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Pharmacological characterization of the human vasopressin receptor subtypes stably expressed in Chinese hamster ovary cells

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1 Three subtypes of human (h) arginine vasopressin (AVP) receptors, hV_{1A}, hV_{1B} and hV_2, were stably expressed in Chinese hamster ovary (CHO) cells and characterized by [^{3}H]-AVP binding studies. In addition, the coupling of the expressed receptor protein to a variety of signal transduction pathways was investigated.

2 Scatchard analysis of saturation isotherms for the specific binding of [^{3}H]-AVP to membranes, prepared from CHO cells transfected with hV_{1A}, hV_{1B} and hV_2 receptors, yielded an apparent equilibrium dissociation constant (K_d) of 0.39, 0.25 and 1.21 nM and a maximum receptor density (B_{max}) of 1580 fmol mg^{-1} protein, 5230 fmol mg^{-1} protein and 7020 fmol mg^{-1} protein, respectively. Hill coefficients did not differ significantly from unity, suggesting binding to homogenous, non-interacting receptor populations.

3 Pharmacological characterization of the transfected human AVP receptors was undertaken by measuring the relative ability of nonpeptide AVP receptor antagonists, YM087, OPC-21268, OPC-31260, SR 49059 and SR 121463A, to inhibit binding of [^{3}H]-AVP. At hV_{1A} receptors, the relative order of potency was SR49059 > YM087 > OPC-31260 > SR 121463A > > OPC-21268 and at hV_2 receptors, YM087 = SR 121463A > OPC-31260 > SR 49059 > > OPC-21268. In contrast, the relative order of potency, at hV_{1B} receptors, was SR 49059 > > SR 121463A = YM087 = OPC-31260 = OPC-21268.

4 In CHO cells expressing either hV_{1A} or hV_{1B} receptors, AVP caused a concentration-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with an EC_{50} value of 1.13 nM and 0.90 nM, respectively. In contrast, stimulation of CHO cells expressing hV_2 receptors resulted in an accumulation of cyclic AMP with an EC_{50} value of 2.22 nM. The potency order of antagonists in inhibiting AVP-induced $[Ca^{2+}]_i$ or cyclic AMP response was similar to that observed in radioligand binding assays.

5 In conclusion, we have characterized the pharmacology of human cloned V_{1A}, V_{1B} and V_2 receptors and used these to determine the affinity, selectivity and potency of nonpeptide AVP receptor antagonists. Thus they may prove to be a valuable tool in further examination of the physiological and pathophysiological roles of AVP.

Keywords: Human vasopressin receptors; YM087; SR 49059; SR 121463A

Introduction

Arginine vasopressin (AVP) has been shown to play important physiological roles in vasoconstriction and antidiuresis and to exert its effect through binding to specific receptors coupled to distinct second messengers and three AVP receptor subtypes (V_{1A}, V_{1B} and V_2) have been identified (Birnbaumer *et al*., 1992; Sugimoto *et al*., 1994; Thibonnier *et al*., 1994). AVP activates phospholipase A_2, C, and D *via* the V_{1A} and V_{1B} receptors (Thibonnier, 1992). This results in the production of inositol 1,4,5-triphosphate and 1,2-diacylglycerol, the mobilization of intracellular calcium, and the activation of protein kinase C resulting in protein phosphorylation (Michell *et al*., 1979). V_{1A} receptors have been shown to be present in vascular smooth muscle cells, hepatocytes, platelets, mesangial cells, cardiomyocytes, brain, testis, adrenal glands, spinal cord, sympathetic ganglia by radioligand binding techniques. These receptors serve to mediate the contraction, proliferation and hypertrophy of cells; platelet aggregation, hepatocyte glycogenolysis; enhancement of learning and memory and steroid secretion (Weingartner *et al*., 1981; Thibonnier & Roberts, 1985; Jard *et al*., 1987; Phillips *et al*., 1990; Howl *et al*., 1991; Guillon *et al*., 1995; Serradeil-Le Gal *et al*., 1995; Tahara *et al*., 1997a; 1998). V_{1B} receptors are located in the anterior pituitary, β-cells of pancreas and adrenal medulla where they stimulate corticotropin, insulin and catecholamine release, respectively (Jard *et al*., 1986; Lee *et al*., 1995; Grazzini *et al*., 1996). In contrast, V_2 receptors stimulate adenylate cyclase resulting in the production of cyclic AMP. V_2 receptors are present in renal epithelial cell lines ($LLC\text{-}PK_1$), as well as in the medulllary portion of the kidney where they control free water and urea reabsorption (Butlen *et al*., 1978; Jans *et al*., 1989).

AVP may be involved in the pathology of several diseases and disorders including heart failure, hypertension, renal diseases, hyponatremia and the syndrome of inappropriate antidiuretic hormone secretion (SIADH). Consequently, AVP antagonists may be effective agents in the treatment of these diseases (Laszlo *et al*., 1991; Fujisawa *et al*., 1993; Naitoh *et al*., 1994; Nishikimi *et al*., 1996). Recently, orally effective nonpeptide AVP receptor antagonists have been discovered (Yamamura *et al*., 1991; 1992; Serradeil-Le Gal *et al*., 1993; 1996). More recently, the pharmacological profile of one such antagonist, YM087 (4′-[(2-methyl-1,4,5,6-tetrahydroimidazo[4,5-d][1]benzazepin-6-yl)carbonyl]-2-phenylbenzanilide

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mono hydrochloride), was examined in rats and dogs (Yatsu *et al*., 1997; Tahara *et al*., 1997b). YM087 is an orally effective nonpeptide antagonist of V_{1A} and V_2 receptors which exhibits high affinity and potency. However, because there are marked species differences in AVP receptor specificity (Tence *et al*., 1990; Pettibone *et al*., 1992; Serradeil-Le Gal *et al*., 1993), it was necessary to investigate the effects of AVP receptor antagonists on human AVP receptors. Furthermore, a cellular system expressing human AVP receptors would be very useful for the development of new AVP receptor ligands, and would also allow detailed investigation of the regulation of AVP receptor function. Consequently, the stable expression and pharmacological characterization of human AVP receptor subtypes (hV_{1A}, hV_{1B} and hV_2) in Chinese hamster ovary (CHO) cells is herein described. Furthermore, these CHO cells were used to characterize and compare the affinity, selectivity and potency of nonpeptide AVP receptor antagonists, including YM087, using receptor binding and second messenger assays.

Methods

Materials

AVP and oxytocin were from Peptide Institute Inc. (Osaka, Japan). d(CH_2)$_5$Tyr(Me)AVP ([β-mercapto-β,β-cyclopentamethylenepropionyl^1,O-Me-Tyr^2,Arg^8]-Vasopressin, SKF-100273), dDAVP ([deamino-Cys^1,D-Arg^8]-Vasopressin, desmopressin), dPTyr(Me)AVP ([deamino-Pen^1,O-Me-Tyr^2,Arg^8]-Vasopressin), dGTyr(Et)VAVP (des-Gly^9-[β-mercapto-β,β-cyclopentamethylenepropionyl^1,O-ET-Tyr^2,Val^4,Arg^8]-Vasopressin), [Thr^4,Gly^7]-Oxytocin, cyclic AMP were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). YM087, OPC-21268 (1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2(1*H*)-quinolinone), OPC-31260 (5-dimethylamino-1-{4-(2-methylbenzoyl-amino) benzoyl}-2,3,4,5-tetrahydro-1*H*-benzazepine), SR 49059 ((2S) 1-[(2R 3S)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide) and SR 121463A (1-[4-(N-tert-butyl-carbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy) cyclohexane]indol-2-one; equatorial isomer) were synthesized at Yamanouchi Pharmaceutical Co. (Ibaraki, Japan). The structures of these compounds were determined by ^{1}H-nuclear magnetic resonance, mass spectometry and elemental analysis. Their purity was measured by high-pressure liquid chromatography and was >98%. These nonpeptide antagonists were initially dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and diluted to the desired concentration with the assay buffer. The final concentration of DMSO in the assay buffer did not exceed 1%, at which specific [^{3}H]-AVP binding was not affected. [^{3}H]-AVP (specific activity, 80 Ci mmol^{-1}) and [^{3}H]-cyclic AMP (specific activity, 27 Ci mmol^{-1}) were obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.). CHO cells were from the American Tissue Culture Collection (Rockville, MD, U.S.A.). Minimum essential medium (MEM)-alpha, LipofectAMINE, fetal calf serum (FCS), antibiotics (penicillin and streptomycin) and trypsin-EDTA were from Gibco (Grand Island, NY, U.S.A.). Bovine serum albumin (BSA) was from Nacalai Tesque Inc. (Kyoto, Japan). Fura 2-acetoxymethyl ester (AM) was from Dojindo Laboratories (Kumamoto, Japan) and EGTA, ionomycin, 3-isobutyl-1-methylxanthine (IBMX) and bovine heart tissue were from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest reagent grade available.

Stable expression of human AVP receptors in CHO cells

CHO cells deficient in dihydrofolate reductase were maintained at 37°C in a humidified atmosphere with 5% CO_2 in MEM-alpha supplemented with nucleosides and 10% FCS. CHO cells were stably transfected with the mammalian expression vector pEF-BOS (Mizushima & Nagata, 1990) which contains the dihydrofolate reductase gene, using LipofectAMINE. After 2 weeks of selection in MEM-alpha without nucleosides and supplemented with 100 nM amethopterin (a dihydrofolate reductase inhibitor) and 10% FCS, surviving colonies of cells were isolated by ring cloning and further amplified in culture medium supplemented with amethopterin (up to 1 μM) Surviving cells were assayed for the production of AVP receptors using receptor binding assay. Cells that expressed the highest level of receptors were selected, then grown in MEM-alpha without nucleosides and supplemented with 1 μM amethopterin and 10% FCS.

Preparation of plasma membranes from CHO cells

Confluent CHO cells were washed with phosphate-buffered saline (PBS) and harvested into ice-cold 10 mM Tris-HCl, pH 7.4, containing 5 mM EDTA followed by homogenization and centrifugation at $35{,}000 \times g$ for 20 min at 4°C. The pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$ and stored in small aliquots at −80°C until use. Protein was determined by the Coomassie blue method using BSA as a standard.

Binding assay

For saturation binding studies, plasma membrane preparations were incubated with various concentrations of [^{3}H]-AVP (0.1–5.0 nM). For competition studies, [^{3}H]-AVP (0.5–1.0 nM) was added to each membrane preparation, which were then incubated with various concentrations of compounds in 250 μl of assay buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 0.1% BSA. Binding reactions were initiated by the addition of the membrane preparations. After the incubation period (60 min, 25°C), the reaction was terminated by the addition of 3 ml of ice-cold Tris buffer (50 mM Tris-HCl, pH 7.4 and 10 mM $MgCl_2$) followed immediately by rapid filtration through 96-well GF/B UniFilter Plates using a MicroMate Cell Harvester (Packard Instrument Company, Meriden, CT, U.S.A.). The filters were rinsed twice and the radioactivity retained on the filters was counted with a TopCount Microplate Scintillation Counter (Packard Instrument Company). Nonspecific binding was determined using 1 μM unlabeled AVP. The radioligand binding data were analysed by GraphPad PRISM (GraphPAD Software, Inc., San Diego, CA, U.S.A.). Each data point derived from at least three separate experiments was analysed. The inhibitory dissociation constant (K_i) was calculated from the following formula: $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of radioligand present in the tube and K_d is the dissociation constant of radioligand obtained from Scatchard plot analysis (Cheng & Prusoff, 1973). For some compounds, which did not show over 50% displacement at 10 μM, the percent displacement at this concentration was shown.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

CHO cells expressing hV_{1A} and hV_{1B} receptors were plated on coverglasses (13.5 mm in diameter) and serum-starved for 24 h.

Cell monolayers were loaded in MEM-alpha with Fura 2-AM (2 μM/coverglass) for 30 min at 37°C. They were then washed with PBS, transferred to a Fura 2-free medium and incubated for an additional 30 min at 37°C. The loaded monolayers were then stored in Krebs-Henseleit-HEPES buffer containing (mM): NaCl 130, KCl 5, $CaCl_2$ 1.25, $MgSO_4$ 0.8, glucose 5.5, HEPES 20 and 0.1% BSA, pH 7.4). The coverglass was placed into a quartz cuvette containing 2 ml Krebs-Henseleit-HEPES buffer and maintained at 37°C with continuous stirring. When thermal equilibrium was reached, the fluorescence signal was recorded with a CAF-110 spectrofluorometer (Japan Spectrometer Co., Tokyo, Japan) at both 340 and 380 nm excitation wavelengths, and 500 nm emission wavelength. After recording the baseline signal for 3 min, AVP was added to the cuvette to stimulate the mobilization of intracellular calcium in the presence or absence of antagonists. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (R_{max}) with the nonfluorescent Ca^{2+} ionophore, ionomycin (25 μM), after which minimal fluorescence (R_{min}) was obtained by adding 3 mM EGTA. From the ratio of fluorescence at 340 and 380 nm, the $[Ca^{2+}]_i$ was determined using the following equation (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i(nM) = K_d \times [(R - R_{min})/(R_{max} - R)] \times b$$

The term b is the ratio of fluorescence of Fura 2 at 380 nm in zero and saturating Ca^{2+}, K_d is the dissociation constant of Fura 2 for Ca^{2+}, assumed to be 224 nM (Grynklewicz *et al*., 1985).

Cyclic AMP production assay

CHO cells expressing hV_2 receptors were grown in 24-well culture plates to confluence and serum-starved for 24 h. The cells were then incubated in MEM-alpha supplemented with 0.5 mM IBMX and 0.1% BSA for 10 min at 37°C. This media contained either the vehicle alone or various concentrations of AVP with or without antagonists. At the end of exposure, the cell monolayers were washed three times with PBS followed by lysis with boiling 50 mM sodium acetate, pH 6.2, containing 2 mM IBMX. Extracts were then boiled for 3 min and kept at −40°C before determination of cyclic AMP. The amount of cyclic AMP in the CHO cells was measured as previously described (Takeda *et al*., 1989) although some minor modifications were introduced. Briefly, a crude binding protein was prepared from bovine heart tissue. Approximately, 100 g of bovine heat tissue was homogenized in 4 volumes of 20 mM sodium phosphate buffer containing 2 mM EDTA and 25 mM 2-mercaptoethanol (PEM buffer), pH 7.4, followed by centrifugation at 11,000 × g for 30 min. The supernatant was precipitated with ammonium sulfate (400 g/liter) for 1 h with stirring. The suspension was centrifuged at 12,000 × g for 20 min and the resulting supernatant discarded. The pellet was resuspended in a minimum volume of PEM buffer, followed by dialysis for 2 h against the PEM buffer. The dialysate was centrifuged again to remove insoluble proteins. The resulting supernatant was used immediately as the crude binding protein. For competitive protein binding assays, [^{3}H]-cyclic AMP (2 nM) was added to crude binding protein (0.05 mg). This mixture was incubated with extract samples or standard cyclic AMP solutions (0–80 pmol) in 250 μl of PEM buffer (pH 7.4) containing 0.5 mg/ml BSA and 1.5 mM IBMX. After incubation for 1 h at 25°C, the reaction was terminated by addition of 3 ml of ice-cold 20 mM sodium phosphate buffer (pH 7.4), containing 2 mM EDTA, followed immediately by rapid filtration through 96-well GF/B UniFilter Plates presoaked in 0.5% polyethylenimine using a MicroMate Cell Harvester. The filters were rinsed twice and the radioactivity retained on the filters was counted with Topcount Microplate Scintillation Counter.

Analysis of data

Apparent K_i values for antagonists were calculated from the Cheng and Prusoff relationship, apparent $K_i = IC_{50}/(1 + [L]/EC_{50})$, whereas IC_{50} is the concentration of antagonist required for 50% inhibition of the maximum response, [L] is the concentration of AVP and EC_{50} is the concentration of AVP needed to cause 50% of the maximum response. Experimental results are expressed as the mean ± s.e.mean or the mean with 95% confidence limits. All experiments were repeated at least three times, and comparable results were obtained.

Results

Saturability of [^{3}H]-AVP binding

Saturation experiments employing increasing concentrations of [^{3}H]-AVP and hV_{1A}, hV_{1B} and hV_2 receptors on CHO membranes preparations showed that specific binding was saturable. Scatchard analysis of these data gave linear plots consistent with the presence of a single class of high affinity binding site for each receptor. The apparent dissociation constant (K_d) was 0.39 ± 0.13 nM ($n = 5$) for hV_{1A}, 0.25 ± 0.02 nM ($n = 5$) for hV_{1B} and 1.21 ± 0.37 nM ($n = 5$) for hV_2. The calculated maximum binding capacity (B_{max}) was 1580 ± 148 fmol mg^{-1} protein (1.5×10^5 receptors cell^{-1}) for hV_{1A}, 5230 ± 686 fmol mg^{-1} protein (4.9×10^5 receptors cell^{-1}) for hV_{1B} and 7020 ± 452 fmol mg^{-1} protein (6.4×10^5 receptors cell^{-1}) for hV_2 (Figure 1). Hill coefficients (n_H) for binding of [^{3}H]-AVP to hV_{1A} (1.04 ± 0.12), hV_{1B} (1.02 ± 0.03) and hV_2 (1.06 ± 0.03) receptors were not significantly different from unity.

Characterization of human cloned AVP receptors

Several peptide AVP receptor agonists and antagonists were tested for their effects on [^{3}H]-AVP binding to plasma membranes prepared from CHO cells transfected with the human AVP receptor subtypes. AVP showed a concentration-dependent inhibition of [^{3}H]-AVP binding to hV_{1A}, hV_{1B} and hV_2 receptors, with K_i values of 0.56 ± 0.11 nM, 0.51 ± 0.07 nM and 3.27 ± 0.68 nM, respectively (Table 1). The V_{1A}/V_{1B} selective antagonist, dPTyr(Me)AVP showed high affinity for hV_{1A} and hV_{1B} receptors, with K_i values of 0.71 ± 0.15 nM and 6.32 ± 0.83 nM, respectively, and exhibited much lower affinity for hV_2 receptors with a K_i value of 410 ± 56.8 nM. The V_{1A}/V_2 selective antagonist, dGTyr(ET)VAVP was at least 2000- and 170 fold more selective for hV_{1A} and hV_2 receptors with K_i values of 0.87 ± 0.30 nM and 10.3 ± 1.64 nM, respectively, than for hV_{1B} receptors ($K_i = 1800 \pm 370$ nM). dDAVP showed high affinity for hV_{1B} receptors, with a K_i value of 5.81 ± 1.28 nM, and exhibited moderate affinity for hV_{1A} and hV_2 receptors with K_i values of 62.4 ± 17.6 nM and 23.3 ± 2.66 nM, respectively. The oxytocin receptor selective agonist, [Thr^4,Gly^7]-Oxytocin was found to have only weak affinity for all human AVP receptor subtypes ($K_i > 300$ nM).

Characterization of YM087, OPC-21268, OPC-31260, SR 49059 and SR 121463A for human AVP receptors

YM087 inhibited the specific binding of [^{3}H]-AVP to hV_{1A} and hV_2 receptors in a concentration-dependent manner, with K_i

values of 4.30±0.99 nM and 1.92±0.24 nM, respectively (Table 1). OPC-31260 was 18 fold weaker than YM087 at inhibiting [^{3}H]-AVP binding to hV_{1A}, showing a K_i of 77.6±9.00 nM. It was also 13 fold weaker inhibiting hV_2 receptors, with a K_i of 24.7±5.05 nM. SR 49059 exhibited high affinity, with a K_i value of 0.53±0.08 nM, and was 330 fold selective for hV_{1A} than for hV_2 receptors (K_i=178±40.6 nM). SR 121463A showed high affinity, with a K_i value of 2.93±0.59 nM, and was at least 130 fold more selective for hV_2 than for hV_{1A} receptors (K_i=398±9.73 nM). In contrast, YM087, OPC-31260 and SR 121463A did not reduce specific binding to hV_{1B} receptors at all (K_i>10 μM), but SR 49059 exhibited a moderate affinity for hV_{1B} receptors (K_i=48.4±10.3 nM). Under the same experimental conditions, OPC-21268 exhibited low affinity for all three AVP receptor subtypes (K_i>25 μM). The slopes of the inhibition curves for YM087, OPC-31260, SR 49059 and SR 121463A did not differ significantly from unity.

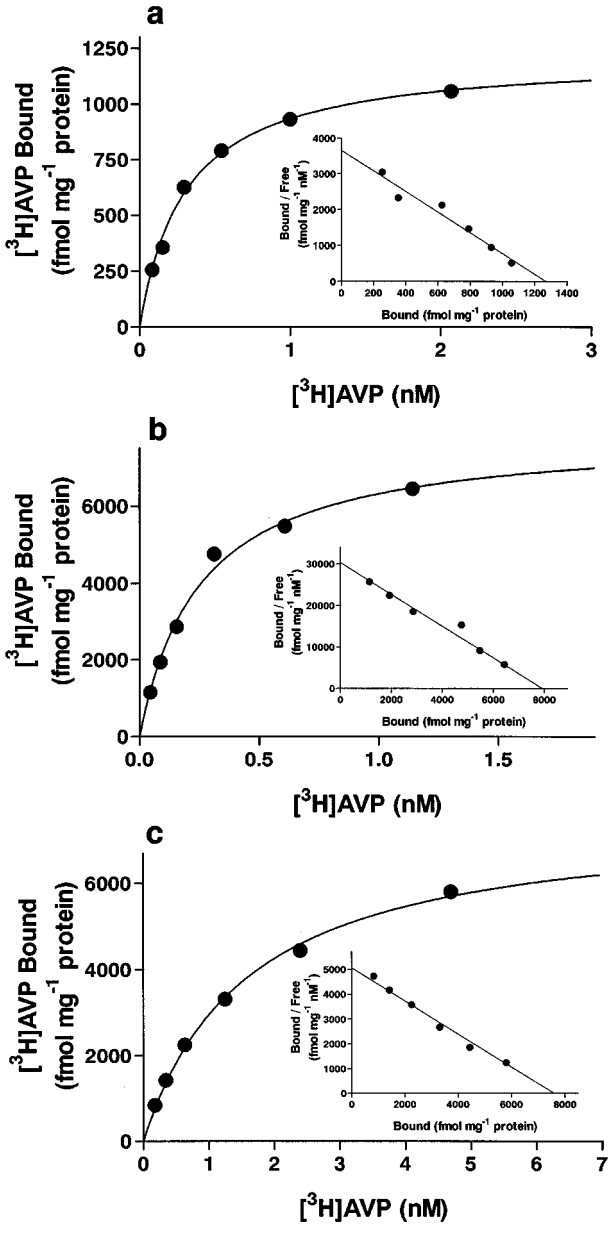

Figure 1 Saturation equilibrium specific binding of [^{3}H]-AVP to plasma membranes prepared from CHO cells transfected with human V_{1A} (a), V_{1B} (b) and V_2 (c) receptors. Inset, Scatchard linear transformation of the data. Results are representative data from 5 independent experiments performed in duplicate.

$[Ca^{2+}]_i$ increase in CHO cells expressing hV_{1A} receptors

Addition of AVP or oxytocin to CHO cells expressing hV_{1A} receptors resulted in an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. Agonists-induced rise of $[Ca^{2+}]_i$ was biphasic and an immediate and transient spike was observed (time to peak <3 s), followed by a prolonged plateau of elevated $[Ca^{2+}]_i$ which was still above basal values 5 min after addition of the stimulus. The EC_{50} values of AVP and oxytocin were 1.13 (0.91–1.40) nM and 20.3 (16.8–24.4) nM. Maximal stimulation was 2200 and 1300% over basal levels, respectively (Figure 2, Table 2). On the contrary, dDAVP did not cause an increase in $[Ca^{2+}]_i$. YM087 inhibited an increase in $[Ca^{2+}]_i$ stimulated by AVP in a concentration-dependent manner with an IC_{50} value of 4.27 (2.75–6.62) nM (Table 2). The inhibitory potency of YM087 was 20 and 50 times higher than that of OPC-31260 (IC_{50}=87.5 (52.8–145) nM) and SR 121463A (IC_{50}=223 (167–298) nM). In contrast, SR 49059 strongly inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner with an IC_{50} value of 0.34 (0.24–0.48) nM; this inhibitory potency was 12 times higher than that of YM087. These IC_{50} values correspond well with the K_i values obtained from the [^{3}H]-AVP displacement studies.

$[Ca^{2+}]_i$ increase in CHO cells expressing hV_{1B} receptors

Addition of AVP, oxytocin and dDAVP to CHO cells expressing hV_{1B} receptors resulted in an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. Agonists evoked a single long-lasting $[Ca^{2+}]_i$ spike (time to peak <10 s), followed by a sustained plateau. The EC_{50} values of AVP, oxytocin and dDAVP were 0.90 (0.70–1.16) nM, 139 (81.8–236) nM and 8.42 (5.10–13.9) nM, respectively. Maximal stimulation was 540, 420 and 420% over basal levels, respectively (Figure 3, Table 2). SR 49059 inhibited the increase in $[Ca^{2+}]_i$ stimulated by AVP in a concentration-dependent manner, with an IC_{50} value of 65.3 (39.8–107) nM. In contrast, YM087, OPC-31260 and SR 121463A did not inhibit the increase in $[Ca^{2+}]_i$ for hV_{1B} receptors (IC_{50} >10 μM). These IC_{50} values correspond well with the K_i values obtained from the [^{3}H]-AVP displacement studies.

Cyclic AMP production in CHO cells expressing hV_2 receptors

Addition of AVP and dDAVP to CHO cells expressing hV_2 receptors resulted in the rapid production of cellular cyclic AMP in a concentration-dependent manner with EC_{50} values of 2.22 (1.69–2.92) nM and 506 (332–770) nM and maximal stimulation of 470 and 590% over basal level, respectively (Figure 4; Table 2). In contrast, oxytocin exhibited much lower potency in the production of cyclic AMP. SR 121463A and YM087 potently inhibited the production of cyclic AMP stimulated by AVP in a concentration-dependent manner, with IC_{50} values of 1.66 (1.18–2.33) nM and 2.13 (1.42–3.18) nM, respectively. The inhibitory potency of SR 121463A was 11 and 110 times higher than that of OPC-31260 (IC_{50}=19.0 (12.2–29.6) nM)) and SR 49059 (IC_{50}=186 (104–330)), respectively. These IC_{50} values correspond well with the K_i values obtained from the [^{3}H]-AVP displacement studies.

Table 1 K_i values of various AVP receptor agonists and antagonists for human AVP receptors

Compounds	hV_{1A}		hV_{1B}		hV_2	
	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H
AVP	0.56±0.11	−1.14±0.06	0.51±0.07	−1.18±0.09	3.27±0.68	−1.02±0.05
Oxytocin	5.46±1.97	−0.97±0.04	160±29.8	−0.94±0.04	1700±340	−1.02±0.17
dDAVP	62.4±17.6	−1.09±0.19	5.81±1.28	−0.90±0.04	23.3±2.66	−0.91±0.05
$d(CH_2)_5$Tyr(Me)AVP	0.77±0.18	−0.95±0.09	121±12.7	−1.04±0.06	113±29.9	−1.02±0.04
dPTyr(Me)AVP	0.71±0.15	−1.02±0.02	6.32±0.83	−1.09±0.08	410±56.8	−1.00±0.04
dGTyr(Et)VAVP	0.87∓0.30	−1.10±0.06	1800±370		10.3±1.64	−1.03±0.08
[Thr^4,Gly^7]-Oxytocin	305±85.1	−1.02±0.01	>10000 (89%)		>10000 (92%)	
YM087	4.30±0.99	−1.09±0.14	>10000 (93%)		1.91±0.24	−1.11±0.08
OPC-21268	>10000 (88%)		>10000 (103%)		>10000 (87%)	
OPC-31260	77.6±9.00	−1.00±0.05	>10000 (96%)		24.7±5.05	−1.04±0.03
SR 49059	0.53±0.08	−1.03±0.10	48.4±10.3	−0.95±0.04	178±40.6	−0.98±0.03
SR 121463A	304±7.29	−1.19±0.02	52000±14000		2.75±0.62	−1.02±0.13

Inhibitory constant (K_i) values were determined from competition experiments calculated according to the equation of Cheng & Prusoff (1973) ($K_i = IC_{50}(1 + C/K_d)$). Values are means±s.e.mean obtained from 3–5 independent experiments performed in duplicate. The parentheses data are presented for compounds which did not show over 50% displacement at 10 μM, and indicate % displacement at this concentration.

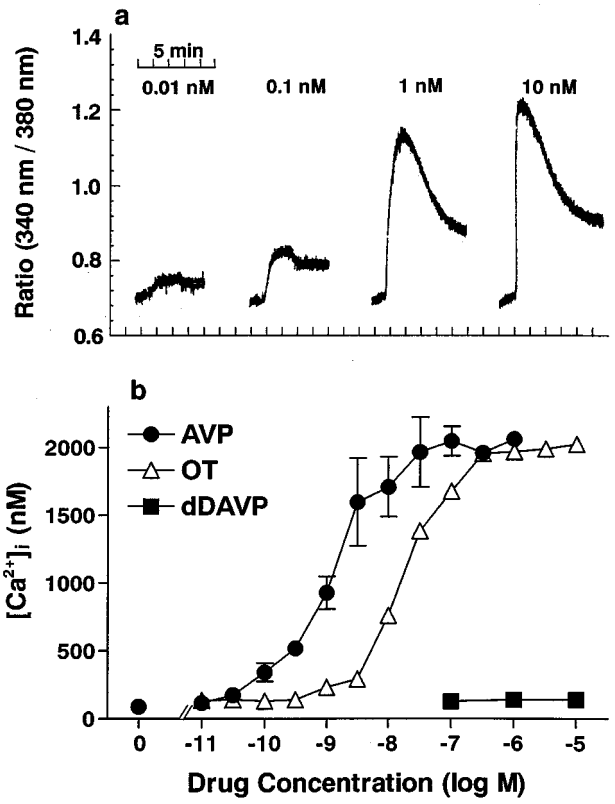

Figure 2 (a) Concentration-response pattern to AVP-induced increases in fura 2 fluorescence in human V_{1A} receptors expressing CHO cells. Typical recording of a single experiment showing the ratio of fluorescence intensity at 340 nm/380 nm excitation of fura 2 fluorescence in response to AVP. (b) Effect of AVP, oxytocin and dDAVP on $[Ca^{2+}]_i$ increase in human V_{1A} receptors expressing CHO cells. Values are means±s.e.mean from 3–5 independent experiments.

Discussion

In this study, receptor binding and second messenger assays were used to characterize cloned human AVP receptors stably expressed in CHO cells. These cells were then employed in the profiling of nonpeptide AVP receptor antagonists. Saturation experiments with increasing concentrations of [^{3}H]-AVP binding to plasma membranes prepared from CHO cells expressing cloned hV_{1A}, hV_{1B} and hV_2 receptors showed that specific binding was saturable. Scatchard analysis of these data gave linear plots with the presence of a single class of high affinity and capacity binding site for each receptor. Hill coefficients did not differ significantly from unity, suggesting binding to homogenous, non-interacting receptor populations.

Using peptide ligands for AVP receptor subtypes, we were able to confirm, in competition experiments, that the three subtypes of human AVP receptor corresponded to V_{1A}, V_{1B} and V_2 receptors, respectively. The V_{1A}/V_{1B} selective antagonist, dPTyr(Me)AVP, showed high affinity for hV_{1A} and hV_{1B} receptors, but exhibited much lower affinity for hV_2 receptors. In addition, the V_{1A}/V_2 selective antagonist, dGTyr(Et)VAVP, also inhibited binding of [^{3}H]-AVP to hV_{1A} and hV_2 receptors, with very weak affinity for hV_{1B} receptors. In contrast, the oxytocin receptor selective agonist, [Thr^4,Gly^7]-Oxytocin, proved to be a very weak inhibitor of [^{3}H]-AVP binding to all human AVP receptors. These profiles and affinities of ligands in cloned human AVP receptors correlate closely with studies undertaken using the same ligands on AVP receptors in their native location (Howl *et al.*, 1991; Laszlo *et al.*, 1991; Pettibone *et al.*, 1992), suggesting that the binding characteristics of these human recombinant AVP receptors are functionally identical to those of AVP receptors found in human tissue. Using these human cloned AVP receptors, the affinity and selectivity of nonpeptide AVP receptor antagonists were determined. The V_{1A} receptor selective antagonist, SR 49059, and the V_2 receptor selective antagonist, SR 121463A, displayed highly competitive and selective affinity for hV_{1A} and hV_2 receptors, respectively, with a potency comparable to that described previously (Serradeil-Le Gal *et al.*, 1993; 1996). In contrast, the previously described V_{1A} selective antagonist, OPC-21268, exhibited much lower affinity for hV_{1A} receptors (K_i = 25,000 nM). For rat V_{1A} receptors, OPC-21268 displayed moderate affinity with a K_i value of 23.5 nM (Tahara *et al.*, 1997b), in agreement with the original published value (Yamamura *et al.*, 1991). These observations confirmed the existence of great species differences between rat and human V_{1A} receptors as already reported (Tence *et al.*, 1990; Pettibone *et al.*, 1992). Next, the properties of YM087, a newly synthesized dual V_{1A} and V_2 receptor antagonist, were compared with those of OPC-31260, a previously described V_2 selective antagonist (Yamamura *et al.*, 1992). YM087 showed high affinity for both hV_{1A} and hV_2

Table 2 Activities of AVP receptor agonists and antagonists for $[Ca^{2+}]_i$ and cyclic AMP responses in human AVP receptors expressing CHO cells

	EC_{50}/K_i (nM)		
	$[Ca^{2+}]_i$ measurement		cyclic AMP production
Compounds	hV_{1A}	hV_{1B}	hV_2
Agonists			
AVP	1.13 (0.91–1.40)	0.90 (0.70–1.16)	2.22 (1.69–2.92)
Oxytocin	20.3 (16.8–24.4)	139 (81.8–236)	5690 (2490–13000)
dDAVP		8.42 (5.10–13.9)	8.76 (6.43–11.9)
Antagonists			
dDAVP	2.24 (1.42–3.52)		
$d(CH_2)_5$Tyr(Me)AVP	0.03 (0.03–0.04)	31.8 (20.9–48.4)	41.5 (21.9–78.4)
YM087	0.43 (0.28–0.67)	>10000 (99%)	0.39 (0.26–0.58)
OPC-21268	>10000 (105%)	>10000 (97%)	>10000 (101%)
OPC-31260	8.88 (5.36–14.7)	>10000 (101%)	3.46 (2.22–5.38)
SR 49059	0.03 (0.02–0.05)	5.39 (3.29–8.86)	33.7 (19.0–60.0)
SR 12146A	22.6 (17.0–30.2)	>10000 (98%)	0.30 (0.21–0.42)

The half-maximal effective concentration (EC_{50}) was calculated from the concentration-response curve obtained for each agonist. The half-maximal concentration inhibiting 10 nM AVP-induced responses (IC_{50}) is given for each antagonist. Apparent K_i values were calculated from Cheng & Prusoff (1973) relationship, $K_i = IC_{50}/(1+[L]/EC_{50})$. Values are mean with 95% confidence limits of 3–8 independent experiments. The parentheses data are presented for compounds which did not show over 50% inhibition at 10 μM, and indicate % inhibition at this concentration.

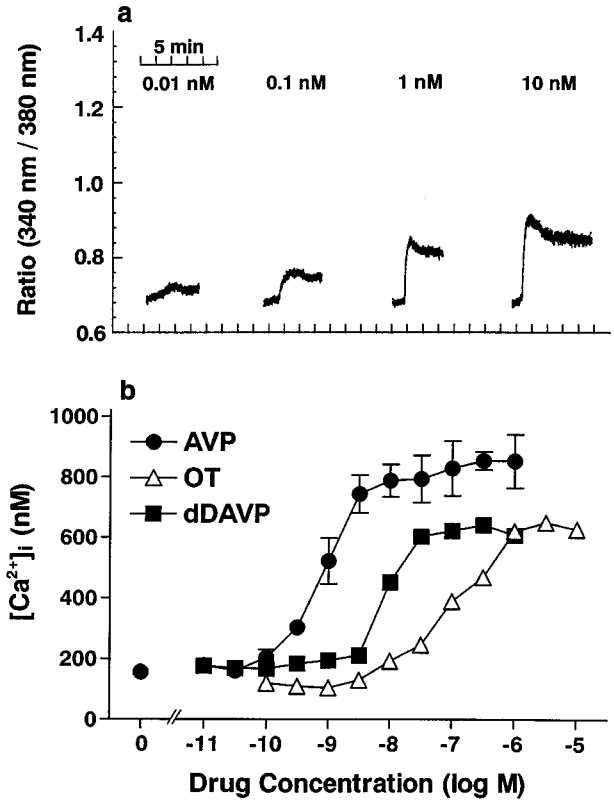

Figure 3 (a) Concentration-response pattern to AVP-induced increases in fura 2 fluorescence in human V_{1B} receptors expressing CHO cells. Typical recording of a single experiment showing the ratio of fluorescence intensity at 340 nm/380 nm excitation of fura 2 fluorescence in response to AVP. (b) Effect of AVP, oxytocin and dDAVP on $[Ca^{2+}]_i$ increase in human V_{1B} receptors expressing CHO cells. Values are means ± s.e.mean from 3–5 independent experiments.

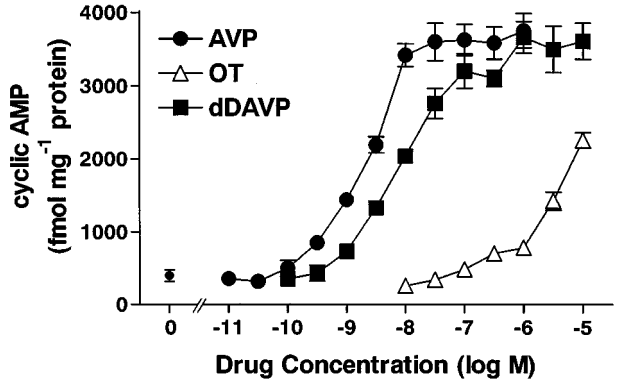

Figure 4 Effect of AVP, oxytocin and dDAVP on production of cellular cyclic AMP in human V_2 receptors expressing CHO cells. Values are means ± s.e.mean from 6–8 independent experiments.

receptors with K_i values comparable to those of AVP. In contrast, OPC-31260 showed much lower affinity for hV_{1A} and hV_2 receptors and exhibited a poor hV_2 receptor selectivity profile and could be considered as a nonselective V_{1A} and V_2 receptor antagonist. Similar results have been reported previously for human kidney V_2 receptors (Serradeil-Le Gal *et al*., 1996).

Following the binding characterization of the expressed receptors, the signal transduction pathway of the human AVP receptors in CHO cells was investigated. AVP activates phospholipase C-mediated hydrolysis of polyphosphoinositides *via* the V_{1A} and V_{1B} receptors to generate two second messengers, inositol-1,4,5-triphosphate, which induces an increase in free intracellular calcium from the endoplasmic reticulum, and 1,2-diacylglycerol, which activates protein kinase C (Michell *et al*., 1979). AVP administered to CHO cells expressing hV_{1A} and hV_{1B} receptors increased $[Ca^{2+}]_i$ in a concentration-dependent manner and these responses were effectively blocked by AVP receptor antagonists. At hV_{1A} receptors, the relative order of potency was SR 49059 > YM087 > OPC-31260 > SR 121463A > > OPC-21268 while at hV_{1B} receptors, the order was SR 49059 > > SR 121463A = YM087 = OPC-31260 = OPC-21268. In contrast, AVP stimulates adenylate cyclase in hV_2 receptors resulting in the production of cyclic AMP (Birnbaumer *et al*., 1992). In the present experiments using CHO cells expressing hV_2 receptors, AVP stimulated intracellular cyclic AMP production in a concentration-dependent manner. Under the same experimen-

tal conditions, AVP receptor antagonists inhibited the production of cyclic AMP induced by AVP. The order of potency was SR 121463A = YM087 > OPC-31260 > SR 49059 > > OPC-21268. Furthermore, these antagonistic potencies are consistent with the affinities obtained from the [^{3}H]-AVP binding studies. It is noteworthy that none of antagonists increased $[Ca^{2+}]_i$ and cyclic AMP production significantly, indicating a complete lack of agonistic activity for these receptors at concentrations up to 10 μM (data not shown).

dDAVP is considered to be a potent and selective V_2 receptor agonist, and is sometimes utilized as a standard ligand of V_2 receptor selective agonists in investigation of the physiologic and pathophysiologic roles of AVP. In the present study using various human AVP receptor-expressing CHO cells, dDAVP more strongly inhibited [^{3}H]-AVP binding to V_{1B} receptors than to V_2 receptors (K_i: 5.81 nM vs 23.3 nM). Furthermore, dDAVP not only induced an agonistic response in V_2 receptors, but also induced an equally potent agonistic response in V_{1B} receptors. On the contrary, dDAVP had no agonistic activity in V_{1A} receptors. These results clearly suggest that dDAVP is not the V_2 receptor selective agonist but acts on V_{1B} receptor in the same manner as on V_2 receptors and is a useful tool for identifying V_{1A} receptors because it clearly lacks agonistic activity.

Because AVP may play a role in several disease conditions, including heart failure, hypertension, renal diseases, hyponatremia and syndrome of inappropriate antidiuretic hormone secretion (SIADH) *via* the V_{1A} and/or V_2 receptors (Laszlo *et al*., 1991; Fujisawa *et al*., 1993; Naitoh *et al*., 1994; Nishikimi *et al*., 1996), AVP receptor antagonists may be useful in the treatment of these diseases. The development of AVP receptor antagonists appears essential for investigating the pathophysiological roles of AVP and could lead to new therapeutic agents for many circulatory and hypertensive disorders. The results of this study indicate that SR 49059 and SR 121463A are the most potent and selective hV_{1A} and hV_2 receptor antagonist, respectively, and YM087 is a potent and nonselective hV_{1A} and hV_2 receptor antagonist with K_i values comparable to those to AVP and, thus, may serve as a useful pharmacological tool to examine the role of these receptor subtypes.

In conclusion, using receptor binding and second messenger assays, we have pharmacologically characterized human V_{1A}, V_{1B} and V_2 receptors stably expressed in CHO cells. These cloned, expressed AVP receptors should prove useful in studying the binding and biochemical function of AVP and its analogues, as well as aid in the evaluation of potential human AVP receptor antagonists. In addition, we have determined the affinity, selectivity and potency of nonpeptide AVP receptor antagonists. Of these, YM087 is, to our knowledge, the highest affinity and potency nonpeptide antagonist to be described for human V_{1A} and V_2 receptors. Potent nonpeptide AVP receptor antagonists, including YM087, should prove to be a novel and valuable tool that can be used to define the physiological and pathophysiological roles of AVP.

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