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Differential distribution of functional α_1-adrenergic receptor subtypes along the rat tail artery[§]

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Abbreviations: A-61603, *N*-[5-(4,5-Dihydro-1*H*-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide; BMY-7378, 8-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride; DRTA, rings from distal segments of the tail artery ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PRTA, rings from proximal segments of the tail artery.

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Abstract

The rat tail artery has been used for the study of vasoconstriction mediated by α_{1A}-adrenoceptors (ARs). However, rings from proximal segments of the tail artery (within the initial 4 cm, PRTA) were at least 3-fold more sensitive to methoxamine and phenylephrine (n=6 to 12; $p<0.05$) than rings from distal parts (between the 6th and 10th cm, DRTA). Interestingly, the imidazolines *N*-[5-(4,5-Dihydro-1*H*-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide (A-61603) and oxymetazoline, which activate selectively α_{1A}–ARs, were equipotent in PRTA and DRTA (n=4 to 12), while buspirone, which activates selectively α_{1D}-AR, was ≈70-fold more potent in PRTA than in DRTA (n=8; $p<0.05$). The selective α_{1D}-AR antagonist 8-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY-7378) was ≈70-fold more potent against the contractions induced by phenylephrine in PRTA (pK_B≈8.45, n=6) than in DRTA (pK_B≈6.58, n=6), although the antagonism was complex in PRTA. 5-methylurapidil, a selective α_{1A}-antagonist, was equipotent in PRTA and DRTA (pK_B≈8.4), but the Schild slope in DRTA was 0.73±0.05 (n=5). The non-competitive α_{1B}-antagonist conotoxin ρ-TIA reduced the maximal contraction induced by phenylephrine in DRTA, but not in PRTA. These results indicate a predominant role for α_{1A}-ARs in the contractions of both PRTA and DRTA, but with significant co-participations of α_{1D}-ARs in PRTA and α_{1B}-ARs in DRTA. Semi-quantitative RT-PCR revealed that mRNA encoding α_{1A}- and α_{1B}-ARs are similarly distributed in PRTA and DRTA while mRNA for α_{1D}-ARs is twice more abundant in PRTA. Therefore, α_1-ARs subtypes are differentially distributed along the tail artery. It is important to consider the segment from which the

tissue preparation is taken to avoid misinterpretations on receptor mechanisms and drug selectivities.

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Introduction

Three α_1-adrenoceptor (AR) subtypes (α_{1A}-, α_{1B}- and α_{1D}-ARs) are involved in the contractions of vascular smooth muscle in response to AR agonists. However, depending on the vessel, the participation of one particular subtype may predominate over the other two α_1-ARs. The use of subtype selective α_1-AR agonists and antagonists have allowed the identification of a predominant role for α_{1A}-ARs in the contractions of the rat tail artery in response to norepinephrine (Gisbert et al., 2003), phenylephrine (Piascik et al., 1995; Piascik et al., 1997), methoxamine, (Villalobos-Molina and Ibarra, 1996), (R)-(-)-3'-(2-amino-1-hydroxyethyl)-4'-fluoromethanesulfonanilide hydrochloride (NS-49) (Murata et al., 1999) and *N*-[5-(4,5-Dihydro-1*H*-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide (A-61603) (Lachnit et al., 1997; Chang et al., 2000; Jahnichen et al., 2004) suggesting that this artery is an interesting model for the study of the mechanisms involved in the vasoconstriction mediated by α_{1A}-ARs and for the characterization of the properties of selective ligands.

Interestingly, studies evaluating the expression of mRNA encoding α_1-ARs reveal that the α_{1A}-AR is probably not the unique subtype expressed in the rat tail artery, since mRNA encoding α_{1B}- and α_{1D}-AR has been detected in this vessel (Piascik et al., 1995; Taki et al., 2004). In fact, radioligand binding studies have shown the presence of α_{1B}-ARs in addition to α_{1A}-ARs (Taki et al., 2004; Tanaka et al., 2004) and functional studies have identified a minor but definite component in the contractions in response to norepinephrine as being mediated by the activation of α_{1B}-ARs (Jahnichen et al., 2004).

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While studying the contractions of rings of the rat tail artery in response to phenylephrine in an attempt to establish in our lab a model of vasoconstriction mediated by α_{1A}-ARs, we noticed considerable variation in the sensitivity of preparations taken from different segments along this artery. The rings cut from segments located within the initial 4 cm of the tail (proximal segments) where much more sensitive to phenylephrine than rings taken from segments more distally located (within the 6^{th} and the 10^{th} cm of the tail). This led us to investigate the identity of the α_1-ARs mediating the contractions of arterial rings isolated from proximal (PRTA, rings of the tail artery isolated from proximal segments) and distal (DRTA, rings of the tail artery isolated from distal segments) regions of the tail of the rat. Experiments employing selective agonists and antagonists reveal that the subtypes of α_1-ARs are differentially distributed along the rat tail artery. Also, semi-quantitative RT-PCR using primers for the specific amplification of mRNAs encoding each of the α_1-ARs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed variation in the amount of transcripts for α_1-ARs between these two regions.

Although some studies have described heterogeneous distribution of α_1- and α_2-ARs along the rat tail artery (Rajanayagam and Medgett, 1987), this is, to our knowledge, the first report on differential distribution of functional α_1-AR subtypes along an artery.

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Methods

Functional studies. The experimental procedures were approved by the *Ethics Committee for the Use of Experimental Animals* from UNESP – Botucatu and are in accordance with the *Guide for the Care and Use of Laboratory Animals (NIH)*. Male Wistar rats weighing between 250-380 g (16 to 20 weeks old) were killed by decapitation in guillotine. Segments of the ventral artery of the rat tail were removed carefully, cleaned of adhering tissue and of endothelium by gentle rubbing, cut into 3 to 4 mm rings and mounted under 19.6 mN of optimal tension in 10 ml organ baths containing a physiological solution of the following composition (mM): NaCl 119; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2 and dextrose 11, prepared in glass-distilled, de-ionized water, maintained at 37^{o}C and bubbled with 5% CO_2/95% O_2. The segments of the rat tail artery were identified according its location along the length of the tail of the rat as proximal (PRTA, located within the initial 4 cm) and distal (DRTA, located between the 6th and 10th cm of the tail). A cocktail containing cocaine (6 μM), corticosterone (10 μM), yohimbine (0.1 μM) and propranolol (0.1 μM), was included in all experiments to block neuronal uptake, extraneuronal uptake, α_2-ARs and β_1-and β_2-ARs, respectively. After an equilibration period of 60 min with adjustments of basal tension and changes of physiological solution at each 20 min, the arteries were repeatedly challenged with phenylephrine 10 μM at 20 min intervals until contractions of similar magnitude were obtained (usually 3 times). Then, concentration response-curves to α_1-AR agonists were obtained in absence or presence of increasing concentrations of the α_1-antagonists prazosin, BMY-7378 and 5-methylurapidil previously incubated for at least 45 min. Not more than four consecutive concentration-response curves to agonists were obtained from each

preparation, since preliminary experiments showed there are significant changes in the sensitivity of the tail artery to AR agonists.

The pA_2 values for competitive antagonists were calculated by Schild regression analysis (Arunlakshana and Schild, 1959). The ratios between the half-maximal concentrations of agonists (concentration-ratios, *r*) were calculated only when the maximal amplitude of the concentration-response curve in the presence of the competitive antagonists was similar to that obtained in its absence. Data were plotted as log antagonist concentrations (M) vs log (*r* - 1). For calculation purposes the slope parameter was constrained to 1.0 when statistically not different from unity. When the slope parameter differed from the theoretical unity or when only one antagonist concentration was tested, the antagonist affinity was estimated as pK_B calculated through the formula pK_B = -log [lowest effective concentration of the antagonist (M)] + log (*r* - 1).

Curve fitting and pD_2 calculation was performed with the software package GraphPad Prism version 3.00, San Diego, California, USA. All values are shown as means ± standard error of mean (S.E.M.) of *n* experiments. Differences between mean values were tested for statistical significance ($p < 0.05$) using Student's paired or unpaired *t*-tests or ANOVA followed by Newman-Keuls for multiple comparisons.

Drugs were obtained from the following sources: A-61603, (*N*-[5-(4,5-Dihydro-1*H*-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide), Tocris Cookson Ltd, UK, cocaine (Cocainum Hydrochloricum puriss.) C.H. Boehringer, Germany; buspirone hydrochloride, corticosterone, noradrenaline [(±)-arterenol HCl]; from Sigma Chemical Co, USA; BMY-7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), oxymetazoline HCl, prazosin HCl, (±)-propranolol HCl and

yohimbine HCl from Research Biochemicals Inc. (RBI/SIGMA), U.S.A. Conotoxin ρ-TIA was a generous gift from Dr. Richard J. Lewis, Xenome Ltd., Indooroopilly, Queensland, Australia.

Total RNA extraction

The ventral artery of the rat tail was rapidly dissected, cleaned of connective and fat tissue in ice-cold saline (0,9% NaCl), and the endothelium was removed by gentle rubbing with stainless steel wire in the lumen.

A proximal segment of the tail artery was taken from the initial 4 cm of the rat tail while a distal segment was taken from a region located between the 6^{th} and 10^{th} cm of the tail. These segments corresponded to those isolated for functional studies.

Proximal and distal segments of the tail artery were homogenized in TRIzol® (Invitrogen, São Paulo, Brazil) with a Polytron and total RNA was extracted according to the recommended protocol from a pool of samples composed by tissues from 4 animals. Total RNA concentration was measured by absorbance at 260 nm (BioPhotomether - Eppendorf®).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

The sequence of the specific primers were described by Queiroz et al. (2002) as follow: α_{1a} *sense*, GTA GCC AAG AGA GAA AGC CG (628-647) and α_{1a} *antisense,* CAA CCC ACC ACG ATG CCC AG (820-839); α_{1b} *sense*, GCT CCT TCT ACA TCC CGC TCG (629-649), and α_{1b} *antisense,* AGG GGA GCC AAC ATA AGA TGA (908-928); α_{1d} *sense,* CGT GTG CTC CTT CTA CCT ACC (759-779), and α_{1d} *antisense,* GCA CAG GAC GAA GAG ACC CAC (1042-1062); GAPDH *sense,* CGG

GAA GCT TGT GAT CAA TGG (258-277) and GAPDH *antisense,* GGC AGT GAT GCC ATG GAC TG (614-595). The size expected for the amplified products are 212, 300, 304 and 357 base pairs for α_{1a}, α_{1b}, α_{1d} and GAPDH genes, respectively. The GAPDH gene was used as an internal control for normalization of the data.

To remove contaminating genomic DNA, 1μg of total RNA was treated with DNAse I (Invitrogen, São Paulo, Brazil) and reverse transcription was then performed with 8 μl of the DNAse-treated RNA solution using a SUPERScript III™ RT kit (Invitrogen, São Paulo, Brazil) according to manufacturer's instructions.

PCR was performed on 2 μl (each of the α_1- ARs subtypes) or 0.5 μl (for GAPDH) of cDNA in a PCR mastermix containing 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotides (dNTPs), 0.4 μM (for α_1-ARs subtypes) or 1.6 μM (for GAPDH) specific primers, 1.25 units Taq DNA polymerase (Invitrogen, São Paulo, Brazil) and H_2O in a total volume of 25 μl. PCR conditions for α_1-ARs subtypes were adapted from Queiroz et al. (2002) as follow: one cycle of denaturation 96°C, 30 s, followed by 30 (α_{1a} , α_{1b}) or 29 (α_{1d}) cycles of denaturation 96°C, 10 s; annealing 58°C (α_{1a}) or 55°C (α_{1b} , α_{1d}), 10 s; extension 74 °C, 40 s (α_{1b} , α_{1d}) or 15 s (α_{1a}). A final extension of 74 °C, 2 min was performed for all samples. GAPDH was amplified under one cycle of denaturation 94°C, 3 min; followed by 33 cycles of denaturation 94°C, 45 s; annealing 60°C, 45 s; extension 70 °C, 1 min.

PCR products (15 μl) were separated in 1,5% agarose gel, stained with ethidium bromide and visualized under U.V. light and digitalized with Image Master VDS® (Pharmacia Biotech®, D&R Israel). Band intensities were measured by computerized densitometry using the Image Gauge V.3.12 software (FujiFilm) and were expressed as arbitrary units (A.U.)

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Semi-quantitative RT-PCR was validated by choosing number of PCR cycles and amount of RNA within the linear range of the amplification curve for each gene. Experiments were performed on three independent pools and the mean ± S.E.M were compared.

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Results

Contractions of different segments of the rat tail artery to agonists. Fig. 1 shows concentration-response curves obtained in rings from different segments (proximal, PRTA and distal, DRTA) of the rat tail artery in response to the phenethylamines phenylephrine and methoxamine (Fig. 1A), to the selective α_{1A}-AR agonists the imidazolines A-61603 and oxymetazoline (Minneman et al., 1994; Knepper et al., 1995) (Fig. 1B), and to the selective α_{1D}-AR agonist buspirone (Eltze et al., 1999, Yamamoto and Koike, 2001) (Fig. 1C). The respective pD_2, maximal contractions (in mN) and relative intrinsic activities (calculated as a fraction of the absolute maximal response to phenylephrine in each segment) are shown in Table 1.

The phenethylamine agonists phenylephrine and methoxamine were approximately 3-5 times more potent in PRTA than in DRTA (Fig. 1A, Table 1), while the imidazolines A-61603 and oxymetazoline were roughly equipotent in rings from both regions (Fig. 1B, Table 1). Buspirone was almost 70 times more potent in PRTA than in DRTA where it was only a very weak agonist as indicated by its low intrinsic activity (Fig. 1C, Table 1).

The rank order of potency was the same in PRTA and DRTA (A61603 > oxymetazoline > phenylephrine > methoxamine = buspirone). However, some differences were observed in the efficacies of oxymetazoline and methoxamine, which behaved as partial agonists in PRTA but as full agonists in DRTA (Table 1).

It was not possible to include experiments using norepinephrine as an α_1-AR agonist in both PRTA and DRTA due to strong participation of α_2-ARs in the contractions, as indicated by the complex antagonism shown by prazosin, which resulted in Schild plots with slopes much lower than 1.0 (data not shown).

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Effects of AR antagonists on contractions induced by phenylephrine in different segments the rat tail artery. The effects of prazosin, 5-methylurapidil and BMY-7378 where tested against the contractions induced by phenylephrine in PRTA and DRTA. The contractions of both PRTA and DRTA in response to phenylephrine were competitively antagonized by prazosin (Figs. 2A and 2B) with similar affinities (Table 2). Similarly, 5-methylurapidil, a selective antagonist of α_{1A}-ARs, was equipotent in PRTA and DRTA (Figs. 2C and 2D), although the antagonism in DRTA was complex, since the slope in the Schild plot was lower than the theoretical unity (Fig. 3 and Table 2). The α_{1D}-AR selective antagonist BMY-7378 antagonized the contractions of the PRTA and DRTA in response to phenylephrine (Figs. 2E and 2F). However, BMY-7378 was much more potent in PRTA than in DRTA, as characterized by the effectiveness of the low concentrations of BMY-7378 in PRTA (10 to 300 nM, Fig. 2E), which induced rightward shifts in the concentrations-response curves to phenylephrine that where concentration-dependent but not linearly related; i.e. in this concentration range, a 3-fold increase in BMY-7378 did not result in a 3-fold reduction in the potency of phenylephrine, as predicted by the dynamics of the competitive antagonism at a single receptor. The resulting slope in the Schild plot was lower than 1.0 (Fig. 3, Table 2). On the other hand, the contractions of DRTA in response to phenylephrine were not affected by low concentrations of BMY-7378 (10 to 300 nM, Fig. 2F) and the resulting straight line in the Schild plot had slope not different from 1.0 (Figs. 2F, 3 and Table 2).

Effects of BMY-7378 on contractions induced by other AR agonists in PRTA. We tested the effects of BMY-7378 on contractions induced by other AR agonists in PRTA in order to investigate whether the shallow slope in the Schild plot observed for

this antagonist against phenylephrine is related or not to the presence of multiple functional α_1-AR subtypes in proximal segments of the rat tail artery. The contractions induced by methoxamine, another phenethylamine agonist, were also sensitive to low concentrations of BMY-7378 (10 to 300 nM, Fig. 4A) and similarly to what was observed when the contractions where induced by phenylephrine, the resulting slope in the Schild plot was much lower than the unity (Fig. 4E, Table 3).

In contrast, when the contractions were induced by the imidazolines A-61603 and oxymetazoline, the antagonism presented by BMY-7378 was competitive (Figs. 4B, 4C, 4E) and yielded low pA_2 values (≈6.4, Table 3). Interestingly, the contractions induced by buspirone where also competitively antagonized by BMY-7378 (Fig. 4D), but with affinity approximately 80-fold higher (pA_2≈ 8.3, Table 3).

The nature of the receptor activated by buspirone. Buspirone is classically known as a partial agonist at 5-HT_{1A} receptors. However, it is also known that most of the vasoconstriction induced by buspirone results from the activation of α_1-ARs (Gurdal et al., 1992, Terron et al., 1996; Osei-Owusu and Scrogin, 2004) and that this drug activates selectively α_{1D}-ARs (Eltze et al., 1999, Yamamoto and Koike, 2001). In spite of this, it is important to determine whether the contractions of the rat tail artery to buspirone recorded in the present study are due to activation of α_1-ARs. To determine this, the potency of prazosin was determined against the contractions induced by buspirone in both PRTA and DRTA. Prazosin (1 to 10 nM) antagonized with high affinity the contractions of the PRTA in response to buspirone (pA_2 = 9.39 ± 0.05, Schild slope = 1.0 ± 0.1; n = 4, data not shown) suggesting that α_1-ARs are involved. Buspirone was a much weaker agonist in the DRTA, precluding

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construction of Schild plots. However the pK_B estimated with single concentrations of prazosin (1 nM) was 9.2 ± 0.1 (n = 4), and of 5-methylurapidil (30 nM) was 8.0 ± 0.1 (n = 4) while BMY-7378 (up to 30 nM) was not effective (data not shown). These results indicate that the small contractions induced by buspirone in DRTA also result from the activation of α_1-ARs.

Effects of conotoxin ρ-TIA on contractions induced by phenylephrine in PRTA and DRTA. It has been recently shown that conotoxin ρ-TIA interacts differently with α_1-AR subtypes: it is a competitive antagonist of α_{1A}- and α_{1D}-AR but a non-competitive antagonist of α_{1B}-ARs; also, the toxin shows slight selectivity for α_{1B}-ARs (10-25 fold) over the other two subtypes (Chen et al., 2004; Lima et al., 2005). Therefore, the effects of different concentrations of conotoxin ρ-TIA on contractions induced by phenylephrine in PRTA and DRTA were investigated. In PRTA conotoxin ρ-TIA (3 to 30 μM) induced concentration-dependent rightward shifts in the concentration-response curves to phenylephrine without affecting the maximal contraction induced by this agonist (Fig. 5A). The resulting Schild plot had slope =0.92 ± 0.04 and pA_2 = 7.34 ± 0.08 (n = 4). In DRTA however, although conotoxin ρ-TIA 3 and 10 μM induced concentration-dependent rightward shifts in the concentration-response curves to phenylephrine, conotoxin ρ-TIA 30 μM reduced the maximal contraction induced by phenylephrine by approximately 15% ($p < 0.05$, Fig. 5B).

To check whether the reduction of the maximal contraction in DRTA is related to the participation of α_{1B}-ARs or to some non-specific event, we examined the effect of conotoxin ρ-TIA 30 μM on contractions induced by the selective α_{1A}-AR agonist A-

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61603. Although conotoxin ρ-TIA 30 μM induced a large reduction in the potency of A-61603 in DRTA (pK_B = 7.50 ± 0.07; n = 4), the toxin was not able to affect the maximal contraction induced by this agonist (Fig. 5C).

Expression of mRNA for α_1-AR subtypes in different segments of the rat tail artery. The mRNA encoding each of the α_1-AR subtypes was amplified by RT-PCR from total RNA extracted from proximal and distal segments of the rat tail artery using specific primers for each subtype. The amplified products were separated by agarose gel eletrophoresis, quantified by densitometry and compared to the mRNA encoding GAPDH. This semi-quantitative procedure allows convenient comparisons of the expression levels of mRNA for the same receptor subtype in different segments. However, this procedure does not allow comparisons of the expression levels among different receptor subtypes. Fig. 6A shows representative gels with bands corresponding to the products amplified using primers specific for GAPDH, α_{1A}-AR, α_{1B}-AR and α_{1D}-AR mRNAs while Fig. 6B shows the expression of each α_1-AR subtype mRNA in relation to the respective GAPDH mRNA. The transcripts for α_{1A}- and α_{1B}-ARs are similarly distributed in proximal and distal segments of the rat tail artery (Fig. 6B). However, the relative expression of α_{1D}-AR mRNA in proximal segments is ≈2.4-fold higher than in distal segments (Fig. 6B, $p < 0.05$).

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Discussion

The nature of the α_1-AR subtypes involved in the contractions of rings from proximal and distal segments of the rat tail artery was investigated in the present study. The hypothesis that there might be a differential distribution of functional α_1-AR subtypes along the rat tail artery was raised from the observation that rings taken from proximal segments of this vessel were much more sensitive to some, but not all AR agonists, than rings taken from more distal segments. This was observed during preliminary experiments carried out in an attempt to standardize in our lab a model of vasoconstriction mediated by the activation of α_{1A}-ARs, which would be suitable for the study of the processes resultant from the activation of this subtype and of the properties of selective ligands.

The phenethylamine agonists phenylephrine and methoxamine were 3 to 5-fold more potent in PRTA than in DRTA. Importantly, the selective α_{1A}-AR imidazoline agonists, A-61603 and oxymetazoline, were equipotent in PRTA and DRTA, suggesting that the higher sensitivity to AR agonists is not a general phenomena, but related to which receptor subtype is activated. Strikingly, buspirone, a partial agonist at 5-HT_{1A} receptors and selective agonist at α_{1D}-AR (Eltze et al., 1999; Yamamoto and Koike, 2001), was almost 70-fold more potent in PRTA than in DRTA where its maximal contraction represented only 30% of that induced by the full agonist phenylephrine. Based on the similar potency presented by the selective α_{1A}-AR agonists A-61603 and oxymetazoline and the higher potency and/or efficacy shown by phenylephrine, methoxamine, and buspirone, it is tempting to speculate that PRTA is endowed with functional α_{1A}- and α_{1D}-ARs while this latter subtype is absent in DRTA. However, it is difficult to conclude about distributions of receptor

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subtypes relying only on evidence obtained with agonists, mainly because of the hyperbolic nature of the occupancy-response relationships often observed for α_1-ARs and their agonists. Therefore, this heterogeneous distribution was further investigated using the selective α_{1D}-AR competitive antagonist BMY-7378.

The selective antagonist BMY-7378 discriminated two distinct components in the contractions of PRTA in response to phenylephrine and methoxamine: the first component was similarly blocked by "low" concentrations of BMY-7378 (10 to 300 nM), while the second was competitively antagonized by higher concentrations (1μM to 10 μM); such behavior precluded the determination of a single consistent affinity for this antagonist in PRTA since the slope in the Schild plots was much lower than the theoretical unity. However, the pK_B estimated from the lowest effective concentration of BMY-7378 (10 nM) through the formula pK_B = -log [BMY-7378 (M)] + log (*r* - 1) against either phenylephrine (8.4 ± 0.1, *n* = 6) or methoxamine (8.4 ± 0.1, *n* = 6) indicates that part of the contractions of PRTA in response to these two phenethylamines is due to activation of α_{1D}-ARs. On the other hand, if only the effects of 0.3 μM to 10 μM are used to construct the Schild plots, the resulting slopes are not different from unity (1.0 ± 0.1 *vs* phenylpephrine and 1.0 ± 0.1 *vs* methoxamine), but the derived pA_2 (6.4 ± 0.1 *vs* phenylpephrine and 6.5 ± 0.1 *vs* methoxamine) is much lower than that expected for the involvement of α_{1D}-ARs. These pA_2 values indicate participation of either α_{1A}- or α_{1B}-ARs. However, the high pA_2 values for 5-methylurapidil estimated against either phenylephrine (≅8.3) or methoxamine (≅8.3, data not shown) suggest that the subtype sensitive only to the higher concentrations of BMY-7378 is the α_{1A}-AR. Therefore, these results corroborate those found with agonists suggesting that both α_{1A}- and α_{1D}-ARs are

functional in PRTA and that phenylephrine and methoxamine contract these arterial rings through the activation of these two subtypes.

The competitive behavior and the high pA_2 values found for BMY-7378 against the contractions induced by buspirone in PRTA further support the presence of functional α_{1D}-ARs. Conversely, the results obtained with oxymetazoline and A-61603 indicate that α_{1A}-ARs are functional in PRTA since these imidazolines are selective α_{1A}-AR agonists (Minneman et al., 1994; Knepper et al., 1995) and the contractions induced by these drugs were competitively antagonized with low affinity by BMY-7378 ($pK_B \cong 6.5$).

The interpretation of the behavior shown by BMY-7378 against the contractions induced by phenylephrine in DRTA is much more straightforward. These data suggest that α_{1D}-ARs are not involved in the contractions of rings from distal segments since there was no component sensitive to “low” concentrations of BMY-7378, and the Schild plot had a slope not different from unity, resulting in the estimation of low affinity for this antagonist. The absence of functional α_{1D}-ARs in DRTA is further supported by the very weak agonist activity shown by buspirone in these arterial rings. Therefore, functional α_{1D}-ARs are differentially distributed along the rat tail artery mediating part of the contractions induced by phenylephrine and methoxamine in PRTA but not in DRTA.

The antagonism presented by the selective α_{1A}-AR competitive antagonist 5-methylurapidil also differed in PRTA and DRTA. Although the contractions of both PRTA and DRTA were antagonized by the same concentrations of 5-methylurapidil, the slope of the straight line in the Schild plot derived in DRTA was lower than the theoretical unity. Interestingly, the largest selectivity for α_{1A}-ARs presented by 5-

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methylurapidil is over the α_{1B}-AR subtype (30 to 100 fold), while that over α_{1D}-ARs is only marginal (3 to 10 fold). Therefore, the complex antagonism presented by 5-methylurapidil in DRTA could be related to the presence of functional α_{1B}-ARs in these rings. We took advantage of the differential antagonism presented by ρ-conotoxin TIA at α_1-ARs to further check for the presence of functional α_{1B}-ARs in DRTA. ρ-Conotoxin TIA is a competitive antagonist of α_{1A}- and α_{1D}-AR but a non-competitive antagonist of α_{1B}-ARs, against which the toxin shows slight selectivity (10-25 fold) over the other two subtypes (Chen et al., 2004; Lima et al., 2005). In fact, high concentrations of ρ-conotoxin TIA reduced the maximal contraction induced by phenylephrine in DRTA but not in PRTA where it was a competitive antagonist. In addition, ρ-conotoxin TIA was not able to reduce the maximal contraction induced by the selective α_{1A}-AR agonist A-61603, suggesting that the inhibition of the maximal contraction induced by phenylephrine is related to the involvement of α_{1B}-ARs. These data suggest that α_{1B}-ARs are also differentially distributed in the rat tail artery, taking part in the contractions of rings from distal segments but not from proximal ones. The presence of functional α_{1B}-ARs contracting the rat tail artery has already been shown. Recently, Janichen et al., (2004) described that both α_{1A}- and α_{1B}-ARs are involved in the contractions of rings from the rat tail artery and that a receptor protection protocol using the selective α_{1A}-AR antagonist (+/-)-1,3,5-trimethyl-6-[[3-[4-((2,3-dihydro-2-hydroxymethyl)-1,4-benzodio xin-5-yl)-1-piperazinyl]propyl]amino]-2,4(1H,3H)-pyrimidinedione (B8805-033) and the alkylating agent chloroethylclonidine is necessary to obtain contractions mediated by α_{1A}-ARs, otherwise mixed receptor populations are uncovered.

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The pattern of expression of the mRNA encoding each of the α_1-ARs was investigated by semi-quantitative RT-PCR using specific primers. Interestingly, mRNA encoding α_{1D}-ARs is approximately twice as abundant in proximal than in distal segments of the tail artery, while mRNA encoding α_{1A}- and α_{1B}-ARs are similarly distributed. Therefore, these results show that not only functional α_{1D}-ARs are differentially distributed along the rat tail artery but also their mRNAs. It is difficult to correlate mRNA abundance with functional response, but the similar distribution of mRNA encoding α_{1B}-ARs suggests that these receptors, if present in proximal segments, are not efficiently coupled to contraction. Alternatively, semi-quantitative RT-PCR might not be sensitive enough to detect subtle differences in the expression of α_{1B}-ARs transcripts.

We are not aware of other studies describing heterogeneous distribution of α_1-AR subtypes along different segments of the same vessel. However, differences in the potencies of prazosin and idazoxan in antagonizing contractions induced by epinephrine in proximal and distal segments of the rat tail artery led Rajanayagam and Medgett (1987) to conclude that the participation of α_2-ARs is greater in distal parts.

The idea that the rat tail artery contracts in response to some AR agonists via activation of multiple α_1-AR subtypes is not new. For instance, Lachnit et al. (1997) concluded that one of these subtypes displays the pharmacology of the α_{1A}-AR, while the other remained to be defined. This seemed to be resolved by the previously mentioned study from Jahnichen et al. (2004) describing that α_{1A}- and α_{1B}-ARs are functional in this vessel. Hence, one important contribution of the present study is the observation that depending on the segment of the rat tail artery from which the

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arterial preparation is isolated, either α_{1B}- or α_{1D}-AR can be found to co-participate with α_{1A}-ARs in the contractions induced by AR agonists. It will be interesting to investigate whether such a pattern of differential distribution of receptor subtypes also occurs in other vessels.

It is important to acknowledge the existence of multiple functional α_1-AR subtypes in the rat tail artery mainly because this tissue has been extensively used for the study of the mechanisms involved in the vasoconstriction mediated by α_{1A}-ARs and for the characterization of the properties of selective ligands. Therefore, even the minor participation of α_1-ARs other than the α_{1A}-AR must be taken into account to avoid misinterpretations.

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FOOTNOTES

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Figure Legends:

Fig. 1. Concentration-response curves obtained for phenethylamine (A, phenylephrine and methoxamine) and imidazoline (B, A-61603 and oxymetazoline) agonists and buspirone (C) in rings from proximal (PRTA) and distal (DRTA) segments of the rat tail artery. Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of 4 to 12 experiments.

Fig. 2. Effects of increasing concentrations of prazosin (A and B), 5-methylurapidil (C and D) and BMY-7378 (E and F) on concentration-response curves to phenylephrine in rings from proximal (PRTA) and distal (DRTA) segments of the rat tail artery. Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of 4 to 12 experiments.

Fig. 3. Schild plot for the antagonism of contractions induced by phenylephrine by prazosin, 5-methylurapidil and BMY-7378 in PRTA and DRTA. Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of 4 to 12 experiments.

Fig. 4. Effects of increasing concentrations of BMY-7378 on concentration-response curves to methoxamine (A), A-61603 (B), oxymetazoline (C) and buspirone (D) in rings from proximal segments of the rat tail artery. The Schild plot for the antagonism of these contractions by BMY-7378 is presented in (E). Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of 4 to 12 experiments.

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Fig. 5. Effects of increasing concentrations of conotoxin ρ-TIA on concentration-response curves to phenylephrine (A and B) and A-61603 (C) in PRTA (A and C) and DRTA (B). Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of 4 experiments.

Fig. 6. (A) Representative gels showing the products amplified by RT-PCR from total RNA isolated from proximal and distal segments of the rat tail artery using primers specific for the amplification of GAPDH and each of the α_1-ARs subtypes. In (B) is shown the expression of mRNA encoding each of the α_1-ARs in relation to that for GAPDH. * $p < 0.05$ in relation to the respective mRNA expression found in proximal segments.

Table 1. Parameters of agonist action in rings isolated from proximal (PRTA) and distal (DRTA) segments of the rat tail artery. Values are mean ± S.E.M. of n independent experiments. * different from the respective pD_2 found in rings from proximal segments ($p < 0.05$). # different from the maximal contraction induced by phenylephrine in the respective segment ($p < 0.05$). Intrinsic activity is defined as a fraction of the absolute maximal response to phenylephrine in each segment.

		Phenethylamines		Imidazolines		
		Phenylephrine (n = 6 to 8)	Methoxamine (n = 6 to 12)	A-61603 (n = 4)	Oxymetazoline (n = 6 to 12)	Buspirone (n = 8)
pD_2	PRTA	7.44 ± 0.08	6.45± 0.09	8.64 ± 0.05	7.72 ± 0.07	6.63 ± 0.04
	DRTA	6.72 ± 0.06*	5.99 ± 0.05*	8.53 ± 0.07	7.64 ± 0.05	4.82 ± 0.09*
Emax (mN)	PRTA	36.2 ± 1.4	30.4 ± 1.0#	34.8 ± 0.6	30.4 ± 0.6#	21.0 ± 0.3#
	DRTA	34.8 ± 0.6	35.1 ± 0.7	35.2 ± 0.7	35.3 ± 0.6	10.8 ± 0.09#
Intrinsic activity	PRTA	1.00	0.84	0.96	0.84	0.58
	DRTA	1.00	1.01	1.01	1.01	0.31

Table 2. Parameters of antagonist action against contractions induced by phenylephrine in rings isolated from proximal (PRTA) and distal (DRTA) segments of the rat tail artery.

Values are mean ± S.E.M. of 4 to 6 independent determinations. * different from the theoretical unity ($p < 0.05$). # when the slope was different from 1.0, the potency of the antagonist was estimated through the formula $pK_B = \log (r\text{-}1) - \log$ [lowest effective molar concentration of the antagonist (M)].

	Prazosin		5-methylurapidil		BMY-7378	
	pA_2	slope	pA_2/pK_B[#]	slope	pA_2/pK_B[#]	slope
PRTA	9.70 ± 0.09	0.92 ± 0.06	8.40 ± 0.08	0.84 ± 0.06	8.45 ± 0.07[#]	0.64 ± 0.10*
DRTA	9.47 ± 0.06	0.88 ± 0.04	8.21 ± 0.05[#]	0.73 ± 0.05*	6.58 ± 0.09	0.92 ± 0.03

Table 3. Antagonism of contractions induced by different agonists in proximal segments of the rat tail artery (PRTA) by BMY-7378.

Values are mean ± S.E.M. of 4 to 6 independent determinations. * different from the theoretical unity ($p<0.05$). # when the slope was different from 1.0, the potency of the antagonist was estimated through the formula $pK_B = \log (r\text{-}1) - \log$ [lowest effective molar concentration of BMY-7378 (M)].

	BMY-7378 *vs.*:			
	Methoxamine	A-61603	Oxymetazoline	Buspirone
pA_2/pK_B[#]	8.39 ± 0.07[#]	6.45 ± 0.09	6.33 ± 0.09	8.31 ± 0.10
slope	0.54 ± 0.05*	1.24 ± 0.08	1.04 ± 0.11	0.92 ± 0.07

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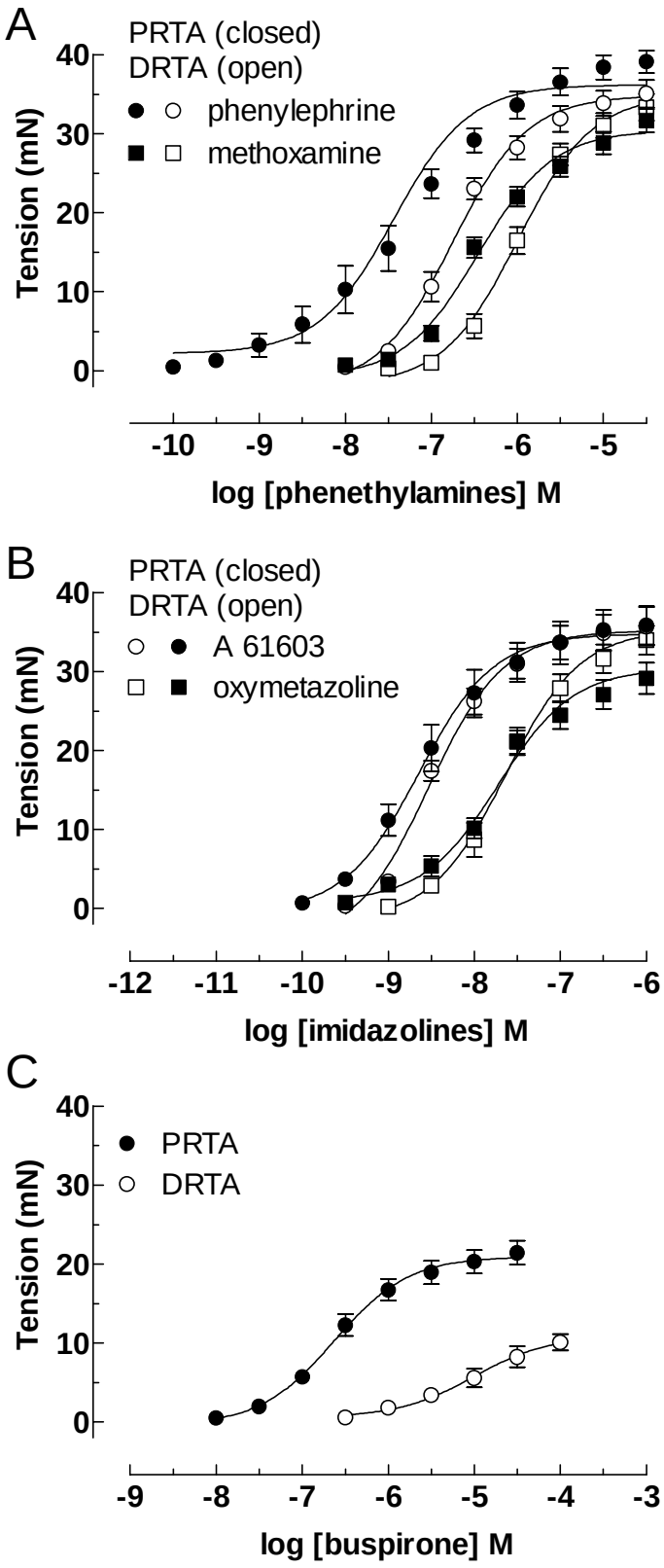

Fig.1 Kamikihara et al

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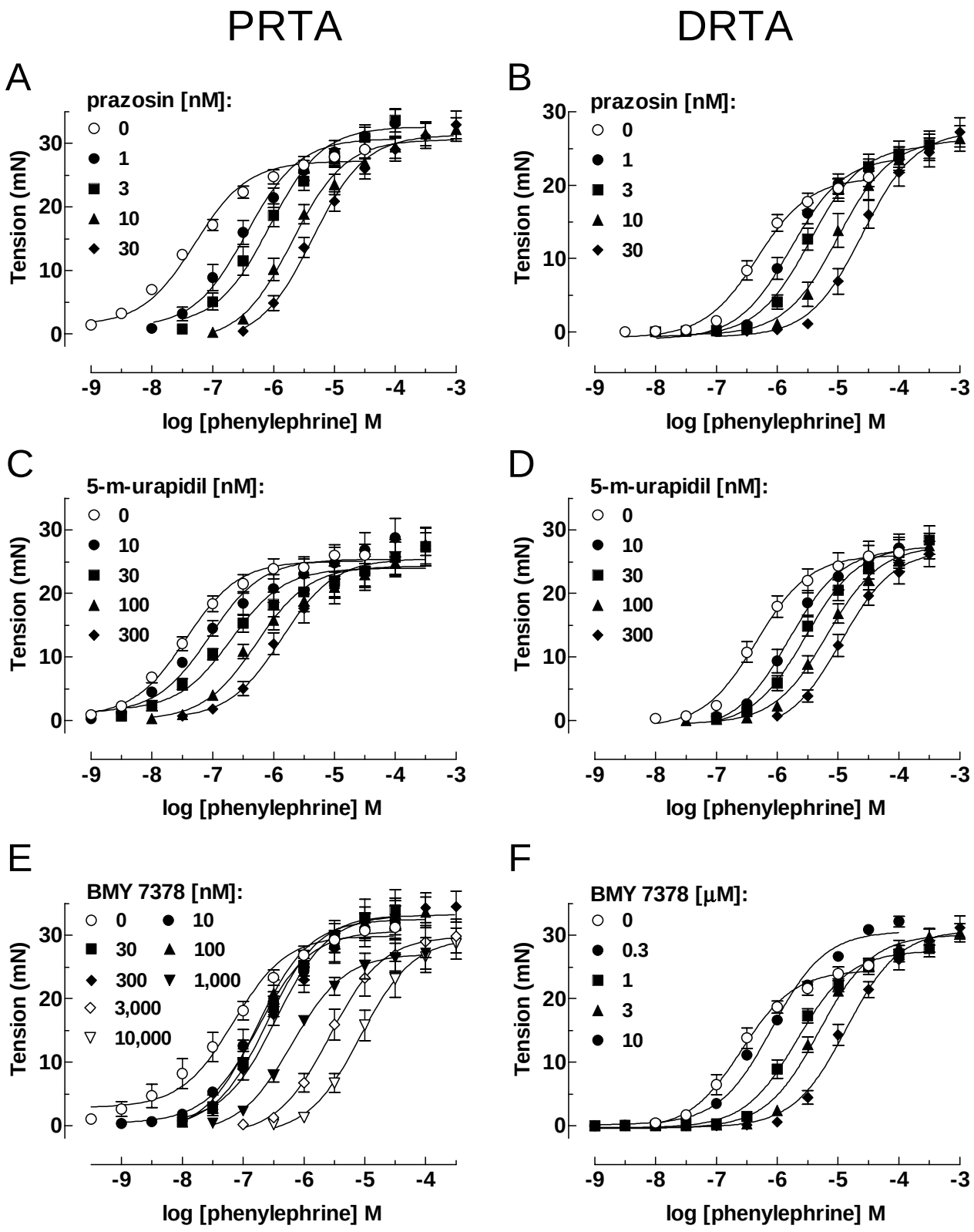

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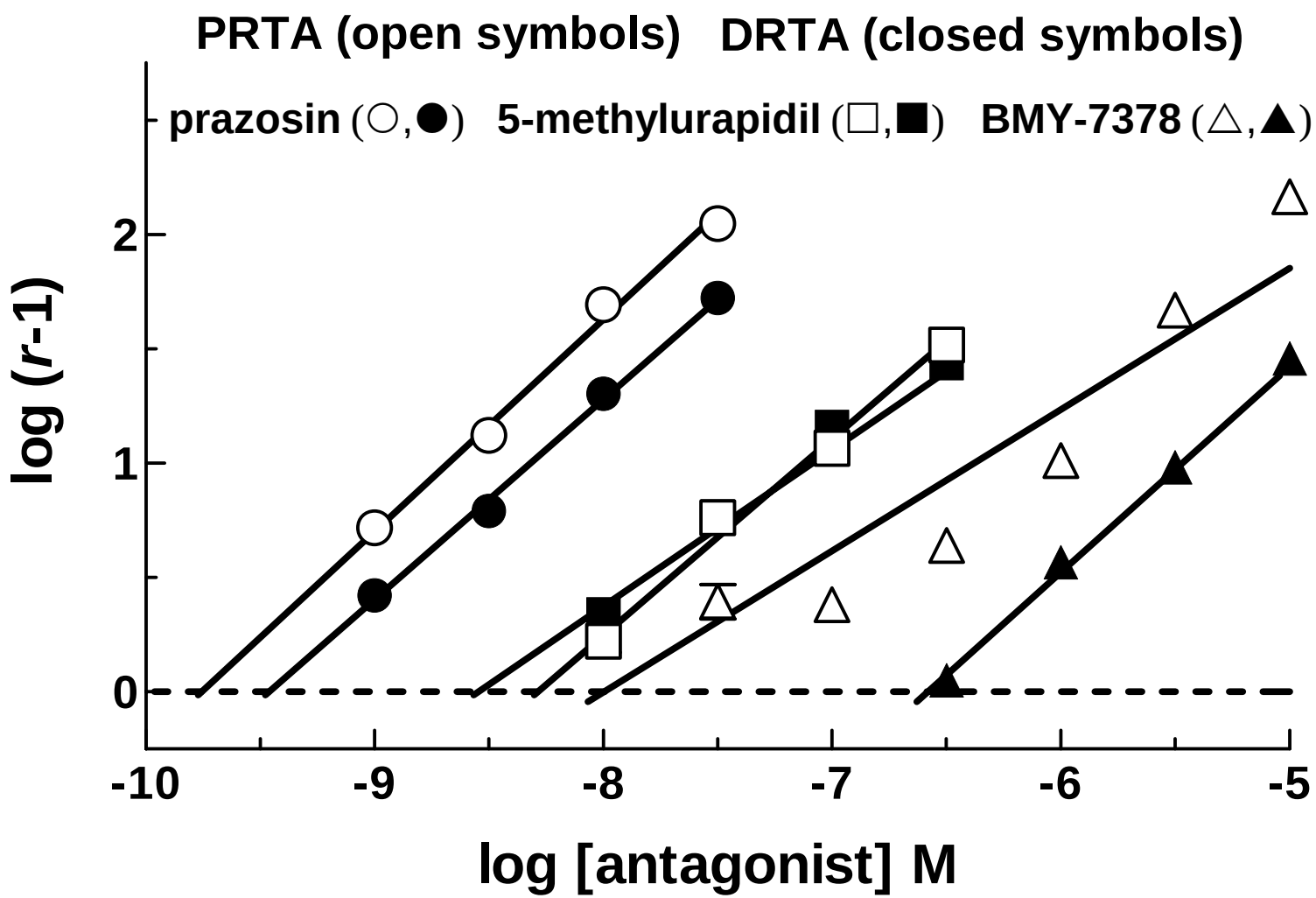

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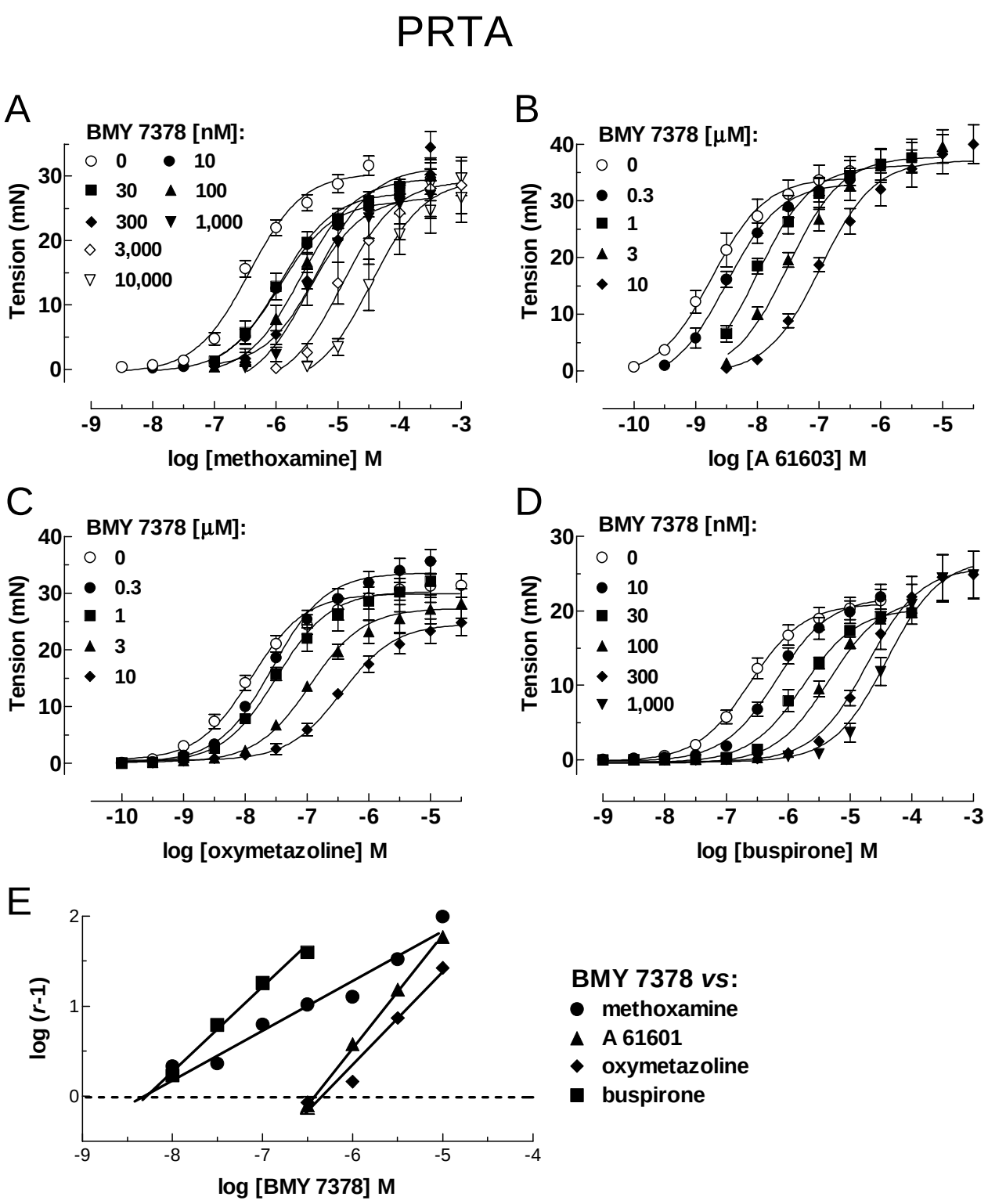

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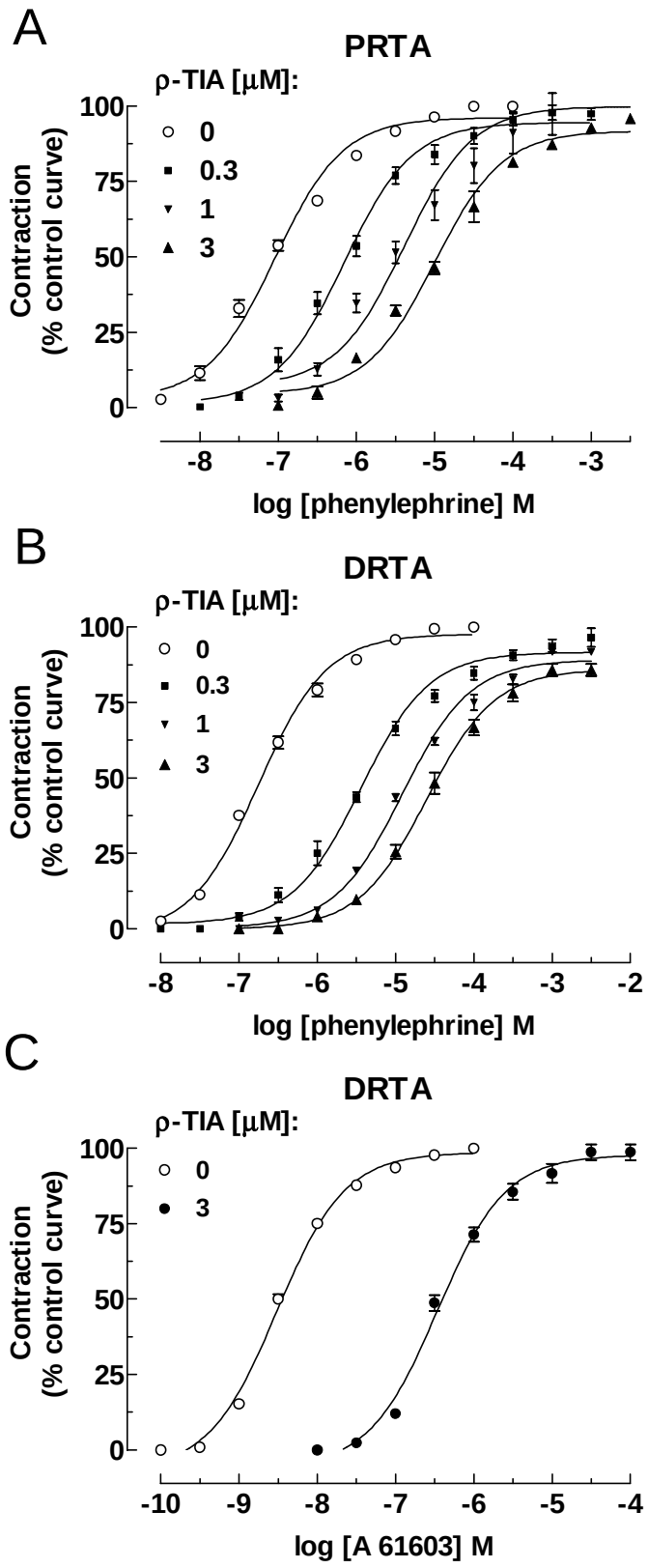

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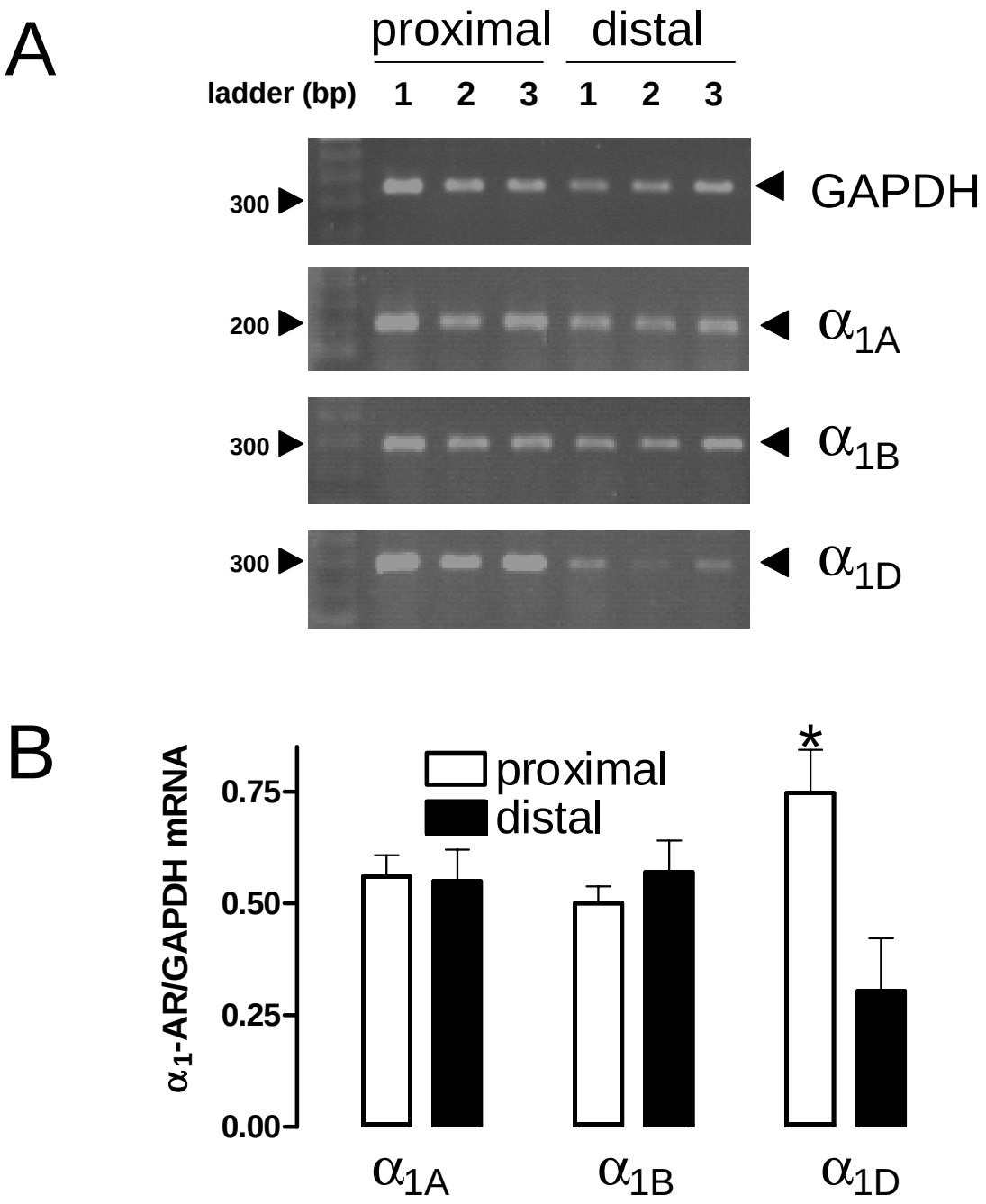

Fig.6 Kamikihara et al