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Agonist-specific activation of the β_2-adrenoceptor/G_s-protein and β_2-adrenoceptor/G_i-protein pathway in adult rat ventricular cardiomyocytes

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1 In rat ventricular cardiomyocytes β_2-adrenoceptors (AR) couple to G_s- and G_i-protein, and evidence has accumulated that β_2-AR agonists can differentially activate either G_s- or G_s- and G_i-protein.

2 In this study, in isolated adult rat ventricular cardiomyocytes, we assessed the effects of pertussis toxin (PTX) on β_2-AR agonist (terbutaline (TER), salbutamol (SAL) and fenoterol (FEN)) evoked inhibition of phenylephrine (PE)-induced increase in the rate of protein synthesis (assessed as [^{3}H]phenylalanine incorporation) to find out which β_2-AR agonist activates selectively G_s- or G_s- and G_i-protein.

3 PE (1 μM) increased the rate of protein synthesis from 100% to 130$\pm$2% ($n=34$). FEN, TER and SAL (1 nM–10 μM) inhibited PE-induced increase in the rate of protein synthesis concentration-dependently. FEN inhibited PE effects almost completely (from 132$\pm$3 to 101$\pm$1%), whereas TER and SAL caused only partial inhibition (from 131$\pm$2 to 114$\pm$2 and 129$\pm$1 to 111$\pm$2%, respectively).

4 Pretreatment of cardiomyocytes with PTX (250 ng ml^{-1} for 16 h at 37°C) did not affect FEN effects, but converted TER- and SAL-evoked partial inhibition into complete inhibition.

5 Inhibitory effects of the three β_2-AR agonists were markedly attenuated by β_1-AR selective antagonist CGP 20712A (CGP) (300 nM); in contrast, β_2-AR selective antagonist ICI 118,551 (55 nM) inhibited the inhibitory effects of the three β_2-AR agonists only in PTX-pretreated cardiomyocytes,-with β_1-AR blocked by CGP.

6 We conclude that, in adult rat ventricular cardiomyocytes, FEN activates selectively the G_s protein-pathway, while TER and SAL activate the G_s- and G_i-protein pathways. Part of the effects of these three β_2-AR agonists appears to be mediated by β_1-AR.
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Abbreviations: AR, adrenoceptors; CGP, CGP 20712A; FEN, fenoterol; ICI, ICI 118,551; PE, phenylephrine; PTX, pertussis toxin; SAL, salbutamol; TER, terbutaline

Introduction

It is now generally accepted that in the heart of various species, including humans, β_1- and β_2-adrenoceptors (AR) coexist (Brodde & Michel, 1999). Both β-AR subtypes couple to the G_s-protein-adenylyl cyclase pathway and mediate increase in heart rate and contractility. However, evidence has accumulated that, at least in the rat heart, stimulation of β_1-AR causes not only positive ino- and chronotropic effects, but can also evoke apoptosis of the cardiomyocytes (Communal *et al*., 1999; Zaugg *et al*., 2000; Zhu *et al*., 2001; Shizukuda & Buttrick, 2002).

Moreover, β_2-AR in the rat heart have been shown to couple not only to the G_s-protein but also to the G_i-protein (Xiao *et al*., 1995), thereby inducing antiapoptosis (Communal *et al*., 1999; Zaugg *et al*., 2000; Zhu *et al*., 2001; Shizukuda & Buttrick, 2002).

Recently, Xiao *et al*. (2003) showed, in isolated ventricular cardiomyocytes from SHR rats with heart failure, that the contractile response to several β_2-AR agonists, including terbutaline (TER), salbutamol (SAL), zinterol and procaterol, could be markedly enhanced when the cardiomyocytes were pretreated with pertussis toxin (PTX), thereby inactivating the G_i-protein. Interestingly, however, the contractile response to another β_2-AR agonist, fenoterol (FEN), was not affected by PTX treatment indicating that FEN might activate solely the β_2-AR–G_s-protein pathway.

We have recently shown that, in adult rat ventricular cardiomyocytes, stimulation of α_1-AR causes increase in the rate of protein synthesis (Schäfer *et al*., 2001; Pönicke *et al*., 2003). This could be inhibited by isoprenaline *via* β_1-AR stimulation and involves cAMP because the isoprenaline effect

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could be mimicked by dibutyryl cyclic AMP (Schäfer *et al*., 2001).

The aim of the present study was to find out whether also in this system action of FEN might be different from that of TER and SAL. We hypothesized that if FEN activates only the β_2-AR–G_s-protein pathway its inhibition of phenylephrine (PE)-induced increase in cardiomyocyte protein synthesis should be insensitive to PTX treatment, while the inhibitory effects of SAL and TER acting in G_s- and G_i-protein (Xiao *et al*., 2003) should be enhanced by PTX treatment. Thus, we determined, in isolated adult rat ventricular cardiomyocytes, the effects of PTX treatment on FEN-, TER- and SAL-induced inhibition of PE-evoked increase in the rate of protein synthesis (assessed as incorporation of [^{3}H]phenylalanine).

Methods

Preparation of cardiomyocyte culture of adult rats

Adult rat left ventricular cardiomyocytes were isolated from 12-week-old male Wistar rats exactly as detailed elsewhere (Pönicke *et al*., 2000; 2003). Freshly isolated cardiomyocytes were gently diluted in sterile culture medium M199, pH 7.4, supplemented with 10% new-born calf serum. The cardiomyocyte suspension was seeded into 12-well plates (16,000 cells per well) which had been coated with 4% fetal calf serum in medium M199 for 24 h at 37°C (in a humidified incubator at 95% air/5% CO_2) and incubated for 16 h at 37°C. Thereafter, the cultures were rinsed with serum-free Hank's balanced salt solution to remove damaged, rounded and nonattached cardiomyocytes, and the rod-shaped cells were cultured in serum-free medium M199 supplemented with 2 mM L-carnitine, 5 mM taurine, 5 mM creatine and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). To prevent growth of nonmyocytes, the culture medium was supplemented with 10 μM cytosine-β-D-arabinofuranoside.

[^{3}H]phenylalanine incorporation

Protein synthesis by cardiomyocytes was assessed as incorporation of [^{3}H]phenylalanine into cells exactly as described previously (Pönicke *et al*., 2000; 2003). Briefly, after addition of [^{3}H]phenylalanine (0.5 μCi ml^{-1}) at 37°C and 1 μM PE, as well as the indicated concentrations of the β_2-AR agonists TER, FEN and SAL with or without the various antagonists (added 10 min prior to β_2-AR agonists), the cells were incubated overnight (16 h) at 37°C in 95% air/5% CO_2. Ascorbate (100 μM) was always present in the medium throughout this incubation period as antioxidant.

In some experiments, 250 ng ml^{-1} PTX was added to the cardiomyocyte suspension 16 h before the cells were exposed to [^{3}H]phenylalanine (Pönicke *et al*., 2003).

To terminate the [^{3}H]phenylalanine incorporation, cardiomyocytes were washed with ice-cold 0.9% NaCl-solution to remove attached radioactivity and incubated for 24 h at 4°C with 10% trichloroacetic acid. Acid-insoluble precipitates were washed again with 10% trichloroacetic acid and twice with 0.9% NaCl. The remaining precipitation on the culture dishes was solubilized in 1 N NaOH supplemented with 0.1% sodium dodecyl sulfate at room temperature for 24 h, and incorporation of radioactivity into acid-insoluble cell mass was determined by the use of a liquid scintillation counter (Beckman LS 6000). We have recently shown that under these experimental conditions [^{3}H]phenylalanine incorporation was paralleled by increases in protein mass, cell volume and cross-sectional area of the cells, indicating growth of the cardiomyocytes (Schäfer *et al*., 2001).

Statistical evaluations

The data given in text and figures are expressed as means ± s.e.m. of *n* experiments. Experimental data for agonist-induced inhibition of 1 μM PE-evoked [^{3}H]phenylalanine incorporation were analyzed by fitting sigmoidal curves to the experimental data using the GraphPad Prism 3.0 program (GraphPad software, San Diego, CA, U.S.A.); the bottom of the curves was fixed to 100% (i.e. complete inhibition of 1 μM PE-evoked [^{3}H]phenylalanine incorporation into the cardiomyocytes), the Hill slopes were fixed as 1.0. Statistical significance of differences was analyzed by paired two-tailed Student's *t*-test. A *P*-value <0.05 was considered to be significant. All statistical calculations were performed with the GraphPad Prism 3.0 program.

Drugs

L-[2,3,4,5,6-^{3}H]phenylalanine (spec. activity: 5.03 TBq mmol^{-1}) was purchased from Amersham Bioscience Europe GmbH (Freiburg, Germany). PTX was from Calbiochem (Merck Biosciences GmbH, Schwalbach, Germany). L-PE hydrochloride, L-phenylalanine, cytosine-β-D-arabinofuranoside, sodium dodecyl sulfate, trypsin (crude), L-carnitine, taurine, creatine, ICI 118,551 (ICI), TER sulfate and laminin were purchased from Sigma-Aldrich (Deisenhofen, Germany). FEN hydrobromide were kindly donated by Boehringer Ingelheim (Ingelheim, Germany). SAL sulfate was a generous gift of the Glaxo Group Research Ltd (Greenford, U.K.).

(1-[2-((3-carbamoyl-4-hydroxy)phenoxy)-ethyl-amino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol methanesulfonate) (CGP 20712A (CGP)) was kindly donated by Ciba-Geigy (Basel, Switzerland). Hank's balanced salt solution, and culture medium M199 and penicillin–streptomycin were obtained from Life Technologies (Eggenstein, Germany).

All other chemicals were of the highest purity grade commercially available.

Results

Effects of FEN and TER on PE-induced increase in rate of protein synthesis

PE (1 μM) caused, in isolated adult rat cardiomyocytes, an increase in rate of protein synthesis ([^{3}H]phenylalanine incorporation) of about 30% above control. This effect is not affected by the β_1-AR antagonist CGP (300 nM), the β_2-AR antagonist ICI (55 nM) and by PTX treatment, but is completely blocked by the α_1-AR antagonist prazosin (100 nM) (Pönicke *et al*., 2003).

The β_2-AR agonist FEN (1 nM–10 μM) concentration-dependently decreased 1 μM PE-induced increase in the rate of protein synthesis; at 10 μM FEN, the rate of protein

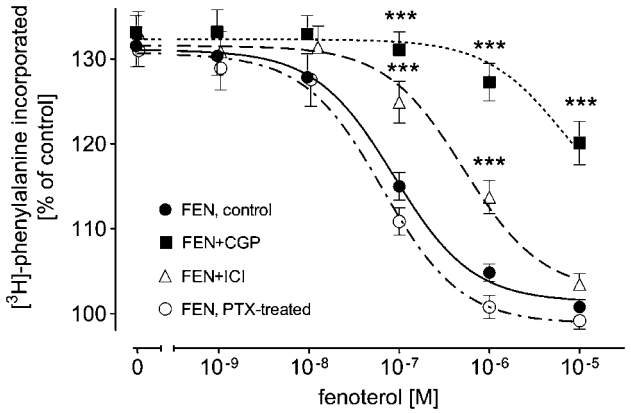

Figure 1 Effects of CGP (300 nM), ICI (55 nM) and PTX pretreatment (250 ng ml^{-1} for 16 h) on FEN-evoked inhibition of 1 μM PE-induced [^{3}H]phenylalanine incorporation in ventricular cardiomyocytes of 12-week-old male Wistar rats ($n=14$). Basal [^{3}H]phenylalanine incorporation amounted to 884 ± 97 c.p.m. in control, 914 ± 105 c.p.m. in the presence of CGP, 874 ± 115 c.p.m. in the presence of ICI and 854 ± 137 c.p.m. after PTX pretreatment. Bars represent mean$\pm$s.e.m. ***$P<0.01$ *versus* FEN.

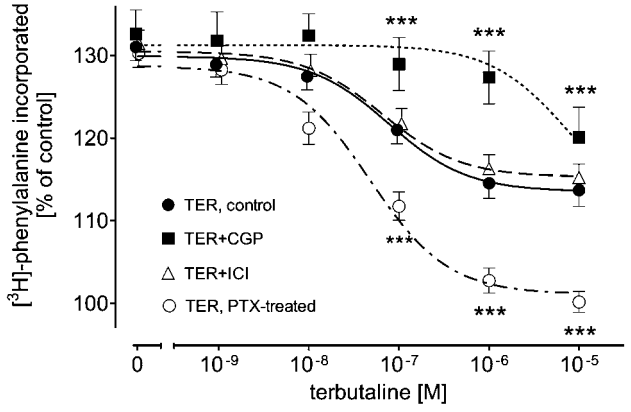

Figure 2 Effects of 300 nM CGP, 55 nM ICI and PTX pretreatment on TER-evoked inhibition of 1 μM PE-induced [^{3}H]phenylalanine incorporation in ventricular cardiomyocytes of 12-week-old male Wistar rats ($n=14$). Basal [^{3}H]phenylalanine incorporation amounted to 1008 ± 131 c.p.m. in control, 1021 ± 107 c.p.m. in the presence of CGP, 998 ± 97 c.p.m. in the presence of ICI and 959 ± 117 c.p.m. after PTX pretreatment. Values are means$\pm$s.e.m. ***$P<0.01$ *versus* TER.

synthesis was nearly completely suppressed (from 132 ± 3 to 101 ± 1% of control, Figure 1).

The β_2-AR agonist TER (1 nM–10 μM) also concentration-dependently decreased 1 μM PE-induced increase in the rate of protein synthesis; however, in contrast to FEN, the highest concentration of TER (10 μM) caused only partial inhibition of the rate of protein synthesis (from 131 ± 2 to 114 ± 2% of control, Figure 2).

Effects of ICI and CGP on FEN- or TER-induced inhibition of rate of protein synthesis

We studied the effects of the highly selective β_2-AR antagonist ICI (55 nM, i.e. a concentration that occupies more than 95% of β_2-AR but less than 5% of β_1-AR; Lemoine *et al*., 1985) on FEN- and TER-induced inhibition of 1 μM PE-induced increase in the rate of protein synthesis. ICI significantly shifted the concentration-inhibition curve of FEN to the right to higher concentrations (Figure 1). In contrast, however, ICI had no significant effect on the concentration–inhibition curve of TER (Figure 2).

On the other hand, the highly selective β_1-AR antagonist CGP (300 nM, a concentration that occupies more than 95% of β_1-AR but less than 1% of β_2-AR; Kaumann & Lemoine, 1987) caused marked shifts to the right of the concentration-inhibition curves of FEN (Figure 1) and TER (Figure 2).

Effects of PTX pretreatment on FEN- or TER-induced inhibition of rate of protein synthesis

We studied the effects of FEN and TER in PTX-pretreated cardiomyocytes. For this purpose cardiomyocytes were treated overnight with PTX (250 ng ml^{-1}) or vehicle at 37°C. We have previously shown that this treatment regimen is sufficient to completely inactivate G_i-protein (Pönicke *et al*., 2003). PTX pretreatment had no significant effect on FEN-induced inhibition of 1 μM PE-induced increase in the rate of protein synthesis (Figure 1). In contrast, TER-evoked inhibition of rate of protein synthesis was significantly enhanced by the PTX treatment: in PTX-treated cardiomyocytes, TER-induced partial inhibition was converted into a complete one (from 130 ± 2 to 100 ± 1% of control; Figure 2).

Effects of ICI on FEN- and TER-induced inhibition of rate of protein synthesis in PTX treated cardiomyocytes in the presence of CGP

In the next series of experiments, we studied, in PTX-pretreated cardiomyocytes with β_1-AR blocked by 300 nM CGP, the effects of ICI (55 nM) on FEN- and TER-induced inhibition of PE-evoked increase in the rate of protein synthesis. In PTX-treated cardiomyocytes, the concentration–response curve for the inhibitory effect of FEN was shifted to the right by CGP (Figure 3), whereby the extent of the rightward shift was comparable with that obtained in not-PTX-treated cardiomyocytes (see Figure 1). Under these experimental conditions, the inhibitory effect of FEN was completely suppressed by ICI (Figure 3).

On the other hand, in PTX-treated cardiomyocytes the rightward shift of the concentration–inhibition curve for TER by CGP (Figure 4) was less than that in not-PTX-treated cardiomyocytes (see Figure 2). Similarly to FEN, the inhibitory effect of TER, too, was seen to be completely suppressed by ICI in PTX-treated cardiomyocytes in the presence of CGP (Figure 4).

Effects of SAL on PE-induced increase in the rate of protein synthesis

In a final set of experiments, we studied the effects of another well-known β_2-AR agonist, SAL, on 1 μM PE-induced increase in the rate of protein synthesis in order to find out whether even under our conditions the pattern of inhibition by SAL resembles that of TER (as described by Xiao *et al*., 2003). SAL (1 nM–10 μM) concentration-dependently decreased 1 μM PE-induced increase in the rate of protein synthesis; however, similar to TER, the highest concentration of SAL (10 μM) caused only partial inhibition in the rate of protein

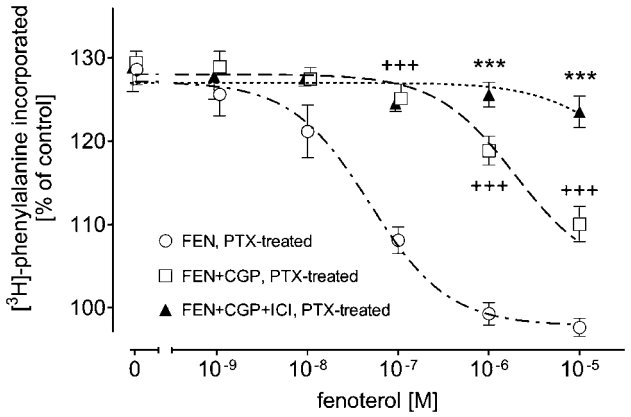

Figure 3 Effects of 300 nM CGP or 300 nM CGP plus 55 nM ICI on FEN-evoked inhibition of 1 μM PE-induced [^{3}H]phenylalanine incorporation in PTX-pretreated ventricular cardiomyocytes of 12-week-old male Wistar rats ($n=8$). Basal [^{3}H]phenylalanine incorporation amounted to 847 ± 86 c.p.m. in PTX-treated control, 871 ± 112 c.p.m. in the presence of CGP and 862 ± 98 c.p.m. in the presence of CGP + ICI. Bars represent mean $\pm$ s.e.m. $^{+++}P<0.01$ *versus* FEN (PTX-treated), $^{***}P<0.01$ *versus* FEN + CGP (PTX-treated).

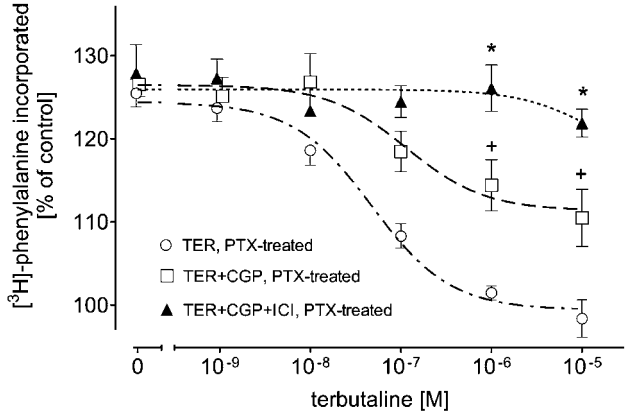

Figure 4 Effects of 300 nM CGP or 300 nM CGP plus 55 nM ICI on TER-evoked inhibition of 1 μM PE-induced [^{3}H]phenylalanine incorporation in PTX-pretreated ventricular cardiomyocytes of 12-week-old male Wistar rats ($n=6$). Basal [^{3}H]phenylalanine incorporation amounted to 912 ± 98 c.p.m. in PTX-treated control, 939 ± 111 c.p.m. in the presence of CGP and 928 ± 95 c.p.m. in the presence of CGP + ICI. Bars represent mean $\pm$ s.e.m. $^{+}P<0.05$ *versus* TER (PTX-treated), $^{*}P<0.05$ *versus* TER + CGP (PTX-treated).

synthesis (from 129 ± 1 to 111 ± 2% of control; Figure 5). The inhibitory effect of SAL was not at all affected by 55 nM ICI, but markedly attenuated by 300 nM CGP. In PTX-treated cardiomyocytes the SAL-induced partial inhibition of rate of protein synthesis was converted into a complete inhibition (Figure 5). Hence, these data confirm that the pattern of SAL effects on the rate of protein synthesis is very similar to that of TER.

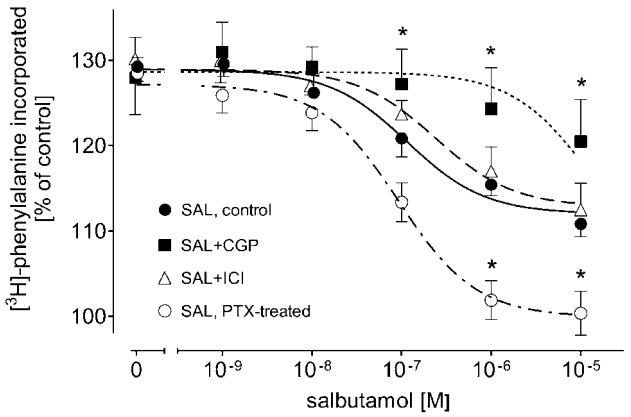

Figure 5 Effects of 300 nM CGP, 55 nM ICI and PTX pretreatment on SAL-evoked inhibition of 1 μM PE-induced [^{3}H]phenylalanine incorporation in ventricular cardiomyocytes of 12-week-old male Wistar rats ($n=7$). Basal [^{3}H]phenylalanine incorporation amounted to 784 ± 121 c.p.m. in control, 795 ± 105 c.p.m. in the presence of CGP, 764 ± 137 c.p.m. in the presence of ICI and 752 ± 92 c.p.m. after PTX pretreatment. Bars represent mean $\pm$ s.e.m. $^{*}P<0.05$ *versus* SAL.

Discussion

Xiao *et al*. (2003) had recently shown, in isolated ventricular cardiomyocytes from SHR rats with heart failure, that the contractile response to several β_2-AR agonists, including TER, SAL, zinterol and procaterol, could be markedly enhanced when the cardiomyocytes were pretreated with PTX, thereby inactivating the G_i-protein. On the other hand, the contractile response to another β_2-AR agonist, FEN, was not affected by PTX treatment.

In rat heart, increase in contractile force is brought about by the β-AR–G_s-protein pathway, and it had been shown that β_2-AR can activate both the rat cardiac G_s- and G_i-protein pathways (Lohse *et al*., 2003; Pönicke *et al*., 2003; Xiao *et al*., 2004). The results from Xiao *et al*. (2003) indicate that, in the isolated ventricular cardiomyocytes from SHR rats with heart failure, most β_2-AR agonists are partial agonists because they stimulate the G_s- and G_i-protein pathways, and they are converted to full agonists after G_i-protein has been inactivated. On the other hand, FEN is a full agonist evoking maximal increases in the force of contraction, which is not affected by PTX treatment. From this, Xiao *et al*. (2003; 2004) had concluded that FEN activates, in isolated adult rat cardiomyocytes, the β_2-AR–G_s-protein pathway selectively.

We have recently shown that, in isolated ventricular cardiomyocytes from adult rats, the increase in protein synthesis evoked by activation of α_{1A}-AR (Pönicke *et al*., 2001) can be inhibited by drugs that activate the G_s-protein–adenylyl cyclase pathway and thereby increase intracellular cAMP content: activation of β_1-AR inhibited PE-evoked increase in the rate of protein synthesis; this was mediated by cAMP because it could be mimicked by dibutyryl cAMP and could be abolished by the cAMP antagonist Rp-cAMPS (Schäfer *et al*., 2001). The mechanism underlying this β-AR-mediated inhibition of the α_1-AR-induced hypertrophic response is not completely clear.

However, it appears that the β_1-AR–G_s- and β_2-AR–G_s-protein induced apoptosis counteracts the α_1-AR-mediated increase in the rate of protein synthesis. This hypothetical view is supported by our recent findings that, in isolated ventricular cardiomyocytes of adult rats, there was a significant negative correlation between β_1- and β_2-AR-mediated apoptosis and

α_1-AR-mediated increase in the rate of protein synthesis (Pönicke *et al*., 2003).

In the present study, we have used this system to test whether also under these conditions FEN might be a selective activator of the β_2-AR–G_s-protein pathway, whereas other β_2-AR agonists such as TER or SAL activate both the β_2-AR–G_s-protein and the β_2-AR–G_i-protein pathways.

As shown in Figures 1, 2 and 5, indeed FEN was a full agonist in inhibiting PE-induced increase in protein synthesis, while TER and SAL were only partial agonists; moreover, FEN effects were not at all affected by PTX pretreatment of the cardiomyocytes, while PTX treatment converted the partial agonist activities of TER and SAL into full agonist activities. These data are compatible with the view that FEN activates only a β-AR–G_s-protein pathway, whereas TER and SAL activate both the β-AR–G_s-protein and the β-AR–G_i-protein pathway. Thus, so far our data are in good agreement with those published by Xiao *et al*. (2003).

However, when we tried to further classify the β-AR subtype involved in the effects of FEN, SAL and TER, we found that the highly selective β_1-AR antagonist CGP, used in a concentration (300 nM) that does not at all act at β_2-AR (Kaumann & Lemoine, 1987), caused a marked shift to the right of the concentration–inhibition curves of all the three β_2-AR agonists. This effect was only marginally affected when the G_i-protein–adenylyl cyclase pathway was inactivated by PTX. Thus, it appears that, in adult rat cardiomyocytes, part of the action of the β_2-AR agonists FEN, TER and SAL is mediated by activation of the β_1-AR–G_s-protein pathway. The issue whether or not in isolated ventricular cardiomyocytes from adult rats effects of β_2-AR agonists might (at least partly) be mediated by β_1-AR is still being controversially discussed, with some authors finding a strong participation of β_1-AR (Kuznetsov *et al*., 1995; Laflamme & Becker, 1998) and others finding no involvement of β_1-AR (Xiao *et al*., 2003; for further references, see Xiao *et al*., 2004).

In contrast to CGP, in the present study, the highly selective β_2-AR antagonist ICI, used at a concentration (55 nM) that does not at all act at β_1-AR (Lemoine *et al*., 1985), caused a significant shift to the right only of the concentration–inhibition curve of FEN, but did not significantly affect the concentration–inhibition curves of TER and SAL. This untypical behavior of ICI might be explained as follows: if the agonist activates the β_2-AR–G_s-protein pathway, protein synthesis is inhibited and that is antagonized by ICI, that is the concentration-inhibition curve of that agonist should be shifted to the right by ICI. If the agonist activates in addition to the β_2-AR–G_s-protein pathway the β_2-AR–G_i-protein pathway, inhibition of protein synthesis is attenuated; now ICI inhibits this attenuating effect of the agonist, that is inhibition of protein synthesis is enhanced and hence the concentration–inhibition curve of that agonist should be shifted to the left by ICI. In the present study, FEN activates – besides β_1-AR (see above) – solely the β_2-AR–G_s-protein pathway; accordingly, its concentration–inhibition curve should be shifted to the right by ICI, which is indeed the case (see Figure 1). On the other hand, SAL and TER activate – besides β_1-AR – the β_2-AR–G_s-protein pathway (its inhibition by ICI should lead to a rightward shift of the concentration–inhibition curve) and the β_2-AR–G_i-protein pathway (its inhibition by ICI should lead to a leftward shift of the concentration–inhibition curve). The fact that ICI does not significantly affect the concentration–inhibition curves of these two agonists, therefore, implies that activation of β_2-AR by both agonists involves the G_s- and G_i-protein pathways to nearly equal amounts.

Taken together so far, in isolated adult rat ventricular cardiomyocytes inhibition of PE-evoked increase in the rate of protein synthesis by the β_2-AR agonists FEN, TER and SAL is (partly) mediated by β_1-AR stimulation; in addition, the effects of TER and SAL involve a PTX-sensitive inhibitory component. Hence, the effects of these three β_2-AR agonists *via* the pure β_2-AR–G_s-protein pathway can be only demonstrated when β_1-AR are blocked and the G_i-protein pathway is inactivated. And this is indeed the case: as shown in Figures 3 and 4, in cardiomyocytes pretreated with PTX, inhibition of protein synthesis by FEN and TER, in the presence of 300 nM CGP, is now completely inhibited by the highly selective β_2-AR antagonist ICI.

Conclusion

In adult rat ventricular cardiomyocytes FEN activates only the β_2-AR–G_s-protein pathway, while TER and SAL activate the β_2-AR–G_s- and β_2-AR–G_i-protein pathways. Part of the effects of these three β_2-AR agonists in the rat cardiomyocytes appear to be mediated by β_1-AR.

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