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ORIGINAL ARTICLE

β_1 Adrenoceptor antagonistic effects of the supposedly selective β_2 adrenoceptor antagonist ICI 118,551 on the positive inotropic effect of adrenaline in murine hearts

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Abstract

Studies on the relative contribution of β_1- and β_2-adrenoceptors (AR) generally employ selective β_1- and β_2-AR antagonists such as CGP 20712A and ICI 118,551, respectively, and assume that antagonism by one of these compounds indicates mediation by the respective AR subtype. Here, we evaluated the β_2-AR-selectivity of ICI 118,551 in ventricular muscle strips of transgenic mice lacking β_1-AR (β_1-KO), β_2-AR (β_2-KO), or both (β_1/β_2-KO). Strips were electrically driven and force development was measured. In wild type (WT), ICI 118,551 (100 nmol/L) shifted the concentration–response curve (CRC) for adrenaline by about 0.5 log units to the right, corresponding to the known affinity of ICI 118,551 to β_1-AR but not to β_2-AR. Conversely, the phosphodiesterase inhibitor rolipram (10 μmol/L) shifted the CRC to the left, but did not enlarge the ICI 118,551 shift, indicating exclusive β_1-AR mediation even when PDE4 is inactive. In line with this, rolipram and ICI 118,551 had similar effects in β_2-KO than in WT. In contrast, β_1-KO did not show any inotropic reaction to adrenaline (+/− rolipram). In WT, the β_1-AR selective antagonist CGP 20712A (100 nmol/L) shifted the CRC for isoprenaline by 2.1 log units, corresponding to the affinity of CGP 20712A to β_1-AR. Rolipram increased the sensitivity to adrenaline independently of the presence of CGP 20712A. We conclude that effects sensitive to the β_2-AR antagonist ICI 118,551 are not necessarily β_2-AR-mediated and CGP 20712A-resistant effects cannot be simply interpreted as β_2-AR-mediated. Catecholamine effects in murine ventricles strictly depend on β_1-AR, even if PDE 4 is blocked.

Abbreviations

cAMP, cyclic adenosine monophosphate; CGP, CGP 20712A; CRC, concentration–response curve; CR, concentration ratio; FRET, Förster resonance energy transfer; ICI, ICI 118,551; PDE, phosphodiesterase; PI3Kγ, phosphoinositide-3-kinase-γ; PKA, protein kinase A; ROL, rolipram; β-AR, β-adrenoceptor.

Introduction

The use of pharmacological tools to determine the relative contribution of receptor subtypes of the same class can be difficult due to the limited selectivity of most compounds and the fact that competitive receptor antagonists do not prevent binding of other ligands (="block"), but just shift their binding curve to the right. A recent study showed that phosphoinositide-3-kinase-γ (PI3Kγ) acts not only as a kinase in the heart but also as a scaffold that provides contact between protein kinase A (PKA) and phosphodiesterase 4 (PDE4; Perino et al. 2011). Genetic ablation of

PI3Kγ (KO) sensitized the heart to β-AR stimulation by disrupting the PKA/PDE4 complex and diminishing PDE4-mediated degradation of cAMP (Ghigo et al. 2012). Accordingly, adrenaline had larger effects on heart rate in PI3Kγ KO mice than in WT. Whereas these data are consistent with the proposed scaffolding effect of PI3Kγ on PKA and PDE4, another experiment and its interpretation stoked our attention. The greater chronotropic effect of adrenaline in PI3Kγ KO was sensitive to the β_2-adrenoceptor-selective antagonist ICI 118,551. Additionally, isoprenaline-induced cAMP accumulation in the presence of the β1-adrenoceptor-selective antagonist CGP 20712A was elevated by rolipram. The two latter findings were interpreted as proof of involvement of β_2-AR.

Since the prevailing evidence suggested inotropic effects in murine hearts to be solely mediated by β_1-AR, we compared log dose shifts of CRCs for adrenaline and isoprenaline by ICI 118,551 and CGP 20712A with published affinity data for both antagonists on β_1-AR and β_2-AR. The (moderate) size of the shift of the adrenaline CRCs induced by ICI 118,551 (100 nmol/L) in the above study (Ghigo et al. 2012) fitted well to the affinity of this compound to β_1-AR, but not to its affinity to β_2-AR. As expected, CGP 20712A (100 nmol/L) shifted the CRC of isoprenaline to the right. However, the shift size was not different in the presence of rolipram.

To solve this issue and avoid confounding effects of the limited subtype-selectivity of the two β-AR antagonists, we tested here with genetic tools (mice lacking β_1-AR [β_1-KO], β_2-AR [β_2-KO] or both [β_1/β_2-KO]) whether block of adrenaline effects by ICI 118,551 necessarily indicates β_2-AR mediation and if isoprenaline effects that persist in the presence of CGP 20712A can indeed be interpreted as mediated via β_2-AR. Experiments were done under control conditions and after pharmacological inhibition of PDE4 by rolipram.

Materials and Methods

Experimental animals

Experiments with adrenaline: Homozygous deletions of β_1- or β_2-AR were generated in Dr. Brian Kobilka's laboratory as described (Rohrer et al. 1996; Chruscinski et al. 1999). We bred β_1-KO and β_2-KO mice in mixed C57BL6J/FVB/N backgrounds. For experiments with isoprenaline: WT animals were on an inbred C57BL6J background.

Contractility studies

Mice were killed following the protocols approved by the local ethics committee (permit number: ORG 365) in accordance with the guidelines of the European Community. Strips of free wall of right and intact left ventricular papillary muscles were rapidly dissected and mounted as pairs in an organ bath. Since inotropic potencies and potentiation upon the PDE4-inhibitor rolipram did not differ between right ventricle strips and left papillary muscles (Christ et al. 2009), we did a pooled analysis. However, we did experiments in pairs of right ventricle strips and left papillary muscles to get almost equal proportions. Muscles were paced at 2 Hz and stretched to 5 mN. Experiments were performed in oxygenated, modified Tyrode's solution containing (mmol/L): NaCl 126.7, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 22.0, NaH_2PO_4 0.42, EDTA 0.04, ascorbic acid 0.2, and glucose 5.0. The solution was maintained at pH 7.4 by bubbling a mixture of 5% CO_2 and 95% O_2. Experiments were performed at 37°C.

All tissues were incubated with 5 μmol/L phenoxybenzamine for 90 min to block α-adrenoceptors and neuronal and extraneuronal uptake of catecholamines (Gille et al. 1985). Contractile force was recorded through PowerLab amplifiers on a Chart for Windows, Version 5.0, recording programme (ADInstruments Pty Ltd., Castle Hill, NSW, Australia). Muscles were allowed to equilibrate for 90 min. Experiments were performed under control conditions (no antagonist present), after 30-min pretreatment with the selective PDE4 inhibitor rolipram (10 μmol/L) and/or in the presence of the selective β_2-AR antagonist ICI 118,551 (100 nmol/L) or the selective β_1-AR antagonist CGP 20712A (100 nmol/L). A single cumulative concentration–effect curve was established for (−)-adrenaline or isoprenaline by the sequential addition of agonist to the organ bath. At the end of each experiment with KO mice, 8 mmol/L $CaCl_2$ was applied to verify proper inotropic response of each muscle strip.

Drugs

Rolipram and ICI 118,551 (1-[2,3-dihydro-7-methyl-1H-inden-4-yl)oxy-3-[(1-methylethyl)amino]-2-butanol) were purchased from Tocris (Bristol, UK). CGP 20712A, isoprenaline and (−)-adrenaline were purchased from Sigma Chemie (Deisenhofen, Germany). Phenoxybenzamine was purchased from Röhm Pharma (Darmstadt, Germany).

Statistics

−Log EC_{50} values for the catecholamines were estimated from fitting a Hill function with variable slopes to concentration–response curves (CRC) from individual experiments. The data are expressed as mean ± SEM of *n*/*n* = number of right ventricular strips/number of left papillary muscles. Significance of differences between means was assessed with the use of Student's *t*-test. *F*-test

was used to compare CRC. GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) was used for curve fitting and statistical analysis. Expected concentration ratios (CR) of agonists in the presence and absence of antagonists' concentration ([B]) were calculated from CR = 1 + [B]/*K*B, where *K*B is the equilibrium dissociation constant for the antagonist.

Results

Effects of adrenaline in WT mice

In ventricular muscle strips from WT mice, adrenaline increased force in a concentration-dependent manner (Fig. 1A). There was no indication for a biphasic CRC. The β_2-AR-subtype-selective antagonist ICI 118,551, employed at a commonly used concentration of 100 nmol/L (Ghigo et al. 2012), shifted the CRC to the right by about half a log unit. To mimic the situation in PI3Kγ KO where PDE-4 action is blunted, we also did the experiments in the absence or presence of a high concentration of the selective PDE4-inhibitor rolipram (10 μmol/L). PDE inhibition alone did not induce a positive inotropic effect, but significantly increased the sensitivity to adrenaline by about half a log unit (Figs. 1A, 3). The additional presence of ICI 118,551 shifted the CRC back to the right, that is, the CRC in control and rolipram+ICI 118,551 were superimposable. Importantly, the ICI 118,551-induced rightward shift of the adrenaline CRC was of similar size in the absence of rolipram, suggesting that it was independent of PDE4 activity.

Effects in β_2-KO mice

To evaluate the β-AR subtype responsible for adrenaline and ICI 118,551 effects, we employed genetically targeted mice. Basal force was not different in β_2-KO compared to WT. β_2-KO mice showed lower sensitivity to adrenaline ($-\log EC_{50}$ 6.07 vs. 6.64 for WT), but maximum force did not differ from WT (pooled analysis for all experimental groups $P = 0.58$; Fig. 1). Interestingly, the magnitude of the rolipram-induced shift to the left was significantly larger in β_2-KO than in WT (0.76 $\pm$ 0.12 vs. 0.27 $\pm$ 0.16 log units [$P < 0.05$] in the absence and 1.1 $\pm$ 0.12 vs. 0.49 $\pm$ 0.05 in the presence of ICI 118,551 [$P < 0.01$]; for summary see Fig. 2). However, as seen before in WT mice, ICI 118,551 (100 nmol/L) shifted the CRC for adrenaline in β_2-KO muscle strips by about half a log unit to the right, both in the absence and presence of rolipram, not consistent with a generation of a β_2-response by rolipram.

Effects of adrenaline in β_1-KO and β_2/β_1-KO mice

Basal force of contraction in β_1-KO and β_2/β_1-KO mice did not differ from WT or β_2-KO (Fig. 3). Remarkably, the inotropic response to adrenaline was completely absent in both β_1-KO and β_2/β_1-KO mice, while high concentrations of Ca^{2+} were similarly effective in WT and β_2-KO. Inhibition of PDE4 by rolipram failed to recover positive inotropic effects in β_1-KO and β_2/β_1-KO. Force in the presence of 8 mmol/L Ca^{2+} was smaller in β_1-KO and in β_1/β_2-KO compared to WT (1.66 $\pm$ 0.22 and 1.39 $\pm$ 0.24 vs. 2.62 $\pm$ 0.33 mN, $P < 0.05$ each).

Effects of isoprenaline in WT mice

In order to validate their results with ICI 118,551 and adrenaline, Ghigo et al. (2012) used a single concentration of the nonselective β-AR agonist isoprenaline in the presence of the β_1-AR selective antagonist CGP 201712A (100 nmol/L). The remaining effect of isoprenaline in the presence of CGP 201712A was interpreted as a β_2-AR effect and the larger effect size in PI3Kγ KO (in which compartmentalization of PDE4 was disturbed) as evidence for PDE4 restricting a β_2-AR effect. Given the absence of any inotropic effect of adrenaline in β_1-AR-KO, we thought to challenge this interpretation by performing full CRCs for isoprenaline in the absence and presence of 100 nmol/L CGP 20712A with or without inhibition of PDE4 by rolipram (Fig. 4). In WT, isoprenaline increased force with a potency of -7.23 (log (M)). Rolipram (10 μmol/L) shifted the curve by about half a log unit. 100 nmol/L CGP 20712A shifted the curve by about 2.1 log units to the right, both in the presence and in the absence of rolipram, fully compatible with a pure β_1-AR-mediated effect of isoprenaline.

Discussion

Subtype-selective β-AR-signaling in the heart has gained large interest since the effects of β_1-AR and β_2-AR may differ not only quantitatively but also directionally with evidence for (chronically) adverse effects of β_1-AR and protective effects of β_2-AR (Xiao and Lakatta 1993; Communal et al. 1999). The most commonly employed tools are the β_1-AR selective antagonist CGP 20712A (500- to 1000-fold greater affinity for β_1- than β_2-AR) and the β_2-AR selective antagonist ICI 118,551 (70-fold greater affinity for β_2- than β_1-AR; Hoffmann et al. 2004; Baker 2005). These two compounds have therefore become widely used, and the notion of the high selectivity of CGP 20712A and ICI 118,551 is in fact so widespread that

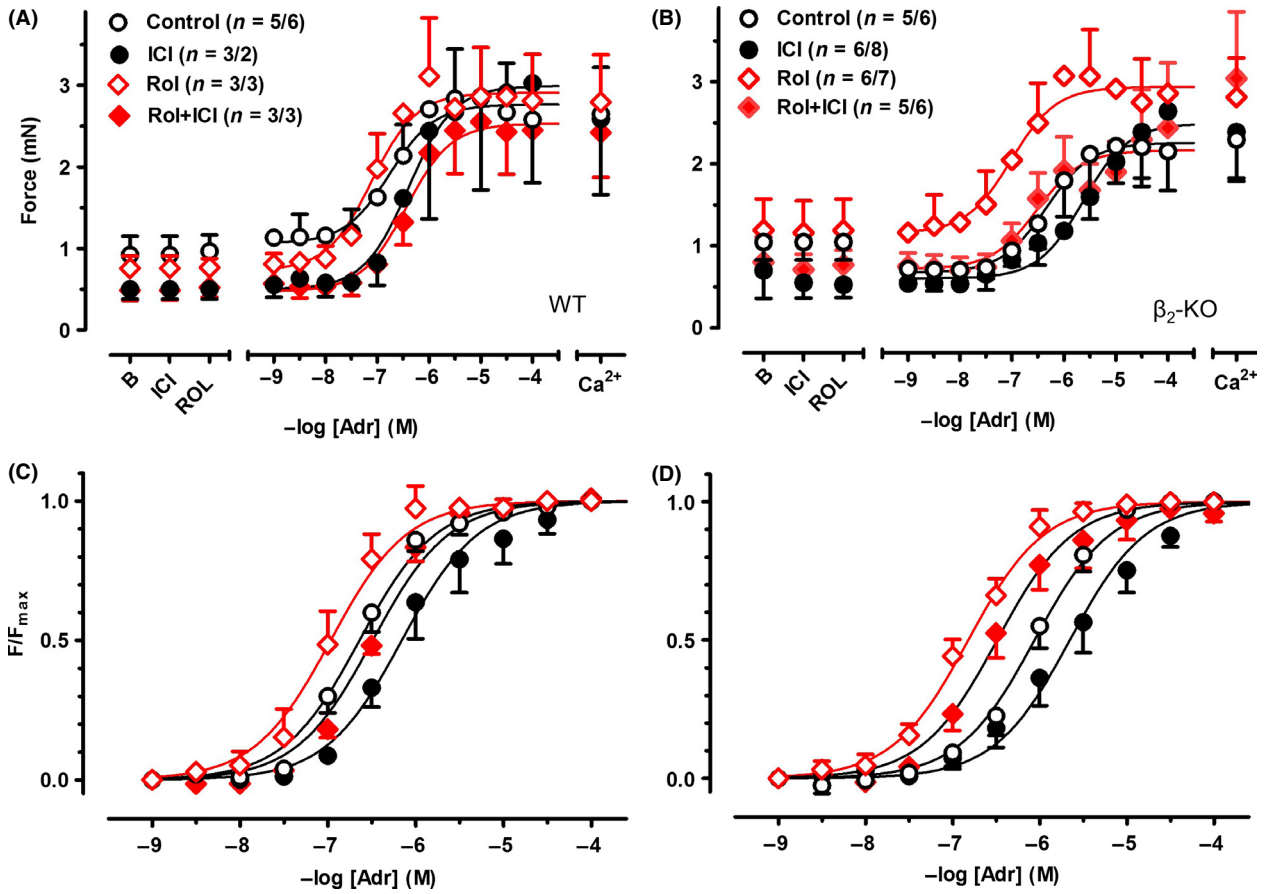

Figure 1. Positive inotropic effects of adrenaline in WT and β_2-KO. Mean values ± SEM of force obtained in WT and β_2-KO (*n*/*n* indicates number of right ventricular strips/left papillary muscles). Data are given in absolute values (A, B) or normalized to its individual maximum response to adrenaline (C, D). Data obtained under basal conditions are labeled as "B." "ICI" and "ROL" indicate force after exposure to 100 nmol/L ICI 118,551 and 10 μmol/L rolipram or respective time-matched controls. At the end of each experiment, 8 mmol/L Ca^{2+} (Ca^{2+}) was given. Please note that for better clarity some error bars are omitted in A and B. β-AR, β_2-adrenoceptors; ROL, rolipram, WT, wild type.

researchers often assume that these compounds "block" only one or the other β_1/β_2-AR.

The present study shows that ICI 118,551, at a concentration usually employed to selectively block β_2-AR, shifts the CRC for the positive inotropic effect of adrenaline to the right as expected. Importantly, however, it did this not only in WT, but to the same degree in β_2-AR-KO mice. And, consistent with previous results (Rohrer et al. 1996), adrenaline had no inotropic effect in cardiac preparations of β_1-AR-KO mice. These data demonstrate that even this highly selective β_2-AR antagonist has residual β_1-AR antagonistic effects and confirm that positive inotropic effects of adrenaline in mice are mediated exclusively via β_1-AR (Rohrer et al. 1996; Heubach et al. 2002), even when PDE4 is pharmacologically blocked (Galindo-Tovar and Kaumann 2008).

β_2-ARs exist in rodent cardiomyocytes, but their relevance in the control of contractile force is a matter of debate for more than two decades. While some groups could not demonstrate inotropic actions in mice ventricles (Heubach et al. 2002), others reported positive inotropy accompanied by cardioprotective effects (Xiao and Lakatta 1993; Communal et al. 1999; Chesley et al. 2000). There is consensus that cAMP signals evoked by β_2-AR in rodents are rather small and locally restricted, compared to those by β_1-AR (Nikolaev et al. 2006). PDE enzymes are likely involved in local control of cAMP pools. Block of PDE increases the potency, but not the efficacy for β_1-AR stimulation. Small cAMP signals (β_2-AR in rat heart), hardly detectable under control condition, can be increased by concomitant block of PDE3 and PDE4 (Christ et al. 2009). However, results from rats cannot be extrapolated to mice, since even under almost identical experimental conditions (concomitant block of PDE3 and 4, adrenaline as agonist), mice ventricles did not show any inotropic response to adrenaline via β_2-AR (Galindo-Tovar and Kaumann 2008). It should be noted that in mice ventricles inotropic responses to adrenaline are

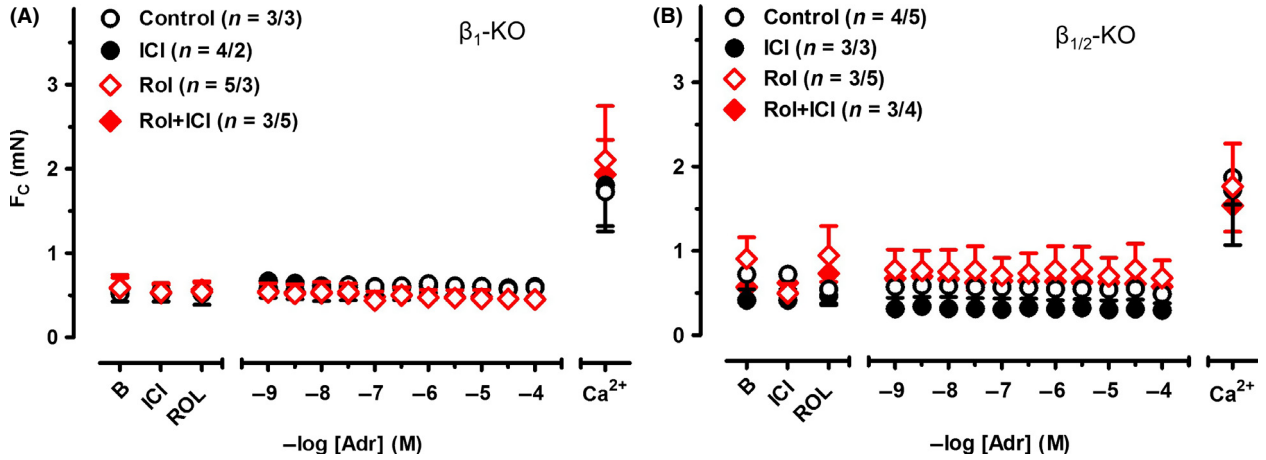

Figure 2. Lack of inotropic effects of adrenaline in β_1-KO and β_1/β_2-KO. Same layout as in Figure 1A and B. Please note that no normalization to adrenaline effect was applied.

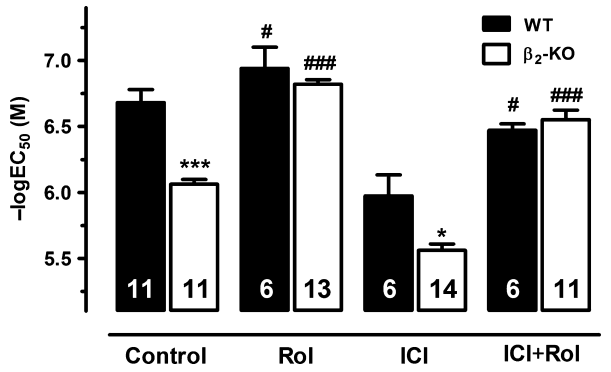

Figure 3. Sensitization to adrenaline by rolipram in WT and β_2-KO. Mean values ± SEM of $-\log EC_{50}$ values for the positive inotropic effect of adrenalin in the absence and presence of ICI 118,551 (100 nmol/L, ICI), rolipram (10 μmol/L, Rol) or both. *Versus respective data obtained from wild type (WT); #Versus the respective control for rolipram (control or ICI). Number in bars indicates total number of experiments.

regulated predominantly by PDE4, but not by PDE3 (Galindo-Tovar and Kaumann 2008).

In the present study, ICI 118,551 was used at a concentration of 100 nmol/L, which has been employed in numerous β-AR subtype-selective studies over the past three decades (Bilski et al. 1983; Kaumann and Lemoine 1987; Schafers et al. 1994; Communal et al. 1999; Chesley et al. 2000). Though counterintuitive at first glance, the rightward shift induced by ICI 118,551 in β_2-AR-KO mice is fully compatible with binding studies. ICI 118,551 bound to human ventricular membrane preparations with a pK_D of 9.0 at β_2-AR and 7.3 at β_1-AR (Kaumann and Lemoine 1987). Expression systems roughly confirmed these values (pK_D 9.2 at β_2-AR and 6.5 at β_1-AR (Hoffmann et al. 2004; Baker 2005). Catecholamine-evoked adenylyl cyclase stimulation was antagonized by ICI 118,551 with almost identical potency: pK_D 8.7 for β_2-AR and 6.9 at β_1-AR (Lemoine et al. 1988). Thus, there is no doubt that ICI 118,551 can bind to β_1-AR and antagonize β_1-AR-mediated catecholamine responses. Based on these affinities, calculations according to Schild law predict that 100 nmol/L ICI 118,551 shifts β_2-AR effects by 2.6 log units, and β_1-AR effects by 0.48 log units. Coincidentally, the predicted and the observed size of the shift by ICI 118,551 was almost as large as the (directionally opposing) shift induced by PDE4 inhibition on β_1-AR-mediated CRC. As a result, the stimulatory effect of rolipram was functionally reversed by concomitant application of ICI 118,551 in our experiments, even in the case where only β_1-AR were present (β_2-AR-KO).

Almost the same counterintuitive results can be predicted from the use of the β_1-AR selective antagonist CGP 201712A. Early functional studies employing Schild plots gave pK_D values of 9.5 at native β_1-AR in rat tissue (Kaumann 1986). More recent direct binding studies in expression systems for human β-AR reported lower pK_D values for CGP20712A at the β_1-AR: 8.8 and 8.3 (Hoffmann et al. 2004; Baker 2005). Consequently the expected shift of the CRC for ISO by 100 nmol/L CGP should account to 2.5 log units for a pK_D value of 9.5 and 1.8 and 1.3 log units for pK_D values of 8.8 and 8.3, respectively. We observed a 2.1 log unit shift by 100 nmol/L CGP 20712A. Our results indicate a pK_D value of 9.0 for CGP20712A at β_1-AR, well in between the reported affinity data.

The study by Ghigo et al. (2012) employed 100 nmol/L CGP 120712A in all experiments and the nonselective β-AR agonist isoprenaline, assuming that isoprenaline exerts only β_2-AR effects under these conditions. Unfortunately,

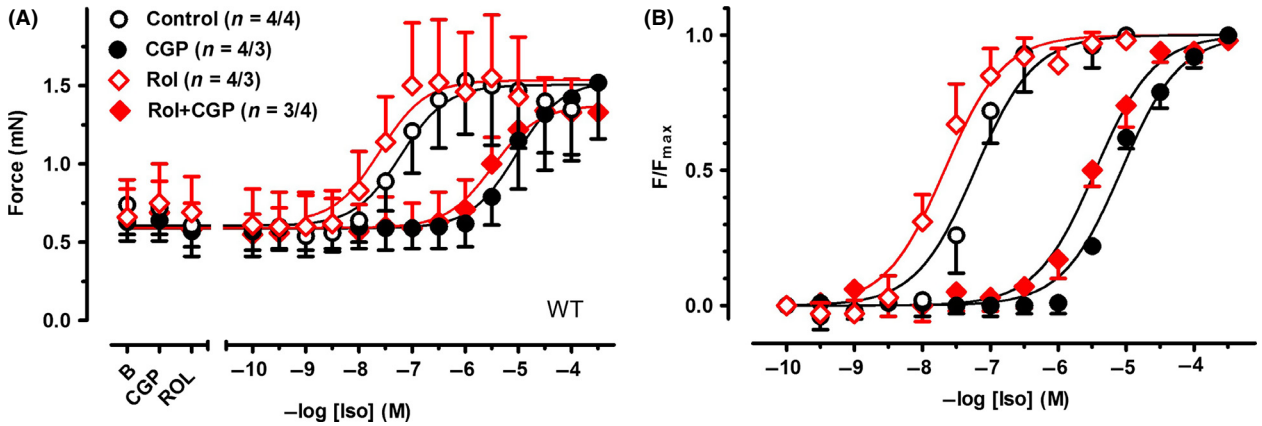

Figure 4. Positive inotropic effects of isoprenaline in WT. Mean values ± SEM of force obtained in WT (*n/n* indicates number of right ventricular strips/left papillary muscles). Data are given in absolute values (A) and normalized to its individual maximum response to adrenaline (B). Data obtained under basal conditions are labeled as "B." "CGP" and "ROL" indicate force after exposure to 100 nmol/L CGP 20712A and 10 μmol/L rolipram or respective time-matched controls. CGP, CGP 20712A; ROL, rolipram; WT, wild type.

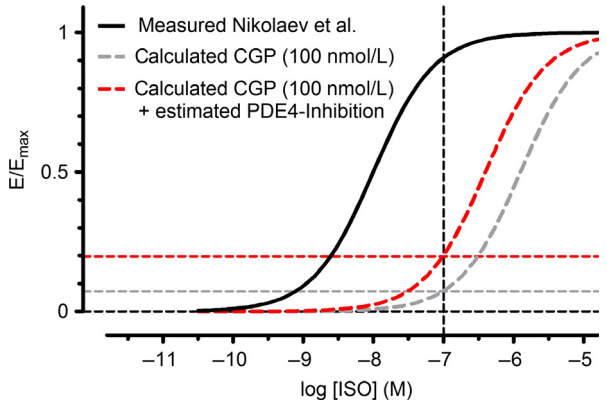

Figure 5. Expected shift of the CRC for the nonselective β-AR agonist isoprenaline on cAMP accumulation by rolipram in the presence of CGP 20712A (100 nmol/L) in WT. Straight black line: Isoprenaline CRC constructed from EC_{50} values reported by Nikolaev et al. (2006). Gray dotted line: calculated isoprenaline CRC in the presence of CGP 20712A. Data are based on experimental findings depicted in Figure 4. Red dotted line: estimated isoprenaline CRC in the presence of CGP 20712A and rolipram. Details see Discussion. Lines indicate calculated effect size for a given concentration of isoprenaline (100 nmol/L) in the presence of CGP 20712A (gray) alone or in the concomitant presence of CGP 20712A and rolipram (red). cAMP, cyclic adenosine monophosphate; CGP, CGP 20712A; CRC, concentration–response curve; DMSO, dimethyl sulfoxide; WT, wild type; β-AR, β_2-adrenoceptors.

only single concentration effects of 100 nmol/L isoprenaline on cAMP accumulation were evaluated. From seminal work in the field of cAMP-focused FRET-sensor by Nikolaev et al. (2006), we have to assume an EC_{50} value of 10 nmol/L for isoprenaline in mice ventricular myocytes. A total of 100 nmol/L isoprenaline alone should therefore give almost maximum effects and additional PDE4 inhibition cannot augment the effect anymore (Fig. 5). Assuming a CGP 20712A-induced shift of the CRC by 2.1 log units (Fig. 4), 100 nmol/L isoprenaline in the presence of 100 nmol/L CGP 20712A should still induce a small increase in cAMP, which can be enlarged by PDE inhibition. Importantly, the relative effect of PDE inhibition would be larger under this condition than under isolated isoprenaline stimulation (Fig. 5). Thus, the data presented by Ghigo et al. perfectly match theoretical considerations predicting the response to be mediated by β_1-AR even when PDE4 is inactive (Ghigo et al. 2012).

Notably, β_2-AR-KO exhibited subsensitivity to adrenaline (~0.5 log units), which was fully reversed by inhibition of PDE4 (Fig. 2), suggesting an overactive to play a causative role of PDE4. Other known mechanisms underlying reduced sensitivity for catecholamines involve G-protein-coupled receptor kinases (GRKs; Santulli et al. 2011; Santulli and Iaccarino 2013) and/or β-arrestin (Lymperopoulos et al. 2009; Santulli 2014). We are not sure how to interpret the blunted adrenaline response in β_2-AR-KO. Studies with β_2-AR-KO mice in different models of heart failure gave conflicting results: β_2-AR-KO was deleterious in some models (Bernstein et al. 2005; Fajardo et al. 2011), but protective in other models (Fajardo et al. 2013; Voltarelli et al. 2014).

Our present results are in line with earlier work which showed that cardiac preparations of β_1-KO and β_1-/β_2-KO mice do not respond to catecholamines (Rohrer et al. 1996, 1999) and indicate exclusive mediation of adrenaline effects via β_1-AR in mice. This differs from a robust positive inotropic effect of β_2-AR stimulation in the human heart (Bristow et al. 1986; Hall et al. 1990; Schafers et al. 1994). As expected, inhibition of PDE4 by roli-

pram increased the sensitivity to adrenaline via β_1-AR, but did not unmask inotropic effects when β_1-AR was absent (β_1-AR-KO and β_1-AR/β_2-AR-KO). The results should be a reminder that subtype-selective β-AR antagonists do not block the receptors and should take into account that even highly selective β-AR subtype antagonists cannot give 100% certainty to restrict effects exclusively to the β-AR subtype of interest.

Limitations

We measured positive inotropic effects instead of cAMP accumulation, chronotropic effects, and arrhythmia induction (Ghigo et al. 2012). We cannot provide a meaningful explanation for the reduced sensitivity for adrenaline and the larger rolipram-induced leftward shift of the CRC of adrenaline in β_2-KO mice compared to WT. Reduced sensitivity to β_1-AR could contribute to cardioprotective effects in β_2-KO in mice models of cardiomyopathy (Bernstein et al. 2005; Fajardo et al. 2013). β_1-KO may provide a helpful pharmacological model to study β-AR pharmacology. However, it should be noted that in addition to PDE other regulatory proteins like GRK2 have been implicated as an important negative regulator of inotropy of β_1-KO (Salazar et al. 2013).

Inotropic responses to high concentrations of Ca^{2+} were reduced in tissue missing an inotropic response to catecholamines. This finding clearly indicates that inotropic response to high Ca^{2+} cannot be used as an independent reference for maximum force generation of cardiac muscle preparations. Most probably, cAMP/PKA-dependent regulation of partners involved in electromechanical coupling may influence the effects of high Ca^{2+}-concentrations.

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Author Contributions

T. E., K. L., and T. C. participated in research design. S. P., F. F., K. D. S., and T. C. conducted the experiments and performed data analysis. S. P., K. L., F. F., T. E., and T. C. wrote the paper.

Disclosure

The authors declare no conflict of interest.

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