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Research Article

A molecular recognition hypothesis for nonpeptides: Na^+K^+ATPase and endogenous digitalis-like peptides

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Abstract. The molecular recognition hypothesis for peptides is that binding sites of ligands and their receptors are encoded by short, complementary segments of DNA. A corollary hypothesis for nonpeptide ligands posited here is that peptide replicas may be encoded by the DNA segment complementary to the receptor binding sites for nonpeptides. This corollary was tested for digitalis, a family of cardiotonic and natriuretic steroids including ouabain. A hexapeptide (ouabain-like peptide, OLP) complementary to a ouabain binding site on sodium/potassium dependent adenosine triphosphatase (Na^+K^+ATPase) exhibited activity in a digitalis bioassay. Antisera to the complementary peptide (OLP) stained the neurohypophysis in an immunocytochemical procedure. The complementary peptide was found to share an identical 4-amino acid region with the 39-amino acid glycopeptide moiety of the vasopressin-neurophysin precursor. This glycopeptide was isolated from pituitary extracts; it exhibited digitalis-like activity in the submicromolar range and cross-reacted with complementary peptide antibodies. Another digitalis-like substance with high activity also was detected in the extracts. These results demonstrate that the vasopressin-neurophysin glycopeptide has digitalis-like activity. Moreover, the findings are consistent with the hypothesis that peptide mimetics of nonpeptides are encoded in the genome.

Key words. Molecular recognition; ouabain; Na^+K^+ATPase; vasopressin-neurophysin; glycopeptide 1-39; complementary peptide; ouabain-like peptide.

The molecular recognition hypothesis for peptides is that binding sites of ligands and their receptors are encoded by short, complementary segments of DNA [1, 2]. Thus, two peptides represented by complementary RNAs were hypothesized to assume mirror-image conformations which resulted in binding of the pair [2]. As predicted by the hypothesis, a complementary peptide to adrenocorticotropic hormone (ACTH) displayed the affinity and specificity characteristics of an ACTH receptor [3]. Antibodies prepared against complementary peptides to ACTH [3] and luteinizing hormone releasing hormone (LHRH) [4] also were shown to recognize the binding sites of their cell receptors. Antibodies to ACTH and to its complementary peptide bound each other, suggesting that the molecular recognition hypothesis is applicable also to the idiotype-antiidiotype immune network [5]. In all, approximately 40 different complementary peptide pairs have been shown to bind with specificity and moderate affinity [2, 6]. However, it should be noted that theoretical as well as

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experimental objections [7] have been raised to the complementary peptide hypothesis.

We posit here a corollary hypothesis for nonpeptide ligands such as steroid hormones, catecholamine neurotransmitters, immune haptens and drugs that are not encoded in the genome, namely that they may have peptide mimetics encoded on the DNA segment complementary to that encoding their receptor binding sites, and in the case of nonpeptide drugs such mimetics may be expressed endogenously. The digitalis family of cardiotonic steroids that inhibit the ubiquitous cellular enzyme, sodium/potassium-dependent adenosine triphosphatase (Na^+K^+ATPase) [8], was chosen to test this corollary. The existence of endogenous substances in the hypothalamus having actions similar to digitalis (ouabain is a potent member of the digitalis family) is supported by a plethora of evidence [9]. The proposed function of this endogenous digitalis-like substance is to stimulate renal sodium excretion (natriuresis) by inhibiting Na^+K^+ATPase [9], and elevation of its secretion is thought to contribute to the development of human hypertension [10]. The recent report that a ouabain binding site was located in the six-amino acid sequence comprising the second extracellular loop of Na^+K^+ATPase [11] presented with us an opportunity to determine whether a delimited complementary peptide to a ouabain-binding site would bear resemblance to an endogenous digitalis-like substance and, if so, whether antibodies to it would lead to the identification of such a substance.

Materials and methods

ATP hydrolysis assay. The assay for detection of ouabain-like activity was based on inhibition of ATP hydrolysis catalyzed by Na^+K^+ATPase [12]. ATP hydrolysis was measured in a linked-enzyme assay whereby nicotinamide adenine dinucleotide (NADH) is consumed during regeneration of ATP from adenosine diphosphate (ADP) [12]. The assay was performed at 37 °C in 25 mM Tris HCl (pH 7.4) containing 2.5 mM Tris ATP, 2.5 mM phosphoenolpyruate, 0.25 mM NADH, 5mM $MgCl_2$, 100 mM NaCl and 10 mM KCl. The reaction solution also contained 2.7 μg/ml purified dog kidney Na^+K^+ATPase (Sigma, Grade IV; 1.0 unit/mg protein), 12.5 units/ml lactic dehydrogenase and 8.75 units/ml pyruvate kinase. The peptides and cardiotonic steroids were dissolved in Tris HCl and added to the mixture immediately before the reaction was initiated by addition of Tris ATP. When immunoglobulins were assayed, they were preincubated with the Na^+K^+ATPase but without ATP for 1 h at 37 °C before initiating the hydrolysis reaction with ATP. The optical density at 340 nm (representing NADH) was recorded in a spectrophotometer at appropriate intervals.

Preparation of peptides and antibodies. Peptides were synthesized using standard solid phase methods, and purified to $>95\%$ homogeneity by high pressure liquid chromatography at the Protein Analysis Core Facility of the Cancer Center, University of Alabama at Birmingham. Antisera were prepared in three rabbits to each peptide coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde (3.0 mg of peptide in 3.0 ml of phosphate buffered saline, pH 7.0, was mixed with 1.2 mg of keyhole limpet hemocyanin and reacted with 1.5 ml of 20 mM glutaraldehyde for 120 min). After dialysis of the mixture, we injected 400-μg peptide equivalents of the conjugate emulsified in complete Freund's adjuvant at multiple subcutaneous sites over the back of each rabbit. Booster injections of 200-μg peptide equivalents per rabbit in incomplete Freund's adjuvant were made at three 10-day intervals thereafter. The first blood sample was taken at 20 days after the initial immunization and at 3–4 day intervals thereafter on eight occasions. Immunoglobulins were isolated from the antisera by ammonium sulfate precipitation, dialysis and DEAE cellulose chromatography. Antibodies to KLH were then removed by chromatographing the immunoglobulin G (IgG) fraction on a column containing sepharose-coupled KLH. The resulting antibodies were dialyzed against phosphate buffered saline, and concentrated by hydro-extraction against polyethylene glycol. The protein concentrations of these antibody preparations were measured using the Bradford dye test.

Immunocytochemistry. Porcine pituitary gland and hypothalamus were cut into small pieces and immersed in Bouin's fixative for 24 h, embedded in Epon-Araldite 812, cut into 1-μm sections, the plastic removed with 50% NaOH in ethanol, the sections rehydrated and subjected to immunohistochemical staining with three different ouabain-like peptide (OLP; see fig. 1) antisera using an avidin-biotin-peroxidase immunohistochemical procedure (Vectastain ABC kit; Vector Labs) as described [13]. The antisera were incubated with the sections for 48 h at 4 °C at 1:500–1:1000 dilutions. All three antisera gave similar results. The antisera (diluted to 1:500-1:1000) were preincubated with complementary peptide (OLP) (50–100 μg/ml) for 5 days at 4 °C to block staining.

Enzyme-linked immunosorbent assay (ELISA). Five micrograms of peptide dissolved in 0.2 ml of pH 9.0 carbonate buffer were added to duplicate wells of an Immulon II, 96-well flat-bottomed micro-ELISA plate (polystyrene; Dynatech) and incubated overnight at 4 °C. The wells were then washed in pH 7.4 buffer (0.1 M sodium phosphate, 0.25 M NaCl) at room temperature and then blocked by a 2-h incubation with 1 gm/100 ml bovine serum albumin in the same buffer.

After washing, a 1:500 dilution of the complementary peptide antiserum (OLP) was added to the wells and incubated for 3 h, followed by a wash, and a 1-h incubation with alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (Sigma). Color was developed with p-nitrophenyl phosphate disodium (1 mg/ml in glycine buffer, pH 10.4) for 2 h, and the wells were scanned for absorbances at 405 nm in an ELISA reader.

^{3}H-ouabain/Na^+K^+ATPase radioreceptor assay. This assay [14] was performed in 0.5 ml of 50 mM Tris HCL (pH 7.4) containing 0.5 mM EDTA, 4 mM $MgS0_4$, 80 mM NaCl, 5mM ATP, 7.5 μg of dog kidney Na^+K^+ATPase (Sigma), 7.8 nC_i of [21, 22-^{3}H]ouabain (specific activity = 15.6 C_i/mmol; Amersham) and 0.1% gelatin. Test samples were incubated for 2 h at 37 °C; ATP and ^{3}H-ouabain were absent during the first hour but present during the second hour. ^{3}H-ouabain bound to Na^+K^+ATPase was recovered on Whatman GF/F glass microfiber filters using vacuum filtration. The filters had been soaked in 0.3% aqueous polyethylenimine for 2 h immediately before filtration. The filters were added to scintillation fluid in vials and counted in a liquid scintillation spectrometer.

Fractionation of posterior pituitary extracts. Vasopressin-neurophysin glycopeptide 1-39 was prepared by extraction of fresh-frozen pig posterior pituitary glands (Pel-Freeze, Rogers, Arkansas, USA) into 0.1 N HCl, and fractionation on Sephadex G-75 in 0.1 M formic acid [15]. Fractions eluting in the appropriate molecular weight range and showing immunoreactivity in an ELISA for OLP were pooled and dialyzed against 0.02 M ammonium acetate (pH 4.6) [16]. This solution was subjected to ion-exchange chromatography (CM Sephadex C-25) [16], and the unretained fraction was absorbed to a concanavalin A affinity column [17]. Glycopeptides were desorbed with 0.05 M α-methyl-D-mannoside in 0.01 M ammonium acetate containing 0.15 M NaCl (pH 5.7) [17] and pumped onto a C_{18} reversed-phase high-pressure liquid chromatography column (rpHPLC; Rainin Dynamax; semipreparative) where the components were resolved in 0.1% trifluoroacetic acid (TFA)/0–60% acetonitrile. The tubes containing the highest immunoreactivity were pooled, diluted to 2% acetonitrile with 0.1% TFA and subjected again to HPLC. To determine immunoreactivity of the samples, 10 μl were evaporated in the wells of an ELISA plate, and the OLP-ELISA was performed. Amino acid analysis of the sample was done by rpHPLC of phenylthiohydantin (PTH) derivatives using a Waters Amino Acid Analyzer; the composition expected for glycopeptide 1-39 was found. The amino acid sequence determined for the first 20 residues using a gas-phase sequenator also was that

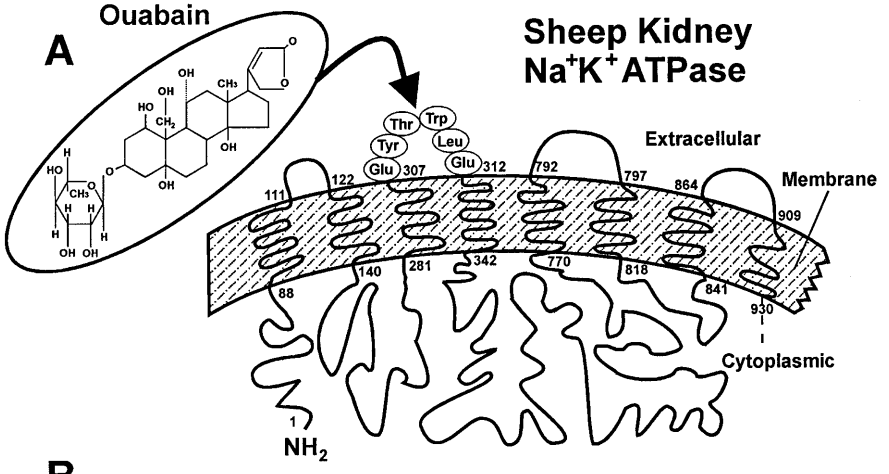

Figure 1. (*A*) A partial model of the transmembrane topology of the sheep α1 subunit of Na^+K^+ATPase. A ouabain-binding site has been reported to reside in the second extracellular loop (amino acid residues 307–312). (*B*) Amino acid sequence of a complementary peptide (OLP) encoded by an RNA that is complementary to the messenger RNA (mRNA) representing a putative ouabain binding site on Na^+K^+ATPase.

expected except for the asparagine at position 6 which was equivocal, as expected, since it is glycosylated.

A partially purified, high-affinity endogenous digitalis-like substance (eOLP) was purified from porcine posterior pituitary glands by extraction and chromatography on Sephadex G-75 as described above. The appropriate immunoreactive fractions were chromatographed by reversed-phase C_{18} HPLC using a linear gradient of acetonitrile (0–60%) in 0.1% TFA. Digitalis-like activity in the eluates was determined with ^{3}H-ouabain radioreceptor assay. The main peak of activity eluting at 30–34% acetonitrile was pooled for further study. A smaller, second immunoreactive peak eluting at 38% acetonitrile represented glycopeptide 1-39, and was excluded from the pool.

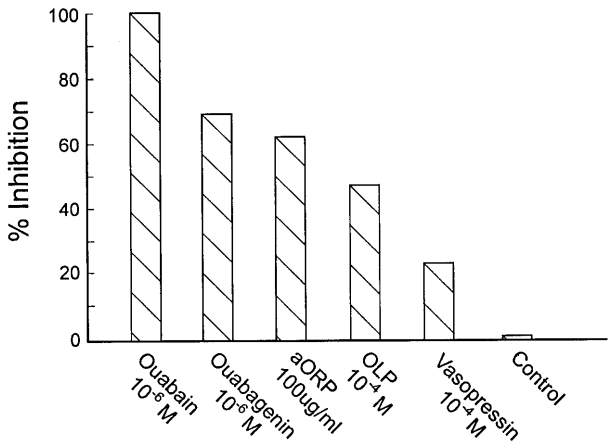

Figure 2. Digitalis-like activity of the complementary peptide (OLP) and of an antibody to a putative ouabain-binding site (aORP-IgG purified as described in 'Materials and methods' and used at a protein concentration of 100 μg/ml) to inhibit ATP hydrolysis catalyzed by Na^+K^+ATPase. ATP hydrolysis was measured in a linked-enzyme assay whereby NADH is consumed during regeneration of ATP from ADP. The control group was assigned 0% inhibition, whereas ouabain was assigned 100% inhibition. Results presented are means of triplicate determinations from a single experiment representative of $n \geq 3$ independent experiments; the rank order of activities shown was identical in all experiments.

Results

Construction and activity of a complementary peptide to a ouabain binding site. A partial model of the transmembrane topology of the sheep α1 subunit of Na^+K^+ATPase is shown as figure 1A (see Palasis et al. [18]. Although several regions of Na^+K^+ATPase have been suggested to interact with ouabain [11, 18], we concentrated on the second extracellular loop comprised of six amino acids implicated by Canessa et al. [11] to bind ouabain. By comparison with sequences in protein databases, we identified the complementary peptide (OLP) (fig. 1B) as bearing a four-amino acid region of identity with the glycopeptide moiety of the vasopressin-neurophysin precursor [19]. A previous study has suggested a possible neurohypophysial source for an endogenous digitalis-like substance [20].

To test the hypothesis that the extracellular loop comprising amino acid residues 307–312 contains a ouabain binding site, we determined whether antibodies directed toward that sequence would antagonize ^{3}H-ouabain binding to Na^+K^+ATPase, and whether they were active in an adenosine triphosphate (ATP)-hydrolysis assay. The hexapeptide (Glu-Tyr-Thr-Trp-Leu-Glu; fig. 1) was synthesized, coupled to keyhole-limpet hemocyanin and used to immunize rabbits. Immunoglobulins (abbreviated aORP-IgG) were isolated from the antisera as described in 'Materials and methods' and tested for activity in a spectrophotometric assay measuring ATP hydrolysis (fig. 2). At a protein concentration of 100 μg/ml, the aORP-IgG significantly inhibited ATP hydrolysis, whereas the same amount of similarly prepared IgG from normal rabbit serum (NRS-IgG) had no effect (fig. 2). In a ^{3}H-ouabain radioreceptor assay using purified dog kidney Na^+K^+ATPase, the aORP-IgG (1 mg/ml protein) produced an approximate 55% inhibition of ^{3}H-ouabain binding, again with the same amount of NRS-IgG protein having no effect (data not shown). These findings corroborate the assignment of amino acid residues 307–312 of Na^+K^+ATPase as containing a ouabain-binding site (fig. 1A).

The presumed digitalis-like peptide (OLP: Leu-Gln-Pro-Gly-Val-Leu) complementary to a putative ouabain binding region on Na^+K^+ATPase (fig. 1A) was derived as shown in figure 1B and synthesized. This hexapeptide (OLP) was tested for digitalis-like activity in the ATP hydrolysis assay (fig. 2) for comparison with ouabain and ouabagenin (ouabagenin is identical to ouabain except that it lacks the carbohydrate moiety) (fig. 1A). OLP exhibited activity in the assay at a concentration of 10^{-4} M, whereas eight control peptides were inert at the same or lower doses (oxytocin; angiotensin II; synthetic fragments of the complementary peptide: OLP 2-5 and OLP 2-6; ORP, the putative ouabain binding site hexmer from Na^+K^+ATPase; a synthetic complementary hexapeptide, Leu-Met-Trp-Thr-Asn-Leu, derived from reading the complementary RNA in the 3′ to 5′ direction; and two synthetic unrelated peptides, Leu-Leu-Glu-Arg-Leu, and Tyr-Glu-Pro-Lys-Lys-Tyr-Tyr-Gly-Phe-Gly-Ala). Tris HCL buffer and NRS-IgG (100 μg/ml protein) also were inactive. Although the digitalis-like activity of the complementary peptide (OLP) is low, it

is significantly higher than that exhibited by vasopressin (fig. 2), an acknowledged weak natriuretic agent [21]. Specificity of the complementary peptide effect to inhibit ATP hydrolysis by Na^+K^+ATPase is supported by its failure to affect ATP hydrolysis by actomyosin Ca^{++}ATPase (data not presented). These findings suggest that a complementary peptide (OLP) derived from a ouabain binding site resembles digitalis in its ability to inhibit ATP hydrolysis catalyzed by Na^+K^+ATPase.

Complementary peptide antibodies immunohistochemically stain the neurohypophysis. A previous study has suggested a possible neurohypophysial source for endogenous digitalis-like substances [20]: hypophysectomy blocked saline-induced natriuresis, which could be restored with extracts of the posterior but not the anterior hypophysial lobe [20]. Thus, we used OLP antisera for immunohistochemical studies of the pituitary gland and hypothalamus. Pig tissues were used so that an abundant source for purification would be available if immunohistochemical localization proved successful. An avidin-biotin immunocytochemical procedure with three different OLP antisera resulted in intense staining of the posterior lobes (fig. 3); the intermediate lobes were completely devoid of stain, but small numbers of anterior lobe cells were stained (fig. 3). In the hypothalamus, magnocellular neurons in the paraventricular (fig. 3) and supraoptic nuclei (not shown) were also intensely stained. Normal rabbit serum (NRS) did not stain these structures, and preincubation of the antisera with OLP abolished all staining (fig. 3). This pattern of staining is strikingly similar with that observed for vasopressin and neurophysin II [22], the classic secretory products of the posterior pituitary gland which are synthesized by magnocellular neurons in the hypothalamus and then transported through long axons which terminate in the posterior lobe from which they are released into the circulation. These findings are consistent with a resemblance between the complementary peptide and an endogenous digitalis-like substance.

To determine if the immunoreactive product detected by the complementary peptide antiserum was identical with peptides established to reside in the neurohypophysis, we used one of the antisera in an ELISA to test for immunoreactivity of the complementary peptide (OLP). We also tested lysine vasopressin, neurophysins I and II, oxytocin, vasoactive intestinal peptide (VIP), thyrotropin releasing hormone (TRH), atrial natriuretic factor (ANF), angiotensin II and dynorphin A (all obtained from Sigma, St. Louis, MO, USA) (fig. 4). Three peptides related to the complementary peptide also were synthesized and used: OLP 3-5, OLP 2-5 and OLP 2-6 (see fig. 1B). Only OLP was immunoreactive in a direct assay where the peptides were attached to the plastic plate (5 μg/well) and reacted with the complementary peptide antiserum (1:500 dilution) (fig. 4) or in a competitive assay where the complementary peptide (5 μg) was absorbed to all wells of an ELISA plate and the peptides (doses from 10^{-7} to 10^{-5} M in one-log increments) were mixed with the antiserum and incubated overnight before being placed in the wells (data not shown). In the latter case, complete inhibition of color development by the complementary peptide occurred at 10^{-5} M, and the smallest detectable inhibition occurred at 10^{-7} M. Ouabain and ouabagenin also were not immunoreactive in either assay. These findings demonstrate that the apparent digitalis-like immunoreactive substance in the neurohypophysis is not identical with peptides of previously established function or with some members of the digitalis family.

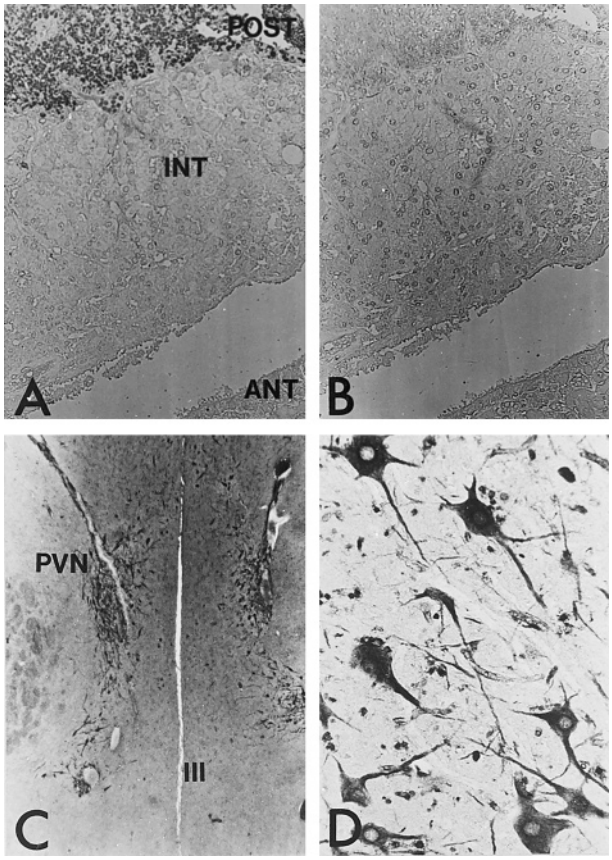

Figure 3. Immunocytochemical staining of the neurohypophysis and hypothalamus with complementary peptide (OLP) antisera. (*A*) The posterior lobe (POST) of the pituitary gland was strongly stained, but the intermediate (INT) and anterior (ANT) lobes remained unstained. (*B*) Preincubation of the OLP antisera with complementary peptide abolished staining of the posterior lobe. This panel is also representative of the results obtained with normal rabbit serum. (*C*) Staining of magnocellular, neurosecretory neurons of the hypothalamic paraventricular nucleus (III is the third ventricle). (*D*) Higher magnification view of stained magnocellular, neurosecretory neurons from the supraoptic nucleus.

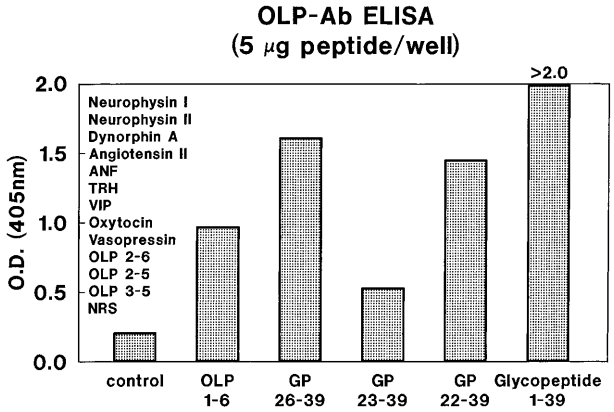

Figure 4. Immunoreactivity of neurohypophysial peptides with complementary peptide (OLP) antiserum in an ELISA. The peptides showing immunoreactivity were the immunogen (OLP), the glycopeptide 1-39 moiety of the vasopressin-neurophysin precursor (fig. 5) isolated from porcine posterior pituitary glands and three synthetic fragments derived from the carboxy terminus of glycopeptide 1-39 (GP22-39, GP23-39 and GP26-39). Nonimmunoreactive peptides (control) used were three derivatives of the complementary peptide (OLP 3-5, OLP 2-5 and OLP 2-6; fig. 1B), lysine vasopressin, neurophysins I and II, oxytocin, vasoactive intestinal peptide, thyrotropin releasing hormone, atrial natriuretic factor, angiotensin II and dynorphin A. Normal rabbit serum substituted for OLP-antiserum was a final control. The results shown are from a single experiment but are typical of $n = 3$. The rank order of the peptide immunoreactivities was the same in all three assays.

Similarity between the complementary peptide and the vasopressin-neurophysin precursor. A computerized search of protein databases was conducted to determine the possible similarity of the complementary peptide with previously sequenced proteins. The vasopressin-neurophysin precursor (bovine, porcine and ovine species) [19] was identified as bearing a region of identity with OLP and is an established secretory protein of neurohypophysial origin (fig. 5). The sequence identified was Gln-Pro-Gly-Val, the central four amino acids in the complementary peptide (fig. 5). The region of identity between the complementary peptide and the vasopressin-neurophysin precursor was located near the carboxy-terminus of the glycopeptide moiety [23] of the precursor (fig. 5). This-39 amino acid glycopeptide is posttranslationally cleaved from the precursor, and two fragments residing at its carboxy-terminus (17 and 14 amino acids) also have been isolated from porcine pituitary extracts [23] (fig. 5). No functions have been assigned to the glycopeptide and its derivatives, but immunocytochemical studies of the glycopeptide show a localization in the posterior pituitary gland and hypothalamus that is identical with vasopressin and neurophysin II [22].

To determine the functional significance of the region of identity shared by the complementary peptide and the glycopeptide moiety of the vasopressin-neurophysin precursor, we tested synthetic peptides representing the carboxy-terminal 18, 17 and 14 amino acids (abbreviated GP22-39, GP23-39 and GP26-39, respectively) of the glycopeptide for activity in the ATP-hydrolysis assay (fig. 6A). GP26-39 showed significantly higher activity than the complementary peptide (OLP), whereas GP22-39 and GP23-39 showed less activity than OLP but more activity than control peptides (fig. 6A) including those (OLP2-6 and OLP2-5) containing the four amino acid region of identity. GP26-39 also was active in a ^{3}H-ouabain radioreceptor assay, but the complementary peptide (OLP), GP23-39 and GP22-39 were not active at the same dose levels (fig. 6B). The apparent discrepancy of OLP and GP26-39 sharing the four-amino acid sequence (Gln-Pro-Gly-Val) and having digitalis-like activity, whereas the-four amino acid sequence itself (OLP 2-5) had no activity, may be explained as follows: the additional amino acids in OLP and GP26-39 constrained the tetrapeptide to a conformation that the tetrapeptide by itself could not assume, and this constrained conformation was compatible with binding to Na^+K^+ATPase.

ELISAs also were performed to determine cross-reactivity between the OLP antiserum and the synthetic peptides representing the carboxy-terminal region of the glycopeptide. GP22-39, GP23-39 and GP26-39 all showed significant immunoreactivity whereas OLP2-6, OLP2-5 and OLP3-5 did not (fig. 4). GP22-39 and GP26-39 exhibited significantly higher activity than did the complementary peptide (the immunogen), whereas GP23-39 exhibited significantly less. These differences may reflect differential absorption to the plastic.

These results demonstrate that a naturally occurring neurohypophysial peptide (GP26-39) which is a derivative of the glycopeptide moiety of vasopressin-neurophysin precursor [23] has digitalis-like activity. Although low, the activity of GP26-39 is greater than the synthetic complementary peptide (OLP) in two bioassays and is potently recognized by OLP antibody. Thus, the findings are consistent with the hypothesis that a peptide mimetic of a nonpeptide ligand may be encoded on the DNA strand complementary to the ligand binding site, and expressed endogenously. In addition, the data suggest that such expressed endogenous peptide ligands may be identified with reagents generated by application of the molecular recognition hypothesis.

Purification of endogenous digitalis-like peptides from neurohypophysial extracts. The digitalis-like properties of GP26-39 suggested that its precursor, the 39-amino acid glycopeptide (fig. 5), also might have digitalis-like

activity. Therefore, we purified glycopeptide 1-39 from porcine posterior pituitary extracts using a variation on established approaches [15–17, 23] and monitored its purification with an ELISA using the complementary peptide antiserum (fig. 7). Amino acid sequencing and composition analysis confirmed that the glycopeptide was identical with that previously reported [23] and shown here in figure 5. Glycopeptide 1-39 was intensely immunoreactive in an ELISA using the complementary peptide antiserum (fig. 4) and was active at submicromolar doses in inhibiting the hydrolysis of ATP by Na^+K^+ATPase (fig. 6A). In this respect, it was about 100-fold more active than its naturally occurring derivative, GP26-39, and about as active as ouabagenin (fig. 6A). Similar absolute and relative activities of glycopeptide 1-39 were also observed in a ^{3}H-ouabain radioreceptor assay (fig. 6B). Thus, glycopeptide 1-39 represents a second naturally occurring neurohypophysial peptide [23] with digitalis-like properties discovered with reagents generated by application of the molecular recognition hypothesis. In contrast to the other digitalis-like neurohypophysial peptide, GP26-39, it has activity near that of ouabagenin. Nevertheless, its role as a circulating form of endogenous digitalis-like substance is questionable, since activity in the nanomolar range probably is required [18].

Another digitalis-like substance was detected in neurohypophysial extracts which seemed to be more potent than glycopeptide 1-39; it has not been completely purified or identified (fig. 8). This activity was detected in pituitary extracts that had been chromatographed on Sephadex G-75 as described for the isolation of glycopeptide 1-39 and then subjected to reversed-phase C_{18} HPLC. Digitalis-like activity in the acetonitrile eluates was determined with the ^{3}H-ouabain radioreceptor assay (fig. 8), and the fractions showing high activity were pooled. Material in this pool, presumably representing an endogenous digitalis-like substance, displayed a steep dose-response curve in the ^{3}H-ouabain radioreceptor (fig. 6B). Also, it was a potent inhibitor of ATP hydrolysis catalyzed by Na^+K^+ATPase (data not shown).

To provide data that the inhibitory effects of this unidentified endogenous digitalis-like substance in the radioreceptor and ATP hydrolysis assays were not artifactual, we have shown: (i) that the binding of the unidentified substance is reversed by excess Na^+ in the ATP-hydrolysis assay, suggesting that the substance binds to a ouabain binding site on Na^+K^+ATPase [24]; (ii) that 3 mM norepinephrine does not prevent inhibition by the digitalis-like substance in the ^{3}H-ouabain radioreceptor assay, ruling out vanadate as the inhibitor [24] (data not shown); and; (iii) that K^+ and Na^+ concentrations measured by flame photometry in the pool of digitalis-like substance were far below the minimally effective concentrations of these potent inhibitory ions in the ATP-hydrolysis and radioreceptor assays (results not presented).

Discussion

Endogenous digitalis-like hormone and hypertension. Since the original report of a naturally occurring hu-

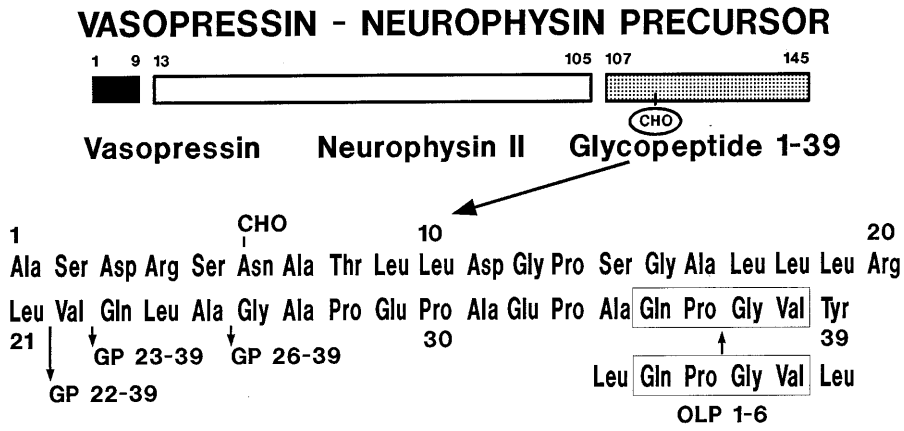

Figure 5. The glycopeptide 1-39 moiety of the vasopressin-neurophysin precursor identified as sharing a four-amino acid region of identity with the complementary peptide (OLP). The complete amino acid sequence of glycopeptide 1-39 and two of its naturally occurring carboxy-terminal fragments (GP23-39 and GP26-39) are shown [27]. The four-amino acid regions of identity shared by glycopeptide 1-39 and the complementary peptide (OLP) are boxed. The organization of the bovine vasopressin-neurophysin-glycopeptide precursor established by nucleotide sequencing of the complementary DNA (cDNA) is shown [19].

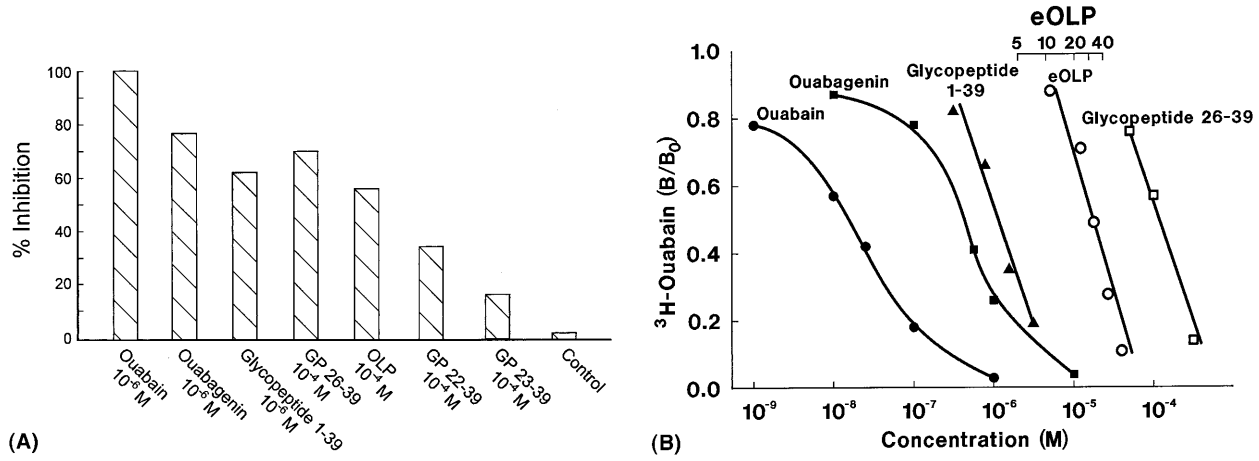

Figure 6. Digitalis-like activities of vasopressin-neurophysin precursor-derived glycopeptide 1-39 and its posttranslational products. (*A*) Inhibition of ATP hydrolysis catalyzed by Na^+K^+ATPase. The linked-enzyme, spectrophotometric assay measuring hydrolysis of ATP was performed as described in figure 2. (*B*) ^{3}H-ouabain-Na^+K^+ATPase radioreceptor assay. Peptides used in the ^{3}H-ouabain radioreceptor that showed no displacing activity at 10^{-4} M concentrations were lysine vasopressin, oxytocin, angiotensin II, OLP, OLP 3-5, OLP 2-5, OLP 2-6, GP23-39 and GP22-39. The data points are means of triplicate (ATP hydrolysis) or duplicate (radioreceptor assay) samples from experiments representative of ≥ 3 independent experiments. The rank order of activities shown was the same in all experiments. Amounts of eOLP are expressed as microliters (see top of graph) rather than as molarities because its concentrations were unknown.

moral agent that stimulated natriuresis [9], many investigators have confirmed its existence [see 9, 10, 24, 25–27] and shown that, like digitalis, its molecular site of action is the ubiquitous cellular enzyme Na^+K^+ATPase [10]. This endogenous digitalis-like hormone was shown to be localized in the hypothalamus [see 24] and suggested to be an important central nervous system component in the genesis of human hypertension [see 10, 24, [25–27]].

Our findings suggest the existence of an endogenous digitalis-like substance which may be related to the glycopeptide 1-39 moiety of the vasopressin-neurophysin precursor. Although the glycopeptide 1-39 moiety itself has digitalis-like activity, neither it nor its two known posttranslationally generated products (GP26-39 and GP23-39) probably has sufficient activity to be the circulating substance reported to exist in plasma [24]. The partially purified but unidentified eOLP we found in the posterior pituitary gland does not appear to be artifactual, because it acts at a ouabain binding site on Na^+K^+ATPase, and it is neither vanadium nor a monovalent cation (Na^+, K^+).

Consistent with the hypothesis of a role for the vasopressin-neurophysin glycopeptide family being involved in natriuresis and hypertension are two studies involving the Brattleboro strain of rat, which has a genetic defect in the vasopressin-neurophysin-glycopeptide gene [28, 29]. Sodium chloride infusions into the cerebral ventricles of this rat do not induce natriuresis [28]. Also, hemi-nephrectomy with corticosteroid and salt administration, which is a classical approach to production of experimental hypertension, does not produce hypertension in this strain of rat [29]. Another experiment consistent with ours is the report that hypothalamic lesions reducing the function of the hypothalamo-neurohypophysial system in rats ameliorate experimental hypertension and reduce plasma levels of the digitalis-like substance [25].

The relationship of the vasopressin-neurophysin-glycopeptide family to other reported endogenous digitalis-like substances, if any, remains to be explored. The most recent work on digitalis-like substances has concentrated on the presence in human plasma, hypothalamus and adrenal cortex of authentic ouabain or an isomer of ouabain [[30–33]; also see 34]. Bufodienolides, cardiotonic steroids originally described in the skin of Bufonidae toads [35, 36], have also been described as existing in mammals [33, 37], including human plasma [38]. The physiological significance of these steroids remains unsettled [34, 39]. The potent digitalis-like substance we detected in purified neurohypophysial extracts (fig. 8) probably is not a cardiotonic steroid because it is detected by the OLP antibody, which does not recognize ouabain and ouabagenin, and because its inhibition in the ^{3}H-ouabain radioreceptor assay was nonparallel to ouabain and ouabagenin but parallel with glycopeptide 1-39 and synthetic glycopeptide 26-39 (fig. 6B).

Peptide mimetics of nonpeptides. Many neurotransmitters (norepinephrine, acetylcholine etc.), hormones (estradiol, cortisol, thyroid hormones etc.) and drugs are nonpeptides but in all instances have peptide binding sites on receptors. Similarly, antibodies can be produced to nonpeptide haptens. Thus, peptide recognition molecules for nonpeptides are encoded in the genome, but their ligands are not. The recognition hypothesis for peptides, however, demands symmetrical arrays of ligand and receptor binding sites on complementary segments of DNA [1–3]. Therefore, we hypothesized that a peptide mimetic of such nonpeptide ligands would be found on the DNA strand complementary to their receptor binding sites. This hypothesis was tested in the current study for a steroid ligand of plant origin whose receptor binding site on its receptor/enzyme was well characterized and confined to a short sequence. The complementary peptide to a ouabain binding site appears to be a peptide mimetic of ouabain since it inhibited Na^+K^+ATPase, and its antibodies led to the identification of even more potent peptide digitalis-like substances. Peptide mimetics have been identified previously for several nonpeptides: (i) reovirus hemagglutinin and an antiidiotypic antibody for norepinephrine [40]; (ii) Elapid snake neurotoxins, rabies virus [41] and antiidiotypic antibodies [see 40] for acetylcholine; (iii) endorphins and enkephalins [42] for morphine; and (iv) thaumatin and monellin (proteins from African berries) for sucrose and the 'sweet' taste receptor [43].

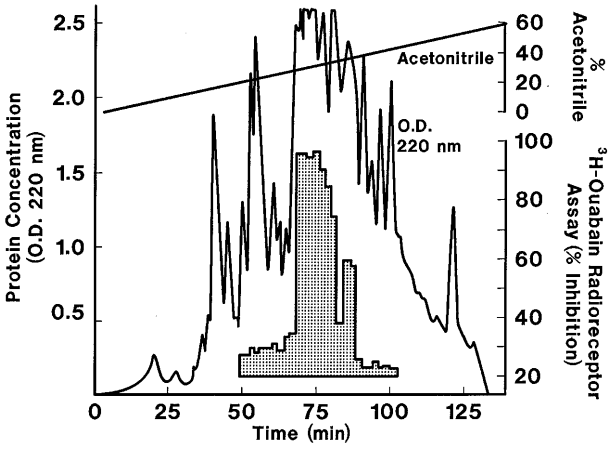

Figure 8. Detection of a partially purified, high-affinity endogenous digitalis-like substance (eOLP) in posterior pituitary extracts. Porcine posterior pituitary glands were extracted and chromatographed on Sephadex G-75. The appropriate fractions were chromatographed by reversed-phase C_{18} HPLC using a linear gradient of acetonitrile (0–60%) in 0.1% TFA. Digitalis-like activity in the eluates was determined with ^{3}H-ouabain radioreceptor assay as described. The main peak of activity eluting at 30–34% acetonitrile was pooled for further study. The smaller, second peak eluting at 38% acetonitrile represents glycopeptide and was excluded from the pool.

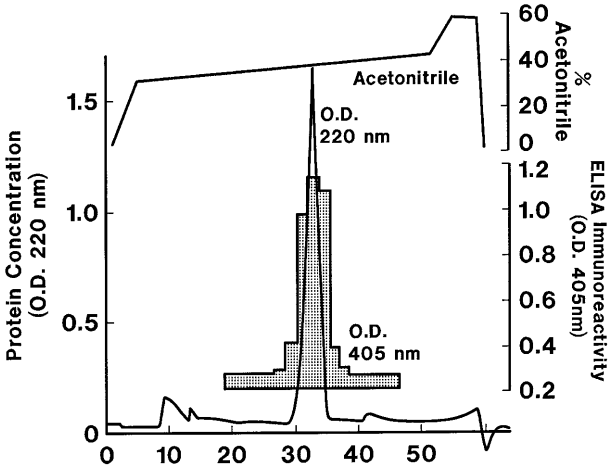

Figure 7. Purification of vasopressin-neurophysin glycopeptide 1-39 by HPLC. To determine immunoreactivity of the samples, 10 µl was evaporated in the wells of an ELISA plate, and the OLP-ELISA described in figure 4 was performed. Amino acid analysis of the sample was done by rpHPLC of PTH derivatives using a Waters Amino Acid Analyzer; the composition expected for glycopeptide 1-39 was found. The amino acid sequence determined for the first 20 residues using a gas-phase sequenator was also that expected except for the asparagine at position 6 which was equivocal, as expected, since it is glycosylated (see fig. 5).

We propose that peptide replicas of nonpeptides in general may be found encoded on the DNA strand complementary to their receptor binding sites. In the case of drugs, such replicas would be expected to be expressed as endogenous peptide ligands such as we have shown for an endogenous digitalis-like hormone. However, in the case of endogenous neurotransmitters and hormones, the replicas might exist only as untranscribed segments of DNA and hence not be expressed, although it is worthy of note that such segments may be expressed in the immune system in the case of antiidiotypic antibodies having acetylcholine- and norepinephrine-like activities (see [40]). Additional work will be required to test this point.

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