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Specific vasopressin binding to rat adrenal glomerulosa cells

Relationship to inositol lipid breakdown

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Cells from the zona glomerulosa of rat adrenals were isolated and maintained for 3 days in primary culture. Specific vasopressin binding was determined by using [^{3}H]vasopressin. [^{3}H]Vasopressin binding was time-dependent (half-time of about 2 min for 6 nM free ligand) and reversible on addition of unlabelled vasopressin (80% dissociation within 30 min). Dose-dependent [^{3}H]vasopressin binding at equilibrium indicated that vasopressin interacted with two populations of sites: high-affinity sites (dissociation constant, K_d = 1.8 nM; maximal binding capacity = 10 fmol/10^6 cells) and low-affinity sites. Vasopressin increased the cellular content of labelled inositol mono-, bis- and tris-phosphate in cells prelabelled with *myo*-[^{3}H]inositol. The vasopressin concentration eliciting half-maximal inositol phosphate accumulation was very close to the K_d value for vasopressin binding to high-affinity sites. Competition experiments using agonists and antagonists with enhanced selectivity for previously characterized vasopressin receptors indicated that vasopressin receptors from rat glomerulosa cells are V1 receptors of the vascular or hepatic subtype. The detected specific vasopressin-binding sites might represent the specific receptors mediating the mitogenic and steroidogenic effects of vasopressin on glomerulosa cells from rat adrenals.

INTRODUCTION

Corticotropin and angiotensin are the two major hormonal regulators of aldosterone secretion by adrenal cells from the zona glomerulosa. We have previously shown that vasopressin is also involved in this regulation. Administration of vasopressin *in vivo* increases the plasma concentrations of aldosterone and corticosterone in the rat (Payet & Lehoux, 1979). Vasopressin also potentiates the effect of low doses of corticotropin on aldosterone secretion (Payet & Lehoux, 1982) and has marked mitogenic and steroidogenic effects in rat glomerulosa cells in primary culture (Gallo-Payet *et al.*, 1984).

The mechanism by which vasopressin exerts its effects has not been elucidated. Preliminary experiments (Gallo-Payet *et al.*, 1985) indicated that vasopressin increases the concentrations of Ins*P*, InsP_2 and InsP_3 in rat glomerulosa cells in primary culture. This observation suggested that Ca^{2+} might be the intracellular messenger of vasopressin on the adrenals. Indeed, there is a general agreement that enhanced production of Ins(1,4,5)P_3 is an early post-receptor step in the mechanisms by which several hormones and neurotransmitters increase the concentration of cytosolic free Ca^{2+} in their target cells (for review, see Berridge & Irvine, 1984). It also provided a clue for characterization of vasopressin receptors in the adrenals, since the relation of specific vasopressin binding to an early post-receptor event could be studied. In addition, vasopressin analogues with enhanced selectivity for the different subtypes of vasopressin receptors so far characterized allowed a precise examination of the ligand specificity of vasopressin receptors from adrenal cells.

In the present study, we demonstrate that rat glomerulosa cells in primary culture express vasopressin receptors of the V1 subtype. This conclusion was based on the observations that: (1) vasopressin receptors from glomerulosa cells have a ligand specificity very similar to that of previously characterized V1 receptors such as vascular or hepatic receptors (for review see Jard, 1983); (2) a close correlation could be demonstrated between vasopressin binding to glomerulosa cells and vasopressin-induced inositol lipid breakdown.

MATERIALS AND METHODS

Chemicals

[^{3}H]Vasopressin (40 Ci/mmol) and *myo*-[^{3}H]inositol (16.5 Ci/mmol) were obtained from New England Nuclear. The purity of [^{3}H]vasopressin was checked by h.p.l.c. on a μBondapack C18 column from Waters. Other chemicals were of A-grade purity and were obtained from the following sources: collagenase from Worthington; deoxyribonuclease from Sigma; Eagle MEM from Gibco; AVP and LVP from Bachem; Dowex 1 X 10 (100–200 mesh; Cl^- form) from Fluka. The vasopressin analogues dVDAVP, d$(CH_2)_5$[Tyr(Et)^2]VAVP and desGly^{9}d$(CH_2)_5$AVP were kindly provided by Dr. M. Manning (Toledo, OH, U.S.A.). Purified labelled Ins*P*, Ins(1,4)P_2 and Ins(1,4,5)P_1 were kindly provided by Dr. C. J. Kirk (Birmingham, U.K.).

Abbreviations used: Ins*P*, inositol monophosphate; InsP_2, inositol bisphosphate; InsP_3, inositol trisphosphate; [^{3}H]vasopressin, [[^{3}H]$_3$Phe^3]-[arginine]vasopressin; AVP, [arginine]vasopressin; LVP, [lysine]vasopressin; dVDAVP, 1-desamino-[4-valine,8-D-arginine]vasopressin; d$(CH_2)_5$[Tyr(Et)^2]VAVP, [(1-β-mercapto-ββ-cyclopentamethylenepropionic acid),2-*O*-ethyltyrosine,4-valine][arginine]vasopressin; desGly^{9}d-$(CH_2)_5$AVP, des-9-glycine-[1-β-mercapto-ββ-cyclopentamethylenepropionic acid][arginine]vasopressin; MEM, minimum essential medium; PBS, phosphate-buffered saline.

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Cell culture

Glomerulosa cells were prepared from the adrenal glands of male Long–Evans rats (200–250 g body wt.). Cells were isolated as previously described (Payet & Lehoux, 1982) with slight modifications. After collagenase and deoxyribonuclease digestion in MEM, the cells were plated in tissue-culture dishes (35 mm diameter) at a density of about 5×10^4 cells/dish. They were grown in MEM supplemented with 10% (v/v) fetal-calf serum, 100 units of penicillin/ml and 100 units of streptomycin/ml. Cultures were incubated at 37 °C in an humidified atmosphere of air/CO_2 (19:1). The culture medium was renewed 24 h after seeding, and the cells were used after 3 days in culture. At that time, cell density was about 4×10^5–6×10^5 cells per dish (determined by direct counting with a haemocytometer).

[^{3}H]Vasopressin-binding assay

Hormone binding was assayed as described by Penit *et al.* (1983) by using cells attached to the plastic substratum of the Petri dishes in which they were grown. At the beginning of the experiment, the culture medium was aspirated and the cells were washed once with 2 ml of PBS ($CaCl_2$, 0.44 mM; KCl, 2.7 mM; $MgCl_2$, 0.5 mM; NaCl, 138 mM; Na_2HPO_4, 8.1 mM; KH_2PO_4, 1.5 mM; pH 7.4) supplemented with glucose, 1 g/l (PBS–glucose). Cells were then kept at 37 °C. The hormone-binding reaction was initiated by rapidly aspirating the PBS solution and adding to each Petri dish 0.7 ml of PBS–glucose containing bovine serum albumin (1 mg/ml), and various amounts of [^{3}H]vasopressin. Except when otherwise specified, cells were incubated for 15 min at 37 °C in the presence of [^{3}H]vasopressin. The reaction was stopped by rapid removal of the incubation medium and addition of 2 ml of ice-cold PBS. Cells were rapidly detached by scraping with a rubber policeman. The cell suspension was layered on to the surface of a Millipore filter (EAWP; 0.45 μm pore size) under continuous aspiration. Then 2 ml of ice-cold PBS was added to rinse the Petri dishes and transferred on to the filter, which was washed with 3×3 ml of ice-cold PBS. The whole procedure, including cell suspension and filtration, lasted 50 s. The radioactivity retained on the filter was counted by liquid-scintillation spectrometry. All determinations were performed in triplicate. Non-specific binding was determined by incubating the cells in the presence of labelled hormone plus an excess of unlabelled vasopressin (2 or 20 μM).

The dissociation constants for binding of unlabelled peptides to glomerulosa cells were determined from competition experiments. Cells were incubated in the presence of a constant amount of [^{3}H]vasopressin (6 nM) and increasing amounts of the unlabelled analogue to be tested.

Inositol lipid labelling and determination of inositol lipid breakdown

Cells were cultured for 3 days as described above in the presence of *myo*-[^{3}H]inositol (2 μCi/ml). At the beginning of the experiment, the culture medium was removed and the cells were incubated for 60 min in Eagle MEM to remove as much as possible of the free *myo*-[^{3}H]inositol. Cells were then washed with 3×2 ml of PBS–glucose, incubated for 15 min at 37 °C in the presence of LiCl (10 mM) and then for an additional 15 min in the presence of LVP and LiCl. The incubation was terminated by rapidly removing the medium and adding 1 ml of 5% (v/v) $HClO_4$ and 200 μl of bovine serum albumin solution (20 mg/ml). The pH of the extract was adjusted to 7.0 by adding the appropriate volume of a solution containing 1.5 M-KOH/75 mM-Hepes. After rapid centrifugation of the $HClO_4$ extracts, Ins$P$, InsP_2 and InsP_3 in the supernatant were separated by chromatography on Dowex 1 X 10 (100–200 mesh; formate form) columns (4.0 cm × 0.6 cm) as described by Berridge *et al.* (1983). Inositol, glycerophosphoinositol, Ins$P$, InsP_2 and InsP_3 were sequentially eluted in 24 ml fractions of: (1) water; (2) 60 mM-ammonium formate/5 mM-disodium tetraborate; (3) 150 mM-ammonium formate/5 mM-disodium tetraborate; (4) 400 mM-ammonium formate/100 mM-formic acid; (5) 1 M-ammonium formate/0.1 M-formic acid. This procedure was established by using purified labelled Ins$P$, Ins(1,4)P_2 and Ins(1,4,5)P_3 (standards obtained from Dr. C. J. Kirk). Radioactivity found in the Ins$P$, InsP_2 and InsP_3 fractions was determined by scintillation counting in gel phase. All results were corrected for quenching and are expressed in d.p.m.

RESULTS AND DISCUSSION

To test the stability of [^{3}H]vasopressin during the course of the binding assay, glomerulosa cells were incubated for 15 min in the presence of [^{3}H]vasopressin (5 nM). A small sample of the culture medium was collected at the end of the incubation period and submitted to h.p.l.c. analysis. The elution profile of ^{3}H radioactivity failed to reveal the presence of [^{3}H]vasopressin degradation products, indicating that glomerulosa cells did not inactivate [^{3}H]vasopressin added to the incubation medium.

After a 15 min incubation period in the presence of [^{3}H]vasopressin (6 nM), glomerulosa cells retained an amount of radioactivity equivalent to about 17 fmol of [^{3}H]vasopressin per 10^6 cells. When the incubation was conducted in the presence of unlabelled vasopressin (2 μM), the amount of bound radioactivity was decreased by about 60%. No further decrease was observed when the concentration of unlabelled vasopressin was raised to 20 μM. These preliminary experiments clearly demonstrated the presence on glomerulosa cells of high-affinity vasopressin-binding sites.

Specific [^{3}H]vasopressin binding to glomerulosa cells was time-dependent and reversible (Fig. 1). For 6 nM-[^{3}H]vasopressin, specific binding was maximum after 5 min incubation and remained stable thereafter up to 15 min. More than 80% of [^{3}H]vasopressin bound during an 8 min incubation period of cells in the presence of [^{3}H]vasopressin (6 nM) was displaceable within 30 min by unlabelled vasopressin (2 μM). To a first approximation, [^{3}H]vasopressin binding to glomerulosa cells had the characteristics expected from a reversible interaction of the labelled hormone with a limited number of high-affinity binding sites. However, the association and dissociation curves could not be fitted with pure monoexponential relationships, as might have been expected from a pseudo-first-order reaction involving reversible vasopressin binding to a single population of binding sites.

As shown in Fig. 2, specific vasopressin binding to glomerulosa cells was saturable. Again, the observed dose-dependent binding indicated the existence of some heterogeneity in the population of vasopressin-binding

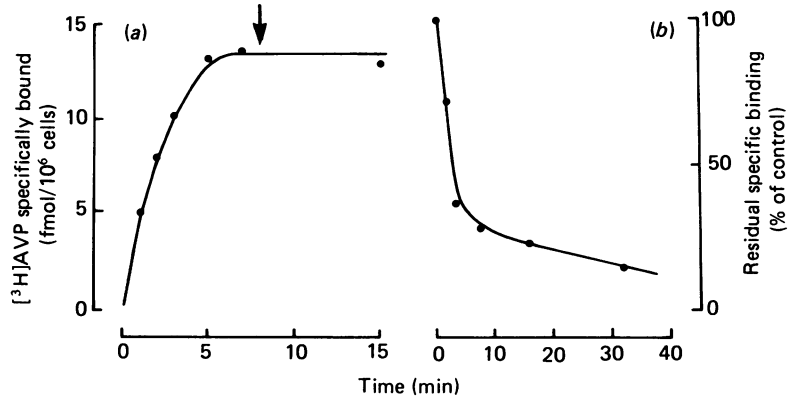

Fig. 1. Time-dependent [^{3}H]vasopressin binding to glomerulosa cells

(*a*) Rat adrenal glomerulosa cells were incubated for the indicated periods of time in the presence of [^{3}H]vasopressin (6 nM). Values on the graph are means of two independent sets of determinations. Non-specific binding was measured in the presence of unlabelled vasopressin (2 μM). (*b*) Glomerulosa cells were incubated for 8 min at 37 °C in the presence of [^{3}H]vasopressin (6 nM) (arrow). The incubation medium was then rapidly aspirated and replaced by a medium without [^{3}H]vasopressin but containing unlabelled vasopressin (2 μM). Cells were then further incubated at 37 °C. Residual specific binding was measured as a function of time after addition of unlabelled vasopressin. Data are expressed as % of specific binding measured immediately before addition of unlabelled vasopressin.

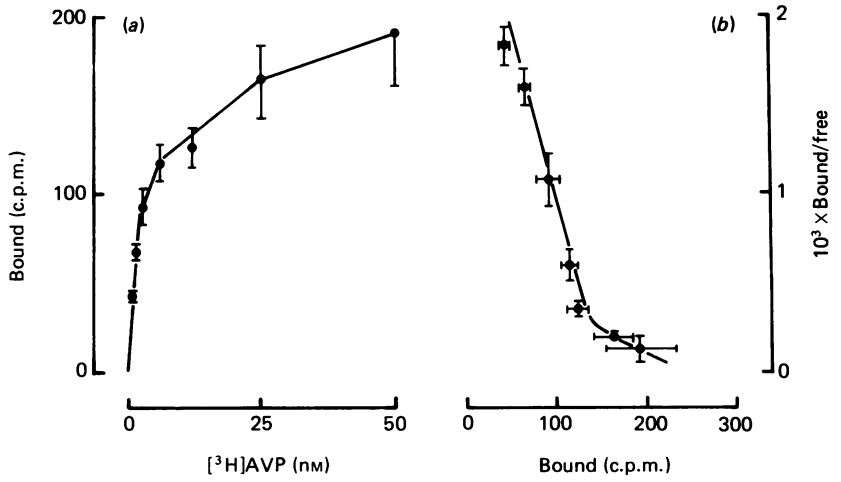

Fig. 2. Dose-dependent specific binding of [^{3}H]vasopressin to glomerulosa cells

(*a*) Glomerulosa cells (0.5×10^6 cells/dish) were incubated for 15 min at 37 °C in the presence of [^{3}H]vasopressin at the indicated concentrations. Radioactivity bound to the cells was measured as indicated in the Materials and methods section. Results were corrected for non-specific binding. Values are the means ± S.E.M. of three independent determinations. The Scatchard plot of the dose–binding curve is shown in (*b*). The experimental curve was fitted with the following two-sites model:

$$B = B_{max.1}/[1+(K_{d1}/H)]+B_{max.2}/[1+K_{d2}/H)]$$

in which $B_{max.1}$, $B_{max.2}$, K_{d1} and K_{d2} represent the maximal binding capacities and dissociation constants for [^{3}H]vasopressin binding to high- and low-affinity sites respectively and H is the concentration of free [^{3}H]vasopressin in the incubation medium. The computed values were $B_{max.1} = 10$ fmol/10^6 cells, $K_{d1} = 1.8$ nM, and $B_{max.2}/K_{d2} = 0.04$ l/10^6 cells.

sites. The Scatchard plot of the data revealed a slight but reproducible deviation from linearity. The experimental curve could be adequately described by a model involving reversible binding of vasopressin to two populations of sites, of high and low affinity. As estimated from the results of three independent experiments, the dissociation constant (K_d) for vasopressin binding to high-affinity sites was 1.8 nM. The maximal binding capacity of these sites was 10 fmol/10^6 cells. The apparent affinity and capacity of the low-affinity sites could not be adequately determined, owing to a lack of precision in determining specific binding at high [^{3}H]vasopressin concentrations.

The K_d value of 1.8 nM found for high-affinity binding sites on glomerulosa cells is in the range of values determined for vasopressin receptors from several other tissues and species (for review, see Jard, 1983). The density of high-affinity sites on glomerulosa cells (6×10^3/cell) is small compared with the corresponding values of 200×10^3, 25×10^3 and 68×10^3 sites/cell found for rat hepatocytes (Cantau *et al.*, 1980), rat aortic

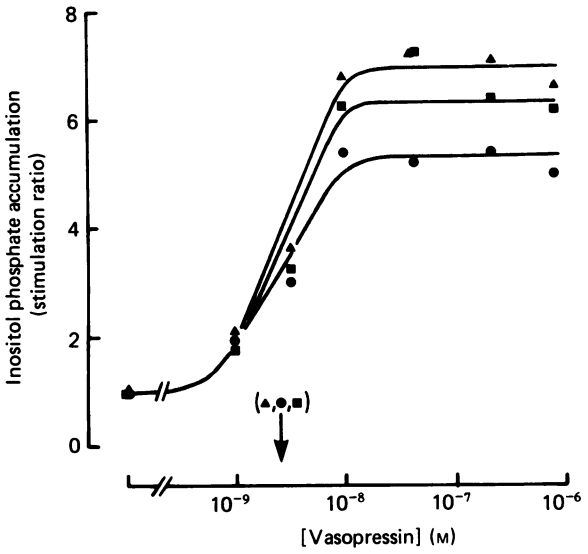

Fig. 3. Dose-dependent accumulation of Ins$P$, InsP_2 and InsP_3 by glomerulosa cells stimulated by vasopressin

Glomerulosa cells prelabelled with *myo*-[^{3}H]inositol were used. Cells were incubated for 15 min as described in the Materials and methods section in the presence of LVP at the indicated concentrations. Data on the graph are means of triplicates from two independent experiments. They are expressed in terms of stimulation ratio (stimulated production/basal production). The basal values were: 1236±81, 669±105 and 282±60 d.p.m./10^6 cells for Ins$P$ (●), InsP_2 (■) and InsP_3 (▲) respectively. Arrow indicates the concentration of vasopressin giving half-maximal stimulation of inositol phosphate accumulation.

myocytes (Penit *et al.*, 1983) and the pig kidney cell line LLCPK1 (Roy & Balestre, 1982) respectively. It is similar to that (5×10^3/cell) determined on Leydig cells from rat testis (Meidan & Hsueh, 1985). Therefore, it could be concluded that the high-affinity vasopressin-binding sites detected on rat glomerulosa cells had characteristics similar to those of well-characterized vasopressin receptors from several other vasopressin-sensitive tissues.

As indicated above, preliminary results from our group (Gallo-Payet *et al.*, 1985) indicated that vasopressin increases the concentration of Ins$P$, InsP_2 and InsP_3 in adrenal cells in primary culture. We therefore decided to compare the dose-dependency for vasopressin binding to glomerulosa cells with that of the vasopressin-induced increase in Ins$P$, InsP_2 and InsP_3 intracellular concentrations; the results are shown in Fig. 3. Under experimental conditions identical (except for the presence of LiCl) with those used for the binding assay, vasopressin increased the cellular contents of Ins$P$, InsP_2 and InsP_3. The relative increases in InsP_2 and InsP_3 were slightly higher than that in Ins$P$. However, Ins$P$ represented the major component of all phosphoinositols. Half-maximal increase in Ins$P$, InsP_2 and InsP_3 contents was obtained for a vasopressin concentration of 5.8±1.7 nM (mean±S.E.M. for three independent experiments). Vasopressin used for those experiments was LVP, which has a slightly lower affinity for glomerulosa cells ($K_d = 3.6 \pm 0.8$ nM) as compared with AVP. The observed similarity in the dose-dependencies for vasopressin binding to high-affinity sites and vasopressin-induced accumulation of Ins$P$, InsP_2 and InsP_3 by glomerulosa cells suggests that these sites are the receptors involved in the measured biological response. These results clearly confirm the conclusions

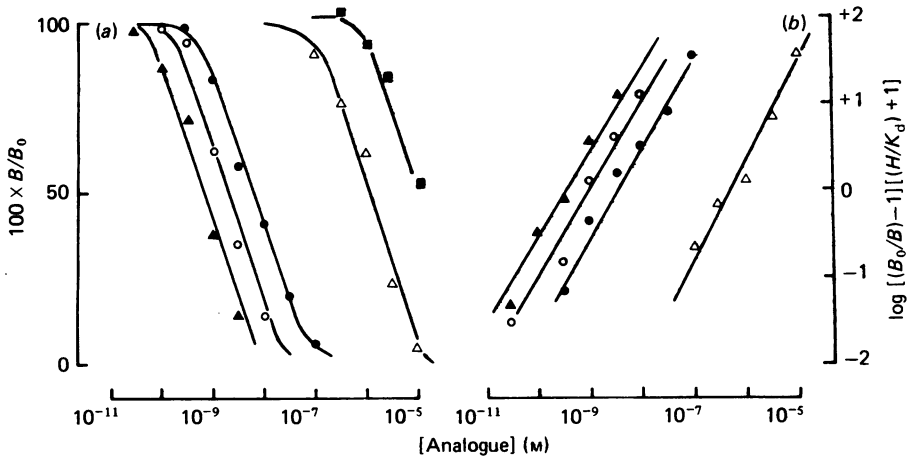

Fig. 4. Dose-dependent inhibition of [^{3}H]vasopressin binding to glomerulosa cells by unlabelled vasopressin analogues

(*a*) Glomerulosa cells were incubated for 15 min at 37 °C in the presence of [^{3}H]vasopressin (6 nM) and increasing amounts of unlabelled peptide: ●, AVP; ○, desGly^{9}d(CH$_2$)$_5$AVP; △, dVDAVP; ▲, d(CH$_2$)$_5$[D-Tyr(Et)^2]VAVP; ■, oxytocin. Residual specific binding measured in the presence of unlabelled peptide (B) was expressed as a percentage of specific binding measured in the absence of competitor (B_0). Values on the graph are means of two independent sets of determinations. (*b*) The binding dissociation constants for unlabelled peptide (K_i) were deduced by fitting the experimental data with the expected linear relationship:

$$\log\{[(B_0/B)-1][(H/K_d)+1]\} = \log I - \log K_i$$

in which I is the concentration of unlabelled peptide. K_i values were deduced from regression lines shown in panel (*b*). They were 1.8±0.6, 263±64, 0.2±0.03 and 0.6±0.06 nM for AVP, dVDAVP, d(CH$_2$)$_5$[D-Tyr(Et)^2]VAVP and desGly^{9}d(CH$_2$)$_5$AVP respectively.

Table 1. Relative affinities for glomerulosa cells and other receptors of vasopressin analogues with enhanced selectivity

$K_{d(AVP)}/K_{d(analogue)}$ is a measure of the affinity of the tested analogue relative to that of [arginine]vasopressin. Data for liver, kidney and adenohypophyseal membranes were derived from Cantau *et al.* (1980) and from S. Jard, R. Gaillard, G. Guillon, J. Marie, A. Muller, M. Manning & W. Sawyer (personal communication).

Vasopressin or analogue	$100 \times (K_{d(AVP)}/K_{d(analogue)})$			
	Liver	Kidney	Adenohypophysis	Glomerulosa cells
AVP	100	100	100	100
dVDAVP	1	158	1.3	0.7
$d(CH_2)_5$[Tyr(Et)^2]VAVP	4000	700	1.9	900
desGly^{9}d$(CH_2)_5$AVP	2554	0.3	0.5	300

derived from parallel measurements of vasopressin binding and vasopressin-induced inositol phosphate accumulation by isolated rat hepatocytes (Creba *et al.*, 1983). Thus, according to the functional criteria proposed by Michell *et al.* (1979) to classify vasopressin receptors, those from glomerulosa cells appeared to be of the V1 type, i.e. receptors mediating Ca^{2+} mobilization through enhanced inositol lipid breakdown, as opposed to V2 receptors, which mediate adenylate cyclase activation.

Numerous pharmacological studies have clearly established that V1 and V2 vasopressin receptors exhibit strikingly different ligand specificities (for reviews, see Sawyer *et al.*, 1981; Jard, 1983). It was also clearly demonstrated that V1 receptors from several tissues (in particular, liver and blood vessels) have closely similar, if not identical, ligand specificities. However, the possibility that several subtypes of V1 receptors might exist emerged from studies on adenohypophyseal vasopressin receptors (Antoni, 1984; Baertschi & Friedli, 1985; S. Jard, R. Gaillard, G. Guillon, J. Marie, A. Muller, M. Manning & W. Sawyer, personal communication). It was shown that the relative affinities of several vasopressin antagonists for hepatic or vascular V1 receptors, on the one hand, and for adenohypophyseal V1 receptors on the other, differed by several orders of magnitude. In an attempt further to characterize vasopressin receptors from glomerulosa cells, we determined their affinity for several vasopressin analogues selected on the basis of an enhanced selectivity for the three types of vasopressin receptors already well characterized (Table 1). dVDAVP was chosen as one of the most selective agonists for renal V2 receptors, $d(CH_2)_5$[Tyr(Et)^2]VAVP as an antagonist with very high affinity for hepatic V1 and renal V2 receptors and decreased affinity for V1 adenohypophyseal receptors, and desGly^{9}d$(CH_2)_5$AVP as a high-affinity antagonist for hepatic V1 receptors, a weak agonist for renal V2 receptors and a low-affinity antagonist for adenohypophyseal V1 receptors. The affinity of these analogues was deduced from the competition experiments shown in Fig. 4; [^{3}H]vasopressin was used at a concentration of 6 nM. At that concentration, one can estimate from the data shown in Fig. 2 that more than 95% of [^{3}H]vasopressin specific binding corresponded to [^{3}H]vasopressin bound to the so-called high-affinity binding sites. The dose-dependent displacement of [^{3}H]vasopressin by the analogues tested therefore mainly reflected their binding to the high-affinity sites. The displacement curves were parallel and corresponding semi-logarithmic plots were linear, with slopes close to 1. Oxytocin was added in this series of experiments in order to ascertain the vasopressic character of the binding sites detected; it exhibited a very low affinity for these sites. The dissociation constant measured for unlabelled AVP (1.8 nM) was identical with that deduced from the determination of dose-dependent [^{3}H]vasopressin binding. As clearly apparent from Fig. 4 and Table 1, the ligand specificity of glomerulosa-cell receptors is similar to that of V1 receptors of the hepatic or vascular type and different from those of renal V2 and adenohypophyseal V1 receptors.

Summing up, the present study demonstrates the presence on glomerulosa cells from rat adrenals of vasopressin receptors showing striking similarities to V1 receptors of the vascular type. It is tempting to consider that these receptors might be those involved in the mitogenic and steroidogenic effects of vasopressin on rat adrenals.

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