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Purification and Characterization of the Bovine Pituitary Luteinizing Hormone Releasing Hormone M_r 60000 Binding Protein

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Luteinizing hormone releasing hormone (LHRH) regulates the release of luteinizing hormone and follicle stimulating hormone from the pituitary. This process takes place through interaction with high affinity membrane receptors. In addition LHRH inhibits the growth of several cancer cell lines through the interaction with M_r 60000 LHRH receptors. Here we describe the purification to homogeneity of the M_r 60000 bovine pituitary LHRH binding protein in amounts allowing *N*-terminal sequencing and peptide mapping.

The procedure describes solubilization of luteinizing hormone releasing hormone receptors from homogenized bovine pituitaries in an active form by using the detergent Triton X-114. The receptors were retained in the Triton X-114 phase during temperature-dependent phase separation. Preparative phase separations were performed directly on solubilized bovine pituitary extracts. SDS-PAGE of the purified LHRH receptor after LHRH-immobilized affinity chromatography showed the presence of a single band with M_r 60000. Partial sequencing of this band after trypsin digestion of gel pieces revealed unknown sequences with a possible homology to other receptors including some G-protein coupled receptors.

The structure of LHRH was elucidated by Schally *et al.* in 1971[1] and the hormone was later found to be synthesized in the hypothalamus as a 92 amino acid precursor.[2,3] The mature hormone is released from nerve endings in the hypothalamus to the portal blood stream[4,5] and thus transported to the pituitary. Binding of this hormone in the pituitary to a specific cell surface receptor on gonadotrophs initiates the process that leads to release of luteinizing hormone (LH) and follicle stimulating hormone (FSH).[1] The process leading to release of gonadotropins is known to involve receptor dimerization,[6–8] G-protein activation,[9] phosphoinositide hydrolysis,[10–12] Ca^{2+} release from internal stores[10–12] and possibly also activation of phosphatase activity.[13]

LHRH has been found to be synthesized in some non-pituitary tissues including placenta and spleen[2,14] and LHRH has been shown to have growth inhibitory effects on several cancer cell lines.[15–18] Consistent with this, receptors for LHRH and LHRH agonists have been reported to exist not only in the pituitary[19–24] but also in extrapituitary tissues,[25–29] cancer cell lines[18,30] and tumors.[31] The receptors for LHRH have generally been found to be proteins of relative molecular weight (M_r) 60000,[19,21,23,26,27,31,32] but M_r values of 40000 have also been reported.[21,31] However, none of these proteins have previously been isolated in pure form or in amounts sufficient to allow protein chemical characterization. A cDNA clone for a murine pituitary LHRH binding protein has recently been identified by expression cloning in Xenopus oocytes[33] and the predicted M_r of this protein is 37684. The reason for the large differences in the reported relative molecular weights cannot be explained at present and must await purification of the authentic murine pituitary receptor for LHRH. In this report we describe a simple procedure for isolating the bovine pituitary M_r 60000 binding protein for LHRH. This procedure has been used to obtain pure binding protein for microsequence analysis and characterization of LHRH binding, and the procedure might be suitable for purification of LHRH receptors from other species and tissues.

Materials and methods

Chemicals. CH_3CN, glycerol, *N,N*-dimethylformamide (DMF), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide, ethanolamine, diethyl ether, CH_3COOH,

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K_2HPO_4, ammonium peroxodisulfate, glycine, MeOH, $(NH_4)SO_4$, $CaCl_2$ and polyethylene glycol (PEG) 6000 were from Merck (Darmstadt, Germany). Divinyl sulfone (DVS)–agarose (Mini Leak) was from Kem-En-Tek (Copenhagen, Denmark). CNBr agarose and Con A Sepharose were from Pharmacia (Uppsala, Sweden). Triton X-114, bromophenol blue, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), mercaptoethanol, 3-mercaptopropionic acid, phenylmethylsulfonyl fluoride (PMSF), tris(hydroxymethylamino)methane (TRIS), sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), bovine serum albumin (BSA) and pentane-1,5-diamine were from Sigma (St. Louis, USA). *N*-Tosylphenylalanine chloromethyl ketone (TPCK)–trypsin was from Worthington (Freehold, USA). Trasylol was from Bayer (Leverkusen, Germany). Luteinizing hormone releasing hormone (LHRH), LHRH(1–11) and D-Trp^6-Des-Gly-Pro-*N*-ethylamide LHRH were from Carlbiotech (Copenhagen, Denmark). ^{125}I-LHRH was from NEN or was made by the procedure of Bolton.[34] Polyvinyl difluoride (PVDF)-membranes were from Millipore (Boston, Massachusetts, USA). Trifluoroacetic acid (TFA) was from Applied Biosystems (Foster City, USA). Acrylamide, bisacrylamide and *N,N,N′,N′*-tetramethylethylenediamine were from BioRad (Richmond, USA).

Amino acid analysis. Amino acid analysis (AAA) was done according to Barkholt and Jensen.[35]

SDS–Polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out as described by Laemmli[36] using 0.75 mm × 6 cm × 10 cm gels with 7.5–12% T, 2.6% C separating gels and 4.6% T, 2.6% C stacking gels. Samples were boiled for 2 min in sample buffer with 40 mM dithioerythritol (DTE) before electrophoresis. Gels were stained with 0.1% (w/v) Coomassie Blue R-250, in 40% MeOH–10% CH_3COOH and destained with 6% (v/v) CH_3COOH–15% (v/v) ethanol.

Electroblotting. Gels were placed on top of a PVDF membrane which had been wetted in MeOH and equilibrated in transfer buffer (10 mM CAPS pH 11) and sandwiched between two layers each consisting of three pieces of Whatman No. 1 paper and three pieces of Whatman No. 3 paper which had been equilibrated in transfer buffer. The current density was 0.1 mA cm^{-2} for 20 h using a semi-dry electroblotting apparatus (JKA Biotech, Copenhagen, Denmark).

Pituitary preparations and solubilization of LHRH binding proteins. Bovine pituitaries from a local slaughter house were dissected and the glands homogenized for 10 min at 1000 rpm with a Kinematica homogenizer (Janke und Kunkel, Breisgau, Germany) at 0°C in 50 mM sodium phosphate buffer–0.1 M NaCl pH 7.2 (PBS), using 4 ml buffer per pituitary. Triton X-114 was then added to 1% and solubilization was allowed to proceed overnight at 4°C.

Preparative Triton X-114 phase separation. Solubilized LHRH binding protein preparations were centrifuged for 30 min at 31000*g*. The supernatant was diluted four times with PBS containing 1% (v/v) Triton X-114, and placed 1 h at 37°C.[37] After centrifugation (560 g) for 30 min at room temperature the Triton X-114 phase was saved and kept at 4°C. The water phase was poured into a separatory funnel and kept at room temperature for 30 min whereafter the separated remaining Triton phase was isolated from the separatory funnel. The Triton X-114 phases from the first and the second phase separations were pooled and insoluble material was removed by centrifugation (30 min at 10000 rpm). The isolated Triton X-114 phase was diluted to 0.1% (v/v) with PBS at 4°C and filtered under vacuum through Whatman No. 1 filters before immobilized LHRH affinity chromatography.

Synthesis of LHRH–DVS–agarose. LHRH was coupled to divinyl sulfone–agarose (Kem-En-Tec, Copenhagen, Denmark) in 0.1 M carbonate buffer pH 10 (1 mg peptide per ml matrix) by incubation 48 h at 25°C. Blocking of excess vinyl groups using 0.1 M mercaptoethanol at pH 9 was done for 3 h at 25°C. The matrix was then washed extensively with 0.1 M sodium phosphate buffer (PB), pH 7.2, and stored at 4°C until required for use.

Synthesis of D-Trp^6-Des-Gly^{10}-Pro^9-N-ethylamide–LHRH–DVS–agarose. D-Trp^6-Des-Gly^{10}-Pro^9-*N*-ethylamide–LHRH was coupled to DVS–agarose as described for LHRH, except that the peptide was first dissolved in DMF and then diluted with 10 volumes of coupling buffer (0.1 M potassium carbonate, pH 10.0).

Synthesis of LHRH(1–11)–pentane-1,5-diamine–DVS–agarose. Six mg LHRH(1–11) (4.8 μmol) were dissolved in 5 ml DMF and 1.1 mg DCC (1.1 mol equiv.) and 1.1 mg *N*-hydroxysuccinimide (2 mol equiv.) was added. After 1 h at 0°C 5 mol equiv. putrescine dihydrochloride and 10 mol equiv. triethylamine were added and the mixture was stirred overnight at room temperature. After filtration the reaction product was precipitated by addition of 20 ml diethyl ether and leaving the mixture overnight at room temperature. The precipitate was collected by centrifugation, redissolved in 5 ml 0.1 M carbonate buffer, pH 9.0, and mixed with 5 ml DVS–agarose, prewashed with coupling buffer (0.1 M sodium carbonate, pH 9.0). The mixture was agitated gently overnight at room temperature and when A_{280} of the supernatant was sufficiently low (24 h), any remaining vinyl groups were blocked by addition of ethanolamine (50 mol equiv. in excess of vinyl groups) and incubation at room temperature for 2 h. The matrix was washed extensively with 0.1 M PB, pH 7.2, and stored at 4°C until required for use.

Synthesis of LHRH–CNBr–agarose. Five mg LHRH were dissolved in 5 ml 0.1 M carbonate buffer, pH 10.0, and added to 1.5 g CNBr Sepharose 4 B, prewashed according to the manufacturers instructions. The mixture was agitated gently until A_{280} of the supernatant was sufficiently low (approximately 48 h) and any remaining reactive groups were blocked by addition of 50 mol equiv. of ethanolamine in excess of the reactive groups. After 2 h the matrix was washed extensively with 0.1 M PB, pH 7.2, and stored at 4°C until required for use.

Synthesis of LHRH–ethylenediamine–silica gel. LHRH–ethylenediamine–silica gel was synthesized by the solid phase method using Fmoc amino acids and DCC with Dhbt activation.[38]

Affinity chromatography. The affinity chromatography was performed by using 0.5 ml immobilized LHRH matrix in a 10 ml Polyprep column (Bio-Rad) at a flow rate of 35 ml h^{-1}. The column was washed with 500 ml PBS, 0.01% (v/v) Triton X-114, pH 7.2 (flow rate 20 ml h^{-1}) and eluted with 1 mg LHRH per ml PB or 1 M acetic acid. The eluate was either neutralized immediately with 1 M K_2HPO_4 before binding assays or lyophilized before analysis by SDS-PAGE.

Binding assays. A 0.1 M NaCl, 0.2% BSA, 50 mM sodium phosphate buffer, pH 7.2, was used as the diluent. Assays with affinity purified LHRH binding protein were performed in 11 mm × 55 mm nunc tubes in a total volume of 1 ml with 100000 cpm (20 pM) ^{125}I-LHRH (NEN) in the absence or presence of various concentrations of unlabelled LHRH. Duplicate determinations were carried out. After incubation for 2 h at 22°C, 1 ml ice cold 50% (w/v) PEG 6000 was added and the tubes were centrifuged 30 min at 10000 *g*. The resulting pellet, containing bound ligand, was counted in a γ-counter. In each assay condition, non-specific binding was assessed in parallel by addition of 10 μg LHRH to the medium, and specific binding was the difference between total and non-specific binding.

Trypsin digestion in SDS–polyacrylamide gel pieces. After staining of SDS-PAGE gels with Coomassie Blue (R-250), 19 LHRH receptor-containing gel pieces (from 132 pituitaries) were sliced out and placed in 200 μl 0.1 M PB, pH 8.0 each, and 1 μl of 0.1 mg ml^{-1} TPCK–trypsin (Worthington) was added in 0.1 M PB–10 mM $CaCl_2$, pH 8.0 and then again after a 20 h interval. Incubation was for 20 h at 37°C after each trypsin addition (E:S ratio was approximately 1:10).

Peptide sequencing. Sequencing of C18 reversed-phase high performance liquid chromatography (RP-HPLC) purified peptides after trypsin digestion of SDS–polyacrylamide gel pieces and electroblotted samples was done on an Applied Biosystem (Foster City, USA) Model 477 A sequencer with on-line analysis of phenylthiohydantoin (PTH) amino acids using standard programs supplied with the sequencer.

Results

Since LHRH does not contain any free lysine ε-amino groups or aspartic acid or glutamic acid carboxy groups which are usually used for coupling peptides and proteins to affinity matrices, we initially tested several methods for coupling LHRH to affinity matrices. Table 1 shows amino acid analyses of the ligands used for coupling and of the resulting affinity matrices. LHRH itself was coupled directly to divinyl sulfone–agarose and cyanogen bromide–agarose. The coupling of the ligands to the various matrices were in all cases monitored by absorbance measurements of the supernatant exemplified in Fig. 1 for coupling of LHRH to divinyl sulfone–agarose. As seen in Fig. 1 the coupling proceeds smoothly and is complete after 1 h. Amino acid analysis indicated that coupling took place primarily through the tyrosine hydroxy group since the value for tyrosine was the only one significantly lowered (50%), and consistent with this was the finding of an additional peak in the amino acid analysis chromatogram eluting before tyrosine at a position corresponding to DVS–tyrosine, and being present in a 1:1 ratio compared with tyrosine (not shown). Similarly, coupling of LHRH to cyanogen bromide activated agarose and of D-Trp^6-Des-Gly^{10}-Pro^9-*N*-ethylamide–LHRH to DVS–agarose probably takes place through tyrosine. Two additional affinity matrices were made: LHRH(1–11) coupled through the extended glycine at position 11 to DVS–agarose using pentane-1,5-diamine as a linker and LHRH synthesized directly on a silica matrix using ethylenediamine as a non-cleavable linker from the *C*-terminal glycine to the matrix. The reasons for testing several matrices were that they had varying degrees of ease of preparation and that they might have different abilities to bind LHRH-binding proteins taking into account that LHRH is a small ligand and that the immobilization might lower the affinity for the receptor considerably.

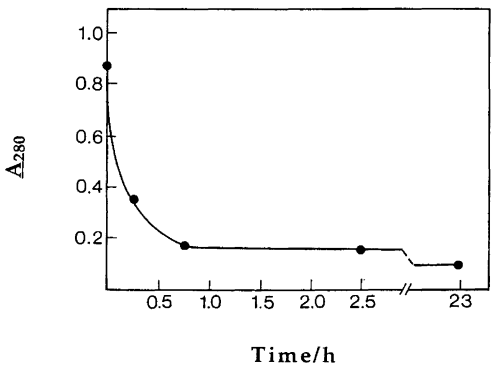

Fig. 1. Time course for immobilization of LHRH to DVS–agarose. The absorbance was measured in the supernatant at the indicated intervals after a brief centrifugation.

Table 1. Amino acid composition[a] of ligands before and after immobilization on the matrices used and LHRH binding capacity of the resulting matrices.

Amino acid	LHRH	LHRH–DVS–agarose	LHRH–Sepharose 4B	LHRH–Silica gel	LHRHa[b]	LHRHa–DVS–agarose	LHRH (1–11)	LHRH (1-11)–DVS–agarose
Ser	1.04(1)	0.82	0.99	0.96	1.01(1)	0.87	0.99(1)	0.94
Glx	1.06(1)	0.93	1.10	0.90	1.00(1)	0.90	1.06(1)	0.85
Pro	1.01(1)	0.92	0.94	1.11	0.96(1)	1.26	1.02(1)	0.99
Gly	2.06(1)	1.98	2.07	2.06	0.01(0)	0.02	3.06(3)	3.09
Leu	1.00(1)	1.00	1.00	1.00	1.00(1)	1.00	1.00(1)	1.00
Tyr	0.96(1)	0.77[c]	0.94(1)	0.93[c]				
His	1.04(1)	0.88	1.01	0.93	1.04(1)	0.95	1.03(1)	0.96
Arg	1.08(1)	0.77	1.03	0.98	1.02(1)	0.86	1.06(1)	0.90
Trp	ND(1)	ND	ND	ND	ND(2)	ND	ND(1)	ND
µmol ligand per ml matrix	0.73	0.61	76.0[c]	0.61	0.47			
LHRH binding ability[d]	+	+	+	+	+			
Specific binding in eluates from affinity chromatography (pmol mg^{-1})	12.0	ND	ND	6.0	7.5			

[a] 110 °C, 6 M HCl, 24 h. [b] D-Trp[6]-Des-Gly[10]-Pro[9]-*N*-ethylamide–LHRH. [c] µmol g^{-1} matrix. [d] Measured as the ability fully to remove LHRH binding material from homogenates with solubilized receptor (10 ml homogenate/ml matrix). [e] The value represents the sum of the value for tyrosine and a derivative of tyrosine eluting at a position corresponding to DVS–tyrosine.

However, all of the affinity matrices were capable of removing LHRH receptor binding material from pituitary homogenates as shown in Table 1. Owing to the above result LHRH–DVS–agarose was selected for further use in the purification of the LHRH receptor and furthermore this matrix was the most easily prepared and contains the natural LHRH immobilized through a very stable ether bond.

Using the LHRH–DVS–agarose in the affinity chromatography step the bovine pituitary M_r 60000 LHRH binding protein was purified as shown schematically in Scheme 1 and Table 2.

In the initial homogenization and solubilization step the detergent Triton X-114 was used in order to allow subsequent temperature-induced phase separation of membrane proteins from soluble proteins,[37] and it was shown by ultracentrifugation experiments (100000 *g*) that membranes had been solubilized effectively since all LHRH binding activity remained in solution. Furthermore, although the protease inhibitors PMSF and BPTI were used in initial homogenization experiments, it was found that omitting these did not decrease the yield of solubilized LHRH binding protein appreciably, and they were consequently excluded in large-scale solubilizations. Using this solubilization procedure (50 mM PB, 0.1 M NaCl, pH 7.2, 1% Triton X-114, 5 °C, 12 h) yields of 1–5 pmol binding protein were obtained per pituitary. After removal of unsolubilized material by centrifugation (30 min at 30000*g*), membrane proteins were separated

Homogenization. 4 ml PBS/pituitary
⇓
Solubilization. 1% Triton X-114
⇓
Phase separation. 37 °C
⇓
Affinity chromatography. LHRH–DVS–agarose column

Scheme 1.

Table 2. Summary of purification of the bovine pituitary M_r 60000 LHRH binding protein as outlined in Scheme 1.[a]

Purification step	Protein (mg/pituitary)	Receptor (pmol/pituitary)	Specific binding (pmol/mg)
1 Homogenate after centrifugation	106	2.90	0.027
2 Triton X-114 phase after phase separation	8.3	1.14	0.137
3 Eluate from LHRH affinity column	1.4×10^{-4}	0.04	2.9×10^{2}

[a] The table shows the mean of three different purifications. The standard deviation was less than 20% of the mean.

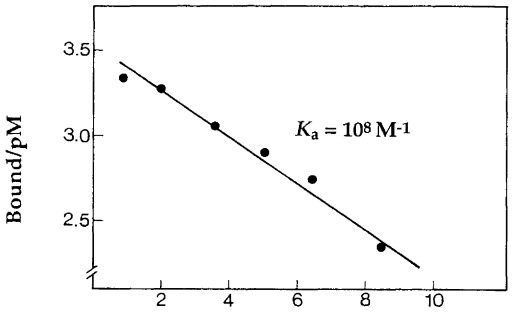

Fig. 2. Scatchard analysis of LHRH binding activity present in the Triton X-114 detergent phase after temperature-induced phase separation. Each point represents the mean of duplicates.

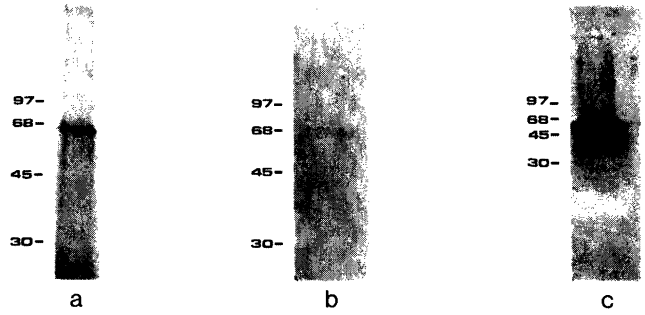

Fig. 4. SDS-PAGE analysis of purified LHRH-receptor and electroblotting to PVDF membrane for *N*-terminal sequencing. Following elution with acetic acid from an affinity column the eluates were lyophilized and reconstituted in a minimal volume of sample buffer (20–50 µl) and analysed on 12% mini gels. Proteins were visualized by Coomassie staining: (a) SDS-PAGE gel representative of samples used for binding experiments; (b,c) electroblots on PVDF membranes used for *N*-terminal sequencing.

from soluble proteins by temperature-induced phase separation at 37°C for 1 h followed by centrifugation. All LHRH binding activity partitioned into the detergent phase. The LHRH binding activity in homogenates before phase separation and in the detergent phase was found to be of high affinity with a K_a of $10^8\ M^{-1}$ (Fig. 2), and yields of 1–2 pmol per pituitary were obtained (approximately 20% of solubilized LHRH binding protein). Further chromatography on Con A columns was found to result in relatively low yields of binding protein, possibly due to heterogeneity in glycosylation, however direct chromatography of the detergent phase on LHRH–DVS–agarose resulted in reasonably good yields of LHRH binding protein (0.02–0.1 pmol per pituitary). The affinity purified protein retained high affinity LHRH binding (Fig. 3), and had a specific binding of 2.9×10^2 pmol mg^{-1}. While this figure is lower than the theoretically possible value (167×10^2 pmol mg^{-1}), it should be considered a minimum value since the amino acid analysis system used for quantitating protein overestimates the amount of protein present due to a high background level compared with the amount analysed. SDS-PAGE analysis of the affinity purified protein showed a single band of M_r 60000 [Fig. 4(a)]. No such band was seen in eluates from control columns in which no LHRH was coupled and whereupon an equal amount of detergent phase was chromatographed. To obtain further information about the purified binding protein, *N*-terminal sequence analyses were performed on electroblotted samples of the M_r 60000 protein [Fig. 4(b,c)]. These results are shown in Table 3. The sequences obtained were not derived from any known sequence as revealed by database searches. In order to obtain more information about the sequence of the purified protein the M_r 60000 band was cut out from 19 gel lanes of purified protein preparations representing 132 pituitaries. *In situ* digestion with trypsin followed by HPLC analysis yielded the chromatograms shown in Fig. 5. Individual peaks were collected and sequenced

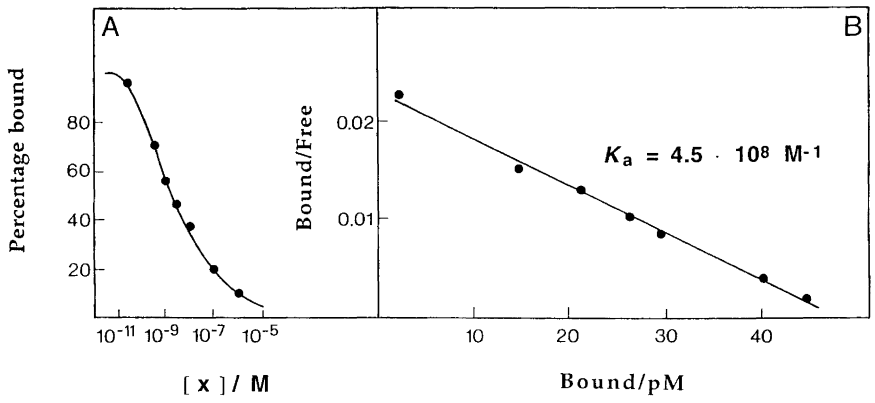

Fig. 3. Dose-dependent inhibition curve of ^{125}I-LHRH binding by LHRH to affinity purified LHRH-binding protein eluted by acetic acid from the affinity column: A, typical displacement curve obtained by incubating fixed amounts of ^{125}I-LHRH (20 pM) and purified LHRH receptor (1/10 of a pituitary equivalent) with increasing amounts of unlabelled LHRH. Each point represents the mean of duplicates; B, corresponding Scatchard analysis of data from A. The calculated binding affinity, K_a was $4.5 \times 10^8\ M^{-1}$ and the total binding capacity was 0.48 pmol/pituitary.

Table 3. *N*-Terminal and 'internal' amino acid sequences obtained from the M_r 60000 bovine pituitary LHRH binding protein.[a]

	Cycle No.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
N-terminal	–	F	F	n	–	r	n	(g)	–	–	–	–	–	–
N-terminal	–	F	F	n	–	(r)	–	(w)	–	–	–	–	–	–
HPLC Fr.6	a/y	F	M	a	–	–	–	–	–	–	–	–	–	–
HPLC Fr. 8	L	V	Y	Q	L	I	A	Q	K	–	–	–	–	–
HPLC Fr. 20	s	q	n	a	q	e	i	n	(I)	(i)	–	(v)	–	–
HPLC Fr. 22	g/a	I	L	V	F	y	G	T	g	d	g	q	f	(I)

[a] The one letter amino acid code is used. Capital letters indicate 100% sure assignment of residues, lower-case letters indicate less than 100% sure assignment of residues and lower-case letters in brackets indicate tentative assignments. – indicates that no residues could be assigned.

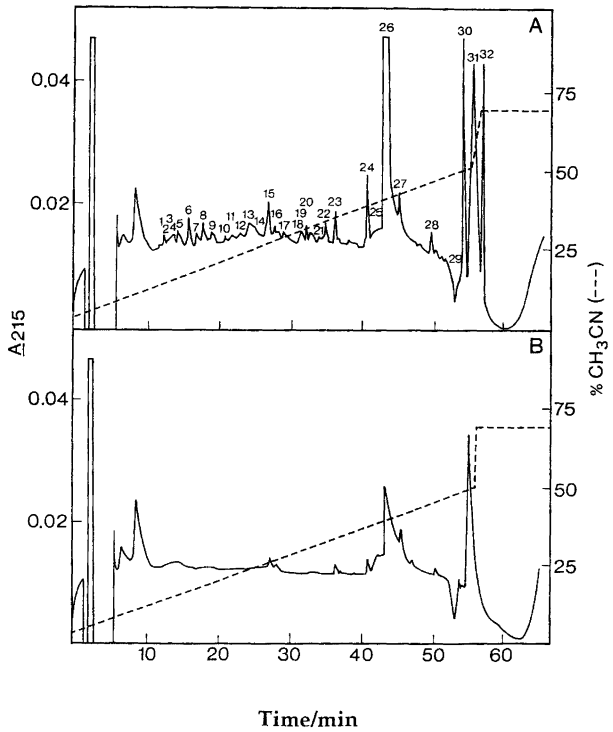

Fig. 5. Peptide map of the LHRH binding protein after trypsin digestion of SDS–polyacrylamide gel pieces: A, the peptide purification was prepared on an RP-HPLC C18 column with a TFA–acetonitrile solvent system (see Methods) and compared with the chromatogram generated after identical trypsin treatment of blank gel pieces (B) before sequencing. The numbers above the tops were manually collected fractions.

yielding the sequences shown in Table 3. These were not derived from known sequences but did show a possible homology to some membrane receptors including some G protein coupled receptors (not shown).

Discussion

In this report we have presented data on the purification of the bovine M_r 60000 pituitary binding protein for LHRH. The criteria for the identification and characterization of the protein purified as a binding protein for LHRH are the following. 1 The protein can be purified from a pool of integral membrane proteins on the basis of its affinity toward LHRH. 2 Affinity chromatography yields a homogeneous protein with M_r 60000 determined by SDS-PAGE under reducing conditions, which is identical with the M_r for the LHRH receptor published by others.[8,9] 3 The purified protein is active and binds LHRH with high affinity, $K_a = 4.5 \times 10^8\ M^{-1}$, as determined by Scatchard plot analysis. 4 The purified protein can be eluted from a column with LHRH–divinyl sulfone–agarose but not from a control column with ethanolamine blocked divinyl sulfone–agarose. 5 Sequence information of the purified protein from peptides after trypsin digestion in SDS–polyacrylamide gel slices reveals unknown sequences as expected for a protein not previously sequenced.

The results presented here together with results presented by others reveal a discrepancy with regard to the M_r for LHRH binding proteins, since the predicted M_r for the recently cloned mouse pituitary LHRH receptor is only 37684. The discrepancy between the predicted M_r and the observed M_r of 60000 is too large to be explained by small sequence-dependent deviations in binding of SDS. Furthermore, although the receptor might be glycosylated the protein purified here does not show the usually observed heterogeneity and resulting band broadening in SDS-PAGE of glycoproteins. However, glycosylation could be argued to be a possible explanation perhaps in combination with phosphorylation which also shifts the M_r towards higher values. The cDNA clone isolated by Reinhart *et al.*[33] shows the tissue distribution expected for the LHRH receptor, but was not present in placenta where M_r 60000 LHRH receptors have been detected by affinity labelling. An unusual feature of the recently cloned, M_r 37684 murine LHRH receptor is its apparent lack of *N*- and *C*-terminal extensions beyond the 7-transmembrane helix sequence. This also holds for other recently cloned pituitary LHRH receptors with homology to the murine receptor.[39–42] These receptors encode proteins of the same size and with about 90% identity in the amino acid sequence and all lacking the *N*- and *C*-terminal extensions usually found in

7-transmembrane receptors. Whether this can explain the discrepancy in relative molecular weights or whether the M_r 60000 protein is a different LHRH binding protein may be resolved using antibodies to the cloned murine LHRH receptor or using affinity labelling of the cloned murine LHRH receptor expressed in oocytes or a mammalian cell line. The sequences obtained in this study can be aligned with the murine cDNA derived sequence, but only with ambiguity and the final decision of whether these sequences are derived from a protein homologous to the cloned murine LHRH receptor must await cloning of the bovine LHRH receptor.

An alternative explanation which seems attractive is that the M_r 60000 protein and the recently cloned M_r 37684 receptor represent different LHRH binding proteins, and in fact we have observed an M_r 40000 protein in addition to the M_r 60000 protein in some eluates from LHRH affinity columns (not shown). This explanation would resolve all the discrepancies between published data on the M_r of the LHRH receptor and data on biological effects, such as growth inhibition of cells not possessing the M_r 37684 receptor. For example, placenta tissue expresses the M_r 60000 receptor but not the M_r 37684 receptor, the affinities of the placental receptor for different LHRH agonists and antagonists are clearly different from the affinities of the pituitary receptor,[43] and breast cancer cells derived from a tissue not expressing the M_r 37684 receptor are inhibited by LHRH. This inhibition presumably takes place through the M_r 60000 receptor, which these cell lines do possess. This situation also seems to hold true for pancreatic carcinoma cells. Thus many different tissues seem to express the M_r 60000 protein with LHRH binding capacity, and which could very well be responsible for the growth inhibitory effects of LHRH, whereas the hormone releasing effects of LHRH on the pituitary take place through the M_r 37684 receptor. This theory will be testable following cloning of the M_r 60000 binding protein and expression of the protein in a cell line not expressing this protein.

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