V₂ Receptor Antagonism of DDAVP-Induced Release of Hemostasis Factors in Conscious Dogs

Sanofi Recherche, Toulouse, France
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ABSTRACT

The synthetic arginine vasopressin (AVP) analog 1-desamino-8-d-arginine vasopressin (DDAVP) is used in a variety of hemorrhagic disorders. The present experiments were designed to further characterize the mechanism of DDAVP-induced release of hemostasis factors. The [³H]AVP-labeled AVP receptor in canine renal medullary membranes exhibited an AVP V₂ profile because the V₂ receptor agonist DDAVP displayed similar subnanomolar affinities as the natural hormone AVP, whereas the two selective V₁a compounds SR 49059 and d(CH₂)₅Tyr(Me)-AVP as well as the selective V₁b agonist d-Pal and oxytocin were much less potent. The rank order of the binding affinities of three V₂ receptor antagonists was SR 121463 (a newly described selective V₂ receptor antagonist) > OPC 31260 >> d(CH₂)₅[deamino-Cys₁, D-3-(pyridyl-1)-Ala²,Arg⁸]AVP. In conscious dogs, DDAVP (0.1–1 mg/kg IV) caused a dose-related increase (maximum, 43–52% at 30 min) in plasma levels of factor VIII (FVIII), von Willebrand factor (vWF) and tissue-type plasminogen activator (t-PA), but not in levels of plasminogen activator inhibitor-1. A DDAVP-induced hemostasis factor release was also observed in bilaterally nephrectomized dogs. Pretreatment with SR 121463 inhibited DDAVP-induced (1 μg/kg IV) increases in FVIII, vWF and t-PA plasma levels in a dose-dependent manner (ID₅₀ = 14.0 ± 4.0, 12.4 ± 3.0 and 16.7 ± 1.0 μg/kg IV, respectively). OPC 31260 (300 μg/kg IV) revealed a lower activity than SR 121463, and d(CH₂)₅[d-lle²,lle⁴]AVP (30 μg/kg IV) was without effect on the DDAVP response. Pretreatment with SR 49059 (1 mg/kg IV) and SR 27417 (a platelet-activating factor receptor antagonist) (1 mg/kg IV) had no effect on the DDAVP-induced (1 μg/kg IV) increases in FVIII, vWF and t-PA plasma levels. The present results, therefore, strongly suggest that the effect of DDAVP on hemostasis factors occurs via a specific interaction with extrarenal V₂ receptors.

The synthetic AVP analog DDAVP is used in a variety of hemorrhagic disorders. It is used as an antihemorrhagic agent in hemophilia and in von Willebrand disease (Manucci et al., 1977), and it has been used to reduce bleeding side effects caused by various compounds, including aspirin (Flordal and Sahlin, 1993), streptokinase (Johnstone et al., 1996). Despite the wide use of DDAVP in these clinical situations, the exact mechanism of DDAVP-induced release of hemostasis factors is not fully understood. DDAVP is a relatively V₂-specific AVP agonist with minimal smooth muscle activity and strong and prolonged antidiuretic action, but it also reveals AVP V₁ₐ (Wun et al., 1995) and Vₙ (also called V₁ₙ) (Ammar et al., 1994) receptor agonist activity. DDAVP stimulates the release of FVIII, vWF and t-PA from endothelial cells (Abreg et al., 1979; Johnson et al., 1986; Lethagen, 1994). The DDAVP-induced clotting factor release has been postulated to involve extrarenal V₂-like receptors (Bichet et al., 1988). Reversal of the DDAVP clotting factor response by using selective V₂ receptor antagonists was employed as a method to test this hypothesis. However, only data with peptide V₂ receptor antagonists have been available, and reported effects of these substances on DDAVP-induced hemostasis factor release are controversial: SKF 105494 was found to be active in the monkey (Kinter et al., 1992), whereas d(CH₂)₅[d-lle²,lle⁴]AVP was inactive in the dog (Vilhardt and Barth, 1991). Peptide AVP V₂ receptor antagonists also showed interspecies differences in antagonizing the antidiuretic AVP action (Bichet et al., 1988). In addition, AVP analogs are not very selective V₂ antagonists, and chronically administered AVP analogs lose their antagonistic properties and show an agonistic activity (Hofbauer et al., 1986). Furthermore, peptide AVP V₂ receptor antagonists are limited to parenteral use. SR 121463 and OPC 31260 are two novel, highly potent and selective nonpeptide antagonists for V₂ AVP receptors. They possess a high affinity for renal V₂ receptors, inhibit AVP-induced cAMP formation and reveal an aquaretic effect in rats, dogs and monkeys (Serradeil-Le Gal et al., 1996; Yamamura et al., 1992).

Animal models to investigate the mechanism of DDAVP-

ABBREVIATIONS: AVP, arginine vasopressin; DDAVP, 1-desamino-8-d-arginine vasopressin; PAF, platelet-activating factor; FVIII, factor VIII, t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; vWF, von Willebrand factor; BSA, bovine serum albumin; d-Pal, [deamino-Cys₁, d-3-(pyridyl-1)-Ala²,Arg⁸]AVP.
induced effects on the coagulation and fibrinolytic systems are lacking. Unlike humans, rats and pigs do not show a DDAVP-induced clotting factor response, but experiments with V$_2$ receptor agonists suggest that dogs and rhesus monkeys may be appropriate (Kinter et al., 1992; Vilhardt and Barth, 1991). The present experiments were designed to further characterize the mechanism of DDAVP-induced release of hemostasis factors in conscious dogs.

Methods

**Drugs and dosage.** DDAVP, oxytocin, d(CH$_2$)$_5$Tyr(Me)-AVP, bacitracin, d(CH$_2$)$_5$[D-lle$_2$,lle$_4$]AVP and p-Pal were from Sigma Chemical Co. (Lisle d’Aubeau, France). BSA (type V) was obtained from IBF (Villeneuve La Garenne, France). EDTA, Tris and dimethylsulfoxide were from Merck-Clevenot (Nogent sur Marne, France). $[^{3}H]$AVP (80 Ci/mmol) was purchased from New England Nuclear (Les Ulis, France). The V$_2$ receptor antagonist d(CH$_2$)$_5$[D-lle$_2$,lle$_4$]AVP and $[^{3}H]$AVP were from Peninsular Lab Ltd. (Belmont, CA). The V$_1a$ receptor antagonist SR 49059 (Serradeil-Le Gal et al., 1993), the V$_2$ receptor antagonists SR 121463 and OPC 31260 and the PAF receptor antagonist SR 27417 (Herbert et al., 1991) were from Sanofi Recherche (Toulouse, France). For the in vitro studies, DDAVP, d(CH$_2$)$_5$[D-lle$_2$,lle$_4$]AVP and SR 121463 were dissolved in saline. SR 49059 was dissolved in a solution containing ethanol, H$_2$O, glycerol and polyethylene glycol (60:30:5.5 v/v). SR 27417 and OPC 31260 were solubilized in 0.1 N HCl in saline (1:40 v/v). All substances were administered intravenously as solutions prepared daily before the administration. Control dogs were treated with saline. For in vivo binding experiments, SR 121463, SR 49059 and OPC-31260 were dissolved in dimethylsulfoxide (10$^{-2}$ M) and then diluted in the test solvent.

**Membrane preparations.** Both kidneys from pentobarbital-anesthetized male mongrel dogs were chilled in ice-cold saline. The renomedullary regions, which constitutively express AVP V$_2$ receptors, were immediately dissected. Membranes were prepared according to the method of Stassen et al. (1982) as described recently (Serradeil-Le Gal et al., 1996) and stored as aliquots in liquid nitrogen at a final concentration of $\sim$10 mg of protein/ml. Protein concentration was determined according to Bradford (1976) with BSA as a standard.

**AVP V$_2$ binding assay.** Renomedullary membranes (100–150 $\mu$g/assay) were incubated for 45 min at 25°C in a 50 mM Tris HCl buffer, pH 8.1, containing 2 mM MgCl$_2$, 1 mM EDTA, 0.1% BSA, 0.1% bacitracin, 3 nM $[^{3}H]$AVP and increasing amounts of the test compounds. The reaction was stopped by the addition of 4 ml of ice-cold buffer followed by filtration through GF/B Whatman glass microfiber filters. Filters were washed twice with 4 ml of ice-cold buffer and counted by liquid scintillation using a beta scintillation counter (Packard, Tricarb). Saturation experiments were performed with increasing concentrations of $[^{3}H]$AVP (0.03–15 nM). Nonspecific binding was determined by incubation with 1 $\mu$M AVP. Data for equilibrium binding ($K_d$, apparent equilibrium dissociation constant, and $B_{max}$, maximum binding density) and competition experiments ($IC_{50}$, $n_{p}$) were analyzed by an iterative nonlinear regression program using the software RS.1 (Munson and Rodbard, 1980). The $IC_{50}$ value was defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding. Inhibition constant ($K_i$) values were calculated from the $IC_{50}$ values using the Cheng and Prusoff equation (1973).

**Animals and procedures.** Twelve-month-old male mongrel dogs weighing 18 to 26 kg were used. The animals were fed a standard laboratory chow (Doko, Fontaine-les-Vervins, France), and tap water was available ad libitum. Before the experiments, the dogs were fasted overnight. They were trained to stand quietly on a table. Blood (4 ml) was collected through venipuncture of cephalic veins immediately before and at indicated intervals after intravenous injections of DDAVP in tubes containing trisodium citrate (3.8%, 1/9 v/v) for measurements of vWF, FVIII and PAI-1 plasma concentrations. At <2 min after withdrawal, 300 $\mu$l of sodium acetate (0.2 M) was added to 300 $\mu$l of blood for determination of t-PA activity. Blood samples were immediately centrifuged at 4°C (1000 × g for 15 min). The plasma was kept at −80°C until use. Antagonists were injected intravenously 5 min before administration of DDAVP. Some dogs repeated the protocol with a minimum interval of 1 week between studies. In these dogs, no tolerance to DDAVP-induced release of hemostasis factors was observed.

In another series of experiments, dogs were anesthetized with pentobarbital (30 mg/kg i.p.), and both kidneys were removed. Blood collections after DDAVP injection to the anesthetized dogs were performed as described above.

The protocol of this study has been approved by the Animal Care and Use Committee of Sanofi Recherche, Toulouse.

**Analytical methods.** FVIII (VIII:C) levels in plasma were measured using an immunodepleted plasma from Diagnostica Stago (Asnières, France). vWF was measured by means of an enzyme immunosorbent assay procedure with the Asserachrom vWF kit (Diagnostica Stago, Asnières, France). Activity of t-PA was determined with CoaTest t-PA by measuring the amidolytic activity of plasmin from the chromogenic substrate S-2251. The PAI plasma activity was determined with Coatest PAI (Chromogenix, Mölndal, Sweden) by measuring the plasmin formed from plasminogen in the presence of t-PA from the chromogenic substrate S-2403.

**Data analysis.** Results are expressed as mean ± S.E.M. Statistical analyses were performed using the Mann-Whitney U test, and P < 0.05 was accepted as a significant difference. ID$_{50}$ values were calculated by fitting the logistic equations to the data by linear regression.

Results

**Binding studies.** AVP receptors in canine renomedullary membranes were characterized using $[^{3}H]$AVP as a ligand. Saturation experiments, performed with increasing concentrations of $[^{3}H]$AVP, showed that the specific binding was saturable. Scatchard analysis of the data (fig. 1) gave a linear plot consistent with the presence of a single class of high-affinity binding sites with a $K_d$ and a $B_{max}$ value of 0.32 ± 0.03 nM and 100 ± 4 fmol/mg of protein, respectively. A
the four parameters were stable for the duration of the study. Initial plasma activities for t-PA and PAI-1 plasma activity were recorded during the 4-hr observation period (data not shown). No significant changes in PAI-1 plasma activity were recorded during the 4-hr observation period (data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Inhibition constant (Kᵢ) (nM)</th>
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<tbody>
<tr>
<td>AVP</td>
<td>0.31 ± 0.04</td>
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<tr>
<td>DDAVP</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>dP-Pal</td>
<td>50 ± 11</td>
</tr>
<tr>
<td>d(CH₂)₅[lle²,lle⁴]AVP</td>
<td>122 ± 11</td>
</tr>
<tr>
<td>d(CH₂)₅[Tyr(Me)-AVP]</td>
<td>251 ± 5</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>122 ± 7</td>
</tr>
<tr>
<td>Nonpeptide</td>
<td></td>
</tr>
<tr>
<td>SR 121463</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>OPC 31260</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>SR 49059</td>
<td>258 ± 20</td>
</tr>
</tbody>
</table>

Inhibition constants (Kᵢ) were calculated according to Cheng and Prusoff (1973). Values are mean ± S.E.M. (n = 6).
dose of 300 \( \mu g/kg \) i.v., DDAVP-induced increases in FVIII plasma concentrations were inhibited by only 57%. As can be seen in figure 4, pretreatment with \( d(CH_2)_5[D-lle^2,lle^4]AVP \) at 30 \( \mu g/kg \) i.v. did not reveal any effect on the DDAVP FVIII response. Similarly, \( d(CH_2)_5[D-lle^2,lle^4]AVP \) (30 \( \mu g/kg \) i.v.) did not inhibit DDAVP-induced (1 \( \mu g/kg \) i.v.) increases in vWF and t-PA plasma concentrations, whereas OPC 31260, at 300 \( \mu g/kg \) i.v., exhibited a moderate inhibitory activity of 42% and 49%, respectively, on these parameters (\( n = 3 \) in the SR 27417-treated groups, \( n = 16 \) in the control group). 100% refers to the values measured before DDAVP administration. For clarity, S.E.M. bars are presented for the saline-treated group only. * \( P < .05 \) compared with the groups treated with saline.

This dose, SR 49059 alone did not influence the measured parameters during the 4-hr observation period.

**Influence of PAF receptor antagonism on DDAVP effects.** Another series of in vivo experiments was performed to examine whether PAF was involved in the DDAVP-induced release of hemostasis factors as suggested by Hashemi et al. (1993). At 5 min before DDAVP (1 \( \mu g/kg \) i.v.) administration, dogs were pretreated i.v. with 1 mg/kg of the PAF receptor antagonist SR 27417. As can be seen in figure 5, SR 27417 pretreatment did not modify DDAVP-induced increases in FVIII, vWF and t-PA levels in plasma. At this high dose, SR 27417 alone did not influence the measured parameters during the 4-hr observation period.

**Discussion**

The present results confirm that the intravenous administration of DDAVP induces a release of FVIII, vWF and t-PA in conscious dogs. Comparable increases in plasma levels of these proteins with similar kinetics have been previously described in conscious dogs (Vilhardt and Barth, 1991) and anesthetized monkeys (Kinter et al., 1992), whereas humans are more sensitive to DDAVP-induced release of vWF and t-PA (Mannucci et al., 1975). The possible influence of DDAVP on PAI-1 plasma activity has not previously been reported. Present results in conscious dogs do not demonstrate evidence of a involvement of PAI-1 in the effect of DDAVP to release hemostasis factors.

SR 121463 and OPC 31260, two novel, selective nonpeptide antagonists for \( V_2 \) receptors, possess a high affinity for renal \( V_2 \) receptors and inhibit AVP-induced cAMP formation (Manning et al., 1984; Serradeil-Le Gal et al., 1996; Yamamura et al., 1992). Both compounds also revealed potent in vivo activities: OPC 31260 exerted an aquaretic effect in rats, dogs,
monkeys and humans, and SR 121463 showed aquaretic activity in rats (Fujisawa et al., 1993; Serradeil-Le Gal et al., 1996; Yamamura et al., 1992). In the present study, SR 121463 inhibited DDAVP-induced releases of FVIII and vWF in a dose-dependent manner with ID50 values of 14.0 ± 4.0 and 12.4 ± 3.0 µg/kg i.v., respectively. OPC 31260 revealed a lower activity than SR 121463 in inhibiting DDAVP-induced clotting factor release. In addition, the present study is the first demonstration of an inhibition of DDAVP-induced t-PA release via V2 receptor antagonism.

The inhibitory actions of SR 121463 and OPC 31260 on DDAVP-induced increases in plasma concentrations of FVIII, vWF and t-PA. Saline (○), SR 49059 (1 mg/kg i.v., □) or SR 27417 (1 mg/kg i.v., ▼) were administered 5 min before DDAVP (1 µg/kg i.v.). Plasma concentrations of FVIII, vWF and t-PA were measured as described in the text (n = 3 in the groups treated with SR 49059 or SR 27417, n = 16 in the control group). 100% refers to the values measured before DDAVP administration. Differences between the SR 49059- or SR 27417-treated groups and the control group were not significant (P > .05).

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Contrary to this, Vilhardt and Barth (1991) reported failure to block DDAVP-induced release of FVIII and t-PA by the V2 receptor antagonist d(CH2)5[D-lle2,lle4]AVP in dogs and concluded that the ability of DDAVP to release FVIII and t-PA does not involve V2 receptors. This conclusion, however, must be considered premature. Despite close structural similarities between AVP V2 receptors cloned in several species such as rat, pig, cow and humans (Lolait et al., 1994; Birnbaumer et al., 1992; Gorbulev et al., 1993), marked interspecies differences exist for AVP V2 receptors on the basis of affinity and efficacy of certain AVP analogs (Ufer et al., 1995; Guillen et al., 1982). Peptide V2 receptor antagonists with branched side-chain amino acid substitutions at positions 2 and 4, such as d(CH2)5[D-lle2,lle4]AVP, show substantial interspecies variability in affinity for renal V2 receptors; they are active as antiaduretic antagonists in dogs (Kinter et al., 1992). In line with these findings are the present in vivo observations that d(CH2)5[D-lle2,lle4]AVP did not reveal an inhibitory effect on DDAVP-induced release of hemostasis factors. These data suggest a low affinity of d(CH2)5[D-lle2,lle4]AVP to canine V2 receptors.

The present in vitro results with canine renomedullary membrane preparations confirm this conclusion. The affinity of d(CH2)5[D-lle2,lle4]AVP was more than 2 orders of magnitude lower than that of SR 121463. d(CH2)5[D-lle2,lle4]AVP strongly discriminates between dog and rat V2 receptors because this compound, which is well known for its potent anti-V2 diuretic properties in rats (pA2 = 8.04), was found in this study to be ~100-fold less potent at dog (Kd = 122 nM) than at rat (Kd = 1.1 nM) V2 receptors (Manning et al., 1984). The present in vitro findings also give a good explanation for the differences in in vivo activity between SR 121463 and OPC 31260 to inhibit the release of hemostasis factors. The observed affinity of OPC 31260 in this study is in agreement with previous results obtained in rat, bovine and human preparations (Serradeil-Le Gal et al., 1996).

Although the agonist activity of DDAVP is generally considered to be V2 mediated, DDAVP also possesses V1a and V3 receptor agonist activity (Wun et al., 1995; Ammar et al., 1994). A V1a receptor-mediated mechanism has been described for DDAVP-induced platelet activation (Wun et al., 1995). In the current model, however, the V1a receptor antagonist SR 49059 had no effect on the DDAVP-induced increases in FVIII, vWF and t-PA plasma levels. Taken together, these data strongly suggest that the stimulation of extrarenal V2-like receptors is the main mechanism involved in the DDAVP-induced release of FVIII, vWF and t-PA. This conclusion is also in agreement with the clinical findings that male patients with congenital X chromosome-linked nephrogenic diabetes insipidus do not exhibit a rise in FVIII plasma concentrations when treated with DDAVP (Kobrinsky et al., 1985). Diuresis in these patients is due to mutation of renal V2 receptors; perhaps the missing DDAVP-clotting factor response is due to the same mutation of extrarenal V2(-like) receptors. There is no evidence as yet to the location of the V2 AVP receptors that are involved in the DDAVP response and intracellular signaling mechanisms. Because bilateral nephrectomy did not prevent the effect of DDAVP in the present experiments, it appears that the receptors responsible for the increase in hemostasis factors are extrarenal. Further evidence for the existence of extrarenal V2 receptors has been previously provided. The DDAVP-induced releases of cAMP.
in anephric dogs and of hemostasis factors in surgically anephric patients were not different from those in the control groups with intact kidneys (Liard, 1992; Mannucci et al., 1975). It is tempting to speculate that the extrarenal V2 receptors accounting for the DDAVP-induced increase in hemostasis factors might be on endothelial cells; however, there is no proof of the existence of V2 receptors on endothelial cells.

The involvement of PAF in the DDAVP-clotting factor response has been postulated by Hashemi et al. (1993). Their in vitro findings indicated that the stimulation of endothelial cells by DDAVP may be an indirect effect mediated through stimulation of a monocyte V3 receptor. In turn, PAF would be secreted by DDAVP-treated monocytes and enhance the release of vWF from endothelial cells. Present in vivo experiments with the PAF receptor antagonist SR 27417, however, do not indicate an involvement of such a mechanism in the DDAVP-induced release of vWF or in the release of FVIII and t-PA.

In summary, the present in vivo results in conscious dogs, together with the in vitro findings in canine renomedullary membranes, strongly support the conclusion that the effect of DDAVP on hemostasis factors occurs via a specific interaction with extrarenal V3 or V2-like receptors, whose localization remains to be further explored. The present data argue that the effect of DDAVP does not involve PAF or V1a receptors.

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References


