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SPECIAL REPORT

Negative inotropic effects of isoprenaline on isolated left atrial assays from aged transgenic mice with cardiac over-expression of human β_2-adrenoceptors

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The action of isoprenaline has been evaluated in an isolated, left atrial assay, from aged transgenic mice with cardiac-specific over-expression of the β_2-adrenoceptor. In the assay, isoprenaline produced a negative inotropic concentration-response curve that was not altered by incubation with CGP-20712A (1 μM), a β_1-adrenoceptor antagonist. However, after incubation with ICI-118,551 (300 nM), a selective β_2-adrenoceptor antagonist, isoprenaline produced a positive inotropic concentration-effect curve that was located to the left of the negative inotropic curve. This suggests that the negative inotropic effect was mediated by a homogenous population of negatively-coupled β_2-adrenoceptors. In the presence of CGP-20712A (300 nM), the positive curve was shifted to the right, suggesting that the positive inotropic effect was mediated, at least in part, by β_1-adrenoceptors. These results differ substantially from those previously obtained in young transgenic mice. An outline of an explanatory model, based on a concept of over-expressed receptors 'stealing' G-proteins, is suggested.

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Abbreviation: TG, transgenic

Introduction In 1994 Milano *et al*., reported the creation of transgenic mice with cardiac specific over-expression of human β_2-adrenoceptors. In young adult mice (8–10 weeks), this over-expression resulted in an increased basal adenylyl cyclase activity, enhanced atrial contractility and increased left ventricular function *in vivo*. Bond *et al*. (1995), used this transgenic model to examine the phenomenon of inverse agonism. According to the two-state-model of agonist action (see Leff, 1995), there is such a marked over-expression of receptors (200 fold) in the TG-4 mice that a significant population of constitutively-active receptors (R*) exists. In the electrically stimulated, isolated, left atrial preparation, Bond *et al*. (1995) reported that the left atrial basal force of contraction was 3 fold higher in the transgenic animals than in their littermate controls. This meant that TG-4 basal tension was already maximal, as shown by the absence of a positive inotropic response to isoprenaline. ICI-118,551 caused concentration-dependent inhibition of left atrial tension defining it as an inverse agonist. Pre-antagonist levels could be restored by the addition of isoprenaline ($p[A]_{50} = 7.5$). However, this isoprenaline curve was not shifted to the right in the presence of the β_1-adrenoceptor-selective antagonist CGP-20712A, suggesting that the response was mediated by a homogeneous population of β_2-adrenoceptors.

As part of our own studies on the phenomenon of inverse agonism, we examined the effect of isoprenaline, the β_2-adrenoceptor-selective antagonist ICI-118,551 (Bilski *et al*., 1983) and β_1-adrenoceptor-selective antagonist CGP-20712A (Doolet *et al*., 1986), in TG-4 mice that were older (i.e. aged 4–11 months) than those previously studied. Initially there was no rationale for the use of older animals, we simply accumulated a stock due to a problem with the probe for the transgene that caused an interruption of the genotyping and breeding programme. Because the transgene expression can be heterozygous, we needed accurate genotyping before we could use the mice.

Methods The data presented in this study were obtained in left atrial preparations from mice that were at least 4 months old. The age distribution was as follows: 25% aged between 4–5 months; 5% between 6–7 months; 25% between 8–9 months; 45% between 10–11 months.

The heart was rapidly excised and placed in pre-warmed, oxygenated, Krebs-Henseleit solution (composition (mM)): NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, Na_2HPO_4 1.0, $NaHCO_3$ 25 and D-glucose 10. The left atrium was removed, trimmed of any extraneous tissue near the atrio-ventricular junction and divided into two roughly equal strips. The tissues were mounted in 20 ml organ baths containing Krebs-Henseleit solution thermostatically controlled at 32°C and gassed with 95% O_2, 5% CO_2. The tissues were placed under an initial resting tension of 0.8 g and electrically stimulated *via* a punctate electrode, at a frequency of 1 Hz, 1 ms duration and a voltage of threshold + 30%. The tissues were then allowed to stabilize for 40 min, during which time the organ bath fluid was replaced with pre-warmed Krebs at 10 min intervals. All tissues were incubated with phentolamine (3 μM), an α-adrenoceptor antagonist, desipramine (100 nM) and corticosterone (10 μM), blockers of catecholamine Uptake 1 and 2, respectively. Any β-adrenoceptor antagonists used were incubated for 90 min prior to obtaining an isoprenaline concentration-effect curve by cumulative dosing at 0.5 log unit intervals. Only one curve was obtained per preparation. Tissue responses were measured using a peak-height detector, which provided a continuous recording of the difference between the resting force and total force at the peak of each electrically-stimulated contraction.

Where possible, the concentration-effect curve data from individual preparations were fitted to a three parameter logistic

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function by means of an interactive, least squares, computer programme, providing estimates of the mid-point location parameter (log $[A]_{50}$), maximum asymptote (α) and mid-point slope (n_H), as described by Black & Shankley (1985). For display purposes (Figure 1), the computed parameter estimates for each treatment group were expressed as means and a single logistic curve was generated and superimposed upon the experimental data. Where individual, replicate curves could not be fitted, simple arithmetic means were calculated for each data point and these values fitted to the logistic function.

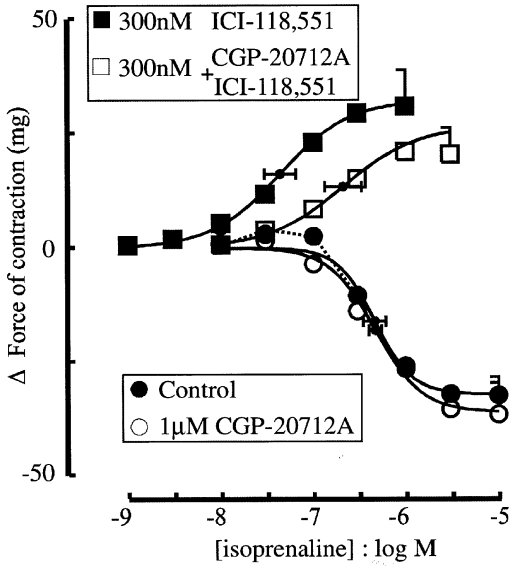

Figure 1 Effect of the β_1-adrenoceptor antagonist CGP-20712A and/or the β_2-adrenoceptor antagonist ICI-118,551 on the response to isoprenaline of the aged TG-4 left atrial strip.

Results The aged transgenic mice appeared to match their corresponding wildtype (WT) controls in terms of weight, activity and mobility. However, there were more cases of sudden death in transgenic animals 8 months or older (17%, as opposed to 4.5% in control mice). The hearts of some of these animals (approximately 20%) demonstrated clear macroscopic abnormalities. The left atrium was frequently hypertrophied and the muscle itself was either partially or totally necrotic, as judged by yellow-white infarcts.

Baseline atrial force of contraction was significantly lower (494 ± 14 mg) in the aged TG-4 mice compared to (568 ± 13 mg) aged-matched WT controls. A 90 min incubation with 300 nM ICI-118,551 (β_2-adrenoceptor antagonist, $pA_2 = 9.3$, Bilski *et al*., 1983; 9.1, Goldie *et al*., 1984) or 1 μM CGP-20712A (β_1-adrenoceptor antagonist, $pK_B = 9.5$, Schild analysis in controls, data not shown, see also Dooley *et al*., 1986) produced only small decreases in baseline atrial tension, 2.2% and 1.7%, respectively. Isoprenaline produced a concentration-dependent inhibition of the left atrial tension in all TG-4 preparations tested (Figure 1). Although it was not possible to fit all the individual replicate curves, possibly because there was some asymmetry about the midpoint location, a fit of the mean data gave a $p[A]_{50}$ of 6.3. Pre-incubation with 1 μM CGP-20712A (3000 fold its K_B at β_1-adrenoceptors) had no effect on the isoprenaline curve ($p[A]_{50} = 6.33 \pm 0.07$), indicating this response is not mediated by β_1-adrenoceptors. In contrast, in the presence of β_2-adrenoceptor blockade, a positive inotropic concentration-effect curve was restored, located to the left of the negative inotropic 'control' curve ($p[A]_{50} = 7.36 \pm 0.15$). This curve was subsequently shifted 0.68 ± 0.26 log units to the right by 300 nM CGP-20712A (1000 fold its pK_B) (Table 1).

The negative inotropic response to isoprenaline is characterized by considerably slower time courses than exhibited by the positive inotropic responses (Figure 2).

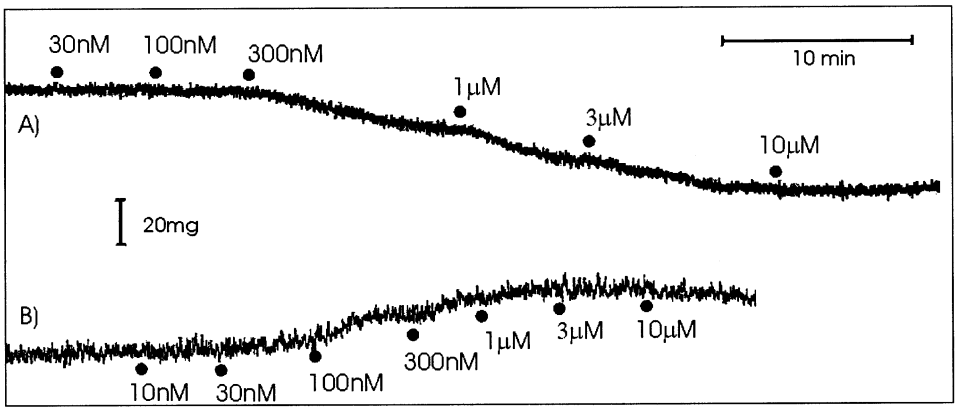

Figure 2 Experimental traces showing (A) an isoprenaline concentration-response curve and (B) an isoprenaline concentration-response curve in the presence of 300 nM ICI-118,551, in the aged TG-4 mouse left atrial strip assay.

Table 1 Curve-fitting parameters for the isoprenaline curves shown in Figure 1.

	n	$p[A]_{50}$	*s.e.mean*	α (mg)	*s.e.mean*	n	*s.e.mean*
Control*	7	~6.3		~−32		~2.1	
1 μM CGP-20712A	5	6.33	0.07	−36.24	8.3	1.73	0.29
300 nM ICI-118,551	4	7.36	0.17	32.32	8.0	1.16	0.11
300 nM ICI-118,551 + 300 nM CGP-20712A	3	6.68	0.20	26.76	5.8	1.07	0.18

n = numner of replicate curves. *Values obtained by fitting the mean data points (see text for details).

Discussion Clearly the results obtained in the aged Tg-4 mouse left atrial assay are different from those reported by Bond *et al*. (1995) in the same assay prepared from young TG-4 mice. We have evidence that the differences cannot be attributed to a change in transgene expression over successive generations of the mouse since the original studies were performed. Thus, although the detailed examination of the time-dependent changes in atrial contractility, pharmacology and histology has not been completed, in preliminary radioligand binding experiments, the level of β_2-adrenoceptor expression in young mice from our colony was found to be $\sim$200 fold higher in TG-4 mice compared to age-matched controls and ICI-118,551 behaves as an inverse agonist in the left atrial assay (data not shown; A. Hasseldine & E. Harper, personal communication). Thus, the level of over-expression and pharmacological behaviour is similar to that originally reported by Milano *et al*. (1994); Bond *et al*. (1995).

Baseline atrial force of contraction was significantly lower in the aged TG-4 animals than in their corresponding WT controls. This differs substantially from results obtained by other researchers (Bond *et al*., 1995; Milano *et al*., 1994), where the baseline atrial tension in young TG-4 mice was reported to be approximately three times greater than in young controls. Presumably, the absolute basal tension will be largely determined by the amount of healthy tissue present in the preparation. In fact, the macroscopic evidence of significant hypertrophy of the TG-4 hearts suggests that only a fraction of each aged tissue sample may have been responding to the electrical stimulus, and perhaps explains why we observed no striking difference between the (healthy) WT controls and (ailing) transgenics at baseline. Interestingly, Engelhardt *et al*. (1999) have reported that overexpression of the cardiac β_1-adrenoceptor may lead to a short-lived improvement of cardiac function, but that ultimately, the increased β_1-adrenoceptor signalling is detrimental, with marked myocyte hypertrophy and a 50% decrease in contractility in 35-week-old mice. So essentially, there is a progressive heart failure with all the functional and histological deficits typical for humans with heart failure. Further studies are in progress to determine if similar, time-dependent, phenotypic changes occur in the TG-4 mice.

It has previously been reported (Bond *et al*., 1995) that ICI-118,551 is an inverse agonist in this system and that it produces a 20–80% reduction in the basal atrial tension in the young TG-4 mice. However, in this study, preincubation with the β_2-adrenoceptor antagonist ICI-118,551 (and the β_1-adrenoceptor antagonist CGP-20712A) had only a small effect on the baseline force of contraction. Thus, in the aged TG-4 mice there was no evidence of β_2-adrenoceptor constitutive activity or inverse agonism by ICI-118,551.

In the absence of β-adrenoceptor blockade, isoprenaline produced a concentration-dependent negative inotropic response ($p[A]_{50}$ approximately 6.3) that was apparently not mediated by β_1-adrenoceptors, as a high concentration of CGP-20712A failed to shift the curve. Interestingly, there was a suggestion that the isoprenaline curve became more symmetrical, as though small positive inotropic responses to low concentrations (30–300 nM; see Figure 1 where this deviation is highlighted with a hatched line connecting the mean data points) were inhibited. When the β_2-adrenoceptors were blocked, the predominant negative inotropic response to isoprenaline was converted to a more potent positive one. Thus, it appeared that block of the β_2-adrenoceptors released a brake on a β_1-adrenoceptor positive inotropic response. The finding of a positive inotropic response is in itself evidence of a significant effect of aging of the TG-4 mouse, as previously, in young mouse atria, Bond *et al*. (1995) found the addition of isoprenaline could, under no circumstances, increase the force of contraction above basal levels.

The positive inotropic response to isoprenaline was blocked by a high but selective concentration of CGP-20712A. However, the log dose-ratio measured (0.68 ± 0.26) was significantly less than that expected for the concentration of antagonist used (300 nM = 1000 fold its K_B). It is unlikely that there will be a simple explanation for this underestimation because the location of the isoprenaline curve obtained in the presence of β_2-adrenceptor blockade (the 'control' curve for the dose-ratio estimation) is located over 1 log unit to the right of the corresponding wildtype β_1-adrenceptor-mediated isoprenaline curve ($p[A]_{50}$ $\sim$8.3). Notwithstanding the underestimation of the activity of CGP-20712A, it appears that under control conditions isoprenaline, even at the low concentrations that do not produce the negative inotropic response (1–100 nM) *via* the β_2-adrenoceptors, prevents any β_1-adrenoceptor positive inotropy. β_1- and β_2-adrenoceptors are usually assumed to couple positively to adenylyl cyclase *via* G_s protein (Dohlman *et al*., 1991; Strader *et al*., 1989). However, it is now apparent that β_2-adrenoceptors can be promiscuous in their interactions with G proteins and can couple to G_i as well as G_s (Xiao *et al*., 1995, 1999). We are currently developing and testing an explanatory model in which it is assumed that β_1-adrenoceptors couple solely to G_s whereas β_2-adrenoceptors couple both to G_s and G_i. In the model, β_2-adrenoceptors and β_1-adrenoceptors compete for G_s and although β_2-adrenoceptors express high affinity for G_s, the complexes formed are less effective than β_1-adrenoceptor-G_S complexes. Thus, the reversal of the inotropic response in the presence of β_2-adrenoceptor blockade is due not only to block of the β_2-adrenoceptor-G_i mediated negative inotropy but also removal of the β_2-adrenoceptor 'steal' of G_s from the β_1-adrenoceptor. Interestingly, Rockman *et al*. (1998) have previously implicated abnormal β-adrenoceptor-G protein coupling in the pathogenesis of the failing heart.

In conclusion, these preliminary results indicate that there is a time-dependent phenotypic change in the coupling of both β_1 and β_2-adrenoceptors in the TG-4 mice that has a dramatic influence on the expression of the pharmacology of ligands for these receptors.

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