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Effects of neurohypophyseal hormone analogues on blood clotting factor VIII and fibrinolytic activity in sheep

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Abstract

The increase in blood clotting factor VIII (antihaemophilic factor, F-VIII) and fibrinolytic activity induced by the administration of neurohypophyseal hormone analogues, was assayed in sheep. Peptides with high selectivity for vasopressin V_1, V_2 or myometrial oxytocin receptors in the dose range of 0.1–10 μg/kg body weight were investigated. The main conclusions are as follows. The time-course of the F-VIII plasma levels following the administration of the peptides was biphasic, with one surge at about 20 min, a rebound phase, and another increase with the maximum at 60–90 min. The time-course of the fibrinolytic response, expressed as biological activity of plasminogen activator in the plasma euglobulin fraction, displayed a single maximum within 60 min. The baseline responses were reached within 90–120 min. Responses were expressed as integrals of the time-concentration curves in a predetermined time range (90–120 min). F-VIII and plasminogen activator enhancing effects seemed to be tightly linked to the specific vasopressin V_2 receptor activities. [Val^4,D-Arg^8]Vasopressin displayed higher plasminogen activator activities than the standard substance, deamino[D-Arg^8]vasopressin. The vasotocin analogue [Phe^2,Orn^8]oxytocin, a specific vasopressin V_1 receptor agonist, also displayed high antihaemophilic and fibrinolytic potencies, expressed in terms of ED_{50} values, but did not reach the same maximal response as vasopressin V_2 receptor agonists. Oxytocin and its highly selective uterotonic analogue, [Thr^4,Gly^7]oxytocin, displayed low antihaemophilic, and virtually no plasminogen activating potencies. Surprisingly, vasopressin V_2 and V_1V_2 receptor antagonists studied in our experiments showed both enhanced F-VIII and fibrinolytic responses. Dose–response curves frequently displayed a decrease of the F-VIII, and sometimes also decreased fibrinolytic responses, at higher peptide doses. Strong decreases of the packed cell volume (haematocrit) and somewhat lower decreases of the total plasma protein concentration were observed shortly after administration of the peptides.

Keywords: Vasopressin; Oxytocin; Factor VIII (F-VIII); Antihemophilic factor; Blood clotting; Plasminogen activator; Neurohypophyseal hormone analog; Dose–response analysis; Time response; Non-monotonic profile

1. Introduction

Vasopressin and some of its structural analogues enhance, after parenteral administration, plasma levels of blood clotting factor VIII (antihaemophilic factor, F-VIII) in humans (Cash et al., 1974; Mannucci et al., 1975; Prowse et al., 1979), dogs (Vilhardt et al., 1987; Vilhardt and Barth, 1991), sheep (Heiniger et al., 1988; Neuenschwander et al., 1989), macaca (Vilhardt and Barth, 1992) and marmosets (Vilhardt et al., 1993). This 'antihaemophilic activity' is particularly pronounced in deamino[D-Arg^8]vasopressin, a peptide with a strong antidiuretic and a negligible vasopressor potency. Deamino[D-Arg^8]vasopressin has therefore been used for treatment of patients with medium severe and mild forms of haemophilia A, von Willebrand-Jürgens syndrome and some types of haemorrhagic diathesis (Mannucci et al., 1977a,b). Clinical experience has generally been very efficacious.

Pharmacologically, however, these substances have been investigated only insufficiently, owing to serious difficulties associated with the assay of antihaemophilic activity. First, the antihaemophilic response is obviously not elicited by a single mechanism, letting alone the question of a specific receptor. In particular, there seems to be some correlation to the antidiuretic activity of these peptides (Vilhardt et al., 1993; Pliška and Neuenschwander, 1994).

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Second, deamino[D-Arg^8]vasopressin and other peptides known to possess antihaemophilic activity also increase the fibrinolytic activity of blood plasma (Cash et al., 1978; Vilhardt and Barth, 1992; Pliška and Neuenschwander, 1994), probably by activating the tissue type plasminogen activator (Keber et al., 1990). It can be expected that the simultaneous occurrence of two counteracting activities in one single peptide would have an impact on the accuracy of the assay, regardless of the animal model employed. This may be the reason why no dose–response analyses have been presented in the literature so far. Third, the selection of a suitable animal model poses additional difficulties. Besides species differences in clotting systems (particularly in plasma levels of individual blood clotting factors) and in responses to deamino[D-Arg^8]vasopressin, one is confronted with technical difficulties: blood sampling must meticulously follow an optimised protocol, any stress of the animal must be avoided, and plasma samples must be analysed in the shortest possible time. The use of small laboratory rodents is therefore hardly possible. Experiments on larger animals, on the other hand, require a relatively high amount of the substance to be tested, the sources of which are usually limited.

For reasons given in our earlier papers (Heiniger et al., 1988; Neuenschwander et al., 1989), we have carried out our assays on sheep, since several observations speak in favour of this species. With this animal model, we have assayed a series of peptides known to act upon vasopressin V_1 or V_2 receptors, or peptides specifically possessing oxytocin-like effects, with the aim of obtaining more secure data about structural requirements associated with antihaemophilic and fibrinolytic activities. We have attempted to obtain dose–response curves for individual peptides, and to find suitable numeric descriptors of their biological activities.

2. Materials and methods

2.1. Peptides

Table 1 reviews the peptides assayed in this study and their antidiuretic, vasopressor, and uterotonic potencies. Arginine vasopressin, oxytocin and deamino[D-Arg^8]vasopressin were products of Ferring (Malmö, Sweden); all other peptides ([Val^4,D-Arg^8]vasopressin, [Phe^2,Orn^8]oxytocin, [Thr^4,Gly^7]oxytocin, [Mep^1,Tyr(Me)^2,D-Arg^8]vasopressin, and [Mpp^1,D-Tyr(Me)^2,Val^4,Arg^8]vasopressin), designed by Manning and Sawyer (1993), were synthesised in the laboratory of Dr. M. Manning. Table 1 also indicates the current classification of these peptides, as acting on vasopressin V_1, V_2 and myometrial oxytocin receptors. The source of additional 'standard' activities was reviewed by Hruby and Smith (1987). Receptor selectivities of the peptides for vasopressin V_1, V_2 and oxytocin receptors were expressed as a ratio of the characteristic potency to the sum of vasopressor + antidiuretic + uterotonic potencies (see Table 1; Pliška and Neuenschwander, 1994). The corresponding receptor selectivity indices (Table 1) reach values between zero and unit.

2.2. Animals

Non-pregnant female White Alpine sheep (WAS), 50–70 kg body weight, were used in the experiments. Animals were kept under standard farm conditions together with the rest of the flock until the day before the actual experiment, when they were separated into smaller compartments of the sheep fold. Their free movement was not restricted during any phase of the experiment except for short periods of injections and blood sampling (each about 2–4

Table 1
Assayed neurohypophyseal peptides, their standard potencies and receptor selectivities

Peptide [a]	Potency [b]			Receptor selectivity index [c]		
	BP	AD	UT	V_1	V_2	OT
Arginine vasopressin	100	100	2.2	0.495	0.495	0.011
Oxytocin	0.5	0.6	100	0.005	0.006	**0.989**
Deamino[D-Arg^8]vasopressin (dDAVP)	0.1	190	0.3	0	**0.998**	0.002
[Val^4,D-Arg^8]Vasopressin	0	130	0.1	0	**0.999**	0.001
[Phe^2,Orn^8]Oxytocin [d]	24.6	0.1	0.2	**0.988**	0.004	0.008
[Thr^4,Gly^7]Oxytocin	0	0	30	0	0	**1**
[Mep^1,Tyr(Me)^2,D-Arg^8]Vasopressin	A_{V1}	0.02	A_{OT}			
[Mpp^1,D-Tyr(Me)^2,Val^4,Arg^8]Vasopressin	A_{V1}	A_{V2}	A_{OT}			

[a] Nomenclature according to IUPAC-IUB rules. Additional abbreviations of amino acids: Mep, β-mercapto-β,β-diethylpropionic acid; Mpp, β-mercapto-β,β-cyclopentamethylenepropionic acid; Orn, ornithin.

[b] Rat vasopressor (BP), antidiuretic (AD), and in vitro uterotonic (UT) potencies are expressed in percentage of the corresponding standards [Arg^8]vasopressin and oxytocin respectively (underlined). Potency data taken from Hruby and Smith (1987). A_{V1}, vasopressin V_1 receptor antagonist; A_{V2}, vasopressin V_2 receptor antagonist, A_{OT}, myometrial oxytocin receptor antagonist.

[c] Estimated receptor selectivities for vasopressin V_1, V_2 and myometrial oxytocin (OT) receptors. Indices were defined as BP/*Sum* for V_1, AD/*Sum* for V_2, and UT/*Sum* for OT receptors; *Sum* = BP + AD + UT. High selectivities (> 0.95) are given in bold letters.

[d] Analogue of vasotocin.

min), and the animals had unlimited access to hay and water. Basal levels of F-VIII in these animals were roughly 2–3-times higher as compared to the standard human plasma pool. The selection of animals for experiments was based on their response to a 1 μg/kg body weight (b.w.) dose of deamino[D-Arg^8]vasopressin: an animal was excluded from the experiment when less than a 20% increase of F-VIII (compared to basal level) was registered 15 or 45 min after injection (Heiniger et al., 1988).

2.3. Experimental protocol

Peptides dissolved in physiological saline were injected into the external jugular vein at a volume of 2 ml. Blood sampling (2 ml) followed from the same vein; 2–3 ml of the blood were discarded prior to sample collection. Blood was mixed during the collection with 0.11 M sodium citrate (final dilution 1:10). Blood plasma was separated by centrifugation at $2000 \times g$ and 4°C for 10 min, and kept on ice. Individual punctures were made at distances of at least 1 cm from the next nearest one, in order to avoid any major damage to the vessel wall which might cause an activation of the blood coagulation cascade through the extrinsic pathway. Injection needles, 40 mm long, inner diameter 1.2 mm, were used for both injection and blood sampling. Three blood samples were collected during a period of 15 min prior to injection (usually -15, -10 and -5 min), and another one immediately before. Average antihaemophilic and fibrinolytic activity levels of these samples were taken as basal levels. Further blood samples were collected at 5–10-min intervals within the first 30 min, and later at 15–30-min intervals until the end of the experiment (120 min).

Each substance was assayed in a group of five responder animals. Individual doses of the investigated peptide were administered in three repetitions to randomly selected animals within the group. In order to achieve a replenishment of the F-VIII and plasminogen activator pools, the time span between two doses was at least 6 days.

2.4. Assay of factor VIII activity in plasma samples

A one-step procedure was used for assays of F-VIII. Test kits were products of Baxter Diagnostics (Merz & Dade, Düdingen, Switzerland). 50 μl of diluted plasma (1 volume part of plasma + 9 parts of Owren's veronal buffer pH 7.35) were mixed with 50 μl of a F-VIII depleted (human haemophilic) plasma and 50 μl of Actin (mixture of cephalin and 10^{-4} M ellagic acid), and incubated for 4 min at 37°C. Clotting time was measured after recalcification with 50 μl 0.02 M calcium chloride, in a Schmittger & Gross coagulometer (H. Amelung, Lemgo, Germany), at 37°C. All measurements were carried out in duplicates and repeated when the observed clotting times deviated for more than 1 s. 'Residual' response was determined for each assay kit as the clotting time T_0 in the absence of the assayed plasma which was substituted by Owren's veronal buffer. Plasma concentration of F-VIII (C) is proportional to the inverse of the clotting time T (Pliška et al., 1982; Neuenschwander and Pliška, 1994),

$$\log C = a + \alpha(1/T - 1/T_0) \quad (1)$$

where a and α are empirical constants. Arbitrary values of C in the time series were assessed by means of a calibration based on the above Eq. (1). Each animal served as its own control: several dilutions of the plasma withdrawn 5 min prior to injection (see Section 2.3) were used as a reference. Coefficients a and α were obtained by regression analysis of this dilution curve. F-VIII concentrations estimated in this way were corrected for the observed plasma dilution based on the difference of haematocrit values at time zero and at the given time (see Section 2.8).

2.5. Assay of fibrinolytic activity (plasminogen activator activity)

The fibrinolytic potency of the euglobulin fraction was estimated by the fibrin plate method (Astrup and Müllerts, 1952). Sheep fibrinogen (Sigma) in 0.15 M Tris buffer pH 7.8 containing 1.50% thrombin (Thrombostasine, Hoffmann-La Roche, 20 NIH/ml) was used to produce a 1 mm layer of fibrin on a glass plate (native plates). Denatured fibrin plates were prepared by subsequent heating to 85°C for 45 min in saturated water vapour atmosphere (wet chamber). 30 μl of resuspended euglobulins (Chakrabarti and Fearnley, 1962) were placed on the surface of native and denatured plates (double estimates) and incubated for 15 h at 37°C in a wet chamber. Trypsin, used as a standard, was applied in the same volume in amounts of 0.1–1 μg per spot. The lytic plaques were then photographed, their area measured and compared with standard plaques of trypsin. The fibrinolytic activities were expressed in amounts of trypsin per ml of plasma. Denaturation prevents plasminogen-fibrin interactions and thereby the action of plasminogen activators; the lytic plaques are caused by directly acting fibrinolytic substances in the plasma sample. Thus, the difference of fibrinolytic activities measured on native and denatured plates is proportional to the biological activity of plasminogen activators present in the sample.

2.6. Response assessment

In order to account for the multiphasic time-course and individual variability, we have expressed the response (E) to a peptide dose (D) as the mean value of the F-VIII or plasminogen activator activities within a time range of zero to τ ($\tau = 120$ min),

$$E = (1/\tau)\int_0^{\tau} \psi(C)\,\mathrm{d}t \quad (2)$$

$\psi(C)$ is a function describing the time-course of the F-VIII or plasminogen activator plasma concentrations (C). For these purposes, C was measured at several times (t) and the integral was substituted by the sum of trapezoids:

$$E = (1/\tau) \sum_{i=1}^{n-1} (C_i + C_{i+1})(t_{i+1} - t_i)/2 \quad (3)$$

where C_i are concentrations (Eq. (1)) measured in corresponding times t_i (n values per time curve). Finally, the response E was standardised with respect to the basal concentration (C_b), obtained as an average of C values measured prior to peptide administration, and expressed as relative response

$$e = E/C_b - 1 \quad (4)$$

(cf., Figs. 1–3).

2.7. Assessment of dose–response relationships

Some of the dose–response curves presented above display a bell-shaped character or a rebound phase commencing at higher doses of the peptides. Such curves indicate a self-inhibition process elicited by the agonistic peptide in the secreting cells. They cannot be evaluated by the current dose–response analysis which assumes a parallel course of dose–response curves for both the tested and standard substances. The significance of the response difference between individual doses in such instances has to be tested prior to the further dose–response analysis. A corresponding statistical test was presented by Simpson and Dallal (1989). The actual dose–response analysis was subsequently carried out in cases for which the test indicated a significant dose dependence at at least 10% probability level P ($P \leq 0.1$, see Table 2). We have employed the dose–response analysis based on the 'tetragonal' dose–response model presented earlier (Pliška, 1994) and expressed as

$$e = E_0 + E_m/\left((K_a/D)^{\nu_a} + (D/K_{ai})^{\nu_{ai}} + 1\right) \quad (5)$$

where D stands for the injected dose, E_m for the maximal attainable effect (maximum release of F-VIII or plasminogen activator, respectively), K_a and K_{ai} for equilibrium constants of release activating and inhibiting processes, respectively (K_a corresponds roughly to the half-maximal dose, ED_{50}), ν_a and ν_{ai} are corresponding power coefficients ('Hill coefficient'), and E_0 the baseline of response e in the absence of stimulation. The parameters of Eq. (4) were estimated by means of non-linear regression using the NONLIN routine of the SYSTAT statistical software program. The Simplex procedure was used. Alternatively, a simplified function without the inhibition term $(D/K_{ai})^{\nu_{ai}}$

$$e = E_0 + E_m/\left((K_a/D)^{\nu_a} + 1\right) \quad (6)$$

was employed for the fit in order to test the significance of the inhibition term by means of an F-test. Residual variances Q_1, Q_2, resulting from the fits to Eqs. (4) and (5), respectively, were used for these purposes:

$$F = (Q_2 - Q_1)/Q_1 \times (n - c_1)/(c_1 - c_2) \quad (7)$$

where n is the number of data pairs, $c_1 = 6$ number of constant parameters in the first, $c_2 = 4$ number of constant parameters in the second run. Dose–response curves displaying a significant rebound effect are marked in Table 2 (footnote c).

The potency (biological activity) of the peptides (κ) was expressed in terms of K_a ratios, on molar basis, of the standard (deamino[D-Arg^8]vasopressin (dDAVP)) and the assayed substance,

$$\kappa(\text{peptide}) = K_a(\text{dDAVP})/K_a(\text{peptide}) \quad (8)$$

Parallelism of log dose–response curves for the standard and the tested substance only rarely occurs in in vivo assays. In consequence, the ratio of responses to the standard and to the tested peptides κ is not constant and depends on the administered doses; it therefore possesses only an arbitrary character. Moreover, some peptides investigated in this study showed a maximum effect (E_m) different (usually lower) from the standard and might be designated 'partial agonists' by the customary nomenclature. Ratios

$$e_m(\text{peptide}) = E_m(\text{peptide})/E_m(\text{dDAVP}) \quad (9)$$

are also presented in Table 2.

2.8. Measurement of haemodilution

The haematocrit (packed cell volume) was determined in citrate treated blood samples using standardised microhaematocrit tubes (Capilet, Merz&Dade) sealed with sealing cement (Dade Miniseal, Merz&Dade). The capillaries were centrifuged for 7 min at $15\,000 \times g$ in a microhaematocrit centrifuge. The biuret assay was used for the determination of the plasma protein concentration (human serum standard: Preciset, Boehringer-Mannheim, Mannheim, Germany). The plasma osmolarity was measured in an automated Knauer osmometer (Knauer, Belmont sur Lausanne, Switzerland) standardised with a 400 mosmol/kg NaCl solution.

3. Results

3.1. Time-course of antihaemophilic and fibrinolytic responses

The characteristic time response of F-VIII, assessed from a decrease in clotting times, to the neurohypophyseal peptides is biphasic (Fig. 1, upper panel). A surge of F-VIII occurs within the first 20 min after administration

of the peptide, followed by a rebound phase during which the F-VIII level may decrease considerably (sometimes to basal levels). The second increase starts after 30–45 min and reaches a maximum approximately at 60–90 min. We have observed certain individual differences in this behaviour: about 30% of animals showed no response to deamino[D-Arg^8]vasopressin (non-reactants, excluded from further experiments); some animals from the reactant group showed a monophasic response, usually at around the first F-VIII surge. The biphasic shape of the time response was influenced neither by the opioid analgesic DAMME ([D-Ala^2,MePhe^4,Met(O)-ol^5]enkephalin, FK 33824, Sandoz, Basel, Switzerland; Römer et al., 1977; 250 mg per animal i.v. prior to the experiment), nor by sedation with Rompun (Bayer, i.v. xylazin 20 μg/kg b.w.). The non-specific protease inhibitor, Trasylol (Bayer, 400 000 kallikrein inhibition units i.v., corresponding to about 0.56 μg of crystalline Aprotinin), suppressed the fibrinolytic activity to a minimum, however, without any effect on the F-VIII time-response curve.

The fibrinolytic response was less regular. When considering average time values, the time-response curve displayed, after a rapid increase, almost a constant level of plasminogen activity within the first 60 min. On the other hand, plasminogen activator peak values within 45 min after injection were regularly found in individual animals. Occasionally, a single 'early' peak (5–10 min) was registered. Time-response curves for three animals are shown in Fig. 1 (lower panel).

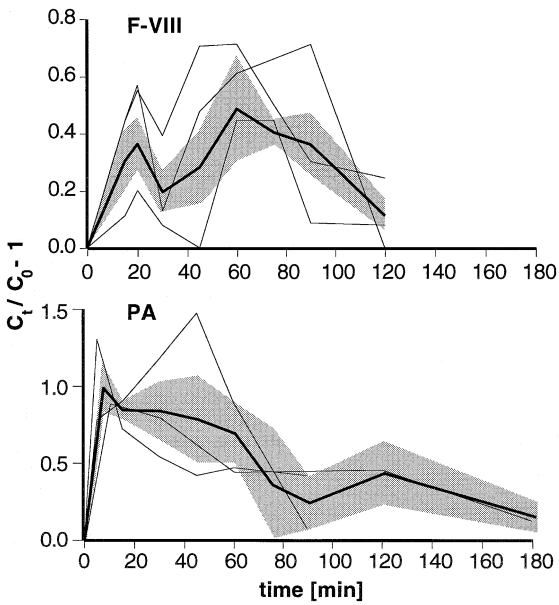

Fig. 1. Time-response curves of factor VIII (F-VIII, upper panel) and plasminogen activator (PA, lower panel) to 1 μg/kg b.w. deamino[D-Arg^8]vasopressin (dDAVP) in sheep. Full lines represent selected female animals, bold lines are averages of all animals in the group, shaded areas show limits of standard errors (5–8 values). Ordinate: change in F-VIII or plasminogen activator levels following peptide administration, relative to respective baseline levels (scale normalised according to Eq. (4)).

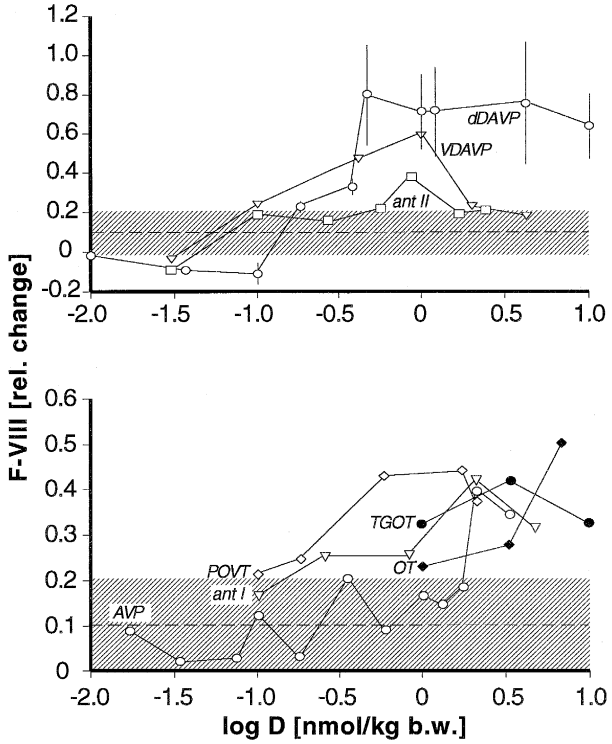

Fig. 2. Dose–F-VIII response curves of neurohypophyseal peptides in female sheep (for abbreviations see Table 1). Response in mean values within 120 min (see text) relative to baseline level of F-VIII (see legend to Fig. 1). Responses to physiological saline administration are within the hatched stripe (mean: broken line). Points: arithmetic means. Bars: S.E.M. (indicated for deamino[D-Arg^8]vasopressin only). Vasopressin V_2 receptor agonists are shown in the upper panel, the others in the lower panel. Abbreviations: AVP, arginine vasopressin; dDAVP, deamino[D-Arg^8]vasopressin; VDAVP, [Val^4,D-Arg^8]vasopressin; POVT, [Phe^2,Orn^8]oxytocin; OT, oxytocin; TGOT, [Thr^4,Gly^7]oxytocin; ant I, [Mep^1,Tyr(Me)^2,D-Arg^8]vasopressin; ant II, [Mpp^1,D-Tyr(Me)^2,Val^4,Arg^8]vasopressin.

3.2. Dose–response relationships

3.2.1. Antihaemophilic response

Fig. 2 shows dose–response relationships for the antihaemophilic activities of individual peptides. The curves indicate, in most instances, a 'bell-shaped' course, with a response decrease at higher peptide concentrations. Doses causing the first significant elevation of F-VIII are around 0.1 nmol/kg b.w. Hatched stripes indicate domains of the 'unspecific' response; they are expressed as a range of the arithmetic mean ± S.E.M. of an F-VIII response computed in the time interval 0–120 min, to an injection of physiological saline.

3.2.2. Fibrinolytic response

The fibrinolytic response, assessed as the biological activity of plasma plasminogen activator, was also expressed as an integral effect, using Eqs. (2) and (4). Results for representative members of the vasopressin V_1, V_2 and myometrial oxytocin receptor agonists are shown

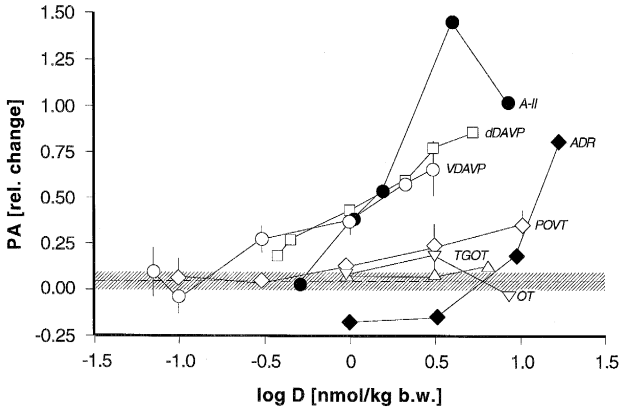

Fig. 3. Dose–plasminogen activator response (PA) curves of neurohypophyseal peptides in female sheep. For explanation see Fig. 2 (mean values within 90 min). Open symbols: neurohypophyseal peptides; closed symbols: angiotensin II (A-II) and adrenaline (ADR).

in Fig. 3 (open symbols). Vasopressin V_2 receptor analogues show markedly stronger plasminogen activator responses than analogues with other specificities. However, it should be noted that the plasminogen activator potency of the vasopressin V_1 receptor analogue assayed here [Phe^2,Orn^8]oxytocin, albeit considerably weaker than within the vasopressin V_2 receptor agonist group, is by no means negligible. Analogues with oxytocin specificity, on the other hand, are very weak, or not at all active.

Two substances of other generic groups known to have a strong plasminogen activator activity, angiotensin II (Cash et al., 1974) and adrenaline (Cash et al., 1969; Gader et al., 1973; Desnoyers et al., 1975), were assayed for purposes of comparison. Their dose–response curves are shown by closed symbols in Fig. 3. The dose range of these two substances (on a molar basis) is altogether comparable with that of the neurohypophyseal hormone analogues but the responses to angiotensin II are about twice as high as those to the most active vasopressin V_2 receptor agonist, [Val^4,D-Arg^8]vasopressin. It must be noted, however, that doses eliciting a maximal response were usually not experimentally determinable, owing to observed or potential side effects (tachycardia), which might have been dangerous for the animal.

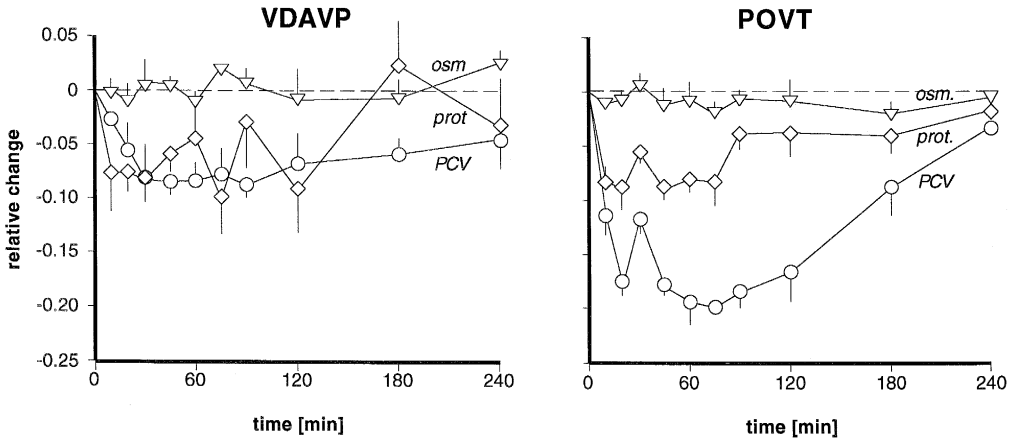

Fig. 4. Time-course of haemodilution following i.v. administration of [Val^4,D-Arg^8]vasopressin (VDAVP; 3 μg/kg b.w.) and [Phe^2,Orn^8]oxytocin (POVT; 0.3 μg/kg b.w.). For ordinate scale see Fig. 1. Triangles (osm), plasma osmolarity; diamonds (prot), plasma proteins; circles (PCV), packed cell volume (haematocrit values). Symbols represent arithmetic means, bars are S.E.M.

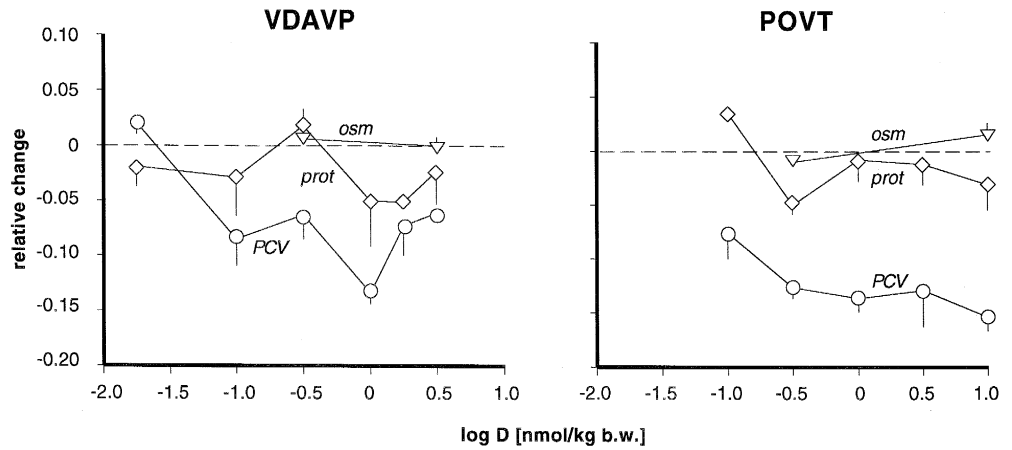

Fig. 5. Dose dependence of haemodilution. Ordinate, mean values within 120 min related to baseline. Also see legend to Fig. 4.

Table 2
Relative antihaemophilic and plasminogen activator enhancing potencies of neurohypophyseal hormone analogues

Peptide	Antihaemophilic potency [a]			Fibrinolytic potency [a] (plasminogen activator)		
	κ	ϵ_m	P [b]	κ	ϵ_m	P [b]
Deamino[D-Arg^8]vasopressin (dDAVP)	**1**	**1**	0.000	**1**	**1**	0.013
[Val^4,D-Arg^8]Vasopressin	0.65	0.85	0.043	2.5	1.09	0.000
Arginine vasopressin	0.07	0.87	0.004		n.d.	
[Phe^2,Orn^8]Oxytocin	3.03	0.54	0.013	0.51	0.42	0.007
Oxytocin	0.12	1.06	0.095 [c]	No fit	No fit	*0.841*
[Thr^4,Gly^7]Oxytocin	0.13	0.79	0.080 [c]	0.47	0.04	*0.500*
[Mep^1,Tyr(Me)^2,D-Arg^8]Vasopressin	0.25	0.45	*0.138*		n.d.	
[Mpp^1,D-Tyr(Me)^2,Val^4,Arg^8]Vasopressin	No fit	No fit	0.017		n.d.	
Adrenaline [d]	0.05	0.59	0.001 [c]	0.11	1.65	0.003
Angiotensin II		n.d.		1.49	1.20	0.001

[a] Potencies κ (see Eq. (8)) are expressed as ratios of K_a constants in Eqs. (4) and (6) (on molar basis) of deamino[D-Arg^8]vasopressin to the respective peptide. ϵ_m is the maximal response to the peptide, in relation to that of deamino[D-Arg^8]vasopressin (cf. Eq. (9)). Cases in which non-linear regression applied to Eq. (5) or Eq. (6) failed to converge are denoted as 'No fit'; n.d., not determined.
[b] Significance of dose–response relationship, including those with a rebound phase: error probabilities P are based on Jonckheere-Terpstra and Mann-Whitney statistics (BUMP program: Simpson and Dallal, 1989). Probability level of $P < 0.1$ was taken as a significance limit.
[c] Denotes significance of a rebound phase on the dose–response curve ($P < 0.05$), as estimated by the F-test (Eq. (7)). The F-test was not performed for cases in which the BUMP routine indicated low significance ($P > 0.1$; in italics).
[d] Computation based on data published by Heiniger et al. (1988).

3.3. Haemodilution

Decreases in packed cell volume and plasma protein concentration were observed after administration of all neurohypophyseal peptides used in this study. Similar effects were absent after administration of adrenaline and angiotensin II. The decrease shown in Figs. 4 and 5 for two peptides has a characteristic time-course (Fig. 4) and an almost regular dose–response relationship (Fig. 5). Decreases in packed cell volume as high as 20% were reached quickly after peptide administration. The decrease in plasma protein concentration was, however, less than that of the haematocrit. Plasma osmolarity was virtually unchanged.

3.4. Antihaemophilic and fibrinolytic potencies

Table 2 summarises κ and ϵ_{m} values (Eqs. (8) and (9)) of the two effects for substances tested in our experiments.

4. Discussion

The choice of sheep as an experimental animal in this study was based on our preliminary experience with this, rather unusual, animal model in haematological experiments. Its advantages with respect to the pharmacology of F-VIII release have been summarised in our earlier communications (Heiniger et al., 1988; Neuenschwander et al., 1989).

Both antihaemophilic and fibrinolytic responses display generally high variations. However, the dose–response analysis used in the present study shows a significant dose–response dependency for most of the peptides. When mean values of the time response are used (Eq. (2)), dose–response relationships are frequently bell-shaped. Such curves are common also in other biological responses when a broad dose range is used for stimulation. Although their nature remains unexplained, these curves can be parametrised. The estimates of parameters can be statistically tested and used to express biological potencies of individual drugs (Simpson and Dallal, 1989; Pliška, 1994).

Using these quantitative characteristics, we were probing for correlations among F-VIII enhancing activity, activation of plasminogen activator and the spectrum of 'classical' biological activities of neurohypophyseal hormones. Although the number of peptides was rather low and restricted to representatives of vasopressin V_1, V_2 and oxytocin receptor agonists (besides a few 'mixed' cases), a clear-cut linkage between the two investigated activities and substances belonging predominantly to the vasopressin V_2 receptor agonists was demonstrated. [Phe^2,Orn^8]Oxytocin, a peptide with a high vasopressin V_1 receptor selectivity, also possesses a high antihaemophilic and fibrinolytic activity in our experiments. Its maximal response, however, was lower when compared to the effects of the vasopressin V_2 receptor agonists. Thus, this analogue seems to act as a 'partial' agonist with regard to the two activities. Substances with the high oxytocin selectivity (see Table 1) elicit rather irregular F-VIII and plasminogen activator responses; dose–response relationships were not significantly different from physiological saline controls.

The time-course of the F-VIII response displays a biphasic form. Its characteristic feature is a surge about 20 min after peptide administration, followed by a decrease to

a minimum at roughly 30–40 min, and a rebound phase with a second maximum at 60–90 min. This form, although commonly encountered in in vivo and also some in vitro responses, cannot be satisfactorily explained at present. An effect of a quick, stress-induced release of F-VIII at the time of injection seems unlikely: pre-treatment by both analgesics and sedatives failed to influence the time-response curves in our experiments. Moreover, the high plasma disappearance rate is simply not consistent with the rather long half-life of F-VIII. This is also valid for the duration of the second decline (90–120 min). The counteracting fibrinolytic response to deamino[D-Arg^8]vasopressin cannot be the only cause of this non-linearity either, as attempts to influence the form of the curve by a strongly acting protease inhibitor were negative. Whereas these findings may not be the final word, given that not all conditions were tested in our experiments, nothing else speaks in favour of such explanations at present. Several other alternative mechanisms can be hypothesised: the depletion of F-VIII after a certain level of clotting response has been reached; the involvement of a non-linear, feedback-driven distribution of F-VIII in the vascular/extravascular body compartments; or a concomitant rise of the von Willebrand factor. None of these, or other, hypotheses have been as yet tested experimentally, but the facts seem to indicate the existence of complex F-VIII kinetics following its stimulated release. The time-course of the plasminogen activator response, on the other hand, was found to be much less regular. It usually displays a single peak at about 30 min after the injection of peptides, reaching baseline values within 60–90 min. Whereas the variation in the shape of this time-response curve is large, the integral values show a satisfactory reproducibility.

The question of side effects is of particular clinical importance. As far as sheep are concerned, we did not observe any strong antidiuretic response even at the highest peptide dosage used. Neither did we observe any strong increases in arterial blood pressure, as measured in the ear artery of some animals (pressure transducers were used). However, our experimental setup was not designed to yield conclusive results in this respect, since the hydration state, etc., of the animals was not quantitatively controlled. Our rough observations indicated that sheep renal and/or vascular responses to neurohypophyseal hormones were less pronounced than, for instance, in rats. On the other hand, we could demonstrate an astonishingly rapid and strong haemodilution shortly after the administration of peptides, predominantly of those with a substantial antidiuretic potency. The haemodilution was reflected in the decrease of the packed cell volume (PCV) and of the total plasma protein concentration. The decrease in PCV was about twice as pronounced as that of the protein concentration. This remarkable difference may indicate the involvement of several mechanisms controlling the haemodilution process, some of them perhaps being specific for ruminants. Reuptake of water through the rumen and/or a redistribution of the circulating blood cells, in response to vasoactive peptides, may contribute to this phenomenon.

Finally, the problem of potency estimates should be addressed. Variations of the two responses among individuals are considerable. Estimates of biological potencies, as given in Table 2, are rather rough but suggest clear-cut structure-activity trends. As such, they can be subjected to robust methods of structure-activity analysis (Rousseeuw, 1984; Hampel, 1986; Rousseeuw and Leroy, 1987), as soon as more comparative data are available (Pliška and Neuenschwander, 1994). Provided that such an analysis allows more insight into their structural dependence, the design of new analogues with enhanced potencies, with dissociated antihaemophilic and plasminogen activator activities, and with diminished renal and other side effects, may be greatly facilitated.

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