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Cardiac-specific overexpression of human β_2 adrenoceptors in mice exposes coupling to both G_s and G_i proteins

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1 Left atrial strips from transgenic (TG4) mice with cardiac-specific overexpression (~200-fold) of the β_2 adrenoceptor (β_2AR) were isolated, and their isometric force of contraction (F_c) in response to electrical stimulation was measured.

2 The βAR agonist isoprenaline elicited negative inotropic responses in all left atrial strips; in 6/11 preparations, it also had a small positive inotropic effect. This 'up-phase' was observed from 0.1 to 10 nM, with the 'down-phase' occurring at higher concentrations. Both phases were mediated by β_2AR, as shown by their sensitivity to the β_2AR antagonist ICI-118,551 (100 nM; pA_2 8.60±0.07, 8.45±0.19, for 'up-phase' and 'down-phase,' respectively), but not the β_1AR antagonist CGP-20712A (100 nM). Conversely, nontransgenic littermate preparations responded to isoprenaline treatment solely by an increase in F_c, which was β_1AR-mediated.

3 Pretreatment of left atrial strips with either 10 nM isoprenaline or 1 mM 8-bromo-cAMP significantly attenuated the TG4 'up-phase', while having no effect on either the TG4 'down-phase' or the littermate controls' responses. *B. pertussis* toxin treatment of the animals prevented isoprenaline's negative inotropic effects in TG4 preparations, but had no effect in littermate controls.

4 The findings imply that the responses of TG4 left atrium to isoprenaline are because of β_2AR coupling to G_s and G_i proteins, consistent with the model of Daaka *et al.*, in which protein kinase A phosphorylation of the β_2AR causes a switch from G_s to G_i protein coupling.

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Abbreviations: F_c, force of contraction; GRK, G protein-coupled receptor kinase; E/[A] curve, plot of effect, E, against agonist concentration [A]; ISO, isoprenaline; LMC, littermate control; NECA, 5′-(*N*-ethylcarboxamido)adenosine; PKA, protein kinase A; PTX, *B. pertussis* toxin

Introduction

TG4 mice were first described by Milano *et al.* (1994), who reported that cardiac-selective high levels of β_2AR overexpression could be attained by using an α-myosin heavy-chain promoter in front of the β_2AR gene. The resulting phenotype was one of elevated right atrial rate and left atrial force of contraction, because of elevated basal cAMP generation. In a subsequent paper, evidence for β_2AR constitutive activity was presented using the TG4 isolated left atrium (Bond *et al.*, 1995). In this preparation, up to 50 nM isoprenaline (ISO) failed to evoke a response. Following the establishment of a TG4 colony by our laboratory in 1997, based on the kind donation of some breeding pairs by R. Lefkowitz, Prendergast *et al.* (2000) reported that the phenotype of the TG4 left atrium differed from the original reports. While ISO evoked increases in force of contraction (F_c) in nontransgenic littermate controls (LMC), it elicited mixed positive and negative inotropy in TG4s. Prendergast *et al.* (2000) hypothesised that the negative inotropic response was because of β_2AR – G_i protein coupling.

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G protein-coupled receptors (GPCR) are capable of coupling to more than a single class of G-protein (for review, see Kenakin, 1995), a phenomenon that has been observed for several disparate receptor classes, such as amine receptors (Berg *et al.*, 1998; Brink *et al.*, 2000), CB_1 cannabinoid receptors (Bonhaus *et al.*, 1998), and δ-opioid receptors (Prather *et al.*, 1994), at both high receptor densities, and physiological expression levels. β_2AR have typically been regarded as G_s protein-coupled receptors. β_2AR coupling to G_i was first described by Asano *et al.* (1984), in reconstituted membranes, where β_2AR, colocalised with G_i protein, were able to stimulate GTPγS binding and GTPase activity. Xiao *et al.* (1995) observed that this was not isolated to artificial systems, and could occur in intact rat cardiomyocytes. Later work (Xiao *et al.*, 1999) uncovered the phenomenon in isolated cells from both TG4 and wild-type mice. The mechanism by which this occurs is reported to depend on the switching of β_2AR signalling from G_s to G_i by protein kinase A (PKA) phosphorylation of the receptor (Daaka *et al.*, 1997).

In this study, we have further investigated the behaviour of ISO in TG4 left atrium, and have endeavoured to elucidate the mechanism by which its positive and negative inotropic effects are mediated. A preliminary report, covering parts of the work, was made at the meeting of the British Pharmacological Society, Dublin, July 2001 (Hasseldine *et al.*, 2001).

Methods

Genotyping of the mice

All procedures involving animals were carried out in accordance with the Animals (Scientific Procedures) Act, 1986. Mouse tail tips were removed under halothane anaesthesia, and used as a source of genomic DNA, which was extracted by phenol/chloroform method (Gross-Bellard *et al*., 1973). The DNA was UV-crosslinked to a nylon membrane and tested for the presence of the transgene using a ^{32}P-labelled (Stratagene Prime-it II Random Primer Kit) oligonucleotide probe, specific for the SV40 intron it contains. The blot was visualised using a phosphorimager.

Measurement of receptor overexpression

Radioligand binding saturation analysis was used to determine the level of β_2AR overexpression. Cell membranes were prepared from whole hearts using two sequential steps of tissue/pellet polytron homogenisation (Kinematica AG; PT-DA 3020/2TS; setting 12, $\sim$8 s), and low-speed centrifugation ($100 \times g$ for 7 min), followed by high-speed centrifugation ($39{,}800 \times g$ for 15 min) of the resulting pooled supernatant. The membranes were suspended in modified Krebs – Henseleit solution (118 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM K_2HPO_4, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose). Selective β_2AR binding was achieved with 0.01 – 3 nM of the β_1/β_2AR antagonist radioligand [^{3}H]-CGP 12177A, by including 5 μM β_1AR-selective antagonist (CGP-20712A; β_1AR pK_I 9.6, β_2AR pK_I 5.7; Dooley *et al*., 1986). 1 μM propranolol was used to determine nonspecific binding. The mixture was incubated for 2.5 h at 32°C, before rapid filtration with a Brandell Cell Harvester. The filters were washed (3×3 ml) with ice-cold 50 mM Tris-HCl (pH 6.9 at 21 ± 3 °C) before bound radioactivity was determined by liquid scintillation counting.

Bioassay of 1/2 left atrium

Methods were as described by Prendergast *et al*. (2000). Briefly, male and female TG4 mice and their nontransgenic LMC (24 – 31 g; 2 – 8 months old) were killed by cervical dislocation. Their hearts were removed, and the left atria resected. The remainder of the heart was immediately frozen on dry ice, and stored at −86°C until required for radioligand binding. The left atria were bisected, and each strip was tied with a loop of cotton at both ends, and suspended within a 20 ml organ bath, in constantly oxygenated (5% CO_2 in O_2) modified Krebs – Henseleit solution (as above) at 32°C. A force of 0.8 g, previously determined to be optimal for this preparation, was then applied. After the application of tension, the muscle strips relaxed to a resting tension of approximately half that applied. Strips were then stimulated electrically via punctate electrodes (1 ms square wave pulses at 1 Hz, 130% threshold V), and their force of contraction measured using an amplitude meter (source: noncommercial), which records dynamic force as the difference between resting and peak force. Experiments were performed in the presence of 100 nM desipramine, 10 μM corticosterone and 3 μM phentolamine, to block catecholamine uptake and αAR. βAR antagonists, where applied, were allowed to equilibrate for 90 min before agonist exposure.

B. pertussis toxin (PTX) treatment

Mice were treated by intraperitoneal injection of PTX in an aqueous suspension (100 μg kg^{-1}; $\sim$3 μg in 0.15 ml), 48 h before assay. This dose was selected on the basis that it inhibited the negative inotropic effects of the adenosine receptor agonist 5′-(*N*-ethylcarboxamido)adenosine (NECA), indicating a loss of G_i protein function.

Analysis

Dose – response data were analysed by nonlinear regression, using Graphpad Prism 3.02, on a plot of effect (force of contraction: F_c) vs agonist concentration (log *M*), referred to as an *E*/[A] curve. A logistic function could be applied to some *E*/[A] curves ('monophasic': Equation (1)). Others were classed as biphasic, as the agonist caused an increase followed by a decrease in F_c, and these were fitted by Equation (2)

$$F_c = Basal + \frac{(Max - Basal)}{1 + 10^{-n_H(p[A]_{50} + \log[A])}} \quad (1)$$

where F_c is the measured force of contraction, *Basal* is the F_c immediately prior to agonist exposure, *Max* is the maximum F_c elicited by the agonist, $p[A]_{50}$ is the midpoint curve location (negative logarithm of the molar concentration of *A*, which elicits half the maximum effect) and n_H is the midpoint slope (Hill slope). The range of the curve was defined as (*Max*−*Basal*)

$$F_c = Basal + \frac{(Max - Basal)}{1 + 10^{-(p[A]_{50u} + \log[A])}} + \frac{(Min - Max)}{1 + 10^{-(p[A]_{50d} + \log[A}} \quad (2)$$

where, in addition to the above definitions, *Min* is the minimum F_c elicited by the agonist, and $p[A]_{50u}$ and $p[A]_{50d}$ are the midpoint curve locations of the 'up' and 'down' phases, respectively. The 'up-range' of the curve was defined as (*Max*−*Basal*), with the 'down-range' as (*Max*−*Min*). Both of these are therefore positive numbers. It was observed that a degree of overlap between the 'up-phase' and the 'down-phase' meant that the fitting programme had trouble discerning the true *Max*. Therefore, *Max* was constrained to the highest observed F_c when fitting biphasic curves.

The Hill slope (n_H) of biphasic curves was not estimated. The inclusion of the slope factor would have introduced two extra parameters into the fitting equation, leading to an unacceptably high degree of redundancy (and interdependency) among the seven parameters.

Receptor saturation by radioligand binding produced data that were well described by a rectangular hyperbola (Equation (3)). These were also analysed using nonlinear regression, with Graphpad Prism3.02.

$$Bound = \frac{[R]_0}{1 + 10^{-(pK_D + \log[L])}} \quad (3)$$

where $[R]_0$ is the receptor density, pK_D is the affinity ($-\log M$ concentration causing 50% occupancy) of the radioligand and [L] is its concentration.

Drugs and chemicals

(−)Isoprenaline, CGP-20712A, ICI-118,551, 8-Br-cAMP, (−)propranolol, phentolamine, corticosterone and desipramine were purchased from Sigma-Aldrich (Dorset, UK). [^{3}H]-CGP 12177A was purchased from NEN Life Science (Cambridge, UK), and *B. pertussis* toxin from CN Biosciences (Nottingham, UK). The Prime-it II Random Primer Kit was purchased from Stratagene (Amsterdam, Netherlands).

Statistical analysis

Best-fit parameters from nonlinear regression of E/[A] curves and other measured parameters are given as mean ± s.e.mean, and were compared by ANOVA or t-test, as stated in the text, using Graphpad Prism 3.02. The P threshold for determination of statistical significance was 0.05, although exact P-values are given where possible.

Results

Radioligand binding

β_2AR density in TG4 cardiac membranes was 55.7 ± 7.8 fmol mg^{-1} (original wet weight; $n = 10/10$ determinations/animals), compared with 0.25 ± 0.02 fmol mg^{-1} in LMC ($n = 6/28$), representing 219 ± 10 fold overexpression of the β_2AR (see Figure 1).

Basal left atrial force of contraction (F_c) and antagonist effects on baseline

There was no statistically significant difference between the baseline F_c of half left atria from TG4 and LMC mice (183 ± 14 mg, $n = 49$ and 172 ± 13 mg, $n = 47$, respectively; $P = 0.6$ by unpaired t-test).

In the absence of antagonists, baseline force of contraction (F_c) in LMC preparations faded by 28 ± 5% ($n = 11$) over the 90 min incubation period. In the presence of 100 nM CGP-20712A, fade in F_c was 41 ± 5% ($n = 8$), with 100 nM ICI-118,551 it was 43 ± 6% ($n = 11$), and with a combination of both of these, it was 49 ± 8% ($n = 9$). There was no statistically significant effect of either antagonist (two-way ANOVA, $P = 0.06$, 0.1 and 0.6 for, CGP-20712A, ICI-118,551 and interaction, respectively). The F_c of untreated TG4 preparations faded by 13 ± 3% ($n = 11$), which was significantly less than that seen in LMC preparations (unpaired t-test, $P = 0.02$). Fade in F_c was 30 ± 3% with ICI-118,551 ($n = 7$), 10 ± 3% with CGP-20712A ($n = 7$), and 26 ± 8% with both antagonists ($n = 6$). Two-way ANOVA revealed a significant effect of ICI-118,551 ($P = 0.0005$), but not of CGP-20712A ($P = 0.5$). There was no significant interaction ($P = 0.9$).

Effect of ISO on left atrial F_c

In LMC preparations ($n = 10$), 0.1 – 30 nM ISO elicited concentration-dependent increases in F_c with no further effects at higher concentrations (see Figure 2). The mean best-fit values for p[A]$_{50}$ and range are shown in Table 1. The β_1AR antagonist CGP-20712A (100 nM; $n = 8$), shifted the ISO E/[A] curve, in parallel, to the right (see Figure 2), yielding a pA_2 of 9.35 ± 0.10 (β_1AR pK_I ~9.6, β_2AR pK_I ~5.7; Dooley *et al.*, 1986). The β_2AR selective antagonist ICI-118,551 (100 nM; $n = 10$) also shifted the ISO E/[A] curve to the right, although to a much lesser extent (0.25 ± 0.08 log units), yielding a pA_2 of 6.89 ± 0.08 (β_1AR pK_B ~7.2, Henry & Goldie, 1990). Each of the antagonists had a significant effect on p[A]$_{50}$ (two-way ANOVA, $P < 0.0001$ and 0.03, for CGP-20712A and ICI-118,551, respectively), but did not affect either the range or Hill slope of the curves (two-way ANOVA $P > 0.05$ for both antagonists in both tests). In none of the three tests was there a significant interaction ($P > 0.05$ for each). The relation between baseline fade in F_c and E/[A] curve range was examined: in ICI-118,551-treated preparations, range correlated with fade (Pearson, $P = 0.008$), but in the absence of antagonist and in CGP-20712A-treated tissues, there was no significant correlation (Pearson, $P = 0.6$, 0.1, respectively).

In all TG4 preparations, ISO caused a decrease in F_c at concentrations in the range 0.1 – 10 μM. It was also observed that, in 6/11 preparations, an increase in F_c was elicited at lower concentrations (0.1 – 10 nM). Therefore, in order to employ the most accurate analytical technique of curve-fitting individual data sets rather than fitting the mean data, they had to be fitted by different equations (see methods, analysis section). Consequently, they are described as biphasic (6/11) or

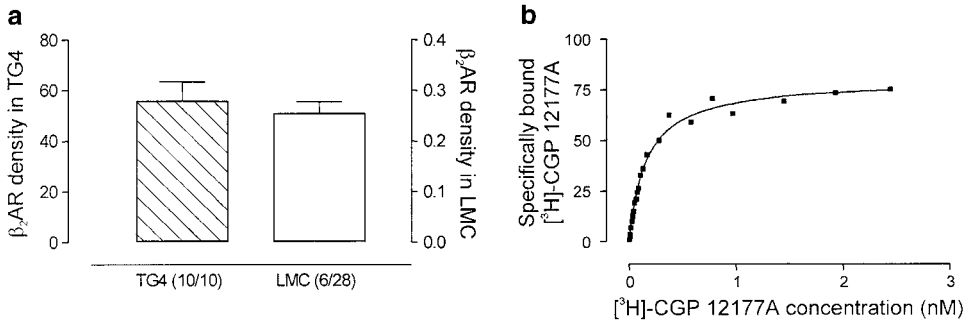

Figure 1 Panel a shows β_2AR density data from TG4 and littermate control (LMC) cardiac cell membrane saturation binding with [^{3}H]-CGP-12177A. Data are expressed as femtomoles specific binding per milligram of tissue wet weight; means are plotted with s.e.mean indicated by the error bars. For TG4 mice, $n = 10$ determinations from 10 animals (10/10), and for LMC, $n = 6$ determinations from 28 animals (6/28). Note that there is a dual ordinate scale, such that the LMC scale is stretched 200-fold relative to that of the TG4. In panel b, a typical β_2AR saturation curve in TG4 heart membranes is shown.

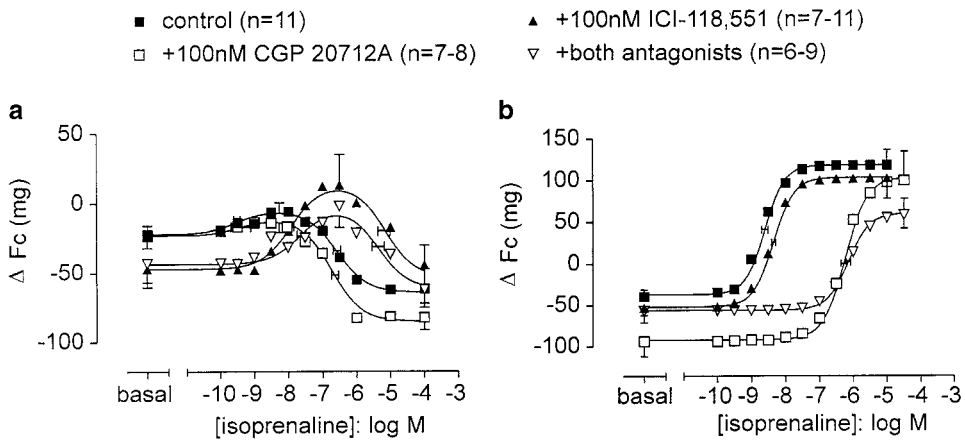

Figure 2 The effect of isoprenaline on littermate control (LMC. in panel a) and TG4 (panel b) left atrial strip contractility, in the presence and absence of antagonists selective for the β_1AR (CGP-20712A) and β_2AR (ICI-118,551). Mean data points are shown with curves that have been simulated using mean best-fit values (s.e.mean indicated by error bars) from nonlinear regression of individual data sets, using a 4 – 5 parameter logistic (see text for equations, Table 1 for mean best-fit data). The units of the ordinate axis are change in force of contraction, expressed in milligram; zero was set as the F_c at the time of antagonist administration (90 min prior to isoprenaline).

Table 1 Best-fit parameters from nonlinear regression of isoprenaline *E*/[A] curves in TG4 and LMC left atrial strips

		p[A]$_{50u}$	*Up range (mg)*	*p[A]$_{50d}$*	*Down range (mg)*	*n*
TG4:	Biphasic controls	9.52±0.08	31±9	6.61±0.16	70±12	6
	Monophasic controls	N/A	0±0	6.54±0.14	46±10	5
	All controls	*9.52±0.08*	17±7	6.57±0.10	59±8	11
	All + ICI-118,551	7.91±0.05	61±22	5.14±0.22	67±23	7
	Biphasic + CGP-20712A	9.43±0.28	19±8	6.70±0.16	63±1	4
	Monophasic + CGP-20712A	N/A	0±0	6.74±0.12	89±9	3
	All + CGP-20712A	*9.14±0.35*	11±5	6.78±0.08	74±6	7
	All + both antagonists	7.60±0.06	42±9	5.36±0.25	61±13	6
LMC:	controls	8.60±0.09	156±18	N/A	N/A	11
	+ ICI-118,551	8.35±0.06	155±25	N/A	N/A	11
	+ CGP-20712A	6.24±0.09	195±31	N/A	N/A	8
	+ both antagonists	6.14±0.06	116±18	N/A	N/A	9

ISO was added to half left atrial preparations in a cumulative fashion, in the presence or absence of 100 nM ICI-118,551, 100 nM CGP-20712A or a combination of the two. The data were fitted to a 4–5 parameter equation (see text for details). Best-fit values are expressed as mean±s.e.mean. Italicised p[A]$_{50u}$ values indicate that the figure is comprised only of those preparations whose responses permit the calculation of a p[A]$_{50u}$ value (i.e. biphasic preparations); the *n* for that row does therefore not apply to the italicised value.

monophasic (5/11). The best-fit parameters from fitting of the individual *E*/[A] curves are shown in Table 1. There was no significant difference between biphasic and monophasic preparations in baseline before ISO, or midpoint location of the 'down-phase' (p[A]$_{50d}$; unpaired *t*-test, $P>0.05$ for each). There was no correlation observed between either initial basal F_c or baseline fade, and the magnitude of the 'up-phase' (Spearman rank test, $P=0.2$, 0.8, respectively). As there was no *a priori* justification for the subgroup separation, statistical comparisons between control and antagonist-treated groups utilised the pooled best-fit parameters ($n=11$; see Table 1), where possible.

In the presence of the β_2AR antagonist ICI-118,551 (100 nM), all ISO *E*/[A] curves in TG4s were biphasic, with ICI-118,551 causing a significant increase in the range of the 'up-phase' (two-way ANOVA, $P=0.004$; see Table 1). ICI-118,551 had no effect on the range of the 'down-phase' (two-way ANOVA, $P=0.8$). It was also observed that, in ICI-118,551-treated preparations, there was a significant positive correlation between the amount of baseline fade and the range of the 'up-phase' (Pearson, $P=0.0008$). Both the 'up' and 'down-phases' of the ISO *E*/[A] curve were significantly shifted to the right by ICI-118,551 (two-way ANOVA, $P<0.0001$ for each), from which pA_2 values of 8.60±0.07 and 8.45±0.19 respectively, were calculated (β_2AR pK_I ~8.7, Bristow *et al.*, 1989). The β_1AR antagonist CGP-20712A (100 nM; β_1AR pK_I ~9.6, β_2AR pK_I ~5.7; Dooley *et al.*, 1986) had no significant effect on any of the parameters measured or fitted, as tested by two-way ANOVA (see Table 1 and Figure 2; $P>0.05$ in every test). In none of the tests was there a significant interaction between the two antagonists ($P>0.05$ in every test).

Desensitisation assay

To study the mechanism of the biphasic response, the assay protocol was modified slightly. The main kinase enzymes that

phosphorylate the β_2AR are PKA and G protein-coupled receptor kinase (GRK). PKA, but not GRK, has been implicated in the switch from β_2AR-G_s to -G_i coupling (Daaka *et al*., 1997). Selective PKA phosphorylation of β_2AR can be achieved by elevating cAMP levels under conditions of low β_2AR occupancy, because GRK, but not PKA, preferentially targets occupied receptors (Lohse *et al*., 1990). To this end, preparations were treated with 10 nM ISO until the positive inotropic response reached a plateau ($\sim$10 min), after which it was washed out (three washes in $\sim$15 min). They were then exposed to cumulative concentrations of ISO. A pretreatment concentration of 10 nM was chosen because it was enough to elicit the maximum increase in F_c in both LMC and TG4, but is predicted to produce only $\sim$2% β_2AR occupancy (Hasseldine *et al*., unpublished data, 1998, pK_I 6.29$\pm$0.11). The second approach to selective activation of PKA was to pretreat with the cAMP analogue 8-bromo-cAMP rather than 10 nM ISO.

In LMC left atrial strips, 10 nM ISO elicited an increase in F_c that was not different from the response to 10 nM ISO as part of a cumulative E/[A] curve (104$\pm$25 mg acute, vs 136$\pm$29 mg cumulative in pre-exposed preparations, paired t-test, $P=0.07$; or vs 129$\pm$11 mg in those not pre-exposed, unpaired t-test, $P=0.8$; $n=5$ each). Pre-exposure to ISO had no effect on the slope, p[A]$_{50}$ and range of the cumulative ISO E/[A] curve (see Figure 3). The best-fit values for pre-exposed and control preparations, respectively, were p[A]$_{50}$ 8.69$\pm$0.07 and 8.82$\pm$0.05, n_H 1.54$\pm$0.12 and 1.31$\pm$0.08 and range 146$\pm$29 and 146$\pm$8 mg.

In TG4 preparations, 10 nM ISO elicited a small increase in F_c (in 6/7 preparations, a positive inotropic response was seen; 16$\pm$5 mg, $n=7$), which was not different from the effect of 10 nM ISO, applied as part of a cumulative curve, in time controls (30$\pm$7 mg, $n=10$; unpaired t-test, $P=0.2$). However, in no TG4 preparation pre-exposed to 10 nM ISO was there a quantifiable increase in F_c upon reapplication of ISO (Figure 3), so that E/[A] curves were all monophasic. By contrast, all time controls (which were also washed) were, in this experiment, biphasic. The p[A]$_{50}$ of the monophasic curves was 6.69$\pm$0.04, with range 59$\pm$8 mg. These estimates were not different (unpaired t-tests, $P>0.05$) from the p[A]$_{50d}$ and range of the 'down-phase' of the control curves (6.77$\pm$0.05, 63$\pm$7 mg). The p[A]$_{50u}$ and 'up-phase' range, in controls, were 9.49$\pm$0.07 and 31$\pm$7 mg.

1 mM 8-Br-cAMP pretreatment (refer Figure 4) for 10 min caused an increase in TG4 left atrial F_c of 48$\pm$17 mg ($n=5$), which was not significantly different from its effect on LMC preparations (39$\pm$12 mg, $n=4$; unpaired t-test, $P=0.7$). Its effects mimicked those of ISO pretreatment: in LMC preparations, exposure to 8-Br-cAMP had no effect on later responses to cumulative ISO, whereas in TG4 preparations, the range of the ISO E/[A] curve's 'up-phase' was significantly decreased (73$\pm$27 mg in time control, 9$\pm$5 mg in pre-exposed preparations; Mann – Whitney U-test, $P=0.03$).

Effect of B. pertussis *toxin (PTX) treatment*

Baseline TG4 left atrial F_c was unchanged by PTX treatment (387$\pm$73 mg, $n=5$ and 370$\pm$86 mg, $n=4$; whole left atria from PTX and vehicle-treated animals, respectively). PTX treatment prevented the negative inotropic effects of ISO ($n=5$; see Figure 5), whereas vehicle treatment ($n=4$) had no effect on the curve shape or location compared with half left atria from untreated TG4. PTX significantly increased the magnitude of the ISO E/[A] curve's 'up-phase' (126$\pm$32 mg with PTX, 21$\pm$10 mg with vehicle; unpaired t-test $P=0.03$), but had no significant effect on its location (p[A]$_{50u}$ 9.21$\pm$0.29, $n=5$ vs 9.37$\pm$0.16, $n=3$; unpaired t-test $P=0.7$). As well as the initial 'up-phase', there was a second increase in F_c in some preparations (see Figure 5), at ISO concentrations greater than 10^{-6} M. The reason for this was not clear, and only the first phase was analysed. Other vehicle-treated TG4 best-fit parameters are as follows (cf. Table 1): p[A]$_{50d}$ = 6.32$\pm$0.07, 'up-range' = 21$\pm$10 mg, 'down-range' = 123$\pm$28 mg – of the four curves, three were biphasic and one monophasic. Following ISO administration, in vehicle-treated TG4 preparations, 10 μM of the adenosine receptor agonist NECA reduced F_c below the minimum elicited by ISO (−226$\pm$98 mg from baseline before ISO). In PTX-treated preparations, NECA was unable even to reverse the positive inotropic effects of ISO, having only a very small negative inotropic

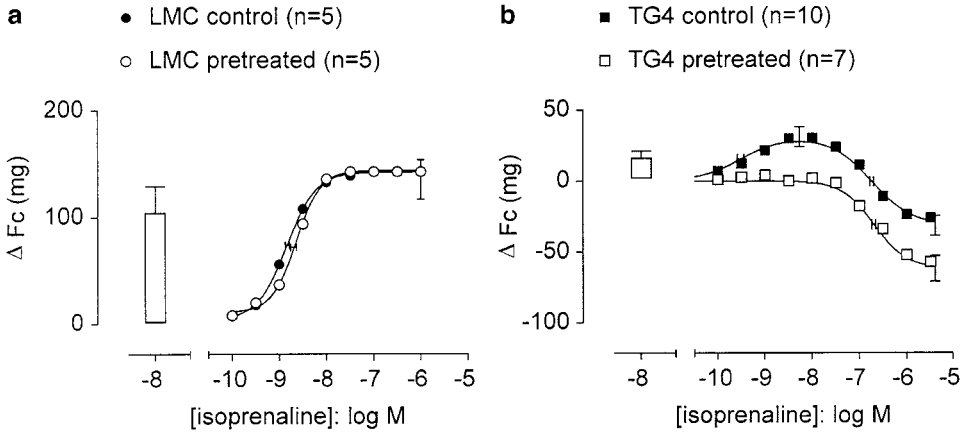

Figure 3 The effect of pretreatment with ISO (10 nM, 10 min) on littermate control (LMC; a) and TG4 (b) left atrial strips to subsequent, cumulative, isoprenaline. Columns indicate the mean effect (with s.e.mean) of acute exposure to 10 nM isoprenaline. Mean data points are shown with curves that have been simulated using mean best-fit values (s.e.mean indicated by error bars) from nonlinear regression of individual data sets, using a 4 – 5 parameter logistic (see text for equations). The units of the ordinate axis are change in force of contraction, expressed in milligram; zero was set as the F_c just prior to isoprenaline administration.

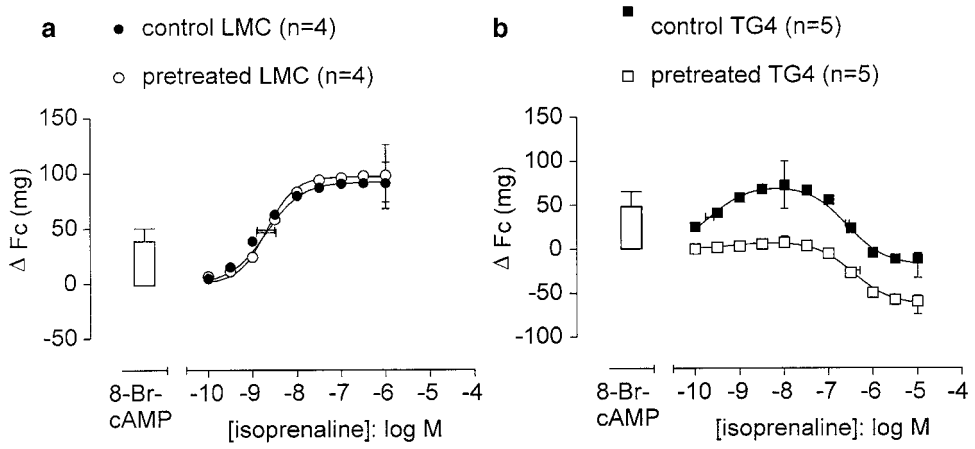

Figure 4 The effect of pretreatment with adenosine cyclic monophosphate analogue 8-Br-cAMP (1 mM, 15 min) on littermate control (LMC; a) and TG4 (b) left atrial responses to subsequent, cumulative, isoprenaline. Columns indicate the mean effect (with s.e.mean) of acute exposure to 1 mM 8-Br-cAMP. Mean data points are shown with curves that have been simulated using mean best-fit values (s.e.mean indicated by error bars) from nonlinear regression of individual data sets, using a 4 – 5 parameter logistic (see text for equations). The units of the ordinate axis are change in force of contraction, expressed in milligram; zero was set as the F_c just prior to isoprenaline administration.

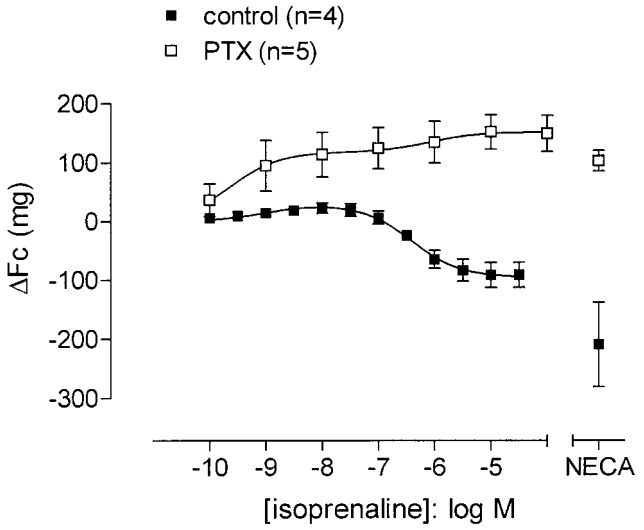

Figure 5 The negative inotropic effects of isoprenaline in TG4 left atrial strips are sensitive to *B. pertussis* toxin treatment. Controls were treated with vehicle (water). Mean data points are shown (s.e.mean indicated by error bars) with curves generated by nonlinear regression, using a five parameter logistic (see text for equations, Table 1 for mean best-fit data). The units of the ordinate axis are change in force of contraction, expressed in milligram; zero was set as the F_c immediately prior to isoprenaline administration. The point labelled 'NECA' is the mean response of each group to 10 μM NECA, administered after the highest dose of isoprenaline.

effect (104 ± 18 mg from before ISO; see Figure 5). PTX had no measurable effects on any parameters measured in LMC preparations (data not shown).

Discussion

Radioligand binding has confirmed that TG4 mice still have ~200-fold overexpression of cardiac β_2AR, as previously reported (Milano *et al*., 1994; Bond *et al*., 1995). This therefore excludes the possibility that discrepancies between the respective bioassay results are because of a change in overexpression.

In isolated TG4 left atrial strips, ISO elicited both increases and decreases in force of contraction, contrasting with its solely positive inotropic effect in LMCs. The effects of ISO in LMC preparations are mediated by the β_1AR, as shown by the high potency of CGP-20712A (pA_2 9.35 ± 0.10; β_1AR pK_I ~9.6, β_2AR pK_I ~5.7, Dooley *et al*., 1986), and the low potency of ICI-118,551 (pA_2 6.94 ± 0.08; β_1AR pK_B ~7.2, Henry & Goldie, 1990; β_2AR pK_I ~8.7, Bristow *et al*., 1989). This is consistent with the behaviour of left atria from other nontransgenic strains, such as Charles River CD1 (data not shown) or C57BL/10ScSn mice (Lu & Hoey, 2000), in which ISO also elicits β_1AR-mediated increases in F_c.

The positive and negative inotropic effects of ISO in TG4 left atria appeared to be solely β_2AR-mediated, as shown by the shift that 100 nM ICI-118,551, but not 100 nM CGP-20712A, produced. It was therefore concluded that β_2AR were coupling to both stimulatory and inhibitory pathways. Although β_2AR are usually considered to be G_s protein-coupled receptors, it has been shown that β_2AR – G_i coupling may occur in several systems (Asano *et al*., 1984; Xiao *et al*., 1995, 1999; Luo *et al*., 1999), and its patho-/physiological importance has been reviewed by Xiao (2000).

From previous work in our laboratory, it had been concluded that β_2AR-G_i coupling occurred in TG4 isolated left atria, but it was thought that the β_1AR also played a part in the positive inotropic effects of ISO (Prendergast *et al*., 2000). This was intuitively attractive, as there is no obvious reason for the disappearance of the functional β_1AR population seen in LMC left atria. However, the shift produced by CGP-20712A was far smaller than expected for a β_1AR-mediated effect (pA_2 7.1 vs β_1AR pK_1 ~9.6, β_2AR pK_1 ~5.7, Dooley *et al*., 1986) – an enigma that was not resolved. The present work has demonstrated that β_2AR mediate both phases of the TG4 response. To explain the apparent disappearance of the β_1AR, it was considered that the excess β_2AR may mask their signal. However, this was discounted on

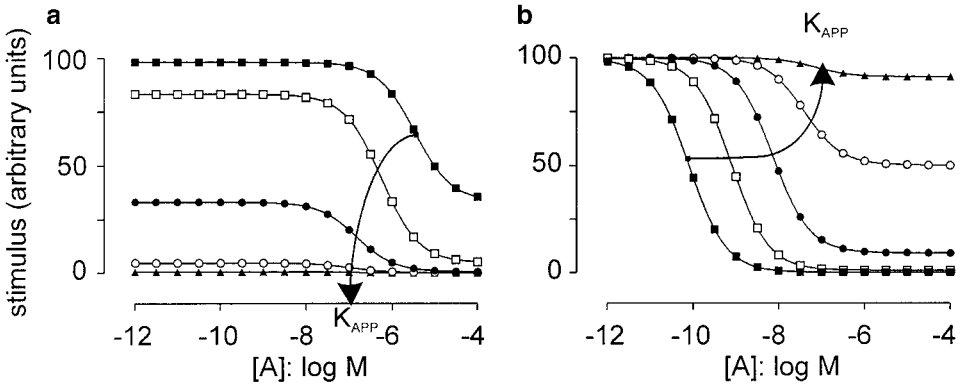

Figure 6 Simulation showing the effect of reducing receptor density in two models of agonism. The large arrow in each graph refers to a progressive reduction in receptor number, which may be, for example, the result of receptor alkylation, or differential expression levels. In a, application of an inverse agonist reduces stimulus by reversing the constitutive receptor activity ('deactivating' receptors, e.g. β_2AR-G_s signalling; two-state model). In b, application of an agonist reduces stimulus by activating receptors (e.g. G_i-coupled receptors, signalling in the presence of elevated basal cAMP; traditional model). Stimulus units are arbitrary, and should not be used for comparison between the models. The E/[A] curve always collapses towards K_{APP}, which is, in both cases, the compound's 'affinity constant,' when receptor density is reduced. An inverse agonist curve may be seen to lie to the right of its K_{APP}, whereas that of an agonist is to the left of its K_{APP}. The effect on curve location of reducing receptor number is thus a qualitative diagnostic, permitting the determination of the appropriate model. The equations and parameters used in the simulations are detailed in the Appendix.

the basis that the β_2AR-mediated positive inotropy can be desensitised by pretreatment with 10 nM ISO or 1 mM 8-Br-cAMP, whereas the β_1AR (in LMC) cannot. Therefore, if β_1AR played a part in the TG4 response, one would expect the positive inotropic effect of ISO to be maintained in TG4 preparations after pretreatment, mediated by the β_1AR. The absence of this effect does not preclude the existence of a β_1AR population in the tissue, but it suggests that β_1AR do not play a part in the TG4 left atrial contractile response to ISO.

β_2AR coupling to both G_s and G_i proteins was first shown by Asano *et al*. (1984). Later, a biochemical mechanism for the switch from G_s to G_i was proposed by Daaka *et al*. (1997): PKA phosphorylation of the β_2AR. This mechanism, described in HEK293 cells, was confirmed in mouse submandibular gland cells (Luo *et al*., 1999). Consistent with this, we found that ISO pretreatment of TG4 left atrial strips was able to repress the 'up-phase' of a subsequent ISO E/[A] curve. Moreover, exposure of the tissue to the cAMP analogue 8-Br-cAMP mimicked this effect, demonstrating that this phenomenon is cAMP-mediated, rather than dependent on a prior interaction between ISO and the β_2AR. The efficacy of the wash out procedure and the β_2AR-specificity of the G_s signalling inhibition were demonstrated by the absence of desensitisation phenomena in LMC preparations treated with ISO or 8-Br-cAMP. The involvement of PKA could not be tested directly in the TG4, as PKA is necessary not only for the switch in coupling to G_i, but also for downstream effects of β_2AR-G_S protein signalling (Zhou *et al*., 1997; Xiao *et al*., 1999).

The hypothesis that β_2AR couple to G_i in TG4 left atrium is further supported by the effect of PTX, which, when administered 48 h prior to sacrifice, prevented the negative inotropic effects of ISO, and enhanced the magnitude of its positive effects. The latter suggests that β_2AR-G_s signalling may be regulated (functionally antagonised) by G_i, consistent with literature reports, in which it has been shown that β_2AR – G_i interactions limit the spread of β_2AR – G_s signalling through the cytosol ('compartmentalisation' of G_s signalling, Kuschel *et al*., 1999). Compartmentalisation, or functional antagonism, of β_2AR-G_s signalling, could explain β_2AR-mediated positive inotropy (ISO in TG4 without PTX) is lesser in magnitude than ISO's effect in LMC (β_1AR mediated), or forskolin in LMC/TG4 (βAR independent; data not shown). Clearly, the small size of the β_2AR-mediated positive inotropic effect is not because of low receptor density, as we have confirmed that β_2AR are overexpressed in this system, and functionally, ISO's $p[A]_{50u}$ in TG4 is approximately 3 log units to the left of its binding affinity. The compartmentalisation hypothesis is also attractive because no left-shift in the 'up-phase' was seen in response to PTX, implying that the functional antagonism of G_s by G_i affects the magnitude of stimulus by altering its range rather than its coupling efficiency (e.g. in Appendix, E_{MAX}, rather than β). The small negative inotropic effect of NECA after PTX treatment indicates that the ADP-ribosylation of G_i was probably incomplete, but this does not alter the conclusion from the experiment, that G_i mediates ISO's negative inotropic action.

The observation that PTX did not affect baseline F_c suggests that there is no tonic G_i protein activation. Hence there is no evidence for β_2AR constitutive activity – had this been present, but curtailed by G_i, PTX treatment would have increased basal left atrial F_c. The absence of β_2AR constitutive activity may be because of enhanced levels of β_2AR phosphorylation, which would also account for the lack of shift in the ISO E/[A] curve 'down-phase' after 'up-phase' desensitisation. In this situation, the phosphorylated β_2AR is present in large numbers, meaning that overexpression of unphosphorylated receptor, in effect, occurs at a considerably lower level than 200-fold. When the tissue is pretreated, the loss of unphosphorylated receptors represents significant receptor depletion (and consequent loss of signal). By contrast, the concomitant increase in phosphorylated β_2AR levels is slight, relative to the high numbers

already present, and hence the $p[A]_{50d}$ does not change appreciably.

There are certain similarities between the present data and those from the original descriptions of TG4 mice (Milano *et al*., 1994; Bond *et al*., 1995), in which the two-state model of receptor activation (for model description, see Leff, 1995) was proposed to account for the observations. That is, some of the previous authors' data are also consistent with β_2AR G_s/G_i coupling. The lack of response to $\leqslant 50$ nM ISO seen in TG4 atria may have been because the 'up-phase' responses are small, when present, and difficult to characterise. Furthermore, it has been suggested that the negative inotropic effect of ICI-118,551 involves β_2AR-G_i-directed agonism (Gong *et al*., 2002). However, despite the similarities, the data of Bond *et al*. (1995) cannot be reconciled with β_2AR-G_i coupling, principally for two reasons. Firstly, force of contraction in the absence of agonist was reportedly three-fold higher in TG4 than LMC mice, implying that constitutive G_s-coupled β_2AR activity existed. Secondly, by depletion of the β_2AR population with pindobind, Bond *et al*. (1995) were able to cause the negative inotropic E/[A] curve of ICI-118,551 (its 'inverse agonist' curve) to collapse to the left, as predicted by the two-state model. By contrast, removal of receptors should cause G_i-directed agonist E/[A] curves to collapse to the right (see Figure 6). This criterion is, in general, useful for distinguishing inverse agonism from 'traditional' agonism phenomena, which may otherwise be difficult to separate.

In this study, we did not use pindobind, as the negative inotropic effects of ICI-118,551 appeared to be independent of β_2AR, for the following reasons. They were not antagonised by the neutral βAR antagonist alprenolol (no significant shift with 100 nM. pK_B 8.62, Hopkinson *et al*., 2000); secondly, the range of concentrations over which they were seen was 0.1 – 10 μM ($p[A]_{50}$ 6.39 ± 0.23, $n=7$) – concentrations considerably higher than required for β_2AR occupancy. Hence, the negative inotropic effects of ICI-118,551 may have been because of another action, perhaps direct inhibition of the G protein-gated inward rectifying potassium channel, which has been reported to occur at concentrations $\geqslant 0.1\,\mu$M (Wellner-Kienitz *et al*., 2001). This channel is regulated by the $\beta\gamma$ subunit of G_i protein, which has also been implicated in β_2AR-G_i signalling (Daaka *et al*., 1997). Gong *et al*. (2002) proposed that the negative inotropic effect of ICI-118,551 is not only G_i- but also β_2AR-dependent, but, as stated, evidence to the contrary was found by the present authors. In addition to the findings outlined above, separate experiments showed that the degree of baseline F_c fade in preparations from PTX-treated TG4 mice, in the presence of ICI-118,551 ($31 \pm 4\%$, $n=4$), was comparable with that from non-PTX-treated half left atria, implying a G_i-independent mechanism of action. In the results presented here, the natural tendency of the left atrial contractions to 'fade' with time, presumably because of fatigue or biochemical run-down of some sort, generally complicated the examination of the negative inotropic actions of ICI-118,551. The experiments undertaken with ICI-118,551 and alprenolol (alluded to above) were conducted over a shorter time course than the 90 min incubation period, after a long equilibration time, to overcome this problem.

Clearly, the phenotype of the TG4 is now different from that which was first described. Although the transgene is still present and functional – β_2AR density has not changed – adaptations may have occurred to compensate for it. The TG4 colony has not been outbred since they were obtained from the USA, but it has been noted that, in our colony, perinatal mortality was initially higher in TG4 litters than in those of the descendants of the original LMCs (C. E. Prendergast, 2001, PhD thesis, University of London). It is possible that natural selection has influenced the TG4 'genetic background', which is an important determinant of phenotype (Wolfer & Lipp, 2000). Alternatively, the change in environment, with different pathogens, food and storage conditions, may have in itself had an effect. Whatever the reason, the phenotype now appears to be stable, with recent data closely resembling observations made 4 – 5 years ago. The data are also similar to those reported by other groups using the same strain of transgenic mouse (Xiao *et al*., 1999; Gong *et al*., 2000), suggesting that either the phenotypic change occurred fairly soon after the initial work was published, before the mice were distributed to other investigators, or that the adaptation is intrinsic to the long-term effect of β_2AR overexpression.

In conclusion, this study has shown, by using selective antagonists, that β_2AR in TG4 left atria mediate both positive and negative inotropic effects of ISO. These responses – as shown by the effects of G_i protein inactivation with PTX, or cAMP preactivation with 10 nM ISO or 1 mM 8-Br-cAMP – are consistent with β_2AR-G_s and G_i proteins, respectively, modulated by a PKA switch, as proposed by Daaka *et al*. (1997).

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References

ASANO, T., KATADA, T., GILMAN, A.G. & ROSS, E.M. (1984). Activation of the inhibitory GTP-binding protein of adenylate cyclase, Gi, by beta-adrenergic receptors in reconstituted phospholipid vesicles. *J. Biol. Chem.,* **259,** 9351 – 9354.

BERG, K.A., MAAYANI, S., GOLDFARB, J., SCARAMELLINI, C., LEFF, P. & CLARKE, W.P. (1998). Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol. Pharmacol.,* **54,** 94 – 104.

BOND, R.A., LEFF, P., JOHNSON, T.D., MILANO, C.A., ROCKMAN, H.A., MCMINN, T.R., APPARSUNDARAM, S., HYEK, M.F., KENAKIN, T.P., ALLEN, L.F. & LEFKOWITZ, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. *Nature,* **374,** 272 – 276.

BONHAUS, D.W., CHANG, L.K., KWAN, J. & MARTIN, G.R. (1998). Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J. Pharmacol. Exp. Ther.,* **287,** 884 – 888.

BRINK, C.B., WADE, S.M. & NEUBIG, R.R. (2000). Agonist-directed trafficking of porcine alpha(2A)-adrenergic receptor signaling in Chinese hamster ovary cells: l-isoproterenol selectively activates G(s). *J. Pharmacol. Exp. Ther.,* **294,** 539 – 547.

BRISTOW, M.R., HERSHBERGER, R.E., PORT, J.D., MINOBE, W. & RASMUSSEN, R. (1989). Beta 1- and beta 2-adrenergic receptor-mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium. *Mol. Pharmacol.,* **35,** 295 – 303.

DAAKA, Y., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature,* **390,** 88 – 91.

DOOLEY, D.J., BITTIGER, H. & REYMANN, N.C. (1986). CGP 20712 A: a useful tool for quantitating beta 1- and beta 2-adrenoceptors. *Eur. J. Pharmacol.,* **130,** 137 – 139.

GONG, H., ADAMSON, D.L., RANU, H.K., KOCH, W.J., HEUBACH, J.F., RAVENS, U., ZOLK, O. & HARDING, S.E. (2000). The effect of Gi-protein inactivation on basal, and beta(1)- and beta(2)-AR-stimulated contraction of myocytes from transgenic mice overexpressing the beta(2)-adrenoceptor. *Br. J. Pharmacol.,* **131,** 594 – 600.

GONG, H., SUN, H., KOCH, W.J., RAU, T., ESCHENHAGEN, T., RAVENS, U., HEUBACH, J.F., ADAMSON, D.L. & HARDING, S.E. (2002). Specific beta(2)AR blocker ICI 118,551 actively decreases contraction through a G(i)-coupled form of the beta(2)AR in myocytes from failing human heart. *Circulation,* **105,** 2497 – 2503.

GROSS-BELLARD, M., OUDET, P. & CHAMBON, P. (1973). Isolation of high-molecular-weight DNA from mammalian cells. *Eur. J. Biochem.,* **36,** 32 – 38.

HASSELDINE, A.R.G., HARPER, E.A. & BLACK, J.W. (2001). Beta adrenoceptor function in transgenic mice with cardiac-specific overexpression of the beta 2 adrenoceptor [Abstract]. *Br. J. Pharmacol.,* **134,** 20P.

HENRY, P.J. & GOLDIE, R.G. (1990). Beta 1-adrenoceptors mediate smooth muscle relaxation in mouse isolated trachea. *Br. J. Pharmacol.,* **99,** 131 – 135.

HOPKINSON, H.E., LATIF, M.L. & HILL, S.J. (2000). Non-competitive antagonism of beta(2)-agonist-mediated cyclic AMP accumulation by ICI 118551 in BC3H1 cells endogenously expressing constitutively active beta(2)-adrenoceptors. *Br. J. Pharmacol.,* **131,** 124 – 130.

KENAKIN, T. (1995). Agonist-receptor efficacy. I: mechanisms of efficacy and receptor promiscuity. *Trends Pharmacol. Sci.,* **16,** 188 – 192.

KUSCHEL, M., ZHOU, Y.Y., CHENG, H., ZHANG, S.J., CHEN, Y., LAKATTA, E.G. & XIAO, R.P. (1999). G(i) protein-mediated functional compartmentalization of cardiac beta(2)-adrenergic signaling. *J. Biol. Chem.,* **274,** 22048 – 22052.

LEFF, P. (1995). The two-state model of receptor activation. *Trends Pharmacol. Sci.,* **16,** 89 – 97.

LOHSE, M.J., BENOVIC, J.L., CARON, M.G. & LEFKOWITZ, R.J. (1990). Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J. Biol. Chem.,* **265,** 3202 – 3211.

LU, S. & HOEY, A. (2000). Changes in function of cardiac receptors mediating the effects of the autonomic nervous system in the muscular dystrophy (MDX) mouse. *J. Mol. Cell Cardiol.,* **32,** 143 – 152.

LUO, X., ZENG, W., XU, X., POPOV, S., DAVIGNON, I., WILKIE, T.M., MUMBY, S.M. & MUALLEM, S. (1999). Alternate coupling of receptors to Gs and Gi in pancreatic and submandibular gland cells. *J. Biol. Chem.,* **274,** 17684 – 17690.

MILANO, C.A., ALLEN, L.F., ROCKMAN, H.A., DOLBER, P.C., MCMINN, T.R., CHIEN, K.R., JOHNSON, T.D., BOND, R.A. & LEFKOWITZ, R.J. (1994). Enhanced 21 myocardial function in transgenic mice overexpressing the beta 2-adrenergic receptor. *Science,* **264,** 582 – 586.

PRATHER, P.L., MCGINN, T.M., ERICKSON, L.J., EVANS, C.J., LOH, H.H. & LAW, P.Y. (1994). Ability of delta-opioid receptors to interact with multiple G-proteins is independent of receptor density. *J. Biol. Chem.,* **269,** 21293 – 21302.

PRENDERGAST, C.E., SHANKLEY, N.P. & BLACK, J.W. (2000). Negative inotropic effects of isoprenaline on isolated left atrial assays from aged transgenic mice with cardiac overexpression of human beta-2 adrenoceptors. *Br. J. Pharmacol.,* **129,** 1285 – 1288.

WELLNER-KIENITZ, M.C., BENDER, K. & POTT, L. (2001). Overexpression of beta 1 and beta 2 adrenergic receptors in rat atrial myocytes. Differential coupling to G protein-gated inward rectifier K(+) channels via G(s) and G(i)/o. *J. Biol. Chem.,* **276,** 37347 – 37354.

WOLFER, D.P. & LIPP, H.P. (2000). Dissecting the behaviour of transgenic mice: is it the mutation, the genetic background, or the environment? *Exp. Physiol.,* **85**, 627 – 634.

XIAO, R.P. (2000). Cell logic for dual coupling of a single class of receptors to G(s) and G(i) proteins. *Circ. Res.*, **87,** 635 – 637.

XIAO, R.P., AVDONIN, P., ZHOU, Y.Y., CHENG, H., AKHTER, S.A., ESCHENHAGEN, T., LEFKOWITZ, R.J., KOCH, W.J. & LAKATTA, E.G. (1999). Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes. *Circ. Res.,* **84,** 43 – 52.

XIAO, R.P., JI, X. & LAKATTA, E.G. (1995). Functional coupling of the beta 2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol. Pharmacol.,* **47,** 322 – 329.

ZHOU, Y.Y., CHENG, H., BOGDANOV, K.Y., HOHL, C., ALTSCHULD, R., LAKATTA, E.G., XIAO & R.P. (1997). Localized cAMP-dependent signaling mediates beta 2-adrenergic modulation of cardiac excitation – contraction coupling. *Am. J. Physiol.,* **273,** H1611 – H1618.

Appendix : Simulation for Figure 6

The simulations were performed using Microsoft Excel 97. For comparison of 'traditional' and inverse agonism, the same equations were used (two-state model) but with constitutive activity set at zero for the former

$$Stimulus = \frac{[R]_0(K_A\alpha[A])}{K_A\alpha(1+L)+(1+L\alpha)[A]}$$

$$Effect = \frac{Stimulus \times E_{MAX}}{Stimulus+\beta}$$

$$K_{APP} = \frac{K_A\alpha(1+L)}{\alpha(1+L)}$$

where $[R]_0$ is the receptor density, [A] is the concentration of the agonist or inverse agonist, K_A is the affinity of A for the receptor in its inactive state, α is the factor by which A is selective for the inactive over the active state and L is the equilibrium constant governing the isomerisation of unliganded receptor. The second equation is a simple sigmoid function, where the conversion of stimulus to effect is governed by the maximum effect parameter, E_{MAX}, and the equilibrium constant, β.

For both simulations, $[R]_0$ was set between 0.1 and 1000, at log unit intervals; K_A was 10^{-7} M; E_{MAX} was 100; and β was 0.2.

For modelling 'traditional' agonism, α was set at 4×10^{-6} and L at 10^6. When modelling inverse agonism, these were both set at 100. The pK_{APP} values are therefore 7.1 and 7.0, respectively.

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