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Contraction of Rat Cauda Epididymis Smooth Muscle to α_1-Adrenoceptor Activation Is Mediated by α_{1A}-Adrenoceptors

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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 α_1-adrenoceptors (α_1-ARs) plays a key role during the seminal emission phase of ejaculation and α_1-AR antagonism has been suggested as a nonhormonal and reversible male contraceptive target. Since the α_1-AR subtype mediating contraction of rat CE is not known, this study investigates the expression and role of α_1-AR subtypes on the proximal and distal rat CE duct contraction to norepinephrine in vitro. Alpha$_{1a}$, α_{1b}, and α_{1d} transcripts were detected by real-time quantitative polymerase chain reaction in proximal and distal CE segments and α_{1a} and α_{1d} were shown to predominate over α_{1b}. The inhibition of [^{3}H]prazosin specific binding to intact CE segments from proximal and distal CE by RS 100329 and 5-methylurapidil (α_{1A}-selective) and BMY 7378 (α_{1D}-selective) showed that α_{1A}- and α_{1D}-ARs are expressed at similar densities. Norepinephrine-induced contractions of CE were competitively antagonized with high affinity by RS 100329 (p$K_B \approx 9.50$) and 5-methylurapidil (p$K_B \approx 9.0$) and with low affinity by BMY 7378 (p$K_B \approx 7.0$) and the α_{1B}-selective L-765,314 (p$A_2 < 7.0$), suggesting contractions are mediated by α_{1A}-ARs. The clinically used $\alpha_{1A/D}$-ARs antagonist tamsulosin potently (p$A_2 \approx 10.0$) inhibited the norepinephrine-induced CE contractions. Altogether, our results show that α_{1A}- and α_{1D}-ARs are expressed in the CE duct and α_{1A}-AR is the main subtype mediating contraction to norepinephrine. Our results highlight the importance of α_{1A}-AR in the peripheral control of ejaculation and strengthen the α_{1A}-AR as a target for a nonhormonal approach to male contraception.

Introduction

α_1-Adrenoceptors [(α_1-ARs); α_{1A}- α_{1B}-, and α_{1D}-ARs], are targeted by the endogenous catecholamines norepinephrine and epinephrine in the control of a large range of biologic functions such as hepatic metabolism, cardiac contractility, and contraction of vascular and nonvascular smooth muscle (Koshimizu et al., 2003). α_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 morphophysiologically 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 α_1-ARs activation (Pholpramool and Triphrom, 1984; Ventura and Pennefather, 1991; Chaturapanich et al., 2002). In fact, α_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 α_1-ARs are expressed in the CE and the α_{1A}-AR protein is known to be present (Queiróz et al., 2002), but the functional α_1-AR subtype(s) mediating CE contractions to norepinephrine is still unknown. The identification of α_1-ARs subtypes mediating CE contraction is of interest because the modulation of the contractility of male accessory organ's smooth muscle during the seminal emission phase of ejaculation has been proposed as a nonhormonal male contraceptive approach (Mulryan et al., 2000; White et al., 2013). Hence, in addition to allowing better understanding of the physiology of the CE, the knowledge of the functional α_1-ARs in the CE is important for the development of pharmacological tools that could be used as male contraceptives by preventing smooth muscle contractions.

In this study we determined the expression and the contribution of α_1-ARs subtypes to the norepinephrine-induced contraction of rat CE duct in vitro. Moreover, since the CE duct usually is morphologically distinguished as proximal and distal

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ABBREVIATIONS: α_1-AR, α_1-adrenoceptor; CE, cauda epididymis; CR, concentration ratio; PCR, polymerase chain reaction.

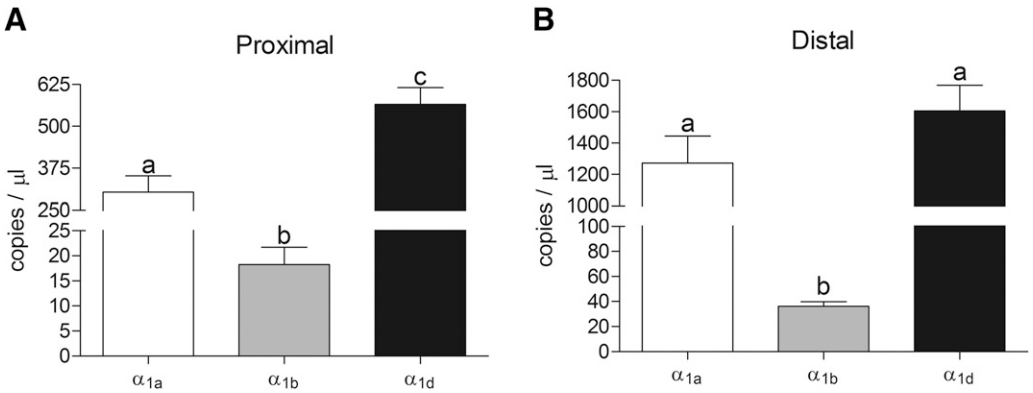

Fig. 1. Real-time quantitative PCR analysis of α_{1a}, α_{1b}, and α_{1d} mRNA expression in the rat proximal (A) and distal (B) CE ducts. Values represent mean ± S.E.M. from tissues taken from eight different rats. Different superscript letters denote statistically different means ($P < 0.05$, analysis of variance + Newman-Keuls).

CE ducts, a comparative analysis of α_1-ARs expression and contractile function in these two regions was performed.

Materials and Methods

Animals. All of the experimental procedures were approved by the Institutional Ethics Committee for the Use of Experimental Animals (protocol number 294) and are in accord with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). Adult male Wistar rats (120–150 days old and 260–380 g) were provided by São Paulo State University. The animals used in this study were maintained under controlled conditions (12-hour/12-hour light/dark cycle, 25 ± 2°C 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 ducts [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 the composition described subsequently) through its lumen. Usually, a 5-cm-long 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.

Real-Time Quantitative Polymerase Chain Reaction (PCR) of α_{1a}, α_{1b}, and α_{1d} mRNA Expression in the Proximal and Distal CE Duct. To accesses the mRNA abundance of α_{1a}, α_{1b}, and α_{1d} in the proximal and distal CE, duct segments were cleaned as described previously and collected in 1 ml Trizol (Invitrogen Life Technologies, Carlsbad, CA) homogenized with a Polytron homogenizer and submitted to total RNA extraction according to the manufacturer's protocol (Polytron, IKA Works, Wilmington, NC). 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.

Real-time quantitative 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 α_1-AR subtypes. Thus, a sample obtained from the distal CE duct was randomly selected and subjected to a PCR reaction in real time (described previously). After the PCR reaction, the products of the three subtypes of α_1-ARs were subjected to agarose gel electrophoresis 2%. Then, the PCR products were purified using the Invisorb Cleanup Kit Fragment according to the manufacturer's instructions (STRATEC Molecular, Berlin, Germany). The purified PCR products were quantified by a spectrophotometer (NanoDrop ND-2000, Thermo Fisher Scientific, Waltham, MA).

Six serial dilutions of the purified PCR products were used to perform the standard curve. The absolute values of the dilutions of the

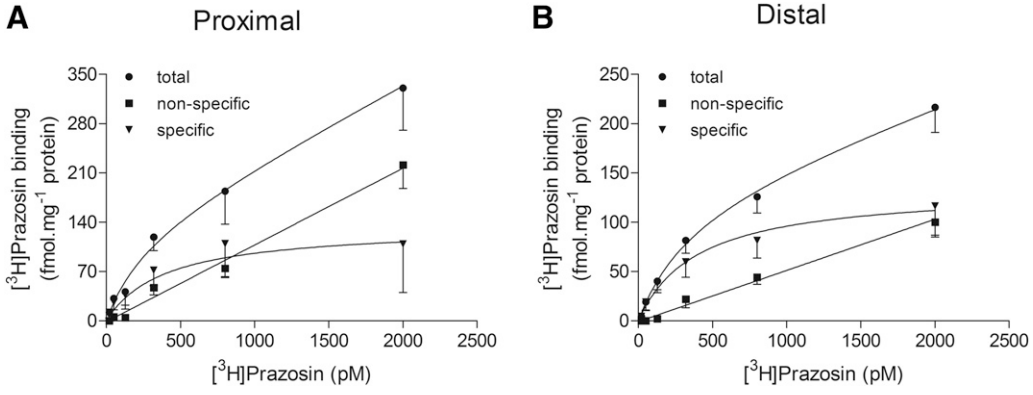

Fig. 2. Binding of [^{3}H]prazosin to intact segments of proximal (A) and distal (B) rat CE ducts. Symbols represent the mean values and the vertical bars represent the S.E.M. values from tissues taken from six different rats.

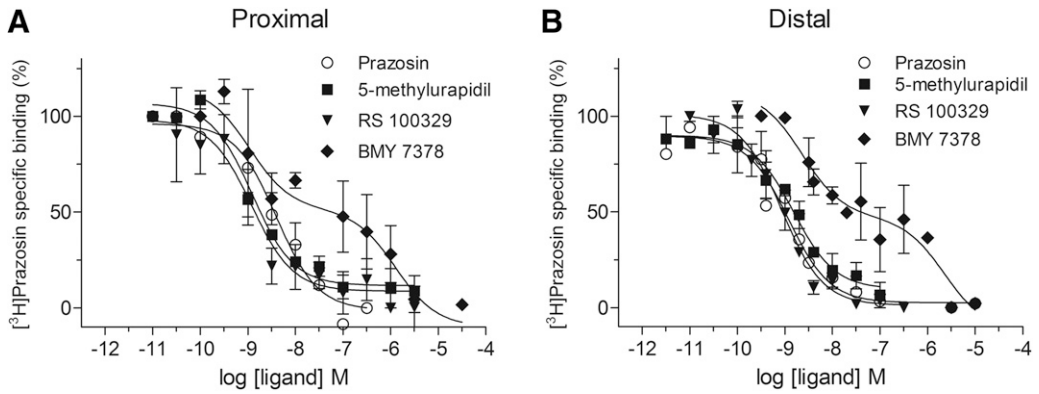

Fig. 3. Inhibition of [^{3}H]Prazosin specific binding to intact segments of proximal (A) and distal (B) rat CE ducts by unlabeled prazosin, RS 100329, 5-methylurapidil, and BMY 7378. Symbols represent the mean values and the vertical bars represent the S.E.M. values from segments taken from four to six different rats.

purified PCR products varied from approximately 32,000 to 3,200,000 copies/μl. Standard curves were obtained by plotting the values of the threshold cycle in the *Y*-axis and the log of the concentration (copies/microliters) 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):

$$\text{Copies}/\mu\text{l} = \left[(6.022 \times 10^{23}\ \text{copies}) \times (\text{plasmid concentrations g}/\mu\text{l})\right] / \left[(\text{number of bases}) \times (660\ \text{Da/base})\right]$$

The absolute quantification was determined by the ratio of the threshold cycle values obtained for each sample of the three α_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 millimolars: 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 previously. Pieces of the proximal and distal CE ducts (5 mm in length) 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 the different concentrations of [^{3}H]prazosin (20–2000 pM) for 16 hours at 4°C. Nonspecific 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 nonspecific binding and dissolved in 500 μl of 0.3 M NaOH at 37°C (tissues usually needed 48–72 hours for complete dissolution in 0.3 M NaOH solution). Aliquots of tissue solution were used to evaluate the protein content by Bradford assay with bovine serum albumin as the standard and the remaining tissue solution was added to a 4-ml scintillation cocktail (Optiphase HiSafe 3; Perkin Elmer, Walthan, MA) for radioactivity measurement in a liquid scintillation counter (1900 TR; PACKARD, Canberra, ACT, Australia). Total radioactivity was quantified and expressed as femtomoles of [^{3}H]prazosin binding/milligrams 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 the different concentrations of prazosin (10 pM–0.3 μM), RS 100329 (10 pM–3 μM; α_{1A}-selective), 5-methylurapidil (10 pM–3 μM; α_{1A}-selective), or BMY 7378 (0.1 nM–30 μM; α_{1D}-selective) for 1 hour at 4°C. After the 1-hour incubation period, [^{3}H]prazosin at a 350-pM final concentration was incubated with the tissues for 16 hours at 4°C. Nonspecific 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 previously. Nonlinear regressions of [^{3}H]prazosin specific binding inhibition curves were analyzed by an one- and two-site models and the preferred fitting was compared by the extra sum-of-squares *F*-test (GraphPad Prism 5; GraphPad Software, San Diego, CA).

In Vitro Contraction Studies. One centimeter segments of uncoiled proximal and distal CE ducts 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 30°C constantly bubbled with 95%O_2/5%CO_2. After a 30-minute stabilization period, the tissues were contracted with

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 (nH) of inhibition curves are presented.

	Proximal				Distal			
	pK_{IH}[a]	pK_{IL}[b]	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
BMY 7378	8.98 ± 0.47 (48%)	6.28 ± 0.42 (52%)	0.53 ± 0.12[c]	4	8.91 ± 0.39 (52%)	5.92 ± 0.57 (48%)	0.42 ± 0.08[c]	5

5-MU, 5-methylurapidil.

[a]pK_I at high-affinity binding site.

[b]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 ± S.E.M. from n experiments with tissues taken from different rats.

[c]Significantly different from 1 ($P < 0.05$).

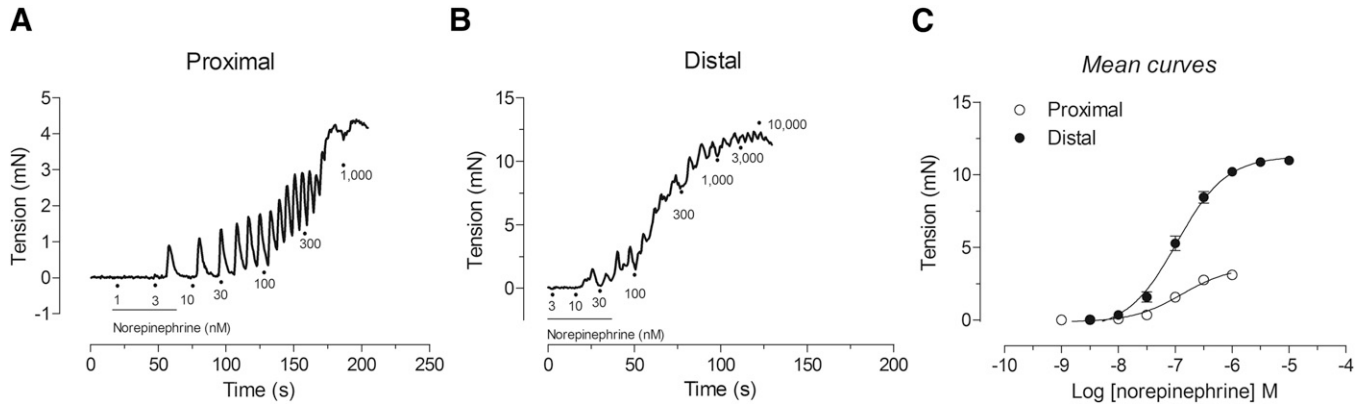

Fig. 4. Representative trace recordings of norepinephrine-induced contractions of proximal (A) and distal (B) rat CE duct segments. The dots denote the approximate time points of norepinephrine administration and the numbers under the dots denote the final norepinephrine concentration attained in the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

80 mM KCl at 30-minute intervals 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; α_{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 of 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 α_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 (GraphPad Software) for the determination of the potency (pEC_{50}, i.e., the log of half-maximal norepinephrine concentration) and the maximal contractions [E_{max}, in milliNewtons (mN)]. Antagonist potencies against norepinephrine-induced contractions were evaluated by Schild analysis (Arunlakshana and Schild, 1959). The rightward displacements of the norepinephrine concentration curves induced by the different antagonist concentrations were used to calculate the concentration ratios (CRs), 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) were 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, the pA_2 values were taken as estimates of antagonist potencies and were calculated through the following equation: $pA_2 = \log (CR\text{-}1) - \log [B]$, where CR is the concentration ratio as defined previously and [B] is the antagonist concentration.

Statistical Analysis. Results are presented as mean ± S.E.M. for segments taken from n rats. Statistical comparisons were performed with Student's t test or analysis of variance followed by Newman-Keuls multiple comparisons test in the GraphPad Prism 5.0 software (GraphPad Inc., La Jolla, CA). 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, and L-765,314 hydrate were obtained from Sigma (St. Louis, MO); RS 100329 was obtained from Tocris Bioscience (Ellisville, MO); and tamsulosin hydrochloride was obtained from IFFECT (Hong Kong, China). 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 were 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

α_1-Subtype mRNA Expression in the CE Duct. The mRNAs encoding for all three α_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 since the mRNA encoding the α_{1a} and α_{1d} mRNA were more abundant than the α_{1b} mRNA (Fig. 1).

[^{3}H]Prazosin Binding to CE Segments. The expression of α_1-AR 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 constants (pK_D) of 9.59 ± 0.28 and 9.40 ± 0.21 in the proximal and distal CE segments, respectively (Fig. 2). There was no difference in the density of α_1-AR 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 α_1-AR 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 the 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 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 to high- and low-affinity binding sites (Fig. 3). The dissociation constants (pK_I) derived from the nonlinear regressions of the inhibition curves are presented in Table 1.

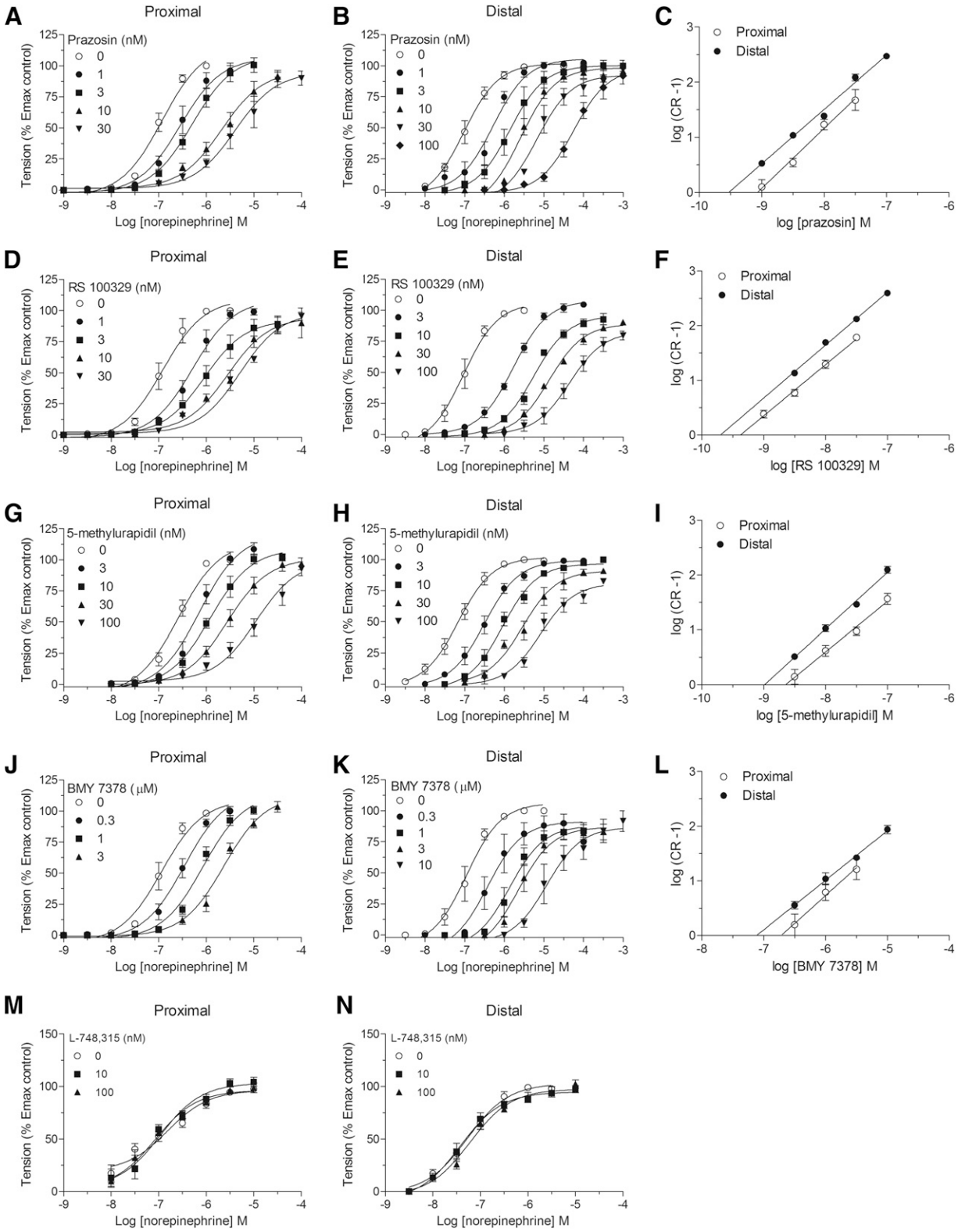

Fig. 5. Antagonism of in vitro norepinephrine-induced contractions of proximal (A, D, G, and J) and distal (B, E, H, and K) rat CE duct segments. The effects of prazosin (A and B), RS 100329 (D and E), 5-methylurapidil (G and H), BMY 7378 (J and K), and L-765,314 (M and N) are presented. For clarity, in (J and K) only the effects of BMY 7378 (0.3–10 μM) are shown since the concentration-response curves for norepinephrine in the presence of BMY 7378 (10–100 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 values and the vertical bars represent the S.E.M. values from four to six different segments taken from different rats.

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. Data represent mean ± S.E.M. from n different segments taken from different rats.

	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)

Functional α_1-AR Mediating CE Duct Contraction. Norepinephrine contracted the proximal and distal CE ducts 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 (Fig. 4A), whereas more sustained contractions were observed at concentrations of norepinephrine higher than 30 nM in both proximal and distal segments of the CE (Fig. 4, A and B). 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, E_{max}: 3.68 ± 0.19 mN, n = 24 vs. distal E_{max}: 11.27 ± 0.21 mN, n = 25; $P < 0.05$, Student's t test).

Contractions to norepinephrine in both portions of the 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 α_1-AR activation. The α_1-AR subtype-selective antagonists RS 100329, 5-methylurapidil, and BMY 7378 inhibited the norepinephrine-induced contractions showing competitive behavior, whereas the α_{1B}-selective antagonist L-765,314 (10 and 100 nM) had no effect (Fig. 5). The pK_B values and the slopes of the Schild plots are shown in Table 2. The α_{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 α_{1A}-AR activation. In contrast, the contractions induced by norepinephrine were antagonized only by high concentrations of the α_{1D}-selective antagonist BMY 7378 (>100 nM), indicating low affinity for this antagonist ($pK_B \approx 6.50$–7.0), not consistent with involvement of α_{1D}-ARs. The antagonist potency order against norepinephrine-induced contractions, RS 100329 > prazosin > 5-methylurapidil > BMY 7378, was the same in both the proximal and distal CE.

As an additional approach to investigate the functional α_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 E_{max} 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 (Fig. 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 ducts were calculated from the effects produced by 0.1 nM (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).

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 α_1-ARs, and the α_{1A}-AR and α_{1D}-AR 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 α_{1A}-AR expression in this tissue (Queiróz et al., 2002). In the present study, by using a different [^{3}H]prazosin assay we demonstrated that the α_{1D}-ARs are indeed expressed in this tissue and at a similar density to the α_{1A}-ARs. Although we have no clear explanation for the failure of Queiróz et al. (2002) to detect α_{1D}-ARs in the CE, one possible explanation 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 α_1-AR subtype antagonists available (Alexander et al., 2015) our results indicate that proximal and distal CE contractions to norepinephrine were mediated by α_{1A}-ARs. In fact, α_{1A}-ARs knockout mice present increased sperm content in the epididymis and decreased vas deferens sperm count, suggesting that the absence of α_{1A}-AR causes impairment in cauda-to-vas deferens sperm transport (Sanbe et al., 2007). The contractions of vas deferens (Burt et al., 1995; Pupo, 1998), prostate, and seminal vesicles (Silva et al., 1999) are similarly mediated by α_{1A}-ARs, making this receptor the main α_1-subtype involved in the seminal emission phase of ejaculation; in fact, knocking out the α_{1B}- or α_{1D}-subtype results in no major effects in mouse fertility (Cavalli et al.,

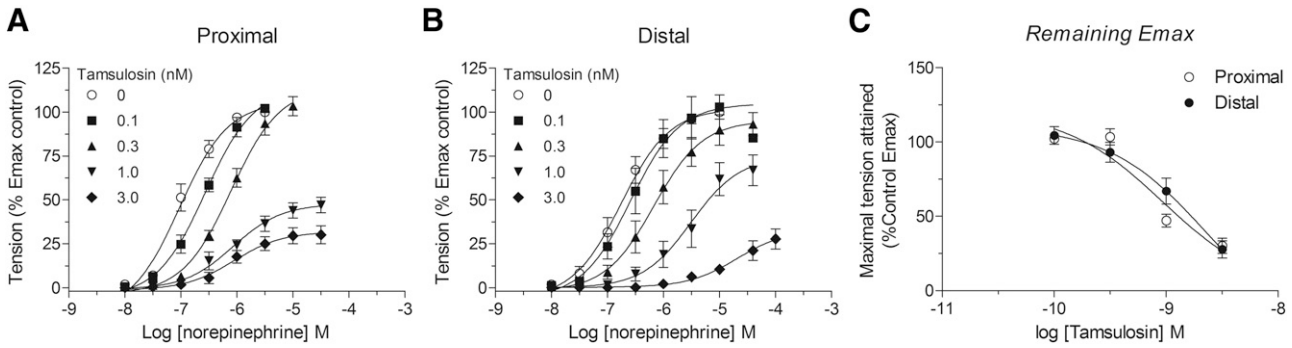

Fig. 6. Antagonism of in vitro norepinephrine-induced contractions of proximal and distal rat CE segments by tamsulosin. (A) Mean concentration-response curves of the proximal CE duct to norepinephrine in the presence of tamsulosin (0.1–30 nM). (B) Mean concentration-response curves of the distal CE 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 concentrations. Symbols represent the mean values and the vertical bars represent the S.E.M. values from six (proximal CE) and seven (distal CE) segments taken from different rats.

1997; Tanoue et al., 2002). Importantly, α_{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 α_{1D}-AR 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 α_{1D}-AR role in CE contraction, but rather assign a predominant role for α_{1A}-ARs. Under treatment with α_1-AR antagonists to relief the symptoms of benign prostatic hyperplasia, a significant number of patients experience ejaculation dysfunction (Giuliano, 2006). In particular, men taking the high-affinity $\alpha_{1A/D}$-AR 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, 2009). The insurmountable antagonism displayed by tamsulosin against α_{1A}-mediated CE and vas deferens smooth muscle contraction (de Almeida Kiguti and Pupo, 2012) emerges as a possible contributing factor to the reported reduced seminal sperm count induced by this drug.

The development of a nonhormonal 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 for such drugs (Murdoch and Goldberg, 2014). In this scenario, the modulations of α_1-induced and purinergic P2X1-induced smooth muscle contractions are the most promising targets since norepinephrine and ATP are the main sympathetic nervous system cotransmitters in the male urogenital system (Burnstock, 2014; Navarrete et al., 2014). Furthermore, the importance of α_{1A}-ARs in the ejaculation reflex and male fertility was explored in a recent study showing complete infertility of male mice with knockout of both α_{1A}-ARs and purinergic P2X1 receptors (White et al., 2013).

Overall, the present study shows that α_{1A}- and α_{1D}-ARs are expressed in the CE and that the α_{1A}-AR is the main α_1-AR subtype mediating contraction of CE smooth muscle to norepinephrine. These results contribute to our understanding of the role of α_1-AR subtypes in male sexual function/fertility and further strengthen the rationale that any male contraceptive approach targeting α_1-AR should rely on the α_{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 experiments: Pacini, Castilho, Hebeler-Barbosa, Pupo, Kiguti.

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

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