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Effects of YM218, a nonpeptide vasopressin V_{1A} receptor-selective antagonist, on human vasopressin and oxytocin receptors

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Abstract

The binding and signal transduction characteristics of YM218 ((*Z*)-4′-{4,4-difluoro-5-[2-oxo-2-(4-piperidinopiperidino)ethylidene]-2,3,4,5-tetrahydro-1*H*-1-benzoazepine-1-carbonyl}-2-methyl-3-furanilide hemifumarate), a newly synthesized, potent arginine vasopressin (AVP) V_{1A} receptor-selective antagonist, were examined using cloned human AVP receptors (V_{1A}, V_{1B} and V_2) stably expressed in Chinese hamster ovary (CHO) cells and human uterine smooth muscle cells (USMCs) expressing oxytocin receptors. YM218 potently inhibited specific binding of [^{3}H] AVP to V_{1A} receptors, exhibiting a K_i value of 0.30 nM. In contrast, YM218 exhibited much lower affinity for V_{1B}, V_2 and oxytocin receptors, exhibiting K_i values of 25,500 nM, 381 nM and 71.0 nM, respectively. In CHO cells expressing V_{1A} receptors, YM218 potently inhibited the AVP-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), exhibiting an IC_{50} value of 0.25 nM. However, in human USMCs expressing oxytocin receptors, YM218 exhibited a much lower potency in inhibiting the oxytocin-induced $[Ca^{2+}]_i$ increase, showing an IC_{50} value of 607 nM, and had no effect on the AVP-induced $[Ca^{2+}]_i$ increase in CHO cells expressing V_{1B} receptors. Furthermore, in CHO cells expressing V_2 receptors, YM218 did not potently inhibit the production of cAMP stimulated by AVP, showing an IC_{50} value of 62.2 nM. In all assays used, YM218 did not exhibit any agonistic activity. These results demonstrate that YM218 is a potent, nonpeptide human V_{1A} receptor-selective antagonist, and that YM218 will be a valuable new tool to gain further insight into the physiologic and pharmacologic actions of AVP.

Keywords: YM218; Vasopressin receptor antagonist; Human V_{1A} receptors

1. Introduction

Arginine vasopressin (AVP), a peptide hormone secreted by the posterior pituitary, is an important regulator of fluid and cardiovascular homeostasis. These actions are mediated through specific AVP receptors located in a variety of tissues and organs including blood vessels, liver, brain and kidney [1–5]. The potential for multiple AVP receptor subtypes has been recognized for some time. The data supporting these proposals were based largely upon the relative affinities or potencies of peptide agonist and antagonist analogs of AVP in various tissues and organs. These AVP receptor subtypes have been classified according to the second messenger system to which they are coupled, and presently at least three AVP receptor subtypes (V_{1A}, V_{1B} and V_2) have been identified. AVP activates phospholipases A_2, C and D via the V_{1A} and V_{1B} receptors [6]. This results in the production of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, the mobilization of intracellular calcium, and the activation of protein kinase C resulting in protein phosphorylation [7]. In contrast, V_2 receptors stimulate adenylate cyclase, resulting in the production of cAMP [4]. These three AVP receptor subtypes have been cloned and stably expressed, and found to belong to a family of seven membrane-spanning receptors that transduce signals through G protein [8–11].

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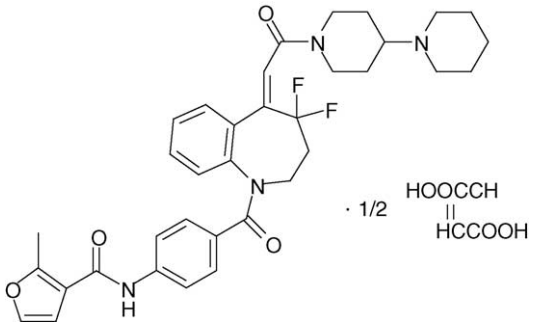

Fig. 1. Chemical structure of YM218, (*Z*)-4′-{4,4-difluoro-5-[2-oxo-2-(4-piperidinopiperidino) ethylidene]-2,3,4,5-tetrahydro-1*H*-1-benzoazepine-1-carbonyl}-2-methyl-3-furanilide hemifumarate.

AVP plays a role in the pathogenesis of several diseases, including heart failure, hypertension, hyponatremia, renal diseases and the syndrome of inappropriate antidiuretic hormone secretion (SIADH). Consequently, AVP receptor antagonists may be useful in treating these diseases. Recently, several orally effective, receptor-subtype-selective nonpeptide AVP receptor antagonists have been discovered, namely the V_{1A} receptor-selective antagonists OPC-21268 and SR 49059 [12,13], the V_2 receptor-selective antagonists OPC-31260, OPC-41061 and SR 121463A [14–16], the V_{1B} receptor-selective antagonist SSR149415 [17] and the V_{1A}/V_2 receptor antagonist conivaptan (YM087) and YM471 [18,19].

In the present study, the effects of YM218 ((*Z*)-4′-{4,4-difluoro-5-[2-oxo-2-(4-piperidinopiperidino)ethylidene]-2,3,4,5-tetrahydro-1*H*-1-benzoazepine-1-carbonyl}-2-methyl-3-furanilide hemifumarate, Fig. 1), a newly synthesized, nonpeptide AVP receptor antagonist, on binding and signal transduction on human AVP and oxytocin receptors were assessed by receptor binding and second messenger assays.

2. Materials and methods

2.1. Material

The radioligands [^{3}H] AVP and [^{3}H] oxytocin with specific activities of 80 Ci mmol^{-1} and 50 Ci mmol^{-1}, respectively, were obtained from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA, USA). AVP and oxytocin were obtained from the Peptide Institute Inc. (Osaka, Japan). YM218, SR 49059 ((2*S*)1-[(2*R*,3*S*)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide), SR 121463A (1-[4-(*N*-*tert*-butylcarbamoyl)-2-methoxybenzenesulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholino ethoxy)cyclohexane]indol-2-one; equatorial isomer), and conivaptan (YM087; 4′-[(2-methyl-1,4,5,6-tetrahydroimidazo[4,5-d][1]benzoazepine-6-carbonyl)-2-phenylbenzanilide monohydrochloride] were synthesized at Yamanouchi Pharmaceutical Co. (Ibaraki, Japan). These antagonists were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10^{-2} M and diluted to the desired concentration with the assay buffer used in the receptor binding and second messenger assays. The final concentration of DMSO in the assay buffer did not exceed 1%, at which neither specific [^{3}H] AVP binding nor specific [^{3}H] oxytocin binding were affected. Fura 2-AM was obtained from Dojindo Laboratories (Kumamoto, Japan) and EGTA, ionomycin, 3-isobutyl-1-methylxanthine (IBMX) and bovine heart tissue were from Wako Pure Chemicals (Osaka, Japan). Minimum essential medium (MEM)-alpha, LipofectAMINE, fetal calf serum (FCS), antibiotics (penicillin and streptomycin) and trypsin–EDTA were from Gibco (Grand Island, NY, USA). Chinese hamster ovary (CHO) cells were from the American Tissue Culture Collection (Rockville, MD, USA). Bovine serum albumin (BSA) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Reagents for protein assay were purchased from Bio-Rad Laboratories (Richmond, CA, USA). All other chemicals were of the highest reagent grade available.

2.2. Membrane preparations

Stable expression of human AVP receptor subtypes in CHO cells and membrane preparations were prepared as described by Tahara et al. [11]. Human uterine smooth muscle cells (USMCs) imported from Clonetics (San Diego, CA, USA) were purchased from IWAKI (Tokyo, Japan). Membrane preparations from human USMCs were prepared as described by Tahara et al. [20,21].

2.3. Binding assays

Binding assays of [^{3}H] AVP to plasma membranes prepared from CHO cells expressing human AVP receptor subtypes were performed as described by Tahara et al. [11]. Binding assays of [^{3}H] oxytocin to plasma membranes prepared from human USMCs expressing oxytocin receptors were performed as described by Tahara et al. [21]. Nonspecific binding was determined using 1 μM unlabeled AVP or oxytocin. Specific binding was calculated as the total binding minus nonspecific binding. The concentration of test compound that caused 50% inhibition (IC_{50}) of the specific binding of [^{3}H] AVP or [^{3}H] oxytocin was determined by regression analysis of displacement curves. The inhibition constant (K_i) was calculated from the following formula [22]: $K_i = IC_{50}/(1 + [L]/K_d)$, where $[L]$ is the concentration of radioligand and K_d is the dissociation constant of radioligand obtained from the Scatchard plot [23]. To investigate whether YM218 is a competitive or noncompetitive inhibitor, saturation binding of [^{3}H] AVP to plasma membranes of CHO cells expressing human V_{1A} receptors in the presence or absence of YM218 was examined. Data were analyzed using the GraphPad PRISM (GraphPAD Software, Inc.; San Diego, CA, USA).

2.4. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cAMP production

Measurement of $[Ca^{2+}]_i$ in CHO cells expressing human V_{1A} or V_{1B} receptors and human USMCs expressing oxytocin receptors were performed as described by Tahara et al. [11,21]. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (F_{max}) with the nonfluorescent Ca^{2+} ionophore, ionomycin (25 μM), after which minimal fluorescence (F_{min}) was obtained by adding 3 mM EGTA. From the ratio (R) of fluorescence at 340 nm and 380 nm, the $[Ca^{2+}]_i$ was determined using the following equation: $[Ca^{2+}]_i$ (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 [24]. Measurement of cAMP production in CHO cells expressing human V_2 receptors were performed as described by Tahara et al. [11].

2.5. Data analysis

Experimental results are expressed as the mean ± standard error of the mean (S.E.M.) or the mean with 95% confidence limits. The IC_{50} values were estimated from concentration–response curves plotted using the nonlinear regression program GraphPad PRISM.

3. Results

3.1. Radioligand binding studies

YM218 potently inhibited specific binding of [^{3}H] AVP to human V_{1A} receptors, exhibiting a K_i value of 0.30 ± 0.02 nM (Fig. 2 and Table 1). The selectivity of YM218 for human V_{1A} receptors was evaluated by measuring the ability of YM218 to inhibit the binding of radioligands to human V_{1B}, V_2 and oxytocin receptors. YM218 exhibited low affinity for V_{1B}, V_2 and oxytocin receptors, exhibiting K_i values of 25,500 ± 2,500 nM, 381 ± 74 nM and 71.0 ± 3.3 nM, respectively, and acted with at least a 200-fold lower potency on V_{1B}, V_2 and oxytocin receptors than on V_{1A} receptors (Table 2). The Hill coefficients (n_H) of YM218 were close to unity, suggesting a single-site, competitive-binding model. [^{3}H] AVP saturation binding experiments performed in the presence or absence of YM218 (0.1 nM, 0.3 nM and 1.0 nM) using CHO cell membranes expressing human V_{1A} receptors revealed increasing concentrations of YM218 caused successive decreases in the slopes of the curves, con-

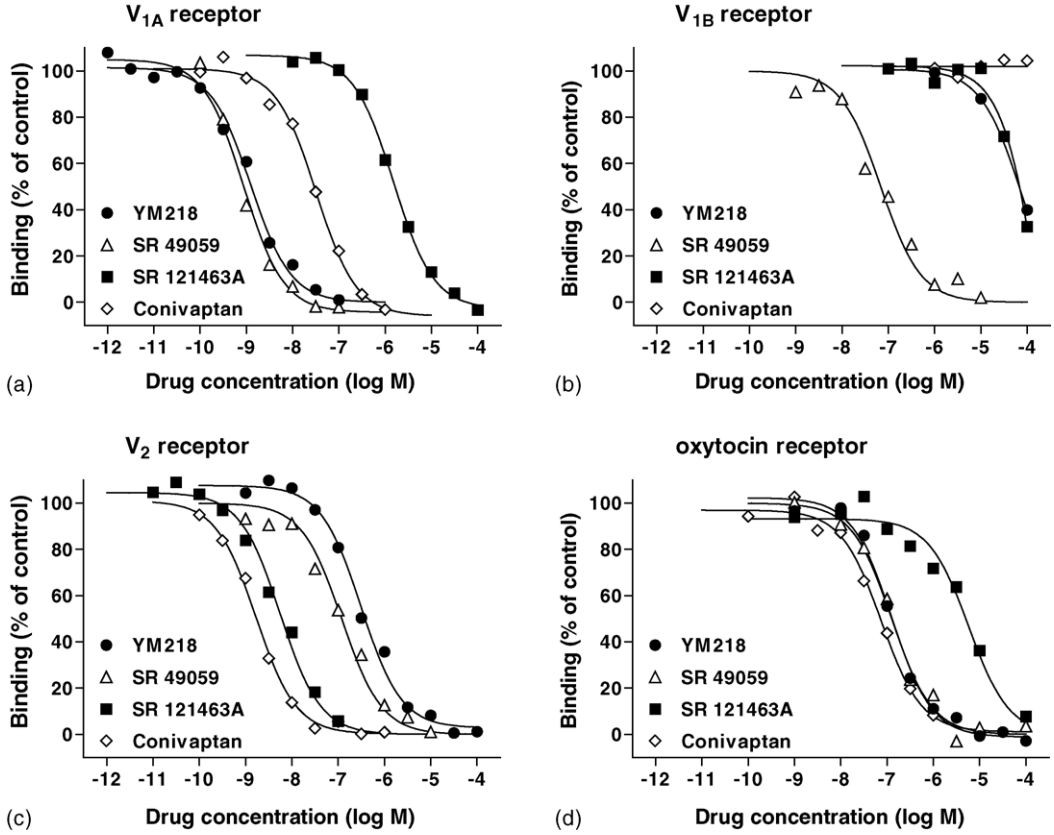

Fig. 2. Inhibition of specific [^{3}H] AVP or [^{3}H] oxytocin bound to plasma membranes prepared from CHO cells expressing human (a) V_{1A}, (b) V_{1B}, (c) V_2, and (d) human uterine smooth muscle cells expressing oxytocin receptors by AVP receptor antagonists. Results are representative data from three to five independent experiments performed in duplicate. The combined results of all experiments are summarized in Table 1.

Table 1
K_i values of nonpeptide AVP receptor antagonists for human AVP and oxytocin receptors

Compounds	V_{1A} receptor		V_{1B} receptor		V_2 receptor		Oxytocin receptor	
	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H
YM218	0.30 ± 0.02	−1.16 ± 0.08	25500 ± 2500		381 ± 74	−1.05 ± 0.07	71.0 ± 3.3	
SR 49059[a]	0.53 ± 0.08	−1.03 ± 0.10	48.4 ± 10.3	−0.95 ± 0.04	178 ± 41	−0.98 ± 0.03	69.3 ± 7.3	−0.94 ± 0.06
SR 121463A[a]	304 ± 7	−1.19 ± 0.02	52100 ± 13800		2.75 ± 0.62	−1.02 ± 0.13	1940 ± 110	−0.91 ± 0.10
Conivaptan[b]	4.30 ± 0.99	−1.09 ± 0.14	>100000		1.91 ± 0.24	−1.11 ± 0.08	29.8 ± 4.1	−1.00 ± 0.12

Values are mean ± S.E.M. obtained from three to five independent experiments performed in duplicate.
[a] Corresponding values of SR 49059 and SR 121463A are taken from previously reported data [19].
[b] Corresponding values of conivaptan are taken from previously reported data [11].

Table 2
Selectivity profile of nonpeptide AVP receptor antagonists, YM218, SR 49059, SR 121463A and conivaptan, for human AVP and oxytocin receptors

Compounds	Selectivity index		
	K_i V_2/K_i V_{1A}	K_i V_{1B}/K_i V_{1A}	K_i oxytocin/K_i V_{1A}
YM218	1300	85000	240
SR 49059	340	91	130
SR 121463A	0.009	170	6.4
Conivaptan	0.44	>20000	6.9

Inhibition constants (K_i) used in the calculation of these ratios are given in Table 1.

sistent with an increase in equilibrium dissociation constant (K_d) with no reduction in receptor density (B_{max}; Fig. 3).

3.2. Measurement of $[Ca^{2+}]_i$

Addition of AVP to fura 2-loaded CHO cells expressing human V_{1A} and V_{1B} receptors increased $[Ca^{2+}]_i$ concentration-dependently with EC_{50} values of 1.13 (0.91–1.40) nM and 0.90 (0.70–1.16) nM, respectively, and submaximal stimulation was observed at a concentration of 10 nM. In CHO cells expressing human V_{1A} receptors, YM218 strongly and concentration-dependently inhibited the increase in $[Ca^{2+}]_i$ stimulated by 10 nM AVP, exhibiting an IC_{50} value of 0.25 (0.20–0.31) nM (Fig. 4 and Table 3). In contrast, in CHO cells expressing human V_{1B} receptors, YM218 did not inhibit the increase in $[Ca^{2+}]_i$ stimulated by 10 nM AVP (IC_{50} > 10,000 nM; Fig. 5). Oxytocin added to fura 2-loaded human USMCs expressing oxytocin receptors resulted in an increase in $[Ca^{2+}]_i$ dependent upon oxytocin concentration with EC_{50} value of 2.09 (1.19–3.65) nM and submaximal stimulation was observed at a concentration of 100 nM. YM218 inhibited the increase in $[Ca^{2+}]_i$ stimulated by 100 nM oxytocin in human USMCs expressing oxytocin receptors, exhibiting an IC_{50} value of 607 (344-1, 070) nM

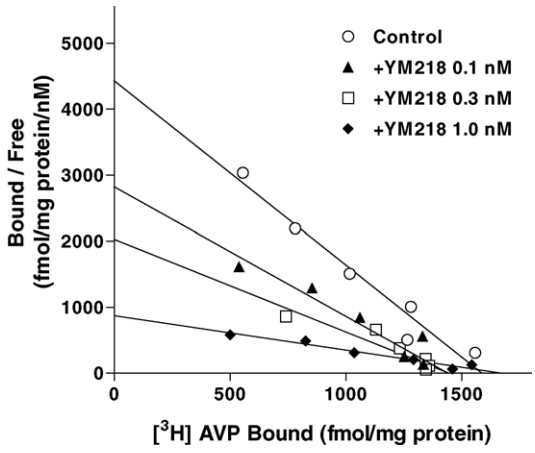

Fig. 3. Scatchard plots of [^{3}H] AVP binding to plasma membranes prepared from CHO cells expressing human V_{1A} receptors in the absence or presence of YM218. Results are representative data from four independent experiments performed in duplicate.

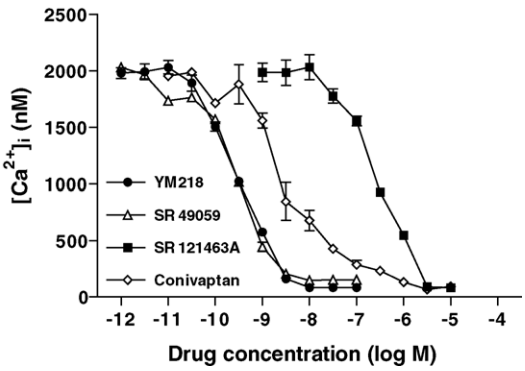

Fig. 4. Effect of AVP receptor antagonists on 10 nM AVP-induced $[Ca^{2+}]_i$ increases in CHO cells expressing human V_{1A} receptors. Values are mean ± S.E.M. from three to five independent determinations.

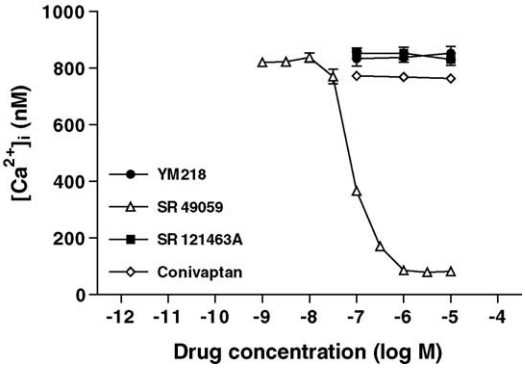

Fig. 5. Effect of AVP receptor antagonists on 10 nM AVP-induced $[Ca^{2+}]_i$ increases in CHO cells expressing human V_{1B} receptors. Values are mean ± S.E.M. from three to five independent determinations.

Table 3
Inhibitory activities of AVP receptor antagonists for $[Ca^{2+}]_i$ and cAMP responses in CHO cells expressing human AVP receptors and in human uterine smooth muscle cells expressing oxytocin receptors

Compounds	IC_{50} (nM)			
	$[Ca^{2+}]_i$			cAMP
	V_{1A} receptor	V_{1B} receptor	Oxytocin receptor	V_2 receptor
YM218	0.25 (0.20–0.31)	>10000	607 (344–1070)	62.2 (45.6–84.8)
SR 49059[a]	0.32 (0.23–0.45)	62.3 (36.5–107)	653 (398–1070)	186 (104–330)
SR 121463A[a]	223 (167–298)	>10000	19500 (13000–29100)	1.66 (1.18–2.33)
Conivaptan[b]	1.32 (0.74–2.36)	>10000	420 (288–612)	2.13 (1.42–3.18)

Values are mean with 95% confidence limits of three to eight independent experiments.
[a] Corresponding values of SR 49059 and SR 121463A are taken from previously reported data [19].
[b] Corresponding values of conivaptan are taken from previously reported data [11].

(Fig. 6). These IC_{50} values correspond well with the K_i values obtained from the binding studies (Tables 1 and 3). In all assays used, YM218 had no agonistic activity at concentrations up to 10 μM.

3.3. Measurement of cAMP production

Addition of AVP to CHO cells expressing human V_2 receptors resulted in a concentration-dependent increase in cellular cAMP production with EC_{50} value of 2.22 (1.69–2.92) nM and submaximal stimulation was observed at a concentration of 10 nM. YM218 concentration-dependently inhibited 10 nM AVP-induced increase in cAMP production by CHO cells expressing human V_2 receptors, exhibiting an IC_{50} value of 62.2 (45.6–84.8) nM (Fig. 7 and Table 3). No change in basal cAMP production occurred when YM218 was tested alone at concentrations up to 10 μM, indicating again the lack of any agonist activity.

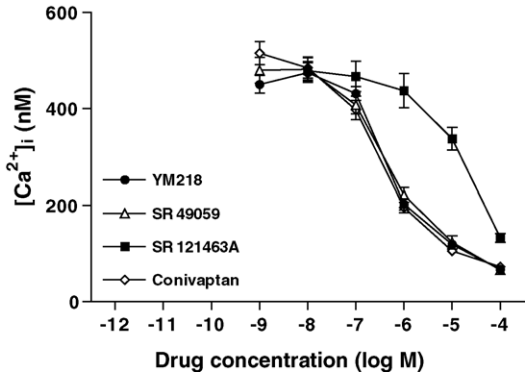

Fig. 6. Effect of AVP receptor antagonists on 100 nM oxytocin-induced $[Ca^{2+}]_i$ increases in human uterine smooth muscle cells. Values are mean ± S.E.M. from four independent determinations.

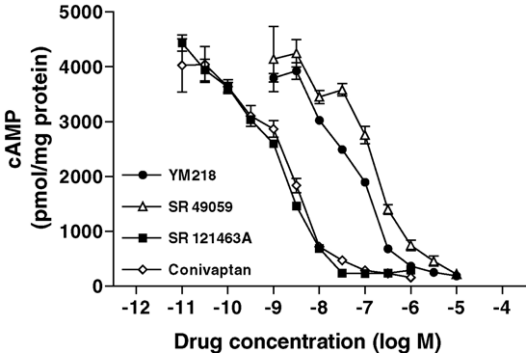

Fig. 7. Effect of AVP receptor antagonists on 10 nM AVP-induced cellular cAMP production in CHO cells expressing human V_2 receptors. Values are mean ± S.E.M. from six to eight independent determinations.

4. Discussion

We investigated the pharmacologic properties of YM218 using radioligand binding and second messenger assays. We first evaluated the affinity of YM218 for human AVP and oxytocin receptors in radioligand binding assay using plasma membranes from CHO cells stably expressing the human AVP receptors (V_{1A}, V_{1B} and V_2) and human USMCs expressing oxytocin receptors. YM218 inhibited [^{3}H] AVP binding to human V_{1A} receptors, exhibiting a subnanomolar affinity. To determine whether YM218 interacts reversibly or irreversibly with V_{1A} receptors, we investigated [^{3}H] AVP saturation binding in the presence or absence of YM218 and analyzed according to Scatchard [23]. In CHO cells expressing human V_{1A} receptors, YM218 concentration-dependently reduced the slopes of Scatchard plots without affecting the intercept, indicating a change in the K_d values of the radioligand for its receptors without a change in the B_{max} values. The data suggest YM218 interacts in a reversible and competitive manner with V_{1A} receptors. In contrast, YM218 showed much lower affinity for human V_{1B}, V_2 and oxytocin receptors. These results indicate that YM218 possesses potent affinity and selectivity for human V_{1A} receptors. We have already confirmed that YM218 possesses potent affinity and selectivity for rat V_{1A} receptors. In the in vitro affinity and potence of several nonpeptide AVP receptor antagonist, we [11,25] and others [1,26,27] have previously demonstrated the existence of great species differences between rat and human V_{1A} receptors. The V_{1A} receptor-selective antagonist, OPC-21268, displayed moderate affinity (K_i = 23.5 nM) for rat V_{1A} receptors, but exhibited low affinity (K_i = 25,000 nM) for human V_{1A} receptors. Therefore, it is important to confirm that YM218 potently interacts with human V_{1A} receptors.

Another important question was whether the affinity observed in these binding studies was translated into a similar potency for inhibition of signal transduction; several in vitro functional studies were performed to characterize the nature of the interaction of YM218 with the human V_{1A}, V_{1B}, V_2 and oxytocin receptors. AVP and oxytocin activate phospholipase C-mediated hydrolysis of polyphosphoinositides via the V_{1A}, V_{1B} and oxytocin receptors to generate two second messengers, inositol-1,4,5-trisphosphate, which induces an increase in free intracellular calcium from the endoplasmic reticulum, and 1,2-diacylglycerol, which activates protein kinase C [7,28–30]. In CHO cells expressing human V_{1A} or V_{1B} receptors, AVP concentration-dependently caused an increase in $[Ca^{2+}]_i$. YM218 potently antagonized the AVP-induced increase in $[Ca^{2+}]_i$ mediated by V_{1A} receptors, but did not inhibit the increase in $[Ca^{2+}]_i$ mediated by V_{1B} receptors. Similarly, in human USMCs expressing only oxytocin receptors [20,21], oxytocin caused an increase in $[Ca^{2+}]_i$ concentration-dependently. While YM218 inhibited the oxytocin-induced increase in $[Ca^{2+}]_i$, the inhibitory potency against oxytocin receptors was more than 200-times lower than that observed against V_{1A} receptors. These results are consistent with the K_i values of YM218 obtained from the [^{3}H] AVP and [^{3}H] oxytocin binding studies. For V_2 receptors, AVP stimulates adenylate cyclase, resulting in the production of cAMP [31]. In the present experiment using CHO cells expressing human V_2 receptors, while AVP concentration-dependently stimulated intracellular cAMP production, YM218 did not potently inhibit the production of cAMP induced by AVP. Furthermore, in the absence of AVP, YM218 neither stimulated $[Ca^{2+}]_i$ mobilization nor induced cAMP production, indicating that YM218 exerts no agonistic activity on AVP or oxytocin receptors. These results suggest that YM218 is a potent human AVP receptor antagonist which possesses V_{1A} receptor-selective antagonistic activities with no agonistic activity.

For the past several years, many reports have described the important role of AVP plays in several regulatory pathways, especially those involving cardiovascular and endocrine functions. These reports provide many clues suggesting AVP might be intimately involved in the pathogenesis of several disorders and diseases including hypertension, congestive heart failure, vasospasm and renal diseases through its effect on the V_{1A} receptor [32–35]. Among these conditions, renal failure and diabetic nephropathy are the most likely treatment indications for V_{1A} receptor antagonists, because these renal diseases are progressively deteriorating condition characterized by glomerular hypertension and mesangial cell hypertrophy and matrix expansion, and AVP can induce potent contraction of renal efferent arterioles and proliferation/hypertrophy of mesangial cells via the V_{1A} receptors [36,37]. Additionally, it has been reported that the plasma level of AVP increases in patients with renal failure and diabetes as well as animal models of these conditions [38–44]. Increased secretion of AVP may induce proliferation/hypertrophy of mesangial cells and glomerular hemodynamic alterations. Indeed, a three-week treatment with OPC-21268, a nonpeptide V_{1A} receptor-selective antagonist, demonstrated that albuminuria significantly decreased without affecting blood pressure and renal functions in patients with diabetic nephropathy [40]. It has already been reported that some antihypertensive drugs including angiotensin converging enzyme inhibitors and calcium blockers decreased albuminuria in diabetic nephropathy. These drugs reduce arterial blood pressure and may cause a fall in glomerular filtration rate. However, OPC-21268 did not reduce the systemic blood pressure in diabetic nephropathy; V_{1A} receptor-selective antagonists may directly attenuate glomerular hemodynamics in diabetic nephropathy. These observations suggest that AVP may play a crucial role in the pathophysiology of diabetic nephropathy and that V_{1A} receptor antagonists could be used to treat or prevent further progression of nephropathy in patients with diabetes mellitus.

In summary, the present data demonstrate that YM218 has the highest affnity, selectivity, and potency of any nonpeptide human V_{1A} receptor antagonist thus far descrived; YM218 should prove to be a novel and valuable tool that can be used to define the physiologic and pathophysiologic role of AVP.

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