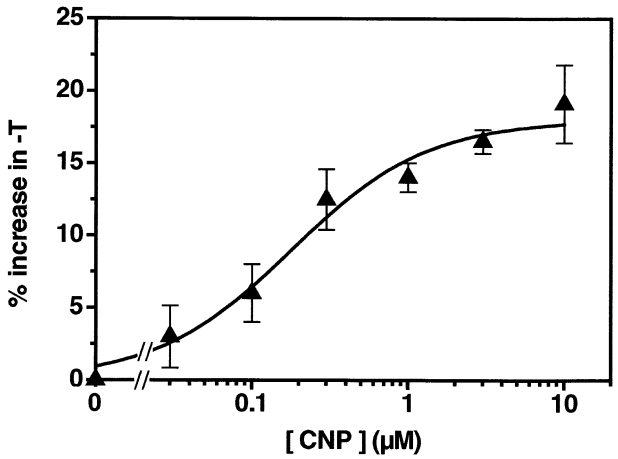

Figure 2 Dose-response curve of C-type natriuretic peptide (CNP) on the maximal velocity of relaxation (−T) of isolated papillary muscles from rat left ventricles. The values of −T shown in the figure were obtained 5 min after addition of the indicated concentrations of CNP. CNP *vs* solvent: NS up to 0.1 μM and $P<0.01$ from 0.3 μM to 10 μM. All the data represent the mean±s.d.mean of results from six to 23 papillary muscles.

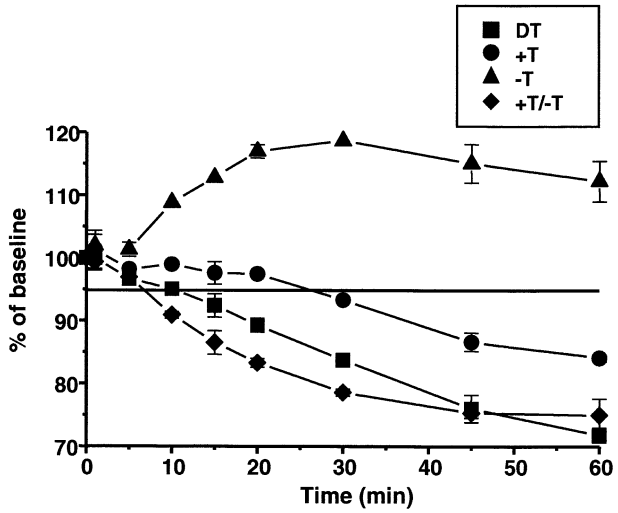

Figure 3 Time course of the effect of 8-bromo-guanosine 3′,5′ cyclic monophosphate (8-Br-cGMP; 50 μM) on developed tension (DT), maximal velocity of tension development (+T), maximal velocity of relaxation (−T) and the +T/−T ratio of isolated papillary muscles from rat left ventricles. All the data represent the mean±s.e.mean of measurements from four papillary muscles isolated from two rats.

CNP has been reported to dilate pre-constricted afferent arterioles *via* the nitric oxide pathway (Amin *et al*., 1996). To investigate the potential involvement of nitric oxide production in the contractile response to CNP, papillary muscles were incubated for 30 min in the presence of 300 μM L-NAME. Baseline parameters were not affected by the nitric oxide synthase inhibitor (data not shown). The presence of L-NAME neither modified the response to the natriuretic peptide: 5 min after addition of 0.3 μM CNP, $-$T was 111.9 ± 2.7 and $111.3 \pm 1.3\%$ of baseline in muscles pre-incubated or not with L-NAME, respectively.

To determine whether the endocardial endothelium is involved in the response to CNP, the natriuretic peptide was added to Triton X-100 treated papillary muscles. This procedure has been shown to remove endothelium from papillary muscles (Brutsaert *et al*., 1988). An increase in $-$T was observed 5 min after the addition of 0.3 μM CNP in both control ($114.0 \pm 2.4\%$ of baseline) and endothelium free ($115.0 \pm 1.3\%$ of baseline) preparations ($n=4$ for each condition). Other contractile properties were neither altered by the detergent treatment even 60 min after addition of the hormone. These results suggest that a functional endothelium is not required for CNP to induce lusitropic or negative inotropic effects.

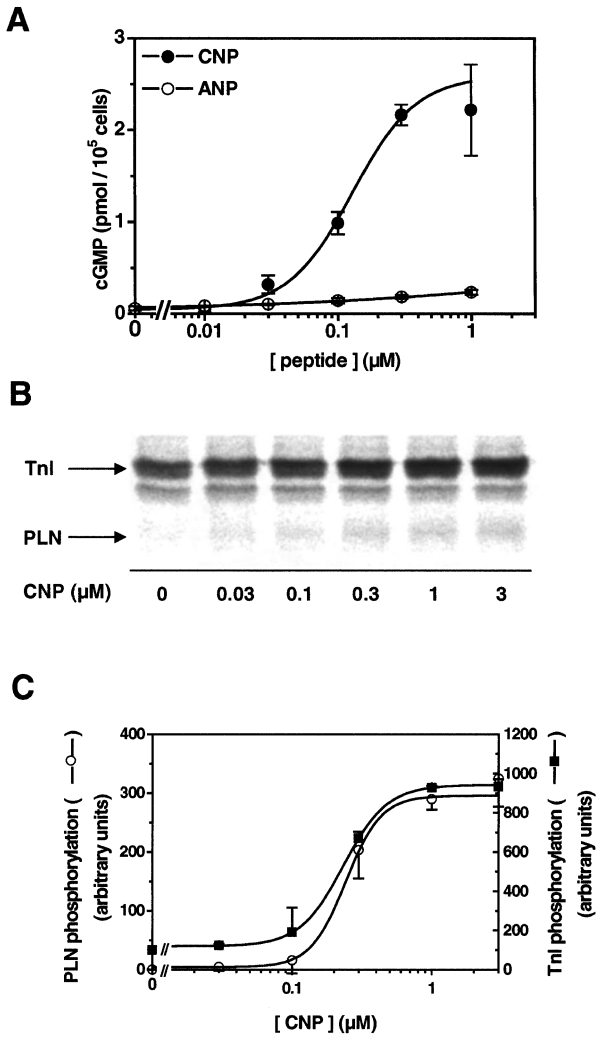

Figure 4 (A) Effect of C-type natriuretic peptide (CNP) and atrial natriuretic peptide (ANP) on guanosine 3′,5′ cyclic monophosphate (cGMP) accumulation. Cardiomyocytes were isolated, incubated with various concentrations of CNP or ANP for 5 min and the levels of cGMP were determined. (B and C) Effect of CNP on TnI and PLN phosphorylation levels. Cardiomyocytes were labelled with ^{33}P-orthophosphate and incubated with various concentrations of CNP. Protein phosphorylation was determined by SDS–PAGE and STORM (Molecular Dynamics) analysis. The values shown on A, B and C are of a representative experiment performed in triplicate and repeated twice. All data are presented as mean $\pm$ s.d.mean.

Effect of natriuretic peptides on cardiomyocyte cyclic nucleotide contents

In order to elucidate the molecular mechanism responsible for the effect of CNP on cardiac contractile function, we studied the alteration of various biochemical parameters in isolated adult rat cardiomyocytes treated with the hormone. The levels of cGMP were increased in a concentration-dependent manner upon treatment of the myocytes with CNP (Figure 4A). Half-maximal effect was obtained at 0.13 ± 0.03 μM of CNP. Cyclic GMP accumulation induced by CNP occurred without any significant modification of cAMP levels: in control and in myocytes treated with 1 μM of CNP for 5 min, the levels of cAMP were 2.6 ± 0.4 and 2.2 ± 0.3 pmol/10^5 cells, respectively. At saturating concentrations of CNP (1 μM), a 25 fold increase in cGMP levels was observed. In contrast, 1 μM ANP hardly produced a 2 fold enhancement of cGMP. Moreover, 50% of the response to ANP was lost when the myocytes were further purified on a percoll gradient, whereas the totality of the CNP response was recovered (data not shown). The results suggest that CNP stimulates the accumulation of cGMP *via* a cardiomyocyte receptor whose pharmacology is closely related to that described for the NPR-B (Koller *et al*., 1991).

Effect of CNP on cardiomyocyte protein phosphorylation

The effect of CNP on the phosphorylation state of intracellular proteins involved in the regulation of myocardial contractility was next studied. In isolated cardiomyocytes, CNP induced the phosphorylation of PLN and strongly enhanced the phosphor-

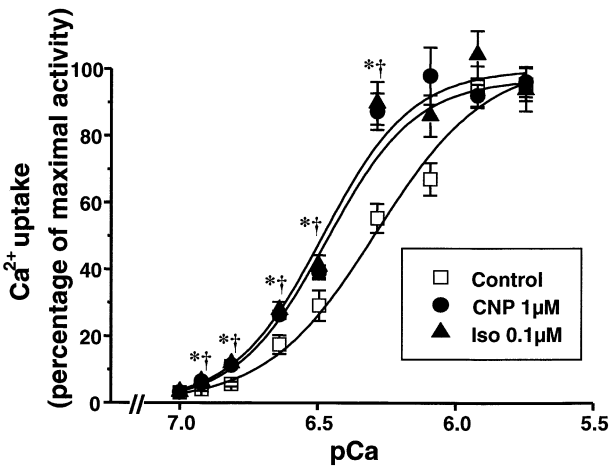

Figure 5 Effect of cardiomyocyte stimulation by C-type natriuretic peptide (CNP) or isoprenaline on the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase. Isolated cells were incubated for 5 min in the presence of 1 μM CNP or 0.1 μM isoprenaline (Iso) or in the absence of any added reagent (control). Then, the sarcoplasmic reticulum was isolated and the rate of oxalate-facilitated, ATP-dependent, $^{45}Ca^{2+}$ uptake was determined. Cell treatments were performed in triplicate wells. The determination of $^{45}Ca^{2+}$ uptake was also performed in triplicate. Data are presented as mean $\pm$ s.e.mean. *$P<0.01$ CNP *vs* control; †$P<0.01$ iso *vs* control. The maximal transport activities for control, CNP- or isoprenaline-treated cells were, respectively, 0.73 ± 0.09, 0.79 ± 0.04, 0.97 ± 0.06 nmol $Ca^{2+}mg^{-1}min^{-1}$.

ylation of the inhibitory subunit of troponin (TnI). Half-maximal effect was obtained with 0.24 ± 0.05 μM and 0.23 ± 0.02 μM of CNP for the phosphorylation of PLN and TnI respectively (Figure 4B, C) whereas ANP, up to 3 μM, did not evoke protein phosphorylation (data not shown). The effects of CNP on protein phosphorylation were mimicked by 10 μM 8-bromo-cGMP (data not shown).

Effect of CNP on the sarcoplasmic reticulum Ca^{2+} pump activity

The sarcoplasmic reticulum Ca^{2+} pump (Ca^{2+}-ATPase) plays a crucial role in the relaxation of the heart (Katz, 1990). PLN controls the uptake of Ca^{2+} by decreasing the affinity of the Ca^{2+}-ATPase for Ca^{2+} (Tada & Inui, 1983). During β-adrenergic stimulation, PLN is phosphorylated by the cAMP-dependent protein kinase, thereby relieving the inhibition of the Ca^{2+}-ATPase (Kranias & Solaro, 1982). We therefore investigated whether the CNP-induced, cGMP-mediated phosphorylation of PLN could also translate into an activation of the Ca^{2+}-ATPase. The enzyme activity was assessed by the measurement of the rate of oxalate-facilitated and ATP-dependent uptake of $^{45}Ca^{2+}$ in homogenates prepared from purified cardiomyocytes treated or not with CNP or isoprenaline. As shown in Figure 5, the uptake of Ca^{2+} in homogenates derived from cells treated with CNP or isoprenaline was greatly increased at low Ca^{2+} concentrations. For instance, at 0.15 μM, a Ca^{2+} concentration found in cardiomyocytes during diastole (Barry & Bridge, 1993), a 2 fold increase in the rate of Ca^{2+} uptake was found for both stimuli compared to control cells ($P<0.01$). The concentrations of free Ca^{2+} required to elicit half-maximal transport activity (EC_{50}) for control, CNP-, and isoprenaline-treated cells were, respectively, 0.52 ± 0.05 μM, 0.34 ± 0.01 μM, and 0.33 ± 0.02 μM. As a control, in parallel wells, and under the same conditions, the phosphorylation of PLN and TnI was assessed and results were similar to those shown in Figure 4.

Discussion

The present study shows that CNP displays a positive lusitropic effect on rat papillary muscles. Catecholamines, such as isoprenaline, have also been reported to exert lusitropic effects which can be illustrated by a decrease in the $+T/-T$ ratio (Grassi de Gende *et al*., 1977). The extent of the positive lusitropic effect of CNP, reflected by a decrease in $+T/-T$ up to 15%, was comparable to that reported for isoprenaline (Mattiazzi *et al*., 1986). However, the nature of the lusitropic effects of CNP and isoprenaline were quite different. Isoprenaline displays lusitropic effects mainly by increasing $-T$ to a higher extent than $+T$ (Grassi de Gende *et al*., 1977). In contrast, as shown here, CNP exerts its lusitropic effect by an immediate enhancement of $-T$ and a slowly developing reduction in $+T$. Moreover, while isoprenaline is a potent positive inotropic agent, CNP was found to be a weak negative inotrope, i.e., it slightly decreases the developed tension (DT). In addition, 8-bromo-cGMP mimicked the effects of CNP on the different contractile parameters whereas the effects of isoprenaline are usually reproduced by cAMP derivatives (Skelton *et al*., 1970). These observations suggest that different mechanisms are involved in the regulation of cardiac contractility by CNP and isoprenaline.

The results presented in this study also show that CNP enhances cGMP accumulation in cardiomyocytes. The EC_{50} values found for $-T$ enhancement, cGMP accumulation and PLN phosphorylation were similar (about 0.2 μM). Therefore, it is tempting to speculate that the increase in $-T$ could be mediated, at least in part, by a cGMP-dependent phospholamban phosphorylation. ANP, at concentrations up to 1 μM, was unable to promote any significant increase in cGMP levels in isolated myocytes. These results are similar to those reported by Koller *et al*., (1991) on the pharmacology of NPR-B. The dose-related cGMP accumulation induced by CNP and the lack of effect of ANP suggest that NPR-B is involved in the response to CNP. Unfortunately, no specific NPR-B antagonist has been described so far, and such a tool would be required to provide a definitive answer to this issue.

Physiologically relevant concentrations of CNP are so far unknown. Circulating concentrations of CNP range between 10^{-9} and 10^{-12} M (Chen & Burnett, 1998; Wei *et al*., 1993). However, the EC_{50} value of CNP for cGMP production found by Koller *et al*. (1991) in cells transfected with NPR-B was 10^{-7} M. Moreover, Amin *et al*. (1996) have shown that CNP dilates pre-constricted arterioles and that the maximal effect of the natriuretic peptide was not attained at 10^{-7} M. Finally, Ueno *et al*. (1997) have demonstrated that CNP can exert antifibroproliferative effects *via* an autocrine/paracrine loop in the absence of enhanced plasma levels of CNP. Based on these observations, we suggest that the EC_{50} values found in the present work for CNP (about 0.2 μM) could be physiologically relevant.

Activation of the sarcoplasmic reticulum Ca^{2+} pump is considered to play a major role in the mechanism of isoprenaline-induced lusitropism. This activation is achieved by the cAMP-dependent phosphorylation of phospholamban, the endogenous inhibitor of the Ca^{2+}-ATPase (Kranias & Solaro, 1982). Our results suggest that the CNP-induced positive lusitropic effect could be mediated, at least in part, by a cGMP-dependent PLN-phosphorylation and the subsequent activation of the sarcoplasmic reticulum Ca^{2+} pump. Indeed, treatment of isolated adult cardiomyocytes with CNP induced the accumulation of cGMP, but not cAMP, and a concomitant phosphorylation of phospholamban. The EC_{50} value of the Ca^{2+}-ATPase for Ca^{2+} was decreased by 35% in CNP-treated myocytes as compared to unstimulated cells. Under the same experimental conditions, isoprenaline raised cAMP, but not cGMP levels, induced phospholamban phosphorylation and produced a similar activation of the sarcoplasmic reticulum Ca^{2+} pump. For comparison, in heart homogenates prepared from phospholamban-deficient mice, the EC_{50} value of the Ca^{2+} pump for Ca^{2+} was reduced by about 50% (Luo *et al*., 1994).

The phosphorylation of TnI observed in cardiomyocytes treated with CNP might also be involved in the relaxant effect of the natriuretic peptide. Indeed cAMP-dependent phosphorylation of TnI desensitizes myofilaments towards Ca^{2+} and thereby increases the rate of cardiac muscle relaxation (Zhang *et al*., 1995). In addition, treatment of intact cardiac myocytes with 8-bromo-cGMP reduces the myofilament response to Ca^{2+} (Shah *et al*., 1994). Thus, it is tempting to speculate that the phosphorylation of TnI induced by a CNP-dependent increase in cGMP also led to myofilament desensitization. In this respect, it will be interesting to study the effect of CNP on calcium transients in isolated, electrically-stimulated, cardiomyocytes.

As pointed out above, both CNP and isoprenaline enhance the relaxation of papillary muscles and activate the Ca^{2+}-ATPase in cardiomyocytes. However, while catecholamines are positive inotropic agents, we found that papillary muscles treated with CNP or 8-bromo-cGMP developed a negative inotropic effect. cGMP-dependent negative inotropic effects

have already been described in the literature. In isolated rat cardiomyocytes (Shah *et al*., 1994) and in cat papillary muscles (Mohan *et al*., 1997), 8-bromo-cGMP also promotes a negative inotropic effect. In addition, negative inotropic effects of various cytokines on papillary muscles were reported to be mediated by the stimulation of the synthesis of nitric oxide, an activator of the soluble guanylate cyclase (Finkel *et al*., 1992). In contrast to these results, MacDonell & Diamond (1997) showed that activation of the cGMP-dependent protein kinase induced by ANP or a NO donor could occur in isoprenaline-treated rat cardiomyocytes without any effect on contractility. However, as mentioned before, no significant increase in cGMP was found in our purified cardiomyocyte preparation treated with ANP.

The difference in the inotropic response to CNP and catecholamines could be due to a differential effect of cGMP and cAMP on the activity of L-type Ca^{2+} channels. cAMP-increasing agents induce the phosphorylation and subsequent activation of these channels leading to a rise in the intracellular Ca^{2+} concentration (Hartzell, 1988). In contrast, in isolated rat cardiomyocytes, addition of 8-bromo-cGMP does not alter peak Ca^{2+} levels (Shah *et al*., 1994). Moreover, cGMP has been shown to inhibit the basal and cAMP-stimulated activity of L-type Ca^{2+} channels in rat cardiomyocytes (Sumii & Sperelakis, 1995; Méry *et al*., 1991). Furthermore, the CNP-dependent phosphorylation of TnI and a subsequent myofilament desensitization, can also contribute to the negative inotropic effect of CNP.

In summary, we have demonstrated that CNP displays a positive lusitropic effect in a rat papillary muscle preparation. In contrast to catecholamines, the lusitropism induced by CNP is associated with a negative inotropic effect. If the present observations extend to the human heart, this might provide a new insight in the comprehension of pathologies associated with impaired relaxation.

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