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# Title Page

## Title

*Contraction of rat cauda epididymis smooth muscle to $\alpha_1$-adrenoceptor activation is mediated by $\alpha_{1A}$-adrenoceptors*

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# Running Title Page

a) Running title:

$\alpha_{1A}$-ARs mediate cauda epididymis smooth muscle contraction.

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*e) Section assignment*

Genitourinary

## Abstract

The cauda epididymis (CE), the site of sperm storage until the ejaculation, is densely innervated by the sympathetic nervous system. Contraction of CE smooth muscle via $\alpha_1$-adrenoceptors ($\alpha_1$-ARs) plays a key role during the seminal emission phase of ejaculation and $\alpha_1$-ARs antagonism has been suggested as a non-hormonal and reversible male contraceptive target. As the $\alpha_1$-ARs subtype mediating contraction of rat CE is not known this study investigates the expression and role of $\alpha_1$-ARs subtypes on the proximal and distal rat CE duct contraction to norepinephrine *in vitro*. Alpha$_{1a}$, $\alpha_{1b}$ and $\alpha_{1d}$ transcripts were detected by qRT-PCR in proximal and distal CE segments and the $\alpha_{1a}$ and $\alpha_{1d}$ were shown to predominate over the $\alpha_{1b}$. The inhibition of [$^3$H]Prazosin specific binding to intact CE segments from proximal and distal CE by RS 100329 and 5-methylurapidil ($\alpha_{1A}$-selective) and BMY 7378 ($\alpha_{1D}$-selective) showed that $\alpha_{1A}$- and $\alpha_{1D}$-ARs are expressed at similar densities. Norepinephrine-induced contractions of CE were competitively antagonized with high affinity by RS 100329 ($pK_B \approx 9.50$) and 5-methylurapidil ($pK_B \approx 9.0$) and with low affinity by BMY 7378 ($pK_B \approx 7.0$) and the $\alpha_{1B}$-selective L-765,314 ($pA_2 < 7.0$) suggesting contractions are mediated by $\alpha_{1A}$-ARs. The clinically used $\alpha_{1A/D}$-ARs antagonist tamsulosin potently ($pA_2 \approx 10.0$) inhibited the norepinephrine-induced CE contractions. Altogether, our results show that $\alpha_{1A}$- and $\alpha_{1D}$-ARs are expressed in the CE duct and $\alpha_{1A}$-AR is the main subtype mediating contraction to norepinephrine. Our results highlight the importance of $\alpha_{1A}$-AR in the peripheral control of ejaculation and strengthen the $\alpha_{1A}$-AR as a target for a non-hormonal approach for male contraception.

## Introduction

$\alpha_1$-Adrenoceptors ($\alpha_{1A}$- $\alpha_{1B}$- and $\alpha_{1D}$-ARs), are targeted by the endogenous catecholamines norepinephrine and epinephrine in the control of a large range of biological functions such as hepatic metabolism, cardiac contractility and contraction of vascular and non-vascular smooth muscle (Koshimizu *et al.*, 2003). $\alpha_1$-ARs are widely expressed in the male reproductive tract and they are essential for male fertility (Sanbe *et al.*, 2007; Avellar *et al.*, 2009). The epididymis, the male reproductive organ responsible for sperm maturation and storage is morpho-physiologically divided in caput, corpus and cauda (Turner, 1995). The cauda epididymis (CE) is the site of storage of spermatozoa until ejaculation and the epididymal duct in this region is encircled by a thick smooth muscle layer (Baumgarten *et al.*, 1971) richly innervated by the sympathetic nervous system, whereas the epididymal duct in the caput and corpus has a thinner and more sparsely innervated smooth muscle layer.

Indeed, the contractions of CE smooth muscle triggered by sympathetic activation are one of the first events in the seminal emission phase of ejaculation (Vignozzi *et al.*, 2008). It is long known that released norepinephrine contracts the CE smooth muscle both *in vivo* and *in vitro* via $\alpha_1$-ARs activation (Pholpramool and Triphrom, 1984; Ventura and Pennefather, 1991; Chaturapanich et al., 2002). In fact, $\alpha_1$-AR antagonists reduce significantly the sperm output in both rats and humans, an effect ascribed to loss of seminal emission (Solomon et al., 1997; Hisasue et al., 2006; Hellstrom and Sikka, 2009). mRNA encoding all three $\alpha_1$-ARs are expressed in the CE and the $\alpha_{1A}$-AR protein is known to be present (Queiroz *et al.*, 2002), but the functional $\alpha_1$-AR subtype(s) mediating CE contractions to norepinephrine is still unknown. The identification of $\alpha_1$-ARs subtypes mediating CE contraction is of interest because the

modulation of the contractility of male accessory organs smooth muscle during the seminal emission phase of ejaculation has been proposed as a non-hormonal male contraceptive approach (Mulryan *et al.*, 2000; White *et al.*, 2013). Hence, in addition to allow a better understanding of the physiology of the CE, the knowledge of the functional $\alpha_1$-ARs in the CE is important for the development of pharmacological tools which could be used as male contraceptives by preventing smooth muscle contractions.

In this study we determined the expression and the contribution of $\alpha_1$-ARs subtypes to the norepinephrine-induced contraction of rat CE duct *in vitro*. Moreover, as the CE duct usually is morphologically distinguished as proximal and distal CE ducts, a comparative analysis of $\alpha_1$-ARs expression and contractile function in these two regions was performed.

# Materials and methods

## Animals

All the experimental procedures were approved by the institutional Ethics Committee for the Use of Experimental Animals and are in accord with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Adult male Wistar rats (120-150 days old and 260-380 g) were provided by the São Paulo State University (UNESP). The animals used in this study were maintained under controlled conditions (12h/12h light/dark cycle, 25±2ºC and 40-70% humidity) with free access to food and water.

## General procedure for CE duct isolation

Rats were killed by decapitation and both epididymides were dissected. The CE duct was uncoiled and segments from the proximal and distal CE duct (corresponding to regions 6 and 7 from (Hinton *et al.*, 1979)) were isolated and cleaned of adherent tissues. The CE duct intraluminal contents were washed away by flushing 1 ml of nutrient solution (see composition below) through its lumen. Usually, a 5 centimeter length uncoiled distal CE duct could be obtained from each epididymis whereas as much as 10 cm was reliably obtained from the proximal CE duct of each epididymis.

## qRT-PCR of $\alpha_{1a}$, $\alpha_{1b}$ and $\alpha_{1d}$ mRNA expression in the proximal and distal CE duct

To accesses the mRNA abundance of α1a, α1b and α1d in the proximal and distal CE, duct segments were cleaned as described above and collected in 1 ml Trizol (Invitrogen Life Technologies®) homogenized with a Polytron and submitted to total RNA extraction according to the manufacturer's protocol. Total RNA (1 µg) from proximal and distal CE duct segments were incubated with DNAse I (1 U/mg RNA; Invitrogen),

and then reverse transcribed with SuperScript III (200 U/ml; Invitrogen) and oligo-d(T) primer.

qRT-PCR analysis was performed with an ABI 7500 thermocycler using Power SYBR Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil). Primers for target genes were designed as described by Yono *et al.* (2008). Reactions were optimized to provide maximum amplification efficiency for each gene. PCR was performed on 0.5-1.0 µl of cDNA in 25 µl reaction volumes in duplicate, and the specificity of each PCR product was determined by melting curve analysis and confirmation of the amplicon size using electrophoresis in 1.5% agarose gels. Negative controls (water replacing cDNA) were run in every plate.

The absolute expression of each target gene was investigated through standard curves generated from serial dilutions of purified PCR products from each of the three $\alpha_1$-ARs subtypes. Thus, a sample obtained from distal CE duct was randomly selected and subjected to a PCR reaction in real time (described above). After the PCR reaction, the products of 3 subtypes of $\alpha_1$-ARs were subjected to agarose gel electrophoresis 2%. After this, the PCR products were purified using the Invisorb® Cleanup Kit Fragment - STRATEC Molecular according to the manufacturer's instructions. The purified PCR products were quantified by spectrophotometer (ND-2000, Nanodrop®).

Six serial dilutions of the purified PCR products were used to perform the standard curve. The absolute values of the dilutions of the purified PCR products varied from approximately 32,000 to 3,200,000 copies/µl. Standard curves were obtained by plotting the values Threshold Cycle (Ct) in the Y-axis and the log of the concentration (copies/µl) of the purified PCR products in the X-axis. Subsequently, the standard curve was analyzed by linear regression. The number of copies was determined by the following formula (Godornes *et al.*, 2007):

Copies/µl = [(6.022 X $10^{23}$ copies) x (plasmid concentration g / µl)]/[number of bases) x (660 daltons/base)]

The absolute quantification was determined by the ratio of the Ct values obtained for each sample of the three $\alpha_1$-AR subtypes amplified with their respective standard curve generated by interpolation of the linear regression obtained.

**[$^3$H]Prazosin binding to intact CE duct segments**

Rats were killed by decapitation, both epididymides were isolated and immersed in ice-cold modified Krebs solution (composition in mM: NaCl 135.7, KCl 4.9, $MgCl_2$ 1.2, $CaCl_2$ 2.0, $NaH_2PO_4$ 1.2, $NaHCO_3$ 10.5, dextrose 11.5, pH 7.4) and cleaned as described above. Pieces of 5 mm length from proximal and distal CE duct were obtained and employed in the saturation or competition [$^3$H]Prazosin binding assays by the tissue segment binding method (Muramatsu *et al.*, 2005).

**Saturation curves for [$^3$H]Prazosin binding to intact proximal and distal CE duct segments**

Cauda epididymis duct segments were incubated in 500 µl of ice-cold modified Krebs solution with one of different concentrations of [$^3$H]Prazosin (20-2000 pM) for 16 hours at 4ºC. Non-specific binding was determined in the presence of 100 µM phentolamine. After the incubation period, the tissues were blotted in filter paper, vortexed for 1 minute in 1 ml of ice-cold modified Krebs solution to reduce the non-specific binding and dissolved in 500 µl of 0.3 M NaOH at 37ºC (tissues usually needed 48-72h for complete dissolution in 0.3 M NaOH solution). Aliquots of tissue solution were used to evaluate the protein content by Bradford assay with BSA as standard and the remaining tissue solution was added to 4 ml scintillation cocktail (Optiphase HiSafe 3; Perkin

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Elmer, Walthan, MA) for radioactivity measurement in a liquid scintillation counter (1900 TR; PACKARD, Canberra, ACT, Australia). Total radioactivity was quantified and expressed as fmol of [$^3$H]Prazosin binding/mg of tissue protein.

**Competition binding assays**

Tissue pieces were incubated in 500 µl of ice-cold modified Krebs solution in the absence or presence of one of different concentrations of prazosin (10 pM-0.3 µM), RS 100329 (10 pM-3 µM; $\alpha_{1A}$-selective), 5-methylurapidil (10 pM-3 µM; $\alpha_{1A}$-selective) or BMY 7378 (0.1 nM-30 µM; $\alpha_{1D}$-selective) for 1 hour at 4ºC. After the 1 hour incubation period, [$^3$H]Prazosin at a 350 pM final concentration was incubated with the tissues for 16 hours at 4ºC. Non-specific binding was determined in the presence of 100 µM phentolamine. Following the [$^3$H]Prazosin incubation period the tissue processing (washing and dissolution) and protein/radioactivity quantification were done as described above.

Non-linear regressions of [$^3$H]Prazosin specific binding inhibition curves were analyzed by an one- and two-site model and the preferred fitting was compared by the Extra sum-of-squares F-test (GraphPad Prism 5, Graph Pad Software, San Diego, CA, USA).

***In vitro* contraction studies**

One centimeter segments of uncoiled proximal and distal CE duct were mounted in 10 ml organ baths under 4.9 mN (proximal CE) or 9.8 mN (distal CE) resting tension in a modified Tyrode's solution (138 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.36 $NaH_2PO_4$,15 mM $NaHCO_3$ and 5.5 mM dextrose), pH 7.4 at 30ºC constantly bubbled with 95%$O_2$/5%$CO_2$ . After a 30 minutes stabilization period, the tissues were contracted with 80 mM KCl at 30 minutes interval until two reproducible contractions

were obtained. After contracture stabilization, a cumulative concentration-response curve to norepinephrine was obtained and taken as a control curve. After washing the tissues, prazosin (1-30 nM), 5-methylurapidil (3-100 nM; ), RS 100329 (1-100 nM), BMY 7378 (10 nM-10 μM) or L-765,314 (10 and 100 nM; $\alpha_{1B}$-selective) were incubated with the tissues for 45 minutes and new concentration-response curves to norepinephrine were constructed in the presence of each antagonist concentration.

All the experiments were done in the presence of a cocktail containing 0.1 μM yohimbine, 0.1 μM propranolol, 0.1 μM desipramine and 10 μM corticosterone to antagonism of $\alpha_2$-adrenoceptors, β-adrenoceptors, and block of neuronal and extraneuronal monoamine uptake systems, respectively.

Norepinephrine concentration-response curves were fitted to a three-parameter concentration-response curve using Prism 5 (Graph Pad Software, San Diego, CA, USA) for the determination of the potency ($pEC_{50}$, i.e. the –log of half maximal norepinephrine concentration) and the maximal contractions (Emax, in milliNewtons - mN). Antagonist potencies against norepinephrine-induced contractions were evaluated by Schild analysis (Arunlakshana and Schild, 1959). The rightward displacements of norepinephrine concentration-curves induced by the different antagonist concentrations were used to calculate concentration ratios (CR), the ratio between the norepinephrine concentration inducing 50% of maximal contraction in the presence and absence of antagonist, and the resulting log (CR-1) values were plotted against the respective antagonist concentrations. Linear regressions of log (CR-1) versus antagonist concentrations were obtained and the slopes were determined. Antagonist affinities ($pK_B$) where defined as the abscissa intercept when the slope of linear regressions was not different from theoretical unity. When the antagonist behavior against norepinephrine-induced contractions was insurmountable, $pA_2$ values were taken as

estimates of antagonist potencies and were calculated through the equation: $pA_2$= log (CR-1) – log [B], where CR is the concentration ratio as defined above and [B] the antagonist concentration.

**Statistical analysis**

Results are presented as mean ± standard error of mean (SEM) for segments taken from n rats. Statistical comparisons were performed with Student's t-test or Analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test in GraphPad Prism 5.0 software (GraphPad Inc., La Jolla, CA, USA). Values of $P < 0.05$ were considered statistically significant.

**Materials**

Prazosin hydrochloride, Yohimbine hydrochloride, (±)-propranolol hydrochloride, BMY 7378 hydrochloride, 5-methylurapidil hydrochloride, desipramine hydrochloride, norepinephrine bitartrate, corticosterone, L-765,314 hydrate (Sigma, St. Louis, MO, USA); RS 100329, (Tocris Bioscience, Ellisville, MO, USA); Tamsulosin hydrochloride (IFFECT, Hong Kong). Corticosterone stock solution (10 mM) was prepared in 100% ethanol. RS 100329 and L-765,314 stock solutions were prepared to 10 mM in dimethylsulfoxide and further dilutions done in distilled water. All other drugs were diluted in distilled water as required. At the maximal concentrations attained dimethylsulfoxide (0.001%) and ethanol (0.1%) had no effect on CE contractions induced by norepinephrine.

# Results

## $\alpha_1$-subtype mRNA expression in the CE duct

The mRNAs encoding for all the three $\alpha_1$-ARs were detected in segments from proximal and distal CE. However, there were large differences in the abundances of mRNAs in both CE regions, as the mRNA encoding the $\alpha_{1a}$ and $\alpha_{1d}$ were more abundant than the $\alpha_{1b}$ mRNA (Figure 1).

## [$^3$H]Prazosin binding to CE segments

The expression of $\alpha_1$-ARs protein was evaluated by [$^3$H]Prazosin binding to intact segments of CE duct. [$^3$H]Prazosin bound to CE segments in a concentration-dependent manner and the specific binding was saturable with equilibrium dissociation constant ($pK_D$) of 9.59 ± 0.28 and 9.40 ± 0.21 in proximal and distal CE segments, respectively (Figure 2). There was no difference in the density of $\alpha_1$-ARs expression between the proximal (132 ± 41 fmol.mg protein$^{-1}$, n=6) and distal (134 ± 28 fmol.mg protein$^{-1}$, n=6) regions of CE ($P > 0.05$, Student's t-test).

To investigate the $\alpha_1$-ARs subtypes expressed in proximal and distal CE ducts, the competition for the [$^3$H]Prazosin specific binding (350 pM) by prazosin and the subtype-selective antagonists 5-methylurapidil, RS 100329 and BMY 7378 was evaluated. At 350 pM the specific binding of [$^3$H]Prazosin amounted to 60 and 73% of total binding in proximal and distal CE, respectively. Figure 3 shows that the specific binding of [$^3$H]Prazosin to proximal and distal CE segments was completely inhibited by prazosin, 5-methylurapidil, RS 100329 and BMY 7378. In CE segments from both regions the inhibition curves for prazosin, 5-methylurapidil and RS 100329 were

monophasic (P > 0.05, F test) with Hill coefficients (*n*H) not different from 1, whereas the inhibition curves for BMY 7378 were better described by a biphasic curve (proximal CE: F(2,20)=4.386, P=0.0263; distal CE: F(2,25)=7.382, P=0.0030) with a similar density of high- and low-affinity binding sites (Figure 3). The dissociation constants ($pK_I$) derived from the non-linear regressions of the inhibition curves are presented in Table 1.

**Functional $\alpha_1$-AR mediating CE duct contraction**

Norepinephrine contracted the proximal and distal CE duct in a concentration-dependent manner. However, the contractions of the proximal and distal CE in response to low concentrations of norepinephrine (<10 nM in proximal CE and <30 nM in the distal CE) were predominantly phasic and tended to wane rapidly (Figure 4A), whereas more sustained contractions were observed at concentrations of norepinephrine higher than 30 nM in both proximal and distal segments of the CE (Figure 4A and 4B). Therefore, contractions to each norepinephrine concentration were measured as the maximal peak before the addition of the consecutive agonist concentration.

There was no difference in the potency of norepinephrine in the contractions of proximal and distal CE (proximal, $pEC_{50}$: 6.88 ± 0.08, n=24; distal $pEC_{50}$: 6.97 ± 0.05, n=25; P > 0.05, Student's t-test), but the maximal contraction was significantly higher in distal CE (proximal, Emax: 3.68 ± 0.19 mN, n=24 vs distal Emax: 11.27 ± 0.21 mN, n=25; P < 0.05, Student's t-test).

Contractions to norepinephrine in both portions of CE were competitively antagonized by prazosin with high affinity ($pK_B$ proximal CE: 9.15 ± 0.06, n=4; $pK_B$ distal CE: 9.51 ± 0.02, n=4) indicating that under the experimental conditions employed the norepinephrine-induced contractions are mediated by $\alpha_1$-ARs activation. The $\alpha_1$-AR

subtype-selective antagonists RS 100329, 5-methylurapidil and BMY 7378 inhibited the norepinephrine-induced contractions showing competitive behavior whereas the $\alpha_{1B}$-selective antagonist L-765,314 (10 and 100 nM) had no effect (Figure 5).The $pK_B$ values and the slopes of the Schild plots are shown in the Table 2. The $\alpha_{1A}$-selective antagonists RS 100329 ($pK_B \approx 9.50$) and 5-methylurapidil ($pK_B \approx 8.50$-9.0) exhibited high affinity against norepinephrine-induced contractions in both CE regions consistent with norepinephrine-induced contractions of both CE regions resulting from $\alpha_{1A}$-ARs activation. In contrast, the contractions induced by norepinephrine were antagonized only by high concentrations of the $\alpha_{1D}$-selective antagonist BMY 7378 (> 100nM) indicating a low affinity for this antagonist ($pK_B \approx 6.50$-7.0), not consistent with involvement of $\alpha_{1D}$-ARs. The antagonist potency order against norepinephrine-induced contractions, RS 100329>prazosin>5-methylurapidil>BMY 7378, was the same in proximal and distal CE.

As an additional approach to investigate the functional $\alpha_1$-AR mediating CE contraction to norepinephrine, the effects of the $\alpha_{1A/D}$-selective antagonist tamsulosin were evaluated. Tamsulosin antagonized the contraction of proximal and distal CE segments to norepinephrine presenting insurmountable behavior, reducing the Emax by 52.90 ± 4.35% (proximal CE; n=6) and 33.08 ± 8.71% (distal CE; n=7) at 1 nM and 69.84 ± 5.11% (proximal CE; n=6) and 72.24 ± 5.75% (distal CE; n=7) at 3 nM (Figure 6). Albeit the insurmountable behavior precluded an affinity estimate for tamsulosin, $pA_2$ values of 10.36 ± 0.15 (n=6) and 10.08 ± 0.07 (n=7) in the proximal and distal CE duct were calculated from the effects produced by 0.1 (proximal CE) and 0.3 nM (distal CE), respectively. The tamsulosin $pA_2$ values in proximal and distal CE were not different ($P > 0.05$, Student's t-test).

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## Discussion

The CE duct contraction is an important step in the seminal emission phase of ejaculation and, in fact, maneuvers that decrease the CE contraction are known to impair male fertility (Ricker *et al.*, 1997; Solomon *et al.*, 1997; Kempinas *et al.*, 1998). The CE expresses transcripts encoding for all three $\alpha_1$-ARs, and the $\alpha_{1A}$-ARs and $\alpha_{1D}$-ARs proteins could be reliably detected in our tissue segment binding assays. A previous study of [$^3$H]Prazosin binding to distal CE membrane preparations showed evidence for $\alpha_{1A}$-ARs expression in this tissue (Queiroz *et al.*, 2002). In the present study, by the use of a different [$^3$H]Prazosin assay we could demonstrate that the $\alpha_{1D}$-ARs are indeed expressed in this tissue and at a similar density of the $\alpha_{1A}$-ARs. Although we have no clear explanation to the failure of Queiroz *et al.* to detect $\alpha_{1D}$-ARs in the CE, one of the possible explanations is that this receptor subtype was lost during the CE homogenization procedure; it is important to mention that one of the advantages of tissue segment binding over the conventional membrane binding method is the preservation of receptor expression as a result of reduced protein loss (Muramatsu *et al.*, 2005).

Using the most selective $\alpha_1$-AR subtype antagonists available (Alexander *et al.*, 2015) our results indicate that proximal and distal CE contractions to norepinephrine were mediated by $\alpha_{1A}$-ARs. In fact, $\alpha_{1A}$-ARs knockout mice present increased sperm content in the epididymis and decreased vas deferens sperm count suggesting that the absence of $\alpha_{1A}$-AR causes an impairment in cauda-to-vas deferens sperm transport (Sanbe *et al.*, 2007). The contraction of vas deferens (Burt *et al.*, 1995; Pupo, 1998), prostate and seminal vesicles (Silva *et al.*, 1999) are similarly mediated by $\alpha_{1A}$-ARs making this receptor the main $\alpha_1$-subtype involved in the seminal emission phase of ejaculation. In fact, knocking out $\alpha_{1B}$- or $\alpha_{1D}$- results in no major effects in mice fertility (Cavalli *et al.*, 1997; Tanoue *et al.*, 2002). Importantly, $\alpha_{1D}$-ARs were shown to play a role in the contraction of rat and mouse vas deferens smooth muscle to endogenous norepinephrine released by electrical field stimulation, and at least in the mouse vas deferens the $\alpha_{1D}$-ARs seems to play a role in exogenous norepinephrine-induced contraction (Mallard *et al.*, 1992; Cleary *et al.*, 2004; Bexis *et al.*, 2008). Therefore, our results do not exclude a $\alpha_{1D}$-ARs role in CE contraction, but rather assign a predominant role for $\alpha_{1A}$-ARs. A significant number of patients under treatment with $\alpha_1$-ARs antagonists to relief the symptoms of benign prostatic hyperplasia experience ejaculation dysfunction (Giuliano, 2006). In particular, men taking the high-affinity $\alpha_{1A/D}$-ARs antagonist tamsulosin report decreased ejaculate volume and laboratorial seminal inspection evidenced reduced ejaculated sperm count (Chapple, 1996; Narayan and Lepor, 2001; Hellstrom and Sikka, 2006; Hellstrom and Sikka, 2009). The insurmountable antagonism displayed by tamsulosin against $\alpha_{1A}$-mediated CE and vas deferens smooth muscle contraction (de Almeida Kiguti and Pupo, 2012) emerge as

possible contributing factor to the reported reduced seminal sperm count induced by this drug.

The development of a non-hormonal male contraceptive pill has attracted significant interest in recent years and the smooth muscle cells of male sexual accessory organs has emerged as potential targets to such drugs (Murdoch and Goldberg, 2014). In this scenario, the modulation of $\alpha_1$- and purinergic P2X1-induced smooth muscle contractions are the most promising targets as norepinephrine and ATP are the main sympathetic nervous system co-transmitters in the male urogenital system (Burnstock, 2014; Navarrete *et al.*, 2014). Furthermore, the importance of $\alpha_{1A}$-ARs on the ejaculation reflex and male fertility was explored in a recent study showing complete infertility of male mice with knockout of both $\alpha_{1A}$-ARs and purinergic P2X1 receptors (White *et al.*, 2013).

Overall, the present study shows that $\alpha_{1A}$- and $\alpha_{1D}$-ARs are expressed in the CE and that the $\alpha_{1A}$-AR is the main $\alpha_1$-AR subtype mediating contraction of CE smooth muscle to norepinephrine. These results contribute to our understanding on the role of $\alpha_1$-AR subtypes on male sexual function/fertility and further strengthen the rationale that any male contraceptive approach targeting $\alpha_1$-AR should rely on $\alpha_{1A}$-AR subtype as the most promising target.

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## Authorship Contributions

*Participated in research design:* Pacini, Castilho, Hebeler-Barbosa, Pupo, Kiguti.

*Conducted the experiments:* Pacini, Castilho, Hebeler-Barbosa, Pupo, Kiguti.

*Performed data analysis:* Pacini, Castilho, Hebeler-Barbosa, Pupo, Kiguti.

*Wrote or contributed to the writing of the manuscript:* Pacini, Castilho, Pupo, Kiguti.

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## Footnotes

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ESAP and LRAK contributed equally to this work.

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## Figure legends

**Figure 1.** qRT-PCR analysis of $\alpha_{1a}$, $\alpha_{1b}$ and $\alpha_{1d}$ mRNA expression in the rat proximal (A) and distal (B) CE duct. Values represent mean ± SEM from tissues taken from 8 different rats. Different superscript letters denote statistically different means ($P < 0.05$, ANOVA + Newman-Keuls).

**Figure 2.** Binding of [$^3$H]Prazosin to intact segments of proximal (A) and distal (B) rat CE duct. Symbols represent the mean and the vertical bars the SEM from tissues taken from 6 different rats.

**Figure 3.** Inhibition of [$^3$H]Prazosin specific binding to intact segments of proximal (A and C) and distal (B and D) rat CE duct by unlabeled prazosin, RS 100329, 5-methylurapidil and BMY 7378. Symbols represent the mean and the vertical bars the SEM from segments taken from 4-6 different rats.

**Figure 4.** Representative trace-recordings of norepinephrine-induced contractions of proximal (A) and distal (B) rat CE duct segments. The dots denote the approximate time points of norepinephrine administration and the numbers under the dots the final norepinephrine concentration attained in the bath. (C) Mean concentration-response curves to norepinephrine in the proximal and distal rat CE duct. Symbols represent the mean and the vertical bars the SEM from 24 (proximal CE) and 25 (distal CE) duct segments taken from different rats.

**Figure 5.** Antagonism of *in vitro* norepinephrine-induced contractions of proximal (A, D, G and J) and distal (B, E, H and K) rat CE duct segments. The effects of prazosin (A and B), RS 100329 (D and E), 5-methylurapidil (G and H), BMY 7378 (J and K) and L-765,314 (M and N) are presented. For clarity, in J and K only the effects of BMY 7378 0.3 to 10 μM are shown, as the concentration-response curves for norepinephrine in presence of BMY 7378 10 to 100 nM were superimposed to the control curves. The resulting Schild plots for the antagonism displayed by prazosin, RS 100329, 5-methylurapidil and BMY 7378 are presented in C, F, I and L, respectively. Symbols represent the mean and the vertical bars the SEM from 4-6 different segments taken from different rats.

**Figure 6.** Antagonism of *in vitro* norepinephrine-induced contractions of proximal and distal rat CE segments by tamsulosin. (A) Mean concentration-response curves of proximal CE duct to norepinephrine in the presence of tamsulosin (0.1-30 nM). (B) Mean concentration-response curves of distal CE duct to norepinephrine in the presence of tamsulosin (0.1-30 nM). (C) Plot of maximal contraction induced by norepinephrine in proximal and distal CE duct segments in the presence of different tamsulosin

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concentrations. Symbols represent the mean and the vertical bars the SEM from 6 (proximal CE) and 7 (distal CE) segments taken from different rats.



## Tables

**Table 1.** Binding affinity values ($pK_I$) of prazosin, 5-Methylurapidil, RS 100329 and BMY 7378 derived from the inhibition of [$^3$H]Prazosin specific binding to proximal and distal rat CE segments. The Hill slopes (*n*H) of inhibition curves are presented.

| | Proximal | | | | Distal | | | |
|---|---|---|---|---|---|---|---|---|
| | $^{\#}pK_{IH}$ | $^{\&}pK_{IL}$ | -nH | n | $pK_{IH}$ | $pK_{IL}$ | -nH | n |
| Prazosin | 9.03±0.17 | --- | 0.84±0.17 | 4 | 9.22±0.16 | --- | 0.83±0.13 | 6 |
| 5-MU | 9.28±0.12 | --- | 0.80±0.16 | 4 | 9.09±0.17 | --- | 0.84±0.08 | 4 |
| RS 100329 | 9.32±0.18 | --- | 0.83±0.17 | 4 | 9.34±0.10 | --- | 1.01±0.13 | 4 |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| BMY 7378 | 8.98±0.47 (48%) | 6.28±0.42 (52%) | 0.53±0.12* | 4 | 8.91±0.39 (52%) | 5.92±0.57 (48%) | 0.42±0.08* | 5 |

5-MU: 5-methylurapidil;

[#]$pK_I$ at high-affinity binding site; [&] $pK_I$ at low-affinity binding site; values between parenthesis in BMY 7378 row represent the total percentage of high and low affinity sites; Data represent mean ± SEM from n experiments with tissues taken from different rats.

*significantly different from 1 ($P < 0.05$)



**Table 2.** Antagonist affinity values ($pK_B$) of prazosin, RS 100329, 5-methylurapidil and BMY 7378 for the norepinephrine-induced contractions of proximal and distal CE segments. The slopes of linear regressions derived from Schild analysis of antagonism of norepinephrine-induced contractions in the proximal and distal CE are also shown.

| | Proximal | | Distal | |
|---|---|---|---|---|
| | $pK_B$ | slope | $pK_B$ | slope |
| Prazosin | 9.15 ± 0.06 | 1.06 ± 0.12 (n=5) | 9.51 ± 0.02 | 0.98 ± 0.03 (n=5) |
| RS 100329 | 9.31 ± 0.03 | 0.93 ± 0.07 (n=5) | 9.63 ± 0.02 | 0.96 ± 0.04 (n=5) |
| 5-methylurapidil | 8.58 ± 0.05 | 0.92 ± 0.09 (n=6) | 9.03 ± 0.03 | 1.02 ± 0.05 (n=5) |

| | | | | |
|---|---|---|---|---|
| BMY 7378 | 6.57 ± 0.13 | 0.98 ± 0.32 (n=6) | 6.99 ± 0.04 | 0.91 ± 0.07 (n=5) |

Data represent mean ± SEM from n different segments taken from different rats.

## Figures

## Figure 1

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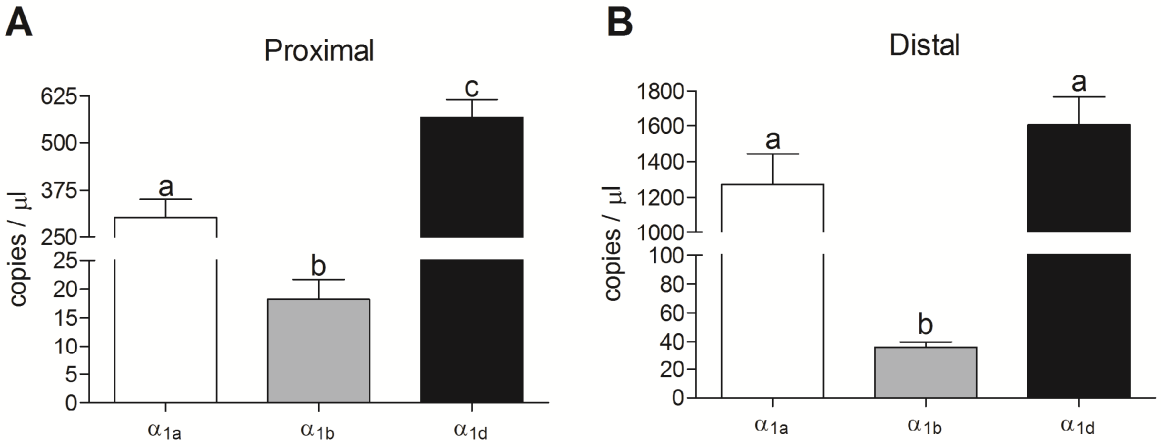


**Figure 2**

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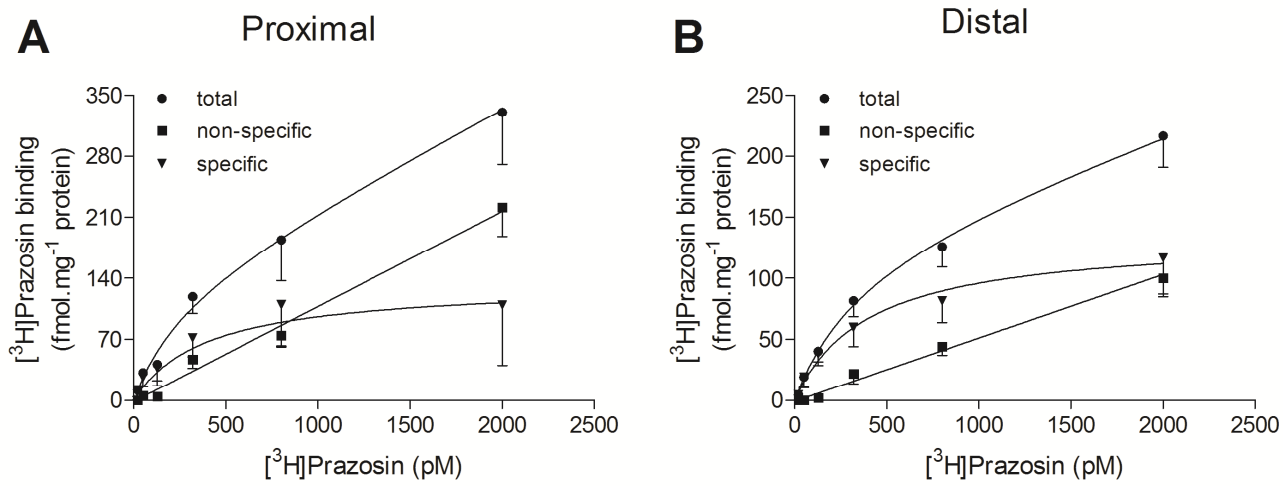


**Figure 3**

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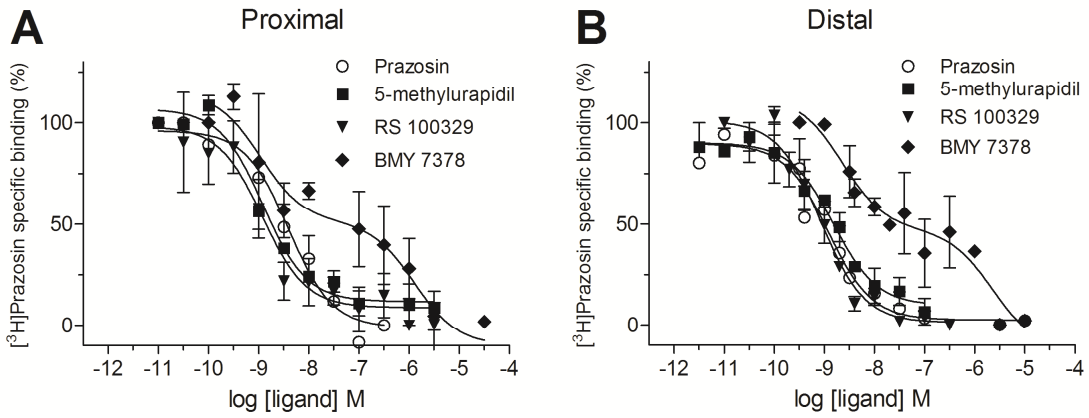


**Figure 4**

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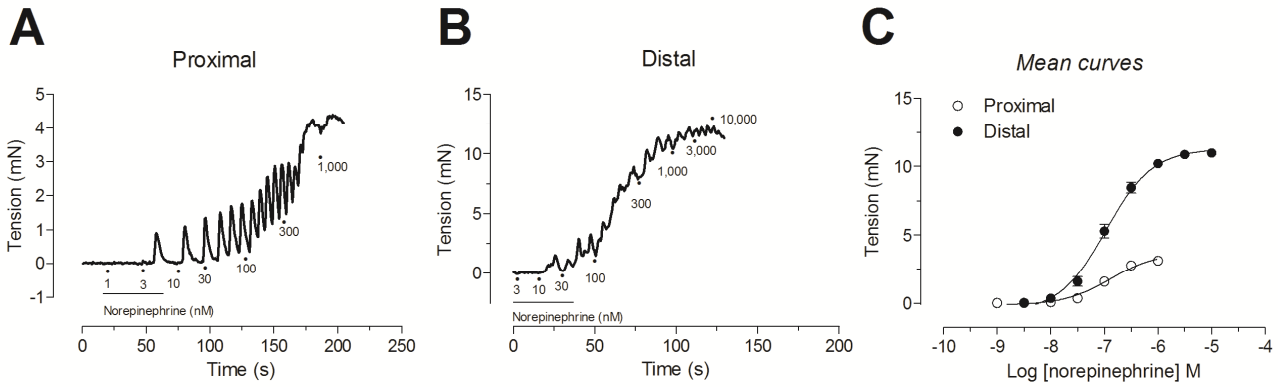


**Figure 5**

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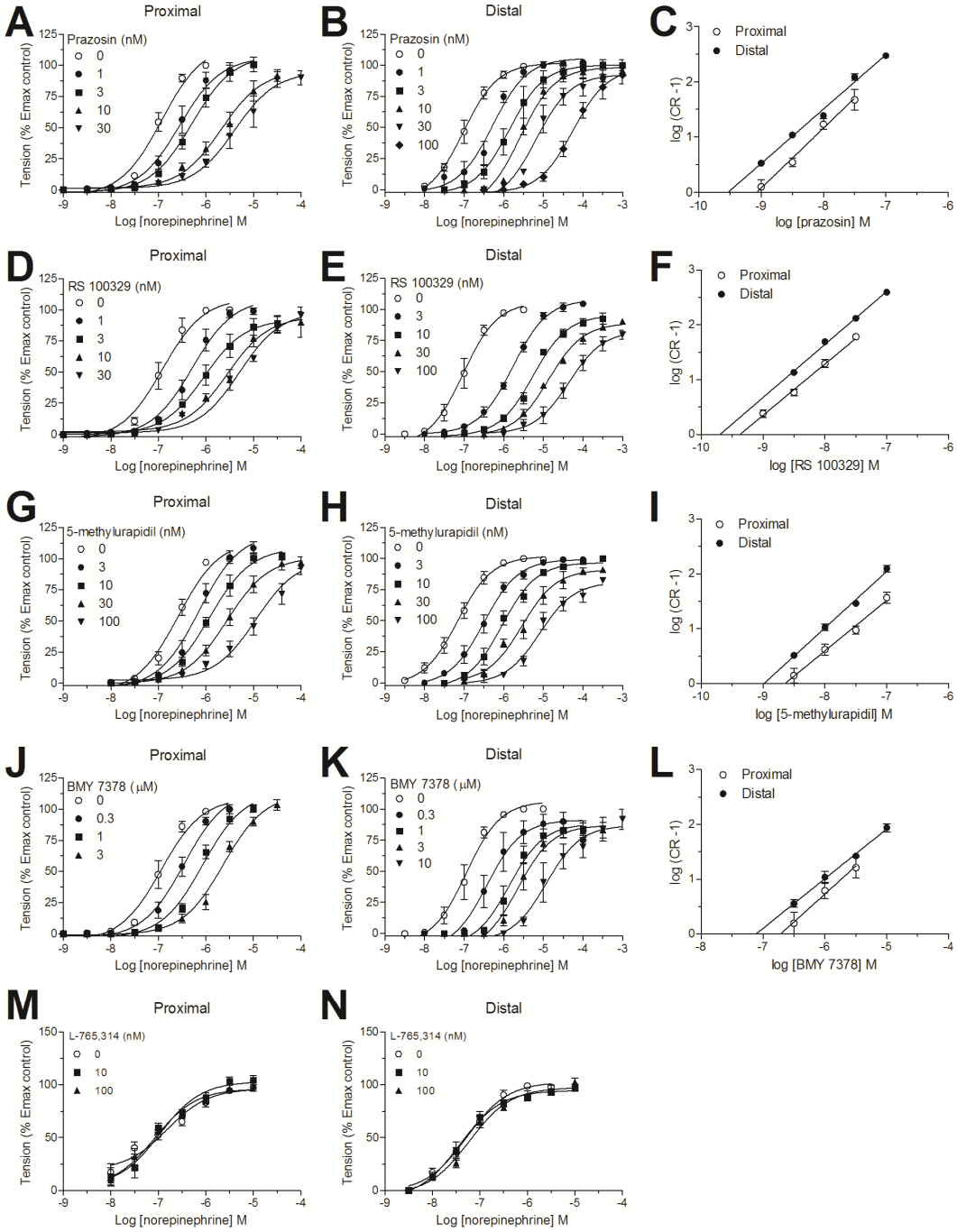


**Figure 6**

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