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β-adrenergic Antagonists Influence Abdominal Aorta Contractility by Mechanisms not Involving β-adrenergic Receptors*

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β-adrenergic receptors (β-AR) are widely distributed in the cardiovascular system, where they considerably contribute to the control of its functions. β-blockers are commonly used in the treatment of disorders of the circulatory system. They act primarily by inhibiting cardiac β-receptors. However, there are also reports of pleiotropic action of β-blockers as well as of new compounds created to study β_3 adrenergic receptors. The study aimed to investigate additional mechanisms of action of β-AR inhibitors in the rabbit abdominal aorta with emphasis on their action on α-adrenergic receptors and calcium influx. Responses to propranolol, betaxolol, metoprolol and SR59230A were evaluated in phenylephrine and PGF_{2alpha} precontracted aortic rings. The effect of propranolol on the phenylephrine concentration-contraction curve was examined. Propranolol (≥10 μM) and SR59230A (≥0.1 μM) induced relaxations in phenylephrine-precontracted rings, while betaxolol and metoprolol had little effect. The β-AR inhibitors produced further contraction of tissues preincubated with PGF_{2alpha}, excluding SR59230A, which after initial contraction, elicited marked relaxation at a concentration above 1 ěM. 100 μM of propranolol caused a significant rightward shift of the concentration-contraction curve to phenylephrine with no reduction in the maximum response. Incubation of aortic rings in phentolamine reduced the maximal contraction to propranolol; verapamil pretreatment by contrast enhanced contractile response. In conclusion, SR59230A and propranolol most probably act as α_1-AR competitive antagonists in the presence of phenylephrine in rabbit abdominal aortic rings. After α-ARs blockade, propranolol exerts a weak relaxing activity connected with Ca^{2+} channel inactivation. SR59230A at a high concentration acts on the rabbit aorta by an additional mechanism needing further investigation.

Key words: abdominal aorta, SR59230A, propranolol, α-adrenergic antagonist, calcium channels, PGF_{2alpha}.

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Aortic wall contractile function is primarily regulated by sympathetic nerve fibres interacting with α_1-adrenergic receptors (α_1-ARs), although multiple other factors also affect vascular smooth muscle contractility. ß-adrenergic receptors (β-ARs) are thought to play a role in vasodilatation. Since vascular beta-receptors were classified as $ß_1$ and β_2 subtypes, their function has been intensively studied (LANDS *et al*. 1967; MALLEM *et al*. 2005). Moreover, in subsequent years, reports have appeared of new subtypes in the vascular bed, namely β_3 and low state affinity β_1 receptors. How-

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ever, existence of the latter in the aorta is still under discussion (MACDONALD *et al.* 1999; BRAWLEY *et al.* 2000; BRAHMADEVARA *et al.* 2004).

β-AR blocking agents may contract the aorta and elicit a rise in blood pressure; conversely, they are widely used in the treatment of hypertension and cardiovascular conditions, mainly because of their influence on cardiac β-AR, which are accelerating in nature (REITER 2004). Negative inotropic and chronotropic effects can result in the lowering of blood pressure, but additional hypotensive mechanisms, like reduction in peripheral vascular resistance, are also involved (VAN DEN MEIRACKER *et al.* 1998; GORRE & VANDEKERCKHOVE 2010). Although recent clinical data suggests that beta-blockers may have less influence on central aortic pressure despite lowering brachial blood pressure, these trials mainly concerned atenolol which does not elicit vasorelaxation *in vitro* (DHAKAM *et al.* 2006; PRIVIERO *et al.* 2006). Third generation β-AR blockers were shown to relax arteries through a nitric oxide pathway and α-AR antagonism. They exert antioxidant properties as well (MONINGKA *et al.* 2012). However, pleiotropic effects of earlier generations of β-blockers have also been described. Mechanisms of action different from inhibition of β-AR are activated at higher concentrations when examined *in vitro* (MAK & WĘGLICKI 1988; DUNXENDORFER & WIEDERMAN 2000), although their role in the therapeutic effects of β-AR blockers cannot be excluded, especially under pathological conditions, since as lipophilic substances they might cumulate in tissues (MASON *et al.* 1991; PACCA *et al.* 2002; ORIJI 2003).

There is some evidence that conventional β-blockers act at calcium channels, but little is known about their possible α-AR antagonistic properties. On the contrary, β_3-AR ligands, especially aryloxypropanolamines, are considered as non-specific α_1-inhibitors (HOSTE & SYS 1998; MELENA *et al.* 1999; KOZŁOWSKA *et al.* 2005). Is a α-AR blockade a more general property of the β-AR antagonists? Are there other mechanisms involved in the non-specific action of β_3-blockers? We have attempted to elucidate these questions in the present work.

Methods

Abbreviations:
α-AR – alpha-adrenergic receptor;
ß-AR – beta-adrenergic receptor;
PGF_{2alpha} – prostaglandin 2 alpha

Animal preparation

The experiments were carried out on ten 16-week-old female New Zealand rabbits. The animals were euthanized by intravenous pentobarbital. Abdominal aortas were isolated immediately afterwards and carefully cleaned of fat and connective tissues and cut into 5 mm wide rings. The study was performed with the approval of the II Local Ethical Committee, consent no. 89/2010.

The prepared aortae rings were mounted on silk threads in 20 ml organ bath chambers filled with Krebs-Henseleit' solution. The Krebs' buffer was composed as follows: NaCl – 118 mM; KCl – 4.7 mM; $CaCl_2$ – 2.5 mM; $MgSO_4$ – 1.6 mM; $NaHCO_3$ – 24.3 mM; KH_2PO_4 – 1.18 mM; glucose – 5.6 mM was bubbled continuously with 95% O_2 and 5% CO_2 in order to obtain a pH value of 7.3-7.5.

The tissues were placed under 1 g resting tension and allowed to equilibrate for 60 minutes. During this period, the resting tension was readjusted several times.

Aortae contractions were recorded by isotonic transducers (Letica Scientific Instruments, Spain) connected with bridge amplifiers (BridgeAmp, ADInstruments, New Zealand) and a data acquisition system (PowerLab, ADInstruments, New Zealand).

Experimental protocol

After the equilibration period, aortae rings with intact endothelium were precontracted either with phenylephrine (0.6 μM) or with $PGF2_{alpha}$ (3 μM). After stabilisation of contraction, cumulative concentration-reaction curves to nonselective and selective ß-AR antagonists: propranolol, betaxolol, metoprolol and SR59230A, were performed. In some experiments, the tissues were pretreated with the α_1-AR antagonist phentolamine (1 μM) or verapamil (1 μM) for 30 min. and then contracted with $PGF2_{alpha}$. In other experiments, the role of α-AR in the relaxant reaction of rabbit aortae to propranolol was evaluated by obtaining cumulative concentration-contraction curves to phenylephrine in rings preincubated for 30 minutes with propranolol (1 μM or 10 μM) and without pretreatment.

Drugs

(±)-Propranolol hydrochloride, Betaxolol hydrochloride, (±)-Metoprolol (+)-tartrate salt, SR59230A (3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-2S-2-propanol oxalate), (*R*)-(-)-Phenylephrine hydrochloride, PGF_{2alpha} tris salt, (±)-Verapamil hydrochloride were purchased from Sigma – Aldrich. A stock solution of PGF_{2alpha} was prepared with ethanol. The remaining drugs were dissolved in distilled water.

Statistical analysis

All results are expressed as mean ± SEM of n rings (sections obtained from n animals). The reaction to the ß-AR antagonists is expressed as the percentage of the contraction induced by phenylephrine or PGF_{2alpha}.

Statistical analyses were performed using Student's t-test and two-way analysis of variance followed by the Tukey multiple comparison post hoc for comparison of three or more groups. $P \leq 0.05$ was considered as significant.

Table 1

Relative relaxant effect of SR 59230A and propranolol depicted as concentrations ($-\log_{10}$ M) eliciting responses equal to 25 and 50% of reaction evoked by 0.6 μM phenylephrine

Antagonist	25% (mean±SEM)	50% (mean±SEM)
SR59230A	5.37±0.54	5.02±0.55
Propranolol	4.90±0.33	4.68±0.36

Results

Effect of ß-AR antagonists on phenylephrine-constricted aortic rings

Propranolol and SR59230A induced dose-dependent full relaxation of phenylephrine precontracted rings. However, both substances were effective at high concentrations: concentrations [$-\log_{10}$ M] eliciting relaxation equal to 50% of reaction induced by phenylephrine was 5.37 (n=10) for propranolol and 4.90 for SR59230A (n=5). In contrast, betaxolol and metoprolol failed to produce marked relaxation: E_{max}= 27.8±9.28% (n=8) and 37.9±38.49% (n=9) of contraction produced by phenylephrine, respectively. ß-AR antagonists elicited a reaction with an order of potency: SR59230A >> propranolol >betaxolol >> metoprolol (Table 1, Fig. 1).

Incubation in propranolol at a concentration of 10 μM had no effect on the phenylephrine cumulative concentration-contraction curve. 100 μM of antagonist caused a significant rightward shift of the concentration-contraction curve with no reduction in the maximum response, suggesting competitive antagonism (Fig. 2).

Effect of ß-AR antagonists on PGF_{2alpha} preconstricted rings

Neither of the examined ß-AR antagonists elicited a relaxant response in PGF_{2alpha} preconstricted aortic rings at low concentrations. On the contrary, all of them elicited a dose-dependent contraction (ranging from 10 nM for betaxolol and SR59230A or 0.1 μM for propranolol and metoprolol to 100 μM for propranolol and betaxolol and 1mM for metoprolol) excluding SR59230A (n=5), which produced a marked relaxation at a concentration >1 μM. The magnitude of reaction induced by propranolol (n=7) and betaxolol (n=5) was comparable (Table 2, Fig. 3). Metoprolol (n=6) was most effective in eliciting contraction. Contraction to the ß-AR an-

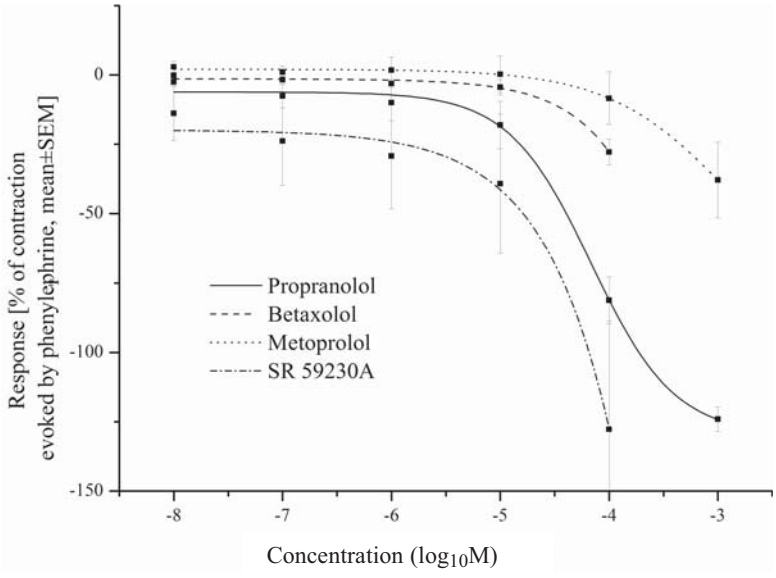

Fig. 1. Relaxation of rabbit abdominal aortic rings elicited by ß-AR antagonists. Propranolol and SR59230A induced marked relaxations, whereas betaxolol and metoprolol were capable of inducing only slight relaxant responses at high concentrations. Features of relaxation curves to propranolol and SR59230A suggest additional mechanism of action at higher concentrations (E_{max} >100%).

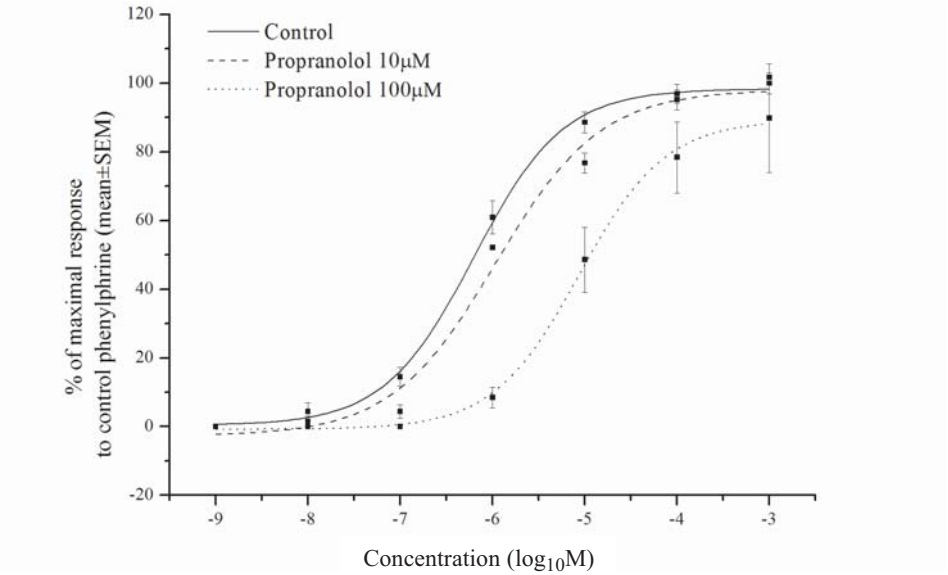

Fig. 2. Concentration-response curve (CRC) to 10-fold increasing concentrations of phenylephrine without and after preincubation with 10 or 100 μM propranolol. Propranolol at concentration of 100 μM markedly shifted rightward CRC to phenylephrine without significant reduction of maximum response, thus indicating competitive antagonism at α_1-adrenergic receptors (* $p \leq 0.001$).

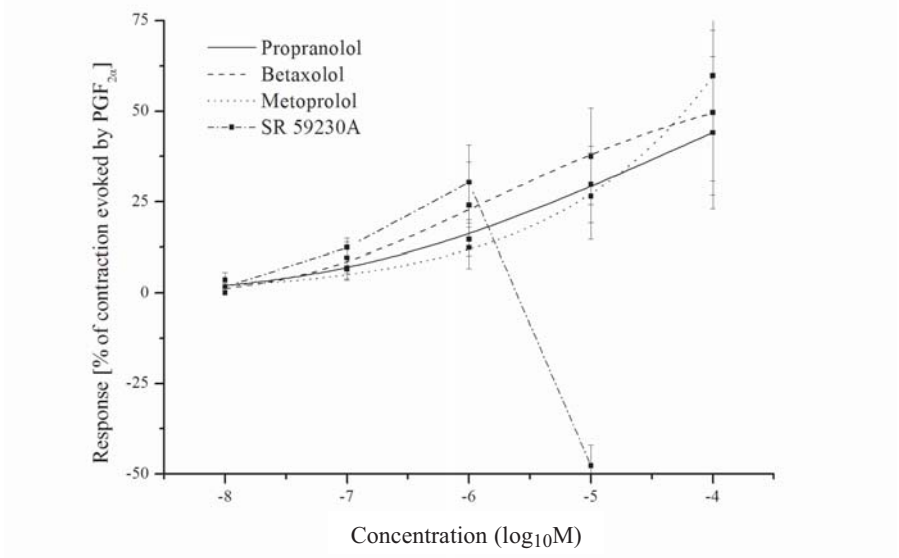

Fig. 3. Reaction of rabbit abdominal aorta to ß-AR antagonists after incubation with PGF_{2alpha}. Propranolol, betaxolol and metoprolol elicited contraction, whereas SR59230A after initial contraction relaxed aortic rings at high concentration (>1μM). Propranolol at 1 mM also elicited relaxation as well as entirely inhibited reaction to phenylephrine (probably by Na^+ channel blockade; data not shown).

tagonists after PGF_{2alpha} pretreatment was diminished by verapamil, which was added at the end of the experiment (data not shown).

Effect of α_1-AR blockade and calcium depletion on reaction to propranolol in PGF_{2alpha} preconstricted rings

In order to determine the possible mechanisms involved in a propranolol elicited reaction, a α_1-AR antagonist or L-type calcium channel blocker were

Table 2

Maximal contractile reaction to ß-AR antagonists* in PGF_{2alpha} preconstricted rabbit abdominal aortic rings (as % of PGF_{2alpha} induced reaction)

Antagonist	E_{max} (mean±SEM)
SR59230A	30.40±6.33 %
Propranolol	44.08±20.95 %
Betaxolol	49.63±22.80 %

*Metoprolol is not listed since it elicited further contraction at concentration of 1mM.

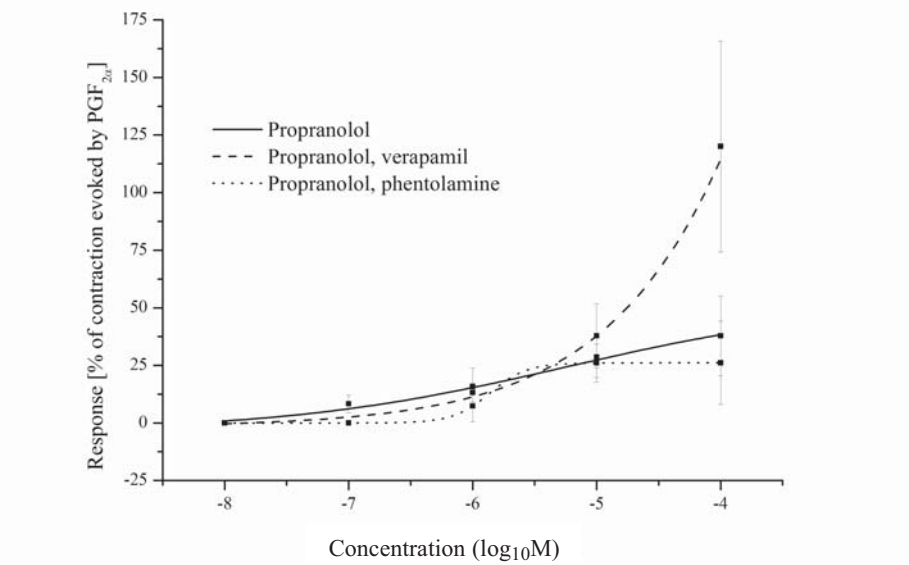

Fig. 4. Reaction to propranolol in PGF_{2alpha} preconstricted abdominal aortic rings after incubation in phentolamine or verapamil. Blockade of α_1-ARs with phentolamine reduced maximum contraction to propranolol. Conversely verapamil preincubation enhanced the contraction.

Table 3

Reaction to propranolol in PGF_{2alpha} preconstricted abdominal aortic rings, effect of α_1-AR blockade and extracellular calcium depletion ($^*P \leq 0{,}05$)

Treatment	E_{max} (mean±SEM)
Propranolol	44.08±20.95 %
Verapamil, propranolol	112.64±38.14 %*
Phentolamine, propranolol	10.34±7.23 %*

added to the organ bath prior to prostaglandin. Incubation of aortic rings in phentolamine (n=5), a α_1-AR antagonist, reduced the maximal contraction to propranolol. Verapamil pretreatment (n=5), by contrast, enhanced the contractile response to propranolol, and the maximal reaction was significantly stronger ($p \leq 0.05$) than after a α_1-AR antagonist (Table 3, Fig. 4).

Discussion

In the present study, we have shown that propranolol and SR59230A dose-dependently relaxed rabbit abdominal aortic rings preconstricted with phenyephrine. Taking into consideration that the abovementioned ß-adrenergic antagonists were effective in concentrations at least 10 times greater than those required to antagonise ß-adrenergic receptors and that there are reports of various mechanisms of the action of ß-AR antagonists, we assume that propranolol and SR59230A relaxed the phenyephrine precontracted rabbit aorta by acting at binding sites other than the beta-adrenoreceptors. Betaxolol and metoprolol also induced relaxation in phenylephrine precontracted aortic rings, but to a lesser extent. Similarly to propranolol and SR59230A, both selective $ß_1$-adrenergic receptor antagonists were effective at high concentrations, indicating a mechanism not related to ß-ARs. To determine whether α-ARs play a role in relaxation of phenylephrine precontracted aortae, the reaction to ß-AR antagonists in PGF_{2alpha} preconstricted preparations was examined. Propranolol, metaxolol and betaxolol caused further contraction of precontracted tissues. SR59230A elicited a contraction which switched to relaxation at higher concentrations (>1 μM). These findings show that SR59230A and propranolol most probably act as α_1-AR competitive antagonists in the presence of phenylephrine, similarly to dobutamine, which relaxes rabbit aorta precontracted with phenylephrine but fails to elicit relaxation after PGF_{2alpha} or K^+ preconstriction (AIKAWA *et al.* 1996). Further evidence provides a parallel rightward shift of the concentration-contraction curve to phenylephrine without significant depression in the maximum response after preincubation with 100 μM propranolol. 10 μM failed to influence the contraction induced by phenylephrine, unlike 1 mM of propranolol, which entirely abolished the reaction. This data suggests competitive antagonism of 100 μM propranolol at α_1-AR.

The α_1-AR antagonistic properties of SR59230A and propranolol were also postulated by other authors. It was found that preincubation of isolated pulmonary arteries with SR59230A (<1 μM) pro-

duced a rightward shift of the concentration-response curve to phenylephrine with no significant changes in the maximum reaction (LEBLAIS *et al.* 2005). Competitive antagonism of SR59230A toward phenylephrine was also reported in rat mesenteric arteries as well as rat aorta. Moreover, SR59230A was able to relax those tissues precontracted with phenylephrine or noradrenaline (BRAHMADEVARA *et al.* 2003; BRIONES *et al.* 2005). Propranolol has been shown to be effective in eliciting the relaxation of phenylephrine or norepinephrine precontracted rat aorta and mesenteric artery rings (MOSTAGHIM *et al.* 1986; BRAHMADEVARA *et al.* 2003; PRIVIERO *et al.* 2006). Finally, it has been shown that SR59230A and propranolol competitively displaced the (^{3}H)-prazosin binding in various tissue preparations. α_1-AR subtype distribution and significance vary depending on vessel type. In the rat mesenteric artery, α_{1A}-AR is predominant; in the aorta - $\alpha_{1D;}$ and in the spleen - α_{1B}-AR. This supports the subtype non-specific action of ß-AR antagonists at α_1-AR (BRAHMADEVARA *et al.* 2004; LEBLAIS *et al.* 2004; BEXIS & DOCHERTY 2009).

Assuming that the examined substances act as α_1-AR antagonists, it is expected that they would fail to induce relaxation in tissues precontracted with agents other than α-AR agonists. In our study, propranolol, betaxolol, metoprolol and SR59230A (<1 μM) did not cause relaxation of rabbit abdominal aorta precontracted with PGF_{2alpha}. A lack of the relaxing effect of different ß-AR antagonists on tissues preincubated with PGF_{2alpha} was also demonstrated in rat aorta. Moreover, propranolol did not elicit relaxation in rat aorta contracted with U-46619 (MOSTAGHIM *et al.* 1986, BRAHMADEVARA *et al.* 2003). However, there is contradicting data about relaxation to propranolol, SR59230A and betaxolol after pretreatment with contracting agents other than phenylphrine. It was shown that propranolol (10-100 μM) relaxed endothelium intact rat mesenteric arteries preincubated with U-46619 as well as endothelin-1 (PRIVIERO *et al.* 2006). SR59230A (≥1 μM) and propranolol (≥10 μM) were able to decrease hypoxic pressure responses in pulmonary vessels which are believed to be elicited by voltage dependent K^+ channel inactivation followed by depolarisation (DUMAS *et al.* 1998).

The relaxant properties of ß-AR antagonists, exerted independently of the preconstrictor used, indicate additional mechanisms of action. We have shown that propranolol, betaxolol, metoprolol and lower concentrations of SR59230A (≤1μM) induced further contraction of the preparations. Similar observations have been made in rat pulmonary arteries, where CGP 12177 and SR59230A enhanced the tension produced by PGF_{2alpha} (LEBLAIS *et al.* 2004, 2005). On the one hand, this supports their α-AR binding properties; on the other hand, it also suggests the possibility of an additional mechanism of action, whereas relaxation induced by high concentrations of SR59230A (>1 μM) directly indicates an additional mechanism of action on the blood vessels.

Contraction in response to ß-AR antagonists after PGF_{2alpha} preincubation could be explained by the enhancement of the contractile reaction. Incubation with PGF_{2alpha} increased the sensitivity of the rat intrapulmonary artery to phenylephrine and uncovered the contractile properties CGP 12177 that had no effect on the basal tone. It was concluded that at least in part, CGP 12177 action could be attributed to α-AR activation, since phenoxybenzamine (an irreversible α-AR antagonist) blunted the contraction (LEBLAIS *et al.* 2004). It was shown that the reaction to some receptor activation may be sensitised by an increased vascular tone, and α-AR can be potentiated by activation of other receptors coupled to the G_q protein (CHOPPIN & O'CONNOR 1995; FABI *et al.* 1998). There is also evidence that PGF_{2alpha} and tromboxane enhance contractile responses by increasing sensitivity to Ca^{2+} and inhibiting myosin light chain dephosphorylation (ITO *et al.* 2003).

In order to reveal if binding of ß-AR antagonists to α-ARs is involved in the contractile response of PGF_{2alpha} preconstricted vessels, we preincubated the aortic rings in phentolamine before propranolol was added. After an initial slight contraction, the aortae tended to relax at high concentrations of propranolol (100 μM). Incubation with verapamil significantly enhanced contraction in response to propranolol as compared with tissues in which α-ARs were blocked. We conclude that after a α-ARs blockade, propranolol exerts weak relaxing activity connected with Ca^{2+} channel inactivation; whereas after extracellular Ca^{2+} deprivation by preincubation of tissues with the L-type Ca^{2+} blocker verapamil, propranolol presumably acted via α-AR activation due to sensitisation of the contractile apparatus after PGF_{2alpha} pretreatment. It seems that the influence on eicosanoid formation is unlikely, since indomethacin did not affect the relaxation of rat aortic and mesenteric rings induced by propranolol (PRIVIERO *et al.* 2006). Moreover, propranolol inhibited thromboxane A_2 production induced by cyclosporine A in rat aortae (ORIJI & SCHANZ 2001). There are numerous reports on the calcium influx blocking properties of ß-AR inhibitors in various vascular beds (CEKIC *et al.* 2013; HOSTE & SYS 1998; HAYASHI-MORIMOTO *et al.* 1999; PRIVIERO *et al.* 2007).

We obtained very interesting results concerning the reaction to SR59230A in PGF_{2alpha} precontracted aortae. High concentrations of SR59230A produced marked relaxation, whereas lower con-

centrations induced contraction. This suggests an additional mechanism of action which needs further investigation. Our results are consistent with data obtained by other authors. SR59230A was found to relax non-precontracted systemic arteries and to reduce the basal tone (BRAHMADEVARA *et al.* 2003; BRIONES *et al.* 2005). It was also capable of eliciting a relaxant response in a hypoxic vasoconstricted lung (DUMAS *et al.* 1998). An inverse reaction was noted in the pulmonary artery in which SR59230A had a contractile effect on non-precontracted arteries and enhanced the contraction elicited by PGF_{2alpha}. The latter property was not prevented by incubation with phenoxybenzamine, indicating a mechanism not related to α-ARs (LEBLAIS *et al.* 2005). Opposing reactions in the pulmonary and systemic arteries suggest that factors with a different physiological role in systemic and pulmonary circulation might be involved. Results showing that SR59230A exerts an inhibitory action on cardiac potassium channels have been recently published (KULZER *et al.* 2012). There is also evidence that it acts as an atypical $ß_3$-AR agonist in various tissues, although it seems unlikely that this mechanism has a role in the aorta, since many reports contradicting the existence of $ß_3$-ARs and the low affinity state of $ß_1$-ARs in systemic arteries have been encountered (BRAHMADEVARA *et al.* 2003; BRIONES *et al.* 2005; HORINOUCHI & KOIKE 2001; HUTCHINSON *et al.* 2005).

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