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Beta 3-adrenoceptor in rat aorta: molecular and biochemical characterization and signalling pathway

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1 We have previously demonstrated that β_3-adrenoceptor (β_3-AR) stimulation induces endothelium-dependent vasorelaxation in rat aorta through the activation of an endothelial NO synthase associated with an increase in intracellular cGMP. The aim of the present study was to localise β_3-AR to confirm our functional study and to complete the signalling pathway of β_3-AR in rat aorta.

2 By RT–PCR, we have detected β_3-AR transcripts both in aorta and in freshly isolated endothelial cells. The absence of markers for adipsin or hormone-sensitive lipase in endothelial cells excluded the presence of β_3-AR from adipocytes. The localization of β_3-AR in aortic endothelial cells was confirmed by immunohistochemistry using a rat β_3-AR antibody.

3 To identify the G protein linked to β_3-AR, experiments were performed in rat pre-treated with PTX (10 μg kg^{-1}), a $G_{i/0}$ protein inhibitor. The blockage of $G_{i/0}$ protein by PTX was confirmed by the reduction of vasorelaxation induced by UK 14304, a selective α_2-AR agonist. The cumulative concentration-response curve for SR 58611A, a β_3-AR agonist, was not significantly modified on aorta rings from PTX pre-treated rats.

4 At the same level of contraction, the relaxations induced by 10 μM SR 58611A were significantly reduced in 30 mM-KCl pre-constricted rings ($E_{max} = 16.7 \pm 8.4\%$, $n = 5$), in comparison to phenylephrine (0.3 μM) pre-constricted rings ($E_{max} = 49.11 \pm 11.0\%$, $n = 5$, $P < 0.05$). In addition, iberotoxin (0.1 μM), glibenclamide (1 μM) and 4-aminopyridine (1 mM), selective potassium channels blockers of K_{Ca}, K_{ATP}, and K_v respectively, decreased the SR 58611A-mediated relaxation.

5 We conclude that β_3-AR is preferentially expressed in rat aortic endothelial cells. β_3-AR-mediated aortic relaxation is independent of $G_{i/0}$ proteins stimulation, but results from the activation of several potassium channels, K_{Ca}, K_{ATP}, and K_v.

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Abbreviations: 4-AP, 4-aminopyridine; β-AR, β-adrenoceptor ; BSA, bovine serum albumin; cDNA, complementary DNA; E_{max}, maximal relaxant response; K_{ATP}, ATP dependent potassium channels; BK_{Ca}, calcium-dependent potassium channels of big conductance; K_v, voltage-dependent potassium channels; PTX, pertussis toxin; RAEC, rat aortic endothelial cells; rβ_3-AR Ab: rat β_3-AR antibody; RT–PCR, reverse transcription-polymerase chain reaction; SR 58611A, ethyl [(7)S)-((2R)2-(3-chlorophenyl)-2-hydroxyethyl]amino]-5,6,7,8-tetrahydronaphtyl2-yl] oxyacetate hydrochloride; TAE, tris acetate EDTA buffer; UK 14304, 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline; vWf Ab, von Willebrand factor antibody

Introduction

Beta-adrenoceptors (β-AR) of the β_1 and β_2 subtypes classically mediate the effects of catecholamines on the relaxation of vascular smooth muscle, β_2-AR subtype being the most important (Guimaraes & Moura, 2001). Recently, the use of β_1/β_2-AR antagonists and β_3-AR agonists in several studies *in vivo* and *in vitro* provided evidence for the functional expression of β_3-AR in some vascular beds in addition to β_1- and β_2-AR subtypes (Gauthier *et al*., 2000). As for other tissues, the effects of β_3-AR stimulation in vessels vary across species. In rat isolated common carotid arteries, BRL 37344, a preferential β_3-AR agonist, produced a significant relaxation, but with a slower kinetic than that induced by isoprenaline, a non selective β-AR agonist (Oriowo, 1994). We have obtained similar results in rat thoracic aorta where isoprenaline produced a rapid relaxation whereas SR 58611A, another preferential β_3-AR agonist, caused slowly developing relaxations (Trochu *et al*., 1999). However, to our knowledge, expression of β_3-AR in rat arteries has not been investigated.

The β_3-AR-signalling pathway in vessels is partially characterized and shows apparent variability according to the vascular bed studied. In rat carotid arteries, β_3-AR-induced relaxation was mainly endothelium-independent (Oriowo, 1994), whereas it was strongly reduced by endothelium removal in the rat thoracic aorta, suggesting a predominant localization of β_3-AR on the endothelial cells (Trochu *et al*., 1999). In this latter model, the β_3-AR

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stimulation activated an endothelial NO synthase leading to an increase in intracellular cGMP (Trochu *et al*., 1999). The G protein linked to β_3-AR in this model is not yet determined. It is now acknowledged that β_3-AR can be coupled with G_s or $G_{i/0}$ protein (Strosberg, 1997). However, the type of G protein depends on tissues studied and/or the pharmacological profile of β_3-AR (Gauthier *et al*., 2000).

Effectors involved in the signalling pathway subsequent to the stimulation of β_3-AR have not yet been identified in the rat thoracic aorta. The vascular tone is regulated by activation of several potassium (K^+) channels (for review, see Sobey, 2001). In perfused rat lungs, β_3-AR agonists induced vasorelaxant effects on the vasoconstriction elicited by hypoxia (Dumas *et al*., 1999). This vasorelaxation resulted from the activation of low and high conductance Ca^{2+}-dependent K^+ channels, SK_{Ca} and BK_{Ca}, respectively (Dumas *et al*., 1999).

The aim of the present study was to characterize β_3-AR in rat aorta. The first objective was to determine the expression and the localization of β_3-AR. The expression of mRNA encoding for this receptor was studied by RT–PCR in intact tissue and in endothelial cells isolated from aorta. Then, the localization of the β_3-AR in this vessel was determined by immunohistochemistry. The second objective was to examine the signalling pathway of β_3-AR i.e. the potential involvement of the pertussis toxin (PTX)-sensitive G protein and the type of potassium channels (BK_{Ca}, K_{ATP}, K_v) involved in the β_3-AR-induced vasorelaxation.

Methods

Animals

Male Wistar rats (200–270 g) were purchased from Janvier (Le Genest St Isle, France). They were housed at 18°C and submit to 0700–1900 h light–dark cycle. Rats were anaesthetized with pentobarbital (30 mg kg^{-1}, i.p.).

Tissue preparation

The thoracic aorta was dissected as previously described (Trochu *et al*., 1999). The descending thoracic aorta was cleared of fat and connective tissues.

Isolation of rat aortic endothelial cells

Rat aortic endothelial cells (RAEC) were dissociated as described by Battle *et al*. (1994). RAEC were isolated by collagenase digestion. Thoracic aorta was removed and rinsed with PSS (Physiological Salt Solution) having the following composition (in mM): NaCl, 130; KCl, 5.6; $MgCl_2$, 1; HEPES, 10; pH adjusted to 7.4 with NaOH. The adventitia was removed. The remaining media and intima were cut in thin rings (1 mm thick). Endothelial cells were obtained by incubating these rings for 30 min in PSS containing 1248 u ml^{-1} collagenase type 1 (Worthington Biochemial Corporation, Lakewood) followed by a mechanical dispersion. The cell suspension was centrifuged ($500 \times g$, 5 min) and RNAs were extracted from the cell pellet. Endothelial cells were identified first by their morphology and secondly by using a rabbit anti-human von Willebrand factor antibody (vWf Ab–DAKO, Trappes). The vWf is a glycoprotein involved in primary hemostasis and synthesized by endothelial cells (Wagner, 1990). All the isolated cells have the endothelial morphology and were immunoreactive (data not shown).

RNA preparation

Total RNAs were extracted from aorta or endothelial cells by a modification of the acid guanidium-thiocyanate-phenol-chloroform method as described by Chomczynski & Sacchi (1987). Briefly, the vessels were homogenized with an ultra-turrax homogenizer in Trizol solution (Gibco Brl, France). After centrifugations and precipitation by addition of isopropanol, the RNA pellets were washed with 75% ethanol and resuspended in RNAse-free water. The concentrations were determined spectrophotometrically at 260 nm and the integrity of the RNA was verified on 0.8% TAE agarose gels.

Oligonucleotide primers and RT–PCR experiments

The oligonucleotides were purchased from Genosis (U.K.). Sequences and positions of the β_3-AR, adipsin and GAPDH oligoprobes on the rat forms and the number of PCR cycles have been described in Table 1. First strand synthesis of cDNA from aorta or endothelial total RNAs was carried out using *moloney murine leukaemia* virus reverse transcriptase (Gibco BRL, France) with oligo(dT) (Sigma, France). The single strand cDNAs were consequently amplified by PCR using Taq DNA polymerase (Pharmacia, France). As well as sample cDNAs, each PCR experiment included a negative control consisting of a RT reaction containing no added reverse transcriptase (NRT on Figure 1) and positive control corresponding to fat cDNA. Following initial heating of samples at 94°C for 3 min, each cycle of amplification consisted of 30 s at 94°C, 45 s at annealing temperature appropriate for the primers used and 30 s at 72°C. Individual annealing temperatures were 65°C for β_3-AR and GAPDH, and 55°C for adipsin primers. Following 35 cycles of amplification, the PCR products were separated through a 2% TAE agarose gels and visualized under UV after staining with ethidium bromide.

Immunohistochemistry analysis

Fixation and tissue processing protocols were performed as described elsewhere (Bittencourt *et al*., 1992). After freezing in 2-methyl butane solution (−50°C), 10 micrometers sections of thoracic aorta were cut on cryostat microtome and stored at −20°C until using. The quality of the endothelial layer of the preparations was performed by incubation with a vWf Ab (DAKO, Trappes, France). The rat β_3-AR antibody (rβ_3-AR Ab) (Santa Cruz Biotechnology Inc., U.S.A.) is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of the β_3-AR of mouse origin. This antibody reacts with β_3-AR of mouse and rat origin, without cross-reactive signal with β_1- and β_2-AR. After the secondary hybridization with an anti-goat Ig G peroxidase conjugated developed in rabbit (Sigma, France), the antibody complexes were revealed for sensitive detection of the enzymatic activity with peroxydase substrate kit AEC (SK4200–Vector, France). Controls for specificity of the rβ_3-AR antiserum involved incubating primary

Table 1 Oligonucleotides used as primers for PCR reactions

Name	*Length*	*Strand*	*Sequence*	*No. of cycles*	*Amplif. size*
Srβ_3B$_1$	25	For.	5′-TAGTCCTGGTGTGGATCGTGTCCGC-3′	35	517 pb
ASrβ_3B$_1$	25	Rev.	5′-GCGATGAAAACTCCGCTGGGAACTA-3′		
SrAdips$_1$	19	For.	5′-ATGAGCAGTGGGTGCTGAG-3′	35	589 pb
ASrAdips$_1$	20	Rev.	5′-AGAACGTTTTCAATCCACGG-3′		
SGapdh	30	For.	5′-ACTGGCGTCTTCACCACCATGGAGAAGGCT-3′	35	720 pb
ASGapdh	30	Rev.	5′-CTCCTTGGAGGCCATGTAGGCCATGAGGTC-3′		

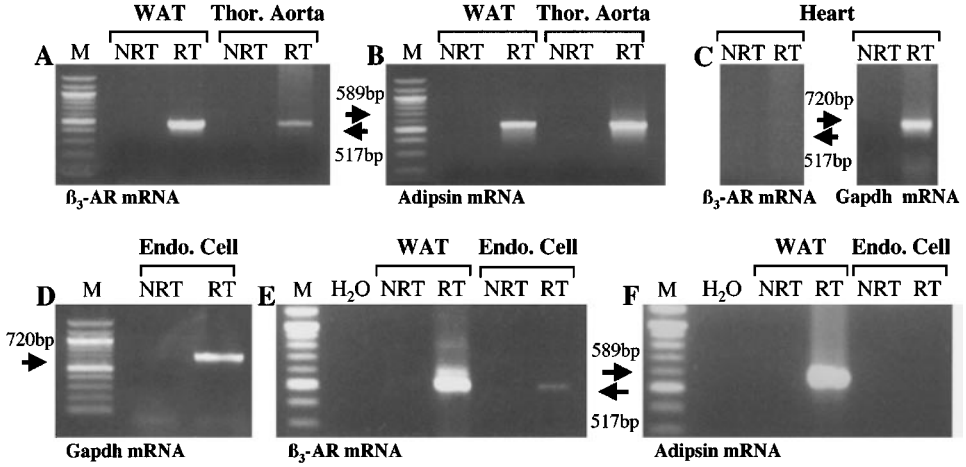

Figure 1 Detection by RT–PCR of β_3-AR and adipsin gene transcripts in white adipose tissue, heart, thoracic aorta and freshly isolated aortic endothelial cells. Amplified cDNA fragments were separed on 2% agarose gels and visualized by staining with ethidium bromide. Sizes of the PCR products are indicated on the left and right of the panels. M: 100 pb ladder (Biolabs, New England); NRT: none reverse transcription; RT: reverse transcription; β_3-AR: β_3-adrenoceptors; WAT: white adipose tissue; Thor. Aorta: thoracic aorta; Endo. Cell: endothelial cells.

antiserum overnight at 4°C with a five excess by weight of blocking peptide, the sequence used for the immunization procedure without the immunogenic carrier.

Pertussis toxin pre-treatment in vivo

Rats were anaesthetized with a mixture of pentobarbital (50 mg kg^{-1}, i.p.) and ketamine (25 mg kg^{-1}, i.m.). A bolus injection of pertussis toxin (PTX, 10 μg kg^{-1}, i.v.) was done in the jugular vein with a 30 gauge needle. Sham rats were injected with the same volume of salt solutions. Seventy-two hours after the PTX injection, animals were sacrificed, and the thoracic aorta was removed (Komatsu *et al*., 1995; Kost *et al*., 1999).

Tension recording

Aorta were cut in 4–5 mm long rings. These rings were suspended in stainless wire in 10 ml organ bath containing Krebs solution composed as follows (mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 20, glucose 11.1, EDTA (ethylenediaminetetraacetic acid) 0.016 and $CaCl_2$ 2.5. The medium was maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. The rings were progressively stretched in order to get a resting tension of 2 g. Functional endothelium was checked by the presence of at least 70% relaxation to acetylcholine (1 μM) in rings pre-contracted with phenylephrine (0.3 μM).

Cumulative concentration-response curves

Some rings were equilibrated in Krebs solution containing potassium channels inhibitors (iberotoxin 0.1 μM, glibenclamide 1 μM, and 4-aminopyridine 1 mM) whereas control rings were not treated during this period. Then, aortic rings were contracted with phenylephrine at concentrations ranging between 0.3 μM and 0.7 μM in order to obtain a similar value of the sustained tension for each ring studied (6.62 ± 0.61 g). Then, cumulative concentration-response curves to SR 58611A or UK 14304 were conducted. For the experiments performed on rings pre-contracted with KCl, only two concentrations of SR 58611A were applied.

As SR 58611A induced a long lasting relaxation (Trochu *et al*., 1999), spontaneous, time-dependent relaxation was evaluated in control rings and subtracted to the corresponding percentage of relaxation induced by SR 58611A. As iberiotoxin, a specific BK_{Ca} potassium channel blocker, was dissolved in bovine serum albumin solution (BSA). The control concentration-response curves of SR 58611A for this experimental protocol were performed in the presence of BSA.

Drugs

Phenylephrine hydrochloride, acetylcholine chloride (Sigma-Aldrich, St Louis, MO, U.S.A.), pertussis toxin (Calbiochem,

La Jolla, CA, U.S.A.), 4-aminopyridine (Research Biochemical International, U.S.A.) and SR 58611A (SR 58611A, ethyl [(7)S)-((2R)2-(3-chlorophenyl)-2-hydroxyethyl]amino]-5,6,7,8-tetrahydronaphtyl2-yl] oxyacetate hydrochloride) (a generous gift from Sanofi Recherche; Montpellier, France) were dissolved in distilled water. Glibenclamide, tolbutamide (Sigma Aldrich) were dissolved in dimethylsulphoxide (DMSO). Iberiotoxin and UK 14304 (Tocris, Oxford, U.K.) were respectively dissolved in BSA 0.1% and ethanol. The final concentration of the solvents in the organ bath was less than 0.1% v v^{-1}.

Statistical analysis

Results are expressed as the means ± s.e.mean of n experiments. The statistical significance of a drug effect was assessed using one way analysis of variance (ANOVA) followed by a Dunnett's test. Comparison of the different cumulative concentration-response curves was performed by a two-way ANOVA. To determine agonist potencies from cumulative concentration-response curves, concentrations producing 50% of maximal effect (EC_{50}) were calculated by fitting curves with the Boltzmann's equation. pD_2 values were then determined according to the equation $pD_2 = -\log$ (molar EC_{50}) and compared using student's t-test ($P<0.05$ being considered as significant).

Results

β_3-AR mRNA in rat aorta

The RT–PCR analysis shown in Figure 1, indicates that an expected amplified product of 517 bp with the rβ_3-AR primer couple could be identified by ethidium bromide staining of the amplified samples of rat adipose tissue (Granneman *et al*., 1991), which was our positive control, and thoracic aorta RNAs (Figure 1A). Moreover, to exclude a non-specific amplification, an experiment was carried out in the same conditions on heart extract (Figure 1C) which is described to lack of expression for β_3-AR transcripts (Gauthier *et al*., 1999). As expected, no transcript was detected for β_3-AR despite the integrity of our reverse transcriptase which was performed by an amplification with GAPDH primer couple. No signal was also observed when the RNA samples were not reverse transcripted into cDNA and directly used for the different PCR experiments (lane NRT for all conditions). As this receptor is highly abundant in the rat adipose tissues, adipsin marker was developed to check for fat contamination in our different preparations. Expression of serine protease adipsin is confined to adipose tissue and the myelin sheath of nerves which are tissues active in lipid metabolism (Cook *et al*., 1987). The RT–PCR analysis with the adipsin primer couple indicated an expected PCR product of 589 bp for amplified samples of adipose tissue but also for thoracic aorta mRNAs (Figure 1B). So the possibility of an adipose contamination in the preparations, prevented us to conclude at a real expression of β_3-AR transcript in vascular cells. However, the same procedure was realized on mRNAs obtained from freshly endothelial cells after enzymatic dissociation. Integrity of our reverse transcriptase was performed by an amplification with GAPDH primer couple (Figure 1D). Under these conditions, the rβ_3-AR mRNA was still present (Figure 1E). The lack of amplification for adipsin transcript (Figure 1F) or other fat markers like the hormone-sensitive lipase (data not shown) suggests an absence of adipose contamination.

Immunohistochemistry

This RT–PCR analysis prompted us to search for rβ_3-AR protein expression in the endothelial layer of rat thoracic aorta. An antiserum raised against the β_3-AR was used in immunohistochemical analysis. The pattern of rβ_3-AR immunoreactivity was compared with the vWf expression profile (Figure 2). The rβ_3-AR Ab highly stained cells from the endothelial layer (Figure 2B) in a similar distribution and form to that revealed with the vWf antiserum (Figure 2A). A light and diffuse signal was also observed in the smooth muscle layer. Furthermore, the pre-absorption of rβ_3-AR antiserum with the synthetic peptide, used for the procedure of immunisation, totally abolished the staining observed for the endothelial layer and only partially in the smooth muscle layer (Figure 2C).

β_3-AR mediated relaxation in rat aorta

SR 58611A, a preferential β_3-AR agonist, for concentration ranging from 0.1 to 30 μM, induced a concentration-dependent vasorelaxation in rat thoracic aorta. This relaxation was characterised by a long lasting response (Figure 4A). The spontaneous relaxation obtained in control rings (10.0 ± 2.1% relaxant effect at the end of the experiment; $n=20$) was subtracted from the corresponding relaxant effect of SR 58611A. In these conditions, the pD_2 value was 5.08 ± 0.02 ($n=21$) and the E_{max} value obtained for a concentration of 30 μM was 55.4 ± 1.6% ($n=21$).

Effect of PTX pre-treatment on β_3-AR induced relaxation

In order to identify the G protein linked to β_3-AR, we performed experiments in rat pre-treated with PTX (10 μg kg^{-1}), a $G_{i/0}$ protein inhibitor. In our experimental conditions, amplitude of contraction in response to phenylephrine was not significantly different in PTX-treated rings (4.39 ± 0.24 g; $n=16$) compared to sham rings (4.74 ± 0.31 g; $n=14$). As the endothelial α_2-AR is known to be coupled with PTX sensitive G-protein in arteries (Vanhoutte, 1997), we evaluated the α_2-AR-induced relaxation in order to verify the PTX pre-treatment efficiency. In isolated aortic rings obtained from sham rats, UK 14304, a selective α_2-AR agonist, produced a concentration-dependent relaxation for concentrations ranging from 0.01 to 1 μM (Figure 3). The E_{max} value obtained at a concentration of 1 μM, was 62.1 ± 6.5% ($n=7$). In aortic rings isolated from PTX pre-treated rats, the response to UK 14304 was blunted ($P<0.01$ *versus* sham rats) and the maximal relaxation induced by 1 μM UK 14304 was significantly reduced ($E_{max} = 36.7 \pm 3.2\%$; $n=8$, $P<0.05$).

At the opposite, the cumulative concentration-response curve for SR 58611A was not significantly modified in aorta rings isolated from PTX pre-treated rats (Figure 4). The potencies of SR 58611A (sham rats: $pD_2 = 5.06 \pm 0.07$, $n=7$; PTX pre-treated rats: $pD_2 = 4.96 \pm 0.06$, $n=8$) and the

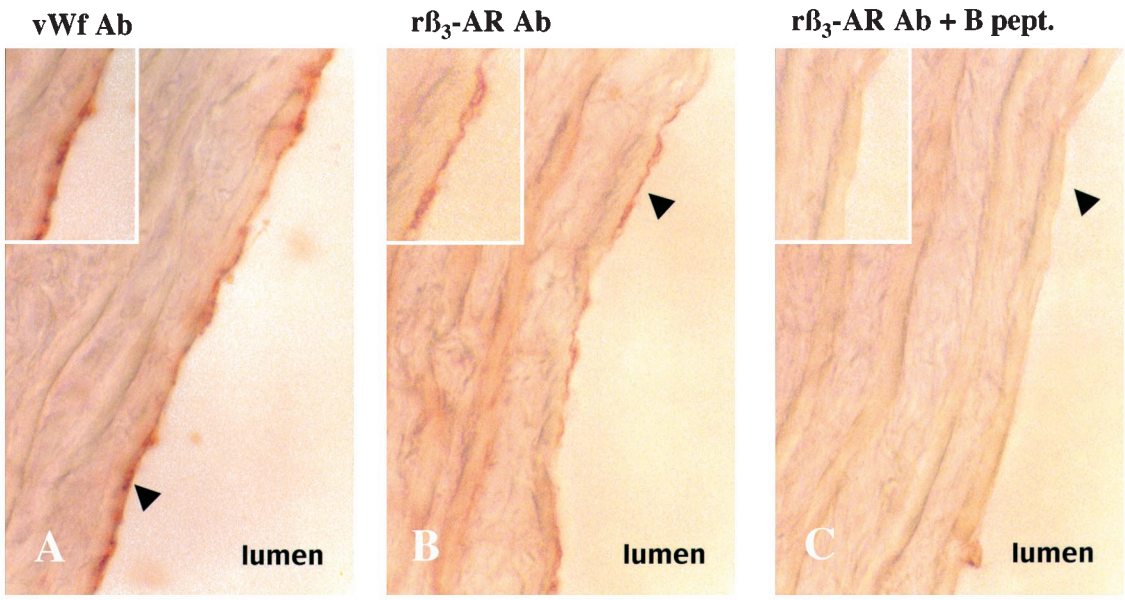

Figure 2 Comparison of von Willebrand factor (A) and β_3-AR expression in rat thoracic aorta (B). Adjacent 10 μm thick sections were incubated with either von Willebrand antibody (vWf Ab; A) or rat β_3-AR antibody (rβ_3-AR Ab; B) revealed by peroxydase-conjugated second antiserum. Black arrowhead shows endothelium intensively stained with vWf and rβ_3-AR Ab (A–B). Same staining after pre-absorption of rβ_3-AR Ab with the blocking peptide (rβ_3-AR Ab +B pept.; C). Micrographs X100.

maximal relaxation induced by 30 μM SR 58611A (sham rats: $E_{max} = 63.4 \pm 5.3\%$; PTX pre-treated rats: $E_{max} = 57.2 \pm 5.8\%$) were similar in both conditions.

Involvement of potassium channels in β_3-adrenergic mediated vasorelaxation in rat aorta

The role of potassium channels in mediating relaxation to SR 58611A were evaluated in vessels pre-constricted with 30 mM KCl before performing a concentration-response curve to SR 58611A. This concentration of KCl blocked potassium efflux and prevented relaxation mediated by opening of potassium channels. The amplitude of contraction was not significantly different in rings constricted with KCl or phenylephrine (5.0 ± 0.4 g and 4.5 ± 0.4 g, respectively; $n = 5$). The decreased driving force for potassium inhibited the relaxant effect induced by 30 μM SR 58611A ($n = 5$; $P < 0.05$), the maximal relaxation being only $16.7 \pm 3.7\%$ ($n = 5$) instead of $49.1 \pm 9.8\%$ in phenylephrine-constricted rings ($n = 5$; Figure 5).

In order to determine which types of potassium channels are involved in the β_3-AR-induced relaxation, aortic rings were pre-treated with a variety of selective potassium channel blockers before applying cumulative concentrations of SR 58611A (Figure 6). In the presence of 0.1 μM iberiotoxin, a specific blocker of BK_{Ca} (Nelson *et al*., 1990), the relaxant effects of SR 58611A were significantly reduced. SR 58611A had a lower potency ($pD_2 = 4.86 \pm 0.02$; $n = 8$; $P < 0.01$) than in the absence of iberiotoxin ($pD_2 = 5.13 \pm 0.11$, $n = 9$) and the maximal effect produced by 30 μM SR 58611A was significantly reduced by 13.5% ($n = 9$; $P < 0.01$ *versus* SR 58611A alone; Figure 6A). Glibenclamide (1 μM), an inhibitor of K_{ATP} (Waldron & Cole, 1999), shifted to the right the concentration-relaxation curve for SR 58611A (SR 58611A alone: $pD_2 = 5.08 \pm 0.02$; $n = 21$; SR 58611A + glibenclamide: $pD_2 = 4.40 \pm 0.08$; $n = 6$; $P < 0.01$ *versus* SR 58611A alone) and reduced the maximal relaxant effect obtained at 30 μM by 14.5% ($P < 0.01$ *versus* SR 58611A alone). Similar effects were obtained in the presence of another K_{ATP} blocker, tolbutamide (100 μM). The potency of SR 58611A ($pD_2 = 4.46 \pm 0.10$; $P < 0.01$ *versus* SR 58611A alone) and its maximal relaxant effect obtained at a concentration of 30 μM ($E_{max} = 43.6 \pm 3.5\%$; $n = 6$; $P < 0.01$ *versus* SR 58611A alone) were significantly reduced (Figure 6B). In arteries, 4-aminopyridine (4-AP) inhibited at millimolar concentrations voltage-dependent potassium channels (K_v). In aortic rings pre-treated with 1 mM 4-AP, the effects of SR 58611A were decreased only at highest concentrations (10 and 30 μM; Figure 6C). In addition, the potency of SR 58611A was reduced ($pD_2 = 4.76 \pm 0.09$; $n = 7$; $P < 0.01$ *versus* SR 58611A alone).

Discussion

In this study, we provided molecular and biochemical evidence for the expression of β_3-AR in endothelial cells of rat thoracic aorta. In addition, in this vascular bed, we demonstrated that β_3-AR-mediated relaxation did not result from the activation of PTX-sensitive G proteins but rather involved the activation of several potassium channel subtypes: BK_{Ca}, K_{ATP} and K_V.

In a previous study, (Trochu *et al*., 1999), we have shown by a pharmacological approach that β_3-AR stimulation produced a relaxation of rat thoracic aorta mediated mainly by endothelium-derived nitric oxide. However, no molecular and biochemical data concerning the expression of this receptor in endothelial cells were available. By RT–PCR, we have detected β_3-AR transcripts both in thoracic aorta and in freshly isolated endothelial cell preparation. No

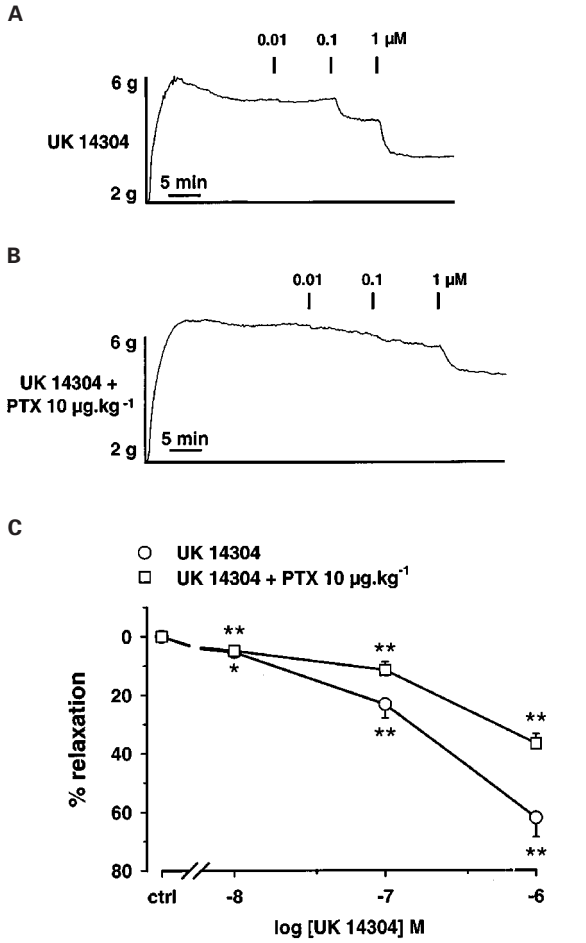

Figure 3 Inhibition of α_2-AR induced relaxation, in thoracic aorta rings from PTX pre-treated rats. The rats were injected i.v. with saline (sham) or PTX 10 μg kg^{-1} (pre-treated) 3 days before experiments. Typical recordings of relaxant effects of UK 14304 for concentrations ranging from 0.01 to 1 μM in aortic rings from sham operated rats (A) and PTX pre-treated rats (B). (C) Concentration-response curves for UK 14304 in aortic rings from sham operated rats ($n=7$) and PTX pre-treated rats ($n=8$). Results are expressed as the percentage of relaxation from the steady-state contraction level induced by phenylephrine. Each point is the mean of n experiments, and the vertical lines show the s.e. of the mean. When no error bar is shown, the error is smaller than the symbol. $*P<0.05$ and $**P<0.01$ indicate significant differences from control. Both curves are significantly different (two-way ANOVA).

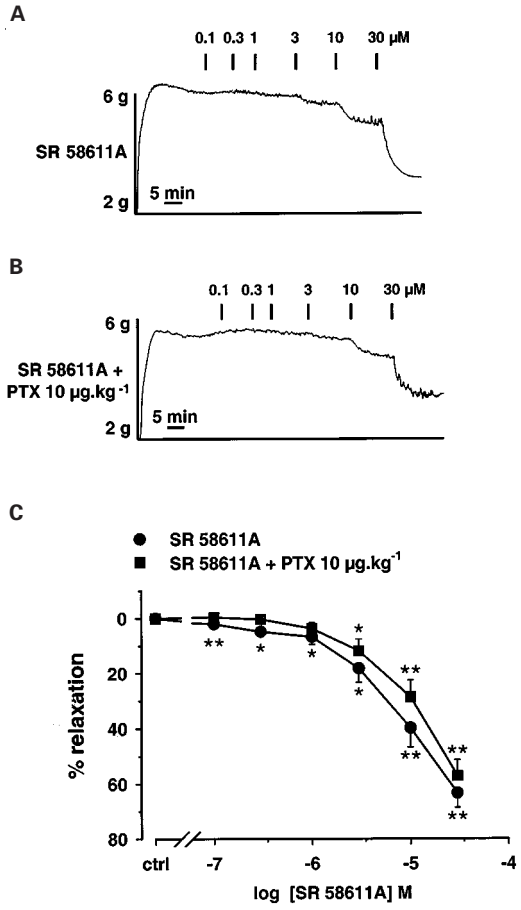

Figure 4 Effects of PTX pre-treatment on β_3-AR-induced relaxation in rat aorta. The rats were injected i.v. with saline (sham) or PTX 10 μg kg^{-1} (pre-treated) 3 days before experiments. Typical recordings of relaxant effects induced by SR 58611A for concentrations ranging from 0.1 to 30 μM on aortic rings from sham operated rats (A) and PTX pre-treated rats (B). (C) Concentration-response curves for SR 58611A in aortic rings from sham operated rats ($n=7$) and PTX pre-treated rats ($n=8$). The mean curves resulting from subtraction of the spontaneous relaxation of control vessels pre-treated or not with PTX are shown. Results are expressed as the percentage of relaxation from the steady-state contraction level induced by phenylephrine. Each point is the mean of n experiments, and the vertical lines show the s.e. of the mean. When no error bar is shown, the error is smaller than the symbol. $*P<0.05$ and $**P<0.01$ indicate significant differences from control.

transcript was detected for β_3-AR on heart extract, which is described to lack of expression for β_3-AR transcripts (Gauthier *et al*., 1999). This observation excludes a non-specific amplification and makes more relevant the result for the freshly isolated endothelial cell preparation. In this latter preparation, we have looking for adipose tissue markers (adipsin and hormone-sensitive lipase) because rat adipocytes are known to express a high level of β_3-AR (Lafontan, 1994). In our conditions, no specific products corresponding to adipsin and hormone-sensitive lipase were detected on reverse transcription performed by a GAPDH primer amplification. Thus, these results indicate that the expression of the β_3-AR in rat thoracic aorta was not due to the presence of adipocytes. Although we have verified the nature of our endothelial cells by a vWf immunocytochemical analysis in order to exclude a putative expression of β_3-AR mRNA from contaminating smooth muscle cells. Immunohistochemical studies performed on intact aorta demonstrated a strong stain in endothelial layer with rβ_3-AR Ab which disappeared when the antiserum was pre-absorbed with the blocking peptide. By contrast, the light staining observed in smooth muscle layer was not totally competed by the antigenic peptide, suggesting a non-specific signal in smooth muscle cells, and/or the presence of a very low expression of β_3-AR in smooth muscle cells, at the limit of detection of this procedure. These results are in agreement with our previous pharmacological study (Trochu *et al*., 1999). To date, although several studies have been performed to characterize the presence of β_3-AR in vessels, only one study reports the expression of β_3-AR mRNA expression in vessels. In rat portal vein, β_3-AR

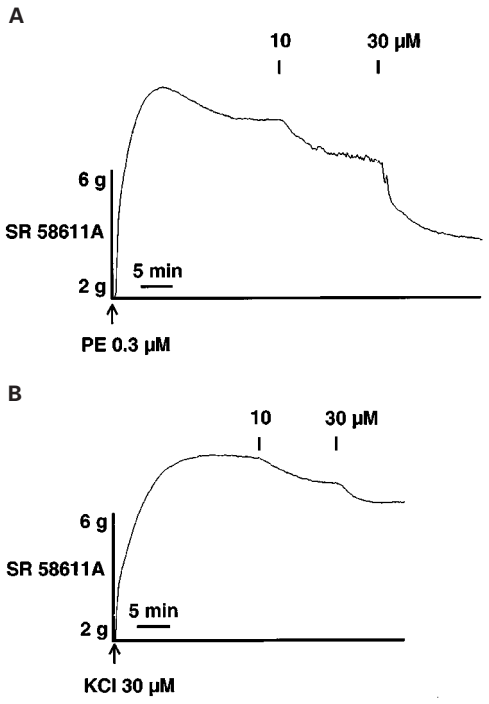

Figure 5 Inhibition of β_3-AR-induced relaxation in rat aorta by high extra-cellular potassium concentration. Typical recordings of SR 58611A for concentrations ranging from 10 to 30 μM on rings pre-constricted with either phenylephrine (PE) (A) or KCl (B). The recordings are representative in each case of five experiments.

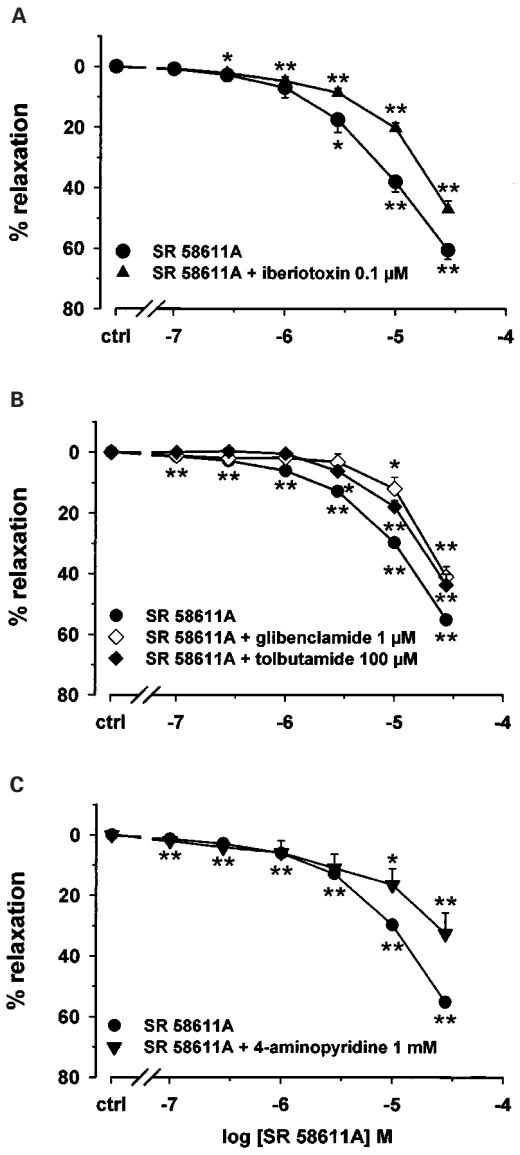

Figure 6 Inhibition of β_3-adrenergic induced relaxation in rat aorta by potassium channel blockers. The mean curves resulting from subtraction of the spontaneous relaxation of control vessel pre-treated or not with potassium channels blockers are shown. (A) Concentration-response curves for SR 58611A in the absence ($n=9$) and presence of 0.1 μM iberiotoxin ($n=8$) in rat thoracic aortic rings constricted with phenylephrine. (B) Concentration response curves for SR 58611A in the absence ($n=21$) and presence of 1 μM glibenclamide ($n=6$) or 100 μM tolbutamide ($n=6$) in rat thoracic aortic rings constricted with phenylephrine. (C) Concentration response curves for SR 58611A in the absence ($n=21$) and presence of 1 mM 4-aminopyridine ($n=7$) in rat thoracic aortic rings constricted with phenylephrine. Results are expressed as the percentage of relaxation from the steady-state contraction level induced by phenylephrine. Each point is the mean of n experiments, and the vertical lines show the s.e. of the mean. When no error bar is shown, the error is smaller than the symbol. $^*P<0.05$ and $^{**}P<0.01$ indicate significant differences from control.

transcripts have been detected in primary culture of smooth muscle cells (Viard *et al*., 2000). This discrepancy could be explained by the type of vessels studied (artery *versus* veins). In the study of Viard *et al*. (2000), a potential contamination by adipocytes has not been evaluated. However, immunohistochemistry with an anti-peptide polyclonal antibody revealed a β_3-AR expression in human gastrointestinal arteries. In this vascular bed, β_3-AR have been localized in smooth muscle cells but not in endothelial cells (Anthony *et al*., 1998). These results suggest that the localization of β_3-AR in vascular wall varies across species. These data corroborate the pharmacological results showing an endothelium-dependent vasorelaxation or not according to the vascular bed or the species studied.

In the rat thoracic aorta, we have previously shown that β_3-AR stimulation produced an endothelium-dependent vasorelaxation by activation of an endothelial NO synthase leading to an increase in intracellular cGMP. This effect is not modified by nadolol, a β_1- and β_2-AR blocker but was abolished in the presence of SR 59230A, a β_3-AR antagonist (Trochu *et al*., 1999). However, the signalling pathway was incomplete, and especially the type of G protein involved was missing. In human cardiac ventricle where β_3-AR are also linked to the NO pathway, we have demonstrated that β_3-AR are coupled to $G_{i/0}$ proteins (Gauthier *et al*., 1996). In the present study, the vasorelaxation induced by β_3-AR stimulation was not modified in PTX-pre-treated rats, suggesting that in rat thoracic aorta, β_3-AR are not linked to $G_{i/0}$ proteins. Indeed, in our experimental conditions, we have verified that $G_{i/0}$ proteins were significantly inhibited by PTX because the relaxant effect induced by UK 14304, a selective α_2-AR agonist, which is known to activate $G_{i/0}$ proteins (Vanhoutte, 1997), was blunted in aorta rings isolated from PTX-pre-treated rats. In native and recombinant systems, it has been shown that β_3-AR could be linked to different types of G proteins and activated different signalling pathways

(Strosberg, 1997; Gauthier *et al*., 2000). In rat portal vein myocytes, β_3-AR are linked to G_s proteins (Viard *et al*., 2000). However, in this study, the compound used to stimulated β_3-AR was CGP 12177, which is known to also activate the state of low affinity of β_1-AR (Granneman, 2001). This atypical β-AR is described to activated cAMP pathway through stimulation of G_s proteins. Clearly, further experiments are needed to identify the type of G proteins involved in the vasorelaxation induced by β_3-AR in rat thoracic aorta.

Potassium channels play an important role in the regulation of calcium influx and vascular tone by affecting the membrane potential in smooth muscle (Sobey, 2001). Activation of potassium channels and subsequent membrane hyperpolarisation contribute to vasorelaxation. The role of the activation of potassium channels in β-AR agonist-induced dilation of mammalian blood vessels has been recently described by a number of research groups. However, only one study performed in rat isolated perfused lung has determined the role of potassium channels in the β_3-AR-induced vasorelaxation (Dumas *et al*., 1999). In the present study, the relaxant effect of SR 58611A was strongly reduced by elevated concentration of extracellular KCl (decreasing the driving force for potassium ions, thereby decreasing potassium efflux and subsequent hyperpolarisation) suggesting the involvement of potassium channels in this effect. This result was confirmed by the use of specific potassium channels blockers. In the presence of iberiotoxin, glibenclamide, tolbutamide or 4-AP, the SR 58611A-induced vasorelaxation was significantly reduced, suggesting that BK_{Ca}, K_{ATP} and K_v respectively, are effectors of the vasorelaxation produced by β_3-AR stimulation in rat thoracic aorta. However, this inhibition was not complete in each experimental condition suggesting the participation of several classes of potassium channels in this global effect. In this model of rat thoracic aorta, it has been shown that the relaxation induced by isoprenaline resulted from the activation of K_{Ca} channels mainly by activation of β_2-AR and K_v channels by activation of β_1-AR (Satake *et al*., 1996). However, as isoprenaline activated all types of β-AR, the analysis of this study is difficult because the effects of β_3-AR were not evaluated. The involvement of potassium channels in the vasorelaxation-induced by β_3-AR stimulation seems different according the vascular bed in one species. Indeed, on the rat hypoxic pulmonary pressure, the activation of BK_{Ca} and SK_{Ca} participated to the vasorelaxation but not K_{ATP}; the involvement of K_v in this effect was not investigated in this study (Dumas *et al*., 1999). The activation of a K_v channel by β_3-AR stimulation has been previously shown in a recombinant expression system (*Xenopus* oocytes) in which β_3-AR stimulation produced by isoprenaline, activated the cardiac KvLQT1/MinK potassium channel through G_s proteins (Kathofer *et al*., 2000). With our technique of organ bath, we cannot distinguish the involvement of endothelial and/or smooth muscle cell potassium channels in the β_3-AR-induced vasorelaxation. For exemple, BK_{Ca} are expressed both in smooth muscle cells and in endothelial cells. In endothelial cells, they regulate the membrane potential and the intracellular calcium level (Marchenko & Sage, 1996). Thus, their activation results in an hyperpolarization of endothelial cells, which increases the electrochemical gradient for calcium and leads to increase intracellular calcium, a prerequisite for the synthesis/release of relaxant factors including NO (Mayer *et al*., 1989). Hence, to determine the localisation of potassium channels involved in the β_3-AR relaxation in rat aorta, further investigations are needed.

Taken together with our previous data, this study demonstrated that functional β_3-AR are mainly localized in rat aorta endothelial cells. Their activation produced a vasorelaxation through an independent activation of $G_{i/0}$ proteins but involved at least three types of potassium channels, BK_{Ca}, K_{ATP} and K_v. As endothelium could be altered in some diseases, this study opens new fields of investigations. In particular, future work will be needed to determine whether β_3-AR stimulation may present a potential interest in hypertension.

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