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Allosteric α_1-Adrenoreceptor Antagonism by the Conopeptide ρ-TIA*

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A peptide contained in the venom of the predatory marine snail *Conus tulipa*, ρ-TIA, has previously been shown to possess α_1-adrenoreceptor antagonist activity. Here, we further characterize its pharmacological activity as well as its structure-activity relationships. In the isolated rat vas deferens, ρ-TIA inhibited α_1-adrenoreceptor-mediated increases in cytosolic Ca^{2+} concentration that were triggered by norepinephrine, but did not affect presynaptic α_2-adrenoreceptor-mediated responses. In radioligand binding assays using [^{125}I]HEAT, ρ-TIA displayed slightly greater potency at the α_{1B} than at the α_{1A} or α_{1D} subtypes. Moreover, although it did not affect the rate of association for [^{3}H]prazosin binding to the α_{1B}-adrenoreceptor, the dissociation rate was increased, indicating non-competitive antagonism by ρ-TIA. N-terminally truncated analogs of ρ-TIA were less active than the full-length peptide, with a large decline in activity observed upon removal of the fourth residue of ρ-TIA (Arg^4). An alanine walk of ρ-TIA confirmed the importance of Arg^4 for activity and revealed a number of other residues clustered around Arg^4 that contribute to the potency of ρ-TIA. The unique allosteric antagonism of ρ-TIA resulting from its interaction with receptor residues that constitute a binding site that is distinct from that of the classical competitive α_1-adrenoreceptor antagonists may allow the development of inhibitors that are highly subtype selective.

α_1-Adrenoceptors, members of the G protein-coupled receptor superfamily, are the predominant mediators of the response to norepinephrine released from the sympathetic nerves that innervate resistance vessels (1). Norepinephrine release modulates vascular tone and, as such, α_1-adrenoreceptors are critically involved in circulatory homeostasis. Several α_1-adrenoreceptor antagonists, such as the quinazoline derivative, prazosin, are widely used for the treatment of hypertension. α_1-Adrenoreceptor antagonists are also used to treat bladder outlet obstruction in benign prostatic hyperplasia (for review, see Ref. 2) because of their ability to relax smooth muscle. Nevertheless, the α_1-adrenoreceptor ligands developed to date interact largely with residues of the transmembrane segments that are homologous between the various receptor subtypes, rather than with residues forming the framework regions (the intra- and extracellular loops). It is not surprising, therefore, that available agonists, and also antagonists, show limited subtype selectivity (affinities differing by 50-fold or less between the various subtypes). For this reason, we sought to identify novel ligands that are likely to interact allosterically and, thus, more likely with the framework residues that are distinct between the three α_1-adrenoreceptor subtypes (α_{1A}, α_{1B}, and α_{1D}).

The venoms of cone snails (marine gastropods of the genus *Conus*) contain bioactive peptides that disrupt neurotransmission. These compounds are referred to generically as "conopeptides" or "conotoxins." Individual conopeptides typically act with a high degree of specificity, yet collectively these toxin peptides possess an extraordinarily diverse spectrum of pharmacological activities. This has made cone snail venoms an attractive resource for the discovery of novel pharmacological agents for use as therapeutics or as research tools. Classes of conopeptides that target voltage-sensitive Ca^{2+} (the ω-conopeptides), Na^+ (μ-, μO-, and δ-conopeptides), and K^+ (κ- and κA-conopeptides) channels, and nicotinic acetylcholine (α-, αA-, and ψ-conopeptides), 5-HT$_3$ (σ-conopeptides), *N*-methyl-D-aspartic acid (conantokins), vasopressin (conopressins), and neurotensin (contulakins) receptors have been identified (for review, see Ref. 3). Two further classes of conopeptides that target the norepinephrine transporter (χ-conopeptides) and the α_1-adrenoreceptor (ρ-conopeptides) have recently been reported by us (4). The prototypical member of the ρ-conopeptide class is ρ-TIA, which acts as an α_1-adrenoreceptor antagonist. This action at the receptor contrasts with other conopeptides presently known to target G protein-coupled receptors, which act as agonists. The endogenous ligands of these receptors are peptides, and, not surprisingly, these conopeptides possess amino acid sequences that display high homologies with them.

Here, we investigated the mode of action of ρ-TIA, its selectivity for α_1-adrenoreceptor subtypes, and its structure-activity relationships. ρ-TIA is a nineteen amino acid peptide, with four cysteine residues and an amidated C terminus. The spacing of the cysteine residues is such that there are two intercysteine regions (*i.e.* in the conformation CC----C----C), and two intramolecular disulfide bonds connect the first and third and the second and fourth cysteine residues, respectively. Structurally, ρ-TIA closely resembles members of the α-conopeptide class. The similarity, however, does not extend to their pharmacological activity. The α-conopeptides act as competitive antagonists of muscle and neuronal nicotinic ACh receptors. The most

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The atomic coordinates and structure factors (code 1IEN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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obvious difference between the sequences of ρ-TIA and the α-conopeptides is at the N termini in the region outside of the cysteine-bracketed loops. The majority of α-conopeptides have only a single N-terminal residue preceding the first cysteine residue. A few other α-conopeptides have been found with zero, two, or three leading residues (5–7), but only one α-conopeptide isolated to date has four pre-cysteine N-terminal residues (8), as found in ρ-TIA. Suspecting that this region may play an important role in conferring α_1-adrenoreceptor antagonist activity on ρ-TIA, we examined the effect of the sequential removal of the first four amino acid residues of ρ-TIA on the activity of the peptide. Further information on the structure-activity relationship of ρ-TIA was provided by examining the effect of systematically substituting each non-cysteine residue with alanine.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—C-terminal amidated ρ-TIA and truncated analogs equivalent to residues 2–19, 3–19, 4–19, 5–19, and 1–5 of ρ-TIA were manually synthesized using *t*-butoxycarbonyl chemistry and cleaved from the resin following procedures described previously (9, 10). The amino acid sequences of the synthesized peptides are listed in Table I. Dinitrophenyl group removal from the histidine residue was accomplished off resin. The reduced peptides (50 mg), dissolved in a 10-ml solution of 6 M guanidine HCl, 100 mM Tris, pH 8.2, containing diisopropylethylamine (1 ml) and β-mercaptoethanol (2 ml), were stirred overnight at 18 °C. The mixture was then acidified to pH 3 with trifluoroacetic acid and purified by preparative HPLC.[1] The pure reduced peptides were oxidized in 0.33 M NH_4OAc, 0.5 M guanidine HCl, pH 7.8, in the presence of reduced and oxidized glutathione as previously described (9). The adoption of the Cys^1–Cys^3, Cys^2–Cys^4 pattern of disulfide connectivity by ρ-TIA and the truncated analogs was verified by NMR techniques. Analogs of ρ-TIA in which single residues were replaced with alanine were synthesized using Fmoc chemistry. The chain assembly of the peptides was performed on a manual shaker system using HBTU activation protocols (10) to couple the Fmoc-protected amino acid to the resin. The Fmoc protecting group was removed using 50% piperidine in dimethylformamide, and dimethylformamide was used as both the coupling solvent and for flow washes throughout the cycle. The progress of the assembly was monitored by quantitative ninhydrin monitoring (11). Peptide was deprotected and cleaved from the resin by stirring at room temperature in trifluoroacetic acid/H_2O/triisopropyl silane/EDT (90:5:2.5:2.5) for 2–3 h. Cold diethyl ether was then added to the mixture and the peptide precipitated out. The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether to remove scavengers. The final product was dissolved in 50% aqueous acetonitrile and lyophilized to yield a fluffy white solid. The crude, reduced peptide was examined by reverse phase HPLC for purity, and the correct molecular weight confirmed by electrospray mass spectrometry. Pure, reduced peptides were oxidized, and the major peak was purified to >95% purity and characterized by HPLC prior to further use.

Isolated Rat Vas Deferens—*Vasa deferentia* were excised from male Wistar rats (250–350 g) killed by a blow to the head, and exsanguinated. Epididymal and prostatic portions were bisected transversely and mounted in 5 ml organ baths under a resting tension of 0.5 g in physiological salt solution containing (mM): NaCl, 119; KCl, 4.7; $MgSO_4$, 1.17; KH_2PO_4, 1.18; $NaHCO_3$, 25.0; D-(+)-glucose, 5.5; $CaCl_2$, 2.5; EDTA, 0.026; maintained at 37 °C, and bubbled with 5% CO_2/95% O_2. Longitudinal tension was measured using isometric force transducers and recorded digitally (MacLab/8s data acquisition system; ADInstruments, Sydney, Australia). In the first set of experiments, a cumulative concentration-response curve for the contraction of the epididymal vas deferens elicited by exogenously applied norepinephrine was established. The preparations were then washed several times with drug-free solution. After 15 min, a dose of ρ-TIA was added to the organ bath and equilibrated with the tissues for 20 min before a second concentration-response curve to norepinephrine was generated. In other experiments, ρ-TIA was assayed for α_2-adrenoreceptor antagonist activity. Prostatic tissue segments were field stimulated with a single electrical pulse of 55 V amplitude and 1 ms duration generated by a Grass S44 stimulator (Grass Instruments, Quincy, MA) at 20 s intervals. Prazosin was added to the baths to a final concentration of 0.5 μM to block α_1-adrenoreceptors, and equilibrated with the tissues for 20 min before doses of norepinephrine were added in half-log unit steps to establish cumulative concentration-response curves. Each dose of norepinephrine was added after the effect of the previous dose had reached its maximum. The tissues were then washed with drug-free solution several times and allowed to recover for ~30 min. At this time, the prazosin (0.5 μM) was replaced and 10 μM ρ-TIA was added. After 20 min, a second concentration-response curve to norepinephrine was generated. In the final set of experiments, the N-terminal truncated analogs of ρ-TIA listed in Table I were assayed for α_1-adrenoreceptor antagonist activity. The bisected prostatic vas deferens segments were stimulated as before, but at 3 min intervals. ρ-TIA or one of its truncated analogs was added to the organ bath to a final concentration of 10 μM, and the peptide's effect on the tissue's contractile response was assessed.

Isolation of Smooth Muscle Cells—*Vasa deferentia* were excised from 10–14-day-old and ≥ 6-week-old Wistar rats killed by exposure to halothane. Adventitia and blood vessels were removed using a pair of fine forceps. The *vasa deferentia* were halved and incubated at room temperature in fresh Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS) for 5 min. Portions of vas deferens were placed in 2 ml Ca^{2+}-free HBSS containing 2 mg/ml collagenase (type B, Roche Applied Science) and bubbled with 95% O_2, 5% CO_2 for 1 h at 30 °C. The tissue pieces were then cut into smaller fragments and further incubated in fresh enzyme-containing solution in three 10 min stages. The vas deferens fragments were slightly agitated to enhance cellular dissociation. At the end of each stage, cells in the supernatant were collected and placed in fresh solution. Bovine serum albumin (1 mg/ml) was added, and the cell suspension was kept at 4 °C for 2 h. Cells were then plated on 25 mm round glass cover-slips in Petri dishes containing Dulbecco's modified Eagle's medium and used within 48–72 h.

Fura-2 Fluorometric Measurements—Cover-slips holding cells were mounted in a recording chamber and constantly perfused with bath solution containing (mM): NaCl, 150; KCl, 5.4; HEPES, 10; D-glucose, 10; $CaCl_2$, 1; $MgCl_2$, 1; pH 7.4, at a rate of 2 ml/min. The loading procedure was similar to that described by Liu *et al.* (12). Briefly, cells were incubated for 1 h in bath solution containing 5 μM Fura-2, AM, and 0.02% Pluronic-127 detergent. After loading, the cells were incubated for a further 30 min in fresh bathing solution to allow de-esterification. Fluorescent signals were acquired using a Photon Technology International photomultiplier detection system driven by the Felix software package, and free Ca^{2+} concentrations were calculated from the ratio of emissions at 510 nm following excitement with wavelengths of 340 nm and 380 nm as described by Grynkiewicz *et al.* (13). Fluorescent responses elicited by the application of norepinephrine or ATP (both 10 μM) in the absence and presence of ρ-TIA were monitored. The experiments were carried out at room temperature (23 °C).

Membrane Preparation and DNA Constructs—COS-1 cells (ATCC; Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, glutamine, and 5% fetal bovine serum. At 60–80% confluency, the cells were transiently transfected with plasmid DNA incorporating α_1-adrenoreceptor cDNA in the modified eukaryotic expression vector pMT2′ using the DEAE-dextran method (14), as previously described (15). In some experiments, COS-7 cells (ECACC; Salisbury, Wiltshire, UK) were used. These were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and transiently transfected with the α_1-adrenoreceptor using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer's protocol. Three different plasmid constructs were used: one for the rat α_{1A}-adrenoreceptor subtype (16), one for the hamster α_{1B}-adrenoreceptor (17), and another for the rat α_{1D}-adrenoreceptor (18). Membranes were prepared 24 h post-transfection as described previously (18), and resuspended in HEM buffer (20 mM HEPES, 1.5 mM EGTA, 12.5 mM $MgCl_2$, pH 7.5) containing 10% v/v glycerol and stored at −80 °C. The protein concentration of the prepared membranes was determined by the method of Bradford (19).

Radioligand Binding Assays—To determine the α_1-adrenoreceptor subtype selectivity of ρ-TIA, the effect of ρ-TIA on the binding of the radiolabelled α_1-adrenoreceptor antagonist [^{125}I]HEAT was examined. Reactions containing [^{125}I]HEAT (70 pM), membranes from α_1-adrenoreceptor-transfected COS-1 cells (1 μg protein), HEM buffer, and increasing concentrations of ρ-TIA were set up in polypropylene tubes and incubated at room temperature for 60 min. The assays were performed in duplicate in a total reaction volume of 250 μl. Nonspecific binding

[1] The abbreviations used are: HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; HBTU, *N,N,N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate); EDT, ethanedithiol; [^{125}I]HEAT, [^{125}I]-2-[β-(3-iodo-4-hydroxyphenyl)-ethylaminomethyl tetralone; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; $GABA_A$, γ-aminobutyric acid, Type A.

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was determined in the presence of 100 μM phentolamine. Reactions were stopped by the addition of ice-cold HEM buffer, and the tubes' contents were filtered under vacuum onto Whatman GF/C glass filters with a Brandel cell harvester. The filters were washed 5 times with ice-cold HEM buffer, and the amount of filter-bound radioactivity was determined using a Packard Auto-γ 500 counter.

In other binding experiments, [^{3}H]prazosin was used as the radioligand. To examine the effect of ρ-TIA on the rate of association of [^{3}H]prazosin to the α_{1B}-adrenoreceptor, reactions containing [^{3}H]prazosin (0.25 nM), membranes from COS-7 cells transiently transfected with the α_{1B}-adrenoreceptor (2.5 μg protein), HEM buffer were set up in duplicate in the absence and presence of ρ-TIA (17.5 nM). The amount of nonspecific binding was determined as described above. Binding was allowed to proceed for 0–90 min. The effect of ρ-TIA on the rate of dissociation of [^{3}H] prazosin from the α_{1B}-adrenoreceptor was investigated in assays where phentolamine (100 μM) with and without ρ-TIA (10 μM) was added to reactions containing [^{3}H]prazosin (0.43 nM) and α_{1B}-adrenoreceptor membranes (7 μg protein) that had previously been incubated for 60 min. In experiments with alanine-substituted analogs of ρ-TIA, [^{3}H]prazosin (0.43 nM) and α_{1B}-adrenoreceptor membranes (7 μg protein) were incubated for 60 min with increasing concentrations of peptide (10^{-11}–10^{-5} M). The reaction volume was 150 μl. Membranes were harvested onto Whatman GF/B filtermats using a Tomtec harvester. BetaPlate scintillant (PerkinElmer Life Sciences) was applied and the filter-bound radioactivity detected using a Wallac MicroBeta counter.

Statistics and Data Analysis—Curves were fitted to the individual data points of sets of 3–6 separate experiments by non-linear regression using Prism 3 software for Macintosh (GraphPad Software, San Diego, CA). Results are expressed as means ± S.E. Student's *t* test was used to compare the curve-fitting parameters between treatments, and analysis of variance with post hoc *t* tests performed by the Tukey method was used for multiple comparisons. Values of $p < 0.05$ were considered significant.

Materials—Adenosine 5′-triphosphate disodium salt, (−)-norepinephrine bitartrate salt, phentolamine hydrochloride, and prazosin hydrochloride were obtained from Sigma. Collagenase type B and 1,4-dithiothreitol were obtained from Roche Applied Science. Fura-2, AM, and Pluronic-127 were obtained from Molecular Probes (Eugene, Oregon). [^{125}I]HEAT (specific activity, 2200 Ci/mmol) and [7-methoxy-^{3}H]prazosin (specific activity, 87 Ci/mmol) were from PerkinElmer Life Sciences. Protected Fmoc-amino acid derivatives were from Novabiochem or Auspep (Melbourne, Australia). Dimethylformamide, dichloromethane, diisopropylethylamine, and trifluoroacetic acid were all peptide synthesis grade supplied by Auspep. HBTU was Fluka no. 12804 supplied by Sigma. HPLC grade acetonitrile and methanol was supplied by Sigma. Resin used was Fmoc-rink amide resin supplied by Polymer Labs. Triisopropyl silane was from Aldrich Chemicals.

RESULTS

α_1-Adrenoreceptor Antagonism—The application of norepinephrine (1 μM) to dissociated smooth muscle cells from the *vasa deferentia* of adult rats elicited an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) from 19 ± 3.3 nM to 130 ± 26 nM (n = 9). In cells from juvenile rats, the same concentration of norepinephrine raised the Ca^{2+} concentration from 32 ± 7.6 nM to 309 ± 46 nM (n = 4). The magnitude of the elicited responses, but not the resting Ca^{2+} levels, were significantly different between adult and juvenile rats. In both age groups, the responses could be abolished by prazosin (10 nM), indicating that they were mediated by α_1-adrenoreceptors. ρ-TIA inhibited the Ca^{2+} responses of adult cells with an IC_{50} of 133 nM (pIC_{50} = 6.88 ± 0.05) and juvenile cells with an IC_{50} of 470 nM (pIC_{50} = 6.33 ± 0.03) (Fig. 1). Neither curve's bottom was significantly different from zero, indicating that ρ-TIA acts as a full inhibitor. The juvenile concentration-response curve was significantly steeper than the adult curve, with a Hill slope parameter of −2.6 ± 0.4 compared with −0.89 ± 0.10. ρ-TIA (1 μM) had no effect on the Ca^{2+} response of the isolated smooth muscle cells that was evoked by 10 μM ATP (n = 3; data not shown).

α_1-Adrenoreceptor But Not α_2-Adrenoreceptor Antagonism—The effect of various concentrations of ρ-TIA on the α_1-adrenoreceptor-mediated contractile responses of isolated segments of rat vas deferens to exogenously applied norepinephrine is shown in Fig. 2*A*. In the absence of ρ-TIA, the EC_{50} of norepinephrine was determined to be 9.3 μM (pEC_{50} = 5.0 ± 0.03). This value was increased to 32 μM (pEC_{50} = 4.5 ± 0.07), 51 μM (pEC_{50} = 4.3 ± 0.09), and to 166 μM (pEC_{50} = 3.3 ± 0.08) in the presence of 1, 3, and 10 μM ρ-TIA, respectively. The maximum response of the tissue to norepinephrine was 99 ± 4.4% of the control response when 1 μM ρ-TIA was present and 82 ± 4.9% and 42 ± 3.4% in the presence of 3 and 10 μM ρ-TIA, respectively. The decline in the maximum response following treatment with the two highest concentrations of ρ-TIA was significant ($p < 0.001$).

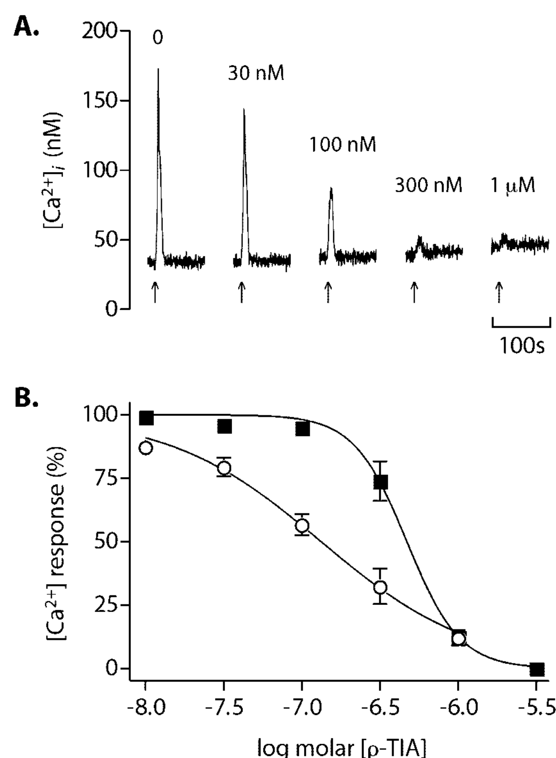

FIG. 1. **Effect of ρ-TIA on the norepinephrine-evoked Ca^{2+} response of isolated rat vas deferens smooth muscle cells.** *A*, the increase in cytosolic Ca^{2+} concentration of an isolated adult rat vas deferens smooth muscle cell elicited by the application of norepinephrine (10 μM; indicated by *arrows*) was attenuated in the presence of ρ-TIA. *B*, concentration-response curves for the inhibition of the Ca^{2+} response by ρ-TIA in adult (○) and juvenile (■) rat cells were constructed. *Symbols* represent the mean ± S.E. of data from six experiments.

The bisected rat prostatic vas deferens responded to electrical field stimulation with a biphasic contraction, reflecting the distinct time courses for the postsynaptic actions of the sympathetic co-transmitters ATP and norepinephrine in the tissue (20). Following the addition of prazosin, the previously biphasic response consisted of only the first component. This prazosin-resistant component could be abolished by activating α_2-adrenoreceptors with exogenously applied norepinephrine. The IC_{50} for the inhibition by norepinephrine was 1.1 μM (pIC_{50} = 6.0 ± 0.10) (Fig. 2*B*). In the presence of ρ-TIA (10 μM), the IC_{50} was not significantly different (pIC_{50} = 5.9 ± 0.13). Neither norepinephrine nor ρ-TIA had a direct effect on the resting tension of the preparation.

α_1-Adrenoreceptor Subtype Selectivity and Effect on [^{3}H]prazosin Binding Kinetics—ρ-TIA inhibited the binding of [^{125}I]HEAT to all three cloned α_1-adrenoreceptor subtypes (Fig. 3). The IC_{50} values for ρ-TIA were 150 nM (pIC_{50} = 6.8 ± 0.04) at the α_{1A}-adrenoreceptor; 70 nM (pIC_{50} = 7.15 ± 0.06) at the α_{1B}-adrenoreceptor; and 340 nM (pIC_{50} = 6.5 ± 0.05) at the

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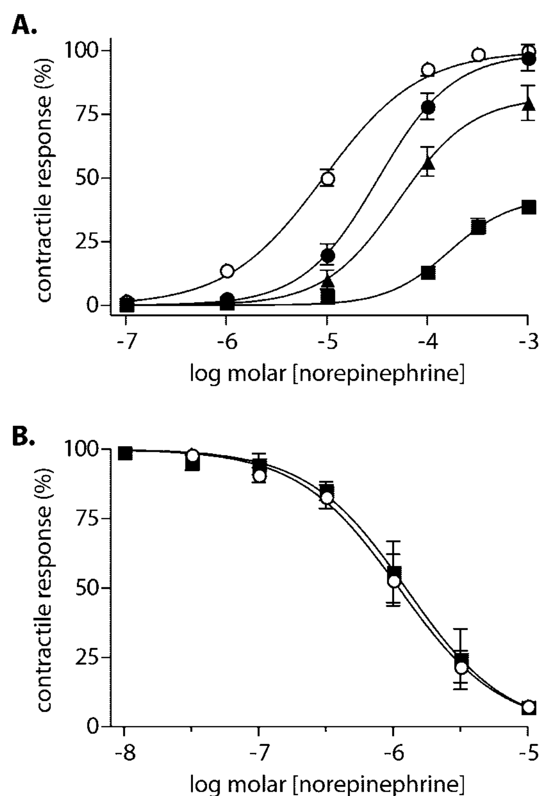

FIG. 2. **Effect of ρ-TIA in functional assays for α_1- and α_2-adrenoreceptor antagonism.** *A*, concentration-response curves for the contractile response elicited by the activation of α_1-adrenoreceptors by exogenously applied norepinephrine measured in the absence (○) and presence of ρ-TIA (●, 1 μM; ▲, 3 μM; ■, 10 μM). *B*, concentration-response curves for the inhibition of the electrically evoked response of the rat vas deferens due to the activation of presynaptic α_2-adrenoreceptors by exogenously applied norepinephrine measured in the absence (○) and presence (■) of 10 μM ρ-TIA. *Symbols* represent the mean ± S.E. of data from five experiments.

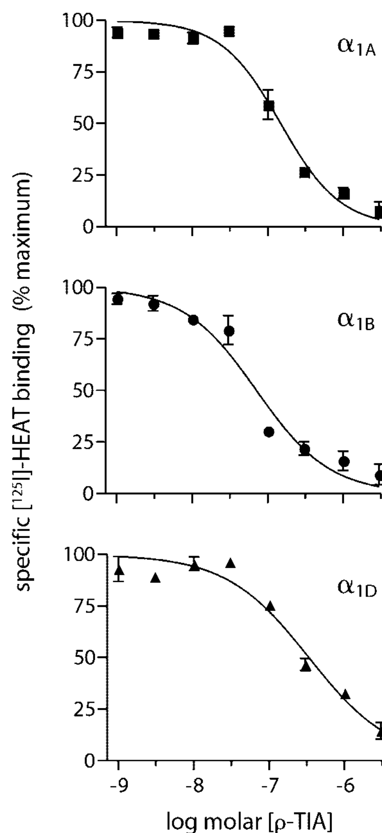

FIG. 3. **Effect of ρ-TIA at α_1-adrenoreceptor subtypes.** Concentration-response curves for inhibition by ρ-TIA of the specific binding of the α_1-adrenoreceptor antagonist [^{125}I]HEAT to membranes from COS-1 cells transiently transfected with the rat α_{1A}- (■), hamster α_{1B}- (●), or rat α_{1D}-adrenoreceptor (▲). *Symbols* represent the mean ± S.E. of data from three separate experiments performed in duplicate.

α_{1D}-adrenoreceptor. The difference in the potency of ρ-TIA between α_1-adrenoreceptor subtypes was significant for all sets of comparisons ($p < 0.001$). The Hill slope parameters for the effect of ρ-TIA were not significantly different from unity.

The effect of ρ-TIA on the association rate of [^{3}H]prazosin binding to α_{1B}-adrenoreceptors is shown in Fig. 4*A*. In the absence of ρ-TIA, the observed rate constant for the association of [^{3}H]prazosin (k_{ob}) was determined to be 0.101 ± 0.008 min^{-1}. The k_{ob} in experiments with ρ-TIA present was 0.119 ± 0.013 min^{-1}, which is not significantly different from the control value. The total specific binding was reduced from 2.50 ± 0.052 pmol/mg protein to 1.52 ± 0.041 pmol/mg protein in the presence of ρ-TIA (17.5 nM). The difference in the amount of total specific binding achieved in the absence and presence of ρ-TIA was highly significant ($p < 0.001$).

The dissociation of [^{3}H]prazosin from the α_{1B}-adrenoreceptors (Fig. 4*B*) proceeded at a rate of 0.50 ± 0.03 h^{-1} in control experiments. In the presence of ρ-TIA (10 μM), the dissociation rate constant (k_{off}) was increased significantly to 1.15 ± 0.06 h^{-1} ($p < 0.001$).

Truncated Analogs of ρ-TIA—All of the N-terminal truncated analogs of ρ-TIA (see Table I) tested were active in the vas deferens assay, causing selective inhibition of the second component of the biphasic contraction as observed for ρ-TIA. However, the N-terminal tail alone, $TIA_{1–5}$, had no effect on either phase of the contraction at a concentration of 10 μM. The deletion of N-terminal residues from ρ-TIA was associated with a loss in activity, demonstrated by the relative extent of the second component that remained after treatment with the analogs and full-length ρ-TIA (all 10 μM; Fig. 5).

Alanine Walk of ρ-TIA—The potencies of ρ-TIA and its alanine-substituted analogs at the α_{1B}-adrenoreceptor are compared in Fig. 6. All of the peptides acted as full inhibitors of specific [^{3}H]prazosin binding and none of the Hill slope parameters of the concentration-response curves were significantly different from unity. The potency of ρ-TIA was the same whether the binding reaction was allowed to proceed for 60 or 120 min (data not shown). Of the fourteen analogs tested, six were found to be significantly less potent than ρ-TIA. The greatest loss of potency was seen upon replacement of the arginine residue at position 4, which resulted in a reduction in potency of ~270-fold compared with the native conopeptide.

DISCUSSION

As a peptide, ρ-TIA is structurally unique among the α_1-adrenoreceptor antagonists described to date, some of which were also originally isolated from natural sources (*e.g.* the plant alkaloids corynanthine, dicentrine, and dehydroevodiamine). We have explored the pharmacology of ρ-TIA in functional and binding assays, and found that the uniqueness of ρ-TIA compared with other α_1-adrenoreceptor antagonists also extends to its mechanism of action.

Functional α_1-adrenoreceptor antagonism by ρ-TIA was demonstrated at both the tissue and cellular level through its ability to inhibit the norepinephrine-evoked increases in cytosolic free Ca^{2+} concentration and contractility. The vast majority of known α_1-adrenoreceptor antagonists act competitively with respect to norepinephrine. ρ-TIA, however, behaves as a non-competitive antagonist. This was indicated in these experiments by the effect of the peptide to inhibit the maximum level

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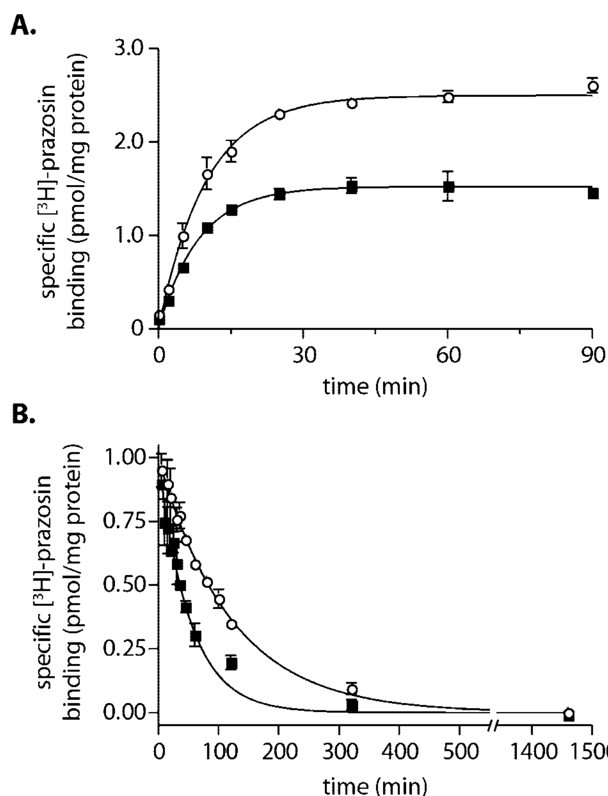

FIG. 4. **Effect of ρ-TIA on the kinetics of [^{3}H]prazosin binding.** *A*, association of [^{3}H]prazosin to α_{1B}-adrenoreceptors over time in the absence (○) and presence (■) of 17.5 nM ρ-TIA. *B*, dissociation of [^{3}H]prazosin from α_{1B}-adrenoreceptors over time in the absence (○) and presence (■) of 10 μM ρ-TIA. *Symbols* represent the mean ± S.E. of data obtained from three separate experiments. Some *error bars* are obscured by the *symbols*.

TABLE I
Amino acid sequence of ρ-TIA and N-terminal truncated analogs

Descriptions of connectivity refer to the presence of disulfide bonds between cysteine residues (in bold) numbered sequentially with respect to the linear sequence. * indicates an amidated C terminus.

Peptide	Amino acid sequence	Connectivity
ρ-TIA	FNWR**CC**LIPA**C**RRNHKKF**C***	1–3, 2–4
$TIA_{2–19}$	NWR**CC**LIPA**C**RRNHKKF**C***	1–3, 2–4
$TIA_{3–19}$	WR**CC**LIPA**C**RRNHKKF**C***	1–3, 2–4
$TIA_{4–19}$	R**CC**LIPA**C**RRNHKKF**C***	1–3, 2–4
$TIA_{5–19}$	**CC**LIPA**C**RRNHKKF**C***	1–3, 2–4
$TIA_{1–5}$	FNWRC*	none

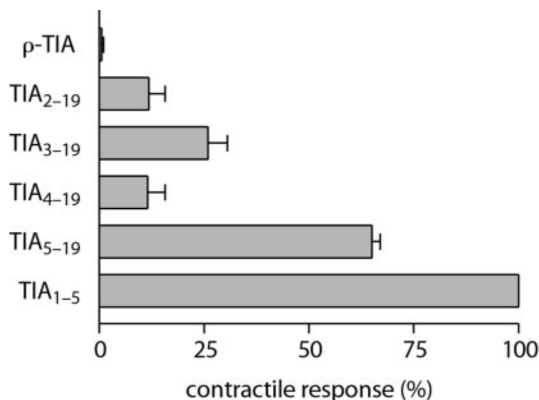

FIG. 5. **α_1-Adrenoreceptor antagonist activity of ρ-TIA and its N-terminal truncated analogs.** Comparison of the effect of ρ-TIA and the five truncated analogs (all 10 μM) on the magnitude of the noradrenergic component of the response of the rat vas deferens to electrical field stimulation. *Bars* represent the mean ± S.E. of results obtained from 3–5 experiments.

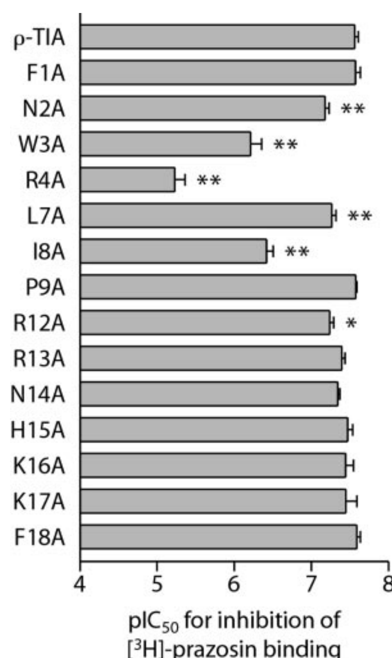

FIG. 6. **Comparison of the potencies of ρ-TIA and its alanine-substituted analogs for the α_1-adrenoreceptor.** A series of analogs of ρ-TIA in which its non-cysteine residues were systematically replaced with alanine were assayed for inhibition of [^{3}H]prazosin binding to the membranes of COS-7 cells transfected with the hamster α_{1B}-adrenoreceptor. *Bars* represent the mean ± S.E. of the analogs' pIC_{50} values determined from three concentration-response curves, with each concentration point on a single curve tested in triplicate. * indicates $p < 0.05$ and ** indicates $p < 0.001$ compared with ρ-TIA.

of the α_1-adrenoreceptor-mediated contractile response proving to be incapable of being surmounted by increasing the concentration of applied norepinephrine.

Changes in various aspects of α_1-adrenergic neurotransmission associated with age have been reported (21–23), so potential age-related differences in the inhibitory action of ρ-TIA were examined. This was investigated in dissociated cells only, because the contractile response of the juvenile rat vas deferens is quite weak (24). ρ-TIA was ~3.5 times more potent at inhibiting Ca^{2+} spikes following α_1-adrenoreceptor activation by norepinephrine in cells from adult rats compared with those taken from juvenile rats. This might be due to an altered pattern of expression of α_1-adrenoreceptor subtypes with age, as has been reported to occur in other tissues (25). Each of the three cloned α_1-adrenoreceptor subtypes play a functional role in the adult rat vas deferens (26–29), with the α_{1A} subtype generally agreed to be the principal mediator of responses to norepinephrine. The difference in potency of ρ-TIA in juvenile and adult rats is similar in magnitude to the range in potency across the three cloned α_1-adrenoreceptors that was observed in the binding experiments. Involvement of the putative prazosin-insensitive α_{1L}-adrenoreceptor described by Ohmura *et al.* (30) in influencing ρ-TIA potency, can, however, be ruled out as both adult and juvenile Ca^{2+} responses were fully inhibited by a low dose of prazosin. Also, because the assays were performed using isolated smooth muscle cells, we can exclude the impact of changes in the effectiveness of the neuronal norepinephrine reuptake system that occur with age (31) as a reason for the disparate potency of ρ-TIA in juvenile and adult rats.

As well as the difference in potency, a change in the Hill slope of the concentration-response curve for ρ-TIA was seen between rats from the two age groups, with the data from

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younger animals exhibiting a much steeper inhibition profile. The relationship between α_1-adrenoreceptor activation and functional response is known to be non-linear in the adult rat vas deferens (32, 33), giving rise to the phenomenon of "spare receptors," and the efficiency of receptor-effector coupling is recognized to vary between α_1-adrenoreceptor subtypes (34). Tighter receptor-effector coupling in the rat vas deferens of juvenile animals, with or without a change in the identity of the adrenoreceptor subtypes mediating the response, would act to reduce the apparent potency of a non-competitive inhibitor like ρ-TIA by requiring that a greater proportion of the receptor pool be inactivated to achieve the same level of inhibition. Furthermore, the concentration-response curve for inhibition would steepen as the relationship between receptor activation and response became more strongly hyperbolic. Our observation that the norepinephrine-evoked Ca^{2+} responses of cells from juvenile animals were substantially larger than those of older animals may indicate a decline in α_1-adrenoreceptor signaling efficiency with age. The presence of spare receptors in the adult rat vas deferens explains why ρ-TIA initially shifted the concentration-response curve for norepinephrine to the right without an accompanying decline in the maximum response, as was evident in the presence of the two higher concentrations of ρ-TIA.

In light of ρ-TIA's modest α_1-adrenoreceptor subtype selectivity, it was of interest to investigate whether α_2-adrenoreceptors were a target of ρ-TIA. The finding that ρ-TIA (10 μM) did not protect the evoked responses from inhibition by norepinephrine, as α_2-adrenoreceptor antagonists such as yohimbine have been demonstrated to do in this assay (35), indicates that the peptide does not block α_2-adrenoreceptors.

To investigate the mode of action of ρ-TIA at the α_1-adrenoreceptor, the effect of ρ-TIA on the kinetics of [^{3}H]prazosin binding to the receptor was examined. The α_{1B} subtype was used as the prototypical α_1-adrenoreceptor in these experiments because it is at this subtype that ρ-TIA displays the highest potency. Confirming that ρ-TIA does not block the α_1-adrenoreceptor through a competitive interaction, the rate constant observed for the association of [^{3}H]prazosin to α_{1B}-adrenoreceptors was unchanged in the presence of ρ-TIA and not reduced as would have been expected if the conopeptide acted competitively. The decline in the amount of equilibrium binding without a change in the rate constant for association when ρ-TIA was included in the binding reaction is consistent with the conopeptide disrupting [^{3}H]prazosin binding through an allosteric interaction. We suggest then that the agonist binding site, which is recognized by norepinephrine and competitive antagonists such as HEAT and prazosin, and the ρ-TIA site on the α_1-adrenoreceptor are distinct. Upon binding of ρ-TIA to its site on the α_1-adrenoreceptor, the receptor loses its ability to recognize agonists and competitive antagonists through an allosteric action of ρ-TIA to disrupt the structure of the agonist-binding site, reducing the size of the pool of available receptors for these ligands.

The increase in the rate of dissociation of [^{3}H]prazosin from the α_1-adrenoreceptor in the presence of ρ-TIA reveals that the conopeptide can still bind to the receptor when [^{3}H]prazosin is already bound and that ρ-TIA promotes the dissociation of [^{3}H]prazosin from the receptor. The R·ρ-TIA·prazosin complex can not be distinguished from the R·prazosin complex in these experiments because both species are radiolabelled. Consequently, the rate of dissociation observed in the presence of ρ-TIA reflects a combination of the rates of [^{3}H]prazosin dissociation from both the R·prazosin and the R·ρ-TIA·prazosin complexes. The relative contribution of each dissociation reaction to the overall rate that is measured depends on the two rate

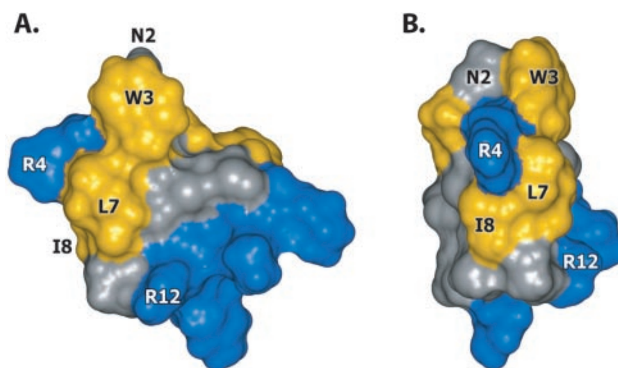

FIG. 7. **Location of amino acid residues of ρ-TIA important for α_1-adrenoreceptor antagonist activity.** The best of twenty structures of ρ-TIA determined by NMR (1) was used to generate a model of the three-dimensional surface of the peptide within INSIGHT II (Accelrys, San Diego, CA). Protein Data Bank accession number for coordinates, 1IEN. The models shown in *A* and *B* are rotated 90° around the *y*-axis. Positively charged residues are colored *blue*, hydrophobic residues *yellow*, and uncharged polar residues *gray*. The cluster of residues whose replacement with alanine caused a significant change in the potency of the peptide at the α_1-adrenoreceptor are *labeled*.

constants and also the relative concentrations of R·prazosin and R·ρ-TIA·prazosin. The modest increase in the observed rate (2.3 times faster) in the presence of a high concentration of ρ-TIA, which fully inhibits specific [^{3}H]prazosin equilibrium binding (10 μM), might indicate that the affinity of ρ-TIA for the α_1-adrenoreceptor is lower when [^{3}H]prazosin is bound than it is for the unoccupied receptor. Such a situation would represent bidirectional negative allosteric modulation between the competitive antagonist and ρ-TIA binding sites.

In addition to the ρ-conopeptides, two other classes of allosteric modulators of the α_1-adrenoreceptor have been reported, but neither act in the same manner as ρ-TIA. The allosteric effect of the benzodiazepines diazepam, lorazepam, and midazolam at the α_1-adrenoreceptor was reported by Waugh *et al.* (36). These agents are better known for their allosteric effect at the $GABA_A$ receptor, where they do not activate the receptor themselves, but act to increase the affinity and efficacy of the endogenous agonist GABA (37, 38). Like ρ-TIA, these three benzodiazepines were found to inhibit [^{125}I]HEAT binding, although much less potently, with IC_{50} values at the human α_1-adrenoreceptor subtypes of ~100 μM (36). Unlike ρ-TIA, however, the benzodiazepines were found to act as weak partial agonists of α_1-adrenoreceptors by themselves in functional assays, and to increase the maximum response and EC_{50} of both full and partial α_1-adrenoreceptor agonists. This indicates a dual allosteric effect of the benzodiazepines to simultaneously reduced the affinity of the agonist-binding site for ligand and to increase the affinity of the agonist-bound form of the receptor for G-protein, effects not observed with ρ-TIA. The other class of α_1-adrenoreceptor allosteric modulators are the amiloride analogs. Leppik *et al.* (39) found that amiloride and its analogs increased the rate of dissociation of [^{3}H] prazosin from the α_1-adrenoreceptor, as ρ-TIA does here. However, the amiloride analogs were found not to inhibit the saturability of radioligand binding as has been shown to occur with ρ-TIA (4). This implies that the allosteric effect of the amiloride analogs on the structure of the competitive antagonist-binding site is more subtle than that of ρ-TIA, with the conopeptide seemingly abolishing the binding site rather than merely changing its structure so that the receptor can still recognize the ligands only with less affinity.

Our attempts to gain an insight into the structural basis for ρ-TIA's α_1-adrenoreceptor antagonist activity initially focused on the N-terminal region of the peptide. This section

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was chosen for study because it displays the least homology to the α-conopeptides, a class whose members are, structurally, otherwise quite similar to ρ-TIA but do not block α_1-adrenoreceptors. We found that the N-terminal region of ρ-TIA alone is not sufficient for α_1-adrenoreceptor antagonist activity, demonstrated by the lack of activity of the TIA_{1-5} analog. The sequential removal of the first three residues of ρ-TIA had a small detrimental effect on activity, but it was upon the removal of the fourth residue of full-length ρ-TIA (in the form of the analog TIA_{5-19}) that the largest impact on activity was seen. Assuming that TIA_{5-19} acts with a Hill slope of unity, the observation that 65% of the response remains after treatment with the analog at a concentration of 10 μM implies that TIA_{5-19} is ~34-fold less potent than full-length ρ-TIA. Thus, a substantial role for the residue located in position 4 of ρ-TIA in conferring α_1-adrenoreceptor activity on ρ-TIA is indicated. This was also the conclusion to be drawn from the results of the alanine walk of ρ-TIA, where the substitution of Arg^4 with alanine had the largest impact on α_1-adrenoreceptor antagonist potency of all the replacements made. Residue replacement has little effect on the robust structures of the α-conopeptides,[2] so it would be expected that ρ-TIA will behave similarly given the structural similarity between the two conopeptide classes. The three-dimensional structure of ρ-TIA (Fig. 7) shows that the Arg^4 sidechain is exposed and surrounded by a cluster of other residues also identified in the alanine walk to contribute to ρ-TIA's α_1-adrenoreceptor antagonist activity. The importance of Arg^4 could reflect an interaction of the positive charge of this side chain with a complementary negatively charged residue on the α_1-adrenoreceptor. The small degree of subtype selectivity possessed by ρ-TIA implies that its binding site is conserved across α_1-adrenoreceptors, and given the size and chemical nature of ρ-TIA, we would expect that this site is located on an extracellularly exposed portion of the receptor. The α_{1A}-, α_{1B}-, and α_{1D}-adrenoreceptor subtypes display 32% sequence identity in their predicted extracellular domains (40), with several conserved negatively charged residues present.

In summary, the venom peptide ρ-TIA acts as a reversible, non-competitive α_1-adrenoreceptor antagonist with some subtype selectivity. The discovery of a peptide ligand that disrupts α_1-adrenoreceptor operation raises the possibility that endogenous peptides or proteins that act in the same manner might exist. The endogenous circulating factor implicated by Shapiro *et al.* (41) in the pathogenesis of sympathotonic orthostatic hypotension may be such a compound. In addition, the elucidation and characterization of a peptide that allosterically inhibits α_1-adrenoreceptors by interacting with residues distinct from those of the poorly selective classical inhibitors suggests that the development of highly subtype-selective compounds should be feasible. Such agents are likely to have major therapeutic advantages over the existing ligands.

[2] R. J. Lewis and P. F. Alewood, unpublished observations.

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