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Identification and Characterization of Vascular (V_1) Vasopressin Receptors of an Established Smooth Muscle Cell Line

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SUMMARY

We report the identification and characterization of specific vasopressin-binding sites on intact cells and membranes of the established vascular smooth muscle cell line A-10, the fate of vasopressin associated with the cells, the role of guanine nucleotides in the regulation of the affinity of the vasopressin-binding sites, and the determination of the vasopressin receptor subtype. We have found specific vasopressin-binding sites on intact cells in monolayer (110,000 sites per cell during log growth and 60,000 sites per cell in stationary culture) with a K_D of 6 nM at 37°. After incubation of [^{3}H]-8-arginine vasopressin ([^{3}H]AVP) and cells for <20 min, cell-associated AVP was intact; with longer incubation times, AVP was progressively degraded. The major metabolites included phenylalanine and a fraction that eluted from a C18 reverse phase high performance liquid chromatography column between AVP and 8-arginine, 9-desglycinamide vasopressin. Extensive degradation also occurred when AVP was allowed to dissociate from the cells. With increased time of incubation, the amount of specifically bound AVP that could dissociate decreased, suggesting receptor-mediated endocytosis. In saturation equilibrium binding experiments with plasma membranes, two affinity states with K_D of 0.7 nM and 379 nM were observed. The number of high affinity binding sites was similar to the number of receptors found on intact cells. Guanosine 5′-(β,γ-imido)triphosphate decreased vasopressin binding to the high affinity sites and did not significantly affect the low affinity sites. Competition binding experiments indicated that the vasopressin-binding sites of A-10 cells belong to the vascular V_1 receptor subtype. We conclude that the established vascular smooth muscle cell line A-10 expressed vasopressin receptors of the vascular V_1 subtype. Vasopressin bound to the receptors reversibly, but could also be degraded by the cells presumably after receptor-mediated endocytosis. The receptors might exist in different affinity states; guanosine 5′-(β,γ-imido)triphosphate decreased the affinity of the high affinity binding state.

The neurohypophyseal hormone vasopressin interacts with at least two types of receptors, the vascular V_1 receptor and the renal V_2 receptor (1). V_2 receptors are coupled to adenylate cyclase and mediate the antidiuretic hormone activity in the kidney (2–5); V_1 receptors are not positively coupled to adenylate cyclase and mediate vasoconstriction. In selected vascular beds, such as the rat mesenteric microcirculation, vasopressin is one of the most potent vasoconstrictors (6). Vasopressin can cause vasoconstriction by a direct action on the vascular smooth muscle, or indirectly by potentiating norepinephrine-induced vasoconstriction (7). We have suggested that vasopressin might also enhance vasoconstriction by inhibiting β-adrenergic receptor agonist and atrial natriuretic factor-induced vasodilation (8, 9). Katusic *et al.* (10, 11) recently showed that vasopressin can also cause vasodilation of the canine basilar and coronary artery in an endothelium-dependent fashion. These reports indicate the potential importance of vasopressin in regulating regional blood flow under normal conditions. In addition, in some pathological conditions, the responsiveness of blood vessels to vasopressin was altered. The vasoconstrictor response to vasopressin was increased in spontaneously hypertensive rats (12–14) and in subjects with a family history of hypertension (15).

A rise in cytosolic free calcium is an essential step in the sequence of events leading to contraction of vascular smooth muscle (16). Inositol 1,4,5-triphosphate, formed by hydrolysis of phosphatidylinositol diphosphate by phospholipase C, may play a key role in intracellular calcium mobilization (17, 18). In vascular smooth muscle, vasopressin has been shown to cause calcium mobilization (19–23) and increase phosphatidyl-

ABBREVIATIONS: DPBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin; AVP, 8-arginine vasopressin; dDAVP, 1-deamino, [8-D-arginine]vasopressin; dAVP, 1-deamino, 8-arginine vasopressin; desGlyNH$_2$AVP, 8-arginine, 9-desglycinamide vasopressin; d(CH$_2$)$_5$Tyr(Me)AVP, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(*O*-methyl)tyrosine]8-arginine vasopressin; d(CH$_2$)$_5$Tyr(Et)VAVP, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(*O*-ethyl)tyrosine, 4-valine]8-arginine vasopressin; d(CH$_2$)$_5$D-IleVAVP, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-isoleucine, 4-valine]8-arginine vasopressin; DMEM, Dulbecco's modified Eagle's medium plus 20% fetal calf serum; EDTA, ethylenediaminetetraacetate; DPBS^{2+}, Dulbecco's phosphate-buffered saline with 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.1% glucose, and 0.2% bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RP-HPLC, reverse phase high performance liquid chromatography; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

inositol turnover (19, 22–24). It seems clear that inositol 1,4,5-triphosphate formation and calcium mobilization are consequences of V_1 receptor activation. However, the molecular events involved in the interaction of vasopressin with its vascular receptor, the receptor activation, and the coupling of the activated receptor to intracellular events are not well understood.

We have previously reported that V_1 receptors mediate inhibition of cAMP and cGMP accumulation by β-adrenergic receptor agonists and atrial natriuretic factor, respectively, and increase phosphatidylinositol turnover and calcium efflux in an established smooth muscle cell line (A-10) of rat thoracic aorta (8, 9, 19). Here we report the identification and characterization of vasopressin-binding sites on intact cells and membranes of the A-10 cell line. We present evidence that these sites represent the V_1 receptor subtype and that the receptor affinity may be regulated by guanine nucleotides.

Materials and Methods

Fetal calf serum was obtained from KC Biologicals (Lanexa, KS); Dulbecco's modified Eagle's medium, DPBS (0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$), and trypsin were from Gibco (Grand Island, NY); BSA (fraction V) and bacitracin were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]AVP (40 Ci/mmol) was purchased from New England Nuclear (Boston, MA); AVP and dDAVP were from Bachem (Torrance, CA). The vasopressin analogs used were dAVP, desGlyNH_2AVP, d$(CH_2)_5$Tyr(Me)AVP, d$(CH_2)_5$Tyr(Et)VAVP, and d$(CH_2)_5$D-IleVAVP. These analogs were synthesized at SK&F Laboratories (Philadelphia, PA).

Cell culture. Rat aortic vascular smooth muscle cells (A-10; ATCC CRL 1476) were obtained from the American Type Culture Collection and cultured in DMEM. Before initiating the experiments, the cells were subcloned twice by limiting dilution. All experiments were performed with cells passaged once a week for no more than 4 months. The cells were removed from the flasks by incubating with 0.25% trypsin containing 0.25% EDTA (pH 7.5). Culture wells (35 mm diameter; six-well Linbro plates) were seeded with 1 ml of medium containing 75,000 cells. Experiments were performed after 3 days, unless indicated otherwise.

Preparation of plasma membranes. Plasma membranes of A-10 cells were prepared as follows. Cells were seeded in T-150 culture flasks (Corning Glass Works, Corning, NY) at 1.5×10^6 cells per flask and cultured for 3 days. The medium was removed and the cells were washed three times with 20 ml of DPBS. The cells were harvested in DPBS by scraping with a rubber policeman. The cell suspension was centrifuged at $600 \times g$ for 10 min, the supernatant was removed, and the cell pellet was frozen in liquid N_2 and stored at −80°. The frozen cell pellet was thawed on ice and the cells were homogenized on ice in 5 mM Tris·HCl, pH 7.5 (at 30°), containing 3 mM $MgCl_2$ and 1 mM EDTA (from 0.5 M EDTA, pH set at 7.3 with Tris base at 20°; hypotonic Tris buffer) with a Dounce homogenizer (25 strokes). The homogenate was centrifuged at $12,000 \times g$ for 10 min. The supernatant was removed and the membrane pellet was suspended in hypotonic Tris buffer (1 ml/2 × 10^7 cells). The membrane suspension was used immediately.

Plasma membranes of liver of male Sprague-Dawley rats (225–275 g) were prepared according to the procedure of Neville (25) up to step 11. The membranes were suspended in hypotonic Tris buffer and stored frozen in liquid nitrogen.

Plasma membranes of rat kidney were prepared as described previously (26).

[^{3}H]AVP binding to cells in monolayer. The culture medium was removed, the cells were washed with DPBS^{2+}, and the binding was initiated with 1 ml of DPBS^{2+} containing [^{3}H]AVP. Nonspecific binding was determined in the presence of 10 μM AVP. At the end of the incubation period the medium was removed and the cells were washed with 1 ml of ice-cold DPBS^{2+}. The cells were scraped in ice-cold DPBS^{2+} and washed rapidly on Amicon filters (0.45 μm), and the cell-associated radioactivity was measured.

[^{3}H]AVP binding to cell membranes. [^{3}H]AVP binding to cell membranes was performed in a mixture (final volume 500 μl) containing 10 mM HEPES buffer, pH 7.4, 10 mM $MgCl_2$, 0.1% BSA, 50–100 μg of membrane protein, and [^{3}H]AVP. Nonspecific binding was determined with 10 μM AVP. The incubation was carried out at 37° for 90 min unless indicated otherwise. Bound and free [^{3}H]AVP were separated by centrifugation at $12,000 \times g$ for 5 min. The membrane pellet was treated with NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL) and the radioactivity was measured. The affinity of unlabeled ligands for vasopressin receptors was determined in competitive binding experiments using 5 nM [^{3}H]AVP after an incubation at 37° for 20 min. The affinities were expressed as IC_{50} values, i.e., the concentration required for 50% inhibition of specific [^{3}H]AVP binding.

[^{3}H]AVP binding to liver membranes. [^{3}H]AVP binding to rat liver membranes was determined in a mixture (final volume 500 μl) containing 100 mM Tris·HCl, pH 8.0 (at 30°), 0.08% BSA, 0.2% bacitracin, 10 mM $MgCl_2$, [^{3}H]AVP, and 12.5 μg of membrane protein for 60 min at 30°. After incubation, bound and free radioligand were separated by centrifugation at $12,000 \times g$ for 5 min. The pellet was solubilized and the radioactivity was determined by liquid scintillation counting. The affinity of unlabeled ligands was determined in competition experiments with 2.0×10^{-9} M [^{3}H]AVP with or without 10 μM AVP. The IC_{50} values and the K_D of [^{3}H]AVP (3.8×10^{-10} M) (27) were employed to calculate the K_{bind} according to the method of Cheng and Prusoff (28).

Adenylate cyclase assay. Adenylate cyclase activity and agonist and antagonist potencies were determined as described previously (26).

Dissociation of [^{3}H]AVP from cells and RP-HPLC analysis. [^{3}H]AVP was bound to cells in monolayer, the medium was removed, the monolayer was washed once with DMEM, and [^{3}H]AVP was allowed to dissociate in DMEM at 37° for 90 min. Dissociated [^{3}H]AVP and [^{3}H]AVP extracted from frozen and thawed cells with ice-cold 1% acetic acid for 5 min were chromatographed by RP-HPLC. A 25-cm ultrasphere ODS 5-μm Altex column (Beckman San Ramon, CA) was loaded with 100 μl of [^{3}H]AVP in 1% acetic acid containing AVP, dAVP, and desGlyNH_2AVP, 2 μg each. The column was developed with a discontinuous acetonitrile gradient containing 0.1% trifluoroacetic acid at 1.5 ml/min. The eluate was monitored at 220 nm and fractions of 0.5 ml were collected. The radioactivity of the fractions was measured. More than 95% of the counts applied were recovered.

Protein determination. Protein was measured with the Folin reagent (29) and BSA was the standard.

Statistical evaluation. Experimental values are reported as the mean ± standard error. The saturation equilibrium binding data were analyzed by a nonlinear least squares curve-fitting procedure using a generalized model for complex ligand-receptor systems (Scatfit) (30). The calculated K_D and B_{max} values are presented with the standard error of the estimate.

Results

Identification and characterization of specific vasopressin-binding sites on intact cells. Specific binding of [^{3}H]AVP (0.75 nM) at 37° to cells in monolayer was maximal within 5 min, the shortest incubation time used, and remained essentially constant for 60 min (data not shown). Nonspecific binding at 7.5 nM [^{3}H]AVP was less than 5%. At 0°, specific binding reached a plateau after 60 min and 120 min, at 4.0 nM and 0.2 nM [^{3}H]AVP, respectively (Fig. 1).

The rate of dissociation of [^{3}H]AVP from cells is shown in Fig. 2. [^{3}H]AVP, associated at 37° for 10 min, dissociated rapidly at 37° ($t_{1/2}$ about 5 min); however, 8.9% of [^{3}H]AVP did not dissociate (Fig. 2A). The amount of nondissociable [^{3}H]

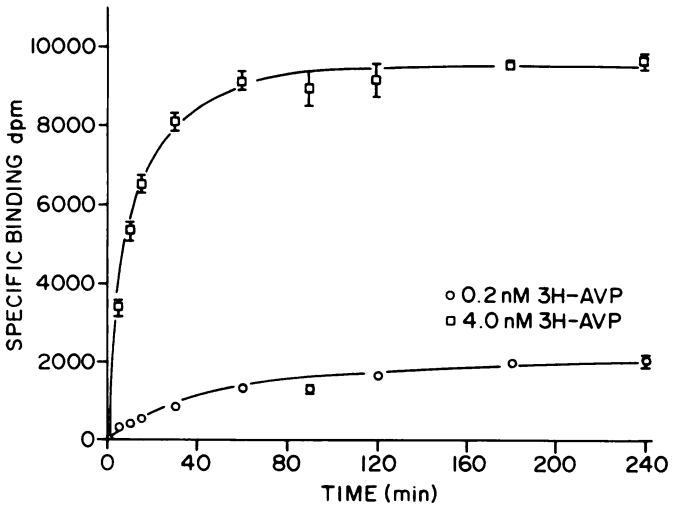

Fig. 1. Specific [^{3}H]AVP binding to cells in a monolayer as a function of time. [^{3}H]AVP was added in 1 ml of DPBS to wells containing 2 × 10^6 cells in monolayer. The incubation was carried out at 0° for the times indicated. Mean values ± standard errors of triplicate determinations are presented.

AVP increased with increased binding time at 37°. After incubation at 37° for 20 min and 120 min, 15% and 40%, respectively, did not dissociate. [^{3}H]AVP, bound at 0° for 120 min, dissociated very slowly at 0° ($t_{1/4}$ > 160 min) but was almost completely dissociated within 30 min at 37° (Fig. 2B).

Saturation equilibrium binding of [^{3}H]AVP to intact cells was performed at 37° for 20 min (from 0.4 nM to 230 nM; Fig. 3A) and at 0° for 4 hr (from 0.4 nM to 55 nM; Fig. 4A). Nonspecific binding was determined in the presence of 10 μM AVP. Specific binding saturated at about 100 nM at 37° and about 20 nM at 0°. Scatchard analysis of these data suggested the presence of a single high affinity binding site (Figs. 3B and 4B) with similar affinities at 37° (K_D was 6.0 nM) and at 0° (K_D was 8.1 nM). However, maximal binding was higher at 0° than at 37° (B_{max} was 137 and 96 fmol/10^6 cells, respectively).

Characterization of cell-associated [^{3}H]AVP by RP-HPLC. To study the fate of [^{3}H]AVP, we developed an RP-HPLC procedure to separate and quantitate potential metabolites of AVP. Free [^{3}H]AVP, bound AVP, and AVP that dissociated from intact cells were analyzed (Fig. 5). Free [^{3}H]AVP in culture medium after incubation with cells for 0 or 20 min at 37°, or for 4 hr at 0°, coeluted with carrier AVP; however, after 2 hr at 37°, 56% of the radioactivity eluted in a peak between AVP and desGlyNH$_2$AVP, whereas only about 15% coeluted with AVP (Fig. 5A). [^{3}H]Phe was also found (0.3%). No significant degradation was observed when [^{3}H]AVP was incubated in medium without cells at 37° for up to 4 hr (data not shown).

Cell-associated [^{3}H]AVP was extracted from freeze-thawed cells with 1% acetic acid containing carrier AVP, desGlyNH$_2$AVP, and dAVP. Prolonged binding at 37° decreased acid extractability; 93% [^{3}H]AVP was extracted after binding for 20 min at 37°, whereas only 69% could be extracted after binding for 2 hr. The acetic acid extracts contained largely intact AVP after binding at 37° for 20 min (Fig. 5, B and C), or at 0° for 2 hr (data not shown). However, after binding for 2 hr at 37°, marked degradation of AVP was found. The AVP peak of the acetic acid extract represented only 18% of the radioactivity eluted off the column, about 47% eluted between AVP and desGlyNH$_2$AVP, and 5% of the radioactivity coeluted with Phe (Fig. 5B).

Dissociated [^{3}H]AVP was markedly degraded. Fig. 5C shows the elution profile of [^{3}H]AVP dissociated from the cells after binding for 20 min at 37°. Only 17% of the radioactivity coeluted with AVP, 24% eluted between AVP and desGlyNH$_2$AVP, and 14% with Phe. Most of the rest of the radioactivity eluted between Phe and AVP.

Vasporessin-binding sites during cell growth. The number of vasopressin-binding sites per cell during culture was determined by [^{3}H]AVP binding at 30 nM for 2 hr at 0° (Fig. 6). During early log growth, we found approximately 110,000 specific vasopressin-binding sites per cell. This number decreased as the cells became confluent and reached a plateau at about 60,000 sites per cell. A similar decrease in number of vasopressin-binding sites was also found when binding was performed at 200 nM [^{3}H]AVP for 20 min at 37°. However, the numbers of binding sites observed were about 70% of those obtained by binding at 0° (data not shown). The decrease in number of vasopressin-binding sites might reflect the decrease

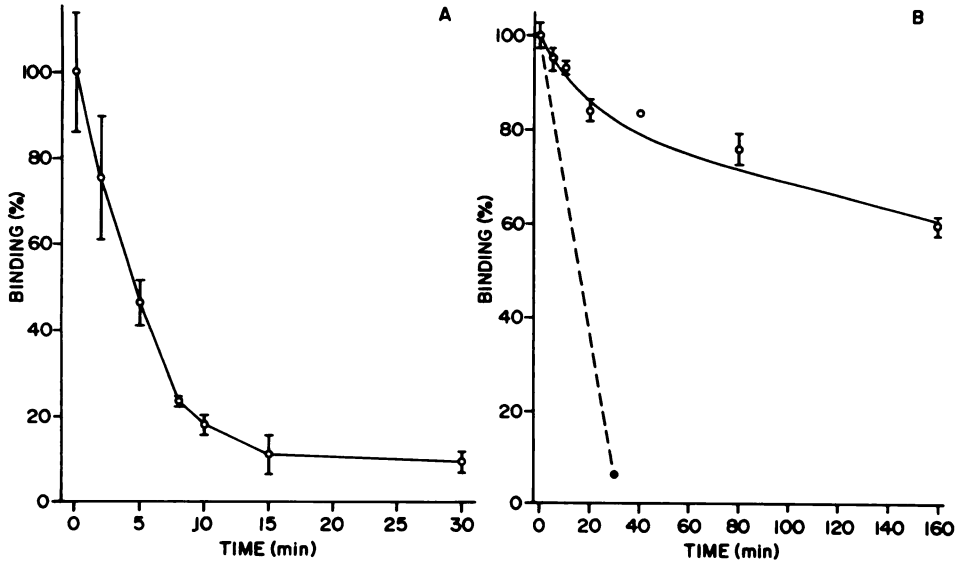

Fig. 2. Dissociation of [^{3}H]AVP from cells in monolayer. A. The cells were incubated with 10 nM [^{3}H]AVP for 10 min at 37° in DMEM. The medium was removed, the cells were washed with DMEM, and subsequently the cells were incubated with DMEM at 37° for the times indicated. B. The cells were incubated with 3.9 nM [^{3}H]AVP for 60 min at 0°. The medium was removed, the cells were washed once with DMEM, and subsequently the cells were incubated with DMEM at 0° (○) for the times indicated or at 37° (●) for 30 min. Mean values ± standard errors of triplicate determinations are presented.

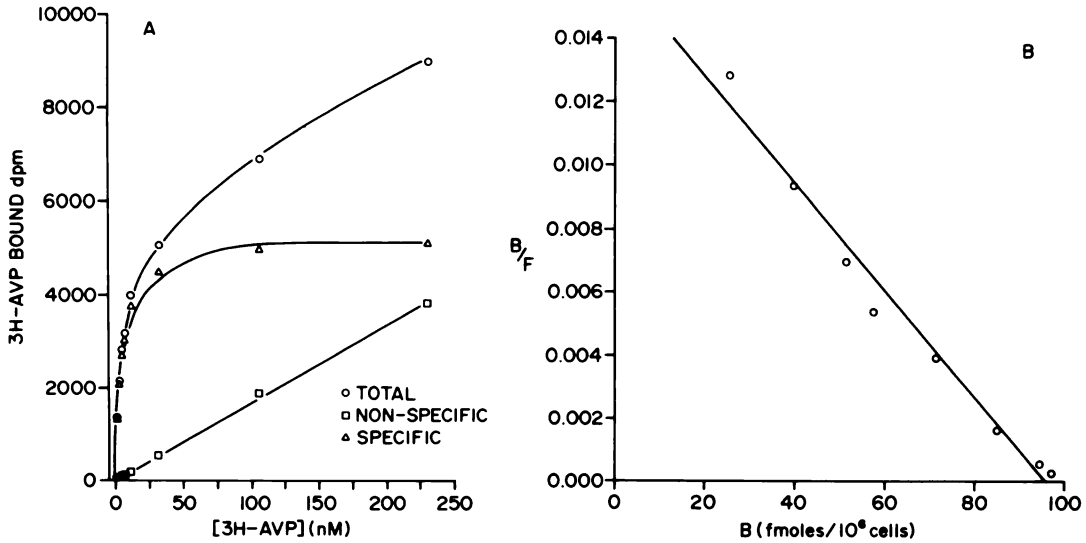

Fig. 3. Saturation equilibrium binding of [^{3}H]AVP to cells in monolayer at 37°. A. The cells were incubated with 0.4–230 nM [^{3}H]AVP for 20 min at 37° in the presence or absence of 10 μM AVP. B. Scatchard analysis of the data (K_D = 6.0 nM; B_{max} = 96 fmol/10^6 cells). Mean values of triplicate determinations are presented. The experiment was repeated with similar results.

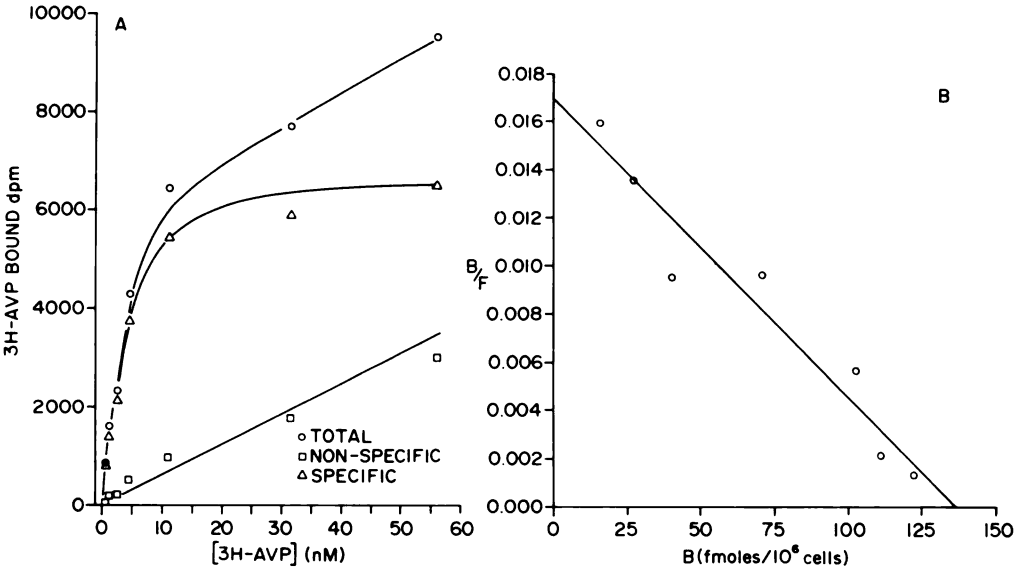

Fig. 4. Saturation equilibrium binding of [^{3}H]AVP to cells in monolayer at 0°. A. The cells were incubated with 0.4–55 nM [^{3}H]AVP for 2 hr at 0° in the presence or absence of 10 μM AVP. B. Scatchard analysis of the data (K_D = 8.1 nM; B_{max} = 137 fmol/10^6 cells). Mean values of triplicate determinations are presented. The experiment was repeated with similar results.

in accessible surface area as the cells reach confluency, or might reflect true loss of receptors.

Identification and characterization of specific vasopressin-binding sites on cell membranes. Specific, saturable vasopressin-binding sites were found on plasma membranes by incubating the membranes with [^{3}H]AVP at 37° for 90 min (Fig. 7A). The Scatchard plot of these data was nonlinear (Fig. 7B), suggesting the presence of more than one affinity state. Two-site fitting of the data of Fig. 7, employing Scatfit, resulted in a K_{D1} = 0.7 ± 0.1 nM and a B_{max1} = 0.051 ± 0.007 nM (or 60 fmol/10^6 cells), and a K_{D2} = 379 ± 1286 nM and a B_{max2} = 1.1 ± 3.2 nM. The high nonspecific binding (70% at 40 nM [^{3}H]AVP) precluded accurate determination of the K_D and B_{max} values of the low affinity sites.

Effect of Gpp(NH)p on vasopressin binding to cell membranes. In the presence of 100 μM Gpp(NH)p, specific and saturable binding of [^{3}H]AVP to plasma membranes was also observed. The Scatchard plot of the data was nonlinear (Fig. 8). Gpp(NH)p decreased the binding at the lower concentrations of [^{3}H]AVP with little apparent effect at the higher concentrations. Whether the decreased vasopressin binding by Gpp(NH)p is due to decreased affinity and/or number of high affinity binding sites could not be determined from these experiments.

The receptor subtype of the vasopressin-binding sites of cell membranes. The affinity of selective vasopressin agonists (dDAVP, V_2; AVP, V_1/V_2) and antagonists [d(CH$_2$)$_5$Tyr(Me)AVP, V_1 (31); d(CH$_2$)$_5$D-IleVAVP, V_2 (32); d(CH$_2$)$_5$Tyr(Et)VAVP, V_1/V_2 (33)] was determined in competitive binding studies (Fig. 9). The V_2-selective analogs were poor competitors for [^{3}H]AVP, whereas the V_1-selective analog was a potent competitor. Similar data were obtained in competition experiments with [^{3}H]AVP and intact cells (data not shown). The rank order of the analog affinities, expressed as IC_{50} values, was the same as that for rat liver vasopressin receptors. The rank orders for rat liver and kidney were different (Table 1). These data indicate that the vasopressin receptor of the A-10 cells belongs to the vascular V_1 receptor subtype.

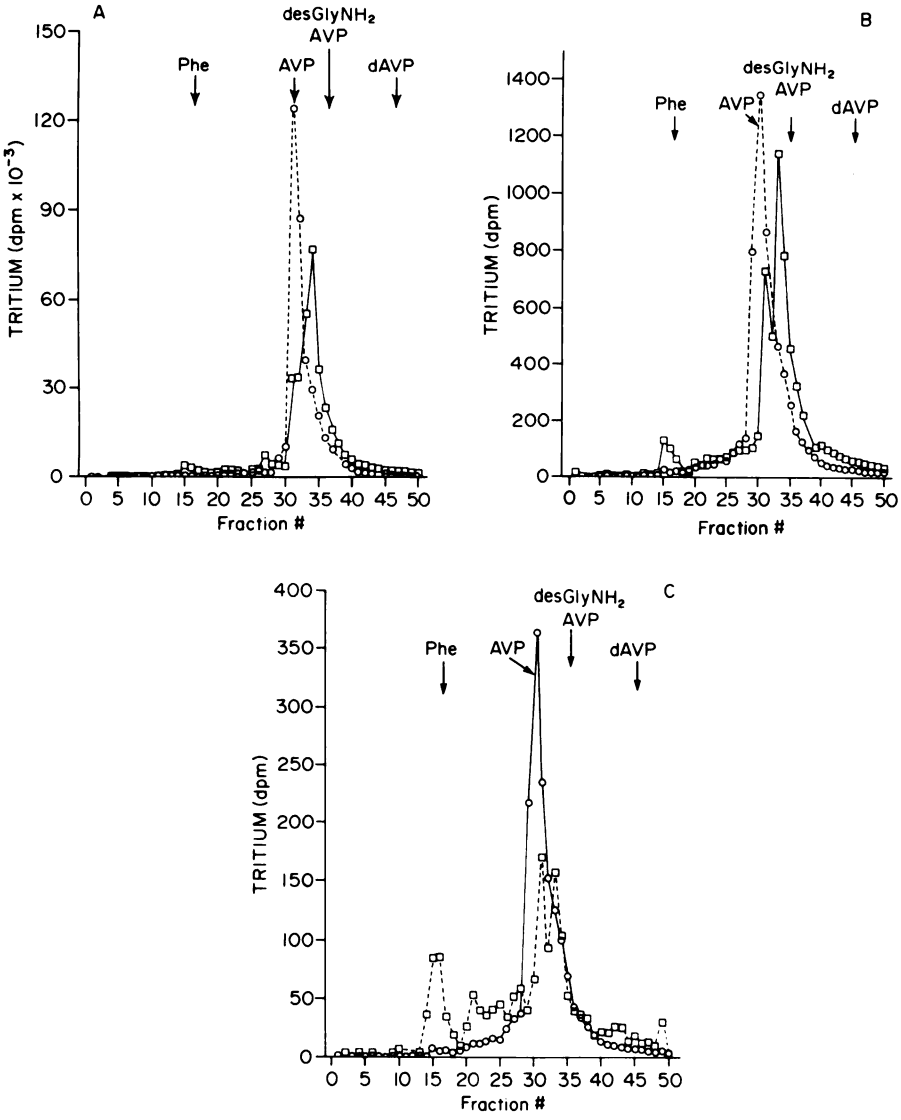

Fig. 5. Analysis of [^{3}H]AVP extracts by RP-HPLC. A. Cells were incubated with 50 nM [^{3}H]AVP at 37° for 0 min (○) or 2 hr (□). The media were chromatographed. B. Cells were incubated with 50 nM [^{3}H]AVP for 20 min (○) or 2 hr (□) at 37°. The cells were washed and extracted with 1% acetic acid. C. Cells were incubated with 50 nM [^{3}H]AVP at 37° for 20 min. The cells were washed and extracted with 1% acetic acid (○), or the ligand was allowed to dissociate for 30 min at 37° (□). Within each panel, the elution profiles represent the same total amount of eluted tritium. The experiments were repeated with similar results.

Discussion

We have previously reported on the molecular mechanisms involved in vasopressin-induced contraction of vascular smooth muscle using the established smooth muscle cell line A-10, derived from rat thoracic aorta (8, 9, 19). We subcloned the A-10 cells to ensure a homogeneous cell population. Here we employed the clonally derived A-10 cells to (i) fully characterize vasopressin-binding sites on intact cells, (ii) determine the expression of vasopressin receptors during monolayer culture, (iii) examine the fate of vasopressin bound to cells, (iv) compare the properties of the vasopressin-binding sites of cell membranes and intact cells, and (v) examine the role of guanine nucleotides in regulating the affinity of vascular vasopressin receptors.

Specific vasopressin-binding sites were found on intact A-10 cells. At 37°, equilibrium binding was reached within 5 min with 0.75 nM [^{3}H]AVP. Bound [^{3}H]AVP also dissociated from the cells rapidly ($t_{1/2}$ about 5 min). However, the extent of dissociation decreased with increased time of binding. At 37°, a single class of specific, saturable vasopressin-binding sites with a K_D of 6 nM and a B_{max} of 96 fmol/10^6 cells was expressed. Similar data were obtained for rat aortic smooth muscle cells in primary culture (34). We have also found specific binding sites at 0°. The affinities at 0° and 37° were similar; however, at 0° about 40% more binding sites were found.

The expression of vasopressin receptors was not associated with a particular phase of cell culture, but the receptors were present during all phases of cell culture (Fig. 6). The highest number of receptors was present during early log phase. The receptor number decreased by about 45% during late log phase and then remained constant during stationary phase. The decrease in receptor number may be caused by a decreased expression of receptors or may result from a decrease in surface area as the cells reach confluency. At present we are unable to distinguish between these possibilities. We observed no change in receptor subtype as a function of cell density.

The fate of [^{3}H]AVP during incubation with intact cells was studied using RP-HPLC to separate potential metabolites of AVP. Little degradation of [^{3}H]AVP occurred during a 20-min incubation period with culture medium and cells; however, after 2 hr about 60% [^{3}H]AVP was degraded. The major metabolite was a peptide that eluted between AVP and desGlyNH$_2$AVP

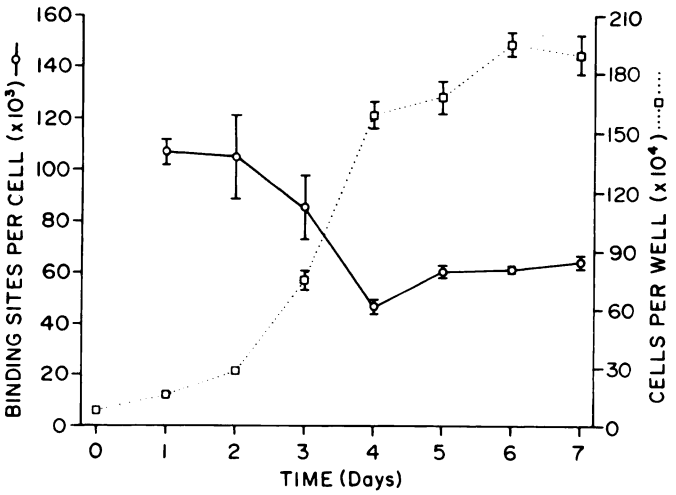

Fig. 6. Vasopressin-binding sites during cell growth. The number of specific vasopressin-binding sites was determined with 30 nM [^{3}H]AVP in the presence or absence of 10 μM AVP for 2 hr at 0°. Mean values ± standard errors of triplicate determinations are presented. The experiment was repeated with similar results.

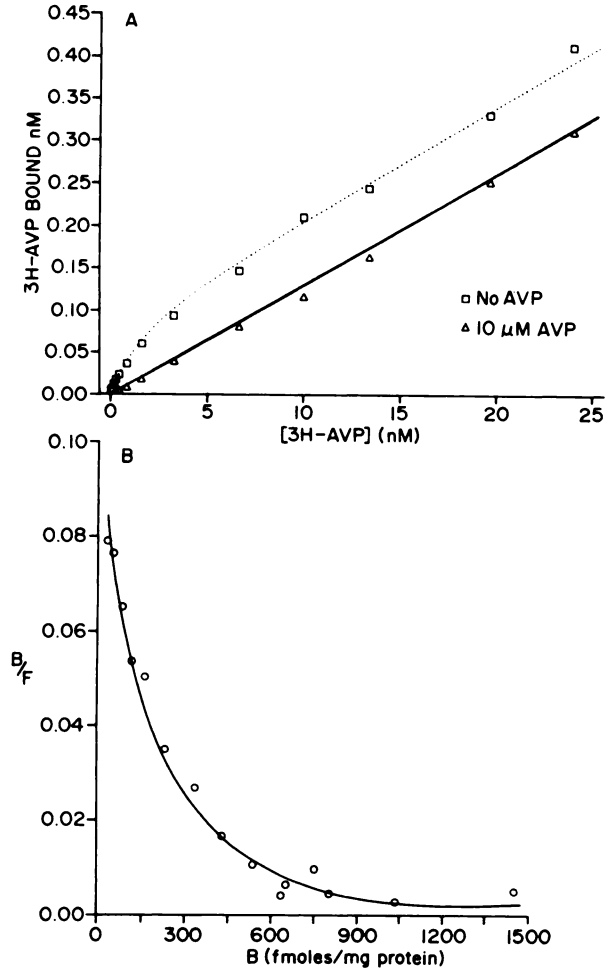

Fig. 7. Saturation equilibrium binding of [^{3}H]AVP to plasma membranes. A. The plasma membranes (62 μg of protein) were incubated with 0.04–50 nM [^{3}H]AVP for 90 min at 37° in the presence or absence of 10 μM AVP. B. Scatchard analysis of the data. The mean values of triplicate determinations are presented. The experiment was repeated four times with similar results.

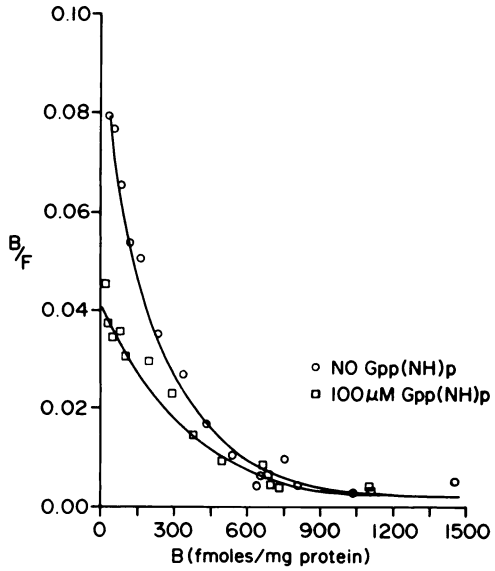

Fig. 8. Effect of Gpp(NH)p on [^{3}H]AVP binding to plasma membranes. Plasma membranes (62 μg of protein) were incubated with 0.04–50 nM [^{3}H]AVP for 90 min at 37° with or without 100 μM Gpp(NH)p. Nonspecific binding was determined in the presence of 10 μM AVP. A Scatchard analysis of the data is presented. Mean values of triplicate determinations are presented. The experiment was repeated four times with similar results.

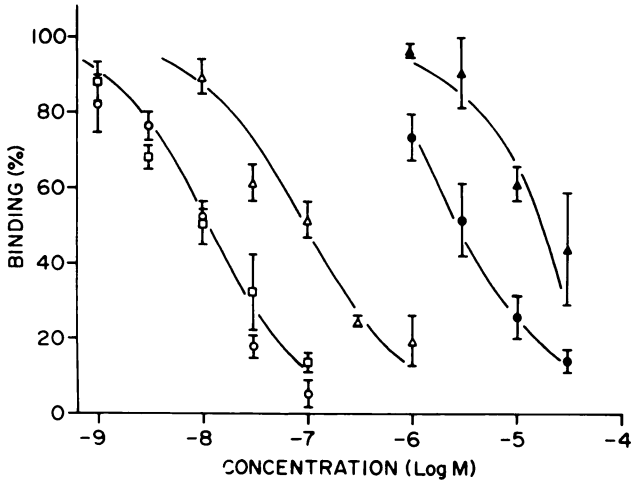

Fig. 9. Inhibition of [^{3}H]AVP binding to plasma membranes by vasopressin analogs. Plasma membranes were incubated for 20 min at 37° with 5 nM [^{3}H]AVP in the presence of vasopressin analogs at the concentrations indicated. Nonspecific binding was determined in the presence of 10 μM AVP. ○, AVP; □, d(CH$_2$)$_5$Tyr(Me)AVP; △, d(CH$_2$)$_5$Tyr(Et)VAVP; ●, d(CH$_2$)$_5$D-IleVAVP; ▲, dDAVP. Mean values ± standard errors of triplicate determinations are presented.

(Fig. 5A). Degradation of AVP in the culture medium required incubation with cells and was temperature and time dependent.

The acetic acid extract of cells incubated for 20 min at 37° with [^{3}H]AVP showed that most of the ligand was intact AVP (Fig. 5, B and C). However, after an incubation for 2 hr, cell-associated AVP was extensively degraded (Fig. 5B). During dissociation not only intact AVP was released, but also degradation products (Fig. 5C). That the degradation products were derived from AVP associated with the cells through nonspecific endocytosis is unlikely, because a similar degradation profile was observed during dissociation at 37° of AVP bound at 0° (data not shown). These data suggest that, in addition to

TABLE 1
Vasopressin receptor subtype of rat aortic smooth muscle cells

Analog structure	Cell membranes IC_{50}[a]	Rat liver K_{bind}[b]	Rat kidney	
			K_i[c]	K_{act}
	nM	nM	nM	
AVP	8.2	0.6		2.0
d(CH$_2$)$_5$Tyr(Me)AVP	10.0	0.5	220.0	
d(CH$_2$)$_5$Tyr(Et)VAVP	93.0	5.9	5.6	
d(CH$_2$)$_5$D-IleVAVP	3300.0	243.0	4.5	
dDAVP	6200.0	150.0		2.1

[a] Data from experiments of Fig. 8.
[b] Affinity of rat liver vasopressin receptors was determined in competition binding assays.
[c] Inhibition (K_i) or activation (K_{act}) of renal medullary adenylate cyclase activity.

dissociation of intact AVP, AVP might also be removed from the receptors by proteolysis. It is not clear whether proteolysis required receptor-mediated endocytosis of AVP. Degradation of AVP may also occur at the receptor on the cell surface.

Receptor-mediated endocytosis of AVP might have occurred because, with increased incubation time, the amount of specifically bound ligand that dissociated from intact cells or could be extracted with acetic acid decreased. Incorporation of [^{3}H]Phe, derived from [^{3}H]AVP, into acetic acid-insoluble proteins could explain decreased extractability of tritium. Taken together, these data are consistent with the following notions: 1) AVP interacts with receptors on the cell surface in a reversible manner; 2) a fraction of receptor-bound AVP is endocytosed and degraded intracellularly; 3) degradation products of AVP are released in the medium and incorporated into acetic acid-insoluble proteins.

To further characterize the vasopressin receptors, we studied [^{3}H]AVP equilibrium binding to plasma membranes at 37° over a large concentration range (from 0.04 nM to 50 nM). The Scatchard plot of the membrane binding data was not linear (Fig. 7B). In contrast, a linear Scatchard fit of saturation binding data was obtained using plasma membranes of rat mesenteric artery (35) and thoracic aorta (36). The linear Scatchard fit of these studies might be due to the use of a limited range of ligand concentrations and incubation at lower temperature (20°); also, the structure of the ligand associated with the tissue membranes was not determined. The nonlinear plot indicated the presence of two affinity states with K_D values of 0.7 nM and 370 nM. The high affinity sites detected on the cell membranes (about 60 fmol/10^6 cells) might represent the receptors found on intact cells (90 fmol/10^6 cells; Fig. 3B). The putative low affinity sites found on cell membranes might mediate arachidonic acid release (37).

An alternative explanation for the nonlinear Scatchard plot of the membrane binding data could be negative cooperativity between the binding sites, consistent with the observed slope of 0.66 of the linear Hill plot ($r = 0.98$) of the data of Fig. 7A. Self-aggregation of the ligand onto the specific binding sites is another possibility (38). In order to be able to better understand the dynamics of the vasopressin-receptor interaction, we are developing radiolabeled antagonists with high affinity for all receptor states.

Our data show that the affinity of vascular vasopressin receptors could be regulated by guanine nucleotides. Binding of [^{3}H]AVP to high affinity sites was decreased in the presence of Gpp(NH)p (Fig. 8), whereas binding to the putative low affinity sites was not significantly affected. We propose that the receptors on intact cells (K_D = 6.0 nM) represent the high affinity receptors of the cell membranes (K_D = 0.7 nM) modulated by endogenous guanine nucleotides. Cantau *et al.* (39) reported similar differences in affinity of receptors of intact hepatocytes and membranes of liver, a tissue expressing the vascular vasopressin receptor subtype (40). We have shown that in A-10 cells vasopressin stimulates phosphatidylinositol turnover (19). Guanine nucleotide-binding proteins are the likely transducers of this vasopressin response, because in *in vitro* experiments GTP was required for vasopressin-induced phosphatidylinositol hydrolysis in membranes of rat hepatocytes (41) and rat mammary tumor cells (WRK1) (42). Regulation of vasopressin receptor affinity by guanine nucleotides supports the hypothesis that activation of phospholipase C of vascular smooth muscle by vasopressin is mediated by guanine nucleotide-binding proteins.

Selective vasopressin analogs were used in competition binding experiments to determine the vasopressin receptor subtype of the A-10 cells. The affinity rank orders of the analogs for cell and rat liver membranes were the same, but different from the potency rank order for inhibition of rat kidney adenylate cyclase. Since the rat liver and vasculature present the same vasopressin receptor subtype (40), we conclude that the binding sites of the A-10 cells represent the vascular V_1 receptor. These binding sites appear to be functional receptors, because, using a similar series of vasopressin analogs, we have previously reported that vasopressin-induced inhibition of cAMP and cGMP accumulation (by β-agonists and atrial natriuretic factor, respectively), and increases in phosphatidylinositol turnover and calcium efflux in the A-10 cells are mediated by V_1 receptors (8, 9, 19).

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