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Discovery of novel selective hypotensive vasopressin peptides that exhibit little or no functional interactions with known oxytocin/vasopressin receptors

[1,3]W.Y. Chan, [1]N.C. Wo, [2]S. Stoev, [2]L.L. Cheng & [2]M. Manning

[1]Department of Pharmacology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021; and [2]Department of Biochemistry and Molecular Biology, Medical College of Ohio, 3035 Arlington Avenue, Toledo, Ohio 43614, U.S.A.

1 Arginine-vasopressin (VP) has both vasoconstricting and vasodilating action. We report here the discovery of four novel selective hypotensive VP analogues: $d(CH_2)_5$[D-Tyr(Et)^2,Arg^3,Val^4]AVP; $d(CH_2)_5$[D-Tyr(Et)^2,Lys^3,Val^4]AVP and their iodinatable Tyr-NH_2^9 analogues.

2 Bioassays in rats for activities characteristic of neurohypophysial peptides showed that the four VP peptides possessed little or no V_{1a}, V_2 or oxytocin (OT) receptor agonistic or antagonistic activities.

3 In anaesthetized rats, these peptides (0.05–0.10 mg kg^{-1} i.v.) elicited a marked fall in arterial blood pressure.

4 Blockade of cholinoceptors, adrenoceptors and bradykinin B_2 receptors, and inhibition of prostaglandin synthesis had little effect on their vasodepressor action.

5 Classical V_{1a}, V_2 and OT receptor antagonists did not block the vasodepressor response.

6 L-NAME, 0.2 mg kg^{-1} min^{-1}, markedly suppressed the hypotensive response to ACh but not the vasodepressor response to the hypotensive VP peptides. However, the duration of the vasodepressor response was shortened. Very high doses of L-NAME attenuated both the vasodepressor response and the duration of action.

7 These findings indicate that the vasodepressor action of these VP peptides is independent of the peripheral autonomic, bradykinin and PG systems and is not mediated by the known classical OT/VP receptors. NO does not appear to have an important role in their vasodepressor action.

8 The discovery of these novel VP peptides could lead to the development of new tools for the investigation of the complex cardiovascular actions of VP and the introduction of a new class of hypotensive agents. The two iodinatable hypotensive VP peptides could be radiolabelled as potential markers for the localization of the receptor system involved.

Keywords: Selective hypotensive vasopressin analogue agonists; hypotensive vasopressin peptides; nitric oxide and vasopressin analogues; vasodilating action of AVP

Introduction

Arginine-vasopressin (AVP or VP) in addition to its well known antidiuretic action mediated by renal V_2 receptors, has also complex cardiovascular actions (Reid & Schwartz, 1984; Share, 1988). The systemic cardiovascular effect of VP is peripheral vasoconstriction, leading to a rise in arterial blood pressure (Altura & Altura, 1977; Reid & Schwartz, 1984; Share, 1988). Regionally, VP can induce vasodilation (Liard, 1988; Naitoh *et al*., 1993; Nakanishi *et al*., 1995; Tagawa *et al*., 1995; van Lieburg *et al*., 1995). It also enhances the arterial baroreflex, leading to a depression of heart rate and cardiac output (Cowley *et al*., 1984; Webb *et al*., 1986; Shimizu *et al*., 1993; Huch *et al*., 1995). It is now generally accepted that VP may play an important role in maintaining systemic blood pressure and in regulating regional blood flows during extreme hemodynamic alterations such as in a hypovolemic state (Schwartz & Reid, 1983; Landry *et al*., 1997; Reid, 1997). The vasopressor effect of VP is mediated by V_{1a} receptors and the phosphatidylinositol-Ca^{2+} signalling pathway (Michell *et al*., 1979; Jard *et al*., 1987). The baroreflex effect of VP appears to be mediated also by V_{1a} receptors (Webb *et al*., 1986; Shimizu *et al*., 1993). It is not clear which receptor subtype V_{1a}, V_2 or a distinctly different receptor subtype, mediates the vasodilating effect of VP. Both OT and VP can induce vasodilation and both V_{1a} and V_2 antagonists have been shown to inhibit the vasodilating action (Nakano & Fisher, 1963; Katusic *et al*., 1986; Liard, 1988; Naitoh *et al*., 1993; Nakanishi *et al*., 1995; Tagawa *et al*., 1995; Hirata *et al*., 1997; Okamura *et al*., 1997). However, a large body of the experimental data suggests that extrarenal V_2 receptors and endothelial nitric oxide (NO) mediate the vasorelaxation action of VP (Kim *et al*., 1988; Yamada *et al*., 1993; Liard, 1994; Rudichenko & Beierwaltes, 1995; Garcia-Villalon *et al*., 1996; Hirata *et al*., 1997). Efforts to delineate the receptor subtype involved in the vasodilating action of VP have been hampered by the lack of receptor-selective vasodilating agonists or antagonists. We now report the first known VP peptides which exhibit selective vasodepressor activity. These hypotensive peptides were discovered during the course of studies on the effects of Phe^3 replacements in $d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP ([1-(β-mercapto-β,β-pentamethylenepropionic acid),2-O-ethyl-D-tyrosine,4-valine]-arginine-vasopressin), a potent non-selective, V_2/V_{1a}/OT antagonist (Manning *et al*., 1982). We had found that the Phe^3 residue could be replaced by a wide variety of conformationally restricted and aromatic amino acids with retention of potent V_2 antagonism (Manning *et al*., 1997). However, in a continuation of this study reported here, we were greatly surprised to find that when the Phe^3 residue was replaced by either Arg^3 or Lys^3, the resulting peptides,

[3] Author for correspondence.

d(CH_2)$_5$[D-Tyr(Et)^2,Arg^3,Val^4]AVP (Peptide I) and d(CH_2)$_5$[D-Tyr(Et)^2,Lys^3,Val^4]AVP (Peptide II), produced unexpected marked hypotension in the rat bioassay. In this paper, we report our findings on the pharmacological properties of these two novel hypotensive VP peptides, together with their Tyr-NH_2^9 (9-tyrosylamide) analogues (Peptides III and IV). The high selectivity of these hypotensive VP peptides suggests that these unique peptides could be deployed as new tools for the investigation and elucidation of the complex cardiovascular actions of VP. Preliminary reports on the hypotensive Peptides I and II have been presented elsewhere (Chan *et al*., 1998; Manning *et al*., 1998).

Methods

Adult Osborne–Mendel or Wistar rats weighing 200–250 g obtained from the institution's Research Animal Resource Center were used in this study. The hypotensive VP peptides were assayed for biological activities characteristic of neurohypophysial peptides. The vasodepressor activities were determined in urethane-anaesthetized rats. The effects of autonomic receptor blockade, bradykinin receptor blockade and inhibition of prostaglandin (PG) and nitric oxide (NO) synthesis on the vasodepressor response were examined. Potential antagonisms of vasodepressor response by classical V_{1a}, V_2 and oxytocin (OT) receptor antagonists were determined.

Synthesis of the novel hypotensive VP peptides

The hypotensive VP peptides d(CH_2)$_5$[D-Tyr(Et)^2,Arg^3,Val^4]AVP (Peptide I), d(CH_2)$_5$[D-Tyr(Et)^2,Lys^3,Val^4]AVP (Peptide II), and their iodinatable analogues, d(CH_2)$_5$[D-Tyr(Et)^2,Arg^3,Val^4,Tyr-NH_2^9]AVP (Peptide III) and d(CH_2)$_5$[D-Tyr(Et)^2,Lys^3,Val^4,Tyr-NH_2^9]AVP (Peptide IV) were synthesized in our laboratories by standard solid phase methods (Merrifield, 1964; Stewart & Young, 1984) according to procedures previously described (Manning *et al*., 1982, 1989, 1997). Details of these syntheses will be presented elsewhere.

Bioassays of peptides

Standard oxytocic, vasopressor and antidiuretic bioassays for neurohypophysial peptides were performed. *In vitro* oxytocic assays were performed on isolated uteri from rats that had been pretreated the previous afternoon with 50 μg diethylstilbestrol in oil per rat injected subcutaneously. The isolated uterine horn was suspended in a Mg^{2+}-free van Dyke-Hasting solution (Munsick, 1960) for isotonic contraction recording. Vasopressor assays were performed in urethane-anaesthetized male rats, 1.0 g kg^{-1} i.p., as described by Dekanski (1952). The carotid artery and jugular vein were cannulated with PE catheters for blood pressure recording and for drug administration respectively. Antidiuretic assays were performed in ethanol-anaesthetized and water-loaded male rats according to the method described by Sawyer (1961). The rat was anaesthetized (surgical depth) with 12% ethanol, 50 ml kg^{-1} p.o. Water diuresis was induced and anaesthesia maintained by a constant water-load equal to 8% of body weight with a 2% ethanol–0.05% NaCl solution p.o. The jugular vein was cannulated for drug administration. The urinary bladder was cannulated through an abdominal incision with a PE catheter for urine collection. In all bioassays, agonistic potencies of the peptides were determined by the four-point (2×2 parallel-line) bioassay design (Holton, 1948). Antagonistic potencies of the antagonists were measured by the pA_2 method (Schild, 1947). AVP and OT standardized against the USP Posterior Pituitary Standard for vasopressor and oxytocic activities by the four-point assay design were used as the working standards in the bioassays of all test samples. At least four independent assays ($n=4$), each in a different animal preparation, were performed for each bioassay. The bioassay value is represented by the mean with s.e.

Determination of vasodepressor activity

The vasodepressor activity of the hypotensive VP peptides was determined in urethane-anaesthetized male rats. Blood pressure was monitored via a canulated carotid artery. The vasodepressor response was measured by the peak fall in mean arterial blood pressure (BP) and/or by the area under the vasodepressor response curve (AUC), determined by a polar planimeter, for the 5 min period following the injection of the hypotensive VP peptide. The vasodepressor responses to the hypotenisve VP peptides were determined in four groups of rats ($n=6$ in each group) with different baseline BP levels. One group, the low baseline BP group, were pretreated with fully blocking doses of atropine (0.6 mg per rat s.c.) and phenoxybenzamine (0.5 mg per rat i.v.). A second group were 'normotensive' rats without pretreatments. The third and fourth groups of rats had baseline BP elevated and maintained at 110–120 mmHg by the infusion of phenylephrine or angiotensin II respectively. Phenylephrine (25 μg ml^{-1}) or angiotensin II (100 μg ml^{-1}) was infused at a rate (0.01–0.05 ml min^{-1}) to maintain the BP at the required range for the 5 min period before the injection of the test peptide. The infusion was continued for another 5 min period following the peptide injection and then ceased. Upon recovery of the vasodepressor response, phenylephrine or angiotensin infusion was reinstituted for the next peptide injection. Two to three peptide injections could be administered in a stable preparation.

Effects of blockade of autonomic receptors and bradykinin B_2 receptors on the vasodepressor response

Cholinoceptor and α-adrenoceptor blockade was produced by fully blocking doses of atropine and phenoxybenzamine in a group of rats ($n=6$) as described above for the low baseline BP group. In a separate group of rats ($n=5$), β-adrenoceptor blockade was produced by propranolol (0.2 mg per rat i.v., injected slowly over a 5 min period). In another group of rats ($n=4$), bradykinin B_2 receptor blockade was produced by the i.v. infusion of a selective B_2 receptor antagonist, B9340 (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Igl-Oic-Arg), for 15 min at its established ED_{50} dose, 0.17 μg kg^{-1} min^{-1} (Stewart *et al*., 1996), and at five times the ED_{50} dose. The vasodepressor responses to the hypotensive VP peptides were determined before and after β-adrenoceptor or bradykinin B_2 receptor blockade in the same animal. The effects of atropine and phenoxybenzamine on the vasodepressor response were compared to a control group of 'normotensive' rats ($n=6$) as described above for determining vasodepressor activity under different baseline BP levels. In a separate group of autonomically-blocked rats ($n=3$), the vasodepressor responses were determined before and again in the same animal after the depressed basline BP was restored to 100–110 mmHg by angiotensin II infusion, 1–5 μg min^{-1}.

Effects of inhibition of PG and NO synthesis on the vasodepressor response

Inhibition of PG synthesis was induced by diclofenac sodium 2.5 mg kg^{-1} i.v., injected slowly over a 5 min period. Diclofenac sodium is a potent water soluble cyclooxygenase inhibitor and has been shown in our previous studies to produce a marked inhibition of PG synthesis *in vivo* in rats (Chan, 1983). Inhibition of NO synthesis was produced by a continuous i.v. infusion of N^{ω}-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, at 0.2 mg kg^{-1} min^{-1} or 1.0 mg kg^{-1} min^{-1}. The vasodepressor responses to the hypotensive VP peptides were determined before diclofenac sodium or L-NAME administration and again in the same animal, 30 min after diclofenac sodium or after 60 min of L-NAME infusion (paired comparisons). For the diclofenac sodium experiment, three rats ($n=3$) were used. For the L-NAME experiment, six rats ($n=6$) were used in each of the two infusion rates.

Effects of OT/VP antagonists on the vasodepressor response

$d(CH_2)_5$[Tyr(Me)^2]AVP (Tyr(Me) = O-methyl-tyrosine), a selective V_{1a} receptor antagonist (Kruszynski *et al*., 1980); $d(CH_2)_5$[D-Tyr(Et)^2,Tic^3,Val^4]AVP (Tic = tetrahydroisoquinoline-3-carboxylic acid), a new selective V_2 receptor antagonist (Manning *et al*., 1997) and desGly-NH_2,$d(CH_2)_5$[Tyr(Me)^2,-Thr^4]OVT (desGly-NH_2 = desglycinamide; Thr = threonine; OVT = ornithine-vasotocin), a selective OT receptor antagonist (Manning *et al*., 1989) were used to assess the ligand binding specificity of the receptor for the hypotensive VP peptides. The effects of equidoses of OT/VP antagonists and agonists (approximately equi-molar concentrations), on the vasodepressor response to the hypotensive VP peptides were determined. Depending on the sensitivity of the rat, the doses of hypotensive peptide used were from 0.10 mg kg^{-1} to 0.15 mg kg^{-1}, to limit the vasodepressor response within the range (< 50 mmHg) that could be quantified by the peak fall in BP. The vasodepressor responses to this dose of the hypotensive VP peptide were determined in the absence and in the presence of the VP/OT antagonist in the same animal (paired comparisons). The antagonist when given was injected 1 min before the hypotensive VP peptide. Five to six rats (n = at least 5) were used in each antagonist experiment.

Drugs

Acetylcholine (Sigma, St. Louis, U.S.A.); angiotensin-II (Sigma); arginine-vasopressin (Sigma); atropine (Sigma); diclofenac sodium (Ciba-Geigy); diethylstilbestrol (Sigma); N^{ω}-nitro-L-arginine methyl ester (Sigma); oxytocin (Sigma); phenoxybenzamine (Smithkline Beecham); phenylephrine (Sigma) and propranolol (Sigma) were purchased. The bradykinin B_2 receptor antagonist, B9340, was a gift from Dr. John Stewart (Stewart *et al*., 1996). The V_{1a}, V_2, and OT receptor antagonists were synthesized in our laboratories (Kruszynski *et al*., 1980; Manning *et al*., 1997, 1989).

Statistical analysis

Bioassay values, sample means and measurements of the vasodepressor response to the hypotensive VP peptides were analysed for variance by one-way ANOVA. Values are reported as means ± s.e., with 'n' indicating the number of experiments for the mean. For bioassays, each bioassay value represents the mean from at least four independent bioassays. Each independent bioassay value represents the average of two to three sets 4-point or pA_2 assays from one animal. In experiments comparing the vasodepressor responses to the hypotensive VP peptides before and after specific treatments, the before and after measurements were paired measurements taken in the same animal. The two sample means were compared by the paired Student's t-test. Differences were considered significant at $P<0.05$ level.

Results

Pharmacological profile of the hypotensive VP peptides

The hypotensive VP peptides (Peptides I – IV) were bioassayed for biological activities characteristic of neurohypophysial peptides. Table 1 compares the pharmacological activity

Table 1 Pharmacological activity profiles of hypotensive VP peptides I – IV compared to AVP and V_2/V_{1a}/OT antagonist, $d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP

	Agonistic Activity (USP U mg^{-1})			*Antagonistic Activity* (pA_2)[a]		
Peptide	*Antidiuretic* (V_2)	*Vasopressor* (V_{1a})	*Oxytocic* (OT)	*Anti-V_2*	*Anti-V_{1a}*	*Anti-OT*
AVP[b]	320	370	14	–	–	–
$d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP[c]	–	–	–	7.81 ± 0.07	8.22 ± 0.12	8.32 ± 0.10
(V_2/V_{1a}/OT antagonist – parent molecule of Peptides I-IV)				ED[d]: 1.1 ± 0.15	0.45 ± 0.11	
Peptide I $d(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP	< 0.005	vasodepressor[e]	< 0.05	ND[f]	ND	ND
Peptide II $d(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4]AVP	< 0.005	vasodepressor[e]	< 0.05	ND	ND	ND
Peptide III $d(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP	< 0.003	vasodepressor[e]	ND	ND	ND	ND
Peptide IV $d(CH_2)_5$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9]AVP	< 0.003	vasodepressor[e]	ND	ND	ND	ND

[a]For the *in vivo* anti-V_{1a} and anti-V_2 pA_2's, the values are estimates since the molar concentrations are estimated by dividing the i.v. dose (ED) required for the pA_2 by an assumed volume of distribution of 67 ml kg^{-1} (Dykes *et al*., 1974). [b]From Sawyer & Manning (1973). [c]From Manning *et al*., (1982). [d]ED = The effective i.v. dose of antagonist required for the pA_2. [e]Peptides I, II, III and IV were approximately equi-potent in vasodepressor activity; threshold doses 0.02 to 0.05 mg kg^{-1}. See text for details. [f]ND = Non-detectable. In anti-V_{1a} assays, up to 20 μg/rat i.v.; higher doses were vasodepressor. In anti-V_2 and anti-OT assays, up to 100 μg no detectable antagonism; estimated $pA_2 << 5$.

profiles of AVP (an agonist); d(CH_2)$_5$[D-Tyr(Et)^2, Val^4]AVP, the parent peptide of the hypotensive peptides, (an OT/VP antagonist) and the four novel hypotensive VP peptides, Peptides I, II, III and IV. Relative to AVP and their parent peptide, the four hypotensive VP peptides were essentially devoid of agonistic or antagonistic activities that are characteristic of neurohypophysial peptides. All four peptides elicited a fall in BP in the vasopressor bioassay rats.

Dose-response characteristics of hypotensive VP peptides

In urethane-anaesthetized rats, Peptides I–IV, 0.05–0.10 mg kg^{-1} i.v., caused a marked fall in BP. Figure 1 shows the characteristic vasopressor response to AVP and the novel vasodepressor response to Peptide I in a vasopressor bioassay rat (low baseline BP due to autonomic blockade by atropine and phenoxybenzamine, standard protocol for vasopressor bioassays of AVP) and the markedly enhanced vasodepressor response to Peptide I in a normotensive rat (no autonomic blockade).

To ascertain whether the lower vasodepressor response to the hypotensive peptides in the 'bioassay' rats was related directly to blockade of autonomic nervous functions or was the result of hypotension due a decreased vascular tone, the vasodepressor response to Peptide II, 0.15 mg kg^{-1} was determined in three autonomically blocked rats during hypotensive state and during angiotensin II-induced vasoconstriction. Under complete α-adrenoceptor blockade, the baseline BP was extremely low 59$\pm$3.2 mmHg. The vasodepressor response, peak fall in BP, was 7.7$\pm$0.9 mmHg. Angiotensin II infusion induced vasoconstriction and elevated BP. However, the low baseline BP could be restored and maintained only partially by a very high infusion rate of angiotensin II (>50 μg kg^{-1} min^{-1}). During angiotensin II infusion, the baseline BP was restored to 101$\pm$1.8 mmHg. The vasodepressor response, peak fall in BP, was increased to 31$\pm$3.6 mmHg.

The relationship between baseline BP and the vasodepressor response to the hypotensive VP peptides was investigated in rats with different baseline BP levels (Figure 2). Because of the difficulty of maintaining elevated BP in autonomic receptor blocked animals, rats with intact autonomic functions were used to induce elevated baseline BP. Four groups of rats (n=6 in each group) with different baseline BP levels were studied. Group 1 (low baseline BP) were pretreated with blocking doses of atropine and phenoxybenzamine. Group 2 (normal baseline BP) were controls without treatments. Group 3 (high baseline BP-phenylephrine) and Group 4 (high baseline BP-angiotensin) were rats with mean arterial blood pressure elevated to and maintained at 110–120 mmHg by the infusion of phenylephrine or angiotensin II respectively. Figure 2 shows the peak falls in BP elicited by Peptide I, 0.10 mg kg^{-1}, in the four groups of urethane-anaesthetized rats with different baseline BP levels. The vasodepressor responses in rats with elevated baseline BP were significantly greater than in rats with normal BP ($P<0.05$) and in rats with low baseline BP ($P<0.01$). The difference between the two groups with elevated baseline BP, phenylephrine-induced or angiotensin-induced, was not statistically significant.

Since the vasodepressor response to the hypotensive VP peptide was baseline BP dependent, the dose-response

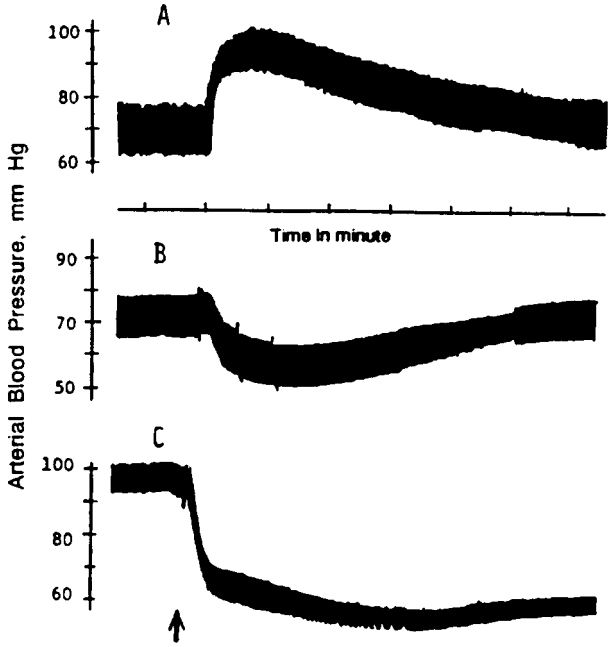

Figure 1 Effects of AVP and Peptide I on the blood pressure in anaesthetized rats. Tracings A and B were blood pressure tracings from the same rat (230 g) prepared for vasopressor bioassays (pretreated with atropine and phenoxybenzamine). Tracing A shows the vasopressor response to 2 mU AVP. Tracing B shows the vasodepressor response to 40 μg Peptide I. Tracing C shows the marked and prolonged vasodepressor response to 40 μg Peptide I in another anaesthetized normotensive (no pretreatment) rat (225 g). The tracings shown are typical tracings representative of the blood pressure responses to injections of AVP and the hypotensive VP peptides in urethane-anaesthetized rats

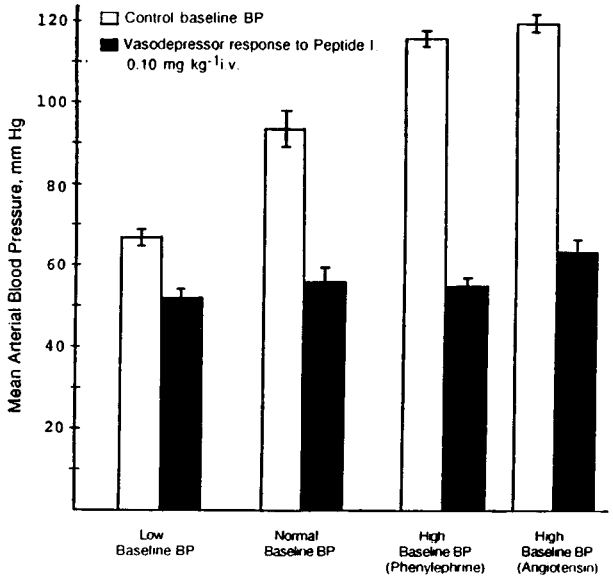

Figure 2 Effects of baseline BP on the vasodepressor response to hypotensive VP peptides. The vasodepressor response to Peptide I, 0.10 mg kg^{-1} i.v., was determined in four groups of anaesthetized rats with different baseline BP levels. The open columns show the baseline BP immediately before injections of Peptide I. The filled (black) columns show the BP at the nadir of the vasodepressor response. Values shown are means$\pm$s.e.mean. $n=6$ rats for each group. The group means (responses) between the two groups with high baseline BP were not significantly different, but they were significantly different from the group mean with normal baseline BP ($P<0.05$) and from the group mean with low baseline BP ($P<0.01$) by Student's t-test. See text for details for animal groups with different baseline BP

characteristics of the hypotensive VP peptides were determined in rats with controlled baseline BP, maintained at 110–120 mmHg by phenylephrine infusion. Figure 3 shows the dose-response curves for Peptides I and II, with the response parameter measured both by the peak fall in BP in mmHg and by the AUC in cm^2 for the 5 min period following the peptide injection. Peptides I and II were approximately equi-potent in vasodepressor activity. They were less than twofold different in potency, with Peptide I being the more potent. The vasodepressor dose-response curves for the hypotensive VP peptides were very steep. In rats with elevated baseline BP induced by phenylephrine, the threshold doses were 0.01 to 0.02 mg kg^{-1} i.v. In rats with normal baseline BP, the threshold doses were at 0.02 to 0.05 mg kg^{-1} i.v. The maximal vasodepressor response (fall in BP of 50 to 60 mmHg) was reached at 0.20 to 0.30 mg kg^{-1}. Higher doses produced no further fall in BP, but prolonged the vasodepressor response. Repeated injections led to a decrease in responsiveness and tachyphylaxis.

The iodinatable Peptides III and IV were also hypotensive with potencies and characteristics similar to their respective parent compounds Peptides I and II.

Effects of blockade of V_{1a}, V_2, OT receptors, β-adrenoceptors and bradykinin B_2 receptors on the vasodepressor response

The vasodepressor responses to Peptides I or II were determined in rats with normal baseline BP before and after administration of a V_{1a}, V_2, or OT receptor antagonist; before and after propranolol or before and after a bradykinin B_2 receptor antagonist ($n=4-6$ in each group). None of the receptor antagonists tested had a significant effect on the vasodepressor response to Peptide I or II. The results are summarized in Figure 4. The effects of receptor antagonists on the vasodepressor response to Peptides III and IV were qualitatively similar.

Effects of inhibition of PG synthesis on the vasodepressor response

The effects of inhibition of PG synthesis were determined in three rats. The vasodepressor responses to Peptide I, 0.10 mg kg^{-1}, were determined before and 30 min after inhibition of PG synthesis by diclofenac sodium. Inhibition of PG synthesis by diclofenac sodium had no significant effects

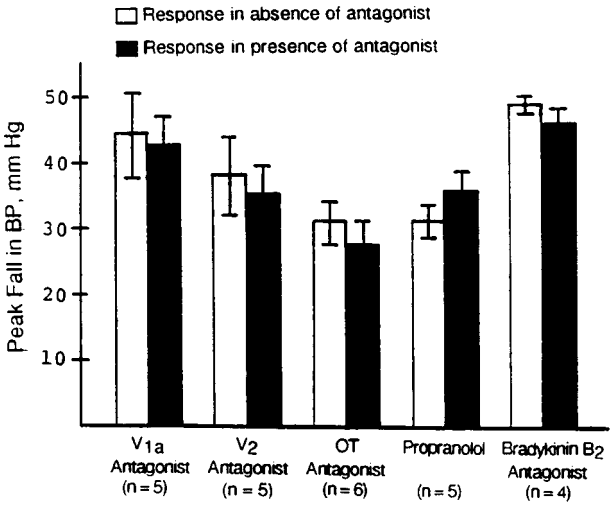

Figure 4 Effects of V_{1a}, V_2, OT receptor antagonists, propranolol and bradykinin B_2 receptor antagonist B9340 on the vasodepressor response to Peptides I and II. Peptide I was used in the V_{1a}, V_2, OT antagonists experiments. Peptides II was used in the propranolol and bradykinin B_2 antagonist experiments. Before and after antagonist responses were compared in the same animal (paired comparisons) in all experiments. Values shown are means ± s.e.mean. n = the number of experiments (rats) for the mean. Differences between the paired means were not statistically significant in all cases (paired Student's t-test). The doses for Peptides I and II were 0.10 to 0.15 mg kg^{-1}. The doses for the antagonists were maximal inhibitory doses with respect to their respective target receptors. Anaesthetized male rats, weight 200–250 g

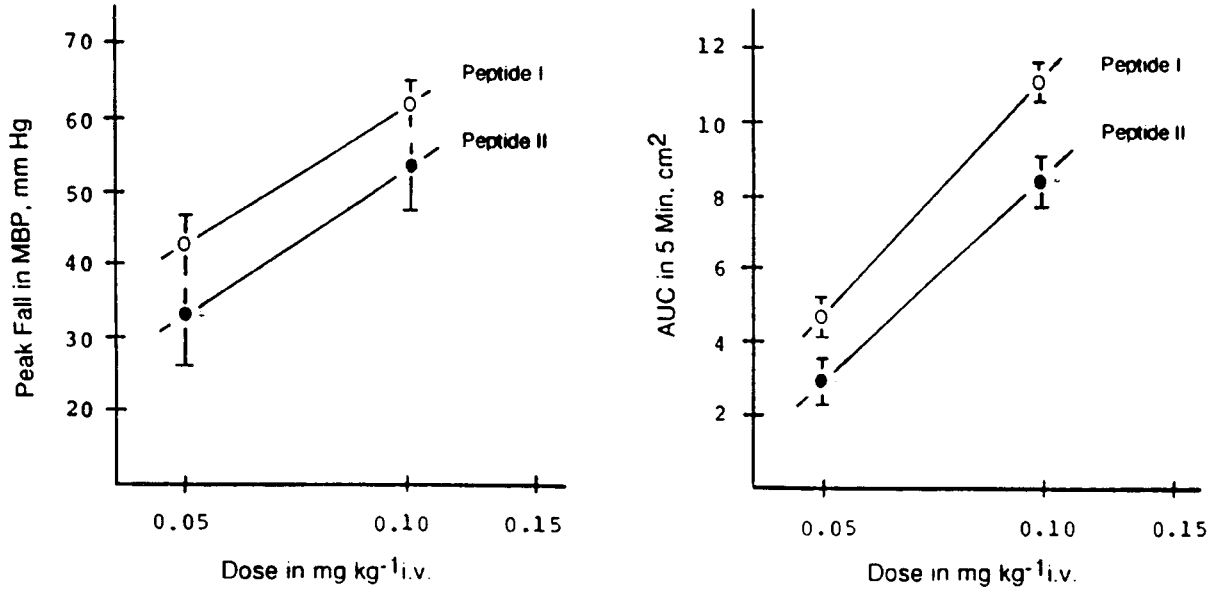

Figure 3 Vasodepressor dose-response curves for Peptides I and II. Vasodepressor responses were measured both by the peak fall in BP in mmHg and by the AUC in cm^2 for the 5-min period following peptide injection. Values shown are means ± s.e.mean $n=6$ for Peptide I; 5 for Peptide II. The baseline BP was elevated and maintained at 110–120 mmHg by phenylephrine infusion in all animals. Anaesthetized male rats, weight 225–245 g

on either the baseline BP or the vasodepressor response to Peptide I. The peak falls in BP induced by Peptide I were 37.3±4.7 mmHg and 32.7±5.0 mmHg before and after diclofenac sodium respectively. The difference between the two means was not statistically significant.

Effects of inhibition of NO synthesis on the vasodepressor response

The effects of inhibition of NO synthase by L-NAME on the vasodepressor response to the hypotensive VP peptides were determined (Table 2). The effects of L-NAME infused for 60 min at 0.2 mg kg^{-1} min^{-1} (low dose) and 1.0 mg kg^{-1} min^{-1} (high dose) on the vasodepressor response to Peptide I were investigated in two groups of rats ($n=6$ in each group). A 'pressure-controlled' control group ($n=6$) receiving angiotensin II infusion to match the elevated baseline BP of the low dose L-NAME group was also included.

Response to acetylcholine (ACh) was monitored in all L-NAME treated animals to verify NO inhibition. Bolus injections of ACh, 2 μg, elicited a transient fall in BP distinguishable in two phases; an initial sharp fall associated with an extremely marked slowing of the arterial pulse (bradycardiac phase) followed by a hypotensive phase presumably endothelial NO-induced (vasorelaxation phase). L-NAME abolished the vasorelaxation phase, but not the bradycardiac phase, of the vasodepressor response to ACh. The inhibition could be demonstrated within 15 min after the start of L-NAME infusion when the baseline BP was elevated. Figure 5 shows the typical inhibitory effect of L-NAME on ACh-induced responses in a representative experiment.

The results of the L-NAME experiments are presented in Table 2. The baseline BP was elevated in all L-NAME treated animals, but only the high dose L-NAME attenuated the vasodepressor response to Peptide I when the response was expressed by AUC. Both the low and the high dose of L-NAME shortened the duration of the vasodepressor action as measured by the response recovery $t_{1/2}$ (time for 50% recovery from the peak fall in BP). Figure 5 shows the BP tracings from a representative experiment examining the effects of low dose L-NAME on the vasodepressor responses to ACh and Peptide I.

Table 2 Effects of L-NAME on the vasodepressor response to hypotensive VP peptides

	Vasodepressor Response to Peptide I (0.15 mg kg^{-1})							
	Before Infusion				*During Infusion*			
Treatment (Infusion)	*Baseline BP* (mmHg)	*Peak Fall in BP* (mmHg)	*AUC in 5 min* (cm^2)	*Recovery* $t_{1/2}$ (min)	*Baseline BP* (mmHg)	*Peak Fall in BP* (mmHg)	*AUC in 5 min* (cm^2)	*Recovery* $t_{1/2}$ (min)
L-NAME, 0.2 mg kg^{-1} min^{-1}								
	89±4.8	38±3.7	7.2±1.0	7.3±0.8	120±6.5**	50±6.0**	7.8±1.1	4.5±0.5**
L-NAME, 1.0 mg kg^{-1} min^{-1}								
	99±1.6	47±1.6	8.7±0.3	5.3±0.5	141±4.0**	42±7.0	4.1±0.7**	2.0±0.3**
Matched-Baseline BP Control (Angiotensin II, 1.0-5.0 μg min^{-1})								
	97±3.5	37±5.3	6.8±1.3	6.6±1.7	123±3.5**	53±2.4**	8.6±0.8*	6.1±1.2

Baseline BP = mean arterial blood pressure immediately before injection of the hypotensive VP peptide. AUC = area under the vasodepressor response curve for the 5-min period following injection of the hypotensive VP peptide. Recovery $t_{1/2}$ = recovery time in min for 50% recovery from the peak fall in BP. Values shown are means±s.e.mean; n = 6. *Group mean significantly different from corresponding pre-injection control mean, $P<0.05$. **Group mean significantly different from corresponding pre-injection control mean, $P<0.01$.

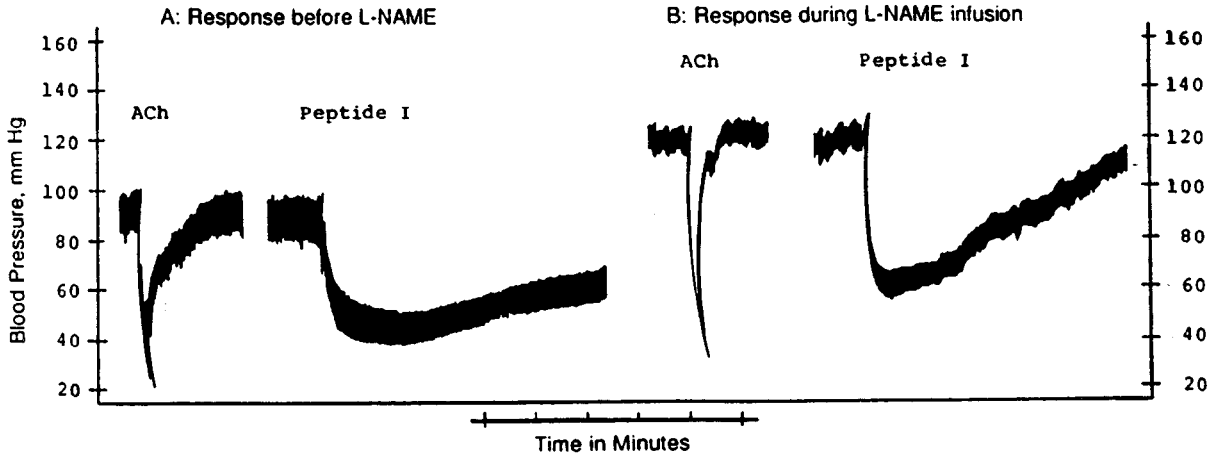

Figure 5 Effects of L-NAME on the vasodepressor responses to ACh and to Peptide I in an anaesthetized rat. (A) shows the control vasodepressor responses to 2.0 μg ACh and 30 μg Peptide I. (B) shows the vasodepressor responses to the repeated doses of ACh and Peptide I in the same animal during L-NAME infusion at 0.2 mg kg^{-1} min^{-1}. L-NAME had been infused for 60 min. Note that L-NAME suppressed the hypotensive response to ACh but not the initial sharp fall in BP associated with a marked slowing of the pulse rate induced by ACh. Under L-NAME, the peak fall in BP elicited by Peptide I was greater compared to pre-L-NAME response, an increase of 36%. The enhanced response, however, could be accounted for by the elevation of baseline BP induced by L-NAME. The duration of action measured by the response recovery $t_{1/2}$ was significantly reduced, from 6.0 min to 3.5 min. Male rat, 220 g

Discussion

In this paper we report the discovery of the first known selective hypotensive VP peptides, d(CH_2)$_5$[D-Tyr(Et)^2,Arg^3, Val^4]AVP (Peptide I); d(CH_2)$_5$[D-Tyr(Et)^2,Lys^3,Val^4]AVP (Peptide II); d(CH_2)$_5$[D-Tyr(Et)^2, Arg^3, Val^4, Tyr-NH_2^9]AVP (Peptide III) and d(CH_2)$_5$[D-Tyr(Et)^2, Lys^3, Val^4, Tyr-NH_2^9] AVP (Peptide IV). The mechanism of action of these hypotensive VP peptides has yet to be elucidated. Our characterizations of the pharmacological properties of these novel VP peptides showed that the hypotensive VP peptides exhibited little or no functional interactions with the known classical OT and VP receptors.

The four VP peptides were found to possess marked hypotensive activity and were essentially devoid of agonistic or antagonistic V_{1a}, V_2 and OT activities that are characteristic of neurohypophysial peptides. Thus, they are the first known selective hypotensive VP peptides. We have not yet determined whether these hypotensive VP peptides interact with the V_{1b} receptors.

In anaesthetized rats, the hypotensive VP peptides elicited a dose-dependent fall in BP. The four hypotensive VP peptides were approximately equi-potent in vasodepressor activity, with threshold doses at 0.02 to 0.05 mg kg^{-1} i.v. In high doses, the hypotensive VP peptides produced a marked and prolonged hypotension, with a peak fall of 50 to 60 mmHg.

The vasodepressor activity appeared to be independent of the autonomic nervous system since the vasodepressor response was not abolished by fully blocking doses of the muscarinic antagonist atropine and the α- and β-adrenoceptor antagonists phenoxybenzamine and propranolol. However, the vasodepressor response in the atropine and phenoxybenzamine treated rats was smaller than in rats with intact autonomic functions. We presented evidence showing that the reduced response was most likely related to the low vascular tone of these animals as a consequence of α-adrenoceptor blockade. Indeed, we demonstrated in a series of experiments that the vasodepressor response to the hypotensive VP peptides was dependent on the baseline BP (Figure 2). The finding that elevation of baseline BP induced by either phenylephrine or angiotensin II produced the same enhancement of vasodepressor response provides further support that the vasodepressor action of the hypotensive VP peptides is independent of the peripheral α-adrenoceptor system. Nevertheless, it should be recognized that cholinoceptor and α-adrenoceptor blockade by atropine and phenoxybenzamine might have interrupted the efferent outflow of central sympathetic inhibition and hence reduced the vasodepressor response. AVP is known to affect baroreflex and inhibit central sympathetic outflow, causing a decrease in heart rate and cardiac output (Cowley *et al*., 1984; Webb *et al*., 1986; Shimizu *et al*., 1993; Huch *et al*., 1995). Thus, a central action for the hypotensive VP peptides could not be ruled out in our experiments.

Eicosanoids and bradykinin do not appear to be involved in the hypotensive action of these hypotensive VP peptides. Inhibition of PG synthesis by diclofenac sodium and blockade of bradykinin B_2 receptor by B9340 had no significant effects on the vasodepressor response to the hypotensive VP peptides. This is consistent with early studies by others on the vasodilating action of AVP and V_2 agonists (Liard, 1988, 1994). Whether other vasodilating mediators, such as atrial natriuretic peptides and adenosine, are involved in the hypotensive action of VP peptides is not known and was not investigated in our study.

Since there is strong evidence in the literature showing that the vasodilating action of VP and OT is mediated by endothelial NO (Katusic *et al*., 1986; Kim *et al*., 1988; Yamada *et al*., 1993; Liard, 1994; Rudichenko & Beierwaltes, 1995; Garcia-Villalon *et al*., 1996; Hirata *et al*., 1997), we investigated the effects of inhibition of NO synthase by L-NAME on the vasodepressor response to the hypotensive VP peptides. We found that L-NAME, 0.2 mg kg^{-1} min^{-1} for 60 min, elevated the baseline BP by 30 mmHg; suppressed the hypotensive response to ACh, but did not reduce the vasodepressor response to Peptide I. In fact, the peak fall in BP was increased by 30% over the pre-L-NAME response in the same animal. The matched-baseline BP controls showed a similar increase in peak fall in BP, consistent with our finding that the vasodepressor response to the hypotensive VP peptides is baseline BP dependent, being greater at higher baseline BP. The duration of action, as expressed by the response recovery $t_{1/2}$, under L-NAME was shorter compared both to the pre-L-NAME value in the same animal and to the matched-baseline BP controls. The difference was statistically significant, $P<0.05$. Thus, there was no significant increase in AUC under L-NAME despite an increase in the peak fall in BP (Table 2).

The high dose L-NAME, 1.0 mg kg^{-1} min^{-1}, elevated the baseline BP by 40 mmHg and attenuated all three response parameters: peak fall in BP, AUC and the response recovery $t_{1/2}$ (Table 2). Although this dose appears excessive for the inhibition of NO release, others had also used high doses of L-NAME and a long period of infusion in the investigation of the vascular actions of AVP and V_2 agonists (Liard, 1994; Dworkin *et al*., 1995; Rudichenko & Beierwaltes, 1995).

Since the vasodepressor action of the hypotensive VP peptides was not significantly inhibited by doses of L-NAME that produced a marked inhibition of NO synthesis/release, as monitored by elevation of baseline BP and suppression of response to ACh, it appears that the vasodepressor action of the hypotensive VP peptides is not mediated by NO. However, the finding that the duration of vasodepressor action was reduced in L-NAME treated rats suggests that endothelial NO may modulate the vasodepressor action of the hypotensive VP peptides.

A large body of data in the literature suggests that the vasodilating action of AVP is mediated by the V_2 receptor (Liard, 1988, 1994; Kim *et al*., 1988; Naitoh *et al*., 1993; Yamada *et al*., 1993; Nakanishi *et al*., 1995; Rudichenko & Beierwaltes, 1995; Tagawa *et al*., 1995; Garcia-Villalon *et al*., 1996; Hirata *et al*., 1997). Of particular interest are reports that vasodilation to dDAVP, a V_2 agonist, seen in normal subjects (increase in forearm blood flow) was not elicited in patients with congenital nephrogenic diabetes insipidus, implying that there may also be a defect in the 'vascular V_2' receptors in these patients (Bichet *et al*., 1988; Tagawa *et al*., 1995; van Lieburg *et al*., 1995). Our findings reported here that the selective hypotensive VP peptides had little or no V_2 activity suggests that their hypotensive action may not be mediated through V_2 receptors.

Therefore, we investigated whether the vasodepressor response to the hypotensive VP peptides would be inhibited by the known classical V_{1a}, V_2 and OT antagonists. We found that d(CH_2)$_5$[Tyr(Me)^2]AVP, a selective V_{1a} receptor antagonist (Kruszynski *et al*., 1980); d(CH_2)$_5$[D-Tyr(Et)^2,Tic^3,Val^4]AVP, a new selective V_2 receptor antagonist (Manning *et al*., 1997) and desGly-NH_2,d(CH_2)$_5$[Tyr(Me)^2,Thr^4]OVT, a selective OT receptor antagonist (Manning *et al*., 1989) at equi-doses with the agonist had no antagonistic effect on the vasodepressor response to Peptides I and II. We used equi-

doses (approximately equi-molar concentrations) for both agonist and antagonist in these experiments. Higher doses of the antagonists were not tested because they are highly potent with pA_2's in the range from 7.7 to 8.3 (Kruszynski *et al*., 1980; Manning *et al*., 1989, 1997). The concentrations of the antagonists used in the experiments already exceeded the maximal inhibitory doses for their respective target receptors. The lack of antagonism of the conventional V_{1a}, V_2 and OT antagonists on the vasodepressor response to the hypotensive VP peptides together with the bioassay findings that the hypotensive VP peptides are essentially devoid of V_{1a}, V_2 and OT agonistic and antagonistic activities indicate that Peptides I and II probably interact either with a hitherto unknown VP receptor subtype or a new receptor outside the VP receptor family. Their high selectivity suggests that they could be developed as specific markers for the localization and identification of this putative new receptor or receptor subtype. We have previously shown that the C-terminal Gly-NH_2 in an OT antagonist could be replaced by a Tyr-NH_2 residue, thus introducing an iodinatable site in the molecule. Radioiodination of this OT antagonist had yielded a radioligand with high affinity (K_d 0.03 to 0.06 nM) for the myometrial OT receptor (Elands *et al*., 1988). We, therefore, effected a Tyr-NH_2^9/Gly-NH_2^9 interchange in Peptides I and II to introduce an iodinatable site. The resulting peptides, Peptides III and IV were found to retain the full vasodepressor activity and selectivity of the parent molecules. Thus, Peptides III and IV are potential candidates for development as specific radioligands for the putative new receptor.

Our discovery of the selective hypotensive VP Peptides I-IV reported here has significant basic and clinical implications. Since they are closely related structural analogues of V_2 receptor ligands, they may serve as lead compounds for the further development of more potent and selective hypotensive VP peptides and, more importantly, antagonists to the vasodilating action of AVP. Such new and improved selective ligands would provide the tools needed to ascertain whether the renal V_2 receptors and the extrarenal (vasodilating) V_2 receptors are two distinct subtypes, and to investigate the purported defects in extrarenal vascular V_2 receptors in nephrogenic diabetes insipidus (Bichet *et al*., 1988; van Lieburg *et al*., 1995). Availability of these unique peptides will also provide a new set of powerful pharmacological probes for the investigation of the complex cardiovascular actions of AVP and its role in the regulation of systemic BP and regional blood flows in hypovolemic states. In addition, the new hypotensive VP peptides may lead to the development of a novel class of antihypertensive agents for the treatment of cardiovascular diseases.

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