ELSEVIER

European Journal of Pharmacology 279 (1995) 33–41

ejp

Pharmacological evaluation of *N*-methyl-actinodaphnine, a new vascular α-adrenoceptor antagonist, isolated from *Illigera luzonensis*

Jih-Hwa Guh [a], Feng-Nien Ko [a], Sheu-Meei Yu [b], Yang-Chang Wu [c], Che-Ming Teng [a,*]

[a] *Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan*
[b] *Department of Pharmacology, Chang Gung Medical College, Kwei-San, Tao-Yuan, Taiwan*
[c] *School of Pharmacy, Kaohsiung Medical College, Kaohsiung, Taiwan*

Received 28 July 1994; revised 27 February 1995; accepted 28 February 1995

Abstract

The pharmacological activity of *N*-methyl-actinodaphnine, isolated from *Illigera luzonensis*, was determined by functional and binding experiments with peripheral tissues. In a functional study, *N*-methyl-actinodaphnine was a simple competitive antagonist of contractions elicited by phenylephrine ($pA_2 = 7.11$) in rat thoracic aorta; it also competitively antagonised the clonidine-induced inhibition of the twitch response of rat vas deferens ($pA_2 = 5.01$). In addition, [^{3}H]inositol monophosphate formation caused by noradrenaline (3 μM) in rat isolated thoracic aorta was concentration dependently inhibited by *N*-methyl-actinodaphnine (1 and 10 μM); however, it had no effect on cyclic AMP and cyclic GMP contents. Additionally, *N*-methyl-actinodaphnine had extremely low affinity for thromboxane receptors, prostaglandin receptors, Ca^{2+} channels, muscarinic receptors, histamine receptors, ß-adrenoceptors, neurokinin and leukotriene receptors in vitro. However, *N*-methyl-actinodaphnine also possessed 5-hydroxytryptamine (5-HT) receptor blocking activity. Its potency for blocking 5-HT receptors was about 14 times less than that for blocking α_1-adrenoceptors. In binding experiments, *N*-methyl-actinodaphnine displaced biphasically the binding of 0.2 nM [^{3}H]prazosin to cultured A10 cells. The selectivity for α_1-adrenoceptor subtypes was also investigated in rat vas deferens and spleens. The contractile response in rat vas deferens to noradrenaline was competitively inhibited by *N*-methyl-actinodaphnine with a pA_2 value of 6.58; *N*-methyl-actinodaphnine also competitively antagonized the phenylephrine-induced contraction in rat spleen with a pA_2 value of 7.38. These results indicate that *N*-methyl-actinodaphnine is a selective α_1-adrenoceptor antagonist. Furthermore, it is more selective for the α_{1B}- than for the α_{1A}-adrenoceptor subtype.

Keywords: α-Adrenoceptor antagonist; α_1-Adrenoceptor subtype; *N*-Methyl-actinodaphnine; (*Illigera luzonensis*)

1. Introduction

α-Adrenoceptors mediate many important physiological functions, and the development of α-adrenoceptor antagonists is important in clinical medicine, particularly for the treatment of cardiovascular diseases. To date, a number of α-adrenoceptor antagonists have been developed and they are structurally diverse. They can be divided into four groups: ß-haloethylamine alkylating agents, imidazoline analogues, piperazinyl quinazolines and indole derivatives. Furthermore, α_1-adrenoceptors have been subdivided into four subtypes: α_{1A}-, α_{1B}-, α_{1C}- and α_{1D}-subtypes (Lomasney et al., 1991; Cotecchia et al., 1988; Schwinn et al., 1990; Perez et al., 1991), but only the α_{1A}- and α_{1B}-subtypes have been identified pharmacologically. The α_{1A}-adrenoceptor subtype has high affinity for 5-methyl-urapidil and phentolamine (Gross et al., 1988; Hanft and Gross, 1989) and the α_{1B}-adrenoceptor subtype is potently inactivated by chloroethylclonidine (Han et al., 1987; Minneman et al., 1988). However, prazosin is a non-selective α_1-adrenoceptor subtype antagonist (Hanft and Gross, 1989; Aboud et al., 1993).

In the present study, we evaluated the pharmacological activity of *N*-methyl-actinodaphnine, an aporphine derivative isolated from *Illigera luzonensis*, in functional and binding experiments with rat thoracic aorta. Furthermore, we used tension tests and calculated pA_2 values for prazosin, 5-methyl-urapidil and

* Corresponding author. Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, 1st Section, Taipei 10018, Taiwan. Fax 886-2-322-1742.

SSDI 0014-2999(95)00135-2

N-methyl-actinodaphnine in rat vas deferens and spleen, known to contain primarily α_{1A}- and α_{1B}-adrenoceptor subtypes, respectively (Han et al., 1987; Hanft and Gross, 1989), to investigate the selectivity of *N*-methyl-actinodaphnine for α_1-adrenoceptor subtypes.

2. Materials and methods

2.1. Rat aortic contraction

Wistar rats (250–300 g) of either sex were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. The vessels were cut into rings of about 5 mm in length and mounted in organ baths containing Krebs solution (5 ml) of the following composition mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11,7, $CaCl_2$ 1.9 and $NaHCO_3$ 25.0. The tissue bath solution was bubbled with a mixture of 95% O_2 and 5% CO_2. Aortae were equilibrated for 90 min with three changes of solution and maintained under a resting tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph. In some experiments, the endothelium was removed by rubbing with a cotton ball. The absence of acetylcholine-induced relaxation was taken as an indicator that vessels were denuded successfully. Aortae were allowed to equilibrate for 15 min with *N*-methyl-actinodaphnine or other agents before the generation of a cumulative concentration-response curve with each agonist for 15–30 min at 3-min intervals. Results are expressed as percentages of the maximal control response for each agonist before the addition of *N*-methyl-actinodaphnine or other agents. The contractile effects of Ca^{2+} were studied in rings stabilized in K^+ (60 mM) solution without Ca^{2+}. Ca^{2+} was then added to reach the final concentrations as indicated. The maximal tension obtained at 3 mM Ca^{2+} was taken as 100%. The high-K^+ solution was prepared by substituting NaCl with KCl in an equimolar amount.

2.2. Twitch contraction of rat vas deferens

Rat vas deferens were trimmed free of fat and connective tissue and were set up in a 5-ml organ bath containing oxygenated Krebs solution and were equilibrated for 30 min. The preparations were field stimulated via a Grass S44 electronic stimulator (square wave, 0.1 Hz, 3-ms duration, threshold voltage +30%). The bathing medium contained desmethylimipramine (10 nM), corticosterone (40 μM) and propranolol (1 μM) to block neuronal and extraneuronal uptake of noradrenaline and ß-adrenoceptors, respectively.

After an equilibration period of 30 min, cumulative concentration-response curves for the inhibitory effects of clonidine were constructed. A fixed concentration of antagonist was then included in the Krebs solution and concentration-response curves for clonidine were constructed.

2.3. Guinea-pig tracheal contraction

Tracheae from guinea-pigs were dissected out, transferred to a dish containing Krebs solution, cut transversely between the segments of cartilage and then mounted in Krebs solution at 37° C, gassed with 95% O_2-5% CO_2. One end of the cartilage was attached to a fixed pin in the bath and the other to a force-displacement transducer connected to a Grass polygraph. Tracheae were equilibrated under the same conditions as rat aortae, and agonist-induced concentration-response curves in the absence or presence of 30 μM *N*-methyl-actinodaphnine were obtained.

2.4. Cyclic AMP and cyclic GMP assay

The contents of cyclic AMP or cyclic GMP were assayed on aortic rings as previously described (Itoh et al., 1982; Kauffman et al., 1987). After incubation of aortic rings in Krebs solution with dimethylsulphoxide (DMSO, 0.1%), forskolin, sodium nitroprusside or *N*-methyl-actinodaphnine for 2 min, the aortic rings were rapidly frozen in liquid nitrogen and stored at −80° C until homogenized in 0.5 ml 10% trichloroacetic acid and 4 mM EDTA, using a Potter glass/glass homogenizer. The homogenate was centrifuged at $10000 \times g$ for 5 min and the supernatant was removed and extracted with 4×3 volumes of ether. The cyclic AMP or cyclic GMP contents were then assayed using EIA kits. The precipitate was used for protein assay (Lowry et al., 1951). Cyclic AMP and cyclic GMP levels were expressed as pmol/mg protein.

2.5. Measurement of [^{3}H]inositol monophosphate

The procedure was described previously (Hirata et al., 1990). Briefly, rat thoracic aortae were incubated in Krebs solution containing 10 μCi/ml of [^{3}H]myo-inositol at 37° C for 3 h and gassed with 95% O_2-5% CO_2 mixture. The tissues were then transferred to tubes containing fresh Krebs solution with DMSO (0.1%) or *N*-methyl-actinodaphnine and incubated for 15 min. Then saline or noradrenaline (3 μM) was added and incubated for another 15 min. LiCl (10 mM) was added 5 min before noradrenaline to inhibit the metabolism of inositol monophosphate (Berridge et al., 1982). Aortae were then frozen in liquid nitrogen and homogenized in 1.3 ml of 10% trichloroacetic acid. After centrifugation, 1 ml of supernatant was collected and

trichloroacetic acid was removed by washing with 4 × 3 volumes of ether. The inositol monophosphate in the aqueous phase was analysed by application of the supernatant to a column of 1 ml Dowex-1 ion-exchange resin according to the method of Neylon and Summers (1987). The pellets of the tissues were resuspended in 1.0 N NaOH and assayed for protein (Lowry et al., 1951).

2.6. Cell culture

The smooth muscle cell line (A10), derived from rat thoracic aorta, was provided by the American Type Cell Collection. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin sulphate at 37° C in a humidified air/CO_2 (95 : 5) atmosphere. To subculture the cells, confluent monolayers were washed with 0.1% trypsin-0.04% EDTA, placed in an equal volume of medium, and centrifuged at 600 × *g* for 5 min. The cells were seeded into T-75 flasks at an initial density of 2×10^4 cells/cm^2.

2.7. Binding study

Cells for receptor binding studies were finally grown in 24-well tissue culture plates. At confluence, the cells were washed with Krebs-Henseleit solution (KHS) (mM: NaCl 117.5, KCl 5.4, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.5 and Hepes 25.0), and then 250 μl of various concentrations of [^{3}H]prazosin (0.01–10 nM) was added to the 24-well plates for 30 min at 37° C. The equilibrium saturation binding of [^{3}H]prazosin and competition with antagonists were investigated. Non-specific binding was defined as binding in the presence of 10 μM phentolamine. Assays were conducted in triplicate.

2.8. Rat vas deferens contraction

Rat vas deferens were mounted and equilibrated under the same conditions as rat thoracic aorta for 90 min under a resting tension of 0.5 g. After the equilibration period, rat vas deferens were contracted twice with 10 μM noradrenaline and then washed and equilibrated for a further 30 min. Non-cumulative concentration-response curves for noradrenaline-induced contractions were determined in the absence or presence of the indicated antagonists. The tissues were allowed to equilibrate with each antagonist for 15 min, except for chloroethylclonidine (30 min).

2.9. Rat spleen contraction

Rat spleens were hemisected and equilibrated under the same conditions as rat thoracic aorta with a resting tension of 1 g. The spleens were primed by making a concentration-response curve for phenylephrine in a cumulative manner in the absence or presence of the indicated antagonists.

2.10. Data analysis

In functional experiments, agonist-elicited concentration-response curves made in the presence of the indicated concentrations of each antagonist were related to the control concentration-response curve, of which the maximal response was taken as 100%. The concentration of agonist necessary to give a half-maximal response in the presence of each concentration of antagonist was divided by the concentration giving a half-maximal response in the absence of antagonist, to determine the dose ratio (DR). Data were plotted by the method of Arunlakshana and Schild (1959)as the −log (antagonist concentration) (M) vs. the log (DR − 1). When DR was 2, the −log (antagonist concentration) was taken as the pA_2 value from the Schild plot (Mackay, 1978).

Saturation and displacement binding data were analysed by the weighted least-squares iterative curve-fitting program LIGAND (Munson and Rodbard, 1980). The data were first fitted to a one- and then to a two-site model, and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site, as determined by *F*-test comparison, then the two-site model was accepted.

The experimental results are expressed as means ± S.E.M. and accompanied by the number of observations. Statistical significance was assessed by Student's *t*-test and *P* values less than 0.05 were considered significant.

2.11. Drugs

N-Methyl-actinodaphnine (Fig. 1) was isolated from the plant *Illigera luzonensis*. Its structure was determined by spectroscopic methods (UV, IR, ^{1}HNMR, ^{13}CNMR, MS and 2DNMR) and chemical transformation, and compared with the authentic compound. The following drugs were used: noradrenaline HCl, iso-

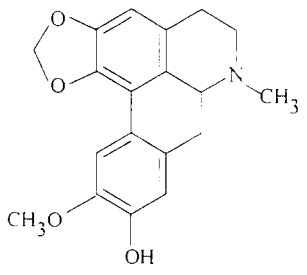

Fig. 1. Chemical structure of *N*-methyl-actinodaphnine.

prenaline HCl, yohimbine HCl, prazosin HCl, phentolamine HCl, clonidine HCl, U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$), carbachol, myo-inositol, histamine dihydrochloride, Dowex-1 resin (100–200 mesh: x8, chloride), nifedipine, propranolol HCl, desmethylimipramine HCl and corticosterone (all from Sigma Chemical Co., St. Louis, USA); phenylephrine HCl (Denmarks Apotekerforening); leukotriene C_4 and prostaglandin $F_{2\alpha}$ (Biomol Research Lab., USA); cyclic AMP and cyclic GMP EIA kits (Cayman Chem. Co., USA), [^{3}H]prazosin (specific activity 81 Ci/mmol) and myo-[2-^{3}H]inositol (Amersham, England) and chloroethylclonidine dihydrochloride and 5-methyl-urapidil (Research Biochemical, Natick, MA, USA). If drugs were dissolved in DMSO, the final concentration of DMSO in the bathing solution did not exceed 0.1% and had no effect on the muscle contraction and cultured cells.

3. Results

3.1. α-Adrenoceptor antagonism in rat peripheral tissue

The α-adrenoceptor antagonistic properties of *N*-methyl-actinodaphnine were evaluated against concentration-response curves for phenylephrine and clonidine in rat peripheral tissues. *N*-Methyl-actinodaphnine (0.1–3 μM) produced a parallel, rightward shift of the log concentration-response curve for phenylephrine in rat aorta without decreasing the maximum contraction and the slope of the Schild plot was close to negative unity (Table 1), indicating that *N*-methyl-actinodaphnine competitively inhibited the contractile responses to the α_1-adrenoceptor agonist. In addition, it was also a competitive antagonist at prejunctional α_2-adrenoceptors situated on the sympathetic nerve terminals of the rat vas deferens. Prazosin, phentolamine and yohimbine were also examined and the pA_2 values as well as the slopes of the Schild plots were obtained (Table 1). The rank order of α_1/α_2-adrenoceptor selectivities for the antagonists studied was prazosin > *N*-methyl-actinodaphnine > phentolamine > yohimbine. In addition, the effect of *N*-methyl-actinodaphnine against phenylephrine-induced contractions in rat aorta denuded of endothelium was also examined and the pA_2 value was 7.18 ± 0.15 ($n = 6$), showing no significant difference from that of intact endothelium. These data indicated that the endothelium did not modify the antagonistic activity of *N*-methyl-actinodaphnine.

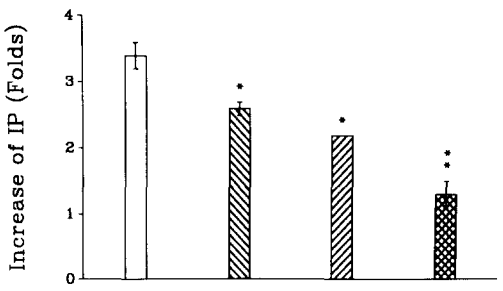

Fig. 2. Effects of *N*-methyl-actinodaphnine on noradrenaline-induced [^{3}H]inositol monophosphate accumulation in rat thoracic aorta. Tissues were preincubated with dimethylsulphoxide (0.1%, control) (first column) or *N*-methyl-actinodaphnine (10^{-6} M, second column; 3×10^{-6} M, third column; 10^{-5} M, fourth column) for 15 min, then noradrenaline (3×10^{-6} M) was added for another 15 min. The increase in [^{3}H]inositol monophosphate (IP) is expressed as the mean ± S.E.M. ($n = 4$). $^*P < 0.01$ and $^{**}P < 0.001$ as compared with the control.

3.2. Effects of N-methyl-actinodaphnine on the formation of [^{3}H]inositol monophosphate and cyclic nucleotides in rat aorta

To investigate if signal transduction after α_1-adrenoceptor activation was blocked by *N*-methyl-actinodaphnine, rat thoracic aorta was labelled with [^{3}H]myo-inositol. The accumulation of [^{3}H]inositol monophosphate in rat aorta was increased in the presence of noradrenaline (3 μM). This increase was concentration dependently inhibited by *N*-methyl-actinodaphnine (1–10 μM, Fig. 2). In addition, the cyclic nucleotide content of rat aorta was measured by enzyme immunoassay. It was found that forskolin and sodium nitroprusside markedly elevated cyclic AMP and cyclic GMP levels in rat aorta, respectively. However, *N*-methyl-actinodaphnine had no effect on the contents of these two cyclic nucleotides (Table 2).

Table 1
α-Adrenoceptor antagonist properties of *N*-methyl-actinodaphnine

Compound	α_1-Antagonism (rat aorta)		α_2-Antagonism (rat vas deferens)		
	pA_2 vs. phenylephrine	Slope Schild plot	pA_2 vs. clonidine	Slope Schild plot	α_1/α_2 ratio
N-Methyl-actinodaphnine	7.11 ± 0.05	−1.19	5.01 ± 0.18	−1.18	126
Prazosin	10.35 ± 0.08	−1.15	6.89 ± 0.20	−1.10	2884
Phentolamine	7.43 ± 0.09	−1.07	7.47 ± 0.15	−1.18	0.91
Yohimbine	6.54 ± 0.11	−1.13	8.02 ± 0.23	−1.19	0.03

Potencies are expressed as pA_2 values of six determinations ± S.E.M.

Table 2
Effects of *N*-methyl-actinodaphnine on the cyclic AMP and cyclic GMP contents of rat thoracic aorta

	Cyclic AMP [a]	Cyclic GMP [a]
Control	0.88 ± 0.19	1.28 ± 0.29
Forskolin 10 μM	4.10 ± 0.59 *	
Sodium nitroprusside 10 μM		9.88 ± 0.63 *
N-Methyl-actinodaphnine		
1 μM	0.78 ± 0.07	1.95 ± 0.51
3 μM	0.74 ± 0.04	1.15 ± 0.13
10 μM	0.65 ± 0.06	1.51 ± 0.33

After preincubation of aortic rings in Krebs solution with dimethylsulphoxide (0.1%, Control), forskolin, sodium nitroprusside or *N*-methyl-actinodaphnine for 2 min, the reaction was stopped by immersing the tissue into liquid nitrogen. Cyclic AMP and cyclic GMP contents in rat aortae were measured. Results are expressed as the means ± S.E.M. ($n = 6$). [a] pmol/mg protein. * $P < 0.001$ as compared with the respective control.

3.3. Selectivity of *N*-methyl-actinodaphnine on various receptors

Agonist-induced contractile or relaxation responses of rat thoracic aorta and guinea-pig tracheal smooth muscles were examined and the concentration ratio was calculated from EC_{50} values in the presence or absence of *N*-methyl-actinodaphnine (30 μM). As shown in Table 3, *N*-methyl-actinodaphnine had little or no influence on the agonists other than α-adrenoceptor agonist-induced effects. However, *N*-methyl-actinodaphnine produced a concentration-related shift of the 5-HT-induced concentration-response curve in rat aorta denuded of endothelium; its potency for blocking 5-HT receptors was about 14 times less than that for α_1-adrenoceptors (Table 3).

3.4. Saturation experiments with [^{3}H]prazosin

[^{3}H]prazosin (0.01–10 nM) was used to label α_1-adrenoceptors of cultured A10 cells. The specific binding was 91% of the total binding at 0.2 nM [^{3}H]prazosin (Fig. 3A). However, the Scatchard plot of the binding data was curvilinear, suggesting more than a single class of binding site (Fig. 3B). LIGAND analysis fitted the data to a two-site model. The pK_D values of high- and low-affinity sites were 9.97 ± 0.11 and 8.60 ± 0.32 and the B_{max} values for both sites were 20.13 ± 5.31 fmol/well and 72.21 ± 9.20 fmol/well, respectively.

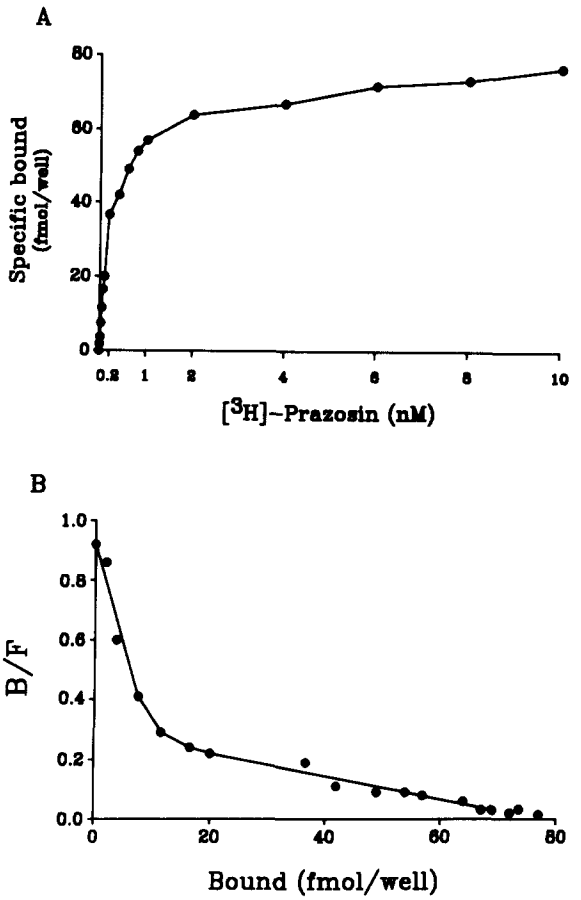

Fig. 3. Saturation experiments of [^{3}H]prazosin binding to smooth muscle cells (A10 cells) of rat thoracic aorta. (A) Saturation curve of specific [^{3}H]prazosin binding to cells. (B) Scatchard plot for specific [^{3}H]prazosin binding to cells.

Table 3
Antagonistic activity of *N*-methyl-actinodaphnine against some agents in rat aorta and guinea-pig trachea

Tissue (response)	Agonist	Concentration ratio	*n*
Rat aorta (contraction)	Phenylephrine	679.12 ± 12.70	6
	5-Hydroxytryptamine	48.94 ± 3.45	6
	U-46619	2.13 ± 0.22	7
	$PGF_{2\alpha}$	1.33 ± 0.12	7
	Ca^{2+}	4.08 ± 0.49	6
Guinea-pig trachea (contraction)	Carbachol	2.13 ± 0.20	6
	Histamine	1.08 ± 0.08	6
	Neurokinin A	1.41 ± 0.11	5
	LeukotrieneC_4	1.14 ± 0.15	5
Guinea-pig trachea (relaxation)	Isoprenaline	1.17 ± 0.09	5

Concentration ratio was calculated from EC_{50} values in the presence or absence of *N*-methyl-actinodaphnine (30 μM) and is presented as mean ± S.E.M. *n* = number of individual experiments.

Table 4
Inhibition of [^{3}H]prazosin binding to α_1-adrenoceptors of cultured A10 cells by *N*-methyl-actinodaphnine, prazosin, phentolamine and yohimbine

Drugs	pK_{IHigh}	pK_{ILow}	Slope factor
N-Methyl-actinodaphnine	7.55±0.32	5.69±0.12	0.56±0.03
Prazosin	10.11±0.20	8.41±0.27	0.60±0.02
Phentolamine	7.74±0.41	6.02±0.22	0.52±0.07
Yohimbine	6.68±0.29	5.12±0.20	0.58±0.03

Displacement curves were individually analysed with the LIGAND program. A two-site fit was accepted only if it was significantly better than a one-site fit. pK_{IHigh} and pK_{ILow}: −log (equilibrium dissociation constant) at high or low affinity sites for antagonists tested. Data are expressed as the means±S.E.M. ($n = 3$).

3.5. Effects of competitive antagonists on [^{3}H]prazosin binding

When 0.2 nM [^{3}H]prazosin was used, unlabelled prazosin, phentolamine, yohimbine and *N*-methyl-actinodaphnine showed shallow displacement curves. Computerized analysis revealed that they all bound to two distinct sites. The pK_I values at high- and low-affinity sites were obtained (Table 4). These results showed a good correlation between the pK_I values of the α_{1High}-affinity sites and the pA_2 values in rat aorta ($r = 0.994$).

3.6. α-Adrenoceptor antagonism in rat vas deferens

Chloroethylclonidine (100 μM) had no significant effect on the concentration-response curve for noradrenaline-induced contractions in rat vas deferens. In contrast, pretreatment with nifedipine (1 μM) almost completely abolished these contractile responses (Fig. 4A). Prazosin, 5-methyl-urapidil and *N*-methyl-actinodaphnine caused concentration-dependent parallel rightward shifts of the concentration-response curve of noradrenaline in a competitive manner without decreasing the maximal contraction (Fig. 4B–D). Schild plots were constructed from the effects of each antagonist at various concentrations; the slopes of these regressions did not differ significantly from negative unity. pA_2 values were calculated to be 9.33, 8.61 and 6.58 for prazosin, 5-methyl-urapidil and *N*-methyl-actinodaphnine, respectively (Table 5).

3.7. α-Adrenoceptor antagonism in rat spleen

Chloroethylclonidine (100 μM) significantly diminished by 54% the maximal contractions induced by phenylephrine. Conversely, nifedipine (1 μM) had no significant effect on the isometric contraction caused by phenylephrine in rat spleen (Fig. 5A). Prazosin, 5-methyl-urapidil and *N*-methyl-actinodaphnine produced parallel rightward shifts in the concentration-response curves of phenylephrine without diminishing the maximal responses (Fig. 5B–D). The slopes of Schild plots were not significantly different from negative unity against phenylephrine. pA_2 values were cal-

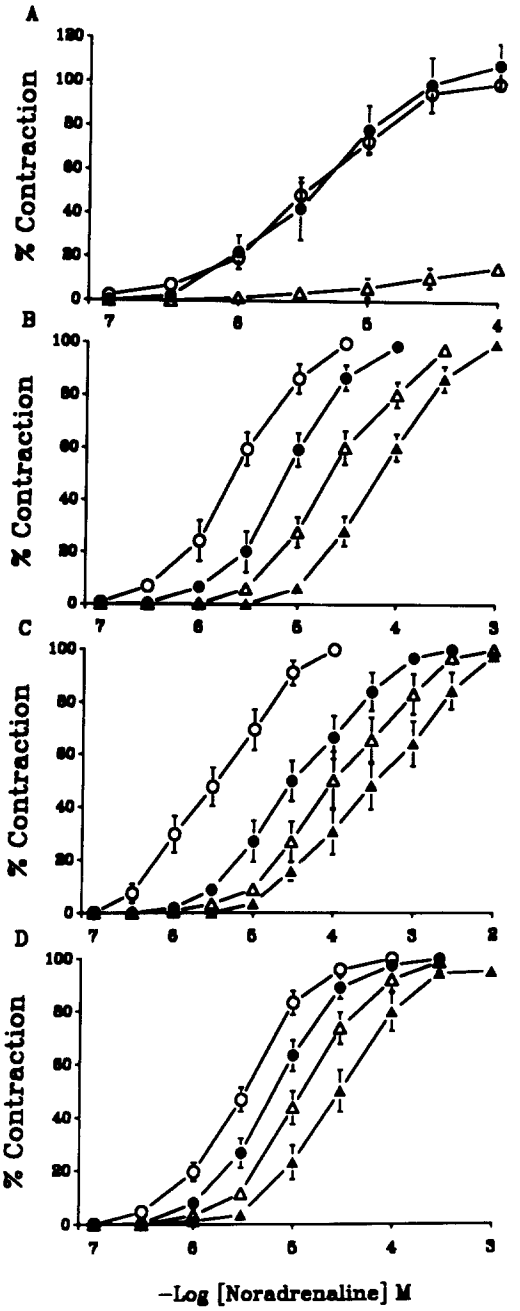

Fig. 4. Effects of nifedipine and various α_1-adrenoceptor antagonists on noradrenaline-induced contractions of rat vas deferens. Tissues were pretreated with dimethylsulphoxide (0.1%, control) (○), or with A: chloroethylclonidine (10^{-4} M, ●) for 30 min or nifedipine (10^{-6} M, △) for 15 min, B: prazosin (10^{-9} M, ○; 3×10^{-9} M, ●; 10^{-8} M, ▲), C: 5-methyl-urapidil (3×10^{-8} M, ●; 10^{-7} M, △; 3×10^{-7} M, ▲), D: *N*-methyl-actinodaphnine (3×10^{-7} M, ●; 10^{-6} M, △; 3×10^{-6} M, ▲) for 15 min, and then concentration-response curves for noradrenaline were determined. Each point is the mean±S.E.M. of eight experiments.

Table 5
Potencies of various antagonists against contractions elicited by noradrenaline in rat vas deferens and by phenylephrine in rat spleen

	Vas deferens (α_{1A})	Spleen (α_{1B})	P
N-Methyl-actinodaphnine	6.58±0.21	7.38±0.11	0.005
Prazosin	9.33±0.31	9.39±0.17	0.641
5-Methyl-urapidil	8.61±0.37	7.18±0.16	0.004
Chloroethylclonidine	no effect	effective	
Nifedipine	effective	no effect	

Potencies are expressed as pA_2 values±S.E.M. (n = 6–8) from the Schild plots constructed from the effects of various concentrations of each antagonist against the agonist-induced contractions in the indicated tissues. Statistical significance was assessed by Student's t-test and P values less than 0.05 are considered significant. The effects of chloroethylclonidine (100 μM) and nifedipine (1 μM) on agonist-induced responses are also listed.

culated to be 9.39, 7.18 and 7.38 for prazosin, 5-methyl-urapidil and *N*-methyl-actinodaphnine, respectively (Table 5).

4. Discussion

The present studies have demonstrated that *N*-methyl-actinodaphnine inhibited the contractile responses of rat aorta to phenylephrine and reversed the inhibitory effect on twitch responses of rat vas deferens elicited by clonidine. The signal transduction after α_1-adrenoceptor activation was also investigated. *N*-Methyl-actinodaphnine concentration dependently inhibited [^{3}H]inositol monophosphate formation caused by noradrenaline (Fig. 2). In addition, it acted as a selective α-adrenoceptor antagonist without affecting the contractile responses of rat aorta caused by high-potassium depolarization, the thromboxane receptor agonist U-46619, and prostaglandin $F_{2\alpha}$. It also had no significant effects on the carbachol-, histamine-, neurokinin A- and leukotriene C_4-induced contractions and isoprenaline-induced relaxation in guinea-pig trachea (Table 3). All these results indicate that *N*-methyl-actinodaphnine is a selective α-adrenoceptor antagonist. However, from the calculated α_1/α_2-adrenoceptor selectivity ratio (Table 1), it appears that *N*-methyl-actinodaphnine is more selective for α_1-adrenoceptors.

N-Methyl-actinodaphnine also possesses 5-HT receptor blocking activity. Its potency for blocking 5-HT receptors was about 14 times less than that for α_1-adrenoceptors. It has been shown that there is a high degree of cross-reactivity between compounds interacting at α-adrenoceptors and 5-HT receptors (Apperley et al., 1976; Black et al., 1981; Purdy et al., 1987). In addition, it has also been demonstrated that α_1-adrenoceptors in the rat have 70% homology with 5-HT_2 receptors (Zifa and Fillion, 1992). The efficiency of *N*-methyl-actinodaphnine at 5-HT receptors can be explained by the close homology of these two receptors.

The vascular endothelium plays an important role in

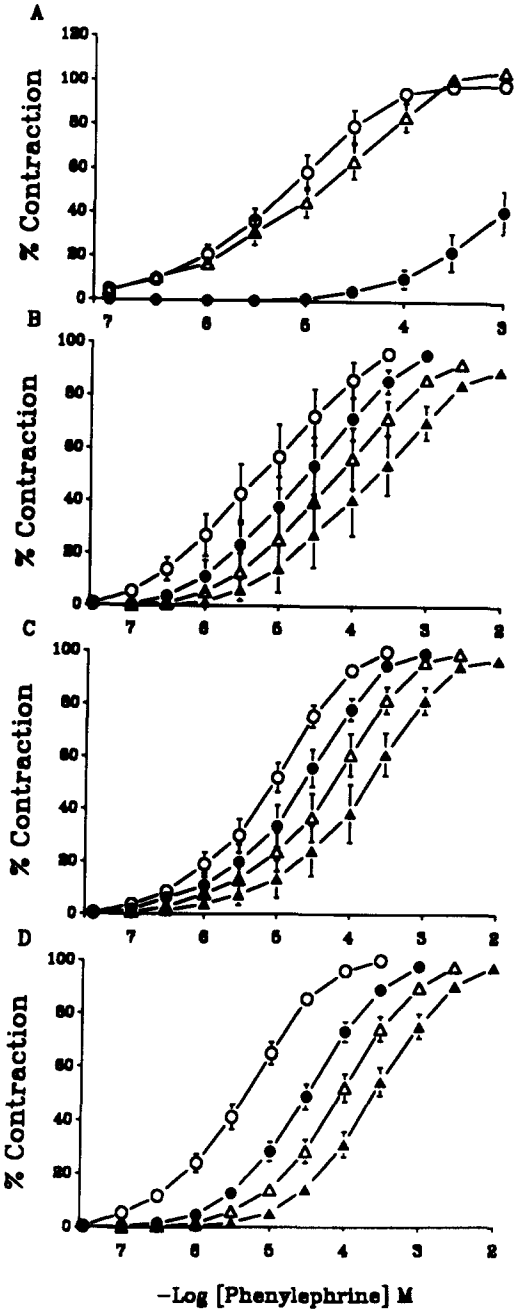

Fig. 5. Effects of nifedipine and various α_1-adrenoceptor antagonists on phenylephrine-induced contractions of rat spleens. Tissues were pretreated with dimethylsulphoxide (0.1%, control) (○), or with A: chloroethylclonidine (10^{-4} M, ●) for 30 min or nifedipine (10^{-6} M, △) for 15 min, B: prazosin (10^{-9} M, ●; 3×10^{-9} M, △; 10^{-8} M, ▲), C: 5-methyl-urapidil (10^{-7} M, ●; 3×10^{-7} M, △; 10^{-6} M, ▲), D: *N*-methyl-actinodaphnine (3×10^{-7} M, ●; 10^{-6} M, △; 3×10^{-6} M, ▲) for 15 min, and then concentration-response curves for phenylephrine were determined. Each point is the mean±S.E.M. of eight experiments.

controlling vascular tone via the secretion of both relaxant and contractile factors (Jaffe, 1985; Vanhoutte et al., 1986). The endothelium can modulate the vasoconstrictor responses to many agonists, and endothelium-derived relaxing factor (EDRF) is mainly responsible for these effects (Egleme et al., 1984; Palmer et al., 1987). It has been reported that the vascular endothelium modifies the mode of antagonism by prazosin and doxazosin but not that by phentolamine and yohimbine of the noradrenaline and phenylephrine concentration-response curves (Alosachie and Godfraind, 1986). However, the pA_2 values of *N*-methyl-actinodaphnine against phenylephrine-induced responses in the absence or presence of endothelium were similar, indicating that the endothelium cannot modify the antagonistic activity of *N*-methyl-actinodaphnine.

Cyclic nucleotides are very important for relaxing vascular smooth muscles (Murad, 1986). Forskolin and sodium nitroprusside have been shown to be potent relaxing agents in vascular smooth muscles. Forskolin increases cyclic AMP levels via activation of adenylate cyclase (Ousterhout and Sperelekis, 1987), and sodium nitroprusside produces a prompt, concentration-dependent increase in the cyclic GMP levels by directly activating guanylate cyclase (Gruetter et al., 1979). In the present study, neither the cyclic AMP nor the cyclic GMP content was changed by *N*-methyl-actinodaphnine (Table 2). This indicates that the α-adrenoceptor antagonistic effects of *N*-methyl-actinodaphnine are not mediated by an increase in cellular cyclic nucleotide concentrations.

The α-adrenoceptor antagonistic effects of *N*-methyl-actinodaphnine were also characterized in binding experiments. The present study clearly demonstrates that [^{3}H]prazosin binds to two distinct populations of binding sites in the cultured A10 cells. The binding affinities of α-adrenoceptor antagonists, prazosin, phentolamine, yohimbine and *N*-methyl-actinodaphnine at high- and low-affinity sites were examined in displacement experiments. The results showed that they all bound to two distinct sites. The results showed a good correlation between the pK_{IHigh} and pA_2 values of these antagonists. In addition, the pK_{IHigh} values of these antagonists were close to the respective pA_2 values in functional experiments. These data suggest that the high-affinity sites of rat thoracic aorta contribute to the functional profile.

α_1-Adrenoceptors are subdivided into α_{1A}- and α_{1B}-adrenoceptor subtypes, based on the affinities of ligands for binding sites in rat vas deferens and spleen (Han et al., 1987; Gross et al., 1988; Hanft and Gross, 1989), and based on the ability of alkylating agents chloroethylclonidine to inactivate the α_{1B}- but not α_{1A}-adrenoceptor subtype. Based on this classification, contractions of rat vas deferens induced by exogenous noradrenaline or adrenaline are mediated predominantly by the α_{1A}-adrenoceptor subtype (Han et al., 1987; Hanft and Gross, 1989), whereas contractions induced by noradrenaline in rat spleen are mediated predominantly by the α_{1B}-adrenoceptor subtype (Han et al., 1987). Recently, the existence of mRNA for α_{1A}-adrenoceptors in the rat vas deferens was confirmed by Northern blotting analysis (Lomasney et al., 1991). In addition, the α_{1A}-adrenoceptor subtype requires the influx of extracellular Ca^{2+} through dihydropyridine-sensitive channels to cause smooth muscle contraction and does not stimulate inositol phosphate formation. Conversely, the α_{1B}-adrenoceptor subtype stimulates the formation of inositol phosphate to cause contractions that are independent of extracellular Ca^{2+} influx through dihydropyridine-sensitive channels (Han et al., 1987).

In this study, chloroethylclonidine (100 μM) had little effect on the noradrenaline-induced contraction in rat vas deferens. In contrast, nifedipine (1 μM) almost completely abolished the effect caused by noradrenaline. These results are consistent with those of Aboud et al. (1993), indicating that the major receptor subtype mediating the noradrenaline-induced contraction in rat vas deferens is the α_{1A}-adrenoceptor subtype. Noradrenaline can also induce contraction in rat spleen. Both α_2- and α_1-adrenoceptors are involved in this response (Kenakin and Novak, 1988), and hence the slopes of Schild plots differed from negative unity for the antagonists tested here (data not shown). Chloroethylclonidine irreversibly and non-competitively inhibited the contraction induced by phenylephrine but nifedipine had no effect on this response. This reveals that the contraction induced by α_1-adrenoceptor agonists in rat spleen is mediated predominantly by the α_{1B}-adrenoceptor subtype and that the opening of the dihydropyridine-sensitive Ca^{2+} channels is not necessary (Han et al., 1987).

Prazosin, 5-methyl-urapidil and *N*-methyl-actinodaphnine were tested in our study. Prazosin is a non-selective α_1-adrenoceptor subtype antagonist (Hanft and Gross, 1989; Aboud et al., 1993) and the pA_2 values we obtained were similar in rat vas deferens and spleen. 5-Methyl-urapidil, the most selective α_{1A}-adrenoceptor subtype antagonist (Gross et al., 1988; Hanft and Gross, 1989), exhibited greater potency (27-fold) in rat vas deferens than in rat spleen against α_1-adrenoceptor agonist-induced contraction. However, *N*-methyl-actinodaphnine showed higher potency (6-fold) in rat spleen than in rat vas deferens ($P < 0.01$, $n = 6–8$).

In conclusion, the present study indicates that *N*-methyl-actinodaphnine is an α-adrenoceptor antagonist with more preferential selectivity for the α_{1B}-adrenoceptor subtype than for the α_{1A}-adrenoceptor subtype.

Acknowledgements

This work was supported by a research grant from the National Science Council of the Republic of China (NSC 83-0425-B002-001).

References

Aboud, R., M. Shafii and J.R. Docherty, 1993, Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen, Br. J. Pharmacol. 109, 80.

Alosachie, I. and T. Godfraind, 1986, Role of cyclic GMP in the modulation by endothelium of the adrenolytic action of prazosin in the rat isolated aorta, Br. J. Pharmacol. 89, 525.

Apperley, E., P.P.A. Humphrey and G.P. Levy, 1976, Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery and aorta, Br. J. Pharmacol. 58, 211.

Arunlakshana, O. and H.O. Schild, 1959, Some quantitative uses of drug antagonists, Br. J. Pharmacol.14, 48.

Berridge, M.J., C.P. Downes and M.R. Hanley, 1982, Lithium amplifies agonist-dependent phosphoinositol response in brain and salivary glands, Biochem. J. 206, 587.

Black, J.L., R.J. French and E.J. Mylecharane, 1981, Receptor mechanisms for 5-hydroxytryptamine in rabbit arteries, Br. J. Pharmacol. 74, 619.

Cotecchia, S., D.A. Schwinn, R.R. Randall, R.J. Lefkowitz, M.G. Caron and B.K. Kobilka, 1988, Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor, Proc. Natl. Acad. Sci. USA 85, 7159.

Egleme, C., T. Godfraind and R.C. Miller, 1984, Enhanced responsiveness of rat isolated aorta to clonidine after removal of endothelial cells, Br. J. Pharmacol. 81, 16.

Gross, G., G. Hanft and C. Rugevics, 1988, 5-Methyl-urapidil discriminates between subtypes of the α_1-adrenoceptor, Eur. J. Pharmacol. 151, 333.

Gruetter, C.A., B.K. Barry, D.B. Mchamara, D.Y. Gruetter, P.J. Kadowitz and L.J. Ignarro, 1979, Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosamine, J. Cycl. Nucl. Res. 5, 211.

Han, C., P.W. Abel and K.P. Minneman, 1987, α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle, Nature 329, 333.

Hanft, G. and G. Gross, 1989, Subclassification of α_1-adrenoceptor recognition sites by urapidil derivatives and other selective antagonists, Br. J. Pharmacol. 97, 691.

Hirata, M., K.P. Kohse, C.H. Chang, T. Ikebe and F. Murad, 1990, Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells, J. Biol. Chem. 265, 1268.

Itoh, T., H. Izumi and H. Kuriyama, 1982, Mechanisms of relaxation induced by activation of ß-adrenoceptors in muscle cells of guinea-pig mesenteric artery, J. Physiol. 326, 475.

Jaffe, E.A., 1985, Physiological functions of normal endothelial cells, Ann. NY Acad. Sci. 454, 279.

Kauffman, R.F., K.W. Schenck, B.G. Utterback, V.G. Crowe and M.L. Cohen, 1987, In vitro vascular relaxation by new inotropic agents: relationship to phosphodiesterase inhibition and cyclic nucleotides, J. Pharmacol. Exp. Ther. 242, 864.

Kenakin, T.P. and P.J. Novak, 1988, Classification of phenoxybenzamine/prazosin resistant contractions of rat spleen to norepinephrine by Schild analysis: similarities and differences to postsynaptic alpha-2 adrenoceptors, J. Pharmacol. Exp. Ther. 244, 206.

Lomasney, J.W., S. Cotecchia, W. Lorenz, W.Y. Leung, D.A. Schwinn, T.L. Yang-Feng, M. Brownstein, R.J. Lefko-Witz and M.G. Caron, 1991, Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor, J. Biol. Chem. 266, 6365.

Lowry, D.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Mackay, D., 1978, How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated?, J. Pharm. Pharmacol. 30, 312.

Minneman, K.P., C. Han and P.K. Abel, 1988, Comparison of α_1-adrenergic receptor subtypes distinguished by chloroethylclonidine and WB 4101, Mol. Pharmacol. 33, 509.

Munson, P.J. and D. Rodbard, 1980, LIGAND: A versatile computerized approach for characterization of ligand-binding systems, Anal. Biochem. 107, 220.

Murad, F., 1986, Cyclic guanosine monophosphate as a mediator of vasodilation, J. Clin. Invest. 78, 1.

Neylon, C.B. and R.J. Summers, 1987, Stimulation of α_1-adrenoceptors in rat kidney mediates increased inositol phospholipid hydrolysis, Br. J. Pharmacol. 91, 367.

Ousterhout, J.M. and N. Sperelekis, 1987, Cyclic nucleotides depress action potentials in cultured aortic smooth muscle cells, Eur. J. Pharmacol. 144, 7.

Palmer, R.J.M., A.G. Ferrige and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor, Nature 327, 524.

Perez, D.M., M.T. Piascik and R.M. Graham, 1991, Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA, Mol. Pharmacol. 40, 876.

Purdy, R.E., D.L. Murray and G.L. Stupecky, 1987, Receptors for 5-hydroxytryptamine in rabbit blood vessels: activation of alpha adrenoceptors in rabbit thoracic aorta, J. Pharmacol. Exp. Ther. 240, 535.

Schwinn, D.A., J.W. Lomasney, W. Lorenz, P.J. Szklut, R.T. Fremeau, T.L. Yang-Feng, M.G. Caron, R.J. Lefkowitz and S. Cotecchia, 1990, Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype, J. Biol. Chem. 265, 8183.

Vanhoutte, P.M., G.M. Rubany, V.M. Miller and D.S. Houston, 1986, Modulation of vascular smooth muscle contraction by endothelium, Annu. Rev. Physiol. 48, 307.

Zifa, E. and G. Fillion, 1992, 5-Hydroxytryptamine receptors, Pharmacol. Rev. 44, 401.