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Introduction

The functions of the bladder include both relaxation, which occurs during bladder filling, and contraction which occurs during the emptying phase of the micturition cycle. Detrusor smooth muscle contraction to muscarinic agonists is mediated via M_3 muscarinic receptors,[1] while noradrenaline relaxes the tissue via β-adrenoceptors, although the subtype involved appears to be species dependent (reviewed by Michel et al., 2006). α-adrenoceptors are also present on the detrusor muscle, but receptor density is low and responses to α-adrenoceptor agonists are small or non-existent.[2]

Over recent years the importance of the inner epithelial lining of the bladder, the urothelium, and the underlying lamina propria in regulating bladder function has been increasingly recognised. The lamina propria, is the layer of tissue between the inner epithelial layer of the bladder wall and the underlying detrusor smooth muscle. This inner region of the bladder wall has a barrier function, but also when stretched it releases a number of factors that influence detrusor contraction and afferent nerve sensitivity.[3] In addition, the urothelium with lamina propria from the dome of the pig bladder[4,5] and also strips of lamina propria from

the rabbit urethra[6,7] have been shown to contract to agonists such as carbachol, neurokinin A and noradrenaline. Isolated strips of urothelium with lamina propria also develop spontaneous contractile activity, with carbachol and also stretch-induced release of acetylcholine mediating increases in spontaneous contractile frequency via M_3 receptors.[8]

The lamina propria of the human bladder has been shown to be immunoreactive for tyrosine hydroxylase[9,10], indicating a sympathetic innervations, and the lamina propria of the rabbit urethra contracts in response to phenylephrine demonstrating that α_1-adrenoceptors are present.[6] Three α_1-adrenoceptor subtypes have been identified at the molecular and functional level (α_{1A},α_{1B},$\alpha_{1D,}$[11]) and another, termed the α_{1L}-adrenoceptor that is a pharmacological phenotype which arises from the α_{1A}-adrenoceptor gene and has a low affinity for prazosin, has been recognised in functional experiments.[12] Which α_1-adrenoceptor subtypes are present in porcine urothelium and lamina propria and their functional role has not been investigated. Similarly, all three β-adrenoceptor subtypes are expressed in the human lamina propria at the mRNA and protein level,[13] but their influence on contractile activity is unknown. Many studies have identified the pig bladder as a suitable model for the human bladder it and demonstrates similarities in the expression of adrenoceptors.[14-16] The present study examines which α- and β-adrenoceptors are expressed in the pig urinary bladder urothelium and lamina propria, and identifies the adrenoceptor subtypes responsible for mediating the spontaneous frequency and tension responses of this tissue.

Material and methods

Functional organ bath studies

Fresh bladders from Large White-Landrace pigs (6 months old, ~80 kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 188.4, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.15, KCL 4.7, KH_2PO_4 1.15 and D-glucose 11.7). The bladders were opened longitudinally and full thickness strips of anterior wall from the dome region were removed. From these tissues, strips of urothelium with lamina propria were prepared (20 x 5 mm) and immersed in Krebs-bicarbonate solution, maintained at 37°C and gassed with 5% CO_2 in oxygen. The tissues were attached to isometric force transducers (ADInstruments MCT050/D) and tensions recorded with a Powerlab system using Labchart v7 software (ADInstruments, Castle Hill, Australia). After washing with fresh Krebs solution the tissues were allowed to equilibrate for 45 minutes under a baseline tension of ~2g before starting drug additions. For studies with antagonists, adjacent pieces of tissue were dissected, set up in pairs under identical conditions and allowed to equilibrate. One tissue was incubated with the antagonist for 30 minutes, and the other remained as a control in the absence of any antagonist. Where antagonists selective for a particular receptor subtype have been used, affinity values were obtained from the literature and the concentration carefully selected to give a maximum antagonism of the receptor without an action on the other receptor subtypes.

RT-PCR studies

Strips of urothelium with lamina propria (n=6) were finely dissected with scissors under magnification and immediately immersed in RNALater solution (Ambion Inc.). The urothelium and lamina propria (~0.2 g each) was then homogenised and RNA extracted with

a Trizol *plus* RNA purification kit (Invitrogen Cat No. 12183-555) as per manufacturer's instructions. All storage of RNA was maintained at -80°C and experiments performed within 3 months of the initial extraction. A NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the total RNA quantity (ng/µL) and to assess the purity (260/280 ratio). The integrity of the RNA was also performed for all extractions with formaldehyde agarose gel electrophoresis in ethidium bromide staining for 28s and 18s RNA band visualisation. cDNA synthesis was then performed using SuperScript III First-Strand Synthesis SuperMix from the qRT-PCR kit (Invitrogen). Initial incubation was in a reaction mix (10µL of 2X RT reaction mix; 2µL of reverse transcriptase enzyme mix; 8µL of eluted RNA) and performed at 25°C for 10 minutes.

An iQTM SYBR Green Super Mix (BioRad) was used to amplify the cDNA targeted genes. Real-time PCR was performed with a Research Rotor-Gene 3000 (Corbett). 5µL of cDNA was used to amplify the genes. PCR for ADRB3 was undertaken in triplicate with the following conditions: 94°C: 10min(x1), Cycle 2, 94°C:30s, 51°C:15s, 72°C:15s(x40). All other genes were investigated with the following conditions: cycle 1, 94°C 10 min(x1), Cycle 2, 94°C:30s, 60°C:30s, 72°C:30s(x45). A post-PCR amplification protocol was preset before the run to obtain melt curve representations, ramping from 50-99°C with 1°C increases every 5s. Two reference genes were examined for internal controls in realtime PCR including 18s-RNA and β-actin. 18s expression was found slightly less optimal as an internal control than β-actin. The actin gene exhibited optimal and identical expression levels across all porcine tissues. Therefore, the expression levels of each target gene were expressed relative to the PCR amplification of β-actin.

Data analysis and statistical procedures

Measurements of frequency and baseline tension were taken at the peak response after the addition of each agonist. The frequency of contractions was expressed as the number of phasic waves per minute (cycles min^{-1}) and the baseline tension as grams (g). The baseline tension was taken as the lowest point of the spontaneous phasic contractions. Mean ± standard error of mean (SEM) values in the absence and presence of drugs were compared using a Student's two-tailed *t*-test with $P < 0.05$ being taken as statistically significant. Prism software (GraphPad v4, San Diego, CA, USA) was used for statistical analysis of data. A one-way analysis of variance (ANOVA) with a Dunnett post test was applied for analysis of more than one sample (Table 1 only). *n* represents the number of different porcine tissues used in the study. Real-time PCR data were calculated using the cycle threshold determination method (Corbett rotor gene 6000 series software v1.7).

Drugs, chemical reagents and other materials

(±)-Noradrenaline hydrogen tartrate, CGP20712A methanesulfonate, clonidine hydrochloride, isoprenaline hydrochloride, phentolamine hydrochloride, (R)-(−)-phenylephrine hydrochloride, and prazosin hydrochloride were purchased from Sigma (St Louis, Missouri, USA). Tamsulosin was a gift provided by Astellas Pharma (Leiderdorp, NL). A61603 hydrobromide, BMY7378 dihydrochloride, BRL37344 sodium salt, CL316243 disodium salt, ICI118551 hydrochloride, (±)-propranolol hydrochloride, RS17053 hydrochloride, RS100329 hydrochloride, SR59230A, salbutamol sulphate, and UK14,304 were purchased from Tocris (Ellisville, Missouri, USA). Oligonucleotide primers were obtained from Geneworks (Thebarton, South Australia).

Results

Spontaneous phasic contractions

The urothelium and lamina propria exhibited spontaneous contractions within 10 minutes of being placed in the organ bath (Fig. 1). This regular phasic activity of the urothelium was present throughout the course of the experiment and occurred at a spontaneous contractile frequency of 3.48 ± 0.07 cycles min^{-1} and an amplitude of 0.68 ± 0.03g (n=211).

Responses to noradrenaline

Noradrenaline (10µM, n=8) significantly reduced the frequency of spontaneous contractions by 15.2 ± 6.5% and baseline tension by 22 ± 4% (Table 1). The frequency responses were significantly enhanced in the presence of phentolamine (10µM), noradrenaline then reducing the frequency of contractions by 39 ± 7%, while relaxation of the tissues to noradrenaline was not significantly affected (Table 1). In contrast, in the presence of propranolol (1µM), responses to noradrenaline (10µM, n=8) were converted to increases in the rate of spontaneous contractions (41 ± 7% increase) and the baseline tension increased by 52 ± 10% (Table 1).

α-adrenoceptor responses

The α_2-adrenoceptor agonists clonidine and UK14,304 are highly potent agonists at α_2-adrenoceptors and produce responses at nano-molar concentrations if these receptors are present in a tissue. However at concentrations up to 1µM, neither agonist had any effect on either the spontaneous contractile frequency or the basal tension developed by tissues (n=8). Higher concentration of these drugs will activate α_1-adrenoceptors and thus were not examined. In contrast, the α_1-adrenoceptor selective agonist phenylephrine (100µM)

increased the spontaneous activity by 41 ± 8% and the baseline tension by 22 ± 5% (n=21, P < 0.001 for both). At a 10-fold lower concentration, the α_{1A}-adrenoceptor selective agonist A61603 (10μM) produced a similar increase in spontaneous contractile frequency 31 ± 4% and also increased baseline tension by 31 ± 4% (n=26, P < 0.001 for both; Fig. 1). Lower concentrations of phenylephrine (1-10μM, n=8) did not cause any significant responses for frequency or tension, yet lower concentrations of A61603 (3μM, n=8) resulted in significant increases for frequency by 27 ± 9% (P < 0.05) and baseline tension by 18 ± 5% (P < 0.01).

The increase in the frequency of contractions produced by phenylephrine was significantly reduced by low concentrations of the α_{1A}-adrenoceptor antagonists, RS100329 (10nM, n=8) and tamsulosin (3nM, n=8), but not by the α_{1D}-adrenoceptor selective antagonist BMY7378 (100nM, n=8; Fig. 2). Increases in basal tension induced by phenylephrine (100μM) were similarly reduced by RS100329 (10nM, n=8, P < 0.05) and tamsulosin (3nM, n=8, P < 0.05), but not BMY7378 (100nM, n=8, Fig. 2). Two antagonists that discriminate between α_{1A}- and α_{1L}-adrenoceptors, prazosin and RS17053 were also examined. Neither prazosin (10nM, n=10) nor RS17053 (1μM, n=8) had any significant effect on either frequency or tension responses to phenylephrine (Fig. 2).

β-adrenoceptor responses

Isoprenaline (1μM, n=62) reduced the spontaneous contractile frequency of tissue strips by 34 ± 2% (1.21 ± 0.08 cycles min^{-1}, P < 0.001) and the baseline tension by 37 ± 2% (0.66 ± 0.44g, P < 0.001). A higher concentration of isoprenaline (10μM, n=16) produced a greater inhibition of the contractile frequency (49 ± 1%, P < 0.01), but the depression of baseline tension was similar to that obtained with the lower concentration of isoprenaline (40 ± 3%). Salbutamol (1μM, n=7) reduced the frequency of spontaneous contractions by 33 ± 6% and

the baseline tension by 22 ± 2% ($P < 0.001$ for both responses). A higher concentration of salbutamol (10μM) did not induce greater inhibition of the tissues. The β_3-adrenoceptor selective agonist, BRL37344, at concentrations up to 10μM (n=8), did not affect the frequency of spontaneous contractions or baseline tension. Another β_3-adrenoceptor selective agonist, CL316243, was similarly without effect over a range of concentrations up to 1μM.

Isoprenaline (1μM; Fig. 2) induced relaxation and a slowing of spontaneous phasic contractions and both these responses were significantly reduced in the presence of propranolol (100nM, n=8) or a combination of the β_1-adrenoceptor antagonist CGP20712A (30nM, n=8) and β_2-adrenoceptor antagonist ICI118551 (70nM, n=11), but were unaffected by CGP20712A (β_1-adrenoceptor antagonist, n=8), or SR59230A (β_3-adrenoceptor antagonist, n=8). The β_2-adrenoceptor antagonist ICI118551 (70nM, n=12) significantly reduced frequency responses to isoprenaline ($P < 0.05$), but not relaxation responses to this agonist (Fig. 2).

RT-PCR

RNA was extracted from isolated urothelium and lamina propria samples (n=6, run in triplicate) and tested for integrity prior to being converted into cDNA. RNA concentrations and 260/280 ratios were as follows: Sample 1: 602ng/μL, 2.06; Sample 2: 699ng/μL, 2.10; Sample 3: 1050ng/μL, 2.08; Sample 4: 620ng/μL 2.09, Sample 5: 823ng/μL; 2.10, Sample 6: 590ng/μL, 2.11. The real-time PCR products for all six adrenoceptor genes and the reference gene β-actin were run in triplicate and the end product visualised on 2% agarose gels to identify that all genes demonstrated the correct fragment sizes. For α_1–adrenoceptors, the greatest expression was observed for the α_{1A}- and α_{1B}-adrenoceptor subtypes, while that for the α_{1D}-adrenoceptor was only half that obtained for α_{1A}- and α_{1B}-adrenoceptors (Fig. 3). All

three β-adrenoceptor subtypes were expressed in these tissues. The expression was highest for β_2-adrenoceptors; which was nearly double that for either β_1- or β_3-adrenoceptors (Fig. 3).

Comment

In the absence of any exogenous agonist, the urothelium with lamina propria samples developed spontaneous phasic contractions. Initial data obtained with noradrenaline suggests that both α- and β-adrenoceptors are present and functional in this tissue: α-adrenoceptors mediating contraction and increases in frequency, whilst β-adrenoceptor activation relaxes the tissue and slows the frequency of phasic contractions. The dominant response to the physiological agonist noradrenaline is β-adrenoceptor mediated relaxation and slowing, since these were the responses obtained to noradrenaline in the absence of antagonists. However, these inhibitory responses to noradrenaline were enhanced in the presence of phentolamine, indicating that α-adrenoceptors were reducing these inhibitory responses to noradrenaline in the absence of any antagonists. Thus both α- and β-adrenoceptors are involved in mediating responses to the endogenous agonist noradrenaline.

α-adrenoceptors

The α-adrenoceptor mediated responses of the urothelium and lamina propria appear to involve the α_1-adrenoceptor subtype since both phenylephrine and A61603 increased phasic frequency, whilst the agonists with predominantly α_2-adrenoceptor agonist selectivity, clonidine and UK14304, had no effect. The tissue expresses mRNA for all three cloned α_1-adrenoceptors, but the levels of α_{1A} and α_{1B}-adrenoceptor mRNA are greater than that for the α_{1D}-adrenoceptor subtype. On isolated tissues, both phenylephrine and the α_{1A}-adrenoceptor selective agonist A61603[17] induced increases in baseline tension and the rate of phasic

contractions. The tension and rate responses for A61603 (10μM) were significantly greater than those to phenylephrine (10μM). Higher concentrations of phenylephrine (100μM) produced a greater response almost identical to that produced by the 10-fold lower concentration of A61603. These agonist data suggest that responses were mediated via the α_{1A}-adrenoceptor subtype.

The antagonist data support these findings. Responses to phenylephrine were reduced by a low concentration of tamsulosin (3nM), an antagonist possessing high affinity for α_{1A}- and α_{1D}-adrenoceptors.[18] Thus responses were not mediated via α_{1B}-adrenoceptors. In addition, rate and tension responses to phenylephrine were potently inhibited by RS100329, a selective antagonist for the α_{1A}-adrenoceptor subtype. RS100329 has a pKi at α_{1A}-adrenoceptors of 9.6 and demonstrates a 126- and 50-fold selectivity over α_{1B} and α_{1D}-adrenoceptor subtypes,[19,20] thus at the concentration used in the present study (10nM) it would only have an action at α_{1A}-adrenoceptors. The lack of involvement of α_{1D}-adrenoceptors was confirmed using BMY7378, an antagonist with a greater than 100-fold high affinity for the α_{1D}-adrenoceptor subtype over α_{1A} and α_{1B}-adrenoceptors.[21,22] The lack of effect at a concentration of 100nM with BMY7378 supports the conclusion that α_{1D}-adrenoceptors are not involved in mediating responses of the urothelium and lamina propria, and that the α_{1A}-adrenoceptor is the functional α_1-adrenoceptor in this tissue.

A number of studies have demonstrated that contractions of the bladder neck and prostate are mediated via α_1-adrenoceptors that, unlike the cloned receptors, have a low affinity for the antagonist prazosin and this putative receptor subtype has been termed the α_{1L}-adrenoceptor or $\alpha_{1A/L}$-adrenoceptor.[11] It is generally accepted that this functional receptor exists as a conformational state of the α_{1A}-adrenoceptor, but the mechanisms involved are unclear and it

has been suggested that experimental conditions[23,24] or specific intracellular proteins[25] may be involved. Both prazosin and RS17053 have a low affinity for this form of the receptor compared to the α_{1A}-adrenoceptor, and the lack of effect for these antagonists in the present study suggests that it is this conformational state ($\alpha_{A/L}$-adrenoceptor) that mediates contraction of this tissue.

β-adrenoceptors

Noradrenaline, isoprenaline and salbutamol all induced relaxation of tissues and a slowing of the frequency of phasic contractions. Propranolol antagonised the inhibitory effects of isoprenaline and abolished those to noradrenaline, converting them to a small contraction and an increase in phasic rate of contractions to noradrenaline. All three subtypes of β-adrenoceptor (β_1, β_2 and β_3) were present at the mRNA level, with the expression of β_2-adrenoceptors being double that of either the β_1- or β_3.-adrenoceptor subtypes. Isoprenaline reduced the rate of phasic contractions and these inhibitory responses were reduced when β_2-adrenoceptors were antagonised with either propranolol (β_1- and β_2- antagonist), ICI118551 (β_2-antagonist) or a combination of ICI118551 and CGP20712A (β_1- antagonist). The responses were insensitive to CGP20712A alone or SR59230A (β_3- antagonist) indicating β_2-adrenoceptors are the predominant functional β-adrenoceptor subtype inhibiting spontaneous contractile frequency in these tissues. For relaxation responses, combined β_1- and β_2-adrenoceptor antagonism appeared to be required to significantly inhibit responses to isoprenaline and CGP20712A alone, ICI118551 alone or SR59230A alone failed to influence responses. In some species, the responses of detrusor smooth muscle to isoprenaline are mediated via β_3-adrenoceptors, but for porcine urothelium and lamina propria, selective concentrations of SR59230A did not affect frequency or tension responses to isoprenaline, and also CL316243 and BRL37344, two selective β_3-adenoceptor agonists did not influence

either response. Thus inhibitory responses of the urothelium and lamina propria to isoprenaline appear to be mediated predominantly by β_2-adrenoceptors with a possible contribution from β_1-adrenoceptors for tension responses.

The mechanisms involved in these responses of the urothelium and lamina propria are not clear. Tension responses to neurokinin A and carbachol are present after fine dissection and removal of smooth muscle from the preparations and it has been suggested that myfibroblasts may be involved.[5,8] In addition, spontaneously generated electrophysiological events have been observed in myofibroblasts located within the lamina propria.[26] The function of this contractile activity is also uncertain. It may act to ensure folding of the urothelium in the empty bladder or alternatively it may act to enhance sensory nerve function.[27] It has been reported that spontaneous calcium and electrical activity arise in the urothelium-lamina propria interface and pass into the detrusor regions[28] and this is increased in tissues from cats with interstitial cystitis.[29] Therefore, in situations where electrical coupling is enhanced, it may be possible that the lamina propria may drive larger spontaneous contractions of the detrusor smooth muscle.

Conclusions

In conclusion, the urothelium and lamina propria of the pig bladder develops spontaneous contractile activity that can be regulated by the sympathetic nervous system. The force of contraction and the frequency of spontaneous contractions are increased by α_1-adrenoceptor stimulation and depressed by β-adrenoceptor stimulation. The predominant α_1-adrenoceptor subtype has the pharmacological characteristics of the $\alpha_{1A/L}$-adrenoceptor subtype, while β_2-adrenoceptors (with possibly a β_1- contribution for tension) appear to be the predominant receptors mediating inhibitory responses.

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Figure legends

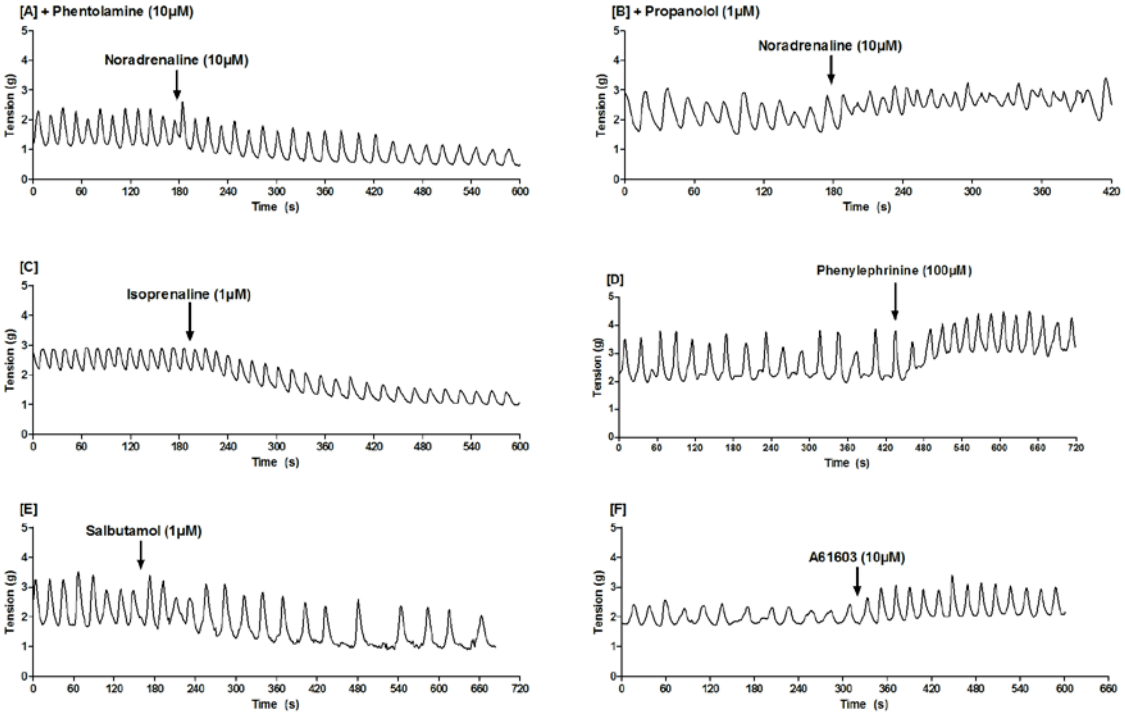


Fig. 1. Typical experimental traces showing the effects of adrenoceptor agonists on urothelium and lamina propria contractile activity. [A] Noradrenaline (10µM) in the presence of phentolamine (10µM), [B] noradrenaline (10µM) in the presence of propanolol (1µM), [C] isoprenaline (1µM), [D] phenylephrine (100µM), [E] salbutamol (1µM) and [F] A61603 (10µM).

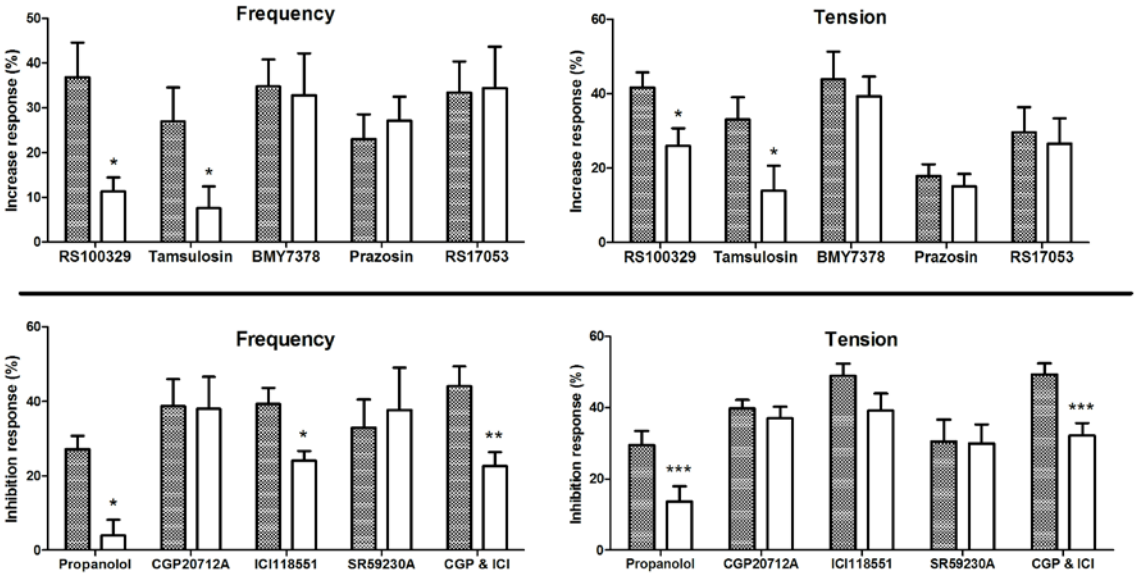
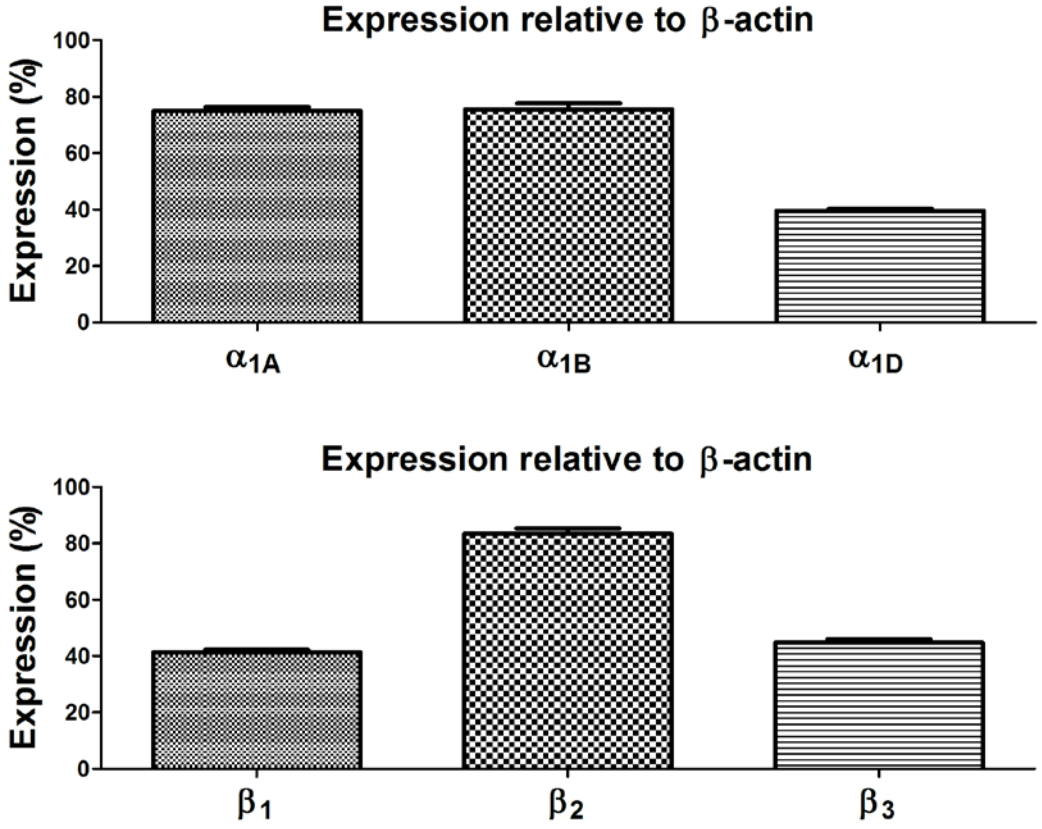


Fig. 2. Upper figure: Frequency and tension responses of urothelium/lamina propria strips induced by phenylephrine (100µM), in the absence (shaded columns) and in the presence (open columns) of antagonists: RS100329 (10nM, n=8), tamsulosin (3nM, n=8), BMY7378 (100nM, n=8), prazosin (10nM, n=10) and RS17053 (1µM, n=8). **Lower Figure**: Frequency and tension inhibitions induced by isoprenaline in the absence (shaded columns) and presence (open columns) of antagonists: propanolol (100nM, n=8), CGP20712A (30nM, n=8), ICI118551 (70nM, n=8), SR59230A (30nM, n=8), and a combination of ICI118551 (70nM) & CGP20721A (30nM, n=11, CGP & ICI). Responses are expressed as the percentage increase (upper figure) or inhibition (lower figure) of rate and tension. Columns represent the mean ± SEM. Student's two-tailed *t*-test: *P < 0.05, **P < 0.01, ***P < 0.001.



Gene	Forward	Reverse	Size
α_{1A}	CCATTGGGTCTTTCTTTCCT	TTTAAACTCTTGACTGGAGCAC	124
α_{1B}	GGTCATTCTGGTCATGTACTG	TCGTGAAAGTTCTTGGAGTG	134
α_{1D}	TTTCTTCGTCCTACCACTG	GTTGAAGTAGCCCAGCC	90
β1	CCAACCTCTTCATCATGTCC	CACAGTACGTCCACCGA	144
β2	GATCCATGACAACCTCATCC	GAAGGCCATCCTGAAATCTG	117
β3	AGACTCCAGACCATGACC	ACAGCTCGCAACAAGTG	132
β-actin	ATGGGCCAGAAGGACTC	GCAGCTCGTTGTAGAAGG	144

Fig. 3. Relative expression of each adrenoceptor gene. Expression values are based relative to β-actin expression for the α-adrenoceptors (upper panel) and β-adrenoceptors (middle panel) and expressed as the mean ± SEM (n=8 for each). Lower panel: Porcine receptor-specific PCR primer sequence and PCR amplicon sizes.

Table 1. Frequency and tension responses of urothelium/lamina propria to noradrenaline.

Drug	Change in frequency (cycles min^{-1})	Change in tension (g)	n
Noradrenaline (10 µM)	-0.57 ± 0.24	-0.64 ± 0.16	8
Noradrenaline (10 µM) + Phentolamine (10 µM)	-1.40 ± 0.25[a]	-0.82 ± 0.06	8
Noradrenaline (10 µM) + Propanolol (1 µM)	1.34 ± 0.20[b]	0.76 ± 0.13[b]	8

Values are mean ± SEM.

[a]Significantly different from noradrenaline alone, $P < 0.05$ (ANOVA)

[b]Significantly different from noradrenaline alone, $P < 0.01$ (ANOVA)