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EFFECT OF A NEW, POTENT, NON-PEPTIDE V_{1a} VASOPRESSIN ANTAGONIST, SR 49059, ON THE BINDING AND THE MITOGENIC ACTIVITY OF VASOPRESSIN ON SWISS 3T3 CELLS

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Abstract—The effects of SR 49059, a new non-peptide, selective arginine vasopressin (AVP), V_{1a} antagonist, were investigated both on AVP's receptors and on the mitogenic effects of AVP on Swiss 3T3 fibroblasts. We characterized the AVP V_{1a} receptors on Swiss 3T3 cell membranes using the new highly specific AVP V_{1a} radioiodinated ligand, ^{125}I-linear AVP antagonist. Specific binding of the ^{125}I-linear AVP antagonist was saturable, time-dependent and reversible. A single class of high affinity binding sites was identified with an apparent K_d of 40 ± 20 pM and a B_{max} of 63 ± 20 fmol/mg protein. ^{125}I-Linear AVP antagonist binding to its receptors was potently inhibited in a concentration-dependent manner by AVP, by the peptide V_{1a} antagonist d(CH$_2$)$_5$Tyr(Me)AVP and by the synthetic V_{1a} antagonist, SR 49059 (IC_{50} in the nanomolar range) while OPC-21268, another non-peptide compound, was about 100-fold less potent. Both DDAVP, a selective V_2 agonist, and oxytocin exhibited low affinity ($IC_{50} > 1$ μM) in agreement with the AVP V_{1a} nature of the site identified on Swiss 3T3 cells. In addition, the broad-spectrum antiproliferative agent [Arg^6, D-Trp^{7,9}, MePhe^8] substance P (6–11), was also able to interact at 3T3 AVP V_{1a} receptors (IC_{50} = 395 ± 170 nM). The mitogenic effects of AVP on quiescent Swiss 3T3 cells, assessed through [^{3}H]thymidine incorporation, were selectively, stereospecifically and strongly inhibited by SR 40959 (IC_{50} = 14 ± 2 nM) while OPC-21268 was inactive up to 220 nM. SR 49059 was even about six times more efficient than d(CH$_2$)$_5$Tyr(Me)AVP in inhibiting AVP-induced DNA synthesis. Moreover, SR 49059 fully inhibited Swiss 3T3 fibroblast proliferation since it completely blocked AVP-stimulated 3T3 cell growth from the G1/G0 into the S/G$_2$M phase, as evidenced by cell cycle analysis using a cytofluorometer. In summary, SR 49059, through direct interaction at AVP V_{1a} receptors, exerts the most potent antiproliferative effect yet described for any V_{1a} antagonist on Swiss 3T3 cells.

AVP‡ is a neuropeptide well known for its pressor and antidiuretic properties in mammals. AVP has also been shown to exert a wide variety of biological activities in stimulating platelet aggregation, liver glycogenolysis and uterine motility (for review, see 1). Among these varying effects, AVP is a potent mitogen on several target tissues such as rat hepatocytes [2], rat glomerula cells [3], Swiss 3T3 fibroblasts [4], human SCLC [5] and rat VSMC [6], suggesting that AVP may be involved in several pathophysiological proliferative states such as lung cancer or atherosclerosis.

The murine fibroblast Swiss 3T3 cells have provided a useful model for testing binding, mitogenic effects and the signal traduction pathways of several mitogenic neuropeptides including AVP [4], bradykinin, vasoactive intestinal peptide, bombesin and GRP [7–10]. So far, three AVP receptor subtypes (V_{1a}, V_{1b} and V_2) have been identified on pharmacological and functional bases. The V_{1a} subtype described on several tissues (e.g. liver, VSMC, platelets) and also on Swiss 3T3 fibroblasts is clearly involved in the mitogenic process of AVP since a specific AVP V_{1a} peptide antagonist such as d(CH$_2$)$_5$Tyr(Me)AVP inhibited the binding, Ca^{2+} mobilization and DNA synthesis by AVP in Swiss 3T3 cells [5, 11].

In the past decade, a large number of potent and selective vasopressin peptide antagonists have been designed [12–14]. However, their lack of oral bioavailability and their short half-life have severely limited their clinical and therapeutical investigations. Novel structural classes of non-peptide AVP V_{1a} structures have been discovered recently; in this field, Yamamura *et al.* [15] described the first orally effective compound, OPC-21268, and more recently we reported the pharmacological characterization of the most potent, selective V_{1a} antagonist yet described, SR 49059, active *in vitro* at rat and human AVP V_{1a} receptors and displaying good oral bioavailability *in vivo* [16].

In this study we investigated the effects of SR 49059 ((2S) 1 - [(2*R* 3*S*) - 5 - chloro - 3 - (2 - chloro-

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‡ Abbreviations: AVP, arginine vasopressin; DDAVP, Desamino-[D-Arg^8]-vasopressin; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; GRP, gastrin-releasing peptide; OT, oxytocin; PMSF, phenylmethylsulfonide fluoride; SCLC, small cell lung cancer; VSMC, vascular smooth muscle cells.

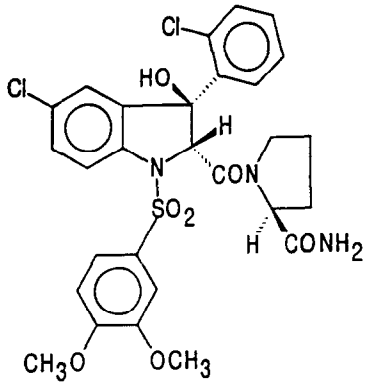

Fig. 1. Structure of SR 49059.

phenyl) - 1 - (3,4 -dimethoxybenzene - sulfonyl) - 3 - hydroxy - 2,3 - dihydro - 1*H* - indole - 2 - carbonyl] - pyrrolidine-2-carboxamide) (Fig. 1) in the murine fibroblast Swiss 3T3 cell model in comparison with OPC-21268. Firstly, we determined the binding characteristics of SR 49059 to AVP 3T3 cell receptors using the recently developed linear radioiodinated ligand, ^{125}I-linear AVP antagonist [17], which displayed highly selective affinity for AVP V_{1a} receptors. Secondly, the activity of SR 49059 was studied on AVP-induced proliferation and cell cycle distribution in cultured Swiss 3T3 fibroblasts.

MATERIALS AND METHODS

Materials. The non-peptide molecules, 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), its enantiomer, SR 49770 ((2*R*) 1 - [(2*S* 3*R*) - 5 - chloro - 3 - (2 - chlorophenyl) - 1 - (3,4 - dimethoxybenzene - sulfonyl) - 3 - hydroxy - 2,3 - di - hydro -1*H* - indole - 2 - carbonyl] - pyrrolidine - 2 - carboxamide) and OPC-21268 (1 - [1 - [4(3 - acetylaminopropoxy)benzoyl] - 4 - piperidyl] - 3,4 - dihydro - 2 - (1*H*) - quinolinone) were synthesized at Sanofi Recherche (Montpellier, France). All compounds were initially dissolved in DMSO at a concentration of 10^{-2} M, then diluted in the appropriate test solvent. AVP, d(CH_2)$_5$Tyr(Me)AVP (SKF-100273), OT, DDAVP, [Arg^6, D-Trp^{7,9}, MePhe^8] substance P (6–11), soy bean trypsin inhibitor, bacitracin, polybrene, PMSF and RNase were from the Sigma Chemical Co. (L'isle d'Abeau, France). Propidium iodide was obtained from Molecular Probes Inc. (OR, U.S.A.). Endothelin-1 and sarafotoxin S6b were purchased from Novabiochem Ltd (U.K.). BSA type V was obtained from IBF (Paris, France). All tissue culture reagents were from Boehringer Mannheim (Meylan, France). EDTA, Tris and DMSO were purchased from Merck-Clevenot (Nogent sur Marne, France). All other chemicals were from Prolabo (France). The radioligand ^{125}I-linear AVP antagonist (phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2) was synthesized as described in [17] and [^{3}H]-thymidine (25 Ci/mmol) was obtained from Amersham (Les Ulis, France).

Swiss 3T3 cell culture. Swiss 3T3 fibroblasts were generously provided by Dr E. Rozengurt (Imperial Cancer Research Fund, London). The cells were maintained in 8% CO_2 in air in 90 mm Nunc dishes by twice weekly passage in DMEM containing 10% FCS.

Membranes preparation of Swiss 3T3 cells. Culture medium was removed and confluent Swiss 3T3 cells in 175 mm^2 flasks were washed with PBS. Cells were then scraped off the flask, resuspended in ice-cold PBS and homogenized with a polytron homogenizer (set at six for 2 × 10 sec). The membrane pellet was washed once in ice-cold buffer A (10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$ and 0.5 mM EDTA), resuspended at a final concentration of approx. 5 mg/mL and stored as aliquots in liquid nitrogen until used. Protein was determined by the method of Bradford [18] with BSA as standard.

Vasopressin binding on Swiss 3T3 membranes using ^{125}I-linear AVP antagonist. Binding assays on Swiss 3T3 membranes were performed in an incubation medium (200 μL) containing 10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 1 mg/mL BSA, 0.05 mg/mL soy bean trypsin inhibitor, 0.5 mg/mL bacitracin, 0.5 mM EDTA, ^{125}I-linear AVP antagonist (1–800 pM for saturation experiments or 40 pM for kinetic and competition studies) and increasing amounts of a test compound. The reaction was started by the addition of Swiss 3T3 membranes (about 20 μg/assay) incubated at 30° for 30 min (except in association–dissociation studies). The reaction was stopped by adding 3 mL of ice-cold buffer followed by filtration through GF/C Whatman glass microfiber filters presoaked in 0.5% polybrene. Filters were washed with 2 × 3 mL of ice-cold buffer and counted for radioactivity in a multiwell gammacounter (LKB Wallac, Finland). Non-specific binding was determined in the presence of 1 μM unlabeled AVP.

Assay of DNA synthesis. Cells were seeded into 96-well microtiter plates (Falcon) at 10^4 cells/well and grown for 6 days at 37° in 8% CO_2, in air, by which time they were confluent and quiescent. The cells were washed twice with serum-free DMEM. The response was then tested in DMEM/Waymouth's medium (1:1) containing 1 μg/mL insulin. The indicated concentrations of drugs were added at culture initiation and left for 48 h. Twenty-four hours prior to harvesting, [^{3}H]thymidine (1 μCi/well) was pulsed. After trypsinization, cells were harvested using a Skatron cell harvester (LKB Wallac, Finland). Radioactivity incorporated into the DNA was measured in a liquid scintillation counter (1205 Betaplate, LKB Wallac, Finland).

Cell cycle measurement. Swiss 3T3 cells were incubated for 48 hr in serum-free DMEM/Waymouth medium. Vasopressin was added at the beginning of the culture in the presence or absence of 10^{-7} M SR 49059. Twenty-four hours later, cells were dissociated with 2.5 mg/mL trypsin and fixed in 70% ethanol overnight. After one wash in PBS without Ca^{2+} and Mg^{2+} cells were resuspended in 1 mL of a solution containing RNase and 50 μg/mL propidium iodide for 30 min at 37°. Analysis of DNA content was

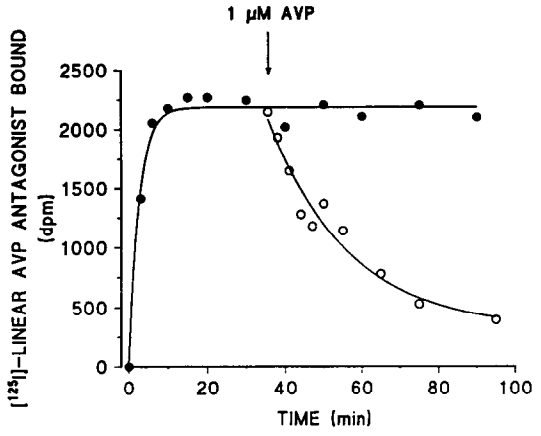

Fig. 2. Time-course of association (●) of ^{125}I-linear AVP antagonist to Swiss 3T3 cells membranes and dissociation (○) after adding unlabeled AVP. Membranes were incubated for various periods of time with ^{125}I-linear AVP antagonist (40 pM) at 30°. The arrow indicates the time at which AVP (1 μM) was added to initiate the dissociation process. Results represent data from a typical experiment which was repeated three times without noticeable modifications.

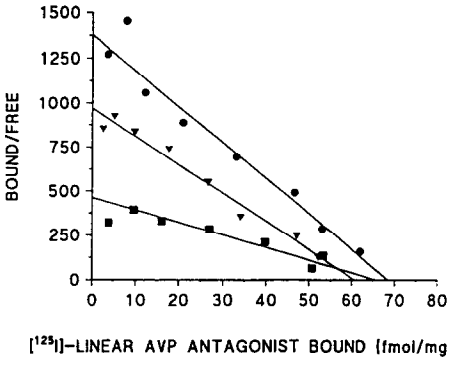

Fig. 3. Scatchard plots of ^{125}I-linear AVP antagonist binding to Swiss 3T3 cell membranes without (●) or with 0.5 (▲), 2 (■) nM SR 49059. Scatchard analyses of data were calculated from saturation isotherms conducted in the presence of increasing ^{125}I-linear AVP antagonist concentrations (1–800 pM) 30 min at 30°, as described in Materials and Methods. Each point is the mean calculated from a typical experiment (N = 2).

performed using a FACSTAR PLUS cytometer. Doublet discrimination was performed using pulse width measurements.

Binding data analysis. 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 [19]. Data for equilibrium binding (K_d, B_{max}), competition experiments (IC_{50}, n_H) and kinetic constants (k_{obs}, k_{-1}) were analysed using an iterative non-linear regression program [20].

RESULTS

Characterization of ^{125}I-linear AVP antagonist binding to Swiss 3T3 membranes

As shown in Fig. 2, specific binding of the ^{125}I-linear AVP antagonist to Swiss 3T3 cell membranes at 30° was time-dependent and reached an apparent equilibrium 15–20 min after the beginning of the experiment. An apparent equilibrium constant (k_{obs}) of 0.301 ± 0.096 min^{-1} and a time of half-maximum association ($t_{1/2}$) of 2.5 ± 1 min (N = 3) were calculated from these time-course binding studies.

After 30 min of incubation, the binding of the ^{125}I-linear AVP antagonist was reversible since the addition of unlabeled AVP (1 μM) to membranes induced rapid dissociation of the peptide–receptor complex (Fig. 2) with an apparent dissociation constant (k_{-1}) value of 0.047 ± 0.023 min^{-1} (N = 3). At equilibrium, under standard conditions, non-specific binding represented 30% of total binding. It is worth noting that carrying out binding studies with this new linear radioiodinated ligand is particularly advantageous since only small protein quantities (approx. 10–20 μg/assay) are required.

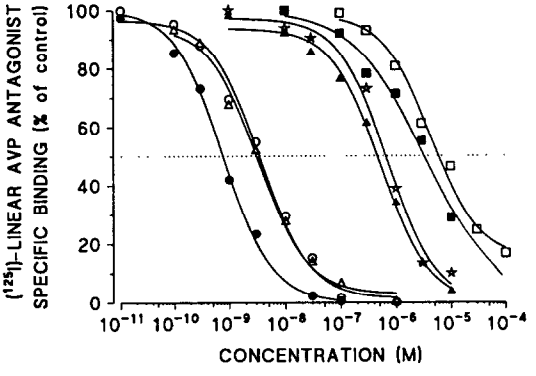

Fig. 4. Inhibition of specific ^{125}I-linear AVP antagonist binding to Swiss 3T3 membranes by AVP (○), $d(CH_2)_5Tyr(Me)AVP$ (●), DDAVP (□), oxytocin (■), [Arg^6, D-Trp^{7,9}, MePhe^8] substance P (6–11) (▲), SR 49059 (△) or OPC-21268 (☆). Membranes (20 μg) were incubated with ^{125}I-linear AVP antagonist (40 pM) and increasing concentrations of the test compound for 30 min at 30°. Each data point represents the mean calculated from three to five experimental determinations performed in duplicate.

Saturation experiments of ^{125}I-linear AVP antagonist to Swiss 3T3 membranes revealed that the specific binding was saturable and Scatchard analysis of data (Fig. 3) revealed a linear plot consistent with the presence of a single class of non-interacting high-affinity binding sites. The apparent equilibrium dissociation constant (K_d) and the maximal binding capacity (B_{max}) were 48 ± 20 pM and 63 ± 20 fmol/mg protein, respectively (N = 4). We could deduce from this B_{max} value an approximate number of binding sites of 10^5 receptors/cell in agreement with values reported by Collins and Rozengurt [21] using [^{3}H]-AVP as a ligand on quiescent Swiss 3T3 intact cells.

Competition experiments (Fig. 4) showed that

Table 1. Inhibition of the binding of ^{125}I-linear AVP antagonist and AVP-induced DNA synthesis in Swiss 3T3 cells by selective non-peptide and peptide AVP V_{1a} antagonists

Specific AVP V_{1a} antagonists	IC_{50} (nM)	
	Binding inhibition*	AVP-induced DNA synthesis inhibition†
SR 49059	5.4 ± 2.8	14 ± 2
SR 49770	140 ± 21	210 ± 30
OPC-21268	584 ± 194	>220
$d(CH_2)_5$Tyr(Me)AVP	1.8 ± 0.9	140 ± 30

* Specific binding was measured on Swiss 3T3 cell membranes using 40 pM ^{125}I-linear AVP antagonist and varying concentrations of antagonists as described in Materials and Methods. Each experiment was performed in duplicate and values are the mean ± SD of at least three independent determinations.

† DNA synthesis inhibition was measured in the presence of 6.24 nM AVP and varying concentrations of antagonists as described in Materials and Methods. Values are the mean ± SD of six independent determinations.

^{125}I-linear AVP antagonist binding was potently inhibited in a dose-dependent manner by AVP and $d(CH_2)_5$Tyr(Me)AVP, the selective AVP V_{1a} peptide antagonist, with IC_{50} values of 3.9 ± 1.0 and 1.8 ± 0.9 nM, respectively (N = 3). The selective AVP V_2 agonist, DDAVP, and OT weakly displaced this radioiodinated ligand, confirming the AVP V_{1a} nature of the sites found on Swiss 3T3 fibroblasts (IC_{50} of 6895 ± 795 and 2184 ± 690 nM, respectively, N = 3). Moreover, binding of the ^{125}I-linear AVP antagonist was highly specific since other mitogenic neuropeptides such as vasoactive intestinal peptide (VIP), bombesin, neuropeptide Y, bradykinin, endothelin-1 and sarafotoxin S6b tested at 10 μM did not interfere with ^{125}I-linear AVP antagonist binding to its receptors (not shown).

It was interesting to note that the hexapeptide substance P analogue ([Arg^6, D-Trp^{7,9}, MePhe^8] substance P (6–11)), known for its broad neuropeptide mitogenic antagonist activity (AVP, substance P, bradykinin, GRP) on Swiss 3T3 cells, exhibited also affinity (IC_{50} = 395 ± 170 nM, N = 3) as already reported in the binding of [^{3}H]AVP to these cells [9] (Fig. 4). Dose–response curves for this non-specific peptide displayed linear Hill plots and pseudo Hill coefficients (n_H) about unity (not shown) indicating competitive antagonism with the ligand.

Binding of SR 49059 to vasopressin receptors on Swiss 3T3 cell membranes

SR 49059 [16] (Fig. 1) dose-dependently inhibited the specific binding of ^{125}I-linear AVP antagonist to AVP V_{1a} receptors from murine Swiss 3T3 fibroblast membranes. As shown in Table 1, IC_{50} value was in the nanomolar range and the Hill coefficient value close to unity, compatible with a single site competitive model. Under the same experimental conditions, OPC-21268 [15] exhibited 100 times lower affinity for Swiss 3T3 AVP V_{1a} receptors than SR 49059 (IC_{50} = 5.4 ± 2.8 versus 615 ± 175 nM). Furthermore, it is important to note that SR 49059 binding to AVP V_{1a} receptors on Swiss 3T3 membranes is highly stereospecific since SR 49059's enantiomer, SR 49770, displayed low affinity in the binding of ^{125}I-linear AVP antagonist to Swiss 3T3 AVP V_{1a} receptors (Table 1).

Finally, saturation binding experiments were performed on Swiss 3T3 cell membranes in the absence or presence of SR 49059 (0.5 and 2 nM). Scatchard analysis of data indicated that SR 49059 inhibited ^{125}I-linear AVP antagonist binding in a competitive manner since in the presence of this molecule the apparent dissociation constant (K_d) was significantly increased, whereas the maximal binding capacity (B_{max}) was not significantly modified (Fig. 3). The K_i value calculated from Scatchard plots (1.5 nM) was consistent with the K_i value obtained according to the Cheng and Prussof equation [19] from competition experiments (2.7 ± 1.4 nM, N = 4).

Effect of SR 49059 on vasopressin-induced mitogenicity

Quiescent Swiss 3T3 fibroblasts responded to AVP in a range between 0.1 and 10 nM by increased [^{3}H]-thymidine uptake [4] (Fig. 5A). It was found that the non-peptide vasopressin V_{1a} antagonist SR 49059 strongly inhibited the stimulation of DNA synthesis induced by a suboptimal concentration of AVP (6.24 nM). The effectiveness of the antagonist was dose-dependent and a half-maximal response (IC_{50}) occurred in a 10 nM range (Fig. 5B).

Under the same experimental conditions, SR 49770 (the enantiomer of SR 49059) and OPC-21268, another non-peptide V_{1a} antagonist [15] which were less potent in inhibiting ^{125}I-linear AVP antagonist binding, were only weak or inactive on AVP-induced DNA synthesis, respectively (Table 1).

Finally, we observed that, in spite of the almost identical potency of SR 49059 and $d(CH_2)_5$Tyr(ME)AVP in inhibiting the binding of the ^{125}I-linear AVP antagonist, SR 49059 was six times more efficient in antagonizing AVP-induced DNA stimulation in Swiss 3T3 cells.

Like AVP, bombesin induced significant stimu-

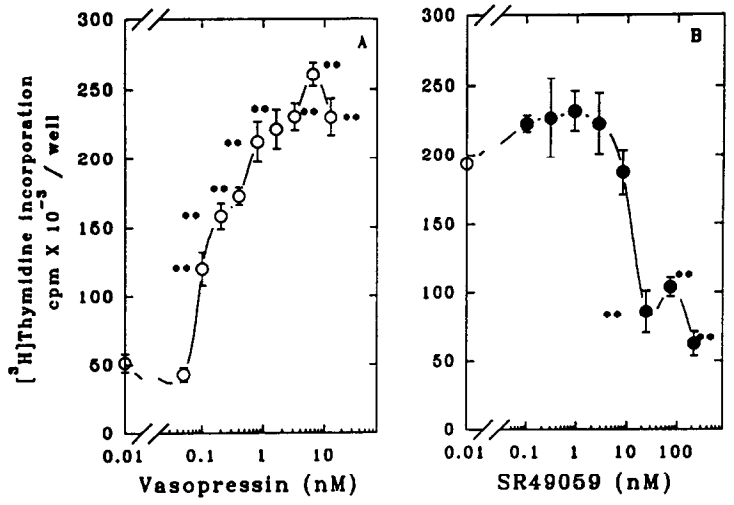

Fig. 5. Inhibition of AVP-induced DNA synthesis in quiescent Swiss 3T3 cells by SR 49059. (A) Stimulation of DNA synthesis by AVP in quiescent Swiss 3T3 cells. Cells were seeded in 96-well microtiter plates and incubated at 37° until they were quiescent. AVP was then added in 0.1 mL of FCS free-DMEM containing 1 μg of insulin/mL. Twenty-four hours later, [^{3}H]thymidine (1 μCi) was added. DNA incorporation of [^{3}H]thymidine was measured at time 48 hr. A control group in which 10% FCS was added in the medium gave 205671 cpm/well. (B) Effect of SR 49059 on the response to a suboptimal concentration of AVP. Incorporation of [^{3}H]thymidine into the cells was measured after 48-hr incubation of quiescent Swiss 3T3 cells with 6.24 nM AVP and varying concentrations of SR 49059 in FCS-free medium containing 1 μg of insulin/mL. Values are the mean ± SEM of six determinations. **$P < 0.01$ compared to controls.

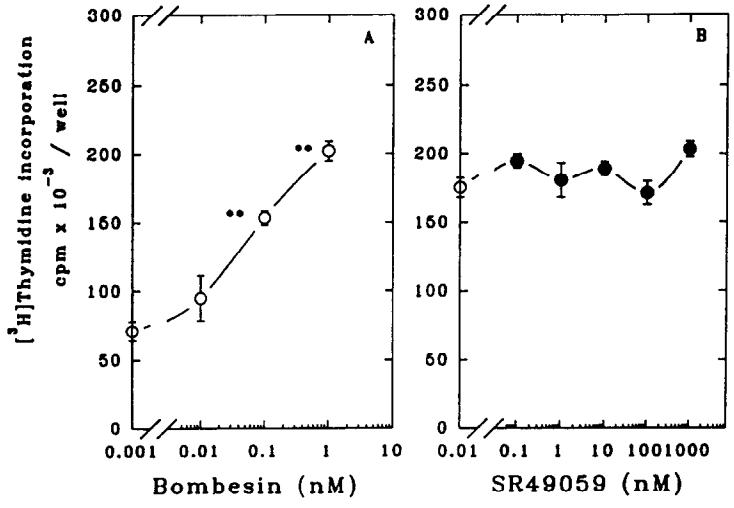

Fig. 6. SR 49059 does not inhibit bombesin-stimulated mitogenesis. (A) Stimulation of DNA synthesis by bombesin in quiescent Swiss 3T3. (B) Effect of SR 49059 on the response to a suboptimal concentration of bombesin (1 nM). The experiment was performed as described in Fig. 5. Values are the mean ± SEM of three determinations. **$P < 0.01$ compared to controls.

lation of [^{3}H]thymidine incorporation in Swiss 3T3 cells in a dose range between 0.01 and 1 nM (Fig. 6A).

Up to 10^{-6} M, SR 49059 did not inhibit the mitogenesis induced by 1 nM bombesin (Fig. 6B), consistent with the lack of affinity observed for this receptor [16]. This result demonstrates that the inhibition of AVP-induced stimulation of DNA synthesis by SR 49059 was due to the specific inhibition of AVP binding to Swiss 3T3 cells. It also indicates that SR 49059 has no deleterious effect *per se* on cell proliferation and that SR 49059 is devoid of any intrinsic mitogenic activity when tested alone.

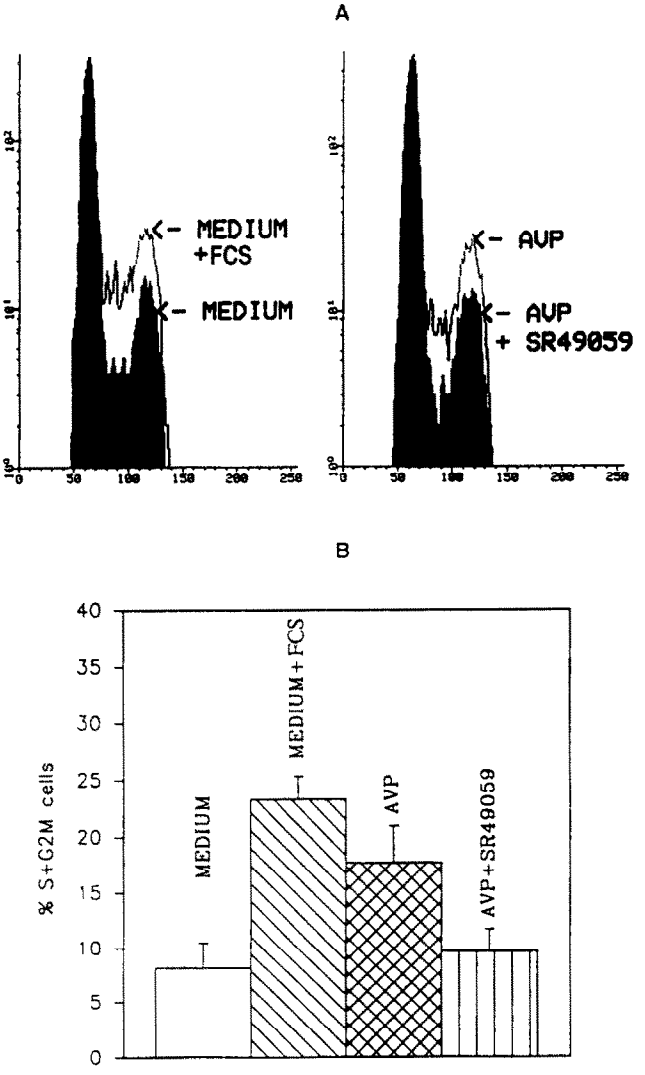

Fig. 7. Effect of SR 49059 on the cell cycle distribution in Swiss 3T3 fibroblasts incubated with AVP. (A) Flow cytometric analysis of DNA contents of Swiss 3T3 cells incubated with medium alone (negative control), medium with FCS (positive control), medium with 6.24 nM AVP and medium with 6.24 nM AVP and 10^{-7} M SR 49059 (vertical axis is shown in a logarithmic scale). (B) Percentages of cells in the S and G_2M phases after 48-hr incubation under the conditions previously described in Materials and Methods.

Effect of SR 49059 on cell cycle distribution in Swiss 3T3 cells stimulated by AVP

Proliferation of Swiss 3T3 cells in serum-free medium was induced by 6.24 nM of AVP. After 48 hr of incubation, the percentage of cells in the S and G_2M phases increased from $8.2 \pm 2.3\%$ without AVP to $17.7 \pm 3.3\%$ with AVP (Fig. 7A and B). This percentage was close to that obtained when cells were maintained in a medium with FCS ($23.3 \pm 2.0\%$). SR 49059, added at 10^{-7} M simultaneously with AVP at the beginning of the culture, decreased the percentage of cells in the S and G_2M phases to a value of $9.7 \pm 2.0\%$. This indicated that SR 49059 fully antagonized AVP-induced Swiss 3T3 cell proliferation.

DISCUSSION

In addition to its well-known pressor and antidiuretic effects via V_{1a} and V_2 receptors, respectively, multiple biological actions have been described for AVP [1]. Among them, AVP is a potent cellular growth factor implicated in a variety of normal and pathophysiological responses in tissue proliferation or regeneration and cellular hypertrophy. AVP has been identified as a powerful mitogen on several cell types including chondrocytes in cell culture [22], bone marrow [23], mouse Swiss 3T3 fibroblasts [4], rat adrenal glomerulosa cells in primary cultures [3], mesangial cells, rat VSMC in which AVP also induces cellular hypertrophy [6], and hepatocytes in the regenerative phase after partial hepatectomy

[2, 24]. More recently, AVP evaluated on human cellular immunity was shown to enhance lymphocyte proliferation [25].

The so-called V_{1a}-vascular (or pressor) receptor subtype, clearly identified on these various tissues both through binding and functional studies, supported the potent mitogenic action of AVP. This is particularly true on the widely studied model of Swiss 3T3 cells by Rozengurt and co-workers [5, 11, 26] where the specific peptide V_{1a} antagonist, $d(CH_2)_5Tyr(Me)AVP$, prevented AVP-induced transient increase in intracellular free calcium ($[Ca^{2+}]i$), protein phosphorylation and DNA synthesis. It was therefore of great interest to investigate the activity of the most potent, non-peptide, orally active, V_{1a} antagonist, SR 49059 [16] both on the AVP receptor and on the proliferative effects of AVP on Swiss 3T3 cells.

The recently designed radioiodinated ligand, ^{125}I-linear AVP antagonist, highly selective for rat and human AVP receptors [16, 17] was used to characterize AVP receptors on Swiss 3T3 membrane preparations. Binding studies using this new linear radioiodinated ligand required an advantageously small quantity of proteins. Specific binding at 30° reached equilibrium rapidly within 15 min and rapid dissociation occurred with 1 μM AVP ($T_{1/2}$ = 2.5 min). A single class of specific, saturable, high affinity AVP-binding sites with a K_d of 48 ± 20 pM and a B_{max} of 63 ± 20 fmol/mg protein were expressed. This high affinity was in agreement with K_d value already observed for rat liver V_{1a} receptors (K_d = 60 pM) with ^{125}I-linear AVP antagonist. It is worth noting that the affinity of this ligand for V_{1a} receptors is much higher than any radiolabeled ligand developed so far for AVP receptors, particularly in comparison with the [^{3}H]AVP generally used (K_i = 0.6–3 nM) [27]. In this respect Rozengurt and co-workers [11, 12] reported a lower 10–18 nM K_d value when they originally characterized the AVP receptors on quiescent Swiss 3T3 intact cells using [^{3}H]AVP as ligand.

The order of potency of several reference vasopressin analogues investigated in competition experiments demonstrated the V_{1a} nature of the receptor identified by this radioiodinated ligand: $d(CH_2)_5Tyr(Me)AVP$ = AVP ≫ oxytocin = DDAVP. Furthermore, the lack of affinity of other mitogens (bombesin, bradykinin, GRP and endothelin) whose receptors have been also identified on 3T3 fibroblasts [7–10] evidenced the high selectivity of this binding assay. Overall, the most significant finding of this study is that SR 49059 displayed full competitive nanomolar affinity for V_{1a} 3T3 receptors as demonstrated in saturation experiments (K_i = 1.5 nM) and subsequently exerted a powerful antimitogenic effect on AVP-induced DNA synthesis on Swiss 3T3 cells (IC_{50} = 14 ± 2 nM). This striking antiproliferative effect resulted in the complete blockage of AVP-stimulated cell growth from G1/G0 into S/GM2 phases as evidenced by cytofluorometer analysis of the cell cycle. Our results clearly demonstrate that the antimitogenic effect of SR 49059 is due to direct interaction with Swiss 3T3 V_{1a} receptors: firstly, SR 49059 has been previously described as a highly selective compound [16] and in this study was unable to counteract bombesin mitogenic effects; secondly, a parallel high stereo-specificity between SR 49059 and its enantiomer, SR 49770, was observed both in binding and proliferative experiments.

Under the same experimental conditions, OPC-21268, another non-peptide orally active V_{1a} antagonist [15] exhibited 200 lower affinity than SR 49059 for Swiss 3T3 V_{1a} receptors and no antiproliferative properties in concentrations up to 220 μM. The affinity of OPC-21268 for the mouse 3T3 V_{1a} fibroblast receptor is quite comparable to that observed on rat liver V_{1a} receptors whereas this compound has been shown to be almost inactive at several human V_{1a} receptors including adrenals, platelets and uterus suggesting great species differences and/or potential AVP V_{1a} receptor subtypes [16, 27, 28].

In this report, it is also important to underline the discrepancies observed between SR 49059 and $d(CH_2)_5Tyr(Me)AVP$ in the binding and proliferative experiments: in spite of the close nanomolar affinity of the two compounds for the Swiss 3T3 V_{1a} receptors (IC_{50} of 5.4 ± 2.8 and 1.8 ± 0.9 nM, respectively), SR 49059 was the most powerful antimitogenic agent being six times more efficient than the peptide molecule in inhibiting AVP-stimulated DNA synthesis. A putative explanation of these data could be different kinetics of interaction of these two compounds at the V_{1a} receptor level. As reported by Jamil *et al.* [29] on rat cultured mesangial cells, $d(CH_2)_5Tyr(Me)AVP$ needs further preincubation to fully antagonize AVP-induced Ca^{2+} entry whereas OPC-21268 added simultaneously with AVP was completely efficient. Other arguments involving the degradation of the peptide by intact cells, V_{1a} receptor down-regulation or internalization could also be raised. Such a difference remains unclear and has to be studied thoroughly.

In the search of novel antiproliferative agents, in particular for SCLC, an intriguing group of substance P analogues with selective antagonist properties for a number of mitogenic neuropeptides (AVP, bombesin, GRP, bradykinin and endothelin) have been designed [9, 11, 30, 31]. Several lines of evidence suggest that these kinds of broad-spectrum peptide antagonists are able to recognize a highly conserved domain of the receptor of these mitogens, thereby preventing their cellular growth response.

Among them, the antagonist [Arg^6, D-Trp^{7,9}, MePhe^8] substance P (6–11) was identified as a good inhibitor of AVP-stimulated DNA synthesis blocking also [^{3}H]AVP binding to Swiss 3T3 fibroblasts and to different SCLC cell lines [9]. In the present study this hexapeptide competed with the radioionated ligand at V_{1a} receptors but with two order lower potency than the selective non-peptide compound SR 49059.

Obviously, this last point highlighted the clinical interest and the therapeutical relevance of a selective synthetic V_{1a} antagonist in tumors and various proliferative states. Complex tumors such as SCLC have the ability to secrete a variety of ectopic hormones or neuropeptides including AVP, bombesin, OT [5, 32] and have several receptors of these growth factors. In this respect, broad-spectrum

inhibitors such as [Arg^6, D-Trp^{7,9}, MePhe^8] substance P (6–11) appear most likely to be suitable as effective antiproliferative agents in SCLC but their lack of oral bioavailability because of their peptide nature will severely hamper their clinical use. Otherwise, since a high plasma level of AVP (and OT) characterizes SCLC tumors [32], a non-peptide V_{1a} antagonist could block part of the clinical syndromes often associated with these tumors.

In conclusion, SR 49059, the most potent, non-peptide, orally effective AVP V_{1a} antagonist yet described [16] expresses powerful and selective AVP-dependent antiproliferative properties by direct interaction at AVP V_{1a} receptors on Swiss 3T3 cells. Therefore, this synthetic compound is a potent pharmacological probe for investigating the pathophysiological role of AVP in several tumoral and proliferative diseases: cancer, atherosclerosis. . . [33]. SR 49059 deserves further investigation.

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