British Journal of Pharmacology (2002) 137, 710–718 

www.nature.com/bjp

# Inotropic responses to human gene 2 (B29) relaxin in a rat model of myocardial infarction (MI): effect of pertussis toxin

[1]Andrew R. Kompa, [2]Chrishan S. Samuel & *,[1]Roger J. Summers

[1]Department of Pharmacology, Monash University, Victoria, 3800, Australia and [2]Howard Florey Institute, University of Melbourne, Victoria, 3010, Australia

**1** Relaxin produces powerful inotropic and chronotropic responses in isolated atria. The effect of relaxin has been examined in a rat model of cardiac failure, induced by myocardial infarction (MI).

**2** Maximum inotropic responses to isoprenaline (sham 5.4±0.3 mN; MI 2.6±0.3 mN; $P<0.001$) and relaxin (sham 5.1±0.6 mN; MI 2.8±0.5 mN; $P=0.013$) were reduced in left atria following MI. No change in chronotropic responsiveness was observed in right atria.

**3** Pertussis toxin (PTX) treatment restored inotropic responses to isoprenaline (sham 5.5±1.3 mN; MI 5.8±1.0 mN; $P=0.850$) but not to relaxin. Instead, PTX reduced inotropic responses to relaxin in sham animals to the same level seen in the MI group (sham 3.2±1.7 mN; MI 2.8±0.6 mN; $P=0.847$). In right atria, PTX treatment did not affect the maximum chronotropic response to isoprenaline, but reduced responses to relaxin in both sham and MI animals.

**4** R3 relaxin and relaxin receptor (LGR7) mRNA was present in atria and left ventricle (LV) from sham and MI animals. R3 relaxin mRNA expression was increased in atria but not LV from MI animals. LGR7 mRNA expression was reduced in atria and LV from MI animals.

**5** PTX treatment in unoperated rats increased chronotropic responses (vehicle 184.3±5.3 beats $min^{-1}$; PTX 211.3±9.5 beats $min^{-1}$; $P=0.029$) and produced a rightward shift in the concentration-response curve to isoprenaline in left atria. PTX reduced inotropic (vehicle 3.3±0.7 mN; PTX 0.8±0.2 mN; $P=0.005$) and chronotropic (vehicle 130.2±8.1 beats $min^{-1}$; PTX 90.6±11.1 beats $min^{-1}$; $P=0.012$) responses to relaxin.

**6** In left atria, relaxin produced a small increase in cAMP compared to those produced by isoprenaline and forskolin. However, PTX treatment significantly reduced relaxin-, isoprenaline- and forskolin-stimulated cAMP accumulation.

**7** Cardiac failure in MI animals caused a reduced inotropic response to both relaxin and (−)-isoprenaline. In non-MI animals, PTX treatment also reduced inotropic responses to relaxin. Differences between responses to (−)-isoprenaline and relaxin can be explained by changes in coupling efficiency occurring at the level of adenylate cyclase.

*British Journal of Pharmacology* (2002) **137,** 710–718. doi:10.1038/sj.bjp.0704922

**Keywords:** Human gene 2 (B29) relaxin; myocardial infarction; pertussis toxin; inotropic response; G-proteins; isoprenaline; heart; LGR7; R3 relaxin

**Abbreviations:** β-AR, β-adrenoceptor; BDM, 2,3.butanedione monoxime; BSA, bovine serum albumin; cAMP, cyclic AMP; c/r, concentration-response; ERK, extracellular-signal regulated kinase; LGR7, Leucine-rich G-protein coupled receptor 7; LV, left ventricle; MAPK, mitogen-activated protein kinases; MI, myocardial infarction; PKA, protein kinase A; PKC, protein kinase C; PKI, protein kinase inhibitor; PTX, pertussis toxin; RT–PCR, Reverse transcription with polymerase chain reaction

## Introduction

Relaxin is a 6 kDa polypeptide predominantly produced by the ovaries to promote connective tissue remodelling of the reproductive tract to facilitate parturition (Sherwood, 1994). Relaxin is also synthesized in the prostate and has been shown to increase motility and egg penetrating ability of sperm *in vitro* (Carrell *et al*., 1995). In addition to its reproductive role, relaxin produces powerful inotropic and chronotropic responses in isolated rat atria (Kakouris *et al*., 1992) and participates in the regulation of blood pressure and fluid balance (Parry *et al*., 1990; Weisinger *et al*., 1993). Relaxin also releases vasopressin and oxytocin (Dayanithi *et al*., 1987; Way & Leng, 1992) from the neurosecretory magnocellular hypothalmic nucleus (Osheroff & Phillips, 1991; McKinley *et al*., 1997). These functions have been supported by the discovery of binding sites in rat atria (Osheroff *et al*., 1992) and brain (Osheroff & Phillips, 1991) where binding assays using receptor autoradiography have shown that relaxin binds to a single high affinity binding site with a $K_D$ in the low nanomolar range. The presence of localized binding in rat atria and cerebral cortex as well as uterus (Tan *et al*., 1999) emphasises the importance of relaxin in non-reproductive tissues. Relaxin has recently been found in myocardial tissue from human heart failure patients (Dschietzig *et al*., 2001). The plasma concentrations of

*Author for correspondence;
E-mail: Roger.Summers@med.monash.edu.au

relaxin and gene expression of the H1 and H2 relaxin gene found in these patients correlates well with the severity of heart failure. Furthermore an elevated left ventricular diastolic pressure in rat heart is associated with increased relaxin gene expression (Dschietzig *et al*., 2001).

We have previously used the rat myocardial infarction (MI) model of cardiac failure to evaluate signal transduction pathways involved in inotropic and chronotropic responses to $\beta$-adrenoceptor ($\beta$-AR) agonists (Kompa *et al*., 1999; Kompa & Summers, 1999). We found that inotropic responses to $\beta$-AR agonists are significantly reduced in this model and that pertussis toxin restores this responsiveness indicating that after MI, there is a component of $\beta$-AR signalling through the inhibitory G-protein, $G_i$. There has been much debate on the signalling mechanism utilized by relaxin. Earlier studies suggested that cyclic AMP (cAMP) (Han *et al*., 1994), inositol trisphosphate (Mayerhofer *et al*., 1995) or ATP-sensitive $K^+$ channels (Noack *et al*., 1992) were involved. Relaxin, like isoprenaline, produces powerful inotropic and chronotropic effects in rat atria (Kakouris *et al*., 1992). The multiplicity of effects and the fact that relaxin is a structural homologue of insulin and insulin-like growth factors suggests that it might also signal *via* a tyrosine kinase receptor. However, a recent study has identified the relaxin receptor as a G-protein-coupled orphan receptor, LGR7, that can couple to adenylate cyclase to increase cAMP (Hsu *et al*., 2002). In this study we demonstrate that inotropic but not chronotropic responses to relaxin and isoprenaline are reduced in a rat model of cardiac failure. Pertussis toxin (PTX) treatment restored the response to isoprenaline but not relaxin, and in fact responses to relaxin after PTX were significantly reduced. Relaxin treatment also produced small but significant increases in cAMP accumulation that were markedly reduced by PTX.

## Methods

### *Rat model of cardiac failure*

Twelve-week-old female Sprague-Dawley rats (250 g) were anaesthetized with Alfaxan *via* tail vein injection (1.5 ml $kg^{-1}$, i.v.), intubated and placed on a respirator. A left thoractomy was performed, the heart exteriorized and the left coronary artery ligated (MI) 2 mm below the left atrium with a 6-0 nylon suture 4 mm in diameter. The chest was closed and animals allowed to recover. On showing signs of consciousness, animals were given buprenorphine (0.01 mg $kg^{-1}$, i.m.) for post-operative pain. Sham animals were treated identically except that the suture was not tied. The protocol used in this study was approved by the Ethics Committee at Monash University and within the guidelines recommended by the National Health and Medical Research Council of Australia. Four weeks after surgery, animals were anaesthetized with 20% $O_2$ in $CO_2$, decapitated, and tissues and infarct size measured. Infarct size (surface area) is expressed as a percentage of the left ventricle surface area for both epicardial and endocardial surfaces, ventricular septum was not included (Kompa & Summers, 2000). Animals with an infarct area covering $>45\%$ of the left ventricular surface area were included in the study. This level of ischaemic damage produces right ventricular hypertrophy and increased lung weight, as well as haemodynamic changes resulting in reduced sustained systolic blood pressure and raised left ventricular end diastolic pressure (Kompa *et al*., 1999; Kompa & Summers, 2000).

### *Atrial bioassay*

Four weeks after surgery sham and MI animals were anaesthetized in 80% $CO_2$ and 20% $O_2$ and decapitated. The heart was removed and the atria dissected in 37°C Krebs bicarbonate (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4.7H_2O$ 1.2, $NaH_2PO_4.2H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.7, pH 7.2) containing 2,3.butanedione monoxime (BDM, 0.03 M), a short-acting calcium antagonist to prevent tissue damage. Right and left atria were mounted separately in organ baths under 5 mN force, at 37°C and aerated with carbogen (95% $O_2$/5% $CO_2$). Left atria were paced at 5 Hz with square-wave pulses of 2 ms duration at 1.5 times threshold voltage, and right atria were allowed to beat spontaneously (Tan *et al*., 1998). Tissues were washed every 5 min over a 30 min period and allowed to equilibrate. Responses were recorded using isometric transducers (FTO3C) connected to a MacLab system. A cumulative concentration-response (c/r) curve to the non-selective $\beta$-adrenoceptor agonist isoprenaline was constructed. Tissues were washed eight times over a 30 min period until responses returned to baseline values and a cumulative c/r curve to human gene 2 (B29) relaxin constructed. At the end of the c/r curves, a single supramaximal dose of isoprenaline (300 nM) was added to the organ baths followed by a single supramaximal dose of calcium (7 mM).

### *RT–PCR analysis of relaxin and relaxin receptor expression (LGR7) in cardiac tissue from sham and MI animals*

Atria and left ventricles (LV) from sham and MI animals were isolated 4 weeks after surgery. Total RNA was extracted by homogenizing tissues in Trizol (Boehringer Mannheim, Australia) according to the manufacturer's instructions. Relaxin and LGR7 gene expression in the atria and LV from sham and MI animals were determined using RT–PCR.

Rats have two relaxin genes designated rat relaxin-1 (R1 relaxin) (Hudson *et al*., 1981) and rat relaxin-3 (R3 relaxin) (Burazin *et al*., 2002). A total of 100 ng of primers and 500 ng of the cDNA template in 50 $\mu$l were used for the R1 and R3 PCR reactions, while 1 $\mu$g of cDNA was used for rat relaxin receptor (LGR7) PCR reactions. All primers used were designed to span intron-exon junctions to control for genomic DNA contamination, while the PCR reactions were carried out in a Perkin Elmer Gene Amplifier. Rat tissues were screened for R1 relaxin using specific forward (5′-AGACTGGCTTTGAGCCAGG-3′) and reverse (5′-GAGTTTAGCAATGGATCTT-3′) primers, which generated a 396 bp product. R3 relaxin expression was also determined using specific forward (5′-GGAATTCTTCGCTGATGGA-GA-3′) and reverse (5′-ATAGCTGACAGCAGGTTGGAC-3′) primers, generating a 292 bp product. Sequences for the rat relaxin receptor (LGR7) were obtained by BLAST searching of the incomplete high throughput genome sequence (NCBI) using the human LGR7 (Genebank

Accession No. AF190500) cDNA sequences. Rat equivalents were identified in a BAC clone sequence (Genebank Accession No. AC098607) which showed high homology to human LGR7 (85%). These sequences were used to design specific forward (5′-CTGACAAACCCCTCTGTCAGC-3′) and reverse (5′-TTCCTTATTGCCAAGTGGAGC-3′) primers, generating a product of 850 bp. For R1 relaxin gene expression, an annealing temperature of 55°C (40 cycles) was used, while an annealing temperature of 52°C (40 cycles) was used for R3 relaxin expression. For LGR7 mRNA expression, the following (touch-down) annealing temperatures were used: 54°C (3 cycles), 53°C (3 cycles), 52°C (3 cycles), 51°C (3 cycles), 50°C (3 cycles), 49°C (3 cycles) and 48°C (25 cycles). GAPDH was used in separate PCR reactions to control for quality and equivalent loading of the cDNA. Aliquots of the PCR products were electrophoresed on 2% (w $v^{-1}$) agarose gels stained with ethidium bromide and photographed.

To confirm the PCR products, the appropriate bands were excised, the DNA eluted from the gel using the Ultraclean TM 15 DNA purification kit (Geneworks Pty Ltd, Adelaide, Australia) and subsequently subcloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.). Multiple subclones were sequenced on both strands using the ABI PRISM 377 automatic DNA sequences, according to the manufacturers instructions (Applied Biosystems, Melbourne, Australia).

### *PTX treatment of animals*

In separate experiments, sham and MI animals were treated with PTX (10 $\mu$g $kg^{-1}$ i.p.) 3 days prior to experimentation to inactivate Gi. Cumulative c/r curves to isoprenaline and human gene 2 (B29) relaxin were constructed in left and right atria as described above. We have previously shown that this protocol inhibits the relaxation response to adenosine (Gi-mediated effect) in submaximally isoprenaline-contracted tissues (Kompa *et al*., 1999).

In a separate set of experiments, unoperated Sprague-Dawley rats (250 g) were treated with PTX (10 $\mu$g $kg^{-1}$, i.p.) or vehicle (0.3 mg BSA in 0.9% saline) 3 days before experimentation. Cumulative c/r curves to isoprenaline and human gene 2 (B29) relaxin were constructed in left and right atria as described above. A single dose of isoprenaline (300 nM) was added after the relaxin c/r curve. Adenosine (100 $\mu$M) was then added to left atria to determine the effectiveness of PTX treatment, this was followed by a maximal dose of calcium (7 mM). In spontaneously beating right atria only isoprenaline (300 nM) was added after the relaxin c/r curve.

### *cAMP assay*

Left atrial tissue was removed from rats pretreated with vehicle or PTX (10 $\mu$g $kg^{-1}$, i.p.) 3 days prior to experimentation. Tissues were loosely placed in warm aerated Krebs bicarbonate and washed every 5 min for 30 min. The phosphodiesterase inhibitor IBMX (1 $\mu$M) was added for 30 min followed by a 30 min incubation with either Krebs (control), relaxin (100 nM), isoprenaline (1 $\mu$M) or forskolin (10 $\mu$M). Tissues were then quickly dried and snap frozen in liquid nitrogen for cAMP analysis.

Tissues were homogenized in 0.8 ml of ice cold 75% ethanol containing 4 mM EDTA, centrifuged for 5 min at $400 \times g$ and the supernatant removed and dried in a speed vacuum for 2 h. The remaining pellet was resuspended in 2 M NaOH and the protein in each sample was determined by a Lowry assay (Lowry *et al*., 1951). The dried supernatant pellet was redissolved in 50 mM Tris with 4 mM EDTA, pH 7.4 at 4°C and sonicated for a few seconds. cAMP was quantitated using the [$^3$H]-cAMP assay system (Amersham TRK 432) and expressed as pmol mg $protein^{-1}$

### *Statistics*

Non-linear regression was used to obtain $pEC_{50}$ values from concentration-response curves. Individual points between sham and MI animals for each dose on the concentration-response curve were analysed using an unpaired Student's *t*-test. Differences between curves were analysed using a two-way ANOVA, to obtain a $P$ value. Statistical significance was achieved with $P<0.05$.

### *Materials used*

Alfaxan (alphaxalone + alphadolone acetate) (Jurox, Silverwater, Australia), adenosine, (−)-isoprenaline bitartrate, pertussis toxin, 3-isobutyl-l-methylxanthine (IBMX), forskolin, 2,3.butanedione monoxime (BDM), Folin & Ciocalteu's Phenol reagent (Sigma, St Louis, MO, U.S.A.), Cyclic AMP (3H) assay system (Amersham International, U.K.), Trizol reagent (Boehringer Mannheim, Australia), human gene 2 (B29) relaxin (Dr J.D. Wade, Howard Florey Institute, Melbourne, Australia).

## Results

### *Model of cardiac failure*

Four weeks after surgery, organ and tissue weights were measured from sham-operated and MI rats and expressed as a proportion of body weight. Body weight was not significantly altered after MI (Table 1). Right ventricular weight (+27%) and lung weight (+18%), expressed as a ratio of body weight, were significantly increased after MI (Table 1), indicating congestion of the lungs and right ventricular hypertrophy representative of cardiac failure. Animals with MI included in this study had a minimum infarct size of 45% as determined by the epicardial left ventricular surface area (Kompa & Summers, 2000). We have previously shown that these morphological changes are associated with reduced systolic blood pressure and raised left ventricular end diastolic pressure (Kompa *et al*., 1999).

### *Comparison of inotropic responses to isoprenaline and relaxin in a rat model of cardiac failure before and after PTX treatment*

Maximum inotropic responses to isoprenaline (sham $5.4 \pm 0.3$ mN; MI $2.6 \pm 0.3$ mN, $n=8$; $P<0.001$; −52%) and relaxin (sham $5.1 \pm 0.6$ mN, $n=6$; MI $2.8 \pm 0.5$ mN, $n=8$; $P=0.013$; −45%) in field stimulated left atria were significantly reduced after 4 weeks MI (Figure 1a,b). Potency of inotropic agents measured from the $EC_{50}$ of the concentration-response curves to isoprenaline ($pEC_{50}$: sham

8.03±0.07, $n=6$; MI 8.11±0.11, $n=8$; $P=0.563$) and relaxin ($pEC_{50}$: sham 8.66±0.10, $n=6$; MI 8.45±0.09, $n=8$; $P=0.147$) were not significantly altered by MI (Figure 1a,b). However, maximal inotropic responses to supramaximal concentrations of isoprenaline (300 nM) and $Ca^{2+}$ (7 mM) were both significantly reduced after MI (Figure 1c).

PTX treatment restored the maximum inotropic response to isoprenaline (sham 5.5±1.3 mN, $n=8$; MI 5.8±1.0 mN, $n=8$; $P=0.850$; Figure 1d) and although it appeared to restore the maximum inotropic response to relaxin, it was evident that the maximum inotropic response to relaxin in the sham group after PTX treatment was in fact lowered to a similar extent to that obtained in the MI group (sham 3.2±1.7 mN, $n=8$; MI 2.8±0.6 mN, $n=8$; $P=0.847$; Figure 1e). The potency of isoprenaline ($pEC_{50}$: sham 8.00±0.12, $n=8$; MI 7.98±0.11, $n=8$; $P=0.881$) and relaxin ($pEC_{50}$: sham 8.55±0.35, $n=8$; MI 8.17±0.11, $n=8$; $P=0.307$) were not significantly altered after MI (Figure 1d,e). As previously reported (Kompa *et al*., 1999), PTX restored the inotropic response to supramaximal concentrations of isoprenaline (300 nM) and $Ca^{2+}$ (7 mM) to levels similarly observed in the sham group (Figure 1f).

**Table 1** Morphological data on sham and MI rats used in this study

| | *Sham* (n=8) | *MI* (n=8) |
|---|---|---|
| Body weight (g) | 295.0±3.3 | 284±5.5 |
| Lung weight/Body weight (mg $g^{-1}$) | 4.31±0.07 | 5.08±0.14* |
| Right ventricle weight/ Body weight (mg $g^{-1}$) | 0.51±0.02 | 0.65±0.01* |
| Infarct size (%) | – | 51.6±1.4 |

Increased lung weight/body weight and right ventricular weight/body weight ratios following MI in rats are indicative of pulmonary oedema and cardiac hypertrophy respectively. Results are expressed in wet weight as mean±s.e.mean, *$P<0.01$.

### *Comparison of chronotropic responses to isoprenaline and relaxin in a rat model of cardiac failure before and after PTX treatment*

Maximum chronotropic responses to isoprenaline (sham 173.4±8.3 beats $min^{-1}$, $n=6$; MI 170.1±10.6 beats $min^{-1}$, $n=8$; $P=0.822$) and relaxin (sham 135.1± beats $min^{-1}$, $n=6$; MI 135.2±16.7 beats $min^{-1}$, $n=8$; $P=0.994$) in spontaneously beating right atria were not changed at 4 weeks MI (Figure 2a,b). The potency of isoprenaline ($pEC_{50}$: sham 8.53±0.08, $n=6$; MI 8.60±0.10, $n=8$; $P=0.578$) and relaxin ($pEC_{50}$: sham 9.27±0.13, $n=6$; MI 8.99±0.15, $n=8$; $P=0.192$) were not significantly altered by MI (Figure 2a,b). Chronotropic responses to a supramaximal concentration of isoprenaline (300 nM) were unaltered by MI (Figure 2c).

PTX treatment failed to change chronotropic responsiveness to isoprenaline (sham 205.4±9.2 beats $min^{-1}$, $n=8$; MI 207.6±10.9 beats $min^{-1}$, $n=8$; $P=0.882$) or relaxin (sham 95.9±14.9 beats $min^{-1}$, $n=8$; MI 97.8±11.8 beats $min^{-1}$, $n=8$; $P=0.707$) in either sham or MI groups (Figure 2d,e), although it appeared to reduce the maximum chronotropic response to relaxin in both sham and MI animals (Figure 2e compared to Figure 2b). The potency of isoprenaline ($pEC_{50}$: sham 8.44±0.08, $n=8$; MI 8.43±0.10, $n=8$; $P=0.896$) and relaxin ($pEC_{50}$: sham 9.05±0.06, $n=8$; MI 8.92±0.05, $n=8$; $P=0.602$) were not significantly altered by MI (Figure 2d,e). Chronotropic responses to a supramaximal concentration of isoprenaline (300 nM) were unaltered by MI (Figure 2f).

### *Relaxin and relaxin receptor (LGR7) mRNA expression in sham and MI rats*

The levels of R1 relaxin, R3 relaxin and rat LGR7 gene transcripts were determined by RT–PCR in atria (Figure 3a) and LV (Figure 3b) tissues from sham and MI animals. R1

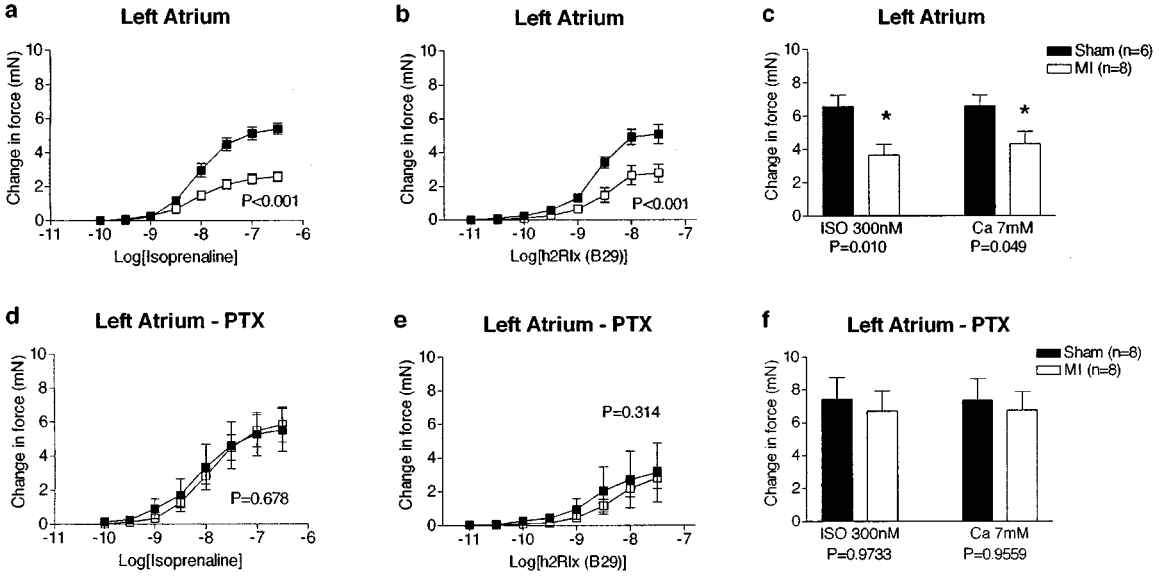


**Figure 1** Inotropic responses in electrically stimulated left atria. (a–c) Responses to isoprenaline (ISO) and relaxin (h2R1x (B29)) in sham (filled symbols) and MI (open symbols) rats. (d–f) Responses to isoprenaline and h2R1x (B29) in sham and MI rats after PTX treatment. *P* values on c/r curves are from a two-way ANOVA performed to determine differences between curves. *P* values on bar graphs indicate significance using an unpaired Student's *t*-test.

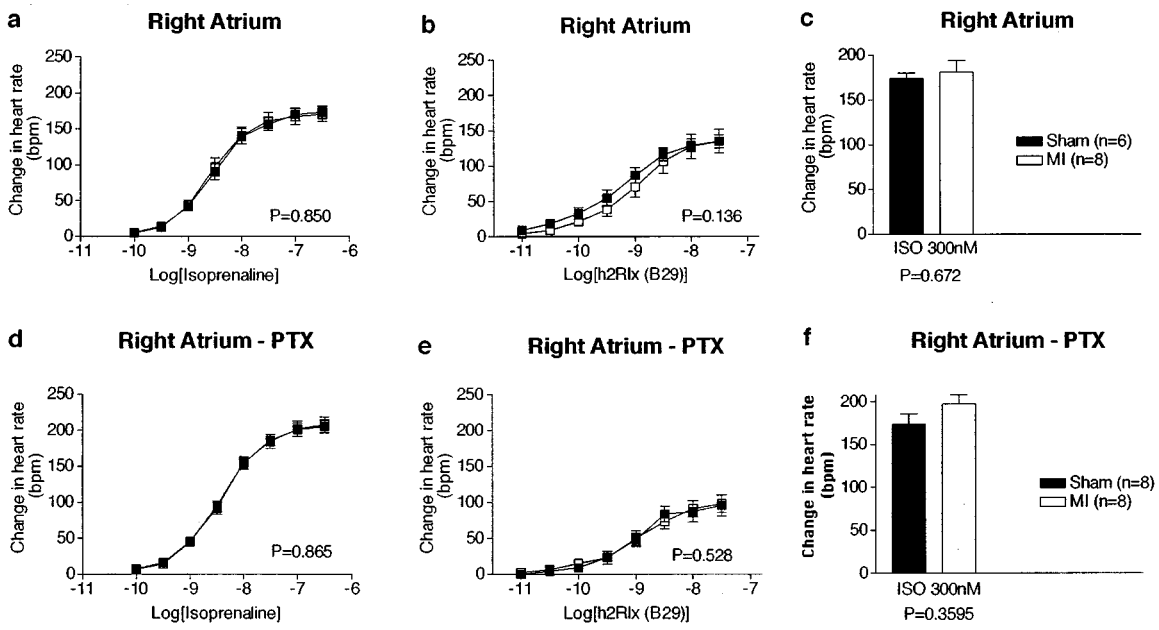


**Figure 2** Chronotropic responses in spontaneously beating right atria. (a–c) Responses to isoprenaline (ISO) and relaxin (h2R1x (B29)) in sham (filled symbols) and MI (open symbols) rats. (d–f) Responses to isoprenaline and h2R1x (B29) in sham and MI rats after PTX treatment. *P* values on c/r curves are from a two-way ANOVA performed to determine differences between curves. *P* values on bar graphs indicate significance using an unpaired Student's *t*-test.

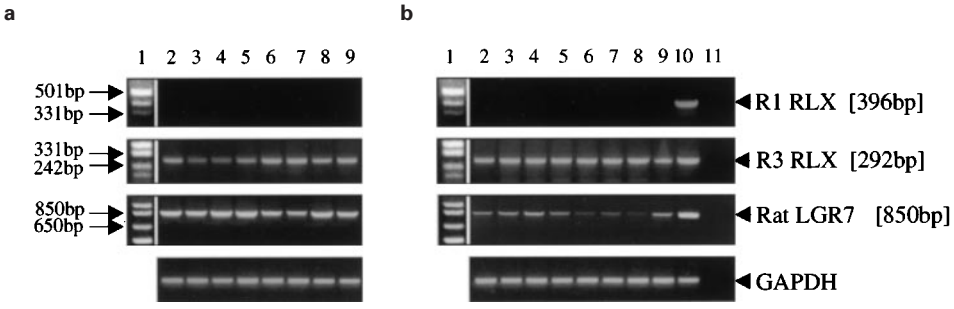


**Figure 3** RT–PCR of R1 relaxin, R3 relaxin and rat LGR7 mRNA expression in atrial (a) and LV (b) tissue from sham (lanes 2–5) and MI (lanes 6–9) animals. Ethidium bromide-stained PCR products of R1 relaxin (396 bp), R3 relaxin (292 bp) and rat LGR7 (850 bp) are shown, while GAPDH products were used as controls for quality and equal loading of the cDNA. Samples consist of a molecular weight marker (lane 1), and gene transcripts from four separate sham atria (a, lanes 2–5), four separate MI atria (a, lanes 6–9), four separate sham LV (b, lanes 2–5) and four separate MI LV (b, lanes 6–9). cDNA from a late pregnant ovary was used as a positive control for R1 relaxin (b, lane 10), while cDNA from the pons/medulla and cortex were used as positive controls for R3 relaxin and rat LGR7 mRNA expression, respectively. Water replaced cDNA in negative control reactions for each PCR (b, lane 11).

relaxin gene expression was absent in the atria and LV from both groups of animals. In contrast, R3 relaxin mRNA expression was clearly detected in atria and ventricular tissues from sham and MI animals, suggesting that R3 relaxin is the predominant form expressed in rat atria. In atria from the MI group (Figure 3a, lanes 6–9), R3 relaxin mRNA expression was greater than in atria from the sham group (Figure 3a, lanes 3–5). LGR7 gene expression was observed in both atrial and ventricular tissues from sham and MI animals with a higher level of LGR7 mRNA expression detected in atria (Figure 3a), compared to LV (Figure 3b). However, the LGR7 gene expression in both atria (Figure 3a, lanes 6,7,9) and LV (Figure 3b, lanes 6–8) was decreased in MI animals compared to tissues from sham-operated animals.

### *Effect of isoprenaline and relaxin after pertussis toxin treatment on inotropic and chronotropic responses*

Since PTX treatment caused a reduction in inotropic and chronotropic responses to relaxin independently of MI, we tested this effect in animals that had not undergone MI or a sham operation.

Sprague-Dawley rats were treated once with either PTX (10 $\mu g\ kg^{-1}$, i.p.) or vehicle 3 days before experimentation. Maximum inotropic responses to isoprenaline, in left atria were unaffected by PTX treatment (vehicle $5.1 \pm 0.5$ mN, $n=7$; PTX $4.3 \pm 0.6$ mN, $n=7$; $P=0.304$; Figure 4a), whereas responses to relaxin were significantly reduced (vehicle $3.3 \pm 0.7$ mN, $n=7$; PTX $0.8 \pm 0.2$ mN, $n=7$; $P=0.005$;

−76% Figure 4b). The potency of isoprenaline ($pEC_{50}$: vehicle 8.51±0.13, $n=7$; PTX 8.04±0.11, $n=7$; $P=0.020$) was significantly altered by PTX but not that of relaxin ($pEC_{50}$: vehicle 8.90±0.08, $n=7$; PTX 8.59±0.18, $n=7$; $P=0.136$) (Figure 4a,b). The effectiveness of PTX treatment was tested by examining the negative inotropic response of adenosine in left atria stimulated with isoprenaline (300 nM). The negative inotropic response to adenosine, a known Gi-coupled receptor, was blocked by prior PTX treatment (Figure 4c).

In right atria, chronotropic responses to isoprenaline were slightly increased by PTX treatment (vehicle 184.3±5.3 beats $min^{-1}$, $n=7$; PTX 211.3±9.5 beats $min^{-1}$, $n=7$; $P=0.029$; +15% Figure 4d), however responses to relaxin were significantly reduced (vehicle 130.2±8.1 beats $min^{-1}$, $n=7$; PTX 90.6±11.1 beats $min^{-1}$, $n=7$; $P=0.012$; −30% (Figure 4e). The potency of isoprenaline ($pEC_{50}$: vehicle 8.63±0.05, $n=7$; PTX 8.33±0.10, $n=7$; $P=0.027$) was significantly altered by PTX but not that of relaxin ($pEC_{50}$: vehicle 9.16±0.07, $n=7$; PTX 9.06±0.01, $n=7$; $P=0.395$) (Figure 4d,e). Chronotropic responses to supramaximal concentrations of isoprenaline (300 nM) were increased by PTX treatment ($P=0.006$, Figure 4f).

### *The effects of relaxin, isoprenaline and forskolin on cAMP production in left atria: alteration by PTX treatment*

Relaxin, isoprenaline and forskolin all produced an increase in cAMP. Relaxin (100 nM) produced a small but significant increase in cAMP from the basal level of 0.023±0.003 to 0.063±0.002 pmol mg $protein^{-1}$, 2.7 fold above basal. Isoprenaline (1 μM; 0.161±0.023 pmol mg $protein^{-1}$) produced a 7 fold increase in cAMP, 2.6 times greater than relaxin. Maximal stimulation of adenylate cyclase with forskolin (10 μM; 0.538±0.054 pmol mg $protein^{-1}$) produced a 23 fold increase in cAMP, 3.3 and 8.5 times greater than isoprenaline and relaxin, respectively (Table 2). Treatment of animals with PTX did not alter basal cAMP levels (control: 0.023±0.003, PTX: 0.018±0.002, $P=0.135$), however cAMP responses to relaxin (≈60%; 0.025±0.001 pmol mg $protein^{-1}$), isoprenaline (≈80%; 0.031±0.005 pmol mg $protein^{-1}$) and forskolin (≈81%; 0.102±0.010 pmol mg $protein^{-1}$) were all significantly reduced (Table 2). However the cAMP response to relaxin, isoprenaline and forskolin after PTX treatment was still significantly increased above basal (Table 2).

## Discussion

In the rat MI model we have demonstrated that the maximum inotropic responses to isoprenaline and relaxin are reduced in cardiac failure. We have also shown that of the two rat relaxin genes, R1 and R3, only R3 relaxin is expressed in both atria and left ventricle. In atria, R3 relaxin gene expression was increased in MI animals suggesting increased production and release of R3 relaxin. This together with the reduction of mRNA expression of LGR7 (relaxin receptor) 4 weeks after MI in atria may be responsible for the desensitization observed in the c/r curves to relaxin.

R3 relaxin but not R1 relaxin is expressed in the rat heart, consistent with the finding that only R3 relaxin is found in normal rat atrial and ventricular tissue and cells with ageing (unpublished data, CS Samuel). In addition to acting as a neuropeptide or neuromodulator (Bathgate *et al*., 2002), R3 relaxin appears to have a role in the heart regulating

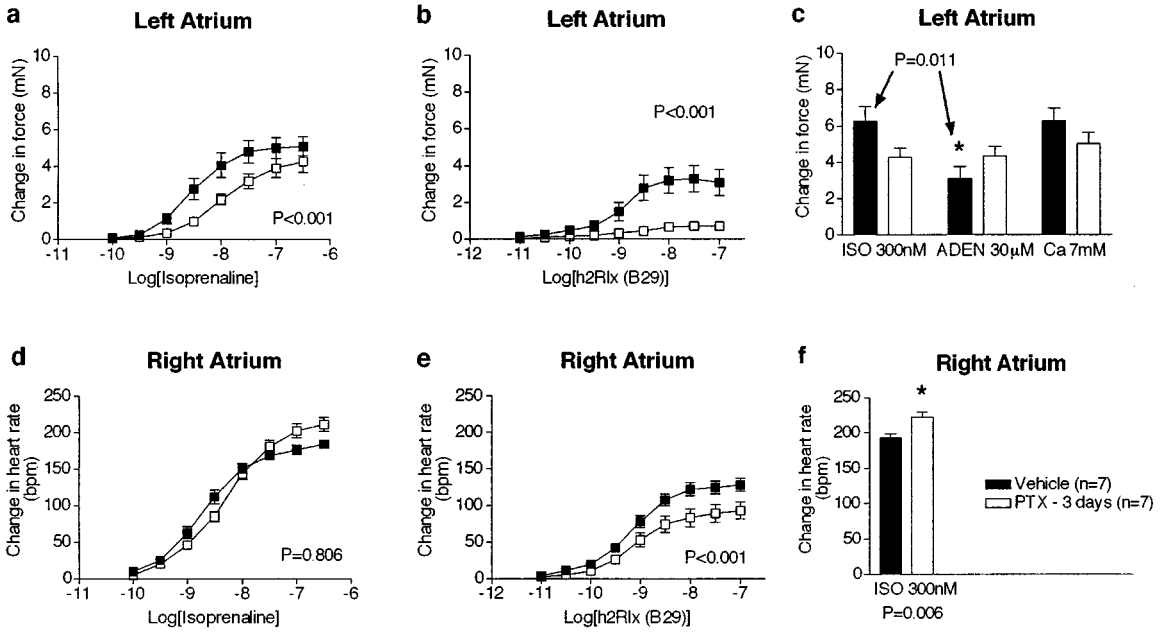


**Figure 4** (a–c) Effect of PTX treatment on inotropic responses to (a) isoprenaline, (b) hR1x (B29) and (c) single consecutive doses of isoprenaline (ISO, 300 nM), adenosine (30 μM) and calcium (7 mM) after the relaxin c/r curve. (d–f) Effect of PTX treatment on chronotropic responses to (d) isoprenaline, (e) hR1x (B29) and (f) single dose of isoprenaline (ISO, 300 nM) after the relaxin c/r curve. Filled symbols represent vehicle-treated animals, whereas open symbols represent PTX-treated animals. *P* values on c/r curves are from a two-way ANOVA performed to determine differences between curves. *P* values on bar graphs indicate significance using an unpaired Students' *t*-test.

**Table 2** Effect of basal, h2R1x (B29), isoprenaline and forskolin on cyclic AMP production in left atria with vehicle and PTX treatment

| | *Vehicle treatment* | *PTX treatment* | P *value* |
|---|---|---|---|
| Basal | 0.023 ± 0.003 | 0.018 ± 0.002 | 0.135 |
| h2R1x(B29) (100 nM) | 0.063 ± 0.002*** | 0.025 ± 0.001** | <0.001 |
| Isoprenaline (1 μM) | 0.161 ± 0.023*** | 0.031 ± 0.005* | 0.002 |
| Forskolin (10 μM) | 0.538 ± 0.054*** | 0.102 ± 0.010** | <0.001 |

Values are expressed in pmol cAMP mg protein$^{-1}$ as mean ± s.e.mean from $n=4$ in each group. $P$ value column indicates significance between vehicle and PTX-treated left atria using an unpaired Student's $t$-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates significant differences compared to the basal group for the same treatment.

inotropic responses. LGR7 mRNA expression was detected in both the atria and LV of sham and MI animals. LGR7 expression in the atria was much higher than in the LV, consistent with the high levels of [$^{33}$P]-relaxin binding in rat atria (Osheroff *et al*., 1992; Tan *et al*., 1999).

In previous studies we have found that PTX treatment restores inotropic responses to isoprenaline (Kompa *et al*., 1999; Kompa & Summers, 1999), whereas in the present study it failed to restore the inotropic response to relaxin. Rather, in sham-operated animals, the inotropic response in left atria was reduced to the level seen in MI-operated animals. In addition, we have confirmed that PTX treatment did not alter the chronotropic response to isoprenaline in the MI model (Kompa *et al*., 1999), but did reduce the chronotropic response to relaxin in both sham and MI animals. Thus it appeared initially that in the heart, relaxin signalling might contain a PTX-sensitive element. This observation was confirmed in experiments on unoperated Sprague Dawley rats treated with PTX, where the maximum inotropic and chronotropic responses to relaxin were significantly reduced. The cAMP data is in agreement with the functional results and shows that PTX treatment significantly reduced the cAMP response to relaxin. The effects of PTX on responses to relaxin did not therefore depend on cardiac failure or on the operating procedure carried out in sham-operated rats. A recent study has suggested a role for relaxin in human heart failure since increased plasma concentrations of relaxin are seen with increased severity of heart failure, and increased relaxin immunoreactivity occurs in myocytes from failing cardiac tissue (Dschietzig *et al*., 2001). Furthermore, under conditions that mimic left ventricular failure in a flow chamber, relaxin inhibited endothelin-1 peptide secretion from pulmonary artery cells and also inhibited the angiotensin II-stimulated increase in endothelin-1 secretion which was abolished by the $ET_B$ receptor antagonist A-192621 (Dschietzig *et al*., 2001). These results suggest that relaxin may be involved as a compensatory mediator in human heart failure inhibiting the vasoconstrictor actions of angiotensin and endothelin. In terms of our results, we have previously shown in the *in vitro* situation that responses to relaxin are difficult to wash out, persisting up to 6 h with constant washing (Summers *et al*., 1995). An increase in plasma relaxin, in our 4 week MI model, could chronically stimulate the receptor leading to the significant receptor desensitization seen in the left atria. Although the plasma concentration of relaxin determined by Dschietzig *et al*. (2001) may be lower than the concentration range of relaxin used in this study, we have shown that M1 increases relaxin gene expression in the atria which may increase local relaxin to concentrations that do cause desensitization.

The chronotropic response to relaxin in right atria was unaffected following MI, which may indicate that relaxin utilizes a different signalling pathway in right atria compared to left atria. Toth *et al*. (1996) reported that relaxin utilizes at least three different intracellular pathways in right atria, protein kinase A (PKA), protein kinase C (PKC) and a calcium/calmodulin dependent protein kinase. The PKA inhibitor H89 (100 nM) inhibited chronotropic responses to relaxin (Toth *et al*., 1996) and although H-89 also is a $\beta_2$-adrenoceptor antagonist at nanomolar concentrations (Penn *et al*., 1999), the protein kinase inhibitor (PKI) and PKI (5-24 amide) prevented inhibition of potassium current by relaxin in rat atrial myocytes (Piedras-Renteria *et al*., 1997a, b), and prevented a dose-dependent increase in L-type calcium current to relaxin in rabbit sino-atrial nodal cells (Han *et al*., 1994). PKI is highly specific for PKA, at 1–5 μM and does *not* inhibit other kinases such as casein kinase I and II, proteolytically activated protein kinase I, $Ca^{2+}$-phospholipid-dependent protein kinase, cGMP-dependent protein kinase, $Ca^{2+}$-calmodulin-dependent protein kinase, or myosin light chain kinase (Cheng *et al*., 1986). In this study, relaxin did produce a significant increase in cAMP, although much less than that produced by isoprenaline. Furthermore there has been recent recognition that the orphan receptor, LGR7, is a relaxin receptor that can produce cAMP when stimulated by relaxin (Hsu *et al*., 2002). Our results confirm that relaxin increases cAMP in left atrial tissue, although this response is small compared to either $\beta$-AR activation or forskolin. Relaxin has also been suggested to signal through PKC. The PKC inhibitor staurosporine inhibited chronotropic responses to relaxin in right atria, as well as atrial ANP secretion in the perfused rat heart (Toth *et al*., 1996). Furthermore, previous studies have described relaxin-induced translocation of PKC from the cytosol to the cellular membrane in human endometrial cells (Kalbag *et al*., 1991). These studies suggest that relaxin *in right atria* may signal through both PKA and PKC-dependent pathways. The calcium/calmodulin-dependent protein kinase may also be involved in relaxin signalling, with KN-62 (1-[N, O-bis (1,5-isoquinolinesulphonyl) - N-methyl-L -tyrosyl] - 4-phenylpiperazine, a calcium/calmodulin dependent protein kinase II inhibitor) significantly reducing the chronotropic response to relaxin (Toth *et al*., 1996). Relaxin had been reported to increase L-type calcium current in single cells isolated from rabbit sino-atrial node (Han *et al*., 1994). This increased calcium influx binds to calmodulin activating the calcium/calmodulin protein kinase, which is involved in the chronotropic response.

These pathways do not readily provide an explanation for the reduced relaxin responsiveness in heart following PTX treatment. If relaxin was signalling through PKA *via* a G protein ($G_s$) to increase cAMP, PTX would be expected to remove any dampening of the cAMP response *via* $G_i$ and increase the response, and not the observed opposite effect. Furthermore, PTX-sensitive G-proteins are not involved in the PKC pathway, which signals predominantly through

$G_{q/11}$-coupled receptors (Gutkind, 1998). The effect of PTX on the relaxin-stimulated cAMP response was in close agreement with the organ bath data, although this was not as obvious for isoprenaline. The effect of PTX pre-treatment on the relaxin, isoprenaline and forskolin stimulated cAMP responses may be explained by compensatory reductions in other components of the signalling pathway and in particular adenylate cyclase. This appears plausible as PTX was administered 3 days before the responses to agonists were tested, and long enough for such changes to occur. The data also showed that basal cAMP levels were 23% lower in PTX-treated tissues, which although not significant ($P=0.135$) may indicate some compensatory down-regulation of adenylate cyclase. This is further supported by studies that showed that 24 h PTX treatment had no effect on maximum responses in relaxin c/r curves (unpublished observations). Following PTX treatment, we speculate that the increase in isoprenaline-stimulated cAMP following PTX treatment (38% increase above non-PTX basal) is adequate to produce maximal responses, whereas the increase in relaxin-stimulated cAMP (11% increase above non-PTX basal) produces a significantly reduced response when compared to responses in the absence of PTX pretreatment. The coupling efficiency of receptor-Gs-adenylate cyclase is higher for isoprenaline than for relaxin. The estimated molar proportions of the elements of the $\beta$-AR-Gs-adenylate cyclase complex in cardiac myocytes are 1 : 200 : 3 (Post *et al*., 1995), suggesting that $\beta$-AR number or the amount of adenylate cyclase may limit $\beta$-AR-mediated transmembrane signalling.

In human endometrial stromal cells and a human monocyte cell line (THP-1), which naturally express the relaxin receptor, increases in relaxin-stimulated cAMP accumulation are inhibited by the non hydrolysable analogue of GDP – GDP-$\beta$-S, an inhibitor of G-protein activation (Bartsch *et al*., 2001). Tyrosine kinase inhibitors such as tyrphostin AG 1478 or AG 527, which are specific for EGF receptor tyrosine kinase, also suppressed the relaxin-stimulated cAMP response in these cells in a dose-dependent manner (Bartsch *et al*., 2001). In contrast, the broad spectrum tyrosine kinase inhibitor tyrphostin AG 213 and the platelet derived growth factor receptor-specific tyrphostin AG 1295 had no effect on the relaxin-stimulated cAMP response (Bartsch *et al*., 2001). These studies suggested that the relaxin receptor may be associated with a membrane tyrosine kinase closely related to the EGF receptor. Many studies have shown that activation of the EGF receptor can lead to the activation of the mitogen-activated protein kinase (MAPK) pathway in heart (Rebsamen *et al*., 2000; Ding *et al*., 2000). Bartsch *et al*. (2001) reported that relaxin-stimulated cAMP accumulation in THP-1 cells is inhibited by the MEK inhibitors PD 98059 and U 0126. There is increasing evidence to suggest a link between MAPK and cAMP pathways at the level of phosphodiesterase (PDE). Extracellular-signal regulated kinase 2 (ERK2) has been shown to phosphorylate PDE4D3 at Ser579 reducing its activity and increasing cAMP (Hoffmann *et al*., 1999). PDE4D3 is the predominant isoform of PDE4D found in rat heart (Kostic *et al*., 1997). In HEK293 and F422A cells, natively expressing PDE4D3, PD 98059 inhibits EGF-induced phosphorylation of PDE4D3 (Hoffmann *et al*., 1999). This provides a potential feedback regulatory system whereby ERK2 inhibition of PDE4D3 allows cAMP levels to rise, linking ERK2 activation to the regulation of cAMP. Therefore if the relaxin receptor is associated with an EGF receptor, it may explain the increase in cAMP to relaxin observed in this study. In rat hepatocytes PTX-treatment is known to inhibit activation of ERK in response to agonists signalling through EGF as well as agents that directly stimulate PKC or elevate intracellular $Ca^{2+}$, suggesting that $G_i$ exerts stimulatory effects on ERK (Melien *et al*., 2000). This effect is thought to occur downstream of receptor coupling as both receptor and direct activation of phospholipase C and D are not affected by PTX (Melien *et al*., 2000). Given that relaxin can associate with an EGF-like receptor (Bartsch *et al*., 2001), this may represent an alternate mechanism for relaxin induced increase in cAMP involving PTX-sensitive G-proteins.

We conclude that the present study provides evidence that in rat atria, both isoprenaline and relaxin couple through a G-protein coupled receptor to $G_s$ and adenylate cyclase but that relaxin receptors are poorly coupled in comparison with the $\beta$-adrenoceptor. Cardiac failure in the MI model causes reduced inotropic responses to both relaxin and isoprenaline. In the case of relaxin this reduced response can be explained by desensitization following the chronic increase in local R3 relaxin levels which causes a reduction in relaxin receptors (LGR7). PTX treatment causes down regulation of the cAMP signalling pathway, most likely at the level of adenylate cyclase and that the differences between isoprenaline and relaxin are related to the differences in their efficiency of coupling.

This study was supported in part by a Howard Florey Institute Block Grant from the National Health and Medical Research Council (NH&MRC) of Australia (Reg Key 983001). We thank Dr R. Bathgate for providing R3 relaxin and LGR7 RT–PCR primers and confirming their sequences. The authors would like to thank Dr J.D. Wade (Howard Florey Institute, Melbourne, Australia) for supplying human gene 2 (B29) relaxin.

## References

BARTSCH, O., BARTLICK, B. & IVELL, R. (2001). Relaxin signalling links tyrosine phosphorylation to phosphodiesterase and adenylyl cyclase activity. *Mol. Human Reprod.,* **7,** 799 – 809.

BATHGATE, R.A.D., SAMUEL, C.S., BURAZIN, T.C.D., LAYFIELD, S., CLAASZ, A.A., REYTOMAS, I.G.T., DAWSON, N.F., ZHAO, C., BOND, C., SUMMERS, R.J., PARRY, L.J., WADE, J.D. & TREGEAR, G.W. (2002). Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene: Novel members of the relaxin peptide family. *J. Biol. Chem.,* **277,** 1148 – 1157.

BURAZIN, T.C.D., BATHGATE, R.A.D., MACRIS, M., LAYFIELD, S., GUNDLACH, A.L. & TREGEAR, G.W. (2002). Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *J. Neurochem.,* (in press).

CARRELL, D.T., PETERSON, C.M. & URRY, R.L. (1995). The binding of recombinant human relaxin to human spermatozoa. *Endocrin. Res.,* **21,** 697 – 707.

CHENG, H.C., KEMP, B.E., PEARSON, R.B., SMITH, A.J., MISCONI, L., VAN PATTEN, S.M. & WALSH, D.A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J. Biol. Chem.,* **261,** 989–992.

DAYANITHI, G., CAZALIS, M. & NORDMANN, J.J. (1987). Relaxin affects the release of oxytocin and vasopressin from the neurohypophysis. *Nature,* **325,** 813–816.

DING, B., HUANG, S.L., ZHANG, S.Q. & LI, Y.X. (2000). Effect of PKC-ζeta mediating Ang II-stimulated activation of CCDPK on rat cardiac fibroblast proliferation. *Acta Pharmacol. Sin.,* **21,** 174–178.

DSCHIETZIG, T., RICHTER, C., BARTSCH, C., LAULE, M., ARMBRUSTER, F.P., BAUMANN, G. & STANGL, K. (2001). The pregnancy hormone relaxin is a player in human heart failure. *FASEB J.,* **15,** 2187–2195.

GUTKIND, J.S. (1998). The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J. Biol. Chem.,* **273,** 1839–1842.

HAN, X., HABUCHI, Y. & GILES, W.R. (1994). Relaxin increases heart rate by modulating calcium current in cardiac pacemaker cells. *Circ. Res.,* **74,** 537–541.

HOFFMANN, R., BAILLE, G.S., MACKENZIE, S.J., YARWOOD, S.J. & HOUSLAY, M.D. (1999). The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. *EMBO J.,* **18,** 893–903.

HSU, S.Y., NAKABAYASHI, K., NISHI, S., KUMAGAI, J., KUDO, M., SHERWOOD, O.D. & HSUEH, A.J.W. (2002). Activation of orphan receptors by the hormone relaxin. *Science,* **295,** 671–674.

HUDSON, P., HALEY, J., CRONK, M., SHINE, J. & NIALL, H. (1981). Molecular cloning and characterization of cDNA sequences coding for rat relaxin. *Nature,* **291,** 127–131.

KAKOURIS, H., EDDIE, L.W. & SUMMERS, R.J. (1992). Cardiac effects of relaxin in rats. *Lancet,* **339,** 1076–1078.

KALBAG, S.S., ROGINSKY, M.S., JELVEH, Z. & SULIMOVICI, S. (1991). Phorbol ester, prolactin and relaxin cause translocation of protein kinase C from cytosol to membranes in human endometrial cells. *Biochim. Biophys. Acta,* **1094,** 85–91.

KOMPA, A.R., GU, X.H., EVANS, B.A. & SUMMERS, R.J. (1999). Desensitization of cardiac $\beta$-adrenoceptor signaling with heart failure produced by myocardial infarction of the rat. Evidence for the role of Gi but not Gs or phosphorylating proteins. *J. Mol. Cell. Cardiol.,* **31,** 1185–1201.

KOMPA, A.R. & SUMMERS, R.J. (1999). Desensitization and resensitization of $\beta_1$- and putative $\beta_4$-adrenoceptor mediated responses occur in parallel in a rat model of cardiac failure. *Br. J. Pharmacol.,* **128,** 1399–1406.

KOMPA, A.R. & SUMMERS, R.J. (2000). Lidocaine and surgical modification reduces mortality in a rat model of cardiac failure induced by coronary artery ligation. *J. Pharmacol. Toxicol. Meths.,* **43,** 199–203.

KOSTIC, M.M., ERDOGAN, S., RENA, G., BORCHERT, G., HOCH, B., BARTEL, S., SCOTLAND, G., HUSTON, E., HOUSLAY, M.D. & KRAUSE, E.G. (1997). Altered expression of PDE1 and PDE4 cyclic nucleotide phosphodiesterase isoforms in 7-oxo-prostacyclin-preconditioned rat heart. *J. Mol. Cell. Cardiol.,* **29,** 3135–3146.

LOWRY, O.H., ROSSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MAYERHOFER, A., ENGLING, R., STECHER, B., ECKER, A., STERZIK, K. & GRATZL, M. (1995). Relaxin triggers calcium transients in human granulosa-lutein cells. *Eur. J. Endocrinol.,* **132,** 507–513.

MCKINLEY, M.J., BURNS, P., COLVILL, L.M., OLDFIELD, B.J., WADE, J.D., WEISINGER, R.S. & TREGEAR, G.W. (1997). Distribution of Fos immunoreactivity in the lamina terminalis and hypothalamus induced by centrally administered relaxin in conscious rats. *J. Neuroendocrinol.,* **9,** 431–437.

MELIEN, Ø., SANDNES, D., JOHANSEN, E.J. & CHRISTOFFERSEN, T. (2000). Effects of pertussis toxin on extracellular signal-related kinase activation in hepatocytes by hormones and receptor-independent agents: Evidence suggesting a stimulatory role of Gi proteins at a level distal to receptor coupling. *J. Cell. Physiol.,* **184,** 27–36.

NOACK, T., DIETMER, P., EDWARDS, G. & WESTON, A.H. (1992). Potassium channel modulation in a rat portal vein by ATP depletion: a comparison with the effects of levcromakalim (BRL 37344 38227). *Br. J. Pharmacol.,* **107,** 945–955.

OSHEROFF, P.L., CRONIN, M.J. & LOFGREN, J.A. (1992). Relaxin binding in the rat heart atrium. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 2384–2388.

OSHEROFF, P.L. & PHILLIPS, H.S. (1991). Autoradiographic localisation of relaxin binding sites in rat brain. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 6413–6417.

PARRY, L.J., POTERSKI, R.S., SUMMERLEE, A.J.S. & JONES, S.A. (1990). Mechanism of the haemotensive action of a porcine relaxin in anaesthetised rats. *J. Neuroendocrinol.,* **2,** 53–58.

PENN, R.B., PARENT, J.L., PRONIN, A.N., PANETTIERI JR, R.A. & BENOVIC, J.L. (1999). Pharmacological inhibition of protein kinases in intact cells: Antagonism of beta adrenergic receptor ligand binding by H-89 reveals limitations of usefulness. *J. Pharm. Exp. Ther.,* **288,** 428–437.

PIEDRAS-RENTERIA, E.S., SHERWOOD, O.D. & BEST, P.M. (1997a). Effects of relaxin on rat atrial myocytes. I. Inhibition of $I_{to}$ via PKA-dependent phosphorylation. *Am. J. Physiol.,* **272,** H1791–H1797.

PIEDRAS-RENTERIA, E.S., SHERWOOD, O.D. & BEST, P.M. (1997b). Effects of relaxin on rat atrial mycotes. II. Increased calcium influx derived from action potential prolongation. *Am. J. Physiol.,* **272,** H1798–H1803.

POST, S.R., HILAL-DANDAN, R., URASAWA, Z.K., BRUNTON, L.L. & INSEL, P.A. (1995). Quantification of signaling components and amplification in the $\beta$-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. *Biochem. J.,* **311,** 75–80.

REBSAMEN, M.C., ARRIGHI, J.F., JUGE-AUBRY, C.E., VALLOTTON, M.B. & LANG, U. (2000). Epidermal growth factor induces hypertrophic responses and Stat5 activation in rat ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.,* **32,** 599–610.

SHERWOOD, O.D. (1994). Relaxin. In *The Physiology of Reproduction*. 2nd Edition. ed. Knobil, E. & Neill, J.D. pp. 861–1009. New York: Raven Press Ltd.

SUMMERS, R.J., TAN, Y.Y., KAKOURIS, H. & EDDIE, L.W. (1995). Cardiac actions of relaxin. In *Progress in Relaxin Research*. ed. MacLennan, A.H., Tregear, G.W. & Bryant-Greenwood, G.D. pp. 487–498. Singapore: Global Publications Services.

TAN, Y.Y., WADE, J.D., TREGEAR, G.W. & SUMMERS, R.J. (1998). Comparison of relaxin receptors in rat isolated atria and uterus by use of synthetic and native relaxin analogues. *Br. J. Pharmacol.,* **123,** 762–770.

TAN, Y.Y., WADE, J.D., TREGEAR, G.W. & SUMMERS, R.J. (1999). Quantitative autoradiographic studies of relaxin binding in rat atria, uterus and cerebral cortex: characterization and effects of oestrogen treatment. *Br. J. Pharmacol.,* **127,** 91–98.

TOTH, M., TASKINEN, P. & RUSKOAHO, H. (1996). Relaxin stimulates atrial natriuretic peptide secretion in perfused rat heart. *J. Endocrinol.,* **150,** 487–495.

WAY, S.A. & LENG, G. (1992). Relaxin increases the firing rate of supraoptic neurones and increases oxytocin secretion in the rat. *J. Endocrinol.,* **132,** 149–158.

WEISINGER, R.S., BURNS, P., EDDIE, L.W. & WINTOUR, E.M. (1993). Relaxin alters the plasma osmolality-arginine vasopressin relationship in the rat. *J. Endocrinol.,* **137,** 505–510.

*(Received May 8, 2002*
*Revised July 31, 2002*
*Accepted August 12, 2002)*