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Effect of atrial natriuretic peptide and cyclic GMP phosphodiesterase inhibition on collagen synthesis by adult cardiac fibroblasts

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1 Cardiac fibroblasts play an important role in the pathophysiology of cardiac remodelling induced by hypertension and myocardial infarction by undergoing proliferation and depositing extracellular matrix proteins such as collagen. We have examined the effects of atrial natriuretic peptide (ANP) on proliferation and collagen synthesis by adult rat and human cardiac fibroblasts in culture.

2 In cells from both species radioligand studies using ^{125}I-ANP suggested that the majority of binding sites (>85%) were non-guanylyl cyclase-linked (NPR-C subtype). Nonetheless ANP (10^{-9} to 10^{-6} M), in the presence of zaprinast, an inhibitor of phosphodiesterase 5 (PDE5), increased fibroblast cyclic GMP levels 3–5 fold in a concentration-dependent manner ($P<0.05$).

3 ANP (10^{-11} to 10^{-6} M), a NPR-C ligand, C-ANF4-23 (10^{-11} to 10^{-6} M) and zaprinast alone had no significant effect on either basal or serum-stimulated DNA synthesis or fibroblast number. In combination with zaprinast (10^{-5} M), however, ANP (10^{-9} to 10^{-6} M) but not C-ANF4-23 (10^{-7} M) inhibited markedly both basal and stimulated fibroblast mitogenesis, an effect reproduced by 8-bromo-cyclic GMP (10^{-5} to 10^{-3} M).

4 Collagen synthesis, determined by measuring hydroxyproline levels, was stimulated with transforming growth factor-β1 (40 pM), angiotensin II (10^{-7} M) or 2% foetal bovine serum. The increase in collagen production, normalised by cell number, was reduced dramatically (to at or near basal production) by ANP (10^{-9} to 10^{-7} M) but not C-ANF4-23 (10^{-7} M) in the presence of zaprinast. Again 8-bromo-cyclic GMP (10^{-5} to 10^{-3} M) reproduced the effect.

5 ANP is capable of inhibiting collagen synthesis in adult rat and human cardiac fibroblasts *via* cyclic GMP, a property unmasked and enhanced by inhibition of PDE5.

Keywords: Cardiac fibroblasts; atrial natriuretic peptide; cyclic GMP; zaprinast; phosphodiesterase 5; collagen synthesis; cell proliferation

Introduction

The cardiac hypertrophy that occurs in response to chronic pressure overload (e.g. systemic hypertension) or myocardial infarction is often associated with a disproportionate increase in collagen deposition (Pearlman *et al*., 1992; Brilla *et al*., 1990). While providing skeletal support for the myocardium and assisting the heart to withstand elevated intracardiac pressure, overproduction of fibrous tissue stiffens the ventricle, impairs myocardial contraction and predisposes to cardiac failure (Weber *et al*., 1990).

Cardiac fibroblasts are the major cell type responsible for synthesising and degrading the fibrillar collagens (types I and III) of the myocardium (Medugoric & Jacob, 1983; Brilla *et al*., 1995). A number of factors have been linked to the stimulation of fibroblast proliferation and collagen deposition in the heart. These include vasoactive factors such as angiotensin II (Villareal *et al*., 1993; Sadoshima & Izumo, 1993; Brilla *et al*., 1994; Kawaguchi & Kitabatake, 1996), aldosterone (Brilla *et al*., 1994, 1995; Zhou *et al*., 1996) and the endothelins (Guarda *et al*., 1993) as well as traditional growth factors such as transforming growth factor-β1 (TGF-β1) and fibroblast growth factor (Eghbali *et al*., 1991; Butt *et al*., 1995). However, little is known about the mechanisms that inhibit collagen production in cardiac tissue.

The heart is a major site of synthesis of atrial and brain natriuretic peptides (ANP and BNP). Recently these peptides have been reported to inhibit agonist and stretch-induced proliferation of rat neonatal cardiac fibroblasts in culture (Cao & Gardner, 1995). Two major sub-classes of natriuretic peptide receptors have been identified to date (Koller & Goeddel, 1992). One sub-class comprises two distinct guanylyl cyclase-linked receptors, NPR-A and NPR-B, which mediate many of the actions of ANP and BNP *via* cyclic GMP. The other comprises NPR-C which is not linked to guanylyl cyclase but may transduce effects through cyclic AMP or phosphoinositides (Anand-Schrivastava & Trachte, 1993). NPR-C is the predominant receptor subtype expressed by fibroblasts in culture and accounts for >70% of the natriuretic peptide receptor population in neonatal and adult rat cardiac fibroblasts (Cao & Gardner, 1995; Lin *et al*., 1995). Interestingly both sub-classes of natriuretic peptide receptor have been associated with inhibition of [^{3}H]-thymidine incorporation and cell division in neonatal rat cardiac fibroblasts (Cao & Gardner, 1995).

These observations suggest that the natriuretic peptides might regulate cardiac fibroblast activity but no data are available on their effect on collagen synthesis. Accordingly, the present studies focused on the effect of ANP on collagen

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production in adult rat and human cardiac fibroblasts in culture and demonstrated a potent inhibitory role for cyclic GMP.

Methods

Cell culture

Cardiac fibroblasts were obtained from the ventricles of (i) 9 male Wistar-Kyoto rats (250–280 g) killed with an overdose of Hypnorm anaesthetic (fentanyl/flunasone) and (ii) a human donor heart not used in cardiac transplantation. Ventricle tissue was placed in Dulbecco's modified Eagle medium (DMEM) containing 0.1% bovine serum albumin (BSA), cut into small pieces and then incubated with 10 mg ml^{-1} collagenase type 2 for 180 min at 37°C in a humidified atmosphere of CO_2 (5%) and air (95%). The resulting cell suspension was washed three times with fresh DMEM containing 0.1% BSA. Cells were then resuspended in culture medium supplemented with 10% foetal bovine serum (FBS) and plated for 30 min at 37°C to allow fibroblasts to attach to the cell culture flasks. After removal of myocardial cells by aspiration, ventricular fibroblasts were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin (100 IU l^{-1} and 100 μg l^{-1}, respectively) and amphotericin B (2.5 μg l^{-1}). Cells were characterised as cardiac fibroblasts by immunostaining with a polyclonal anti-vimentin antibody, which positively stained >99% of the cells. Moreover, the cells had a typical fibroblast morphology, i.e., were thin and triangular with a light cytoplasm. Studies were conducted in cultured rat cardiac fibroblasts from passages 1 to 6 and human cardiac fibroblasts from passages 6 to 10. Preliminary experiments using Trypan Blue staining established that none of the test treatments (ANP, C-ANF4-23, TGF-β1, angiotensin II or zaprinast) alone or in combination affected cell viability in the concentrations studied. Furthermore, all cells were inspected by microscopy at the end of each treatment protocol for evidence of cell loss or damage.

ANP binding experiments

Binding assays were peformed in 48-well plates on confluent cell cultures, as described by Lin *et al.* (1995). Rat cold and labelled ANP were used throughout for both rat and human cells as previous studies have shown that this peptide exhibits equal affinity for rat and human natriuretic peptide receptors (Schoenfeld *et al.*, 1995). Briefly, cells were washed with DMEM containing 0.1% BSA and incubated with ^{125}I-ANP (100,000 c.p.m. per well) and various concentrations of unlabelled ANP or NPR-C-specific analogue C-ANF4-23 in 0.1 ml DMEM plus 0.1% BSA. After 4 h at 4°C, the cells were washed three times with cold DMEM and solubilised in 0.2 ml of 0.25 M NaOH containing 0.5% SDS for 30 min at room temperature. Cell lysates were transferred to polystyrene tubes and counted in a gamma counter. Nonspecific binding was determined by incubating parallel cultures with ^{125}I-ANP in the presence of 10^{-5} M unlabelled ANP. Specific binding was calculated as the difference between total and nonspecific binding. Aliquots from the same plates were used to determine protein content according to the method described by Bradford (1976) using BSA as a standard.

Measurement of cyclic GMP

Fibroblasts were grown to confluence in 24-well plates and then growth-arrested for 24 h. The cells were washed twice with PBS and incubated in DMEM plus 0.1% BSA in the presence of 10^{-5} M zaprinast, an inhibitor of phosphodiesterase 5 (PDE5) (Coste & Grondin, 1995). After 15-min preincubation, different concentrations of ANP or C-ANF4-23 were added to the cells and incubated for further 20 min. After this period, the medium was aspirated and 0.5 ml ice cold 65% (vol/vol) ethanol was added to the cells. The remaining precipitates were washed twice with 0.25 ml 65% ethanol and the washings were added to the appropriate tubes. The extracts were centrifuged at 2000 *g* for 15 min at 4°C, transferred to fresh tubes, evaporated in a speed-vac and frozen at −20°C until use. Intracellular cyclic GMP content was determined using an enzyme immunoassay kit after acetylation (Amersham).

DNA synthesis and cell number

DNA synthesis was assayed by measuring [^{3}H]thymidine incorporation as described previously (Peiro *et al.*, 1992). In brief, fibroblast cultures were grown until confluence, washed and arrested for 24 h in serum-free medium and then incubated with DMEM plus 0.1% BSA containing [^{3}H]thymidine (1 μCi ml^{-1}) and different factors for 24 h. The cells were washed three times with cold phosphate-buffered saline (PBS) and incubated at 4°C for 30 min in 10% trichloroacetic acid. The acid-insoluble material was solubilised by incubation with 0.2 M NaOH at 4°C overnight and counted in a liquid scintillation counter.

Cell number was determined by the method of Gillies *et al.* (1986) with minor modifications. Cardiac fibroblasts were split to 24-well plates at a density of 5×10^4 cells per well, cultured for 4 days with serum and then arrested for 24 h. The medium was then replaced by fresh medium with different compounds and the cells incubated for 24, 48 and 72 h. Media with additives were changed every 24 h throughout the experiment. After these time periods, cardiac fibroblasts were stained with crystal violet, a basic dye that stains cell nuclei, for 30 min at room temperature. The plates were washed with distilled water and 10% acetic acid was added to each well. After 15 min at room temperature, aliquots from each well were read at 595 nm on a multiwell scanning spectrophotometer. A standard curve was carried out to establish the relationship between optic units and cell number determined by haemocytometer counting ($r = 0.98$).

Procollagen synthesis

Procollagen synthesis was assayed by measuring hydroxyproline in intact proteins, as well as hydroxyproline derived from procollagen synthesised and subsequently degraded during the culture period, using the high-pressure liquid chromatography (HPLC) method described by McAnulty *et al.* (1991). Briefly, cardiac fibroblasts were seeded at 6×10^4 cells per well in 12-well plates. On achieving confluence, the medium was replaced with DMEM supplemented with 0.1% BSA, 50 μg l^{-1} ascorbic acid and 0.2 mM proline with or without a factor to stimulate collagen production (TGF-β1 40 pM, angiotensin II 0.1 μM or 2% FBS). After 24 h this medium was replaced with fresh medium with or without stimulant and the test inhibitory compounds of interest (i.e. ANP, C-ANF4-23, zaprinast). At this point (time 0), one of the plates with fresh medium was frozen and the amount of hydroxyproline in these wells was taken as time zero levels (and subsequently substracted from all other sample values). After a further 24 h, the rest of the plates were frozen at −20°C overnight. The frozen plates were then thawed and the cell layer of each well scraped into the medium. The contents of each well were aspirated and transferred to

tubes. Culture wells were washed with 1 ml PBS (which was added to the initial aspirate) and the proteins in each tube were precipitated by addition of ethanol to a final concentration of 67% (vol/vol) at 4°C overnight. The proteins (representing the ethanol-insoluble fraction) were separated by filtration through a 0.45 μm pore filter (type HV, Millipore) using a vacuum filtration unit (Millipore). Supernatants (containing ethanol soluble fractions) were collected and evaporated to dryness on a Dri-Block sample concentrator. The filters and dried supernatants were then hydrolysed in 6 M hydrochloric acid at 110°C for 16 h. Hydrolysates were decolourised with 30 mg charcoal and centrifuged at 2000 *g* for 20 min and these supernatants were then collected and 100 μl-aliquot of each sample dried on a speed-vac before chromatography.

Dried samples were re-dissolved in 100 μl distilled water, buffered with potassium tetraborate (100 μl, 0.4 M, pH 9.5) and reacted with 36 mM 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) in a methanol (100 μl). Samples were protected from light and incubated at 37°C for 20 min. The reaction was stopped by addition of hydrochloric acid (50 μl, 1.5 M), and finally 150 μl sodium acetate (167 mM, pH 6.4) in acetonitrile (26% vol/vol) was added. Samples were filtered (Millipore, type GV, pore size 0.22 μm) and a 100 μl-aliquot was injected onto the HPLC column and eluted with an acetonitrile gradient according to the method described by Campa *et al.* (1990). The hydroxyproline content in each sample was determined by comparing peak areas of samples from the chromatogram to those generated from standard solutions, derivatised and separated under identical conditions.

Hydroxyproline measured in the ethanol-insoluble fraction was taken as an index of procollagen production, whereas the hydroxyproline in the ethanol-soluble fraction represented hydroxyproline derived from procollagen synthesised and subsequently degraded during the culture period. The rate of procollagen synthesis was obtained from the combined values for ethanol-soluble and ethanol-insoluble fractions. In all experiments, parallel cultures treated identically to those for procollagen synthesis were used to determine the cell number per well with crystal violet as described above. Collagen production was then expressed as fmoles hydroxyproline per cell.

Drugs used

[^{3}H]-Thymidine (specific activity, 6.7 Ci mmol^{-1}) was purchased from ICN Biomedicals Ltd (U.K.). ^{125}I-rat ANP (specific activity, 2000 Ci mmol^{-1}) was obtained from Amersham International plc (U.K.). Rat ANP (28 aminoacids) and rat C-ANF4-23 (des-Gln^{18}, Ser^{19}, Leu^{21}, Gly^{22} ANF$_{4\text{-}23}$-NH$_2$) were from Peninsula Laboratories Inc (U.S.A.). Human TGF-β1 was from R&D Systems Europe Ltd (U.K.). Collagenase type 2 (273 U mg^{-1}) was from Worthington Biochemical Corporation (U.S.A.). Angiotensin II, bovine serum albumin, zaprinast and 8-bromoguanosine 3′ : 5′-cyclic monophosphate were from Sigma Chemical Company Ltd (U.K.). TGF-β1 was dissolved in 4 mM hydrochloric acid containing 0.1% bovine serum albumin to prepare a stock solution of 40 nM. Zaprinast stock solution was prepared in dimethyl sulfoxide at 10^{-1} M. All other agents were dissolved in distilled water and diluted to the desired concentration with culture medium. Previous experiments showed that the solvents used had no effect on the cells at the concentrations applied.

Data analysis and statistics

Results are given as mean ± s.e.mean from the number (n) of independent experiments, each one performed at least in triplicate. Binding data were evaluated with computer assisted non-linear regression analysis (GraphPad Prism, GraphPad Software Inc.) to calculate the dissociation constant (K_d). Two-way analysis of variance (ANOVA) and unpaired Student's t-test were used for the statistical evaluations. P values less than 0.05 were considered to be statistically significant.

Results

Natriuretic peptide receptor expression in cultured cardiac fibroblasts

Binding experiments with ^{125}I-ANP were performed using rat cardiac fibroblasts from passage 1 and human cardiac fibroblasts from passage 8 to examine for the presence of natriuretic peptide receptors (Figure 1). In both rat and human cells, non-linear regression analysis of experiments using unlabelled ANP as the competitive ligand suggested a single high affinity binding site affinity (K_d, 0.302 ± 0.02 and 0.135 ± 0.08 nmol/L for rat and human cells, respectively). The truncated ANP analogue, C-ANF4-23, which exhibits selectivity for NPR-C over NPR-A and NPR-B (Maack, 1992), inhibited 85–88% of maximum binding to rat and human fibroblasts, in both cases with a slightly lower affinity than ANP (K_d, 1.28 ± 0.05 and 5.06 ± 0.15 nmol/L for rat and human cells, respectively). These data suggest that approximately 85% of the total receptor population on freshly isolated

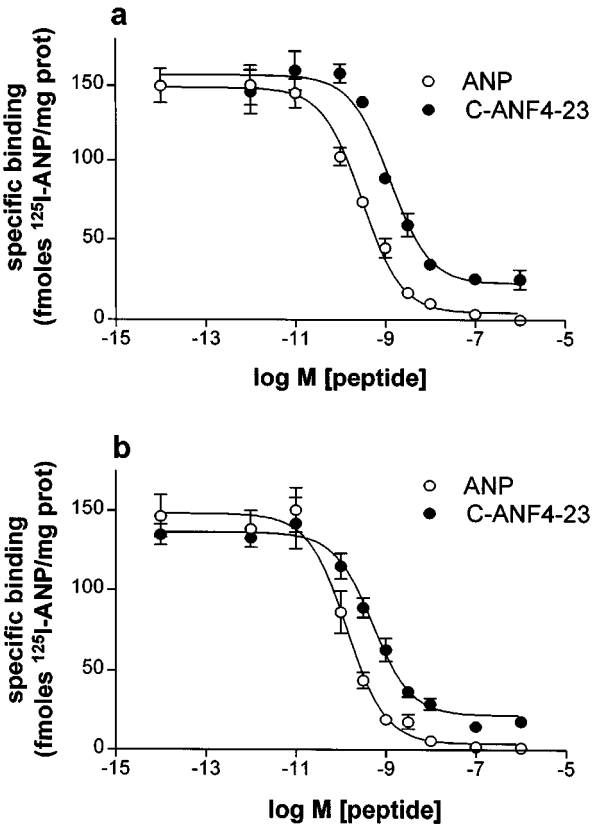

Figure 1 Inhibition of the specific binding of ^{125}I-atrial natriuretic peptide (^{125}I-ANP) to rat (a) passage 1 and human (b) passage 8 cardiac fibroblasts. Cells were incubated at 4°C for 4 h alone or in the presence of increasing concentrations of unlabeled ANP or the NPR-C agonist, C-ANF4-23. Results are expressed as fmoles of ^{125}I-ANP specifically bound at each peptide concentration per milligram protein. Data shown are mean ± s.e.m. of four experiments, each performed in triplicate.

fibroblasts are of the NPR-C subtype and the remaining 15% are NPR-A/NPR-B.

Effect of ANP on cyclic GMP levels in cardiac fibroblasts

Previous experience has indicated that at high concentrations ($\geqslant 10^{-6}$ M) C-ANF4-23 may lose selectivity for NPR-C (Rutherford *et al*., 1994). Therefore we examined the effect of both ANP and C-ANF4-23 on cyclic GMP production in late passage fibroblasts. As shown in Figure 2, in the presence of the PDE5 inhibitor, zaprinast (10^{-5} M), ANP increased cyclic GMP levels in rat (passage 5) and human (passage 10) fibroblasts by about 5-fold and 3-fold respectively over the concentration range 10^{-9} to 10^{-6} M. C-ANF4-23 had no effect on cyclic GMP levels except at the highest concentration (10^{-6} M) studied. These data are consistent with the idea that at high concentrations, C-ANF4-23 may bind to NPR-A and NPR-B receptors and underestimate the prevalence of guanylyl cyclase-linked receptors in radioligand binding studies.

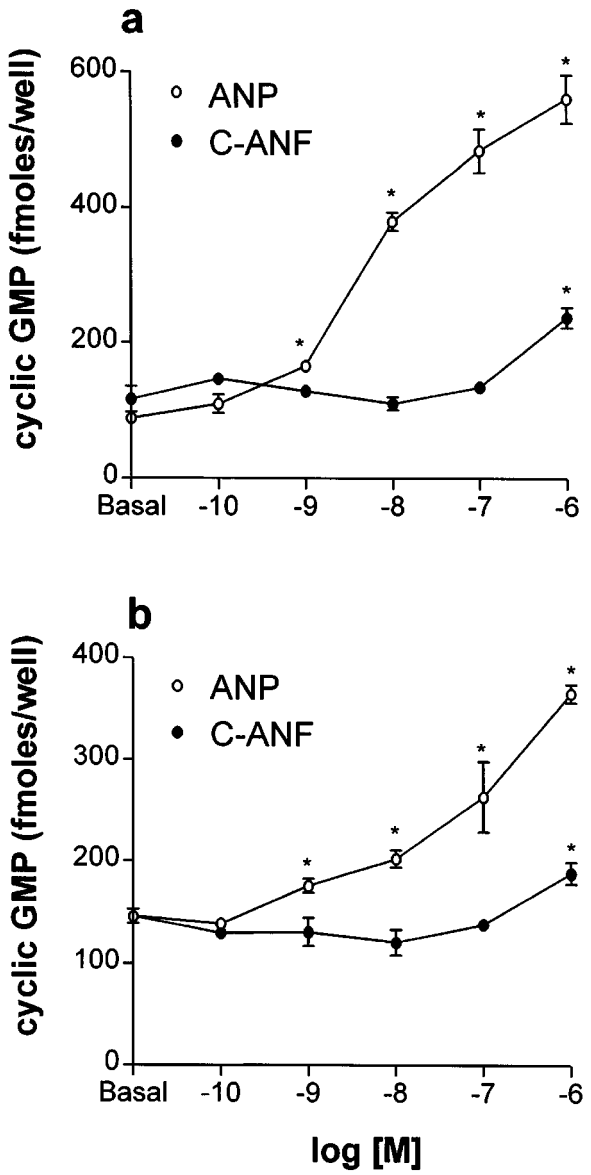

Figure 2 Effect of atrial natriuretic peptide (ANP) and the NPRC-agonist, C-ANF4-23, on cyclic GMP accumulation in rat (a) and human (b) cardiac fibroblasts. The cells were preincubated with 10^{-5} M zaprinast for 15 min and then incubated for a further 20 min with ANP and C-ANF4-23. Data are mean$\pm$s.e.m. of three experiments, each performed in duplicate. *$P<0.05$ vs value in the absence of ANP or C-ANF4-23.

Effect of ANP on cardiac fibroblast mitogenesis

To determine whether ANP possessed the capacity to regulate cardiac fibroblast proliferation, we measured [^{3}H]thymidine incorporation, an indirect assay of DNA synthesis, in our cell cultures. 24-h treatment with ANP (10^{-6} M) alone did not modify significantly basal or serum-stimulated thymidine incorporation in fibroblasts from either species (Figure 3). Similarly, zaprinast had no effect on its own but in combination with ANP produced concentration-dependent inhibition of basal and serum-stimulated thymidine incorporation over the range ANP 10^{-9} to 10^{-6} M (Figure 3) in both rat and human fibroblasts. Moreover, 10^{-6} M ANP with zaprinast abolished the increase in thymidine incorporation induced by 1% serum in both cultures. These effects were reproduced by 8-bromo-cyclic GMP (Figure 4) but not by the combination of C-ANF4-23 (10^{-7} M) and zaprinast (data not shown).

To analyse further the effect of ANP on cardiac fibroblast mitogenesis, we determined the number of cells after 24, 48 and 72-h treatment in the presence or the absence of serum. ANP (10^{-9} M) alone had no significant effect on cardiac fibroblast number under basal conditions or in the presence of 1% FBS.

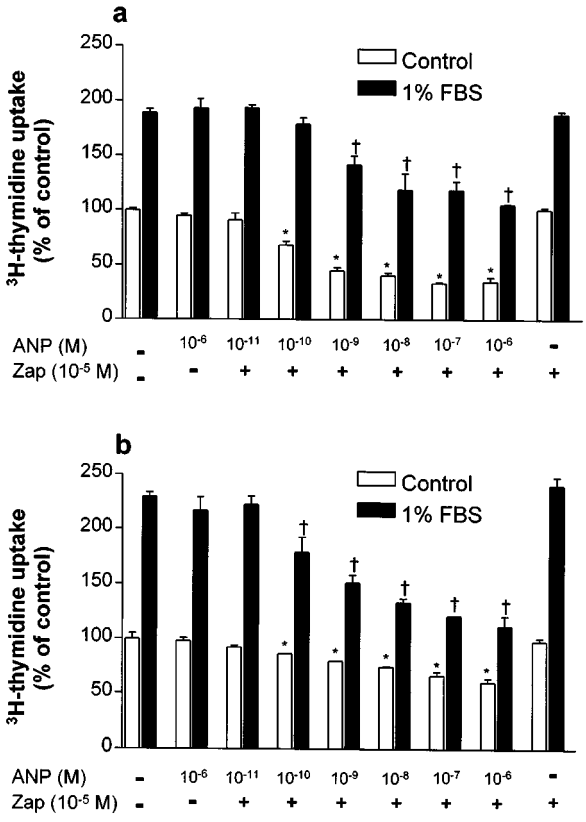

Figure 3 Effect of atrial natriuretic peptide (ANP) and zaprinast on basal and serum-stimulated thymidine incorporation in rat (a) and human (b) cardiac fibroblasts. Cells were incubated with increasing concentrations of ANP with zaprinast in control or 1% foetal bovine serum (FBS)-medium in the presence of ^{3}H-thymidine for 24 h. All values are normalised to the levels found in untreated cells (control). Data are mean$\pm$s.e.m. $n=5$. *$P<0.05$ vs control cells; †$P<0.05$ vs serum-treated cells.

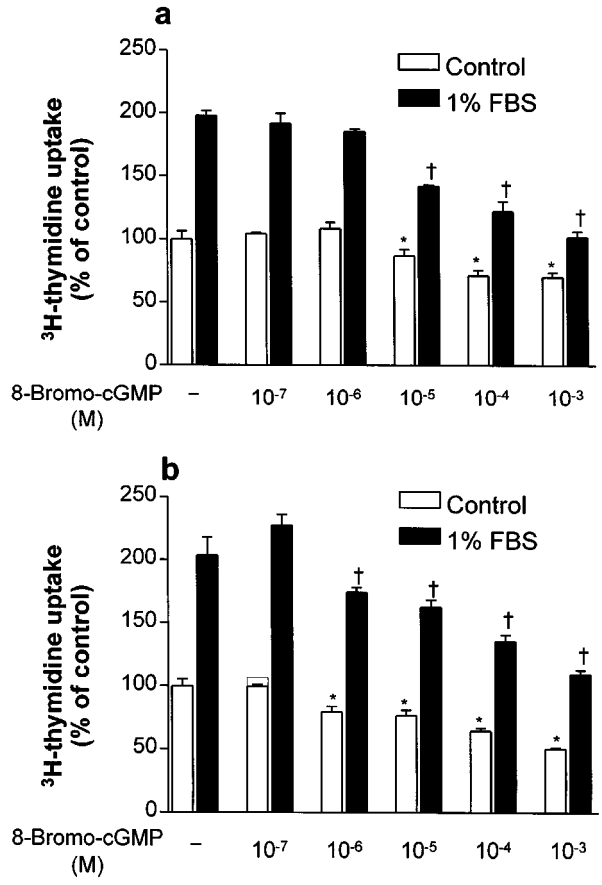

Figure 4 Effect of 8-bromo-cyclic GMP (8-Br-cGMP) on basal and serum-stimulated thymidine incorporation in rat (**a**) and human (**b**) cardiac fibroblasts. Cells were incubated with increasing concentrations of 8-Br-cGMP in control or 1% foetal bovine serum (FBS)-medium in the presence of ^{3}H-thymidine for 24 h. All values are normalised to the levels found in untreated cells (control). Data are mean±s.e.m. $n=4$. *$P<0.05$ vs control cells; †$P<0.05$ vs serum-treated cells.

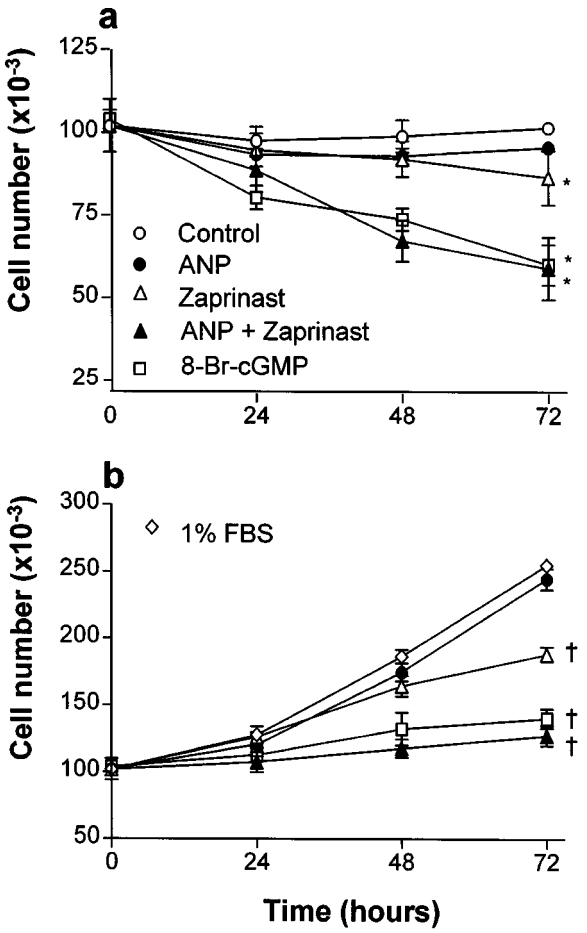

Figure 5 Effect of 10^{-6} M zaprinast, 10^{-9} M atrial natriuretic peptide (ANP), zaprinast with ANP or 10^{-4} M 8-bromo-cyclic GMP (8-Br-cGMP) on rat cardiac fibroblast number in the absence (a) and presence (b) of 1% foetal bovine serum for 24, 48 and 72 h. Data are mean±s.e.m. of four experiments, each performed in triplicate. *$P<0.05$ vs control cells; †$P<0.05$ vs serum-treated cells (two-way ANOVA).

Zaprinast (10^{-6} M) alone had a small inhibitory effect at 72 h but the combination of ANP and zaprinast produced significant inhibition of proliferation, evident from 24 h onwards (Figure 5). This inhibitory effect was again mimicked by 8-bromo cyclic GMP (10^{-4} M) (Figure 5).

Effect of ANP on procollagen synthesis by cardiac fibroblasts

To examine the potential inhibitory effect of ANP on procollagen synthesis, cells were stimulated with TGF-β1 (40 pM). TGF-β1 produced a 1.8 to 3 fold increase in procollagen levels which was not affected by ANP (10^{-7} M) or zaprinast (10^{-5} M) alone in either rat or human cells (Figure 6). However, ANP in the presence of zaprinast (10^{-5} M) achieved a concentration-dependent decrease in stimulated collagen synthesis, and at concentrations around 10^{-7} M abolished the effect of the stimulant (Figure 6). C-ANF4-23 (10^{-7} M) alone or with zaprinast had no effect on procollagen synthesis. To test the generality of the inhibitory action of ANP, human cardiac fibroblasts were stimulated with angiotensin II (10^{-7} M) or 2% foetal bovine serum. Angioten-sin II- and serum-stimulated procollagen synthesis by human cells was also inhibited by ANP (10^{-7} M) and zaprinast (10^{-5} M) (Figure 7). The inhibitory effect of ANP and zaprinast on TGF-β1 stimulated procollagen synthesis was reproduced by 8-bromo cyclic GMP in rat and human cells; 8-bromo-cyclic GMP 10^{-3} M abolished the effect of TGF-β1 (Figure 8). Interestingly, the inhibitory effects of ANP plus zaprinast and 8-bromo-cyclic GMP were confined to procollagen synthesis. Neither of these two treatments influenced the rate of procollagen degradation, as defined by the amount of hydroxyproline in the ethanol-soluble fraction.

Discussion

Collagen is an important component of cardiac tissue, comprising 3–6% of the normal myocardium by dry weight (Weber & Brilla, 1991). Collagen turnover in the myocardium is a dynamic process, an activity co-ordinated largely by the cardiac fibroblast. Several humoral factors, including TGF-β1 (Eghbali *et al*., 1991) and angiotensin II (Sadoshima & Izumo, 1993; Brilla *et al*., 1994; Kawaguchi & Kitabatake, 1996; Zhou *et al*., 1996) have been shown to stimulate cardiac fibroblasts and are thought to participate in the disproportionate increase in collagen deposition in the heart that occurs in response to chronic pressure overload. This is the first demonstration that

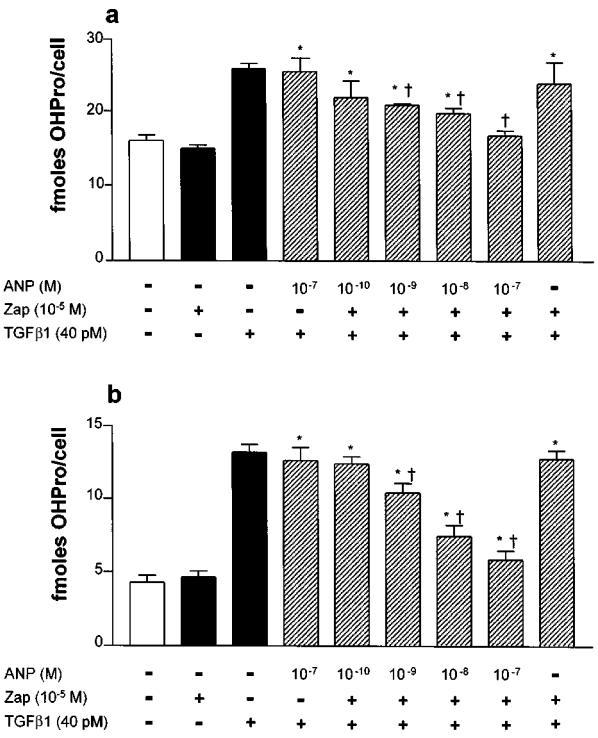

Figure 6 Effect of atrial natriuretic peptide (ANP) and zaprinast (Zap) on TGF-β1-stimulated collagen synthesis in rat (a) and human (b) cardiac fibroblasts. Cells were incubated with TGF-β1 for 24 h in the presence and absence of ANP and zaprinast. Hydroxyproline (OHPro) levels represent net collagen synthesis corrected for cell number. Data are mean$\pm$s.e.m. of three experiments. *$P<0.05$ vs control cells; †$P<0.05$ vs TGF-β1-stimulated cells.

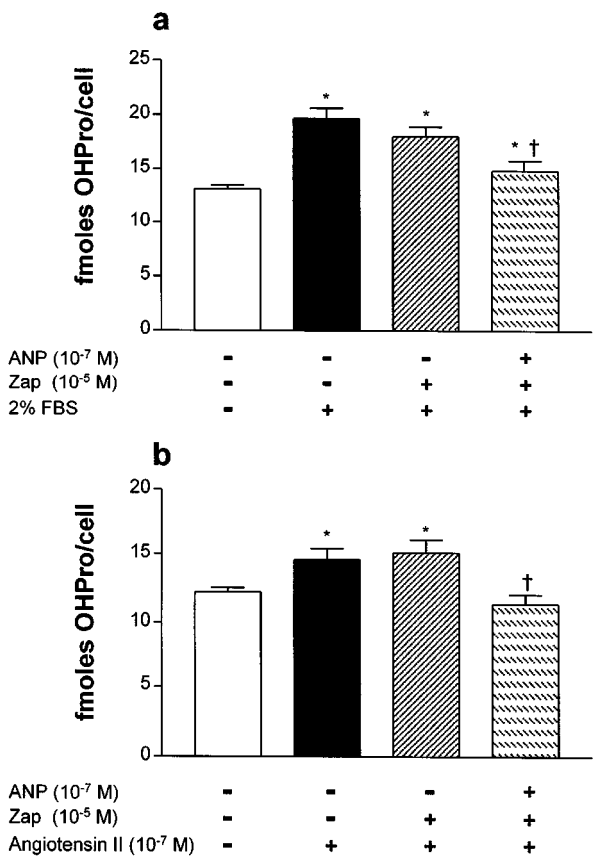

Figure 7 Effect of atrial natriuretic peptide (ANP) and zaprinast (Zap) on serum- (a) or angiotensin II- (b) stimulated collagen synthesis in human cardiac fibroblasts. Cells were incubated with angiotensin II or 2% serum for 24 h in the presence of ANP and zaprinast. Hydroxyproline (OHPro) levels represent net collagen synthesis corrected for cell number. Data are mean$\pm$s.e.m. of three experiments. *$P<0.05$ vs control cells; †$P<0.05$ vs agonist-stimulated cells.

ANP activity can be manipulated pharmacologically to inhibit collagen synthesis by fibroblasts from rat and human hearts.

Previous reports have described anti-trophic effects of ANP in cells cultured from vascular tissue (Abell *et al*., 1989; Itoh *et al*., 1990). For example, ANP (10^{-7} M) has been shown to inhibit serum and growth factor induced mitogenesis and angiotensin II-stimulated hypertrophy of rat aortic smooth muscle cells (Abell *et al*., 1989; Itoh *et al*., 1990). Recently, the natriuretic peptides have been reported to inhibit DNA synthesis in rat neonatal cardiac fibroblasts (Cao & Gardner, 1995). We saw no effect of ANP (10^{-7} M) alone on the proliferation of adult rat and human cardiac fibroblasts in culture but in combination with a PDE5 inhibitor, ANP in nanomolar concentrations attenuated basal and serum-stimulated growth. Moreover, we have shown for the first time that the same combination is a potent inhibitor of a variety of factors (TGF-β1, angiotensin II and serum) that stimulate collagen synthesis in cardiac fibroblasts.

The substrate specificity and kinetics of PDE5 suggest that it is most directly involved in the regulation of cyclic GMP levels and it is traditionally termed cyclic GMP-specific (Beavo, 1995). Zaprinast is a selective inhibitor of PDE5 (Beavo, 1995; Coste & Grondin, 1995) and alone had a small effect on cell proliferation at 72 h, which may reflect augmentation of endogenous cyclic GMP production. The observation that zaprinast is necessary to demonstrate the inhibitory action of ANP on cardiac fibroblast proliferation and collagen synthesis in our cultures suggests that the effect is mediated *via* cyclic GMP. In support of this, ANP and zaprinast increased cardiac fibroblast cyclic GMP concentrations 3–5 fold. Assuming an intracellular volume of 1 μl per 10^6 cells, the rise in intracellular cyclic GMP levels with ANP and zaprinast reaches around 10^{-3} M. The addition of 8-bromo-cyclic GMP to cultured fibroblasts reproduced the effects of ANP and zaprinast over the concentration range 10^{-6} to 10^{-3} M. Our protocol allowed us to examine the effect of these treatments on procollagen degradation as well as synthesis. Significantly, the inhibitory effect was observed only on the rate of synthesis, with no detectable effect on the rate of degradation in culture. The mechanism of cyclic GMP-mediated inhibition of procollagen synthesis, in particular whether it occurs at the level of transcription or translation, is not defined by these experiments but will be the subject of future work.

The lack of effect of ANP alone probably reflects the relatively low expression of guanylyl cyclase-linked receptors in our fibroblasts in culture i.e. the level of expression is insufficient to produce a significant elevation of cyclic GMP without PDE5 inhibition. Our binding studies and those of others (Cao & Gardner, 1995; Lin *et al*., 1995) suggest that the major natriuretic peptide receptor subtype on cardiac fibroblasts is NPR-C. We found no evidence that NPR-C regulates adult rat or human cardiac fibroblast activity: C-ANF4-23 10^{-7} M, a concentration that had no measurable effect on cyclic GMP levels when combined with zaprinast, had no effect on proliferation or collagen synthesis. These data are at variance with studies by Cao & Gardner (1995) in rat

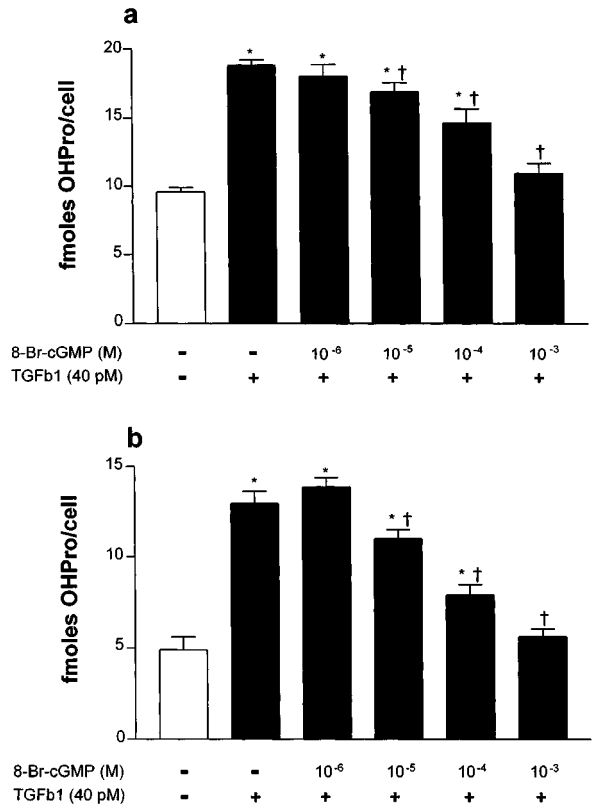

Figure 8 Effect of 8-bromo-cyclic GMP (8-Br-cGMP) on TGF-β1-stimulated collagen synthesis in rat (a) and human (b) cardiac fibroblasts. Cells were incubated with TGF-β1 for 24 h in the presence of increasing concentrations of 8-Br-cGMP. Hydroxyproline (OHPro) levels represent net collagen synthesis corrected for cell number. Data are mean $\pm$ s.e.m. of three experiments. $^*P<0.05$ vs control cells; $\dagger P<0.05$ vs TGFβ1-stimulated cells.

neonatal cardiac fibroblasts where DNA synthesis was inhiited by incubation with C-ANF4-23 at concentrations $\geqslant 10^{-7}$ M. We have found that high concentrations (10^{-6} M) of C-ANF4-23 produce a small increase in cyclic GMP levels, indicating that this ligand may lose selectivity for NPR-C at these concentrations. However, Cao & Gardner (1995) excluded cyclic GMP and cyclic AMP as second messengers for the NPR-C ligand and the mechanism of its effect on rat neonatal cells in their studies was not defined.

Extrapolation of our findings to the regulation of cardiac fibroblast activity *in vivo* is limited by insufficient and conflicting data on natriuretic peptide receptor expression in freshly isolated cardiac tissue. *In vitro* autoradiography studies of fresh-frozen sections of rat and human hearts indicate that binding of ANP is confined to the endothelial cells of the endocardium and epicardium and largely absent from the myocardium (Bianchi *et al*., 1985; Rutherford *et al*., 1992). Nonetheless functional studies on freshly isolated single cells identified unmistakably as myocytes support the idea that myocytes express functional receptors for ANP (Neyses *et al*., 1989). While a recent study demonstrated mRNA for all three receptors in freshly isolated myocytes, insufficient non-myocytes were obtained and these cells were sub-cultured for further examination (Lin *et al*., 1995).

Studies of the effect of natriuretic peptides on collagen deposition in cardiovascular tissue *in vivo* are few but supportive of an inhibitory action. Thus, Mourlon-le-Grand *et al*. (1992) found that continuous infusion of ANP reduced collagen deposition in the arterial wall of spontaneously hypertensive rats. Monopoli *et al*. (1992) have reported that chronic administration of a neutral endopeptidase inhibitor (which inhibits natriuretic peptide degradation) inhibited collagen deposition in the hypertrophied ventricle of these animals. These effects may be in part secondary to the reduction in haemodynamic stress but, in the light of our data, may also be mediated by a direct effect of ANP on cardiac fibroblast activity. Given that ANP and BNP synthesis is increased in cardiac tissue in response to pressure overload from hypertension and aortic stenosis, treatment with a cyclic GMP phosphodiesterase inhibitor to augment their effects on the cardiac fibroblast offers a novel approach to reducing cardiac remodelling in these conditions.

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