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# Differential antagonism by conotoxin ρ-TIA of contractions mediated by distinct $\alpha_1$-adrenoceptor subtypes in rat vas deferens, spleen and aorta

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**Abstract**

The ability of the conotoxin ρ-TIA, a 19-amino acid peptide isolated from the marine snail *Conus tulipa*, to antagonize contractions induced by noradrenaline through activation of $\alpha_{1A}$-adrenoceptors in rat vas deferens, $\alpha_{1B}$-adrenoceptors in rat spleen and $\alpha_{1D}$-adrenoceptors in rat aorta, and to inhibit the binding of [$^{125}$I]HEAT (2-[[β-(4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone) to membranes of human embryonic kidney (HEK) 293 cells expressing each of the recombinant rat $\alpha_1$-adrenoceptors was investigated. ρ-TIA (100 nM to 1 μM) antagonized the contractions of vas deferens and aorta in response to noradrenaline without affecting maximal effects and with similar potencies (p$A_2$~7.2, $n$=4). This suggests that ρ-TIA is a competitive antagonist of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors with no selectivity between these subtypes. Incubation of ρ-TIA (30 to 300 nM) with rat spleen caused a significant reduction of the maximal response to noradrenaline, suggesting that ρ-TIA is a non-competitive antagonist at $\alpha_{1B}$-adrenoceptors. After receptor inactivation with phenoxybenzamine, the potency of ρ-TIA in inhibiting contractions was examined with similar occupancies (~25%) at each subtype. Its potency (p$IC_{50}$) was 12 times higher in spleen (8.3±0.1, $n$=4) than in vas deferens (7.2±0.1, $n$=4) or aorta (7.2±0.1, $n$=4). In radioligand binding assays, ρ-TIA decreased the number of binding sites ($B_{max}$) in membranes from HEK293 cells expressing the rat $\alpha_{1B}$-adrenoceptors without affecting affinity ($K_D$). In contrast, in HEK293 cells expressing rat $\alpha_{1A}$- or $\alpha_{1D}$-adrenoceptors, ρ-TIA decreased the $K_D$ without affecting the $B_{max}$. It is concluded that ρ-TIA will be useful for distinguishing the role of particular $\alpha_1$-adrenoceptor subtypes in native tissues.


*Keywords:* $\alpha_1$-adrenoceptor; Conotoxin ρ-TIA; Vas deferens; Spleen; Aorta

## 1. Introduction

The conotoxin TIA (ρ-TIA) is a 19-amino acid peptide isolated from the marine gastropodus *Conus tulipa* (Sharpe et al., 2001). Most of the toxins from snails of the *Conus* genus target voltage-gated ion channels (such as $Ca^{2+}$, $Na^+$ and $K^+$) and ionotropic receptors (such as nicotinic, 5-$HT_3$ and NMDA glutamate), and are important pharmacological tools in the study of their properties (for review, see Terlau and Olivera, 2004). Interestingly, ρ-TIA interacts with high affinity with $\alpha_1$-adrenoceptors (Sharpe et al., 2003), which are members of the seven transmembrane domain, G protein-coupled receptor superfamily.

There are three subtypes of $\alpha_1$-adrenoceptors ($\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$) (Zhong and Minneman, 1999), and radioligand binding and functional experiments with heterologously expressed human receptors have shown that ρ-TIA has a 10-fold higher affinity for the $\alpha_{1B}$-subtype than for the other two receptors (Chen et al., 2004). Also, ρ-TIA interacts differentially at the human $\alpha_1$-adrenoceptor subtypes, as a non-competitive antagonist at $\alpha_{1B}$-adrenoceptor, and as a

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competitive antagonist at $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors (Chen et al., 2004).

The selective nature and differential modes of inhibition of ρ-TIA suggests that this toxin should prove valuable in elucidating the role of $\alpha_1$-adrenoceptor subtypes in mediating different functional effects. This is particularly important since to date no highly selective $\alpha_{1B}$-adrenoceptor antagonist has become available. However, most previous experiments have been done with recombinant receptors, where a single subtype is expressed in isolation. In addition, the most extensive characterization of the interaction of ρ-TIA with $\alpha_1$-adrenoceptor subtypes has been done with the human clones (Chen et al., 2004). Since the role of individual $\alpha_1$-adrenoceptor subtypes in mediating functional responses is usually done in rodent tissues, often expressing more than a single subtype, it is essential to determine whether similar properties are observed in contractile studies of rat tissues. This will allow the use of this compound in distinguishing responses in tissues expressing mixtures of subtypes. Therefore, the ability of ρ-TIA to inhibit contractions of the rat vas deferens, spleen and aorta by noradrenaline, effects predominantly mediated by activation of $\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptors, respectively, was examined. In addition, the nature of the interactions between ρ-TIA and rat $\alpha_1$-adrenoceptors was further investigated in radioligand binding assays using [$^{125}$I]HEAT (2-[[β-(4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone) and membranes from human embryonic kidney (HEK) 293 cells expressing each of the recombinant subtypes.

## 2. Methods

### 2.1. General

The experimental procedures were approved by the *Ethics Committee for the Use of Experimental Animals* from UNESP-Botucatu. Male Wistar rats (16 to 20 weeks old, 260 to 380 g) were killed by decapitation and selected tissues were carefully excised and prepared for digital recording of isometric contractions as follows: the vas deferens (epididymal portion), spleen (hemi-sections) and thoracic aorta (~5 mm rings, endothelium denuded) were cleaned of adherent tissues and mounted in organ baths under 9.8 mN (vas deferens and spleen) or 14.7 mN (aorta) tension in a nutrient solution with the following composition (mM): NaCl 138; KCl 5.7, $CaCl_2$ 1.8, $NaH_2PO_4$ 0.36, $NaHCO_3$ 15, dextrose 5.5 (for vas deferens); NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, dextrose 5.5 (for spleen and aorta) prepared in glass-distilled, de-ionized water, maintained at 30 °C (vas deferens) or 37 °C (spleen and aorta), pH 7.4 and continuously bubled with 95% $O_2$/5% $CO_2$. All experiments were done in the presence of a cocktail of inhibitors containing cocaine (6 μM), corticosterone (10 μM), idazoxan (1 μM) and propranolol (0.1 μM) to block neuronal uptake, extraneuronal uptake, and $\alpha_2$- and β-adrenoceptors, respectively.

### 2.2. Effect of ρ-TIA on KCl-induced contractions

To check if the effects of ρ-TIA on the contractions of the smooth muscles to noradrenaline might be due to actions not related to $\alpha_1$-adrenoceptors, contractions in response to KCl (80 mM) were recorded (for 3 min in vas deferens and for 5 min in aorta and spleen) in the absence and presence of ρ-TIA (300 nM) added 30 min previously.

### 2.3. Concentration–response curves to noradrenaline in the absence and presence of adrenoceptor antagonists and ρ-TIA

After a 30-min (vas deferens) or 45-min (spleen and aorta) stabilization period, the tissues were challenged with 80 mM KCl. This procedure was repeated (usually two or three times) until reproducible contractions were obtained. Then, a concentration–response curve to noradrenaline was constructed by adding cumulative concentrations of the agonist. After washing and relaxation, antagonists (prazosin, 5-methylurapidil and BMY 7378) or ρ-TIA were incubated with the tissue. After at least 45 min, a concentration–response curve to noradrenaline was then obtained in the presence of the antagonist. This was repeated with increasing concentrations of prazosin (1 to 30 nM), 5-methylurapidil (3 nM to 1 μM), BMY 7378 (10 nM to 3 μM) and ρ-TIA (30 nM to 1 μM). To check for the reversibility of the actions of ρ-TIA, after the concentration–response curve to noradrenaline in the presence of the highest concentration of the peptide (1 μM in vas deferens and aorta, 300 nM in the spleen), the tissues were washed at least five times and a concentration–response curve to noradrenaline was generated 30 min (vas deferens) or 45 min (spleen and aorta) later.

### 2.4. Determination of the affinities of the adrenoceptor antagonists and of ρ-TIA

Estimates of the affinities of the adrenoceptor antagonists and of ρ-TIA ($pA_2$, the negative logarithm of the antagonist dissociation constant) were obtained by Schild regression analysis (Arunlakshana and Schild, 1959) only when the maximal contraction induced by noradrenaline in the presence of the antagonist was not different from that in its absence. For calculation purposes, the slope parameter was constrained to 1.0 when statistically not different from theoretical unity.

### 2.5. Determination of the $pIC_{50}$ of adrenoceptor antagonists and of ρ-TIA

In order to compare the potencies of the adrenoceptor antagonists and ρ-TIA in vas deferens, spleen and aorta,

the negative logarithm of the concentrations of these drugs inhibiting half of the contractions ($pIC_{50}$) induced by similar $\alpha_1$-adrenoceptor occupancies by noradrenaline were determined. The concentrations of noradrenaline occupying similar fractions of $\alpha_1$-adrenoceptors in vas deferens, spleen and aorta were determined through the Hill-Langmuir equation: fractional occupancy=[A]/([A]+$K_a$), where [A] is the agonist concentration and $K_a$ is the full agonist macroscopic equilibrium dissociation constant. The $K_a$ for noradrenaline at the $\alpha_1$-adrenoceptors in the vas deferens, spleen and aorta was calculated according to Furchgott's partial alkylation method using phenoxybenzamine (Besse and Furchgott, 1976). This method is based on the comparison of equiactive concentrations of a full agonist before ([A]) and after ([A′]) partial receptor alkylation. Therefore, equiactive concentrations of noradrenaline were compared before and after treatment with phenoxybenzamine (10 nM/15 min in the vas deferens, 1 μM/10 min in the spleen and 100 nM/10 min in the aorta). At the end of the incubation, the tissues were extensively washed (at least 10 times) and a new concentration–response curve to noradrenaline was obtained after 30 min. Data was plotted as [A] vs. [A]/[A′] to reduce distortion of error and avoid undue weighting of values of lower magnitude observed in double reciprocal plots (Kenakin, 1997). As such, $K_a$=−(slope+intercept) of the resulting straight line. The effects of noradrenaline resulting from occupancy of approximately 25% of $\alpha_1$-adrenoceptors in vas deferens, spleen and aorta were chosen because these are submaximal in the vas deferens and aorta and easily measurable in the spleen. If effects resulting from larger occupancies were chosen, these would be maximal in the vas deferens and aorta; on the other hand, if smaller occupancies were chosen the resulting effects would be difficult to measure in the spleen. Therefore, the $pIC_{50}$ of adrenoceptor antagonists and of ρ-TIA were determined against the tonic contractions induced by noradrenaline at 1 μM in the vas deferens, 3 μM in the spleen and 100 nM in the aorta, which are concentrations of noradrenaline that occupy ~25% of the $\alpha_1$-adrenoceptors in these tissues (see Results). The antagonists and ρ-TIA were incubated at least 30 min before addition of noradrenaline. The effect of antagonists and ρ-TIA was measured on the tonic contraction for 3 min (vas deferens) or 5 min (spleen and aorta) after adding the agonist. Preliminary experiments showed that at least 10 contractions in response to noradrenaline with similar magnitude could be obtained. Data was plotted as −log[adrenoceptor antagonists or ρ-TIA] versus percentage inhibition and the $pIC_{50}$ determined by nonlinear regression using GraphPad Prism.

### 2.6. Constructs

cDNAs for the rat $\alpha_{1A}$- and rat $\alpha_{1B}$-adrenoceptors (Lomasney et al., 1991) were generously provided by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC), and the rat $\alpha_{1D}$-adrenoceptor cDNA (Perez et al., 1991) was kindly provided by Dr. Dianne Perez (Cleveland Clinic, Cleveland, OH). All three cDNAs were subcloned into the mammalian expression vector pcDNA3.

### 2.7. Cell culture and transfections

HEK293 cells were propagated in Dulbecco's modified Eagle's medium with sodium pyruvate supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere with 5% $CO_2$. Confluent 150 mm plates were subcultured at a ratio of 1:4 1 day before transfection, then transfected with 5 μg DNA of each construct using Lipofectamine 2000.

### 2.8. Membrane preparation

48 h after transfection, cells were harvested with PBS (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH

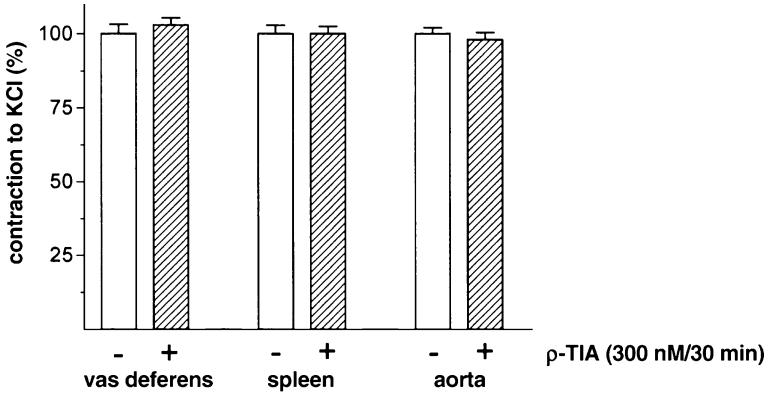


Fig. 1. Effect of ρ-TIA (300 nM/30 min) on tonic contractions induced by KCl (80 mM) in vas deferens, spleen and aorta. Each bar is the mean±S.E.M. of four experiments.

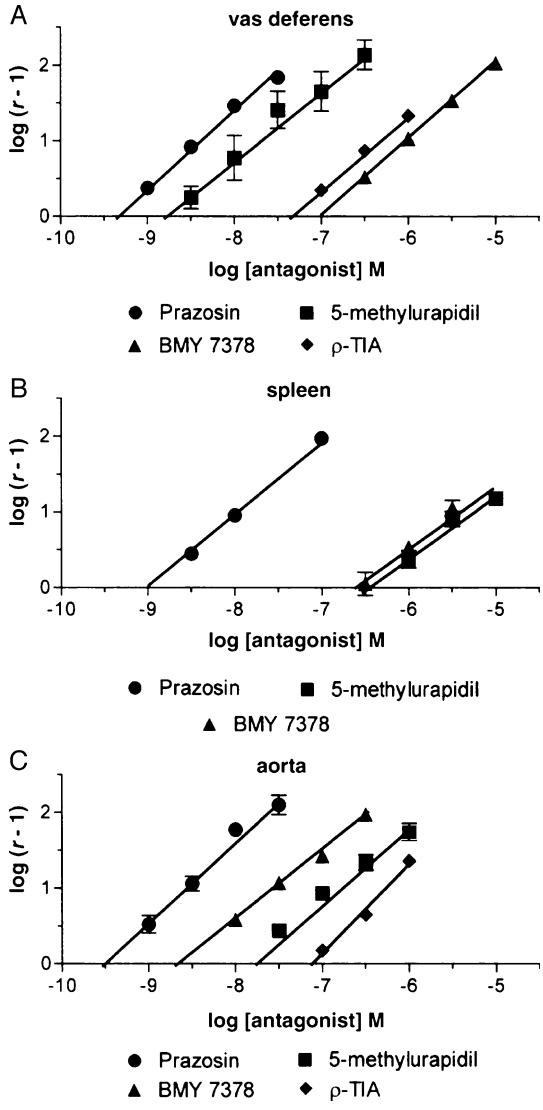


Fig. 2. Schild plots for the antagonism of contractions induced by noradrenaline in vas deferens (A), spleen (B) and aorta (C). Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of four to eight experiments.

7.4), collected by centrifugation at 30,000×*g* for 10 min, resuspended in PBS and homogenized with a Polytron. This process was then repeated and final membranes were collected in 0.5 ml PBS. Total protein concentration was determined by Bradford assay (Bradford, 1976).

### 2.9. Radioligand binding

Receptor density and binding affinity were determined by saturation binding assays with the $\alpha_1$-specific antagonist [$^{125}$I]HEAT (20–400 pM) (Minneman et al., 1983). Membranes were incubated with increasing concentrations of [$^{125}$I]HEAT in the absence or presence of 20 nM or 5 nM ρ-TIA as indicated at 37°C for 20 min. After incubation, samples were filtered through a wet Whatman GF/B paper under vacuum. Filter papers were washed twice with cold wash buffer (10 mM Tris·HCl, pH 7.4) and radioactivity was measured by gamma counting. Non-specific binding was determined as binding in the presence of 10 μM phentolamine. Inhibition curves were determined by displacement of [$^{125}$I]HEAT (50–70 pM) under increasing concentrations of ρ-TIA and data were analyzed by non-linear regression using Prism (GraphPad, CA).

### 2.10. Materials

Drugs, cells and reagents were obtained from the following sources: cocaine (cocainum hydrochloricum puriss.) from C.H. Boehringer, Germany; corticosterone, from Sigma Chemical, USA; BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), idazoxan HCl, prazosin HCl, (±)-propranolol HCl, 5-methylurapidil from Research Biochemicals (RBI/SIGMA), U.S.A.; HEK293 cells, American Type Culture Collection (Manassas, VA); Lipofectamine 2000, Invitrogen Life technologies (Carlsbad, CA); penicillin, streptomycin, phosphate-buffered saline (PBS), phentolamine mesylate, Bradford Reagent, Sigma-Aldrich (St. Louis, MO); carrier-free Na[$^{125}$I], Amersham (Arlington Heights, IL); Dulbecco's modified Eagle's medium with 4.5 g/l glucose and L-glutamine (DMEM), Mediatech (Herndon, VA); HEAT from Dr. Giuseppe Romeo (University of Catania, Italy). ρ-TIA was synthesized at Xenome as described previously (Sharpe et al., 2001).

### 2.11. Statistical analysis

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 ($P<0.05$)

Table 1
p$A_2$ and slope values[a] obtained from Schild plots for the antagonism by adrenoceptor antagonists of contractions induced by noradrenaline in the rat vas deferens, spleen and aorta

| | Prazosin | | 5-Methylurapidil | | BMY 7378 | | ρ-TIA | |
|---|---|---|---|---|---|---|---|---|
| | p$A_2$ | Slope | p$A_2$ | Slope | p$A_2$ | Slope | p$A_2$ | Slope |
| Vas deferens | 9.35±0.17 | 0.98±0.05 | 8.76±0.16 | 0.93±0.07 | 7.05±0.02 | 1.00±0.01 | 7.22±0.05 | 0.98±0.06 |
| Spleen | 8.91±0.06 | 1.01±0.02 | 6.67±0.12 | 0.83±0.08 | 6.56±0.03 | 0.99±0.02 | non-competitive | |
| Aorta | 9.63±0.08 | 1.01±0.10 | 7.63±0.05 | 0.86±0.05 | 8.64±0.05 | 0.91±0.05 | 7.15±0.08 | 1.18±0.13 |

[a] Each value represents the mean and the S.E.M. of four to eight experiments.

using Student's paired or unpaired *t*-tests or analysis of variance (ANOVA) followed by Newman–Keuls for multiple comparisons.

## 3. Results

### 3.1. Effects of ρ-TIA on contractions induced by KCl

ρ-TIA (300 nM) did not affect the maximal contraction induced by 80 mM KCl in vas deferens, spleen or aorta (Fig. 1).

### 3.2. Effects of adrenoceptor antagonists and of ρ-TIA on concentration–response curves to noradrenaline

The adrenoceptor antagonists prazosin, 5-methylurapidil and BMY 7378 antagonized contractions caused by noradrenaline in vas deferens, spleen and aorta. The antagonism was competitive as indicated by the resulting Schild plots (Fig. 2), where the slopes were not different from unity (Table 1). Except for prazosin, which was equipotent in the vas deferens, spleen and aorta with similar $pA_2$ values, significant differences were found in the affinities of 5-methylurapidil (higher in vas deferens than in spleen and aorta) and BMY 7378 (higher in aorta, than in vas deferens and spleen), as reported in previous studies (Buckner et al., 1996; Piascik et al., 1995; Pupo, 1998).

The three concentrations of ρ-TIA tested in the vas deferens and aorta (100, 300 nM and 1 μM) antagonized the contractions induced by noradrenaline in an apparently competitive fashion and with similar potency in these two tissues (Fig. 3, Table 1) as indicated by the respective Schild plots (Fig. 2). However, in the spleen, ρ-TIA (30 to 300 nM) induced significant reductions of the maximal contraction to noradrenaline (Fig. 3).

After washing out ρ-TIA, the concentration–response curve obtained for noradrenaline 30 to 45 min later was not different from that obtained in tissues that had not been exposed to the peptide, suggesting its antagonism, although non-competitive, is reversible (Fig. 3).

### 3.3. $pK_a$ for noradrenaline and occupancy–response relationships

In order to determine the concentrations of noradrenaline which occupies similar fractions of the $\alpha_1$-adrenoceptor populations in vas deferens, spleen and aorta, the $pK_a$ for noradrenaline was calculated using receptor alkylation with phenoxybenzamine (Besse and Furchgott, 1976). The treatment with phenoxybenzamine of the vas deferens (10 nM/15 min), spleen (1 μM/10 min) and aorta (100 nM/10 min) induced rightward shifts in the concentration–response curves associated with similar degrees of reduction in the maximal response to noradrenaline (Fig. 4A, B and C and Table 2). The comparison of equiactive concentrations of

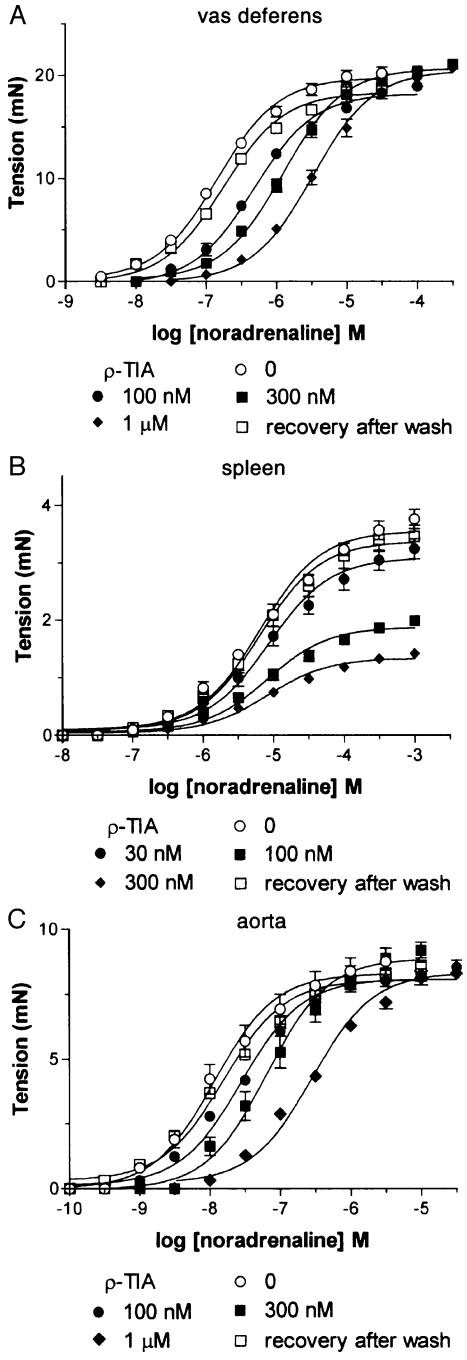


Fig. 3. Concentration–response curves for noradrenaline in the absence and presence of increasing concentrations of ρ-TIA in vas deferens (A), spleen (B) and aorta (C). Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of four experiments.

noradrenaline before and after receptor alkylation with phenoxybenzamine (Fig. 4D, E and F) allowed the determination of the $pK_a$ for noradrenaline in these three tissues (Table 2). Based on these values, the occupancy–response relationship for noradrenaline was determined and is shown in Fig. 4G, H and I. The concentrations of noradrenaline occupying ~25% of the $\alpha_1$-adrenoceptor populations in the vas deferens, spleen and aorta were 1 μM, 3 μM and 100 nM, respectively.

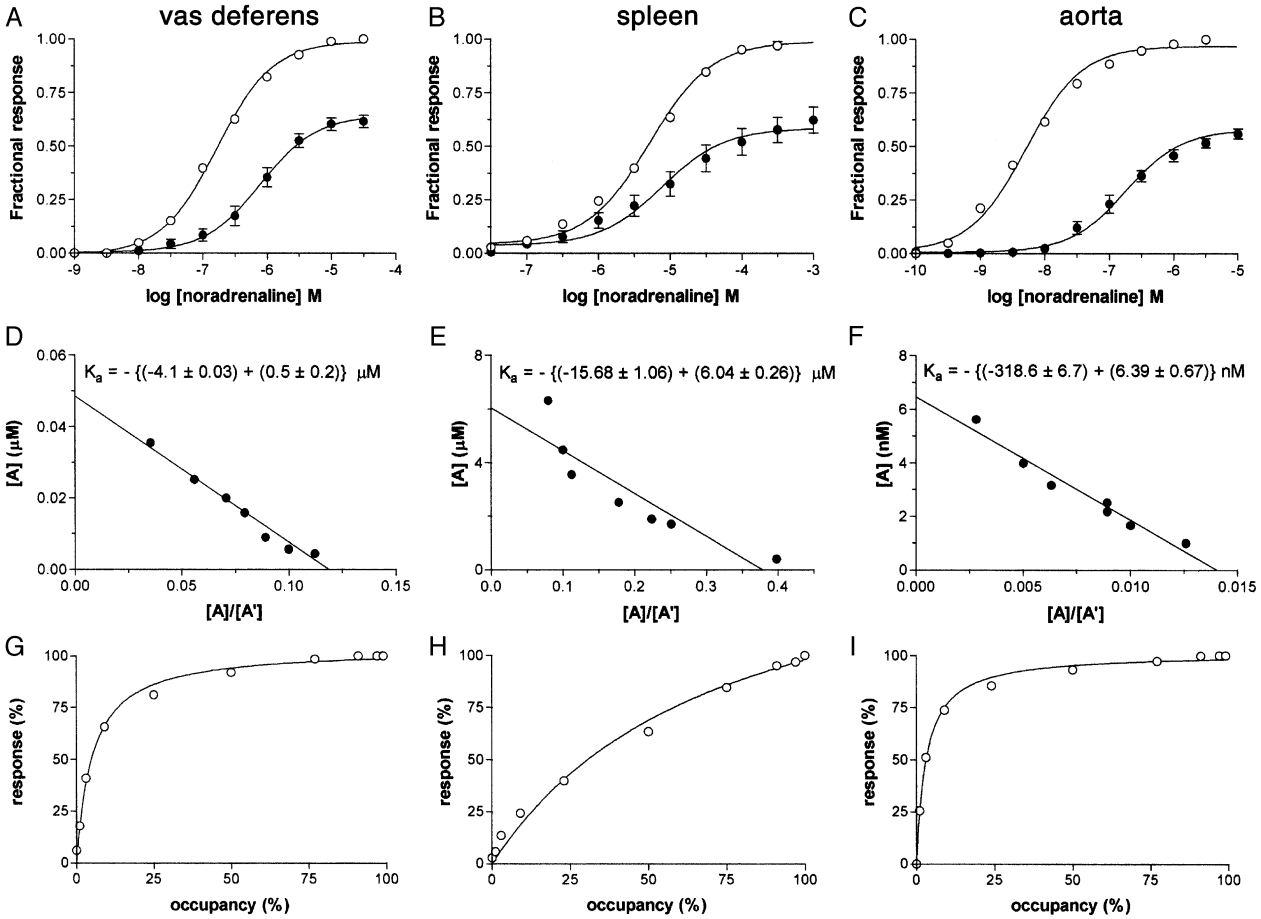


Fig. 4. Concentration–response curves for noradrenaline before and after treatment with phenoxybenzamine of the vas deferens (A, 10 nM/15 min), spleen (B, 1 μM/10 min) and aorta (C, 100 nM/10 min). Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of six experiments. In (D), (E) and (F) are shown typical plots of equiactive concentrations of noradrenaline before and after phenoxybenzamine derived from the mean concentration–response curves in the vas deferens, spleen and aorta, respectively. In (G), (H) and (I) are shown typical occupancy–response relationships for noradrenaline and the $\alpha_1$-adrenoceptors in the vas deferens, spleen and aorta, respectively.

### *3.4. $pIC_{50}$ for adrenoceptor antagonists and of ρ-TIA in the inhibition of noradrenaline contractions*

The inhibitory potencies of the adrenoceptor antagonists and of ρ-TIA were determined against the tonic component of the contractions induced by the concentrations of noradrenaline calculated to occupy 25% of the receptor pool in each tissue: 1 μM, 3 μM and 100 nM noradrenaline in vas deferens, spleen and aorta, respectively. Prazosin inhibited the tonic contractions induced by noradrenaline with similar potencies in all three tissues (Fig. 5A, Table 3). As expected, 5-methylurapidil was more potent in vas deferens than in spleen or aorta (Fig. 5B, Table 3), while BMY 7378 was more potent in aorta than in vas deferens or spleen (Fig. 5C, Table 2). Finally, ρ-TIA was more potent in inhibiting contractions of spleen than vas deferens or aorta (Fig 5D, Table 3).

Table 2
Effect of phenoxybenzamine (POB) treatment on $pD_2$ values and maximal responses[a] for noradrenaline in vas deferens, spleen and aorta and the $pK_a$ derived from the plot of equiactive concentrations before and after POB (10 nM/15 min in vas deferens; 1 μM/10 min in spleen and 100 nM/10 min in aorta)

| | $pD_2$ | | | Remaining contraction after POB (%) | Noradrenaline $pK_a$ |
|---|---|---|---|---|---|
| | Before POB | After POB | Ratio[b] | | |
| Vas deferens | 6.78±0.03 | 6.10±0.04[c] | 4.8 | 64±6 | 5.49±0.08 |
| Spleen | 5.29±0.05 | 5.11±0.07[c] | 1.5 | 58±7 | 5.05±0.08 |
| Aorta | 8.29±0.06 | 6.74±0.07[c] | 35 | 58±6 | 6.48±0.07 |

[a] Each value represents the mean and the S.E.M. of six experiments.
[b] Antilog ($pD_2$ before divided by $pD_2$ after POB).
[c] Different from the respective value found before POB treatment ($P<0.05$).

### *3.5. Effects of ρ-TIA on the binding of [$^{125}$I]HEAT to membranes from HEK293 cells expressing rat $\alpha_1$-adrenoceptors*

To further investigate the nature of the interactions between ρ-TIA and the subtypes of rat $\alpha_1$-adrenoceptors, [$^{125}$I]HEAT saturation binding assays were performed in the absence and presence of the conotoxin. ρ-TIA (20 nM) was unable to affect the maximum binding capacity ($B_{max}$) of [$^{125}$I]HEAT to membrane preparations from HEK293 cells

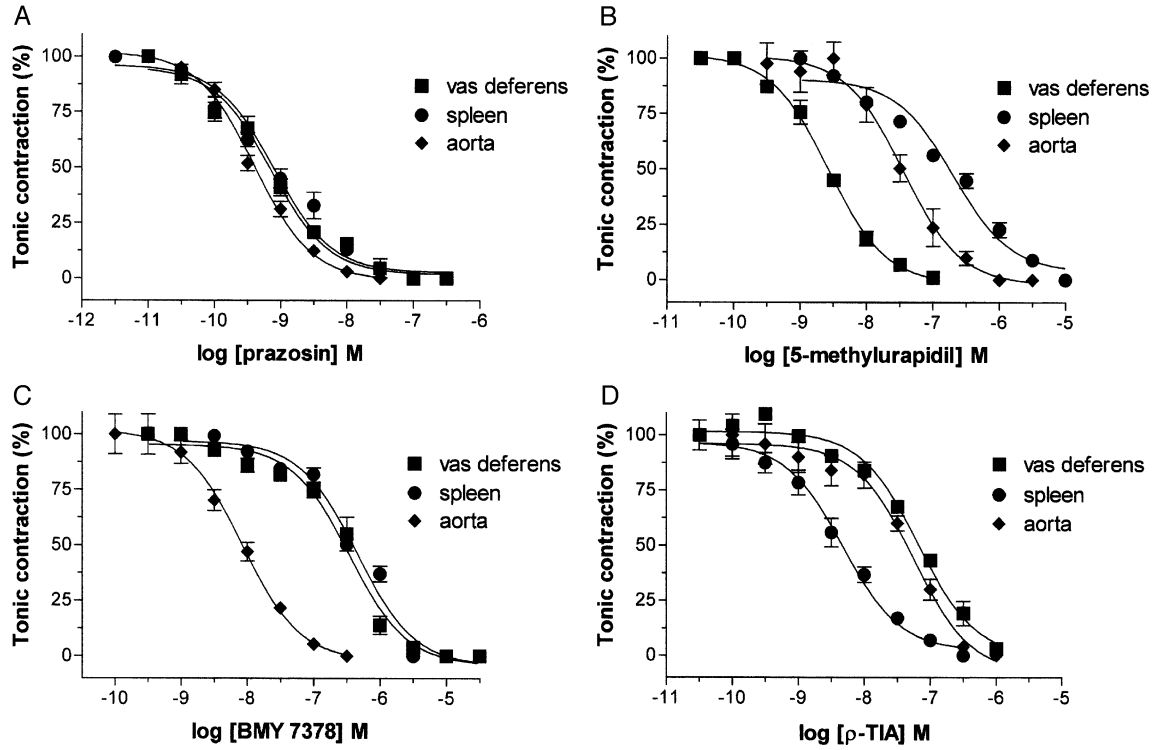


Fig. 5. Concentration–response curves for inhibition of tonic contractions to noradrenaline in vas deferens ( 1 μM/3 min), spleen ( 3 μM/5 min) and aorta ( 0.1 μM/5 min). Each symbol represents the mean and the vertical line, when greater than the symbol, the S.E.M. of four to six experiments.

transiently transfected with rat $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors, but caused a two- to three-fold apparent reduction in the affinity ($K_D$) of radioligand (Fig. 6, Table 4). In contrast, in membrane preparations from HEK293 cells expressing rat $\alpha_{1B}$-adrenoceptors, ρ-TIA (5 nM) reduced by approximately 58% the $B_{max}$ without affecting the $K_D$ (Fig. 6, Table 4).

In competition assays, ρ-TIA was approximately 20- to 25-fold more potent in displacing the binding of [$^{125}$I]HEAT to membranes from HEK293 cells expressing rat $\alpha_{1B}$-adrenoceptors than to membranes from cells expressing rat $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors (Fig. 7, Table 4).

## 4. Discussion

This work compared the actions of ρ-TIA on $\alpha_1$-adrenoceptor mediated contractions in rat vas deferens, spleen and aorta. Noradrenaline causes contraction of these three tissues predominantly through $\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptors respectively. This allowed a determination of the ability of the toxin to antagonize functional responses mediated by each of the $\alpha_1$-adrenoceptor subtypes natively expressed in rat tissues. Also, the potency and selectivity of ρ-TIA with that of other commonly used $\alpha_1$-adrenoceptor antagonists was compared and the effects of ρ-TIA on the binding of [$^{125}$I]HEAT to membranes from HEK293 cells expressing each of the recombinant rat $\alpha_1$-adrenoceptors were also determined to further check for the selectivity and nature of these interactions.

Table 3
$pIC_{50}$ values[a] for adrenoceptor antagonists and ρ-TIA determined from inhibition of contractile responses resulting from occupancies of similar fractions of $\alpha_1$-adrenoceptor subtypes (25%) by noradrenaline in vas deferens, spleen and aorta

| | Vas deferens | Spleen | Aorta |
|---|---|---|---|
| Prazosin | 9.14±0.09 | 9.10±0.10 | 9.40±0.05 |
| 5-Methylurapidil | 8.58±0.04 | 6.69±0.14 | 7.45±0.06 |
| BMY 7378 | 6.46±0.09 | 6.34±0.10 | 8.10±0.10 |
| ρ-TIA | 7.18±0.07 | 8.33±0.07 | 7.26±0.07 |

[a] Each value represents the mean and the S.E.M. of four to six experiments.

The $pA_2$ values estimated for prazosin, 5-methylurapidil and BMY 7378 confirmed that the contractions induced by noradrenaline in vas deferens, spleen and aorta are mediated by activation of different $\alpha_1$-adrenoceptor subtypes. The affinity of 5-methylurapidil, an $\alpha_{1A}$-adrenoceptor selective antagonist, was higher in the vas deferens than in the other two tissues, while that of BMY 7378, an $\alpha_{1D}$-adrenoceptor selective antagonist, was much higher in the aorta. Unfortunately, identification of responses mediated by $\alpha_{1B}$-adrenoceptors is largely based on exclusion, due to the lack of competitive antagonists with significant selectivity at this subtype in functional studies. Accordingly, 5-methylurapidil and BMY 7378 showed low affinities in spleen. These results support the hypothesis that the contractions induced by noradrenaline in vas deferens, spleen and aorta are predominantly mediated by activation of $\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor populations, respec-

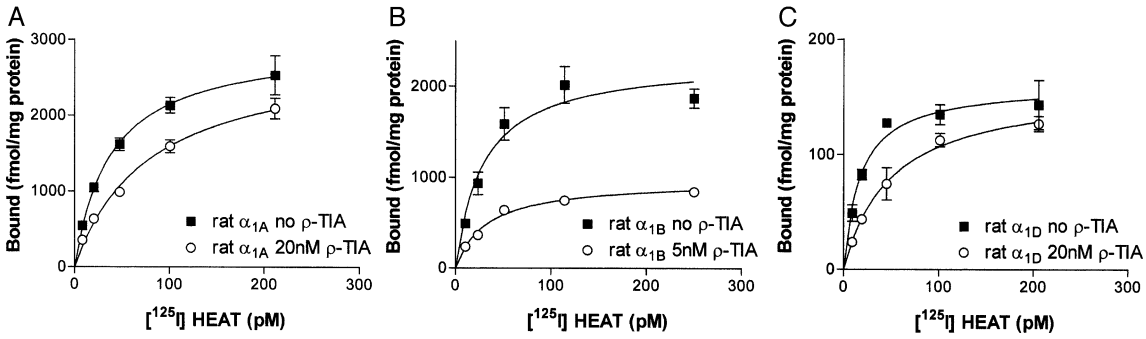


Fig. 6. Saturation binding of [$^{125}$I]HEAT to HEK293 membranes expressing rat $\alpha_1$-adrenoceptor subtypes in the absence or presence of 20 nM ($\alpha_{1A}$ and $\alpha_{1D}$) or 5 nM ρ-TIA ($\alpha_{1B}$). Each symbol represents the mean±S.E.M. of four to six determinations.

tively, and that these effects can be conveniently assumed as functional responses resulting from activation of each the $\alpha_1$-adrenoceptor subtypes. This is consistent with several other studies (Burt et al., 1995; Pupo, 1998) for vas deferens (Buckner et al., 1996; Burt et al., 1995), for spleen and (Buckner et al., 1996; Piascik et al., 1995) for aorta.

ρ-TIA potently inhibited contractions of all three tissues in response to noradrenaline. The potency of ρ-TIA, as estimated by its p$A_2$ values, was very similar in vas deferens and aorta. In the concentration range tested (up to 1 μM), ρ-TIA behaved as a competitive antagonist in these tissues. Unfortunately, the limited amount of ρ-TIA and the relatively large volume of the tissue baths precluded the use of higher concentrations of the peptide. Nonetheless, these results are compatible with those obtained by (Chen et al., 2004) in HEK293 cells expressing recombinant human $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors, where ρ-TIA inhibited accumulation of [$^3$H]inositol phosphates induced by noradrenaline in a manner consistent with competitive antagonism. In fact, the potencies estimated for ρ-TIA in the present study in the rat vas deferens and aorta (p$A_2$~7.2) are very close to those estimated in HEK293 cells expressing recombinant human $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors (p$A_2$~6.9).

The antagonism of the contractions of rat spleen by ρ-TIA was clearly different from that of vas deferens and aorta. In the spleen, ρ-TIA did not reduce the potency of noradrenaline in activating contraction, but reduced its maximal effect. This indicates that the antagonism of noradrenaline by ρ-TIA in spleen is non-competitive. This is in agreement with previous results in HEK 293 cells stably expressing recombinant hamster (Sharpe et al., 2003) or human (Chen et al., 2004) $\alpha_{1B}$-adrenoceptors. Similar to the results obtained here, in studies with recombinant $\alpha_{1B}$-adrenoceptors, ρ-TIA reduced the maximal accumulation of [$^3$H]inositol phosphates stimulated by noradrenaline in the previous studies (Chen et al., 2004; Sharpe et al., 2003).

The effects of ρ-TIA on [$^{125}$I]HEAT saturation binding assays support the conclusion that ρ-TIA is a competitive antagonist of rat $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors and a non-competitive antagonist of rat $\alpha_{1B}$-adrenoceptors. In experiments with recombinant rat $\alpha_{1B}$-adrenoceptors, ρ-TIA reduced the $B_{max}$ without affecting the affinity of the radioligand, indicating that the antagonism is non-competitive in nature; on the other hand, in experiments with recombinant rat $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors, the affinity of the radioligand was affected, while the $B_{max}$ was not. These results are similar to those observed for recombinant human $\alpha_1$-adrenoceptor subtypes (Chen et al., 2004).

It is difficult to compare the potencies of non-competitive antagonists in different tissues because of the existence of different degrees of spare receptors. When there are significant proportions of spare receptors, non-competitive

Table 4
Effects of ρ-TIA 20 nM ($\alpha_{1A}$ and $\alpha_{1D}$) or 5 nM ($\alpha_{1B}$) on the binding of [$^{125}$I]HEAT to membranes from HEK293 cells expressing each of the recombinant rat $\alpha_1$-adrenoceptor subtypes and p$IC_{50}$ determined from displacement curves

| | [$^{125}$I]HEAT | | | | ρ-TIA p$IC_{50}$ (competition assays) |
|---|---|---|---|---|---|
| | $B_{max}$ (fmol/mg protein) | | $K_D$ (pM) | | |
| | No ρ-TIA | Plus ρ-TIA | No ρ-TIA | Plus ρ-TIA | |
| Rat $\alpha_{1A}$ | 2971±184 | 2860±210 | 38.4±7.1 | 79.7±13.7[a] | 7.50±0.12 |
| Rat $\alpha_{1B}$ | 2295±199 | 969.0±49[a] | 29.3±8.5 | 32.8±4.9 | 8.90±0.06[b] |
| Rat $\alpha_{1D}$ | 160.9±9.5 | 159.8±13 | 17.4±3.7 | 50.0±10.9[a] | 7.58±0.11 |

[a] Different from the respective value found in the absence of ρ-TIA ($P$<0.05).

[b] Different from the respective value found in the rat $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor ($P$<0.05). Each value represents the mean±S.E.M. of four to six experiments.

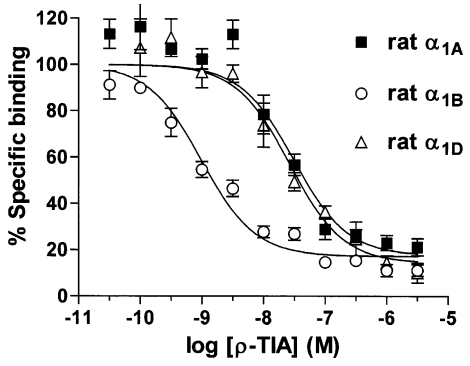


Fig. 7. Concentration-dependent inhibition of specific [$^{125}$I]HEAT binding by ρ-TIA in membranes prepared from HEK293 cells transiently transfected with individual rat $\alpha_1$-adrenoceptor subtypes. Each symbol represents the mean±S.E.M. of four determinations.

antagonists will induce rightward shifts in the concentration–response curves to agonists before any reduction of the maximal response is detected, precluding direct comparisons. On the other hand, in the absence of spare receptors, non-competitive antagonists will reduce the maximal response to an agonist without altering its potency. Despite of this cautionary argument, it was interesting to observe that the potency of ρ-TIA in antagonizing the contractions of the spleen ($pIC_{50}$~8.3) was close to the potency of ρ-TIA in inhibiting binding of [$^{125}$I]HEAT to membranes from HEK293 cells transfected with the cDNA encoding the recombinant human ($pIC_{50}$~8.4, Chen et al., 2004) and rat ($pIC_{50}$~8.9, present study) $\alpha_{1B}$-adrenoceptor. This comparison is strengthened by the fact that the occupancy–response relationship for noradrenaline and $\alpha_{1B}$-adrenoceptors in rat spleen was only slightly hyperbolic, indicating only a small proportion of spare receptors. ρ-TIA was approximately 12 times more potent in inhibiting contractions of rat spleen to noradrenaline than it was in the vas deferens and aorta. However, it should be stressed that the potency of ρ-TIA at $\alpha_{1B}$-adrenoceptors in functional studies will be highly dependent on receptor–effector coupling efficiency. ρ-TIA will tend to be less potent in tissues where the $\alpha_{1B}$-adrenoceptors are more efficiently coupled than in the rat spleen.

The selectivity of ρ-TIA for rat $\alpha_{1B}$-adrenoceptors detected in functional experiments was also observed in radioligand binding assays using recombinant rat receptors, where the affinity of this peptide at this subtype was 20- to 25-fold higher than the affinity at $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors. The reasons why ρ-TIA was slightly more potent in radioligand binding assays than in functional studies are not completely clear, but may be due to the use of peptides from different batches in different experiments.

Importantly, the contractions of the vas deferens, spleen and aorta induced by a submaximal concentration of KCl were not affected by ρ-TIA, suggesting that this peptide is devoid of the characteristic ion channel blocking properties shared by most of the conotoxins; also, its effects were reversible as suggested by the prompt recovery of the responses to noradrenaline after washing. These results indicate that ρ-TIA can be adequately used in functional studies involving isolated tissues. However, it will be important to determine the properties of ρ-TIA at other G-protein coupled receptors.

The modest (10–25-fold) selectivity of ρ-TIA for hamster (Sharpe et al., 2003), human (Chen et al., 2004) and rat (present study) $\alpha_{1B}$-adrenoceptors is not really sufficient for easily differentiating contributions of particular subtypes when combinations of $\alpha_1$-adrenoceptors are present. However, its noncompetitive inhibition of $\alpha_{1B}$-adrenoceptors and competitive inhibition of the other two subtypes will make this task much easier. Since inhibition of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor subtypes by ρ-TIA can be competitively overcome by increasing the concentration of agonist, while the non-competitive inhibition of $\alpha_{1B}$-adrenoceptors cannot; it should be fairly simple to determine whether a particular response is mediated by $\alpha_{1B}$-adrenoceptors simply by examining the effect of ρ-TIA in the presence of high agonist concentrations. Of course, if responses are still seen, it will be important to demonstrate that they are mediated by $\alpha_1$-adrenoceptors by the use of selective antagonists (such as prazosin). However, the identification of ρ-TIA as a slightly selective non-competitive $\alpha_{1B}$-adrenoceptor antagonist rounds out our pharmacological armamentarium for this receptor subfamily, since we now have $\alpha_{1A}$ (5-methylurapidil, (+)niguldipine)-, $\alpha_{1B}$ (ρ-TIA)- and $\alpha_{1D}$ (BMY 7378)-adrenoceptor selective compounds.

In conclusion, our data indicate that ρ-TIA discriminates functional responses mediated by $\alpha_1$-adrenoceptors natively expressed in rat tissues. ρ-TIA is about 10–25-fold more potent at $\alpha_{1B}$- than $\alpha_{1A}$- or $\alpha_{1D}$-adrenoceptors, and is a noncompetitive inhibitor of $\alpha_{1B}$-adrenoceptors but a competitive inhibitor of the other two subtypes. However, the potency of ρ-TIA in antagonizing responses to $\alpha_{1B}$-adrenoceptors is likely to be highly dependent on the receptor–effector coupling efficiency in a particular tissue or cell.

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