Biotinyl Analogues of Vasopressin as Biologically Active Probes for Vasopressin Receptor Expression in Cultured Cells*

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Biotinyl analogues of [Arg3]vasopressin were synthesized with the biotin moiety at position 4. This involved the substitution of 2, 4-diaminobutyric acid (Dab) for Gin1 in [1-deamino-Arg3]vasopressin to give the parent peptide des-[Dab4,Arg3]vasopressin. Two biotinyl analogues with different spacers between the side chain of Dab4 and the biotinyl residue were then prepared and characterized in detail. The analogues retained high binding affinities for the V2-receptor in both bovine kidney membranes and LLC-PK1 renal epithelial cells and for the V1-receptor in rat liver membranes. Both analogues were as potent as [Arg3]vasopressin in stimulating the cAMP-dependent protein kinase and the production of urokinase-type plasminogen activator in LLC-PK1 cells, with concentration dependence consistent with receptor binding affinities. Avidin or streptavidin did not appear to reduce receptor binding or biological activity of the biotinyl analogues. The use of the biotinylated vasopressin analogue des-[Dab-(biotinylamido)hexanoyl]Arg3vasopressin together with fluorescein-labeled streptavidin as a fluorescent probe for the V2-receptor in LLC-PK1 cells demonstrated the following: 1) Specific binding of the biotinyl analogue shown by quantitative single-cell fluorescence measurements using the technique of fluorescence microphotolysis; 2) the V2-receptor visualized by fluorescence microscopy; and 3) the expression of the V2-receptor detected by flow cytometry.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM) was from Flow Laboratories, Inc., and fetal calf serum was from Biochrom. ATP, cAMP, Kemptide fragment, bovine serum albumin (fatty acid-free, fraction V), isobutylmethylxanthine, and Triton X-100 were from Sigma. [5'-γ32P]ATP and [3H][Arg3]vasopressin were from Amersham Corp., and phosphocellulose paper (P-81) was from Whatman.

Analytical Methods

Thin-layer chromatography was performed on precoated Silica Gel 60 F254 plates (Merck). The following solvent system was used: ethyl acetate/butanol/acetic acid/water (1:1:11:1).

For high-performance liquid chromatography (HPLC), peptides were dissolved in 1-10% aqueous acetic acid, applied to a Lichrosorb RP-18 column (10 μm, 250 × 4.6 mm), and eluted by a gradient of 0.09% trifluoroacetic acid in water (solvent system A) and 0.09% trifluoroacetic acid, 9.91% water, 90% acetonitrile (solvent system B)–30% for 30 min. The flow rate was 1.5 ml/min.

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The neurohypophysial hormone vasopressin is a major regulator of water homeostasis, blood volume, and blood pressure. It interacts in a specific manner with membrane receptors present in many target tissues (for review, see Ref. 1). Binding to the renal V1-type receptor leads to the activation of the adenylate cyclase system (2), whereas interaction with the hepatic and vascular V1-type receptors stimulates the phospholipase C system and mobilizes internal Ca2+ (3, 4). Partial characterization of the V1-receptor using membranes prepared from renal medulla and from the V1-receptor using membranes derived from rat liver has been accomplished with radiolabeled photoaffinity vasopressin analogues (5, 6), chemical cross-linking techniques (7–9), and target size analysis (10). Purification of vasopressin receptors in a native form has been prevented by the loss of hormone binding properties after solubilization with detergents (11–13).

Biotinylated vasopressin analogues could be of use in the study of the expression of vasopressin receptors in various cell types and the isolation of the hormone-receptor complex. The particularly high affinity (Kd = 10−15 M) of biotin for egg white avidin and bacterial streptavidin could be exploited in the qualitative and quantitative investigation of hormone receptors (for review, see Refs. 14 and 15), whereby success is critically dependent on the ability of the biotinyl-substituted hormone ligand to bind simultaneously to both avidin and the receptor. Introduction of residues at the α-amino group in vasopressin greatly decreases its biological activity in most target systems. Based on our recent studies (5, 6, 16) of structure-activity relationships of synthetic vasopressin analogues, we chose position 4 in the vasopressin sequence to investigate the V1- and V2-receptor binding properties of these biotinyl analogues in isolated membranes and their binding and biological activity in the renal epithelial LLC-PK1 cell line. Furthermore, we demonstrate that the biotinylated vasopressin analogues together with fluorescein-labeled streptavidin can be used as a fluorescent probe for the V2-receptor in LLC-PK1 cells. Receptor expression can be analyzed by single-cell fluorescence measurements, fluorescence microscopy, and flow cytometry.

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The abbreviation used is: DMEM, Dulbecco's modified Eagle's medium; AVP, [Arg3]vasopressin; BSA, bovine serum albumin; Dab, 2,4-diaminobutyric acid; des-[Dab4]AVP, [1-deamino-Dab4,Arg3]vasopressin; HPLC, high-performance liquid chromatography; fAB, fast atom bombardment; uPA, urokinase-type plasminogen activator; FITC, fluorescein isothiocyanate; IBMX, isobutylmethylxanthine.
Synthesis and Purification of Vasopressin Analogues

Des-[Dab{supscript}4]AVP (Fig. 1, Peptide 1)—The parent peptide was prepared by Merrifield solid-phase synthesis and dialysis formation as recently described (5). Des-[Dab-propionyl]{superscript}4AVP (Fig. 1, Peptide 2)—Synthesis was performed with 1 eq of des-[Dab{superscript}4]AVP (5 μmol, 5.2 mg) and 2 eq of proionic acid N-hydroxysuccinimidoester (17) (10 μmol, 1.7 mg) as described below for BIO-AVP (Fig. 1, peptide 3). Ethyl acetate (5 ml) was added to the reaction mixture after stirring for 24 h, and the precipitated peptide 2 was collected by centrifugation. Analytical data were as follows: HPLC, t{subscript}R = 8.3 min and k' = 4.0; TLC, R{subscript}f = 0.52; FAB mass spectroscopy, m/z = 1097 (M + H){superscript}+.

BIO-AVP (Fig. 1, Peptide 3)—1 eq of des-[Dab{superscript}4]AVP (10 μmol, 10.4 mg) was dissolved in dimethylformamide (400 μl). 1 eq of N-methylmorpholine (10 μl of 10% N-methylmorpholine in dimethylformamide) and 5 eq of 6-(biotinylamido)hexanoic acid N-hydroxysulfosuccinimidoester (50 μmol, 28 mg; NHS-LC-Biotin, Pierce Chemical Co.) dissolved in 1.50 ml of dimethylformamide were added. The solution was stirred for 48 h and maintained at pH 7.5. After evaporation of the solvent, the mixture was dissolved in acetic acid and chromatographed on a Sephadex G-25 (fine) column (200 × 2 cm) with 20% acetic acid as eluant. Fractions of the main peak of UV absorption at 278 nm (fractions 53–58) 15 ml/fraction) were collected and lyophilized. An aliquot was checked for purity by analytical HPLC and found to be homogeneous. Analytical data were as follows: HPLC, t{subscript}R = 9.5 min and k' = 4.1; TLC, R{subscript}f = 0.52; yield, 5.4 mg (determined by UV absorption); FAB mass spectroscopy, m/z = 1380 (M + H){superscript}+.

Des-[Dab-propionyl]{superscript}4,Arg{superscript}8]vasopressin and of its derivatives. Mpa, 3.9 mg (determined by UV absorption); FAB mass spectroscopy, m/z = 1431 (M + H){superscript}+.

Membrane Preparations

Plasma membranes from rat liver containing 2–3 pmol of the V{subscript}1 receptor/mg of protein were prepared using a modification (18) of the method of Lesko et al. (19) using an aqueous two-phase polymer system. Plasma membranes from bovine kidney inner medulla (V{subscript}2 receptor) were prepared by differential centrifugation followed by Percoll density gradient centrifugation as described previously (6, 20). Membrane preparations obtained by this procedure had a specific [H]{superscript}3-HAVP binding capacity of 4–6 pmol/mg of protein.

Cell Culture

The pig kidney epithelial LLC-PK{subscript}1 cell line (21) and the vasopressin V{subscript}2-receptor-deficient mutant M18 (22) were cultured in DMEM supplemented with 10% (v/v) fetal calf serum, 0.2 mg/ml streptomycin, and 50 units/ml penicillin in a humidified incubator at 37 °C using a 95% air, 5% CO{subscript}2 atmosphere.

Receptor Binding Assay

The K{subscript}D values for vasopressin and its analogues were obtained from competitive binding experiments. Plasma membranes from rat or bovine kidney (50 μg of protein) were equilibrated with 10{superscript}–6 M [H]{superscript}3-HAVP and varying concentrations of the nonlabeled peptides for 30 min at 30 °C. The binding assay with liver membranes contained bacitracin (1 mg/ml). Other conditions have been described previously (18).

Vasopressin binding in EDTA-suspended cells was measured as described previously (22, 23). Parameters of binding were determined as described previously (18) by using a weighted nonlinear least-squares fit to logistic curves.

Enzyme Assays

Extracts for the assay of cAMP-dependent protein kinase catalytic activity were prepared and assayed with Kemptide fragment (Leu-Arg-Arg-Ala-Ser-Ala-OH) as substrate as described previously (22, 24). Protein kinase activity is expressed in units/milligram of protein (1 unit of kinase activity is defined as the amount of enzyme that catalyzes the transfer of 1 nmol of phosphate from ATP to peptide/min at 30 °C). The cAMP-dependent protein kinase activity ratio expresses the catalytic subunit activity present in cell extracts assayed in the absence of exogenous cAMP relative to the total stimulatable activity (assayed in the presence of cAMP) (22, 25, 26). Although possibly not a strictly quantitative measure of cellular events, as discussed previously (25, 27, 28), it is assumed that the activity ratio measures events occurring intracellularly rather than during the preparation of cell extracts (25, 29).

Cells to be assayed quantitatively for urokinase-type plasminogen activator (uPA) induction were treated, and uPA activity in medium was measured as outlined previously (22, 24, 25) using the chromogenic substrate S-2251 (D-Val-Leu-L-Lys-p-nitroanilide) (Bachem AG, Bubendorf, Switzerland) in a coupled assay with human plasminogen (Sigma). Human urokinase (EC 3.4.21.31; Calbiochem) was used as standard, with uPA activities expressed as Ploug units/mg of cellular protein. Screening for uPA production after agonist stimulation (usually an 8-h treatment) was performed using an agar spot test as follows. 5 μl of medium from treated cells was spotted onto 10-cm diameter agar plates (8.5 mg/ml agarose, 13.6 mg/ml casein, and 0.014 mg/ml plasminogen) (22). The plate was incubated at 37 °C, and lytic zones of proteolysis could be observed after ~2 h.

Protein was estimated using the dye binding assay of Bradford (30) with BSA (fatty acid-free) as the standard.

Fluorescence Measurements

Cells to be used for fluorescence measurements were grown on coverslips for 3–4 days to ~90% confluence as previously described (31). After incubation with ligands, cells were washed with phosphate-buffered saline (NaCl/P) (containing 0.5 mg/ml BSA), incubated with FITC-streptavidin, and mounted in glycerol containing 2% propyl gallate. The methods used in measurements of fluorescence intensity and the fluorescence microphotography apparatus used have been described previously in detail (32).

Flow Cytometry

Flow cytometry was performed using a FACScan (Becton, Dickinson & Co.); the data were analyzed using the Consort 30 program. LLC-PK{subscript}1 and M18 cells were grown for 3 days to 90–95% confluence in 10-cm Petri dishes prior to suspension with EDTA (0.02% EDTA in NaCl/P) for 30 min at 37 °C), centrifugation, washing with serum-free DMEM, centrifugation, and suspension at a concentration of 1–2 × 10{superscript}5 cells/ml in serum-free DMEM containing 2 mg/ml BSA. All subsequent incubations were performed in 1-ml aliquots at 4 °C. Cells were incubated with or without 10{superscript}–10 M BI0-AVP in the absence or presence (nonspecific binding) of 2 × 10{superscript}–10 M AVP for 1 h. Cells were then pelleted, washed once with NaCl/P (containing 0.5 mg/ml BSA), pelleted, and resuspended in DMEM/BSA. Incubation with 2.5 μg/ml FITC-streptavidin was then performed for 1–2 h prior to direct flow cytometry, where 5000 cells were routinely analyzed.

RESULTS

Synthesis of Biotinyl Analogues of Vasopressin—We have previously found that substitution of the glutamine at position 4 of [1-deamino Arg{superscript}1]vasopressin with 2,4-diaminobutyric acid and acylation of the resultant γ-amino group of Dab{superscript}1 by
arylazido compounds gives rise to photoreactive analogues with high affinity for renal V₂ receptors (5) and hepatic V₁ receptors (6) and with substantial in vivo antidiuretic and vasopressor activity (16). We therefore chose this position to introduce a biotinyl residue into the molecule and used two spacers of different length (Fig. 1), α-aminoehexanoic acid (Fig. 1, peptide 3, BIO-AVP) and 2-aminoethyl-1,3-dithiopionic acid (Fig. 1, peptide 4, BIO-SS-AVP), which are cleavable by thiols. To study more closely the effect of steric hindrance on receptor affinity, we also introduced a shorter propionyl residue at the side chain of Dab¹ (Fig. 1, peptide 2).

The acylation of des-[Dab¹]AVP was performed with the corresponding N-hydroxysuccinimide esters. The resulting analogues were purified by gel chromatography and shown to be homogeneous by HPLC. Their structures were confirmed by amino acid analysis and FAB mass spectrometry.

**Binding Affinities of Biotinyl Analogues of Vasopressin for Membrane-bound V₂ and V₁ Receptors**

The binding affinities of all the analogues shown in Fig. 1 were measured by incubating plasma membranes from bovine kidney (containing V₁-receptors) and from rat liver (containing V₂-receptors) with 10⁻⁸ M [³H]AVP and various concentrations of nonradioactive ligands. The displacement curves of the biotinyl analogues obtained with both kidney and liver membranes are shown in Fig. 2 (A and B, respectively), and the apparent dissociation constants (K₀) for all the analogues are summarized in Table I. The parent peptide, des-[Dab¹]AVP (peptide 1), showed reduced binding affinities for V₂ receptors and more markedly for V₁ receptors as compared to the natural hormone AVP. Acylation of the side chain amino group of Dab¹, thereby removing the positive charge at position 4, increased the affinity of the resulting analogues in a manner dependent on the length and flexibility of the substituents. des-[Dab-propionyl]AVP had K₀ values for both receptor subtypes which were only two times higher than those for AVP, and the K₀ values for des-[Dab-(biontylimidohexanoyl)]AVP (BIO-SS-AVP) were only 3–4-fold higher than those for AVP. BIO-SS-AVP showed 20–25-fold higher K₀ values than AVP.

**Binding Properties of Biotinylated Vasopressin Analogues in LLC-PK₁ Cells**

The binding affinities of BIO-AVP and BIO-SS-AVP for the V₂ receptor in LLC-PK₁ cells were determined by incubating cells with 10⁻⁸ M [³H]AVP and various concentrations of nonradioactive ligands (Fig. 3). The estimated K₀ values for BIO-AVP and BIO-SS-AVP were 2.4 × 10⁻⁸ and 7.1 × 10⁻⁸ M, respectively, about 10–30 times that for AVP (2.2 × 10⁻⁸ M). A slight difference is evident in the K₀ values for BIO-AVP between the V₂ receptor in LLC-PK₁ cells (Table I) and in bovine kidney membranes (Table I). The significance of this 2-5-fold difference is unclear, but is probably the result of species differences, which do not affect the affinities for AVP or BIO-SS-AVP.

**Biological Activities of Biotinylated Vasopressin Analogues**

Agonistic stimulation of adenylate cyclase activity leads to elevation of intracellular cAMP levels and activation of the cAMP-dependent protein kinase (33, 34). The biotin-labeled vasopressin derivatives were compared to AVP for their ability to promote cAMP-dependent protein kinase activation in LLC-PK₁ cells. Cell monolayers were treated with various ligand concentrations in the presence or absence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) (Fig. 4A). In the presence of IBMX, BIO-AVP induced half-maximal response at 6.6 × 10⁻⁸ M. BIO-AVP and BIO-SS-AVP induced comparable responses at higher ligand concentrations (half-maximal response at 1.1 × 10⁻⁸ and 3.7 × 10⁻⁸ M, respectively), consistent with the reduced binding affinities for receptor compared to AVP (see above). As a negative control, we also tested the ligands on the LLC-PK₁ V₂ receptor-deficient mutant M18, which exhibits severely reduced maximal [³H]AVP binding activity (less than 8% wild type) (22, 23, 31) compared to LLC-PK₁ cells. In identical fashion to AVP, which fails to promote cAMP-dependent protein kinase activation in M18 cells (activity ratio of 0.10 after a 30-min treatment with 10⁻⁷ M AVP and 900 μM IBMX), BIO-AVP and BIO-SS-AVP failed to activate the cAMP-dependent protein kinase in M18 cells (ratios of 0.09

![FIG. 2. Competition of [³H]AVP binding and biotinyl analogues to membranes from bovine kidney (A) and rat liver (B). Plasma membranes were incubated at 30 °C for 30 min with 10⁻⁸ M [³H]AVP and varying concentrations of BIO-AVP (●) and BIO-SS-AVP (○). Values of specific binding of [³H]AVP measured in the absence of nonradioactive ligands (Fig. 3) were used to estimate a dissociation constant (K₀) for all the analogues.](image-url)
Biotinylated Vasopressins as Receptor Probes

**TABLE II**

Summary of binding and biological properties of AVP and biotinyl analogues in LLC-PK1 cells

<table>
<thead>
<tr>
<th>Concentration of half-maximal activity</th>
<th>AVP</th>
<th>BIO-AVP</th>
<th>BIO-SS-AVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding (K_{d})</td>
<td>2.23 ± 0.45 × 10^{-9} (3)</td>
<td>2.35 ± 0.23 × 10^{-8} (4)</td>
<td>7.12 ± 3.81 × 10^{-8} (4)</td>
</tr>
<tr>
<td>cAMP-PK (K_{a})</td>
<td>6.20 × 10^{-8}</td>
<td>1.10 × 10^{-8}</td>
<td>3.67 × 10^{-8}</td>
</tr>
<tr>
<td>uPA production (K_{a})</td>
<td>2.05 × 10^{-9}</td>
<td>5.62 × 10^{-9}</td>
<td>1.53 × 10^{-9}</td>
</tr>
</tbody>
</table>

* cAMP-PK, cAMP-dependent protein kinase.

**FIG. 3.** Competition of [3H]AVP binding to LLC-PK1 cells by AVP (○) and biotin-labeled vasopressin analogues BIO-AVP (▲) and BIO-SS-AVP (●). EDTA-suspended LLC-PK1 cells were incubated with 10^{-6} M [3H]AVP and the indicated concentrations of competing ligand for 30 min. Cells were then filtered and washed, and filters were counted as described (22). Results are from a single typical experiment, with standard errors of the mean (n = 3) indicated.

**FIG. 4.** Biological activity of biotinylated vasopressin analogues. A, concentration dependence of activation of cAMP-dependent protein kinase in LLC-PK1 cells by AVP (○), BIO-AVP (▲), and BIO-SS-AVP (●). Monolayers were incubated with the indicated ligand concentrations for 30 min in the absence or presence of 500 μM IBMX and then washed, and cell extracts were prepared and assayed as described under "Experimental Procedures." The cAMP-dependent protein kinase activity ratio represents the cAMP-dependent protein kinase activity measured in the absence of exogenously added cAMP (agonist-induced cAMP-dependent protein kinase activation) relative to that in the presence of 10 μM cAMP (total activatable cAMP-dependent protein kinase) for duplicate determinations from a single typical experiment. B, concentration dependence of induction of uPA production in LLC-PK1 cells. Symbols are as described for A. Monolayers (95-100% confluent) were washed four times with serum-free DMEM and incubated with various concentrations of ligand. Medium was collected after 8 h, and uPA activity was determined as described under "Experimental Procedures." Results represent the average of at least three separate determinations, performed in triplicate, where the standard error of the mean was less than 12% of the value of the mean. Maximal response has been attained in the case of BIO-AVP and BIO-SS-AVP at 10^{-7} M.

**FIG. 5.** uPA production induced by BIO-AVP. Monolayers of cells from the LLC-PK1 and M18 (●) cell lines were treated as described for Fig. 4B with 10^{-4} (8), 10^{-3} (9), 10^{-2} (10), 10^{-1} (11), or 10^{-2} (12) M AVP (○) or BIO-AVP (▲). 0 represents untreated cells. Medium was collected after 8 h, and uPA activity in medium results in a zone of proteolytic clearing in the agar. Results (not shown) for BIO-SS-AVP were identical to those for BIO-AVP.

**TABLE III**

CAMP-dependent protein kinase activation in cell-free extracts from the LLC-PK1 cell line in response to biotinylated AVP analogues in the presence or absence of streptavidin

| Treatment | cAMP-PK activity ratio
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−IBMX +IBMX</td>
</tr>
<tr>
<td>No addition</td>
<td>0.02 0.08 0.03 ND*</td>
</tr>
<tr>
<td>No addition/streptavidin</td>
<td>0.09 ND*</td>
</tr>
<tr>
<td>AVP</td>
<td>0.09 0.70</td>
</tr>
<tr>
<td>AVP/streptavidin</td>
<td>0.09 0.73</td>
</tr>
<tr>
<td>BIO-AVP</td>
<td>0.07 0.59</td>
</tr>
<tr>
<td>BIO-AVP/streptavidin</td>
<td>0.09 0.69</td>
</tr>
<tr>
<td>BIO-SS-AVP</td>
<td>0.06 0.50</td>
</tr>
<tr>
<td>BIO-SS-AVP/streptavidin</td>
<td>0.06 0.42</td>
</tr>
</tbody>
</table>

* As described in the legend to Fig. 4A (cAMP-PK, cAMP-dependent protein kinase).

**and 0.08, respectively, after a 30-min treatment with 10^{-6} M ligand and 500 μM IBMX.**

In addition to activation of adenylate cyclase and cAMP-dependent protein kinase, AVP induces production of the extracellularly secreted protease urokinase-type plasminogen activator (uPA) in LLC-PK1 cells (35). LLC-PK1 and M18 cells were treated for 8 h with various concentrations of ligand, and uPA activity was detected in medium using either an agar spot test (Fig. 5) or a quantitative coupled assay with the chromogenic substrate S-2251 (Fig. 4B). Results were similar to those for cAMP-dependent protein kinase activation in that both BIO-AVP and BIO-SS-AVP induced uPA production, with concentration dependence consistent with receptor binding affinities. Whereas AVP promoted half-maximal response at 2.1 × 10^{-10} M, BIO-AVP and BIO-SS-AVP induced half-maximal activity at 5.6 × 10^{-8} and 1.3 × 10^{-8} M, respectively (Fig. 4B). In identical fashion to AVP, which induces
TABLE IV

Specific binding of the biotinylated vasopressin analogue BIO-AVP to the vasopressin V₂-receptor in LLC-PK₁ cells as determined by single-cell fluorescence measurements

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁶ M BIO-AVP (20 min)/FITC-streptavidin</td>
<td>4516 ± 614 (12)</td>
</tr>
<tr>
<td>10⁻⁶ M BIO-AVP + 10⁻⁶ M AVP (20 min)/FITC-streptavidin</td>
<td>2969 ± 345 (17)</td>
</tr>
<tr>
<td>10⁻⁶ M BIO-AVP (17 h)/FITC-streptavidin</td>
<td>2349 ± 308 (8)</td>
</tr>
<tr>
<td>Autofluorescence</td>
<td>1398 ± 248 (8)</td>
</tr>
</tbody>
</table>

*Total fluorescence.

Non-specific fluorescence. Non-specific cell-associated fluorescence resulting from the binding of FITC-streptavidin in the absence of BIO-AVP (not determined in this experiment) accounted for 85-95% of the non-specific cell-associated fluorescence remaining after the subtraction of autofluorescence.

Fig. 6. Visualization of V₂-receptors in LLC-PK₁ cells. Cells were grown on coverslips for 3 days to 90% confluence and then treated with 10⁻⁶ M BIO-AVP for 20 min at 37°C, prior to washing at 4°C and fixation with acetone (for 2 min at room temperature). After overnight blocking with NaCl/P₄ containing 0.5 mg/ml BSA, cells were incubated for 3 h at room temperature with 2 μg/ml FITC-streptavidin prior to washing at 4°C and mounting in glycerol containing 2% propyl gallate. Measurements were then performed as described under “Experimental Procedures.” Results represent means ± S.E. (n in parentheses).

FIG. 6. Visualization of V₂-receptors in LLC-PK₁ cells. Cells were grown on coverslips for 3 days to 90% confluence and then treated with 10⁻⁶ M BIO-AVP for 20 min at 37°C, prior to washing at 4°C and fixation with acetone (for 2 min at room temperature). After overnight blocking with NaCl/P₄ containing 0.5 mg/ml BSA, cells were incubated for 3 h at room temperature with 2 μg/ml FITC-streptavidin prior to washing at 4°C and mounting in glycerol containing 2% propyl gallate. Cells were photographed under normal (A) and fluorescent (B) illumination using a 100 × oil immersion objective.

Fig. 7. Fluorescence profile of living LLC-PK₁ and M₁₈ (V₂-receptor-deficient) cell lines stained with BIO-AVP and FITC-streptavidin. EDTA-suspended cells were incubated with 10⁻⁶ M BIO-AVP (for 1 h at 4°C), centrifuged, washed, and then incubated with 2 μg/ml FITC-streptavidin (for 2 h at 4°C) prior to analysis as described under “Experimental Procedures” using a FACScan flow cytometer. A.U., arbitrary units.

no uPA production in M₁₈ cells, 10⁻⁸ M BIO-AVP (see Fig. 5) or BIO-SS-AVP (data not shown) induced no detectable uPA production. A summary of the properties of binding and biological activity in LLC-PK₁ cells is presented in Table II.

The interaction of biotin-labeled vasopressin analogues with the V₂-receptor in LLC-PK₁ cells in the presence of streptavidin was investigated. The activation of cAMP-dependent protein kinase was measured in cells incubated with 9 × 10⁻⁸ M BIO-AVP or BIO-SS-AVP in the presence or absence of 2 μg/ml streptavidin (Table III). The presence of streptavidin did not markedly affect cAMP-dependent protein kinase activation; in the case of BIO-AVP, streptavidin appeared to elevate the cAMP-dependent protein kinase activity ratio (0.69 compared with 0.59 in streptavidin’s absence, in the presence of IBMX), whereas streptavidin slightly reduced the cAMP-dependent protein kinase activity ratio (0.42 compared with 0.50 in streptavidin’s absence, in the presence of IBMX) in the case of BIO-SS-AVP. It can be concluded that formation of the streptavidin–biotin complex did not significantly alter the agonistic properties of the biotinylated AVP analogues bound to the V₂-receptor. The effect of avidin on the binding of BIO-AVP to the hepatic V₂-receptor was also examined (data not shown). Avidin at concentrations as high as 4 × 10⁻⁴ M did not prevent the interaction of the biotinyl analogue with the membrane-bound V₂-receptor. In the presence of avidin, the apparent dissociation constant for BIO-AVP was increased only about 2-fold.

BIO-AVP and FITC-Streptavidin as Fluorescent Probe for V₂-receptor in LLC-PK₁ Cells—Subsequent to incubation with a fluorescent ligand, total cell-associated fluorescence is the sum of specific fluorescence (due to receptor-bound ligand), non-specific fluorescence (due to non-receptor-bound ligand), and autofluorescence. To discriminate among these components, LLC-PK₁ cells were treated with 10⁻⁸ M BIO-AVP (for 20 min at 37°C) in the absence or presence of 10⁻⁶ M AVP followed by FITC-streptavidin (2 μg/ml) (for 40 min at 4°C). Cells were then mounted, and single-cell fluorescence measurements were performed on the basal plasma membrane, as previously described (31). Autofluorescence, determined by measuring cell-associated fluorescence in the absence of FITC-streptavidin, accounted for 43% of total fluorescence, whereas specific and non-specific fluorescence accounted for 34 and 23%, respectively (Table IV). After the subtraction of autofluorescence, specific binding accounted for 60% of the remaining fluorescence. Non-specific (non-AVP-competible) fluorescence was largely due to binding of FITC-streptavidin in the absence of BIO-AVP (~90% of the residual non-specific fluorescence, not routinely measured). Cells incubated with 10⁻⁸ M BIO-AVP for 17 h at 37°C prior to treatment with FITC-streptavidin possessed only 16% of the fluorescence of those incubated for 20 min (autofluorescence subtracted) (Table IV), consistent with internalization and down-regulation of the V₂-receptors. The V₂-receptor in LLC-PK₁ cells was visualized by fluorescence microscopy (Fig. 6). Fluorescence was largely concentrated in aggregates, a result similar to that previously obtained with a rhodamine-labeled vasopressin analogue (31).

The BIO-AVP/FITC-streptavidin system was further characterized by using the technique of flow cytometry. EDTA-suspended cells from the LLC-PK₁ and M₁₈ cell lines were incubated with 10⁻⁶ M BIO-AVP (for 1 h at 4°C), washed, and then incubated subsequently with FITC-streptavidin (2 μg/ml) for a further 2 h at 4°C prior to analysis (Fig. 7). The peaks for LLC-PK₁ and M₁₈ (negative control) cells overlap, but indicate a clear difference between the two cell types. Incubation with FITC-streptavidin alone, with BIO-AVP...
alone, or BIO-AVP in the presence of $2 \times 10^{-6} \text{M AVP (data not shown)}$ resulted in a profile resembling that of M18 cells in Fig. 7.

**Discussion**

The results of this study demonstrate that a biotinyl residue can be attached via a spacer arm at Dab in vasopressin without substantial loss of V$_1$- and V$_2$-receptor binding affinity or biological activity. Acylation of the side chain of Dab with a biotinyl carboxyl group and the y-amino group in Dab. BIO-SS-AVP by the (biotinylamido)hexanoyl residue yields an analogue (BIO-AVP) with 7 atoms between the biotinyl carboxyl group and the y-amino group in Dab. BIO-AVP shows a 3-4-fold lower binding affinity for membrane-bound V$_1$, and V$_2$-receptors as compared to AVP, but a higher affinity as compared to the parent peptide des-[Dab]$^4$AVP. The analogue BIO-SS-AVP contains a spacer with a disulfide bridge and 8 atoms between the biotinyl carboxyl group and the side chain of Dab. We find, for this analogue, 20-30-fold reduced affinities for membrane-bound V$_1$- and V$_2$-receptors and for the V$_2$-receptor in LLC-PK$_1$ cells. Both biotinylated vasopressin analogues showed biological activities comparable to those of AVP in LLC-PK$_1$ cell monolayers. They were full agonists in stimulating V$_2$-receptor-dependent, cAMP-dependent protein kinase activity and in stimulating the production of urokinase-type plasminogen activator. The concentration dependence of their biological activity in I.F.C-PK$_1$, cells was consistent with their binding affinity. It has also recently been shown (36) that analogues of [1-deamino-Lys$_4$, Hyp$_7$]AVP with affinity and fluorescence groups at Lys$_7$ retain in vivo antidiuretic activity. No conclusions can be drawn concerning the effect of these modifications on receptor binding affinities, however, since no binding constants were determined for these analogues.

In considering the results presented here, it should be remembered that spacer arms are required for optimizing interaction among avidin, biotinyl ligand, and the receptor. For example, the hydroosmotic activity of a biotinyl analogue (31). The biotinylated vasopressin analogue should therefore be useful for histological analysis of vasopressin receptor expression and for the detailed subcellular localization of vasopressin receptors by electron microscopy.

In this study, the biotin/avidin system was used for the first time to investigate the expression of vasopressin receptors in cultured cells by flow cytometry. The fluorescence profiles of LLC-PK$_1$ cells (~40,000 V$_2$ receptors/cell) and a receptor-deficient mutant (possessing less than 3,000 receptors/cell) (22) showed a clear difference between the two cell types. The use of flow cytometry to measure vasopressin receptor expression in living cultured cells has a number of possible applications. The biotinylated vasopressin analogue could be used either in combination with DNA-staining techniques to study receptor expression during the cell cycle or to enrich and separate subpopulations of cells expressing different numbers of receptors using cell sorting to enable their subsequent analysis in detail.

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