Novel ligands for the affinity labelling of luteinizing hormone releasing hormone receptors

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A number of novel luteinizing hormone releasing hormone (LHRH) analogues incorporating biotin together with potential covalent attachment sites have been synthesized. Those based on the des-Gly¹⁰-[D-Lys⁶]-LHRH ethylamide peptide backbone resulted in the most useful characteristics of binding to the LHRH receptor in rat anterior pituitary gland membranes. Of these, des-Gly¹⁰-[biotinyl-aminoethylglycyl-D-Lys⁶]-LHRH ethylamide (XBAL) gave the best specific:non-specific binding ratio, with 44 ± 6 % (± S.E.M.) of total binding being specific with a K_d of 131 ± 16 pM (± S.E.M., $n = 4$) as determined by Scatchard analysis. Two methods have been used to covalently crosslink these analogues with the LHRH receptor; photoaffinity labelling and the use of homobifunctional *N*-hydroxysuccinimide ester crosslinkers. The photoaffinity analogues gave poor specific:non-specific binding ratios. Of the chemical crosslinkers tested, ethylene glycolbis(succinimidylsuccinate) (EGS) was found to be the most efficient at covalently linking the ¹²⁵I-XBAL bound to the LHRH receptor site. At an EGS concentration of 5 mM, 23 ± 3 % (± S.E.M.) of the specific binding of ¹²⁵I-XBAL was covalently crosslinked.

INTRODUCTION

Luteinizing hormone releasing hormone (LHRH) causes release of gonadotrophins from the anterior pituitary gland by a mechanism involving interaction with a membrane-bound receptor (Clayton & Catt, 1981) and a subsequent increase in the turnover of membrane phosphoinositides (Conn *et al.*, 1986). Information on the structure of the binding site and signal transduction domains of the LHRH receptor may facilitate our understanding of the signalling events occurring. We have recently described a high-yielding method of solubilization for the rat anterior pituitary LHRH receptor (Ogier *et al.*, 1987), the initial step in purification and molecular characterization of this receptor. Several receptor types have been successfully purified using the technique of ligand affinity chromatography (Strosberg, 1984), including the GABA_A (Sigel & Barnard, 1984), muscarinic cholinergic (Haga & Haga, 1985), glycine (Pfeiffer *et al.*, 1982), β_2 -adrenergic (Homcy *et al.*, 1983) and insulin receptors (Cuatrecasas, 1972). This approach requires conditions to be found which retain the receptor in a solubilized state without denaturing the protein or greatly reducing its affinity for the ligand utilized. In the case of the LHRH receptor, an extensive survey of different detergents failed to find conditions compatible with these criteria (Ogier *et al.*, 1987). Although detergent/salt conditions were found to successfully solubilize the receptor, ligand binding could only be observed subsequently by removal of the solubilizing agents (and consequent precipitation of the receptor protein). A novel method for purification of the solubilized LHRH receptor was therefore required.

Hofmann & Finn (1985) and Kohanski & Lane (1985) described a method of receptor affinity chromatography based on the interaction of immobilized avidin/strept-
avidin with biotin incorporated into specific receptor ligands. A similar biotinylated (but still reversible) ligand for LHRH receptors has also been described (Hazum *et al.*, 1986). As described, this approach is strictly dependent on the ligand–receptor interaction being viable in the presence of the detergent (the biotin/avidin interaction appears to be much more resistant to disruption by detergents; Hofmann & Finn, 1985; Kohanski & Lane, 1985). To further develop this approach for LHRH receptor purification we adopted the strategy of covalently linking biotinylated ligands to the LHRH receptor, thereby overcoming the problem of detergent suppression of reversible ligand binding.

The present study describes the design of several LHRH analogues incorporating a biotin molecule. These have been used in conjunction with the two methods of covalent labelling (photoaffinity labelling or affinity cross-linking; Pilch & Czech, 1984) to assess the optimal conditions required for the covalent affinity labelling of LHRH receptors by biotinylated LHRH analogues.

EXPERIMENTAL

Materials

The divalent crosslinkers bis(sulphosuccinimidyl)-suberate (BSSS), disuccinimidyl suberate (DSS), disuccinimidyl tartarate (DST), ethylene glycolbis(succinimidylsuccinate) (EGS) and sulphosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulpho-SANPAH)

Abbreviations used: LHRH, luteinizing hormone releasing hormone; buserelin, des-Gly¹⁰-[D-Ser(Bu)⁶]LHRH ethylamide; PBL, [biotinyl-*p*-azidophenylalanyl-D-Lys⁶]LHRH, PBAL, des-Gly¹⁰-[biotinyl-*p*-azidophenylalanyl-D-Lys⁶]LHRH ethylamide; XBAL, des-Gly¹⁰-[biotinyl-aminoethylglycyl-D-Lys⁶]LHRH ethylamide; sulpho-SANPAH, sulphosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate; EGS, ethylene glycolbis(succinimidylsuccinate); DSS, disuccinimidyl suberate; DST, disuccinimidyl tartarate; BSSS, bis(sulphosuccinimidyl)suberate; Boc, *t*-butoxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; chloramine-T: *N*-chloro-*p*-toluene sulphonamide, sodium salt; PEG, poly(ethylene glycol)8000.

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were purchased from Pierce and Warriner (U.K.) Ltd., Chester, U.K. The LHRH analogue busserelin (des-Gly¹⁰-[D-Ser(Bu^t)⁶]LHRH ethylamide) was a generous gift from Hoechst A. G., Frankfurt, F.R.G. [D-Lys⁶]-LHRH was purchased from Peninsula Laboratories Europe Ltd., St. Helens, U.K. Other LHRH analogues were synthesized as described below. All other chemicals and peptides, unless otherwise stated, were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Synthesis and derivatization of peptide analogues

[des-Gly¹⁰, D-Lys⁶]LHRH ethylamide was synthesized by the solid-phase method using Sheppard's Fmoc-*t*-butyl-polyamide chemistry (Eberle *et al.*, 1986). After purification, the peptide had the expected amino acid composition (Glu₁, 1.02; His₁, 0.96; Ser₁, 0.89; Tyr₁, 0.97; Lys₁, 1.00; Leu₁, 1.00; Arg₁, 1.05; Pro₁, 0.95) and fast atom bombardment mass spectrum (MH^+ , 1225; calc. for C₅₉H₈₅N₁₇O₁₂, 1224).

The biotinylated reagents (Fig. 1, 1 and 2) were prepared in several steps from biotin and either *p*-nitrophenylalanine or 2,4-diaminobutyric acid, and were converted to their *N*-hydroxysuccinimide esters prior to

coupling to the ϵ -amino group of the D-Lys⁶ of the peptide backbone (Bladon *et al.*, 1988).

The synthesis of reagent 2, (Fig. 1) (biotinyl-*t*-butoxycarbonylaminoethylglycine), required the selective protection/deprotection of the two amino groups of L-2,4-diaminobutyric acid. The γ -amino function was protected with the acid-labile *t*-butoxycarbonyl group (Boc) in a two-step process (Schwyzer & Rittel, 1961) which first involved the formation of a copper complex with the α -amino and carboxyl groups. Only the γ -amino function was then left available to react with di-*t*-butyl dicarbonate to yield the protected derivative in 40 % yield. The copper complex was then destroyed by treatment with H₂S. The α -amino function was protected with the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group using the standard procedure (Carpino & Han, 1972), and the carboxylic acid was esterified with diazomethane. The Fmoc group was cleaved by piperidine and then the free amino derivative was treated with biotin pentafluorophenyl ester to yield biotinyl-*t*-butoxycarbonylaminoethylglycine methyl ester in 66 % yield for the two-step process. The methyl ester was hydrolysed to yield reagent 2 (Fig. 1). To facilitate coupling to the peptide, the carboxylic acid was converted to its *N*-hydroxysuccinimide ester. Also, the Boc-protecting group on the γ -amino function was left in position until after the reagent was conjugated to the peptide backbone and the production purified. The same type of procedure was used for synthesis of reagent (1) (biotinyl-*p*-azidophenylalanine).

The conjugates [biotinyl-*p*-azidophenylalanyl-D-Lys⁶]-LHRH (PBL), des-Gly¹⁰-[biotinyl-*p*-azidophenylalanyl-D-Lys⁶]LHRH ethylamide (PBAL) and des-Gly¹⁰-[biotinyl-aminoethylglycyl-D-Lys⁶]LHRH ethylamide (XBAL) were all synthesized in a similar manner. For example, PBL was synthesized by first dissolving D-Lys⁶-LHRH (2 mg, 1.6 μ mol) in freshly distilled dimethylformamide (2 ml) containing triethylamine (2 drops), and then an excess of biotinyl-*p*-azido-phenylalanine-*N*-hydroxysuccinimide ester (reagent 1, Fig. 1) (5 mg, 9.45 μ mol) was added. The resulting mixture was left in the dark for 1 h and then the dimethylformamide was evaporated. The residue was triturated with ethyl acetate (3 $\times$ 2 ml) to remove excess reagent and then the crude product was purified by semi-preparative reverse-phase h.p.l.c. (Column: Aquapore RP300; eluents: A, 0.1 % aqueous trifluoroacetic acid; B, 90 % acetonitrile: 10 % A; the product was eluted with a linear gradient of 10–70 % B over 30 min). Reactions utilizing the azido ligand were carried out in subdued lighting, and during purification the u.v. detector was switched off whilst the peak was collected. Amino acid compositions of the derivatives are shown in Table 1.

Membrane preparation

Male Wistar rats (approx. 250 g) were stunned, decapitated and their anterior pituitary glands rapidly removed. These were placed in 100 vol. of ice-cold HEPES/KOH (25 mM, pH 7.4) and sonicated, followed by centrifugation at 60000*g* for 15 min at 4 °C. The resulting washed membranes were resuspended in the HEPES/KOH buffer.

Equilibrium binding studies

The binding characteristics of the LHRH analogues were determined by equilibrium displacement assays on anterior pituitary membranes. Iodination of all analogues

Fig. 1. Biotinyl-LHRH analogue structures

Structures of the novel biotin ligands (1) and (2) and the three LHRH analogues incorporating them: PBL, [biotinyl-*p*-azidophenylalanyl-D-Lys⁶]LHRH; PBAL, des-Gly¹⁰-[biotinyl-*p*-azidophenylalanyl-D-Lys⁶]LHRH ethylamide; XBAL, des-Gly¹⁰-[biotinyl-aminoethylglycyl-D-Lys⁶]LHRH ethylamide.

Table 1. Amino acid analysis of affinity analogues

Results of amino-acid analysis of the three biotinyl-LHRH analogues PBL, PBAL and XBAL.

Analogue	Amino acid										
	pGlu	His	Ser	Tyr	D-Lys	PheN ₃ *	But	Leu	Arg	Pro	Gly
PBL	1.07	1.00	0.81	1.07	1.01	0.30	—	1.00	1.00	0.96	1.04
PBAL	1.05	0.96	0.83	0.95	1.02	0.20	—	1.00	0.89	0.91	—
XBAL	1.09	1.01	0.91	1.01	1.07	—	0.94	1.00	0.96	0.99	—

* Largely decomposed on hydrolysis.

was by the chloramine-T method (Mitchell *et al.*, 1985) to a specific activity of approx. 1100 Ci/mmol. Assays were usually carried out in triplicate, in a volume of 500 μ l containing 10–60 μ g of protein, unlabelled analogue as appropriate and 50000 c.p.m. of iodinated analogue in 25 mM-Tris/HCl, pH 7.4, containing 0.1 % bovine serum albumin. Non-specific binding was determined in the presence of 1 μ M-LHRH. After incubation for 90 min at 4 °C, equilibrium had been reached and the membrane preparation was subjected to a poly(ethylene glycol) 8000 (PEG) precipitation as previously described (Ogier *et al.*, 1987). Briefly, PEG was added to a final concentration of 15 % and bovine γ -globulin to 0.035 % in a volume of 1.5 ml. After 20 min on ice, samples were centrifuged at 1600 *g*, 6 °C for 15 min. Precipitated (bound) label was then counted by γ spectrometry. Under these conditions, total binding of the iodinated analogue ¹²⁵I-labelled buserelin was typically 21 ± 1 % ($\pm$ S.E.M., *n* = 8) of the total radioligand added, with non-specific binding representing 28 ± 3 % ($\pm$ S.E.M., *n* = 8) of this value. These values are similar to those previously reported for the binding of iodinated LHRH analogues (Marshall & Odell, 1975). For photoreactive analogues, all procedures were carried out in subdued light, using a safety light as necessary. Equilibrium binding for chemical crosslinking studies was carried out as above but in Hepes/KOH instead of Tris/HCl/bovine serum albumin. Saturation and displacement data were analysed by an error-weighted programme (Zivin & Waud, 1982).

Covalent labelling of the LHRH receptor

In all cases, covalent labelling was initiated on membrane preparations which were at binding equilibrium with an iodinated LHRH analogue. For photoaffinity labelling studies, the photoreactive conjugates were prepared prior to iodination, with the exception of ¹²⁵I-labelled-XBAL-sulpho-SANPAH. After an excess of the heterobifunctional reagent sulpho-SANPAH (1.5 mg) had been allowed to react for 40 min with ¹²⁵I-XBAL (approx. 0.03 μ g in Hepes/KOH), at room temperature in the dark (total volume 200 μ l), 200 μ l of 0.2 M-glycine was added for 10 min to quench the reaction. The ¹²⁵I-XBAL-sulpho-SANPAH was then diluted in Hepes/KOH for use in membrane equilibrium binding. When binding equilibrium had been attained, the photoreactive analogue ¹²⁵I-XBAL-sulpho-SANPAH (or in other experiments ¹²⁵I-PBL or ¹²⁵I-PBAL) was activated by exposure to u.v. light (366 nm) at 20 cm from a 4 W mercury lamp for 0–10 min (on ice).

In the divalent chemical crosslinker studies, DSS, DST and EGS were dissolved into dimethyl sulphoxide immediately before use. In the case of BSSS, the crosslinker was dissolved directly into Hepes/KOH. Crosslinkers were routinely added at 1:100 dilutions and left to react for 30 min either on ice or at room temperature, before quenching with 0.25 ml of 0.2 M-glycine.

After photoaffinity or chemical crosslinking, the membrane preparation was centrifuged (45 min at 1600 *g*, 6 °C) to remove excess unbound label and crosslinker, and the membrane-bound radioactivity was determined by γ spectrometry. Photoreactive preparations were kept in subdued lighting.

Assay of crosslinking efficiency

Efficiency of the covalent crosslinking reaction was monitored by comparing the amount of bound ligand in the same samples both at the time of crosslinking and then subsequently after a period of 4 h at room temperature in the presence of 1 μ M-LHRH (sufficient to cause dissociation of at least 95 % of the ligand reversibly bound to the receptor). The crosslinked membrane preparation (or crosslinker-free controls) were resuspended into 0.75 ml of Tris/HCl (25 mM, pH 7.4) containing 1 μ M-LHRH, 0.1 % bovine serum albumin, soyabean trypsin inhibitor (50 μ g/ml) and aprotinin (400 KIU/ml). The radioactivity present was counted. After 4 h at room temperature the membranes were again centrifuged (45 min at 1600 *g*, 6 °C) and recounted. As before, photoreactive preparations were used in subdued light.

Samples for total and non-specific binding were carried through all procedures in parallel. Since the excess amount of membrane binding in the total samples (above that in non-specific samples) initially represented occupancy of the LHRH receptor (being displaceable by LHRH), the effect of crosslinking these samples will most likely include attachment of some of this specifically-bound ligand to part of the receptor protein. Crosslinking the non-specific samples should control for the same level of spurious labelling of non-receptor proteins as occurs additionally to the specific binding in the total samples. After crosslinking, the excess specific covalent labelling in the total samples should be reflected by the value for non-dissociable binding in total samples less that in non-specific samples. The efficiency of receptor-specific crosslinking can then be expressed in terms of the fraction by which reversible specific binding is rendered irreversible after incubation with crosslinker rather than its vehicle control (that is, specific irreversible binding).

RESULTS

Characterization of LHRH analogue binding properties

The affinities of non-radioactive analogues for the LHRH receptor were assessed by the displacement of ^{125}I -buserelin as described previously (Mitchell *et al.*, 1985; Ogier *et al.*, 1987). Displacement of ^{125}I -buserelin from rat anterior pituitary membranes revealed a K_i of $0.92 \text{ nM} \pm 0.16$ ($\pm \text{s.e.m.}$, $n = 4$) for PBL (Fig. 2). This is similar to that reported for the parent peptide [D-Lys⁶]-LHRH (Clayton & Catt, 1980) suggesting that the addition of the photo-biotin group in the D-Lys⁶ position does not impede binding to the LHRH receptor. Binding with the iodinated derivative ^{125}I -PBL could be partially displaced by excess LHRH but only a low ratio of specific to non-specific binding could be observed (Mitchell *et al.*, 1987). Previous reports of binding using iodinated LHRH analogues have suggested that better specific:non-specific ratios are seen using analogues altered at their C-terminal to des-Gly¹⁰-ethylamide (Perrin *et al.*, 1983). The analogue des-Gly¹⁰-[D-Lys⁶]-LHRH ethylamide was constructed. Again, displacement of ^{125}I -buserelin (Fig. 2) showed it to bind specifically to the LHRH receptor site, with a K_i of $98 \pm 10 \text{ pM}$ ($\pm \text{s.e.m.}$, $n = 3$). Addition of the biotin-containing group at D-Lys⁶ (to form the analogue XBAL) apparently enhanced binding affinity [K_i of $14 \pm 1 \text{ pM}$ ($\pm \text{s.e.m.}$, $n = 6$)] (Fig. 2). Hofstee analysis of self-displacement (Hofstee, 1952) gave a K_i value of $57 \pm 14 \text{ pM}$ ($\pm \text{s.e.m.}$, $n = 3$) and Scatchard analysis of saturation (Scatchard, 1949) gave a K_d of $131 \pm 16 \text{ pM}$ ($\pm \text{s.e.m.}$, $n = 3$) (Fig. 3). The apparent receptor number (B_{max}) value for ^{125}I -XBAL binding on male rat anterior pituitary tissue was $63 \text{ fmol/anterior pituitary}$, comparable to that for buserelin of $92 \text{ fmol/anterior pituitary}$. Under the conditions used, specific binding of ^{125}I -XBAL was $11 \pm 1\%$ ($\pm \text{s.e.m.}$, $n = 4$) of total radioligand added and represented $44 \pm 6\%$ ($\pm \text{s.e.m.}$, $n = 4$) of total binding. The specific binding of ^{125}I -XBAL was displaced by buserelin with a K_i value of $0.22 \pm 0.07 \text{ nM}$ ($\pm \text{s.e.m.}$, $n = 4$), similar to the value of $0.47 \pm 0.14 \text{ nM}$ ($\pm \text{s.e.m.}$, $n = 6$) for displacement of ^{125}I -buserelin by buserelin.

Photoaffinity labelling

The receptor specificity of photolabelling will be determined in the first instance by the degree of specific binding of the iodinated ligand prior to photoactivation. In the

Fig. 2. Binding characteristics of the LHRH analogues

(a) Displacement of ^{125}I -buserelin by the photoaffinity analogue PBL (■), the LHRH analogue des-Gly¹⁰-[D-Lys⁶]LHRH ethylamide (◆) and the biotinyl-LHRH analogue XBAL (▲), from male rat anterior pituitary membrane preparations. Specific binding was $66 \pm 5\%$ ($\pm \text{s.e.m.}$, $n = 8$) of total binding; non-specific binding was determined in the presence of $1 \mu\text{M}$ -LHRH. Values are the means $\pm \text{s.e.m.}$, $n = 4-8$. (b) Hofstee plot of PBL displacement of ^{125}I -buserelin from rat anterior pituitary membrane preparation. $K_d = 0.92 \pm 0.16 \text{ nM}$ ($\pm \text{s.e.m.}$, $n = 4$). (c) Hofstee plot of des-Gly¹⁰-[D-Lys⁶]LHRH ethylamide displacement of ^{125}I -buserelin from rat anterior pituitary membrane preparation. $K_d = 0.098 \pm 0.01 \text{ nM}$ ($\pm \text{s.e.m.}$, $n = 3$). (d) Hofstee plot of XBAL displacement of ^{125}I -buserelin from rat anterior pituitary membrane preparation. $K_d = 13.6 \pm 1.2 \text{ pM}$ ($\pm \text{s.e.m.}$, $n = 6$).

Fig. 3. Scatchard analysis of ^{125}I -XBAL binding

^{125}I -XBAL binding to rat anterior pituitary preparation is shown. All determinations were made in duplicate through the range of 5000 to 500000 counts of ^{125}I -XBAL added per assay tube. Non-specific binding was determined in the presence of $1\ \mu\text{M}$ -LHRH; specific binding was 30–60 % of total. $K_d = 131 \pm 16\ \text{pM}$ ($\pm$ S.E.M., $n = 3$), $B_{\text{max.}} = 63.2 \pm 5\ \text{fmol/anterior pituitary}$ ($\pm$ S.E.M., $n = 3$).

Fig. 4. Photoaffinity labelling of the LHRH receptor

The graph shows the percentage of the initial specific binding of ^{125}I -PBAL which had failed to dissociate after a period of 4 h at room temperature in the presence of $1\ \mu\text{M}$ -LHRH (that is, the irreversible specific binding) plotted against exposure time to u.v. light. In samples unexposed to u.v. light, some $95 \pm 12\%$ of initial specific ^{125}I -PBAL binding dissociated under these conditions. Initial specific binding was $10 \pm 3\%$ of total binding; non-specific binding was determined in the presence of $1\ \mu\text{M}$ -LHRH. Values are means $\pm$ S.E.M., $n = 3$.

case of ^{125}I -PBL, the binding displaceable by $1\ \mu\text{M}$ -LHRH (specific binding) was only $12 \pm 4\%$ ($\pm$ S.E.M., $n = 4$) of total binding [$2 \pm 1\%$ ($\pm$ S.E.M., $n = 4$) of total added ligand]. The photoaffinity analogue of des-Gly 10 -[D-Lys 6]LHRH ethylamide, that is ^{125}I -PBAL, gave specific binding of only $10 \pm 3\%$ ($\pm$ S.E.M., $n = 3$) of total binding [$3 \pm 1\%$ ($\pm$ S.E.M., $n = 3$) of total added ligand] and ^{125}I -XBAL-sulpho-SANPAH gave specific binding of only $15 \pm 4\%$ ($\pm$ S.E.M., $n = 4$) of total binding [$2 \pm 1\%$ ($\pm$ S.E.M., $n = 4$) of total added ligand]. These values for ligands containing aryl azido photoreactive groups were all much lower than for the other ligands such as buserelin and XBAL. After activation (0–10 min) with u.v. light (366 nm), only minimal levels of specific irreversible labelling could be detected for either ^{125}I -PBL or ^{125}I -XBAL-sulpho-SANPAH. (Specific irreversible binding was defined as above as the excess binding in total above non-specific samples that was resistant to 4 h dissociation in the presence of excess LHRH.) ^{125}I -PBAL showed a small amount of labelling, reaching a maximum, after 2 min activation, of $28 \pm 5\%$ ($\pm$ S.E.M., $n = 3$) of initial specific binding (Fig. 4). Since however the initial specificity of ^{125}I -PBAL binding was low (10 %), the specific irreversible binding represented only some 3 % of initial total binding and with a poor specific:non-specific binding ratio.

Homobifunctional chemical crosslinkers

The success of specific chemical crosslinking was dependent on two factors: firstly, on the ability of the reagents to allow specific ligand binding to the receptor without detrimental effects, and secondly, on their efficiency at rendering specific binding irreversible (Table 2). Of the reagents used, both DSS and BSSS had detrimental effects on the initial level of specific binding; that is, measured immediately after the 30 min crosslinking incubation, they reduced the difference in bound counts between total and non-specific samples. Control values of specific binding were reduced to 58 % in the presence of 0.2 mM-DSS, and to 55 % in the presence of 0.5 mM-BSSS. Whilst DST had less effect on initial specific binding at low concentrations (0.1–0.5 mM), it too had profound inhibitory effects on specific binding at higher concentrations. However, in contrast, EGS had no significant effect on specific binding until the highest concentration tested, 5 mM, at which there was a small reduction in initial specific binding, to 79 % (Table 2).

Concentrations above 1 mM-EGS were successful at crosslinking a proportion of the specific binding of ^{125}I -XBAL and rendering it resistant to dissociation. Concentration-dependent increases in irreversible specific binding were observed (Fig. 5) with a maximum of $23 \pm 3\%$ ($\pm$ S.E.M., $n = 3$) of the control level of initial specific binding being rendered irreversible after treatment with 5 mM-EGS. Both BSSS and DST also rendered significant proportions of specific binding irreversible but only at concentrations with profound inhibitory effects on specific binding. Under the present conditions, DSS appeared to be ineffective at crosslinking specific binding of XBAL to the LHRH receptor. The levels of non-specific irreversible binding were slightly increased (1.5–2-fold) by the highest concentrations of BSSS, DST and EGS, whereas DSS at concentrations of 1–3 mM caused large increases of greater than 10-fold. It appears therefore that all the reagents were active in these experiments, but that only EGS showed the required ability

Table 2. Crosslinking of ^{125}I -XBAL to rat anterior pituitary membranes using homobifunctional covalent crosslinkers

The effects of the crosslinking reagents DSS, BSSS, DST and EGS on the initial specific binding of ^{125}I -XBAL and their ability to irreversibly crosslink this specifically-bound ligand are shown at various concentrations. Rat anterior pituitary membrane preparations were equilibrated with ^{125}I -XBAL (90 min at 4 °C) prior to exposure to the crosslinkers or relevant solvent alone in the controls. The crosslinking reaction was quenched by the addition of excess glycine (67 mM). After centrifugation the membranes were resuspended into buffer containing 1 μM -LHRH in the presence of peptidase inhibitors and left at room temperature for 4 h. Meanwhile the initial specific binding (a) was determined as the difference between membrane counts in samples with or without 1 μM -LHRH present during the earlier equilibration with ^{125}I -XBAL and the crosslinking incubation. Results in (a) are expressed as percentages of control specific binding determined in the absence of any crosslinker (means $\pm$ S.E.M., $n = 4-12$). After 4 h dissociation of reversibly bound ligand, the same membrane samples were again centrifuged and the amount of specific binding remaining was determined. This irreversible specific binding (b) was expressed as a percentage of the previously measured (initial) specific binding in the presence of crosslinker which was resistant to dissociation (means $\pm$ S.E.M., $n = 4-12$). The irreversible specific binding gives an estimate of the amount of specific crosslinking of ^{125}I -XBAL to the LHRH receptor site achieved. In control sets of samples, routinely over 95% of the initial specifically-bound ligand had dissociated during the 4 h incubation. In the case of DSS, DST and EGS, their control experiments were carried out in the presence of 1% dimethyl sulphoxide (DMSO). The solvent had no deleterious effect on the initial specific binding, values in the presence of 1% dimethyl sulphoxide being $104 \pm 6\%$ ($\pm$ S.E.M., $n = 12$) of solvent-free controls. The overall yield of specifically bound ligand crosslinked to receptor is dependent on both (a) and (b). In the case of 5 mM-EGS, this was $18 \pm 3\%$ ($\pm$ S.E.M., $n = 5$), since there was $23 \pm 3\%$ ($\pm$ S.E.M., $n = 5$) crosslinking of specifically bound ligand (b), and the available specific binding (a) was reduced to $79 \pm 2\%$ ($\pm$ S.E.M., $n = 5$) by the disruptive effect of crosslinker on ligand binding. Since binding of ^{125}I -XBAL under these conditions was $44 \pm 6\%$ ($\pm$ S.E.M., $n = 4$) specific, these figures represent specific crosslinking to the receptor of some 8% of the total binding and since specific binding was $22 \pm 1\%$ ($\pm$ S.E.M., $n = 4$) of total added label, specific crosslinking of around 2% of total added label.

Crosslinker and reaction conditions	Concentration of crosslinker (mM)	(a) Initial specific binding (%)	(b) Irreversible specific binding (%)
DSS	0.025	96 ± 12	-6 ± 2
1% DMSO	0.2	58 ± 6	-3 ± 2
30 min on ice	0.3	35 ± 3	5 ± 5
	0.5	29 ± 8	1 ± 5
	1	23 ± 8	5 ± 11
	2	21 ± 10	4 ± 6
BSSS	0.5	55 ± 9	19 ± 6
No DMSO	1	56 ± 2	17 ± 4
30 min at room temperature	2	33 ± 1	24 ± 4
	5	42 ± 8	16 ± 5
DST	0.1	78 ± 9	-6 ± 4
1% DMSO	0.2	78 ± 13	-9 ± 3
30 min at room temperature	0.5	72 ± 14	17 ± 10
	1	54 ± 22	17 ± 7
	2	35 ± 8	30 ± 20
	5	23 ± 26	68 ± 25
EGS	0.1	99 ± 1	-1 ± 1
1% DMSO	0.2	100 ± 2	-2 ± 2
30 min on ice	0.5	93 ± 2	0 ± 4
	1	100 ± 4	3 ± 2
	2	94 ± 14	11 ± 3
	3	108 ± 4	17 ± 5
	5	79 ± 2	23 ± 3

to crosslink ligand bound specifically to the LHRH without disrupting the ligand-receptor interaction.

DISCUSSION

The present study describes the development of a novel approach for receptor purification. LHRH analogues incorporating biotin together with a covalent attachment site have been designed and synthesized, and methods for their covalent crosslinking to the LHRH receptor have been optimized.

Photoaffinity ligands may be expected to give a more specific labelling of receptor sites than the addition of non-specific bivalent crosslinkers, since the linking group

is already localized to the ligand. Activation of the photoreactive aryl azido group gives rise to a transient aryl nitrene with a non-selective reactivity (Ji, 1976), and so receptor labelling will not be dependent on the local presence of a particular chemical group. In contrast, chemical crosslinking is dependent both on the presence of suitable functional groups on both ligand and receptor and on their appropriate spatial separation (Pilch & Czech, 1984), but generally occurs in higher yield (Strosberg, 1984). The present experiments employed a series of homobifunctional *N*-hydroxysuccinimide ester crosslinkers with selective reactivity for amino groups. Since the separation between the side-chain free amino group of ^{125}I -XBAL and hypothetical amino groups around the receptor binding site is unknown, we used four cross-

Fig. 5. EGS crosslinking of specific binding of ^{125}I -XBAL to anterior pituitary membranes

The graph shows the percentage of the initial specific binding of ^{125}I -XBAL which had failed to dissociate after a period of 4 h at room temperature in the presence of LHRH (that is, the irreversible specific binding) plotted against concentrations of the bivalent crosslinker EGS used. Initial specific binding was $44 \pm 6\%$ ($\pm$ S.E.M., $n = 4$) of total binding; non-specific binding was determined in the presence of $1\ \mu\text{M}$ -LHRH. Values are the means $\pm$ S.E.M., $n = 4$ –12.

linkers of various bridge lengths. These range from DST at 0.5 nm (Smith *et al.*, 1978) to EGS at 1.51 nm (Baskin & Chung, 1980).

Initially, [D-Lys⁶]LHRH was chosen as the parent LHRH ligand for the analogues. Attaching a biotin-photoreactive aryl azido group via the free amine on D-Lys⁶ gave the PBL derivative which retained a high affinity for the LHRH receptor site, but a poor specific: non-specific binding ratio. As in previous reports (Perrin *et al.*, 1983), alteration of the analogue at the C-terminal to des-Gly¹⁰-ethylamide gave better specific binding. However, the photoaffinity ligands ^{125}I -PBAL and ^{125}I -XBAL-sulpho-SANPAH gave much higher non-specific binding than the biotin-free amine derivative ^{125}I -XBAL. The presence of the aryl azido group seemed to increase the non-specific binding of the analogue – perhaps due to low-level activation of the photoreactive group outwith the u.v. illumination. Although care was taken to protect the analogues from exposure to light, low levels were necessary for safe handling of the iodinated compounds. None of the photoaffinity analogues tested therefore

showed sufficient specificity for further use in receptor purification.

Of the bivalent crosslinkers used, EGS was the most successful in covalently crosslinking the ligand ^{125}I -XBAL to the receptor site. This is the longest of the crosslinking reagents tested, at 1.5 nm bridge length. Some 23% of the specific binding of ^{125}I -XBAL was rendered irreversible at the highest concentration of EGS tested, 5 mM. Both the appearance of an inhibitory effect on the ligand–receptor interaction and solubility problems prevented higher concentrations from being tested. This figure represents some 8% of the total binding (and 2% of added ligand) being specifically covalently attached to the receptor; sufficient to develop the strategy further for receptor purification.

Preliminary experiments examining displacement of [^3H]-biotin from avidin–sepharose showed that our biotinylated ligands retained a significant proportion of the potency of biotin even in the presence of detergent–salt solutions required for receptor solubilization (Ogier *et al.*, 1987). For example, PBL retained $45 \pm 5\%$ ($\pm$ S.E.M., $n = 3$) of the potency of native biotin. Using solubilized membranes previously labelled with ^{125}I -PBL, we have further shown that labelled membrane proteins do bind to an avidin–Sephacel column and can be displaced by elution with 2 mM-biotin (Mitchell *et al.*, 1987). This procedure resulted in an approx. 700-fold increase in the specific activity of labelled proteins compared with the initial membrane preparation. However, the low specificity of ^{125}I -PBL binding made it unsuitable for receptor purification. Preliminary experiments showed that membrane proteins specifically labelled with ^{125}I -XBAL and then crosslinked with EGS can be similarly purified by affinity chromatography on streptavidin–agarose. The higher level of intrinsic specificity in ^{125}I -XBAL binding means that its use with covalent crosslinkers such as EGS should facilitate extensive purification of the LHRH receptor.

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