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Epinephrine Activates Both G_s and G_i Pathways, but Norepinephrine Activates Only the G_s Pathway through Human β_2-Adrenoceptors Overexpressed in Mouse Heart

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ABSTRACT

Isoproterenol increases and decreases contractile force at low and high concentrations, respectively, through β_2-adrenoceptors overexpressed in transgenic mouse heart (TG4), consistent with activation of both G_s and G_i proteins. Using TG4 hearts, we demonstrated that epinephrine behaves like isoproterenol, but norepinephrine does not. Epinephrine both increased ($-\log EC_{50}M = 9.4$) and decreased ($-\log EC_{50}M = 6.5$) left atrial force. Pertussis toxin (PTX) abolished the negative inotropic effects of epinephrine, consistent with mediation through G_i protein. Norepinephrine only increased contractile force ($-\log EC_{50}M = 7.5$). Norepinephrine (10–100 μM) prevented the positive inotropic effects but hardly affected the negative inotropic effects of epinephrine. Cardiodepressive epinephrine concentrations (1–10 μM) 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 μM) increased cAMP-dependent protein kinase activity in TG4 left ventricle. Cardiodepressive concentrations of epinephrine (1 and 100 μM) did not increase cAMP-dependent protein kinase activity. The inotropic results were simulated with a model of two β_2-adrenoceptor sites. For one site involved in receptor coupling to G_s, both epinephrine and norepinephrine compete. The other site, recognized by epinephrine but not by norepinephrine, leads to receptor G_i coupling.

β_2-Adrenoceptors participate with β_1-adrenoceptors in the mediation of cardiostimulant 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 cardiostimulation through human β_2-adrenoceptors (Kaumann and Lemoine, 1987; Hall et al., 1990). Human β_2-adrenoceptors, overexpressed ~200-fold in murine heart (TG4), have been reported to constitutively couple to G_s protein (Milano et al., 1994; Bond et al., 1995) but not to G_i protein (Gürdal et al., 1997; Xiao et al., 1999) in the absence of agonist, thereby eliciting continuous cardiostimulation.

In addition to coupling to G_s protein, β_2-adrenoceptors, activated by isoproterenol, can also couple to G_i 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 β_2-adrenoceptors in the left atrium. The cardiodepressant effects of (−)-isoproterenol were abolished by pretreatment with pertussis toxin, consistent with mediation through β_2-adrenoceptors coupled to G_i 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 G_s protein of the β_2-adrenoceptor induces PKA-catalyzed phosphorylation of the receptor, which in turn couples to G_i. This switch of G_s to G_i coupling of the β_2-adrenoceptor

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ABBREVIATIONS: TG4, transgenic mouse with cardiac overexpression of the human β_2-adrenoceptor; CGP20712A, 2-hydroxy-5(2-((2-hydroxy-3-(4-((methyl-4-trifluomethyl)-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_s coupling) and negative (G_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_2-adrenoceptor sites. For the site that couples to G_s, norepinephrine and epinephrine compete. The other site is only recognized by epinephrine, but not by norepinephrine, and leads to coupling to G_i.

Materials and Methods

Materials. [γ-^{32}P]ATP was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). ICI 118,551 was from Tocris 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). Phenoxybenzamine 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_2-adrenoceptors in a heart-specific manner (Milano et al., 1994). Originally, β-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_7. Genotypes were determined as described previously (Heubach et al., 2001).

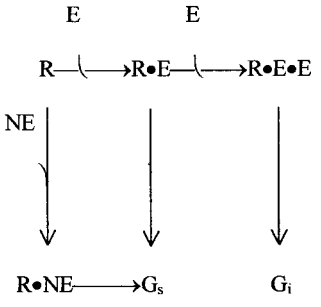
Isolated Cardiac Tissues. Mice of either gender were killed by dislocation of the neck, and the hearts were dissected and placed in oxygenated, modified Tyrode's solution at room temperature containing 126.7 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 22.0 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 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_2 and 95% O_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 baths, were exposed to phenoxybenzamine (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_1-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_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 [^{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-L-methylxanthine and centrifuged at 4°C for 5 min at 6000*g*. PKA was determined by incubating 10 μl of resultant supernatant for 2 min at 30°C with 10 μl of [γ-^{32}P]ATP and 50 μl of assay buffer with final concentrations of 20 μM malantide, 0.3 mM [γ-^{32}P]ATP, 50 mM Na_2HPO_4, 10 mM $MgCl_2$, 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_3PO_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-^{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_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_s protein, whereas binding to the low-affinity site stimulates coupling to G_i protein. Because G_s coupling stimulates whereas G_i coupling inhibits force of contraction, this model can account for the inhibition of the receptor configuration that stimulates G_s 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



$R + NE <=> R•NE$ $\quad K_{NE} = R \times NE/R•NE$

$R + E <=> R•E$ $\quad K_E = R \times E/R•E$

$R•E + E <=> R•E•E$ $\quad K_{EE} = R•E \times E/R•E•E$

Scheme 1. Model for reactions between norepinephrine and epinephrine with the β_2-adrenoceptor.

is fully saturated. As shown in Scheme 1, the R·NE and R·E complexes couple to G_s and the R·E·E complex couples to G_i, with K_{NE}, K_E, and K_{EE} as the corresponding equilibrium dissociation constants and $K_{EE} \gg K_E$. NE and E are concentrations of norepinephrine and epinephrine. The total receptor population $R_t = R + R{\cdot}NE + R{\cdot}E + R{\cdot}E{\cdot}E$. The relative responses of norepinephrine (r_{NE}) and epinephrine (r_E) are

$$r_{NE} = e_{NE} \times R{\cdot}NE/R_t = e_{NE} \times NE/[NE + K_{NE}(1 + E/K_E + E^2/K_{EE} \times K_E)] \quad (1)$$

and

$$r_E = e_E \times R{\cdot}E/R_t = e_E \times E/[E + K_E(1 + NE/K_{NE} + E^2/K_{EE} \times K_E)] \quad (2)$$

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

$$r_{NE} + r_E = (e_{NE} \times NE + e_E \times E)/R_t \quad (3)$$

Data Analysis. Concentration-effect curves for the catecholamines were cumulative. $-\text{Log } EC_{50}M$ and $-\log IC_{50}M$ 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'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. ($-\log EC_{50}M = 9.40 \pm 0.06$ for maximal effects, 9.56 ± 0.06 for steady-state effects, $n = 9$, N.S.) (Fig. 1, A and B). At high concentrations, epinephrine decreased force ($-\log IC_{50}M = 6.45 \pm 0.04$ 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 ($-\log EC_{50}M = 7.53 \pm 0.09$ for maximal effects, 7.44 ± 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 cardiodepressant potency of epinephrine, as shown in Fig. 2, A to C, and simulated in Fig. 2D. The $-\log IC_{50}M$ values for the relaxant effects of epinephrine were 6.45 ± 0.04 ($n = 5$), 6.28 ± 0.25 ($n = 3$, $P = 0.4$) and 5.96 ± 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 $-\log EC_{50}M$ values for the steady-state effects of norepinephrine were 7.49 ± 0.08 ($n = 6$), 4.37 ± 0.10 ($n = 3$, $P < 0.001$), and 3.46 ± 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 α_1-selective prazosin (1 μM) plus α_2-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 $-\log EC_{50}M$ values for the positive inotropic effects of epinephrine were 9.04 ± 0.07 ($n = 4$) and 9.36 ± 0.25 ($n = 3$, $P = 0.2$) in ventricular preparations from PTX-untreated and PTX-treated mice. The $-\log IC_{50}M$ for the relaxant effects of epinephrine was 6.88 ± 0.09. The $-\log EC_{50}M$ 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 28% 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 β_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 β_2-adrenoceptor coupled to G_s protein. In addition, high concentrations of epinephrine but not of norepinephrine induce coupling of the β_2-adrenoceptors to G_i protein. The mode of action of epinephrine resembles that of isoproterenol, which also has cardiostimulant 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 β_2-adrenoceptor induces a switch to G_i coupling from G_s 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

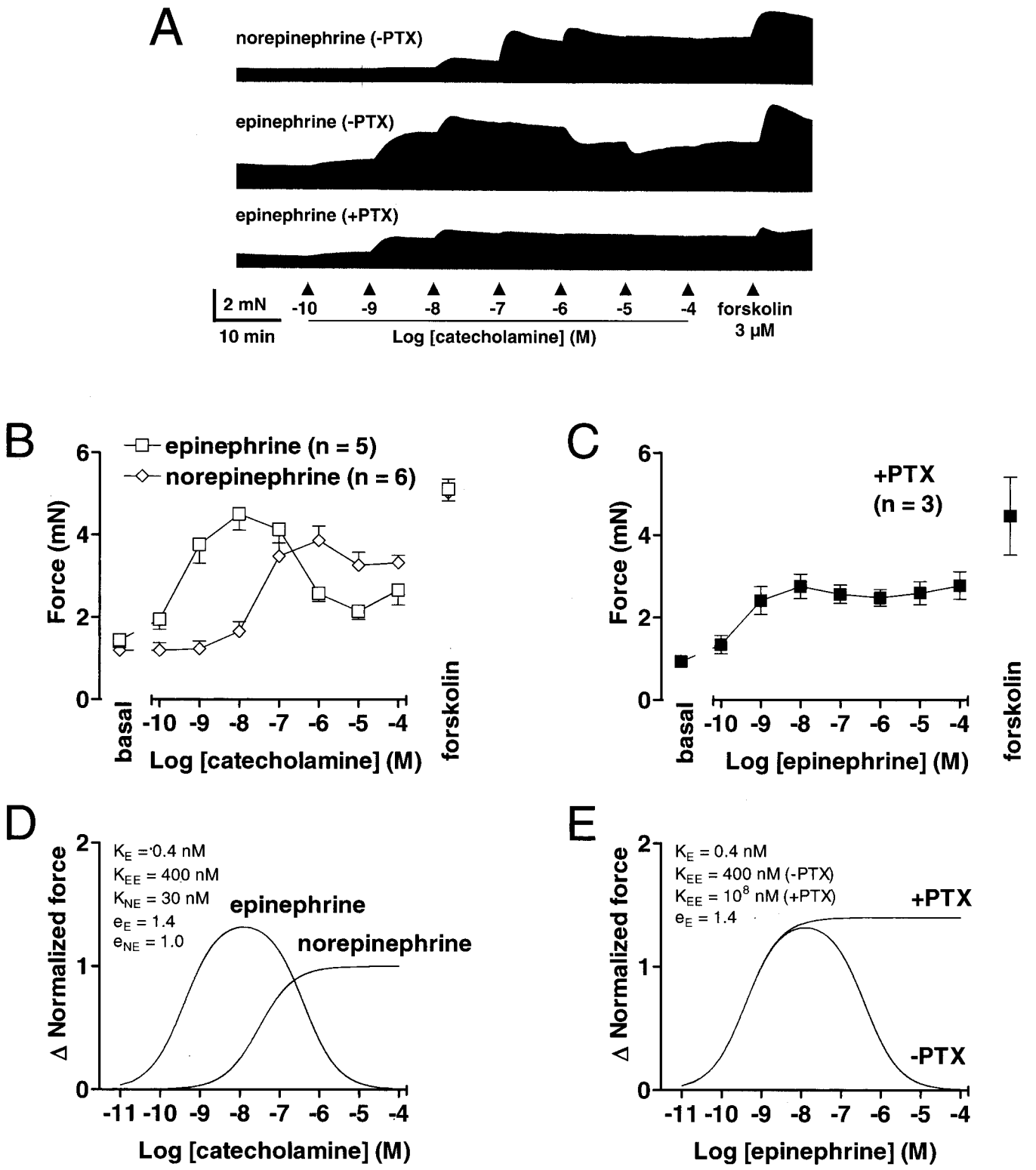


Fig. 1. Effects of norepinephrine and epinephrine on TG4 left atrium and influence of PTX. A, representative experiments. B, comparison of the effects of epinephrine and norepinephrine. Maximum positive inotropic and negative inotropic effects at each agonist concentration were assessed for the construction 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 β_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 β_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 β_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

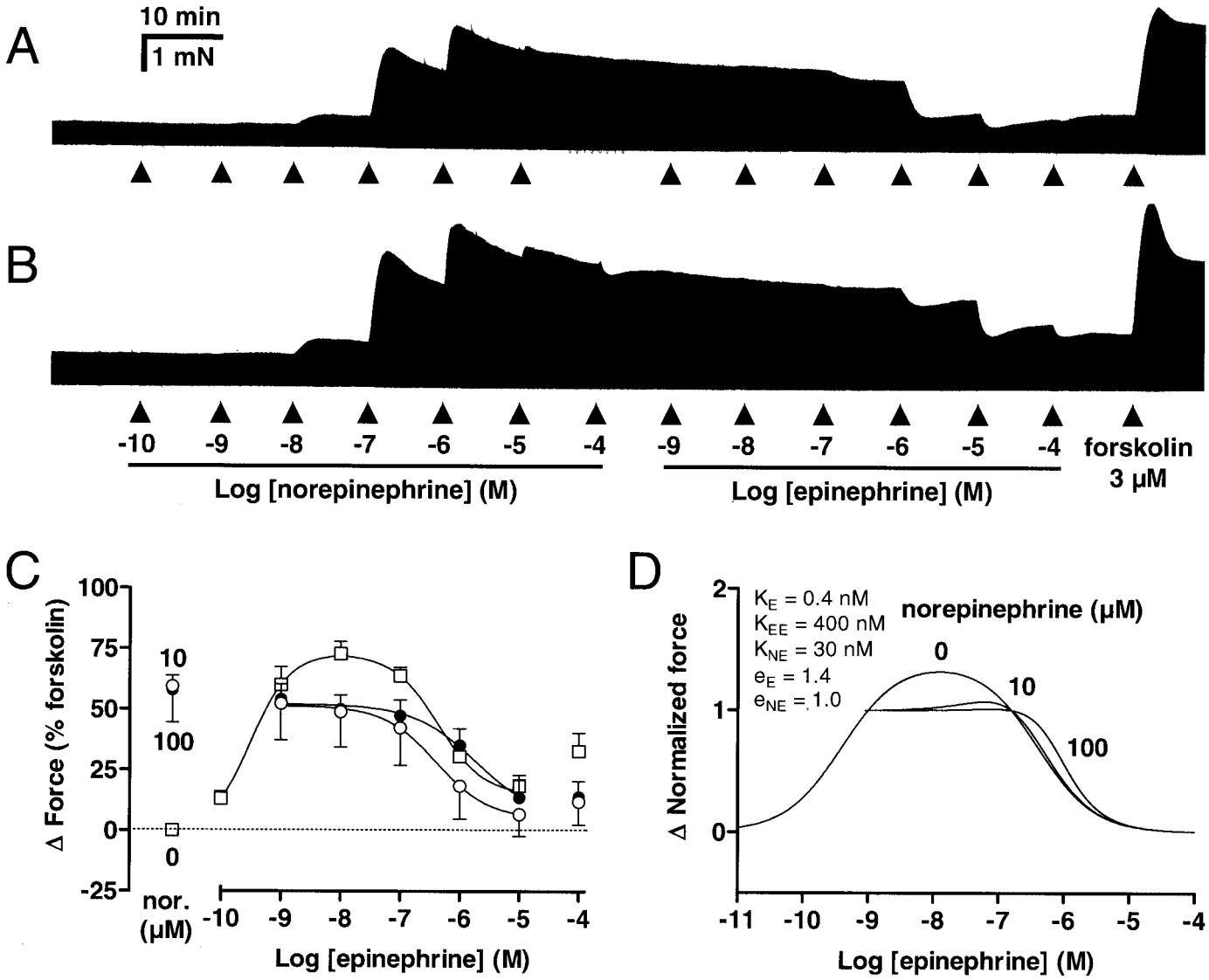
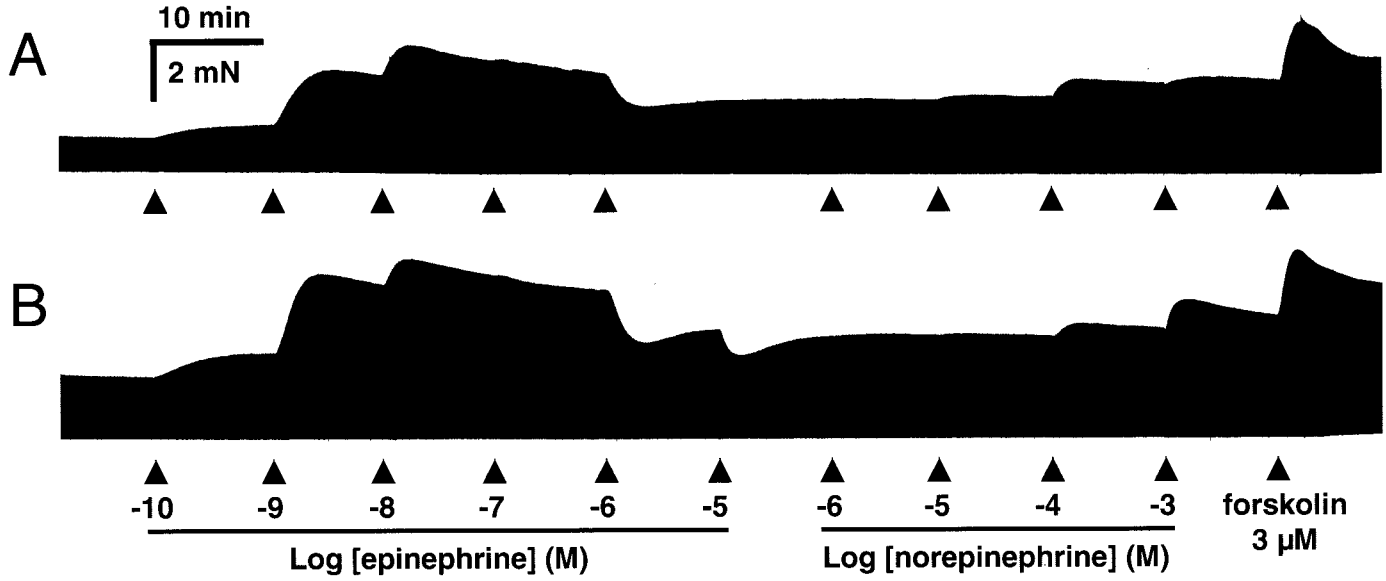


Fig. 2. Antagonism by norepinephrine of the positive inotropic effects of epinephrine but hardly of the negative inotropic effects of epinephrine on TG4 left atrium. A and B are representative experiments. A concentration-effect curve to norepinephrine was carried out up to 10 μM (A) or 100 μM (B), followed by a curve for epinephrine. C, curves for steady-state positive inotropic effects and maximal negative inotropic effects at each epinephrine concentration in the absence and presence of indicated concentrations of norepinephrine. The positive inotropic effects of norepinephrine are steady-state effects. D, simulation of the experiments shown in A, B, and C using eqs. 2 and 3 .



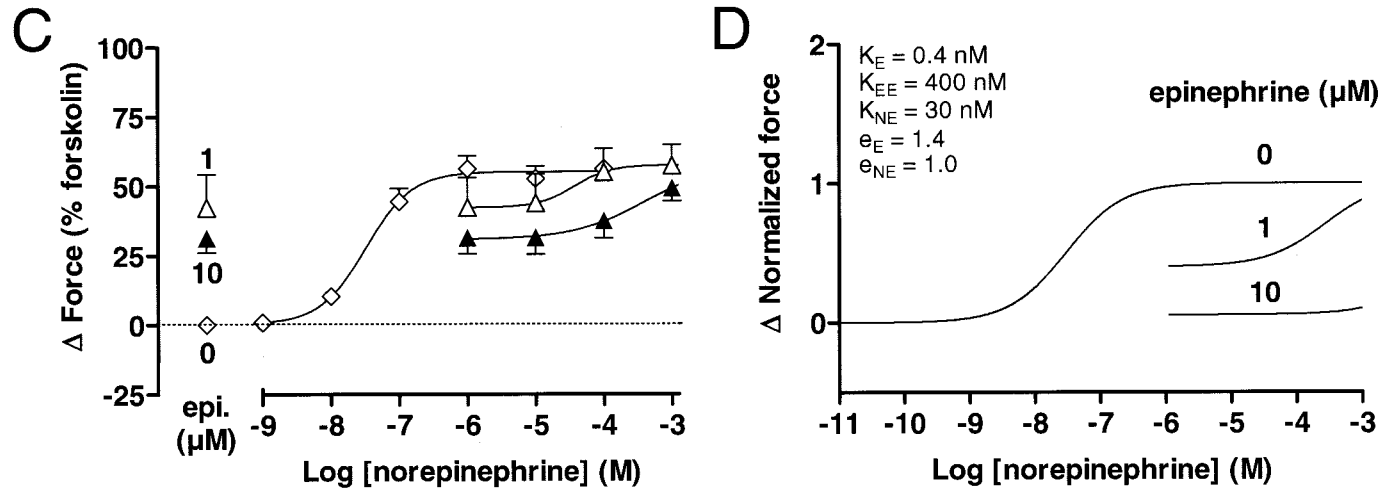


Fig. 3. Antagonism by epinephrine of the positive inotropic effects of norepinephrine on TG4 left atria. A and B are representative experiments. A concentration effect curve to epinephrine up to 1 μM (A) and 10 μM (B) was carried out, followed by a curve for norepinephrine. C, curves for steady-state 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.

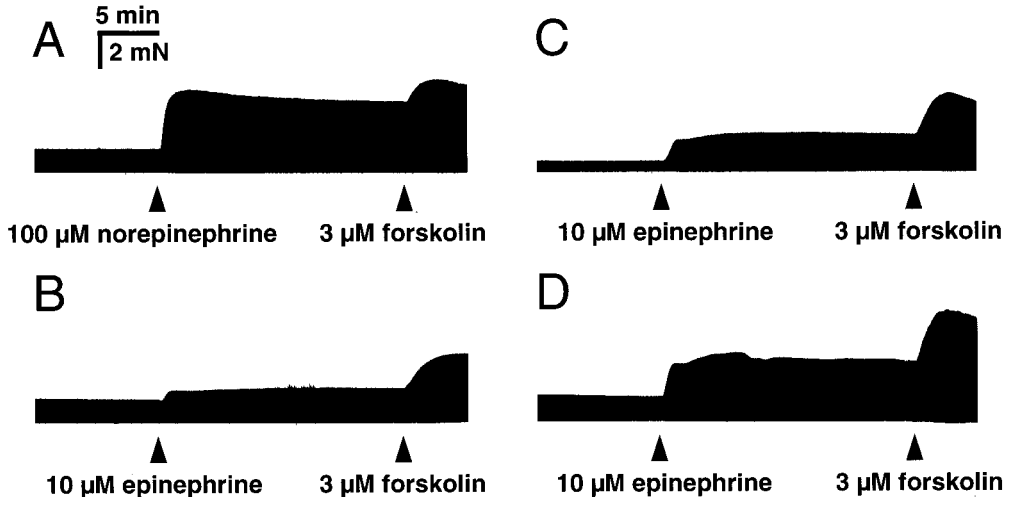


Fig. 4. Kinetics after noncumulative administration of a single high epinephrine concentration (B-D). Comparison of norepinephrine (A) and epinephrine (B). Shown are representative left atria. The effects of 10 μM epinephrine on force of contraction were similar in the absence (C) and presence (D) of the additional α-adrenoceptor blockers prazosin (1 μM) and yohimbine (1 μM).

the concept of agonist-dependent selectivity for coupling of rat cardiac β_2-adrenoceptors was published (Xiao et al., 2003). These authors showed that the positive inotropic effects of the β_2-selective agonists salbutamol, zinterol, and procaterol, but not of fenoterol, are enhanced by PTX. Thus, fenoterol only activates the G_s pathway in rat heart expressing native β_2-adrenoceptors, as found by us for the effects of norepinephrine mediated through β_2-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 $G_s \rightarrow G_i$ switch in the three cardiac regions. On the other hand, the inotropic and PKA data with norepinephrine are consistent with G_s coupling but not with G_i 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., $e_{NE} \leq e_E$). As expected from competition for binding of epinephrine to the G_s-coupled β_2-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 G_i 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 $-\log EC_{50}$ 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 β_2-adrenoceptor site coupled to G_s. The $\log CR = \log(1 + [E]/K_E) = \log(1 + 1000/0.4) = 3.4$, where CR is the EC_{50} ratio of norepinephrine in the presence and absence of 1 μM epinephrine ([E]) (Fig. 3). Similarly, the observed EC_{50} 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

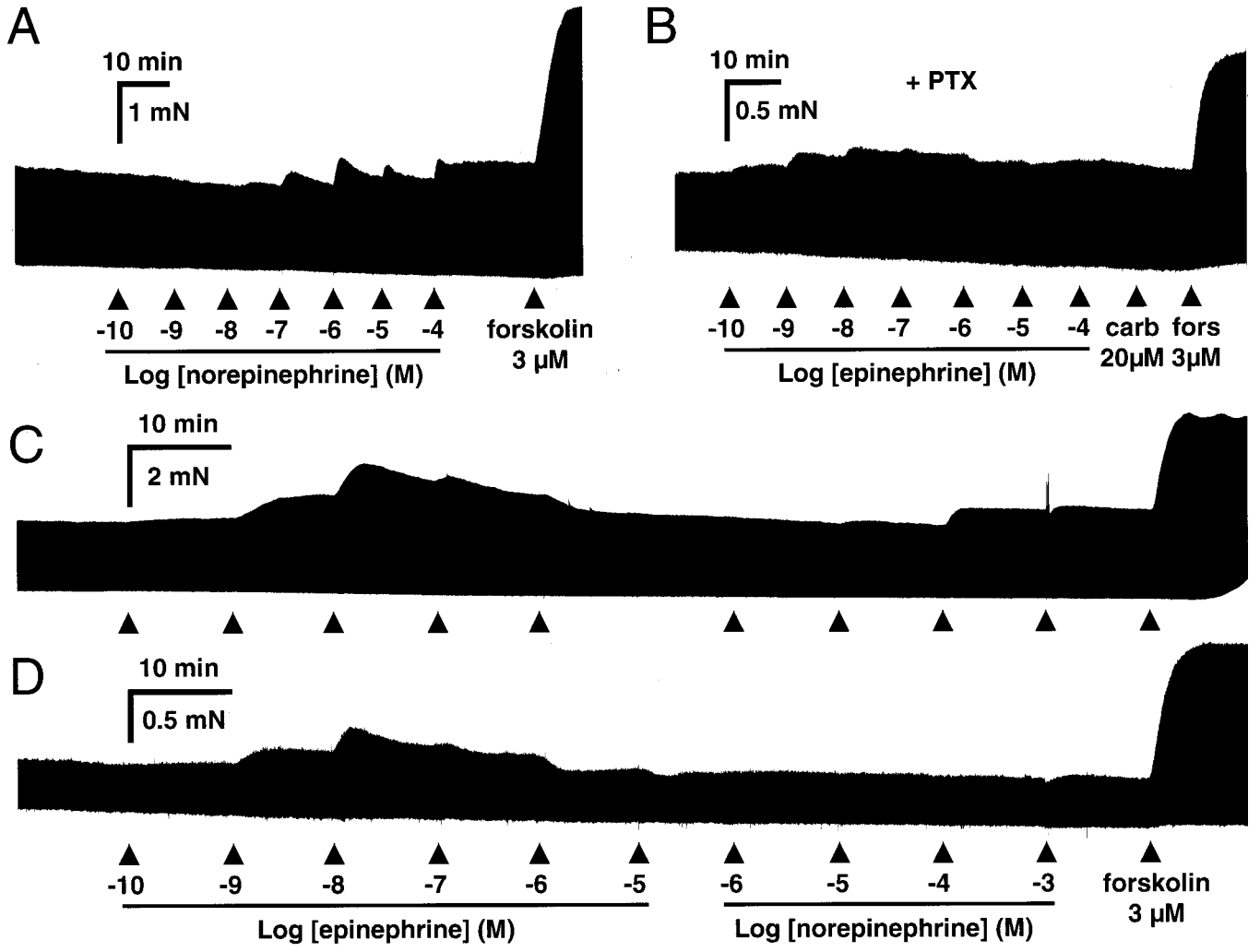


Fig. 5. Representative experiments of the effects of norepinephrine and epinephrine, isolated or in combination, on TG4 right ventricle. A, effects of norepinephrine. B, effects of epinephrine in a ventricle obtained from a PTX-treated TG4 mouse. Carb, carbachol. C, effects of epinephrine (up to 1 μM) and norepinephrine. D, effects of epinephrine (up to 10 μM) and norepinephrine.

agreements support the use of the atrial positive inotropic potency of epinephrine, $EC_{50} \sim 0.4$ nM, as $K_E = 0.4$ nM in the model.

To model our experiments, we assumed as a first approximation that our potency estimates (EC_{50} values) were equivalent to the K values (K_{NE} and K_E) used in the model. Our affinity estimates for norepinephrine, deduced from inotropic EC_{50} values in TG4 atrium ($K_{NE} = 30$ nM) and ventricle ($K_{NE} = 150$ nM) were similar to the dissociation equilibrium constant for norepinephrine ($K_{NE} = 210$ nM), estimated from inhibition of membrane binding of $(-)$-^{125}I-cyanopindolol to human ventricular β_2-adrenoceptors (Kaumann et al., 1995). However, for epinephrine, the inotropic EC_{50} values in TG4 atrium ($K_E = 0.4$ nM) and TG4 ventricle ($K_E = 0.9$ nM) were lower than the corresponding dissociation equilibrium constant from binding inhibition ($K_E = 15$ nM) (Kaumann et al., 1995). The discrepancy between the binding K_E estimate from human ventricle and our EC_{50} for the positive inotropic effects of epinephrine in TG4 myocardium may be caused by the oversimplification of equating $EC_{50} = K_E$. However, for two reasons, our EC_{50} values, estimated functionally, seem to reflect a high-affinity state. First, our EC_{50} values for epinephrine and norepinephrine agree with the corresponding EC_{50} values ($\sim$1 and $\sim$100 nM, respectively) for cAMP accumulation induced by epinephrine and norepinephrine through recombinant β_2-adrenoceptors (Swaminath et al., 2004). Second, the stimulant potency (EC_{50}) and blocking potency of epinephrine against norepinephrine on the left atrium were consistently subnanomolar, suggesting that epinephrine causes half-maximal β_2-adrenoceptor occupancy at the estimated K_E of 0.4 nM, consistent with the mass law assumptions of the model.

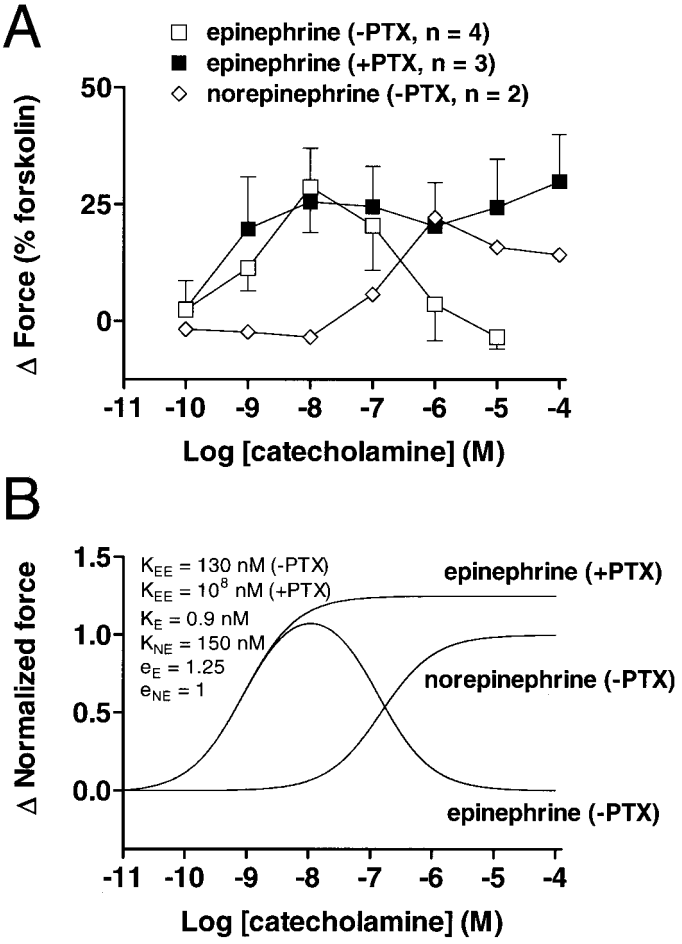


Fig. 6. A, inotropic effects of epinephrine and norepinephrine on TG4 right ventricle, and effects of PTX. B, simulations of the ventricular effects of epinephrine and of PTX treatment using eqs. 1 and 2.

TABLE 1
Comparison of the stimulation of PKA activity by epinephrine, norepinephrine, and forskolin in TG4 left ventricle

	PKA Activity Ratios	*n*	*P*
Basal	0.364 ± 0.011	9	
Epinephrine 10 nM	0.414 ± 0.019	6	0.033
Epinephrine 1 μM	0.363 ± 0.019	6	0.84
Epinephrine 100 μM	0.355 ± 0.030	3	0.64
Norepinephrine 1 μM	0.433 ± 0.033	3	0.026
Norepinephrine 100 μM	0.418 ± 0.020	9	0.043
Forskolin 3 μM	0.545 ± 0.026	10	0.000082

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 μM and abolishment at 100 μM, which was not observed (Figs. 1D and 3D). In the left atrium, the experimental maximum negative inotropic effect occurred at 10 μM 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 μM epinephrine tended to increase the force of contraction with slow kinetics. The slow increase in contractile force was also observed after application of 10 μM epinephrine as a single concentration, and the effect was resistant to α-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 μM 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 μM epinephrine, administered noncumulatively to the left atrium, were biphasic. As expected from the blunting effect of G_i 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 α-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_{EE} and K_E in ventricle was only

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 β_2-adrenoceptor, as reflected through the inotropic effects.

The coupling of the β_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 β_1- but not β_2-adrenoceptors produces apoptosis in rat heart (Communal et al., 1999), and the β_2-adrenoceptor of murine heart seems to deliver antiapoptotic signals through G_i-dependent coupling to phosphatidylinositol 3′-kinase (Chesley et al., 2000; Zhu et al., 2001). Both norepinephrine and isoproterenol have been reported to prevent hypoxia-induced cellular nuclear fragmentation through G_i-coupled β_2-adrenoceptors in cultured myocytes from the hearts of neonatal rats (Chesley et al., 2000). Our results, demonstrating that norepinephrine does not induce coupling of human β_2-adrenoceptors to G_i protein in TG4 ventricle, suggest a difference with murine β_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 β_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 β_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 β_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 β_2-adrenoceptors into murine heart needs to be tested at native human cardiac β_2-adrenoceptors expressed at physiological density.

We conclude that coupling of the human β_2-adrenoceptor to G_i protein is agonist-dependent. Epinephrine and isoproterenol, but not norepinephrine, interact at a binding site of the β_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_i-coupled β_2-adrenoceptor.

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References

Blinks JR (1965) Convenient apparatus for recording contractions of isolated muscle. *J Appl Physiol* **20:**755–757.

Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, McMinn TR, Apparsundaram S, Hyeck MF, Kenakin TP, Allen LF, et al. (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoceptor. *Nature (Lond)* **374:**272–276.

Bristow MR, Hershberger RE, Port JD, Minobe W, and Rasmussen R (1989) β_1- and β_2-adrenergic receptor-mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium. *Mol Pharmacol* **35:**295–303.

Chesley A, Lundberg MS, Toshinobu A, Xiao RP, Seiji O, Lakatta EG, and Crow MT (2000) The β_2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G_i-dependent coupling to phosphatidylinositol 3′-kinase. *Circ Res* **87:**1172–1179.

Communal C, Singh K, Sawyer DB, and Colucci WS (1999) Opposing effects of β_1- and β_2-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein. *Circulation* **100:**2210–2212.

Daaka Y, Luttel LM, and Lefkowitz RJ (1997) Switching of the coupling of the β_2-adrenergc receptor to different G proteins by protein kinase A. *Nature (Lond)* **390:**88–91.

Daul A, Hermes U, Schafers RF, Wenzel R, von Birgelen C, and Brodde OE (1995) The beta-adrenoceptor subtype(s) mediating adrenaline- and dobutamine-induced blood pressure and heart rate changes in healthy volunteers. *Int J Clin Pharmacol Ther* **33:**140–148.

Del Monte F, Kaumann AJ, Poole-Wilson P, Wynne DG, Pepper J, and Harding SE (1993) Coexistence of functioning β_1- and β_2-adrenoceptors in single myocytes from human ventricle. *Circulation* **88:**854–863.

Eason MG, Jacinto MT, and Ligget SB (1994) Contribution of ligand structure to activation of α2-adrenergic receptor subtype coupling to Gs. *Mol Pharmacol* **45:** 696–702.

Eason MG, Kurose H, Holt BD, Raymond JR, and Liggett SB (1992) Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. *J Biol Chem* **267:**15795–15801.

Gille E, Lemoine H, Ehle B, and Kaumann AJ (1985) The affinity of (−)-propranolol for β_1- and β_2-adrenoceptors of human heart. Differential antagonism of the positive inotropic effects and adenylate cyclase stimulation by (−)-noradrenaline and (−)-adrenaline. *Naunyn Schmiedeberg's Arch Pharmacol* **331:**60–70.

Graf EM, Heubach JF, and Ravens U (2001) The hyperpolarization-activated current I_f in ventricular myocytes of non-transgenic and β_2-adrenoceptor overexpressing mice. *Naunyn Schmiedeberg's Arch Pharmacol* **364:**131–139.

Gürdal H, Bond RA, Johnson MD, Friedman E, and Onaran O (1997) An efficacy-dependent effect of cardiac overexpression of β_2-adrenoceptor on ligand affinity in transgenic mice. *Mol Pharmacol* **52:**187–194.

Hall JA, Kaumann AJ, and Brown MJ (1990) Selective β_1-adrenoceptor blockade enhances positive inotropic responses of endogenous catecholamines through β_2-adrenoceptors in human atrial myocardium. *Circ Res* **66:**1610–1623.

Hasseldine ARG, Harper EA, and Black JW (2003) Cardiac-specific overexpression of human β_2-adrenoceptors in mice exposes coupling to both G_s and G_i proteins. *Br J Pharmacol* **138:**1361–1369.

Heubach J, Blaschke M, Harding SE, Ravens U, and Kaumann AJ (2003) Cardiostimulant and cardiodepressant effects through overexpressed human β_2-adrenoceptors in murine heart: regional differences and functional role of β_1-adrenoceptors. *Naunyn Schmiedeberg's Arch Pharmacol* **367:**380–390.

Heubach JF, Graf EM, Molenaar P, Jäger A, Schröder F, Herzig S, Harding SE, and Ravens U (2001) Murine L-type Ca^{2+} current is enhanced by zinterol via β_1-adrenoceptors and is reduced in TG4 mice overexpressing the human β_2-adrenoceptor. *Br J Pharmacol* **133:**73–82.

Heubach JF, Rau T, Eschenhagen T, Ravens U, and Kaumann AJ (2002) Physiological antagonism between ventricular β_1-adrenoceptors and α_1-adrenoceptors but no evidence for β_2- and β_3-adrenoceptor function in murine heart. *Br J Pharmacol* **136:**217–229.

Heubach JF, Trebeß I, Wettwer E, Himmel HM, Michel MC, Kaumann AJ, Koch WJ, Harding SE, and Ravens U (1999) L-type calcium current and contractility in ventricular myocytes from mice overexpressing the cardiac β_2-adrenoceptor. *Cardiovasc Res* **42:**173–182.

Kaumann AJ, Bartel S, Molenaar P, Sanders L, Burrell K, Vetter D, Hempel P, Karczewski P, and Krause EG (1999) Activation of β_2-adrenoceptors hastens relaxation and mediates phosphorylation of phospholamban, troponin I and C protein in ventricular myocardium from patients with terminal heart failure. *Circulation* **99:**65–72.

Kaumann AJ, Hall JA, Murray KJ, Wells FC, and Brown MJ (1989) A comparison of the effects of adrenaline and noradrenaline on human heart: the role of adenylate cyclase and contractile force. *Eur Heart J* **10** (Suppl B)**:**29–37.

Kaumann AJ and Lemoine H (1987) β_2-adrenoceptor-mediated positive inotropic

effects of adrenaline in human ventricular myocardium. Quantitative discrepancies between binding and adenylate cyclase stimulation. *Naunyn Schmiedeberg's Arch Pharmacol* **335:**403–411.

Kaumann AJ, Lynham JA, Sanders L, Brown AM, and Molenaar P (1995) Contribution of differential efficacy to the pharmacology of human β_1- and β_2-adrenoceptors. *Pharmacol Commun* **6:**215–222.

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

Kenakin T (1995b) Agonist-receptor efficacy II: agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:**232–238.

Kilts JD, Gerhardt MA, Richardson MD, Sreeram G, Mackensen B, Grocott HP, White WD, Davis D, Newman MF, Reves JG, et al. (2000) β_2-Adrenergic and several other G protein-coupled receptors in human atrial membranes activate both G_s and G_i. *Circ Res* **87:**705–709.

Milano CA, Allen LF, Rockman HA, Dolber PC, McMinn TR, Chien KR, Johnson TD, Bond RA, and Lefkowitz RJ (1994) Enhanced myocardial function in transgenic mice overexpressing the β_2-adrenergic receptor. *Science (Wash DC)* **264:**582–586.

Molenaar P, Bartel S, Cochrane A, Vetter D, Jalali H, Pohlner P, Burrel K, Karczewski P, Krause EG, and Kaumann AJ (2000) Both β_2- and β_1-adrenergic receptors mediate hastened relaxation and phosphorylation of phospholamban and troponin I in ventricular myocardium of Fallot infants, consistent with selective coupling of β_2-adrenergic receptors to G_s-protein. *Circulation* **102:**1814–1821.

Murray KJ, England PJ, Lynham JA, Mills D, Schmitz-Pfeiffer C, and Reeves ML (1990) Use of synthetic dodecapeptide (malantide) to measure the cyclic AMP-dependent protein kinase activity ratio in a variety of tissues. *Biochem J* **267:**703–708.

Oostendorp J and Kaumann AJ (2000) Pertussis toxin suppresses carbachol-evoked cardiodepression but does not modify cardiostimulation mediated through β_1- and putative β_4-adrenoceptors in mouse left atria: no evidence for β_2- and β_3-adrenoceptor function. *Naunyn Schmiedeberg's Arch Pharmacol* **361:**134–145.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C, and Kobilka BK (2004) Sequential binding of agonists to the β_2-adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* **279:**686–691.

Xiao RP (2000) Cell log IC for dual coupling of a single class of receptors to G_s and G_i proteins. *Circ Res* **87:**635–637.

Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter S, Eschenhagen T, Lefkowitz RJ, Koch WJ, and Lakatta EG (1999) Coupling of β_2-adrenoceptor to G_i proteins and its physiological relevance in murine cardiac myocytes. *Circ Res* **84:**43–52.

Xiao RP, Thang SJ, Chakir K, Avdonin P, Zhu W, Bond RA, Balke W, Lakatta EG, and Cheng H (2003) Enhanced Gi signaling selectively negates β_2-adrenergic receptor (AR)- but not β_1-AR-mediated positive inotropic effect in myocytes from failing rat hearts. *Circulation* **108:**1633–1639.

Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, and Xiao RP (2001) Dual modulation of cell survival and cell death by β_2-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci USA* **98:**1607–1612.

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