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FEBS Letters 356 (1994) 215–220

FEBS 14881

# Cloning and characterization of the human V3 pituitary vasopressin receptor

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Received 9 November 1994

**Abstract** Arginine-vasopressin (AVP) plays a determinant role in the normal ACTH response to stress in mammals. We cloned a human cDNA coding a 424 amino acid G-protein coupled receptor structurally related to the vasopressin/oxytocin receptor family . When expressed in COS cells, this receptor binds AVP with a high affinity ($K_d$ = 0.55 ± 0.13 nM) and is functionally coupled to phospholipase C. Competition studies with peptidic or non peptidic AVP analogues reveal that it is pharmacologically distinct from V1a and V2 AVP receptors and therefore it is designated V3. RT-PCR analysis shows that the human V3 receptor is expressed in normal pituitary and also in kidney, but is undetectable in liver, myometrium and adrenal gland. Northern blot analysis reveals a ~4.8 kb messenger in human corticotropic pituitary adenomas.

*Key words:* Arginine-vasopressin; Pituitary; Phospholipase C; G-protein coupled receptor; ACTH

## 1. Introduction

The existence of a pituitary-specific receptor for arginine-vasopressin (AVP) participating in the stress-induced ACTH surge, has been surmised for many years [1]. AVP is a direct ACTH secretagogue which also exerts a synergistic effect on corticotropin releasing hormone (CRH) action in many species including in human [2].

The multiple actions of AVP are mediated by different membrane-bound receptors: the vascular V1a receptor coupled to phospholipase C (PLC) and the renal V2 receptor which activates adenylate cyclase [3]. Several groups recently cloned these receptors in different species and identified a new subfamily including receptors for vasopressin, oxytocin and vasotocin [4–10]. All the members of the AVP/oxytocin receptor family are G protein-coupled receptors with a classical structure made of a single polypeptide chain with seven transmembrane domains (TMD).

AVP action on corticotroph cells is mediated by a specific receptor which activates PLC [11, 12]. However, pharmacological studies suggest that the pituitary-specific AVP receptor is distinct from both the V1a and the V2 receptors, hence its various designations as either V1b or V3 [13,14].

We present here the structure, functional expression and tissue distribution of a cDNA encoding an AVP receptor cloned from a human pituitary corticotropic tumor responsible for a case of Cushing's disease. This new receptor has the characteristics of a pituitary-specific PLC-coupled AVP receptor that should be recognized as the V3 AVP receptor. Surprisingly, it presents similar degrees of identity with the V1a, oxytocin and vasotocin receptors, and therefore is not closely related to the V1a receptor.

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The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under Accession Number L37112 (submitted the 10-25-94).

## 2. Materials and methods

### 2.1. cDNA library construction

Total RNA was prepared by the guanidium/caesium chloride method [15] from a human (h) corticotropic adenoma obtained at surgery from a patient with Cushing's disease. Poly(A)$^+$ RNA was isolated using Dynabeads (Dynal). A cDNA library of $3 \times 10^6$ primary recombinants was generated from 5 μg of poly(A)$^+$ RNA using the λZAPII cDNA cloning kit (Stratagene).

### 2.2. Library screening

A 1.5 kb *Eco*RI fragment containing the entire coding region of the human liver hV1a receptor cDNA [10] was used to screen the library under low stringency conditions (final wash: 2 × SSC, 0.1% SDS, 50°C for 30 min). One partial hV3 receptor cDNA clone was isolated and used for subsequent screening under standard conditions to identify full-length cDNAs. Nucleotide sequencing of both strands was done by the dideoxy chain-termination method using the Sequenase sequencing kit (United States Biochemicals Corp.).

### 2.3. Transient expression in COS-7 cells

The cDNA coding for the entire sequence of the hV3 receptor was inserted as a 2.2 kbp *Eco*RI fragment into the expression vector pECE [16]. Transient expression of the hV3 receptor was obtained by transfection of COS-7 cells, using the DEAE-Dextran method [10]. Expression of the receptors was observed between day 2 and 10 after transfection.

### 2.4. Radioligand binding assays

Saturation binding experiments of hV3 receptors of confluent control and transfected COS-7 were performed as previously described [10] with increasing concentrations of [$^3$H]AVP ± 1 μM unlabeled AVP. Affinity and capacity of the hV3 receptors were calculated by a non-linear least square analysis program (Ebda Ligand; Elsevier-Biosoft, Cambridge, UK). Competition binding experiments were performed on COS-7 cells expressing hV3 or CHO cells expressing the V1a human receptor in the presence of 2 nM [$^3$H]AVP and increasing concentrations of competitors. AVP, oxytocin (Oxy), arginine-vasotocin (AVT) were from Sigma. The V1a antagonist d(CH$_2$)$_5$Tyr(Me)AVP, and the linear V1a antagonist Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH$_2$ (TyrPhaa) were from Bachem. The non peptidic V1a antagonist SR 49059 was provided by Dr. C. Serradeil-Le Gal, Sanofi Recherche, Toulouse, France. The non peptidic rat V1a antagonist OPC21268 (batch number 93F92M), and the non peptidic V2 antagonist OPC 31260 (batch number 93D96M) were provided by Dr. J.F. Liard, Otsuka America Pharmaceutical, Inc. Rockville, MD. The linear V1a

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*SSDI* 0014-5793(94)01268-7

antagonists Phenylacetyl D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-$NH_2$ (PhaaGln), 4-hydroxy-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-$NH_2$ (OHPhaaGln), and tBaa-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Arg-$NH_2$ (tBaa), the V2 antagonist d($CH_2$)$_5$[D-Ile$^2$-Ile$^4$-Ala-$NH_2$] AVP (V2ag), and the V1a/V3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-$NH_2$ were provided by Dr. M. Manning from the Medical College of Ohio in Toledo. All experiments were performed in duplicate and incubated 60 min at room temperature. After extensive washing, cells were solubilized in 0.5 ml of 0.2 N NaOH + 1% SDS and counted in a $\beta$ counter.

*2.5. Inositol phosphate (IP) determination*

Vasopressin-induced IP production was determined in non transfected and transfected COS-7 cells as previously described [17]. Cells were incubated with AVP (1 pM to 1 $\mu$M) in presence of 10 mM LiCl for 30 min.

*2.6. Northern blot analysis*

Pituitary tumors were collected at surgery and normal pituitaries were obtained at autopsy. Total and poly(A)$^+$ RNAs were prepared as described in the library construction paragraph. RNAs were denatured with 15 mM $CH_3HgOH$, separated on a 1% agarose/2.2 M formaldehyde gel and transferred onto BAS-85 nitrocellulose sheet (Schleicher and Schuell). The membrane was hybridized under classical conditions with a 1.3 kb *Eco*RI fragment of hV3 receptor [18]. The probe encompassing 5′ untranslated sequence and most of the coding region (up to TMD VI) was labeled by random priming with [$\alpha^{32}$P]dCTP. After the final wash the blot was autoradiographed on XAR film (Kodak) with intensifying screen (DuPont).

*2.7. RT-PCR analysis*

RT-PCR was performed according to standard protocols [18]. Briefly, random primed DNA were synthesized from 0.5 $\mu$g of total RNA with 20 units of MMLV reverse transcriptase (BRL). Half of the cDNA reaction was used for PCR with two oligonucleotides flanking TMS VI and VII. After 40 cycles of amplification 10% of the PCR products were analyzed on 2% agarose gel, blotted onto Nylon membrane and probed with an internal oligonucleotide.

## 3. Results and discussion

Biochemical and pharmacological studies performed in rat anterior pituitary primary cultures have shown the existence of a pituitary-specific receptor for AVP related to the V1 subfamily [13]. This receptor however is pharmacologically distinct from the already known V1a-receptor present in liver, brain, adrenals and in the vascular smooth muscle cells. For this reason, in an attempt to isolate a V3-receptor cDNA, we used a human V1a cDNA probe to screen a corticotropic pituitary adenoma library under low stringency conditions. This screening resulted in the identification of a 4.5kb cDNA, containing an open reading frame of 1,272 bp coding for a 424 amino acid protein ($M_r$ = 46,977) thereafter named hV3 (Fig. 1).

The hydropathicity profile [19] of hV3 shows the typical features of a G protein-coupled receptor with seven putative transmembrane hydrophobic domains of 20–24 residues linked by three extracellular and three intracellular loops. The N-terminal region preceding the first TMD contains a single putative N-linked glycosylation site at Asn$^{21}$. The third intracellular loop and the C-terminal tail contain numerous seryl and threonyl residues, many of which are in good sequence context for phosphorylation by protein kinase C or casein kinase II [20], and may therefore play a role in the regulation of receptor function. There is conservation of many of the cysteyl residues that are present in other members of the AVP/oxytocin receptor family. Some of them may form disulfide bridges (Cys 107 and 186 between the extracellular loops 1 and 2) or anchor the C-terminal tail into the membrane after palmitoylation (Cys$^{355}$, Cys$^{356}$ or Cys$^{385}$) [21].

A search in the protein data bank (Swissprot) revealed marked sequence identities between hV3 and the other members of the AVP/oxytocin receptor family (Fig. 2A). A relatively high degree of amino-acid sequence identity is observed with the human V1a, oxytocin and V2 receptors (45.5, 44.8 and 37.3%, respectively), as well as with the animal members of this receptor family (46.2% with rat V1a , 37.8% with rat V2, 44.3% with porcine oxytocin, 38.9% with porcine V2 and 45.7% with fish vasotocin receptors). The overall identity with other 7TMD receptors was below 15% (e.g. the human GnRH and gastrin/cholecystokinin-B receptors (data not shown)).

Amino acid identities between hV3 and other member of the AVP/oxytocin receptor family are not evenly distributed throughout the sequences. N- and C-terminal extremities show almost no identity except for a few residues adjacent to TMD I and VII. Within the TMD, identity varies from 36 to 87%, with the most conserved TMD being TMD II, III, VI, and VII. The putative common epitopes which delineate the binding site of the natural hormone AVP within the extracellular loops and TMD are not readily apparent. The first extracellular loop and the distal part of the second extracellular loop are strongly conserved within the AVP/oxytocin receptor family, but sequences within TMDs may also participate in forming the binding site. It is interesting to note that two residues of hV3 (Ala$^{82}$ in TMD II and Val$^{209}$ in TMD V) are strictly conserved in all known vasopressin receptors, but are different in all vasotocin and oxytocin receptors. The sequences involved in G protein coupling and PLC activation are probably located in the second intracellular loop and the proximal and distal parts of the third intracellular loop [22]. These sequences are well conserved between hV3 and the other receptors of the family coupled to PLC (50 to 78% identity) but diverge with those of the V2 receptor subtypes (23 to 30% identity).

At the nucleotide level the strongest homology is found between hV3 and the human oxytocin receptor. This special relationship between hV3 and the oxytocin receptors was further assessed by constructing a phylogenetic tree from the progressive alignment of the receptor sequences (UPGMA method, Fig. 2B). This tree indicates that the hV3 receptor did not diverge recently from the hV1a receptor as suspected, but instead evolved at an earlier stage from a common ancestor.

Since the subtype classification of the hV3 was unknown, radioligand binding characterization was initiated with the native hormone [$^3$H]AVP itself in transiently transfected COS-7 cells. These experiments confirm that hV3 binds AVP with a high affinity. Scatchard plotting (data not shown) indicates a single class of binding sites with a dissociation constant $K_d$ = 0.55 ± 0.13 nM, in good agreement with $K_d$ values of 0.4 nM to 4 nM previously reported in studies on human or animal pituitaries [14,23].

Competition binding experiments with the natural hormones AVP ($K_i$ = 0.72 ± 0.17 nM), vasotocin ($K_i$ = 6.7 ± 3.7 nM), and oxytocin ($K_i$ = 479 ± 99 nM) reproduce the order of potency observed in autoradiographic studies performed by [$^3$H]AVP displacement on human anterior pituitary tissues (vasopressin > vasotocin >> oxytocin) and show that hV3 does not belong to the oxytocin receptor subtype. The V2 specific antagonists d($CH_2$)$_5$[D-Ile$^2$-Ile$^4$-Ala-$NH_2$]AVP and OPC31260 have a poor affinity for the hV3 receptor ($K_i$ = 499 ± 200 nM, and

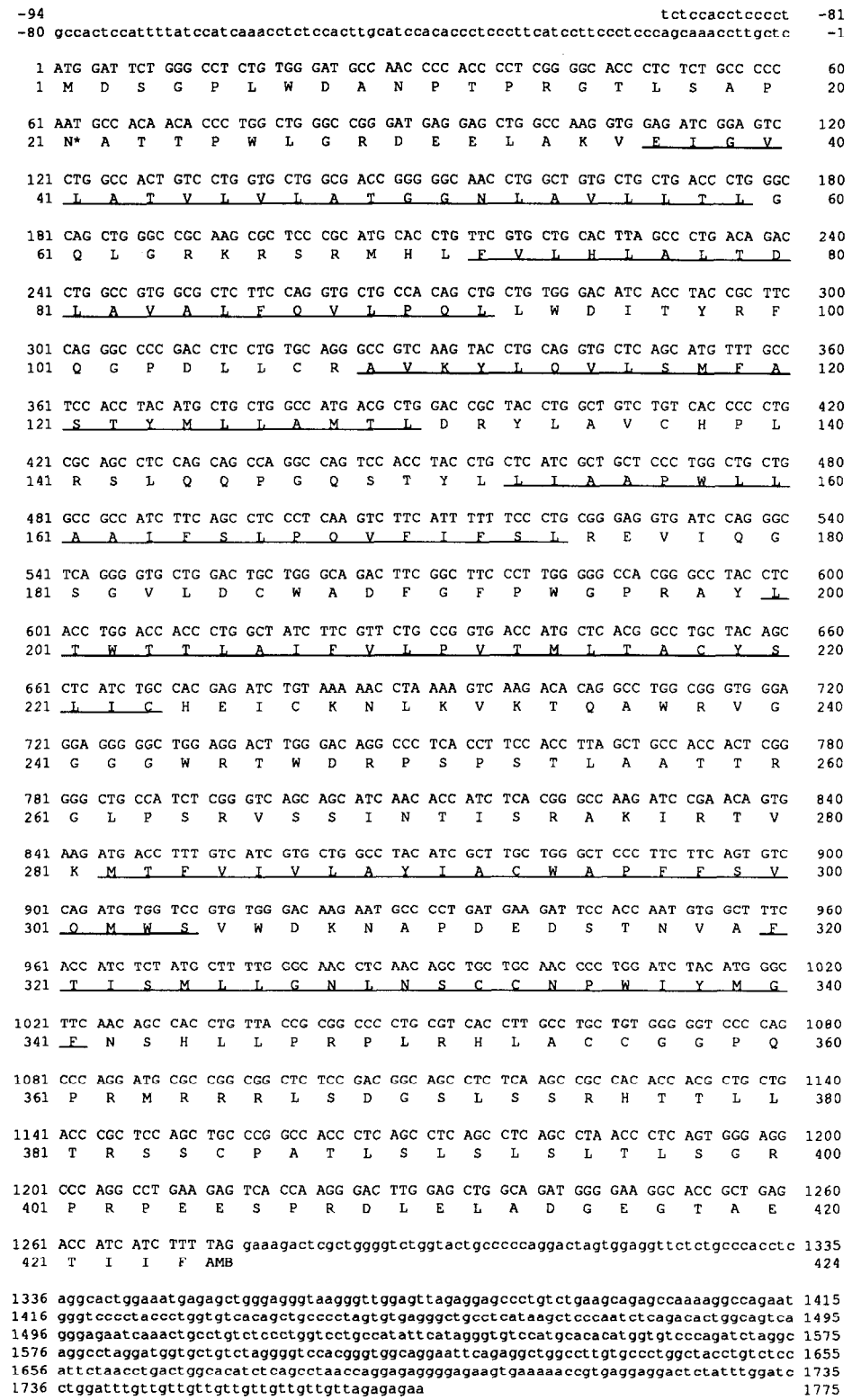

Fig. 1. Primary nucleotide and amino acid sequences of the human V3 receptor. Nucleotides are numbered from the putative starting codon. The putative transmembrane domains are underlined. The potential N-linked glycosylation site is shown by an asterisk.

>1000 nM respectively), which therefore does not belong to the V2 subtype either. The affinity constants for hV3 and hV1a receptors of various peptidic and non peptidic V1 analogues are compared in Table 1. They are in agreement with the pharmacological profiles of the pituitary and liver V1a receptors previously reported for the rat and pig, and readily distinguish hV3 as a new subtype, significantly different from the hV1a subtype.

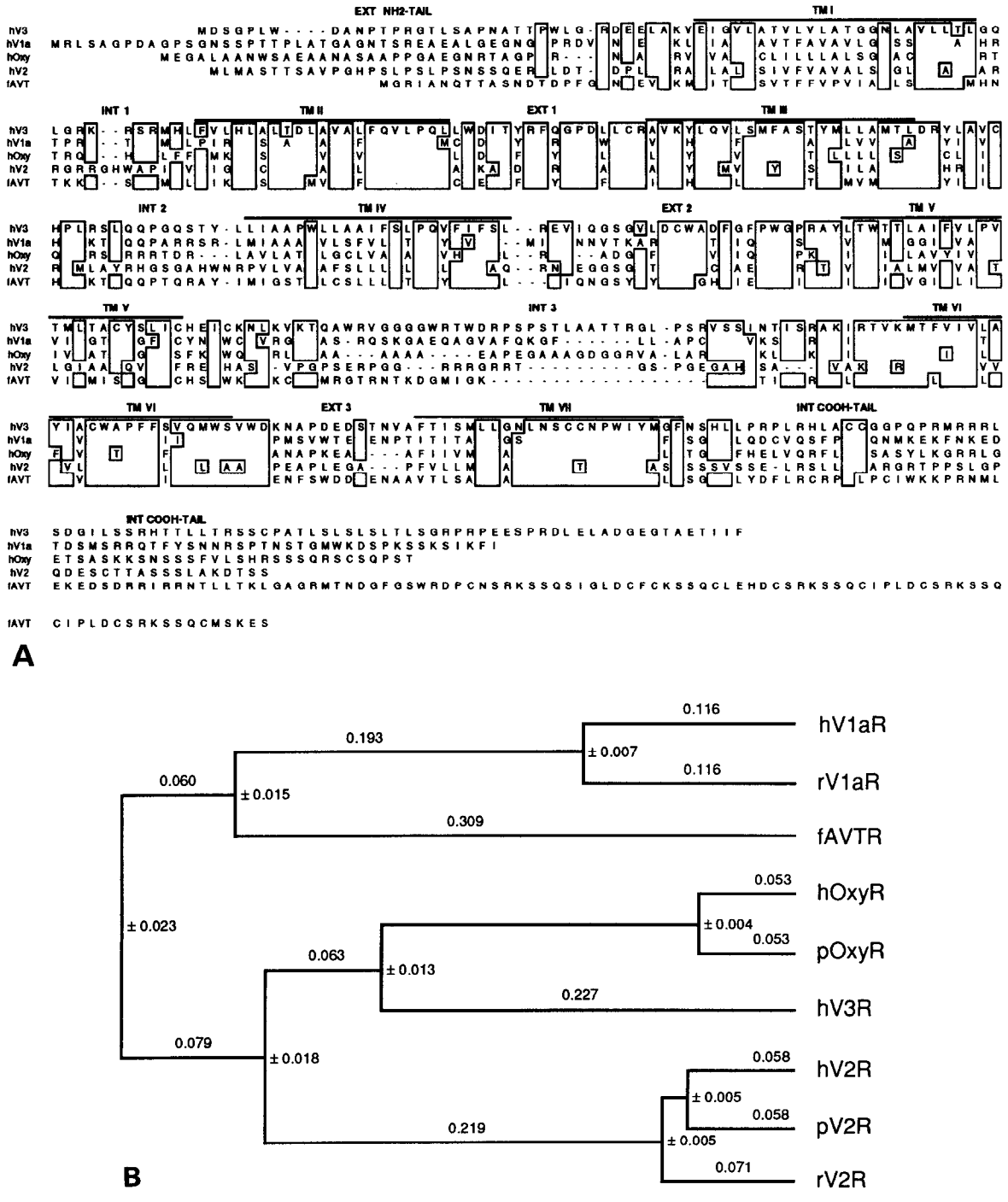


Fig. 2. (A) Alignment of the amino-acid sequences of the AVP/oxytocin family receptors. Human V3 (hV3), V1a (hV1a), V2 (hV2), oxytocin (hOxy) receptors and the fish arginine-vasotocin (fAVT) are compared. Overlined regions correspond to TMD and boxed sequences are conserved in 4 of the 5 sequences. INT: internal, EXT: external. (B) Phylogenetic tree of the family of AVP/oxytoxin receptors. The nucleotide sequences include hV3, hV1a, hV2, hOxy anf fAVT receptors and rat V1a (rV1a), porcine lysine vasopressin (pV2), rat V2 (rV2) and porcine oxytocin (pOxy). This tree was constructed using the UPGMA method. The topology of the tree and the branch lengths give an estimation of the evolutionary distances.

Coupling of the hV3 receptor to intracellular signaling pathways was explored in the transiently transfected COS cells. Nanomolar concentrations of AVP were found to stimulate an increase in phosphoinositide breakdown thus demonstrating a functional coupling to the PLC pathway (Fig. 3). The maximal 5-fold stimulation of inositol phosphate production was detected in these cells after addition of 100 nM AVP ($EC_{50}$ = 1.63 ± 0.32 nM). No stimulation of cAMP production was detected in these cells after addition of 1 $\mu$M AVP (data not shown). Taken together, these data demonstrate that the hV3 cDNA encodes an AVP receptor with binding properties distinct from those of the V1a and V2 receptor subtypes and

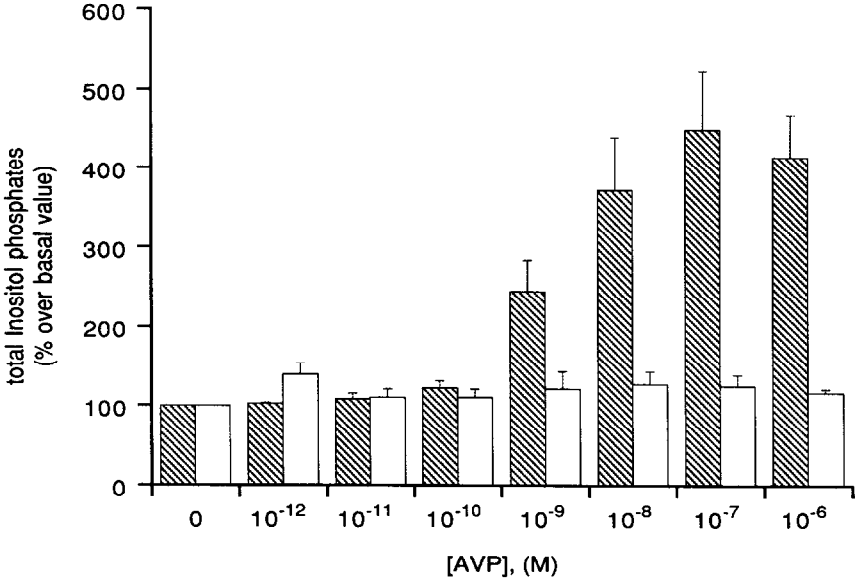


Fig. 3. Vasopressin-induced stimulation of total IP production. IP production was determined in non transfected (□) and transfected (▨) COS-7 cells in presence of indicated concentrations of AVP. Results are expressed as the ratio of the cpm in cells exposed to the buffer (basal value) and represent the mean ± S.E.M. of three different experiments performed in triplicate.

signaling properties identical to those of the V1a and oxytocin receptors. Accordingly and also for phylogenetic reasons, we decided to designate it as the hV3 receptor.

Prior pharmacological studies suggested that hV3 receptor expression was restricted to the anterior pituitary and possibly limited to the corticotroph cells [23]. Consistent with these data, a predominant 4.8 kb mRNA was detected by Northern blot analysis of two corticotropic pituitary adenomas responsible for Cushing's disease (Fig. 4A). Given the low level of expression of hV3 receptor in these homogeneous populations of corticotroph cells, its tissue distribution was further analysed by RT-PCR. As shown in Fig. 4B, a PCR product of the expected size was present in three different normal human pituitaries whereas no such signal was detectable in liver, adrenals or myometrium, which express the V1a and oxytocin receptors, and in a range of other human tissues including brain, spleen, muscle, lung and foreskin fibroblasts (data not shown). Unexpectedly, a faint but distinct signal was detected in kidney, which also expresses V1a [24] and V2 receptors [5]. Thusfar, the kidney is the only organ to express all three known types of AVP receptors. The precise localization of hV3 mRNA in the pituitary and of each receptor in the kidney should be examined by in situ hyridization.

The V3-pituitary receptor plays a major role in the regulation

Table 1
Comparison of the affinity ($K_i$ in nM) of AVP and structural analogs for the human V1a and V3 receptors

| Compound | hV3R | hV1a R | ratio V3/V1a |
|---|---|---|---|
| Natural hormones | | | |
| 1. AVP | 0.72 | 1.66 | 0.42 |
| 2. Vasotocin | 5.4 | 5.0 | 1.1 |
| 3. Oxytocin | 391 | 87.1 | 4.5 |
| V2 antagonists | | | |
| 4. $d(CH_2)_5$[D-Ile$^2$-Ile$^4$-Ala-$NH_2$]AVP | 499 | 52.9 | 9.4 |
| 5. OPC31260 | > 1000 | 142 | > 7 |
| V1 analogues | | | |
| 6. $d(CH_2)_5$Tyr(Me)AVP | 64.3 | 1.59 | 40.4 |
| 7. Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-$NH_2$ | 80.3 | 2.37 | 33.9 |
| 8. Phenylacetyl-D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-$NH_2$ | 10.7 | 0.79 | 13.5 |
| 9. 4-hydroxy-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-$NH_2$ | 1.24 | 0.45 | 2.8 |
| 10. tBaa-D-Tyr(Et)Phe-Val-Asn-Lys-Pro-Arg-$NH_2$ | 113 | 0.79 | 143 |
| 11. SR 49059 | 38.2 | 0.97 | 39.4 |
| 12. OPC21268 | > 1000 | > 1000 | – |
| 13. Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-$NH_2$ | 4.7 | 0.58 | 8.1 |

Affinity constants for the hV3 receptor were derived from competition experiments performed in the present study with 2 nM [$^3$H]AVP and COS-7 cells transfected with the hV3 clone. Affinity constants for the V1a receptors were obtained with the V1a ligand [$^{125}$I]Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-$NH_2$ and CHO cells transfected with an V1a clone [10]. The protocol for these experiments was as previously described [10].

Each value represents the mean ± S.E.M. of at least 3 different experiments. $K_i$ values were calculated according to the Cheng and Prusoff equation, $K_i = IC_{50}/1+(L_t/K_d)$.

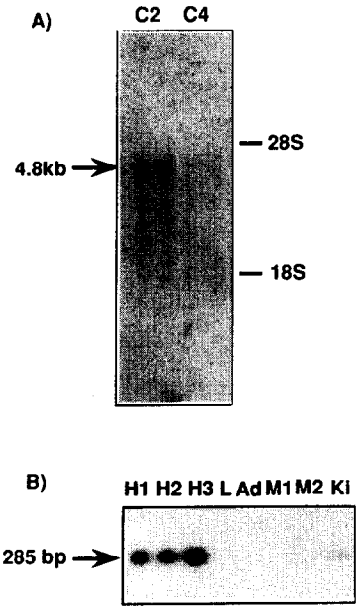


Fig. 4. Tissue expression of the hV3 receptor mRNA. (A) Northern blot analysis of two corticotropic adenomas (C2 and C4). Each lane contains 2 $\mu$g of poly(A)$^+$ RNA. The position of hV3 mRNA is indicated by the arrow and the position of the 28S and 18S rRNA of an adjacent lane are indicated by dashes. (B) hV3 receptor RT-PCR analysis of total RNA from three normal pituitaries (H1, H2, and H3), non-pregnant (M1) and pregnant (M2) myometrium, adrenal (Ad), liver (L) and kidney ($K_i$). The amplified PCR product is shown by the arrow.

of ACTH secretion through its potentiating effect on CRH-stimulation. It is specifically involved in the corticotropic response to stress in many species including man where AVP is clearly required for a full corticotropic response. Elegant physiological studies on ACTH regulation in animals have demonstrated the role of AVP in the response to stress: whereas immunization against CRH strongly diminishes both stress-induced and basal ACTH secretion, immunization against AVP only blunts the stress-induced ACTH surge without affecting the basal level of circulating ACTH [1, 25].

Stable cell lines expressing the hV3 receptor will be instrumental to develop specific V3 analogues with either agonist or antagonist activities. Such molecules should provide a new pharmacological approach to assess and manipulate an essential, endocrine, component of the response to stress.

*Acknowledgements:* We wish to thank Kathleen Curnow for helpful comments and G. Masquelier for preparing Fig. 4.

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