Epinephrine Activates Both Gs and Gi Pathways, but Norepinephrine Activates Only the Gs Pathway through Human \( \beta_2 \)-Adrenoceptors Overexpressed in Mouse Heart

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ABSTRACT

Isoproterenol increases and decreases contractile force at low and high concentrations, respectively, through \( \beta_2 \)-adrenoceptors overexpressed in transgenic mouse heart (TG4), consistent with activation of both Gs and Gi proteins. Using TG4 hearts, we demonstrated that epinephrine behaves like isoproterenol, but norepinephrine does not. Epinephrine both increased (−log EC50\(_{GW} \)= 9.4) and decreased (−log EC50\(_{GW} \)= 6.5) left atrial force. Pertussis toxin (PTX) abolished the negative inotropic effects of epinephrine, consistent with mediation through Gi protein. Norepinephrine only increased contractile force (−log EC50\(_{GW} \)= 7.5). Norepinephrine (10–100 \( \mu \)M) prevented the positive inotropic effects but hardly affected the negative inotropic effects of epinephrine. Cardiodepressive epinephrine concentrations (1–10 \( \mu \)M) antagonized the positive inotropic effects of norepinephrine. In the free wall of TG4 right ventricle, norepinephrine and low epinephrine concentrations caused positive inotropic effects, and high epinephrine concentrations caused PTX-sensitive negative inotropic effects, as observed in the left atrium. Epinephrine (10 nM), a concentration causing maximum increase in contractile force, and norepinephrine (1 and 100 \( \mu \)M) increased cAMP-dependent protein kinase activity in TG4 left ventricle. Cardiodepressive concentrations of epinephrine (1 and 100 \( \mu \)M) did not increase cAMP-dependent protein kinase activity. The inotropic results were simulated with a model of two \( \beta_2 \)-adrenoceptor sites. For one site involved in receptor coupling to Gs, both epinephrine and norepinephrine compete. The other site, recognized by epinephrine but not by norepinephrine, leads to receptor Gi coupling.

\( \beta_2 \)-Adrenoceptors participate with \( \beta_1 \)-adrenoceptors in the mediation of cardiomodulating effects of epinephrine in human atrium (Gille et al., 1985; Hall et al., 1990; Kaumann et al., 1999), ventricle (Kaumann and Lemoine 1987; Gille et al., 1985; Bristow et al., 1989; Kaumann et al., 1999; Del Monte et al., 1993; Molenaar et al., 2000), and sinoatrial node (Daul et al., 1995). Norepinephrine can also cause cardiomodulation through human \( \beta_2 \)-adrenoceptors (Kaumann and Lemoine, 1987; Hall et al., 1990). Human \( \beta_2 \)-adrenoceptors, overexpressed ~200-fold in murine heart (TG4), have been reported to constitutively couple to Gs protein (Milano et al., 1994; Bond et al., 1995) but not to Gi protein (Gürdal et al., 1997; Xiao et al., 1999) in the absence of agonist, thereby eliciting continuous cardiomodulation. In addition to coupling to Gs protein, \( \beta_2 \)-adrenoceptors, activated by isoproterenol, can also couple to Gi protein (recombinant receptors: Daaka et al., 1997; murine heart: Xiao et al., 1999; human heart: Kilts et al., 2000), but the relevance to human heart function is not clear. In a mouse phenotype descendent from the original TG4 mouse described by Milano et al. (1994), we (Heubach et al., 2003) and others (Hasseldine et al., 2003) have shown that isoproterenol both increases and decreases contractility through overexpressed \( \beta_2 \)-adrenoceptors in the left atrium. The cardiodepressant effects of (−)-isoproterenol were abolished by pretreatment with pertussis toxin, consistent with mediation through \( \beta_2 \)-adrenoceptors coupled to Gi protein (Hasseldine et al., 2003; Heubach et al., 2003).

We compared in this TG4 phenotype (Heubach et al., 2003) the effects of the physiological catecholamines norepinephrine and epinephrine on atrial and ventricular contractility. Daaka et al. (1997) proposed that isoproterenol-evoked coupling to Gs protein of the \( \beta_2 \)-adrenoceptor induces PKA-catalyzed phosphorylation of the receptor, which in turn couples to Gi. This switch of Gs to Gi coupling of \( \beta_2 \)-adrenoceptor.

**ABBREVIATIONS:** TG4, transgenic mouse with cardiac overexpression of the human \( \beta_2 \)-adrenoceptor; CGP20712A, 2-hydroxy-5-[2-(2-hydroxy-3-[4-[(methyl-4-trifluoromethyl)-1H-imidazole-2-yl]-phenoxy[propyl]-amino]ethoxy)]-benzamide monomethane sulfonate; PTX, pertussis toxin; PKA, cAMP-dependent protein kinase; ICI 118,551, (−)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[1-methylethyl]amino]-2-butanol; NE, norepinephrine; E, epinephrine.
(Daaka et al., 1997) provides one plausible biochemical explanation for the positive (G\textsubscript{s} coupling) and negative (G\textsubscript{i} coupling) inotropic effects of isoproterenol (Hasseldine et al., 2003; Heubach et al., 2003). To further test the hypothesis of the PKA-induced switch of G protein, we compared the ventricular PKA activity before and during the administration of epinephrine and norepinephrine.

Both catecholamines caused cardiostimulation, but only epinephrine also elicited cardiodepression. The positive inotropic effects of epinephrine and norepinephrine were accompanied by an increase of ventricular PKA activity. The decline of force at high epinephrine concentrations was associated with a decrease of PKA activity. The inotropic results were simulated with a model for two β\textsubscript{2}-adrenoceptor sites. For the site that couples to G\textsubscript{s}, norepinephrine and epinephrine compete. The other site is only recognized by epinephrine, but not by norepinephrine, and leads to coupling to G\textsubscript{i}.

Materials and Methods

Materials. [γ\textsuperscript{32}P]ATP was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). ICI 118,551 was from Toecris Cookson Inc. (Bristol, UK). CGP20712A was from Novartis (Basel, Switzerland). Pertussis toxin, prazosin, yohimbine, forskolin, (−)-epinephrine hydrochloride, and (−)-norepinephrine hydrochloride were from Sigma Chemie (Deisenhofen, Germany). Phenoxbenzamine was from Röhm Pharma (Darmstadt, Germany).

Transgenic Mice. The experiments and the use of pertussis toxin were approved by the German Home Office (Az 75-9168.11-1-2000-10). Transgenic mice were descendents from the TG4 mice at Duke University (Durham, NC) that overexpress human β\textsubscript{2}-adrenoceptors in a heart-specific manner (Milano et al., 1994). Originally, β\textsubscript{2}-adrenoceptor density was increased 200-fold, and the overexpression was verified more recently for our TG4 colony (260- to 435-fold overexpression) (Heubach et al., 1999, 2001; Graf et al., 2001). TG4 mice were propagated in Dresden by breeding TG4 female mice with wild-type C57BL6 male mice until generation F\textsubscript{2}. Genotypes were determined as described previously (Heubach et al., 2001).

Isolated Cardiac Tissues. Mice of either gender were killed by dislocation of the neck, and the hearts were dissected and placed in oxygenated, modified Tyrode's solution at room temperature containing 126.7 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.05 mM MgCl\textsubscript{2}, 22.0 mM NaHCO\textsubscript{3}, 0.42 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.04 mM EDTA, 0.2 mM ascorbic acid, and 5.0 mM glucose. The solution was maintained at pH 7.4 by bubbling a mixture of 5% CO\textsubscript{2} and 95% O\textsubscript{2}. Left atrium and the free wall of the right ventricle were rapidly dissected and mounted in pairs, attached to Swema 4–45 strain gauge transducers in an apparatus (Blinks, 1965) containing the above solution at 37°C, paced at 2 Hz, and stretched as described previously (Oostendorp and Kaumann, 2000; Heubach et al., 2002). Usually four thin left ventricular strips from each heart were also dissected for PKA assays. Contractile force was recorded through PowerLab amplifiers on a Chart for Windows, Version 4.0, recording program (ADInstruments Pty Ltd., Castle Hill, Australia).

All tissues, including left ventricular strips floating freely in the organ bath, were exposed to phenoxbenzamine (6 μM) for 90 min followed by washout to irreversibly block α-adrenergic receptors and both neuronal and extraneuronal uptake of catecholamines (Gille et al., 1985). The experiments were carried out in the presence of CGP20712A (300 nM) to selectively block β\textsubscript{2}-adrenoceptors (Heubach et al., 2002, 2003). We have previously shown in TG4 left atrium that the positive inotropic effects of norepinephrine are resistant to blockade by 300 nM CGP20712A and therefore are exclusively mediated through β\textsubscript{2}-adrenoceptors (Heubach et al., 2003).

In Vivo PTX Treatment. Mice were injected with PTX (600 μg/kg i.p.) or 0.9% NaCl. Twenty-four hours later, left atrial and right ventricular free-walls from PTX-treated mice were set up into the same organ bath. In wild-type C57BL6 mice, this procedure inhibited by 82% in vitro ADP-ribosylation of ventricles as measured by [γ\textsuperscript{32}P]ADP-ribose incorporation (Heubach et al., 2002).

PKA Assay. The PKA activity ratio was assayed in frozen strips of left ventricle as described previously (Kaumann et al., 1989; Murray et al., 1990). Thin left ventricular strips, weighing ~15 mg, were placed into modified Tyrode's solution. The tissues were quickly frozen in liquid nitrogen for PKA assays in control tissues and tissues 10 min after cumulative exposure to the last catecholamine concentration or to forskolin. The tissues were processed with a Polytron homogenizer (Kinematica, Basel, Switzerland) (7-mm probe at speed setting 8 for 10 s) in 40 volumes of ice-cold buffer, pH 6.8, containing 10 mM sodium phosphate, 10 mM EDTA, and 0.5 mM 3-isobutyl-1-methyloxanthine and centrifuged at 4°C for 5 min at 6000g. PKA was determined by incubating 10 μl of resultant supernatant for 2 min at 30°C with 10 μl of [γ\textsuperscript{32}P]ATP and 50 μl of assay buffer with final concentrations of 20 μM maltamide, 0.3 mM [γ\textsuperscript{32}P]ATP, 50 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM MgCl\textsubscript{2}, 1.0 mM EGTA, and 0.010% Tween 20 (w/w) and in the absence or presence of 2 μM cAMP. The reaction was terminated with 10 μl of 1 M HCl, after which 35 μl of sample was spotted onto phosphocellulose (P81) papers. These papers were washed six times for 2 min with 0.05% (w/v) tetraphosphoric acid/38 mM H\textsubscript{2}PO\textsubscript{4}, and then they were dried and counted in water by Cerenkov radiation. The activity ratio was calculated by dividing the radioactivity (counts per minute) obtained in the absence of cAMP by that obtained in the presence of cAMP after subtracting blank values (HCl added before [γ\textsuperscript{32}P]ATP). Samples in the presence and absence of 2 μM cAMP were assayed in duplicate and assays replicated in 3 to 10 tissues.

Simulations. It is assumed that the β\textsubscript{2}-adrenoceptor has one binding site for norepinephrine (NE) and two independent binding sites for epinephrine (E). The affinity of the binding sites for epinephrine differs by three orders of magnitude so that the high-affinity sites will be fully saturated when the low-affinity sites begin to form complexes with epinephrine. Binding to the high-affinity site is assumed to trigger coupling to G\textsubscript{s} protein, whereas binding to the low-affinity site stimulates coupling to G\textsubscript{i} protein. Because G\textsubscript{i} coupling stimulates whereas G\textsubscript{s} coupling inhibits force of contraction, this model can account for the inhibition of the receptor configuration that stimulates G\textsubscript{i} protein. Scheme 1 represents this model by sequential binding of epinephrine because binding to the low-affinity site can only occur to a significant extent once the high-affinity site is occupied.
is fully saturated. As shown in Scheme 1, the R-NE and R-E complexes couple to Gs and the R-E/E complexes couple to Gs, with $K_{NE}, K_E$, and $K_{EE}$ as the corresponding equilibrium dissociation constants and $K_{NE} \gg K_E$. NE and E are concentrations of norepinephrine and epinephrine. The total receptor population $R = R + R-NE + R-E + R-EE$. The relative responses of norepinephrine ($r_{NE}$) and epinephrine ($r_E$) are

$$r_{NE} = \frac{e_{NE} \times R-NE/R}{e_{NE} \times NE/[NE + K_{NE}(1 + E/K_E + E/K_{EE}) \times K_E]}$$

(1)

and

$$r_E = \frac{e_E \times R/E/R}{e_E \times E/[E + K_E(1 + NE/K_{NE} + E/K_{EE} \times K_E)]}$$

(2)

in which $e_{NE}$ and $e_E$ are relative maximum effects: $e_{NE} = 1$ in both left atrium and right ventricle, whereas $e_E = 1.4$ in left atrium and $e_E = 1.25$ in right ventricle. The relative effects of combinations of norepinephrine and epinephrine are given by

$$r_{NE} + r_E = (e_{NE} \times NE + e_E \times E)/R_1$$

(3)

Data Analysis. Concentration-effect curves for the catecholamines were cumulative. $-\log EC_{50M}$ and $-\log IC_{50M}$ values for catecholamines were estimated from fitting a Hill function with variable slopes to concentration-effect curves from individual experiments. The data are expressed as mean ± S.E.M. of n = number of mice. Significance of differences between means were assessed with the use of either Student’s $t$ test or analysis of variance followed by Bonferroni or Dunnett P test or analysis of variance followed by Bonferroni or Dunnett’s post hoc test at $P < 0.05$ using Instat software (GraphPad Software Inc., San Diego, CA). Simulations were calculated with Sigma Plot software version 4 (SPSS Inc., Chicago, IL).

Results

Left Atrium. Epinephrine increased left atrial contractile force at low concentrations. $-\log EC_{50M}$ was $9.40 \pm 0.06$ for maximal effects, $9.56 \pm 0.06$ for steady-state effects, $n = 9$, N.S.) (Fig. 1, A and B). At high concentrations, epinephrine decreased force $-\log IC_{50M}$ was $6.45 \pm 0.04$ for maximal relaxations, $n = 5$), an effect prevented by PTX (Fig. 1C). The maximum negative inotropic effect was observed at 10 μM epinephrine and was followed by a slow increase of force. Norepinephrine only increased contractile force $-\log EC_{50M}$ was $7.53 \pm 0.09$ for maximal effects, $7.44 \pm 0.08$ for steady-state effects, $n = 6$, N.S.) (Fig. 1B). The effects of norepinephrine and epinephrine were simulated on Fig. 1D. The effects of PTX on the epinephrine responses were simulated in Fig. 1E.

Norepinephrine (10–100 μM) prevented the increases in left atrial contractile force by epinephrine and slightly reduced the cardio depressant potency of epinephrine, as shown in Fig. 2, A to C, and simulated in Fig. 2D. The $-\log IC_{50M}$ values for the relaxant effects of epinephrine were $6.45 \pm 0.04$ ($n = 5$), $6.28 \pm 0.25$ ($n = 3, P = 0.4$) and $5.96 \pm 0.04$ ($n = 3, P = 0.05$) in the absence and presence of 10 and 100 μM norepinephrine, respectively.

Force-reducing concentrations of epinephrine (1 and 10 μM) antagonized the positive inotropic effects of norepinephrine in surmountable manner (Fig. 3, A–C). The $-\log EC_{50M}$ values for the steady-state effects of norepinephrine were $7.49 \pm 0.08$ ($n = 6$), $4.37 \pm 0.10$ ($n = 3, P < 0.001$), and $3.46 \pm 0.25$ ($n = 3, P < 0.001$) in the absence and presence of 1 and 10 μM epinephrine, respectively. The blockade of the norepinephrine effects by epinephrine was simulated in Fig. 3D.

The noncumulative administration of 10 μM epinephrine elicited positive inotropic responses (Fig. 4, B and C) that were smaller than the response to norepinephrine (100 μM, Fig. 4A). The responses tended to be biphasic (Fig. 4, B and C), with an initial fast component followed by a brief plateau and a slow component leading to a steady-state increase of $34 \pm 4\%$ of forskolin ($n = 3$). This biphasic pattern was not modified by additional treatment with the combination of $\alpha_1$-selective prazosin (1 μM) plus $\alpha_1$-selective yohimbine (1 μM) (Fig. 4D), leading to a steady-state increase of $36 \pm 14\%$ of forskolin ($n = 3$). The steady-state positive inotropic effect observed with the noncumulative administration did not differ from the steady-state positive inotropic effect of 10 μM epinephrine added cumulatively ($30 \pm 4\%$ of forskolin). Thus, the same conclusions can be drawn from cumulative and noncumulative effects of 10 μM epinephrine on the left atrium.

The maximum contractile force, observed under forskolin (3 μM), administered to terminate the experiments was not significantly different in the experimental groups shown in Figs. 1 to 4.

Right Ventricle. The catecholamines tended to produce arrhythmias on the free wall of the right ventricle. Representative recordings from nonarrhythmic tissues are illustrated in Fig. 5. As observed in left atrium, both norepinephrine (Fig. 5A) and epinephrine (Fig. 5, B–D) produced positive inotropic effects, but only epinephrine elicited negative inotropic effects (Fig. 5, C and D). PTX prevented the negative inotropic effect of epinephrine (Fig. 5B). Also as observed in the left atrium, epinephrine antagonized the positive inotropic effects of noradrenaline (Fig. 5, C and D). Quantitative data and simulations from a limited number of arrhythmia-free ventricles are shown in Fig. 6, A and B. The $-\log EC_{50M}$ values for the positive inotropic effects of epinephrine were $9.04 \pm 0.07$ ($n = 4$) and $9.36 \pm 0.25$ ($n = 3, P = 0.2$) in ventricular preparations from PTX-untrreated and PTX-treated mice. The $-\log IC_{50M}$ for the relaxant effects of epinephrine was $6.88 \pm 0.09$. The $-\log EC_{50M}$ values for the positive inotropic effects of norepinephrine in two ventricular preparations were 6.77 and 6.89.

PKA Activation. A low concentration of epinephrine (10 nM) and micromolar concentrations of norepinephrine (1 and 100 μM) increased the left ventricular PKA activity ratio (Table 1). However, micromolar concentrations of epinephrine (1 and 100 μM) failed to increase the PKA activity ratio (Table 1). The increases of PKA activity ratio by epinephrine (10 nM) and norepinephrine (1–100 μM) amounted to 25% and 38–30%, respectively, of the increase in PKA activity ratio caused by forskolin (3 μM) (Table 1).

Discussion

Our experiments demonstrate that the physiological agonists epinephrine and norepinephrine act differently through human $\beta_2$-adrenoceptors overexpressed in mouse heart. The results and simulations are consistent with a model of interaction of norepinephrine and epinephrine with and competition for the $\beta_2$-adrenoceptor coupled to Gs protein. In addition, high concentrations of epinephrine but not of norepinephrine induce coupling of the $\beta_2$-adrenoceptors to Gs protein. The mode of action of epinephrine resembles that of isoproterenol, which also has cardio stimulant and depres-
sant effects in the TG4 phenotype used in this work (Heubach et al., 2003).

It has been proposed that PKA-catalyzed phosphorylation of the β2-adrenoceptor induces a switch to G<sub>i</sub> coupling from G<sub>s</sub> coupling (Daaka et al., 1997), and our results with epinephrine, as well as previous results with isoproterenol (Hasseldine et al., 2003; Heubach et al., 2003), are apparently in line with this hypothesis. However, our norepinephrine data

Fig. 1. Effects of norepinephrine and epinephrine on TG4 left atrium and influence of PTX. A, representative experiments. B, comparison of the effects of epinephrine and norepinephrine. Maximum positive inotropic and negative inotropic effects at each agonist concentration were assessed for the construction of the concentration-effect curves. C, PTX-treatment of the mice abolished the negative inotropic effects of epinephrine. D, simulation of the effects of epinephrine and norepinephrine using, eqs. 1 and 2. E, simulation of the effects of epinephrine with and without PTX treatment, using eq. 2.
are inconsistent with the hypothesis of the PKA-evoked switch because increases in PKA activity and contractile force were observed in a 100-fold concentration range (1–100 μM), but the high norepinephrine concentration did not decrease PKA activity and contractile force. The increases in PKA activity by 10 nM epinephrine and 1 μM norepinephrine, concentrations causing maximum increases in contractile force, were of similar magnitude. As expected from the $G_s \rightarrow G_i$ switch hypothesis, increasing the concentration of epinephrine 100-fold to 1 μM and even 10,000-fold to 100 μM reduced the PKA activity from its higher level produced by low epinephrine concentrations, consistent with uncoupling from $G_s$ protein and coupling to $G_i$ protein. On the contrary, when the norepinephrine concentration was increased 100-fold to 100 μM, the PKA stimulation persisted, suggesting persistent coupling to $G_s$ without a switch to $G_i$ coupling. Our results therefore suggest that PKA activation through the $\beta_2$-adrenoceptor and the subsequent shift of coupling from $G_s$ to $G_i$ is agonist-dependent: Norepinephrine (1–100 μM) stabilizes a $G_s$-coupled receptor configuration, which is consistently observed with both contractile force and PKA stimulation. Unlike norepinephrine, however, high epinephrine concentrations (1–100 μM) reduced both previously elevated PKA activity and contractility, probably through coupling of the $\beta_2$-adrenoceptor to $G_i$ protein. The negative inotropic effect of epinephrine was prevented by PTX pretreatment, consistent with mediation through the $G_i$-coupled $\beta_2$-adrenoceptor.

Agonist-dependent coupling of a receptor to more than one G protein has been observed previously with other receptor systems (Kenakin, 1995b). Interestingly, epinephrine exhibits a ~200-fold greater potency for activating the $G_i$ pathway than activating the $G_s$ pathway at recombinant $\alpha_{2C(10)}$-adrenoceptors, whereas the imidazoline agonist oxymetazoline only stimulates the $G_i$ pathway (Eason et al., 1994). Furthermore, during submission of this work, an article supporting
Fig. 3. Antagonism by epinephrine of the positive inotropic effects of norepinephrine on TG4 left atria. A and B are representative experiments. A concentration effect curve to epinephrine up to 1 μM (A) and 10 μM (B) was carried out, followed by a curve for norepinephrine. C, curves for steady-state positive inotropic effects of norepinephrine in the absence and presence of the indicated epinephrine concentrations. D, simulation of the experiments shown in A, B, and C using eqs. 1 and 3.

Fig. 4. Kinetics after noncumulative administration of a single high epinephrine concentration (B-D). Comparison of norepinephrine (A) and epinephrine (B). Shown are representative left atria. The effects of 10 μM epinephrine on force of contraction were similar in the absence (C) and presence (D) of the additional α-adrenoceptor blockers prazosin (1 μM) and yohimbine (1 μM).
the concept of agonist-dependent selectivity for coupling of rat cardiac β2-adrenoceptors was published (Xiao et al., 2003). These authors showed that the positive inotropic effects of the β2-selective agonists salbutamol, zinterol, and procaterol, but not of fenoterol, are enhanced by PTX. Thus, fenoterol only activates the Gs pathway in rat heart expressing native β2-adrenoceptors, as found by us for the effects of norepinephrine mediated through β2-adrenoceptors overexpressed in mouse heart.

The increases and decreases in left atrial and right ventricular contractility caused by epinephrine are mirrored by increased and decreased PKA activity in left ventricle at low and high epinephrine concentrations, respectively, consistent with the Gs→Gi switch in the three cardiac regions. On the other hand, the inotropic and PKA data with norepinephrine are consistent with Gs coupling but not with Gi coupling in the three cardiac regions.

The maximum inotropic effects of norepinephrine tended to be smaller than those of epinephrine in both atrium and ventricle (i.e., eNE < eE). As expected from competition for binding of epinephrine to the Gi-coupled β2-adrenoceptor site, increasing concentrations of norepinephrine that partially activate the receptor through this site antagonized the cardiostimulant effects of epinephrine. This pattern resembles that of a classic partial agonist (norepinephrine) antagonizing the effects of a full agonist (epinephrine). As expected from a lack of interaction with the receptor site that would couple to Gi protein, norepinephrine hardly affected the cardiodepressive effects of epinephrine, which are mediated through this site. The small decrease in negative inotropic potency of epinephrine, observed under 100 μM norepinephrine, was anticipated by the model (simulation in Fig. 2D).

Under concentrations that depress contractile force, epinephrine becomes a competitive antagonist of the positive inotropic effects of norepinephrine. The experimental difference of –log EC50 values of norepinephrine in the absence and presence of 1 μM epinephrine (7.49–4.37 = 3.12 log units) is similar to the 3.4 log units theoretically expected using a dissociation equilibrium constant $K_E = 0.4$ nM for epinephrine as antagonist at the β2-adrenoceptor site coupled to Gs. The logCR = log(1 + [E]/$K_E$) = log(1 + 1000/0.4) = 3.4, where CR is the EC50 ratio of norepinephrine in the presence and absence of 1 μM epinephrine ([E]) (Fig. 3). Similarly, the observed EC50 ratio of norepinephrine under 10 μM epinephrine of 4 log units (7.49–3.46 = 4.03) was similar to the expected 4.4 log units. These quantitative

**Fig. 5.** Representative experiments of the effects of norepinephrine and epinephrine, isolated or in combination, on TG4 right ventricle. A, effects of norepinephrine. B, effects of epinephrine in a ventricle obtained from a PTX-treated TG4 mouse. Carb, carbachol. C, effects of epinephrine (up to 1 μM) and norepinephrine. D, effects of epinephrine (up to 10 μM) and norepinephrine.
agreements support the use of the atrial positive inotropic potency of epinephrine, EC50 \sim 0.4 \text{nM}, as K_E = 0.4 \text{nM} in the model.

To model our experiments, we assumed as a first approximation that our potency estimates (EC50 values) were equivalent to the K values (K_NE and K_E) used in the model. Our affinity estimates for norepinephrine, deduced from inotropic EC50 values in TG4 atrium (K_NE = 30 \text{nM}) and ventricle (K_NE = 150 \text{nM}) were similar to the dissociation equilibrium constant for norepinephrine (K_NE = 210 \text{nM}), estimated from inhibition of membrane binding of (\text{-}125\text{I})-cyanopindolol to human ventricular \beta_2\text{-adrenoceptors} (Kaumann et al., 1995). However, for epinephrine, the inotropic EC50 values in TG4 atrium (K_E = 0.4 \text{nM}) and TG4 ventricle (K_E = 0.9 \text{nM}) were lower than the corresponding dissociation equilibrium constant from binding inhibition (K_E = 15 \text{nM}) (Kaumann et al., 1995). The discrepancy between the binding K_E estimate from human ventricle and our EC50 for the positive inotropic effects of epinephrine in TG4 myocardium may be caused by the oversimplification of equating EC50 = K_E. However, for two reasons, our EC50 values, estimated functionally, seem to reflect a high-affinity state. First, our EC50 values for epinephrine and norepinephrine agree with the corresponding EC50 values (\sim -1 and \sim -100 \text{nM}, respectively) for cAMP accumulation induced by epinephrine and norepinephrine through recombinant \beta_2\text{-adrenoceptors} (Swaminath et al., 2004). Second, the stimulant potency (EC50) and blocking potency of epinephrine against norepinephrine on the left atrium were consistently subnanomolar, suggesting that epinephrine causes half-maximal \beta_2\text{-adrenoceptor} occupancy at the estimated K_E of 0.4 \text{nM}, consistent with the mass law assumptions of the model.

Although the simple mass law model yielded satisfactory simulations for several experimental conditions, it predicted nearly complete reversal of the positive inotropic effects of epinephrine 10 \mu\text{M} and abolishment at 100 \mu\text{M}, which was not observed (Figs. 1D and 3D). In the left atrium, the experimental maximum negative inotropic effect occurred at 10 \mu\text{M} epinephrine with a residual force equivalent to 19 to 25% of the forskolin response. This negative inotropic response was followed by a slow increase in force (Fig. 1). Furthermore, 100 \mu\text{M} epinephrine tended to increase the force of contraction with slow kinetics. The slow increase in contractile force was also observed after application of 10 \mu\text{M} epinephrine as a single concentration, and the effect was resistant to \alpha\text{-adrenoceptor} blockade by the combined treatment with phenoxybenzamine, prazosin, and yohimbine. The discrepancy between the prediction of the model and the experimental results could be attributed, at least in part, to the slow positive inotropic effects of epinephrine, of unknown nature, which became apparent at 10 and 100 \mu\text{M} and would partially oppose the predicted cardiodepression. However, it is also plausible that the G_i activation failed to oppose completely the G_s activation produced by epinephrine in the left atrium. In contrast to the left atrium, in ventricle, high epinephrine concentrations completely reversed the positive inotropic effects of low concentrations, as predicted by the model (Figs. 5D and 6, A and B).

The kinetics of the positive inotropic effects of 10 \mu\text{M} epinephrine, administered noncumulatively to the left atrium, were biphasic. As expected from the blunting effect of G_s stimulation, the positive inotropic response to epinephrine was considerably smaller than that of a maximally effective concentration of norepinephrine (Fig. 4). We interpret the fast initial component as residual effects mediated through G_s not completely opposed by G_i, and the late slow component as the unknown effect resistant to \alpha\text{-adrenoceptor} blockade. Consistent with this interpretation is that PTX treatment abolished the negative inotropic effect of epinephrine (Fig. 1).

The inotropic results from the right ventricle were quantitatively similar but not identical with those of the left atrium. The negative inotropic effects of epinephrine seemed more pronounced in the ventricles (Figs. 5 and 6) than in the left atrium (Fig. 1). The negative inotropic effects of epinephrine occurred at lower concentrations in ventricle than in atrium. The ratio between K_E and K_E in ventricle was only

Table 1

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<thead>
<tr>
<th>PKA Activity Ratios</th>
<th>n</th>
<th>p</th>
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<tr>
<td>Basal</td>
<td>0.364 ± 0.011</td>
<td>9</td>
</tr>
<tr>
<td>Epinephrine 10 nM</td>
<td>0.414 ± 0.019</td>
<td>6</td>
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<tr>
<td>Epinephrine 1 \muM</td>
<td>0.363 ± 0.019</td>
<td>6</td>
</tr>
<tr>
<td>Epinephrine 100 \muM</td>
<td>0.355 ± 0.030</td>
<td>3</td>
</tr>
<tr>
<td>Norepinephrine 1 \muM</td>
<td>0.433 ± 0.033</td>
<td>3</td>
</tr>
<tr>
<td>Norepinephrine 100 \muM</td>
<td>0.418 ± 0.020</td>
<td>9</td>
</tr>
<tr>
<td>Forskolin 3 \muM</td>
<td>0.545 ± 0.026</td>
<td>10</td>
</tr>
</tbody>
</table>
Norepinephrine Does Not Induce $\beta_2$-Adrenoceptor G$\alpha_i$ Coupling

147, but it was 1000 in the left atrium. G$_i$-mediated inhibition of contractile force by epinephrine would therefore be expected to oppose G$_s$-mediated increases in contractile force more in ventricle because the cardiodepression occurs at lower epinephrine concentrations than in atrium. Inactivation of G$_i$ with PTX would consequently be predicted to enhance further the positive inotropic effect of epinephrine in ventricle than in atrium, as observed in the simulation (compare Figs. 1E and 6B). However, because of large errors in basal force, the maximum positive inotropic effects of epinephrine were not significantly different in atria and ventricles from PTX-untreated and PTX-tREATED mice.

The experimental errors of the effects of the catecholamines were large. However, using the experimental parameters, the model was able to simulate several effects of the catecholamines, separately and in combination, despite the large experimental errors of the effects. This was possible because of the relatively small errors of the catecholamine concentrations causing half-maximal effects under various conditions. The simple mass law relations of the model agreed reasonably well with some interactions of catecholamines with the $\beta_2$-adrenoceptor, as reflected through the inotropic effects.

The coupling of the $\beta_2$-adrenoceptor to G$_i$ protein has been proposed to exert a cardioprotective role against G$_s$-protein-mediated cardiac overstimulation, especially in patients with heart failure who have high noradrenaline levels (Xiao, 2000). Stimulation of $\beta_1$- but not $\beta_2$-adrenoceptors produces apoptosis in rat heart (Communal et al., 1999), and the $\alpha_1$-adrenoceptor. Hence, they would not result in coupling to G$_i$ protein but would enhance Gi-coupled G$_s$-adrenoceptors in single myocytes from ventricles of PTX-untreated and PTX-treated mice.

From an extrapolation of our results, it would seem that norepinephrine does not induce coupling of human $\beta_2$-adrenoceptors to G$_i$ protein in TG4 ventricle, suggest a difference with murine $\beta_2$-adrenoceptors. The suggestion of Xiao (2000) was derived from data of Kilts et al. (2000), who used isoproterenol to demonstrate coupling of human atrial $\beta_2$-adrenoceptors to G$_i$ protein. However, from an extrapolation of our results, it would seem that sympathetic nerve stimulation is unlikely to cause G$_i$-mediated protection, because interaction of the physiological neurotransmitter norepinephrine with the human $\beta_2$-adrenoceptor would not result in coupling to G$_i$ protein but would actually enhance cardiostimulation through coupling to G$_s$ protein. Further work is necessary to understand the differences between murine and human $\beta_2$-adrenoceptors regarding G$_i$ protein coupling.

Simultaneous coupling of a receptor to more than one G-protein often becomes more evident at a high density of recombinant receptors (Eason et al., 1992; Kenakin, 1995a). The relevance of our finding with overexpressed human $\beta_2$-adrenoceptors into murine heart needs to be tested at native human cardiac $\beta_2$-adrenoceptors expressed at physiological density.

We conclude that coupling of the human $\beta_2$-adrenoceptor to G$_i$ protein is agonist-dependent. Epinephrine and isoproterenol, but not norepinephrine, interact at a binding site of the $\beta_2$-adrenoceptor that will lead to coupling to G$_i$ protein and which is different from the site that induces coupling to G$_s$ protein. The failure of norepinephrine to affect substantially the epinephrine-induced relaxation is consistent with a lack of recognition of the site which, when activated by epinephrine, leads to the G$_s$-coupled $\beta_2$-adrenoceptor.

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References

Graf EM, Heubach JF, and Ravens U (2001) Stimulation of $\beta_1$-adrenoceptors in murine heart seems to deliver antiapoptotic signals through G$_s$-dependent coupling to phosphatidylinositol 3’-kinase. The human $\beta_2$-adrenoceptor produces apoptosis in cardiac myocytes from the hearts of neonatal rats (Chesley et al., 2000). Our results, demonstrating that norepinephrine does not induce coupling of human $\beta_2$-adrenoceptors to G$_i$ protein in TG4 ventricle, suggest a difference with murine $\beta_2$-adrenoceptors. The suggestion of Xiao (2000) was derived from data of Kilts et al. (2000), who used isoproterenol to demonstrate coupling of human atrial $\beta_2$-adrenoceptors to G$_i$ protein. However, from an extrapolation of our results, it would seem that sympathetic nerve stimulation is unlikely to cause G$_i$-mediated protection, because interaction of the physiological neurotransmitter norepinephrine with the human $\beta_2$-adrenoceptor would not result in coupling to G$_i$ protein but would actually enhance cardiostimulation through coupling to G$_s$ protein. Further work is necessary to understand the differences between murine and human $\beta_2$-adrenoceptors regarding G$_i$ protein coupling.

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