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Fluorescent glucagon derivatives. I. Synthesis and characterisation of fluorescent glucagon derivatives

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The synthesis of monofluorescein, monorhodamine, and mono-4-nitrobenz-2-oxa-1,3-diazole (NBD) derivatives of glucagon is reported. The fluorescent groups were introduced by converting tryptophan-25 to 2-thioltryptophan using thiol-specific fluorescent reagents. All derivatives retained the ability to activate adenylate cyclase when compared to glucagon and thus were considered full agonists. IC_{50} values of $6.8 \cdot 10^{-9}$, $1.7 \cdot 10^{-8}$, $1.8 \cdot 10^{-8}$ and $5.4 \cdot 10^{-9}$ M were measured in rat liver membranes for NBD-, fluorescein-, rhodamine-Trp^{25}-glucagon and native glucagon, respectively. From the IC_{50} values K_d values of $2.16 \cdot 10^{-9}$, $4 \cdot 10^{-9}$, $2 \cdot 10^{-9}$ and $1.72 \cdot 10^{-9}$ M were calculated for the binding of NBD-, fluorescein-, rhodamine-Trp^{25}-glucagon and native glucagon, respectively. The highest quantum yield (0.18) of the monomer derivatives was obtained with fluorescein-Trp^{25}-glucagon in phosphate-buffered saline (pH 7.4). Difluorescein-glucagon was also prepared by reacting the amino groups of histidine-1 and lysine-12 with fluorescein isothiocyanate and dimer derivatives were prepared using fluorescein-labelled 2-thiolTrp^{25}-glucagon. Difluorescein-glucagon bound only weakly to glucagon receptors and displayed antagonist properties. The dimer derivative formed from two difluorescein-2-thiolTrp^{25}-glucagon molecules had similar poor binding qualities, whereas the dimer formed from difluorescein-2-thiolTrp^{25}-glucagon and 2-thiolTrp^{25}-glucagon exhibited, at low concentrations, properties similar to monofluorescein-glucagon. Both dimer derivatives were only sparingly soluble in aqueous medium. Specific binding of fluorescein-Trp^{25}-glucagon and difluorescein-glucagon to rat hepatocytes was followed using flow cytometry.

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Abbreviations: PBS, phosphate-buffered saline; EGF, epidermal growth factor; TMRM, tetramethylrhodamine maleimide; IAF, iodoacetamide fluorescein; IANBD, 4-[*N*-(iodoacetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole); NBD, 4-nitrobenz-2-oxa-1,3-diazole; $(S\text{-glucagon})_2$, glucagon dimer; DNPS-glucagon, tryptophan-(2,4-dinitrophenylsulfenyl)glucagon; DTT, dithiothreitol; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; Mops, 4-morpholinepropanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Introduction

The disposition of the receptor in the plasma membrane can be visualised in living cells by the use of suitable fluorescence labelled ligands. Studies with the β-adrenergic antagonist, NBD-alprenolol suggested that β-adrenoreceptors were predominantly preaggregated on Chang liver cells under physiological conditions [1]. These experiments, although directed at a receptor linked to the adenylate cyclase system, were performed with an antagonist. Thus, it is desirable to repeat and extend these studies using a fluorescence-labelled agonist. Glucagon was chosen for these studies because the hormone binds with high affinity to primary hepatocytes in culture, stimulates adenylate cyclase and, being a peptide, readily lends itself to specific modification. As glucagon does not possess any intrinsic sulfhydryl groups, Trp-25 was converted to 2-thioltryptophan [2] in order to provide a specific site for modification with sulfhydryl-reactive fluorescent reagents. In this manner, monomer derivatives of NBD-, fluorescein- and rhodamine-Trp^{25}-glucagon were synthesised. Difluorescein glucagon and glucagon-dimer derivatives were also synthesised. The newly synthesised fluorescent glucagon derivatives were tested with respect to their binding properties and ability to activate adenylate cyclase in primary hepatocytes and membranes. Equilibrium binding was also measured by flow cytometry, a technique which has been successfully applied in other hormone receptor-binding studies [3,4]. Finally, the spectroscopic properties of the fluorescent glucagon derivatives are described.

Preliminary data were presented at the Spring Meeting of the Bunsen-Gesellschaft für Physikalische Chemie, 'Mechanisms of Membrane Transport' Königstein, Taunus, F.R.G., 21–23 March, 1988.

Materials and Methods

Chemicals. Bacitracin, theophylline and bovine serum albumin were Sigma products. Tetramethylrhodamine maleimide, iodoacetamide fluorescein and 4[*N*-(iodoacetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole were obtained from Molecular Probes, Eugene, OR, U.S.A. Creatine kinase ATP and creatine phosphate were purchased from Boehringer Mannheim. 2,4-Dinitrophenylsulfonyl chloride, and fluorescein were obtained from Fluka and Merck, respectively. Dowex and glucagon were obtained from Serva. GF/B and GF/C filters were Whatman products and [^{32}P]ATP was from Amersham. P-2 Gel was obtained from Bio-Rad and Sephadex-G25 was obtained from Pharmacia. [^{125}I]glucagon (New England Nuclear) was a generous gift from Dr. H. Schoene, Hoechst Company, Frankfurt, F.R.G.

Spectroscopic studies. All absorption spectra were measured on a Pye-Unicam SP 8-100 UV/VIS spectrophotometer. Fluorescence measurements were carried out on a Schoeffel Model RRS 1000 fluorescence spectrometer using ratio mode and 5 nm excitation and emission slits at 25 °C.

Synthesis of fluorescent glucagon derivatives. 2-ThiolTrp^{25}-glucagon was prepared as described [2]. This compound spontaneously dimerises to give (*S*-glucagon)$_2$. (*S*-glucagon)$_2$ (6.9 mg) was dissolved in 1 ml 0.5 M Tris-acetate buffer (pH 8.3) and 950 mg (10 mmol) guanidine hydrochloride were added. To this, 30 μl 0.5 M 2-mercaptoethanol were added and stirred for 15 min at 0 °C. The distinctive UV/VIS spectra of 2-thiolTrp^{25}-glucagon and its dimeric form were used to monitor the progress of the reaction [2]. NBD-Trp^{25}-glucagon was prepared by adding 7 mg solid IANBD to the mixture and stirring for 30 min in the dark at 0 °C. On completion of the reaction, the product was separated from the excess unreacted IANBD by passage through a P-2 column. The yield of the orange solid was 64%. The preparation of fluorescein-Trp^{25}-glucagon was identical except that IAF was used in place of IANBD. The yield of the yellow solid was 55%. For the synthesis of rhodamine-Trp^{25}-glucagon, (*S*-glucagon)$_2$ was first reduced as described except that 0.1 M Mops buffer (pH 6.8) was used. The subsequent procedure was identical to that used for the other derivatives except that TMRM was used. A red solid was obtained at a 72% yield.

Difluorescein-(His^{1},Lys^{12})-glucagon was synthesised from glucagon by the following method: 5 mg of glucagon was reacted with 200 μl of 1 mM fluorescein isothiocyanate in the Tris-acetate buffer described above. The reaction mixture was

stirred in the dark at room temperature for 2 h before passage through a dry G25 column to remove unreacted fluorescein isothiocyanate. Difluorescein-(His[1],Lys[12])-2-thiolTrp[25]-glucagon was synthesised from 2-thiolTrp[25]-glucagon in the same manner. Carboxymethyl-(2-thiolTrp[25]-glucagon) and diacetyl(His[1],Lys[12]-glucagon) were synthesised as outlined above using iodoacetate and acetylisothiocyanate, respectively.

Two dimers of glucagon were synthesised using difluorescein-2-thiolTrp[25]-glucagon as an intermediate. In synthesising the fluorescent dimeric forms of glucagon, use was made of the previous observation that 2-thiolTrp[25]-glucagon dimerised rapidly in 0.5 M Tris-acetate buffer (pH 8.3) [2]. The heterodimer was obtained on dimerisation of difluorescein-2-thiolTrp[25]-glucagon with 2-thiol-Trp[25]-glucagon and the homodimer was obtained on dimerisation of two molecules of difluorescein-2-thiolTrp[25]-glucagon.

Purification. The glucagon derivatives were purified by reversed-phase chromatography on a Bondapak/Phenyl column in a Bruker LC 31B HPLC system. Samples were dissolved in 200 μl of 20% acetic acid and loaded onto the column pre-equilibrated in buffer A (distilled water acidified to pH 2.3 with trifluoroacetic acid) before washing with the same buffer for 5 min. This was followed by gradient elution with buffer B (85% acetonitrile in buffer A) up to a concentration of 50% over a 25-min period. This mixture was maintained for a further 10 min before the percentage of buffer B was increased to 100% over the next 10 min and maintained there for another 10 min. All samples were eluted at a flow rate of 2 ml/min.

Quantum yields of glucagon derivatives. The quantum yields of all derivatives were obtained by comparing the integrated fluorescence emission spectra of the derivative with that of a standard [5]. The standard was fluorescein in 0.1 M NaOH for which a quantum yield of 0.92 has been reported [6].

Preparation of rat liver membranes and hepatocytes. Rat liver membranes were prepared [7] and stored in liquid nitrogen until use. Hepatocytes were isolated by enzymatic digestion with collagenase [8]. Non-viable and non-parenchymal cells were separated using a percoll gradient equilibrated with Hepes buffer (137 mM NaCl/5.44 mM KCl/1.17 mM $MgSO_4$/0.15 mM KH_2PO_4/0.79 mM Na_2HPO_4/0.1% glucose/10 mM Hepes/1.0 mM $CaCl_2$, pH 7.4) [9]. Hepatocytes were either used immediately in suspension binding studies or for flow cytometric analysis or seeded in petri dishes at a density of $2 \cdot 10^5$/dish for analysis of adenylate cyclase activity, or at a density of 40000 per well in 24-well plates for monolayer binding studies. In all cases, cells were cultured in Ham's F12 medium (containing 10% fetal calf serum, $5 \cdot 10^{-8}$ M insulin and $1 \cdot 10^{-7}$ M dexamethasone) for two days prior to experimentation. The medium was changed twice daily.

[^{125}I]Glucagon binding. In suspension assays, hepatocytes ($1 \cdot 10^5$ to $5 \cdot 10^5$) were incubated with [^{125}I]glucagon (9 pM–18 nM) at 20°C in Hepes buffer (containing 0.1% BSA/0.1% bacitracin, final volume 0.5 ml) for 2 h with continuous shaking. Cell-bound radioactivity was separated from free by passage through GF/B filters that had been previously soaked for at least 1 h in 5% BSA. Cell-associated radioactivity was determined by gamma counting. The steady-state binding of [^{125}I]glucagon (20 pM–40 nM) to adherent cells was measured in PBS buffer (containing 0.1% BSA/0.1% bacitracin (pH 7.4)) at 20°C. After incubation for 2 h, cells were washed and cell-bound radioactivity was determined.

Fluorescent derivative binding. The ability of the derivatives to bind to glucagon receptor in liver membranes and rat hepatocytes was deduced from their ability to displace [^{125}I]glucagon. Derivatives (10 pM to 10 μM) were incubated with membranes (100 μg) and 0.5 nM [^{125}I]glucagon in 20 mM Tris-HCl buffer (pH 7.4; containing 0.1% bacitracin/0.1% BSA) for 20 min at 30°C. Unbound ligand was separated by passage though GF/C filters as above. For displacement experiments with primary hepatocytes the medium was removed and the cells were washed three times with 2 ml 0.15 M NaCl at 37°C. Cells were then incubated with 0.5 nM [^{125}I]glucagon and 10 pM–10 μM derivative in Hepes buffer (pH 7.4) containing 0.1% BSA and 0.1% bacitracin. Cells were incubated for 2 h at room temperature before being washed three times with 2 ml ice-cold PBS, containing 0.1% BSA (pH 7.4). The adherent cells were removed with 0.2 M NaOH and the

cell-associated radioactivity was measured.

Adenylate cyclase activity. The ability of the glucagon derivatives to stimulate adenylate cyclase was tested with rat liver membranes and hepatocytes in primary culture. Cyclase activity in membranes was measured as described [10]. Prior to the measurement of cyclase activity in adherent cells, the cells were washed three times with 2 ml 0.15 M NaCl at 37 °C. The cells were incubated with each derivative (10 pM–10 μM) in PBS buffer for 20 min at 30 °C, then washed with 2 ml 0.15 M NaCl, and removed from the petri dishes with 1 ml of ethanol. The ethanol was evaporated and the residue was resuspended in 600 μl 0.5 mM 3-isobutyl-1-methylxanthine. This solution was stored at −20 °C until aliquots were assayed for cAMP [11].

Flow cytometry. Cytometry was performed on a Becton-Dickinson FACS equipped with an argon ion laser. The chromophore was excited at 488 nm and emission was collected through standard filters for fluorescein. Percoll-prepared hepatocytes were washed and suspended in either PBS or Hepes buffer containing 0.1% BSA and 0.1% bacitracin (pH 7.4). Cell density was adjusted to 10^6 cells per ml before incubation with fluorescein-labelled derivatives (10^{-8} to 10^{-7} M) at 4 °C for up to 120 min. Non-specific binding was determined in the presence of 10^{-5} M native glucagon. At the end of the incubation, some samples were washed by rapid sedimentation and resuspension in ice-cold buffer prior to cytometric analysis. A total of 10000 cells were counted per sample. Analysis was triggered by the scattering signal in a two-parameter mode. The frequency distribution of the scattering signal was essentially unimodal and remained constant regardless of the cell treatment, indicating that no gross morphological changes had occurred.

Amino acid analysis. Samples of carboxymethyl-(2-thiolTrp^{25}-glucagon) and diacetyl(His^1,Lys^2-glucagon) were prepared for amino acid analysis [12] and analysed on a Biotronik BT6000E amino acid analyser.

Results

Synthesis and purification of derivatives

Synthesis of the 2-thiolTrp^{25}-glucagon and the 2,4-dinitrophenyl derivative (DNPS-glucagon) was monitored on the basis of the UV/VIS spectra of these compounds, since the spectra of DNPS-glucagon and 2-thiolTrp^{25}-glucagon have distinctive shoulders at 360 nm and maxima at 320 nm. The dimerisation of 2-thiolTrp^{25}-glucagon could be followed by the disappearance of the shoulder at 360 nm. The dimer was routinely reduced and converted back to the monomer with a 5-fold excess of 2-mercaptoethanol before alkylation. The reactions with the iodo- and maleimide compounds were carried out at pH 8.3 and pH 6.8, respectively, as these conditions have been shown to favour alkylation of exposed sulfhydryl groups without reacting with amino groups [13]. After separation of excess alkylating agent by rapid gel filtration, successful synthesis was indicated by the formation of coloured solids at high (55–72%) yield. Reversed-phase high-performance liquid chromatography with acetonitrile-water-trifluoroacetic acid mixtures gave rise to three well-resolved peaks. The relative retention times of the various derivatives are given in Table I. The highly coloured fluorescent products were all shown to elute in the region of the second peak. Peaks 1 and 3 were identified as unreacted glucagon and the dimeric form of 2-thiolTrp^{25}-glucagon, respectively. Unreacted monomeric 2-thiolTrp^{25}-glucagon was not seen in the elution profile as a result of dimer formation under the conditions used. In order to ascertain that only Trp-25 was deriva-

TABLE I

HPLC PURIFICATION OF FLUORESCENT DERIVATIVES

Heterodimer contains one molecule of 2-thiolTrp^{25}-glucagon and one molecule of difluorescein-(2-thiolTrp^{25}-glucagon). Homodimer contains two molecules of difluorescein-(2-thiolTrp^{25}-glucagon).

Compound	Retention time (min)
Native glucagon	34.5 ± 0.2
NBD-Trp^{25}-glucagon	36.7 ± 0.1
Fluorescein-Trp^{25}-glucagon	36.8 ± 0.3
Rhodamine-Trp^{25}-glucagon	37.5 ± 0.3
(*S*-glucagon)$_2$	40.2 ± 0.2
Difluorescein(His^1,Lys^{12})-glucagon	38.5 ± 0.2
Heterodimer	39.5 ± 0.3
Homodimer	43.5 ± 0.3

TABLE II

AMINO ACID ANALYSIS OF GLUCAGON AND CARBOXYMETHYL-(2-THIOLTrp^{25}-GLUCAGON) AND DIACETYL(His^{1},Lys^{12}-GLUCAGON)

Values in brackets in column 1 are the theoretical number of each amino acid per mol glucagon. n.d., not determined.

Amino acid	Glucagon	Carboxymethyl-(2-thiolTrp^{25}-glucagon) [a]	Diacetyl (His^{1},Lys^{12}-glucagon) [b]
Lys (1)	0.99	1.14	0.09
His (1)	0.99	0.90	0.25
Arg (2)	1.98	2.20	2.11
Asp (4)	4.10	4.12	4.03
Thr (3)	3.34	3.23	2.63
Ser (4)	3.78	4.06	4.08
Glu (3)	3.08	3.18	3.08
Gly (1)	0.88	1.11	0.99
Ala (1)	0.76	0.92	0.90
Val (1)	1.02	0.82	0.76
Met (1)	1.29	0.73	0.70
Leu (2)	1.69	1.56	2.25
Tyr (2)	1.97	2.17	2.05
Phe (2)	2.09	2.31	1.95
Trp (1)	0.98	0.02	n.d.

[a] 2-ThiolTrp^{25}-glucagon was reacted with iodoacetate.
[b] Glucagon was reacted with acetylisothiocyanate.

tised, amino acid analysis was carried out. Hydrolysis was performed under conditions where the destruction of tryptophan residues was avoided. Since it was not possible to analyse the fluorescent derivatives because of the highly hydrophobic nature of the tryptophan adducts, iodoacetate was reacted with 2-thiolTrp^{25}-glucagon under identical conditions and subsequently purified by HPLC. Amino acid analysis (Table II) indicates that iodoacetate had indeed reacted specifically with the tryptophan thiol residue, since essentially no unreacted tryptophan residue could be detected in the lysate. On the other hand, lysine and histidine, the two other potential reactive groups, were found in equal proportions both in native and in derivatised glucagon. Assuming that iodoacetate reacts like other sulfhydryl reagents used for the derivatisation, it would follow that Trp-25 is the only site on the glucagon molecule to which the fluorescent groups were attached.

Difluorescein-glucagon synthesis resulted in the formation of a highly coloured product. Dimer synthesis relied on the spontaneous association of 2-thiolTrp^{25}-glucagon and was evident even when all the glucagon present had been previously reacted with fluorescein isothiocyanate. Difluorescein-glucagon and the dimers were readily separable by HPLC (Table I) and amino acid analysis of glucagon reacted with acetylisothiocyanate (Table II) confirmed that only His-1 and Lys-12 were likely to be labelled with fluorescein and that only 8% of the available lysine residues and 25% of the histidine residues were unlabelled. Thus, at least 68% of the glucagon isolated in the fluorescently labelled fraction contained two fluorescein groups.

Spectroscopic studies

The molar absorption coefficients and quantum yields of each derivative calculated from their UV/VIS absorption spectra and fluorescent emission spectra are summarised in Table III. The absorption and fluorescence spectra were consistent with the successful coupling of the fluorophores to glucagon. Of the derivatives formed through Trp-25, fluorescein-Trp^{25}-glucagon has the highest quantum yield (0.18) in PBS buffer > rhodamine-Trp^{25}-glucagon (0.07) > NBD-Trp^{25}-glucagon (0.02). Difluorescein-glucagon and the

TABLE III

ABSORPTION AND FLUORESCENCE CHARACTERISTICS OF GLUCAGON DERIVATIVES

Spectra were measured in PBS buffer (pH 7.4). Heterodimer and homodimer are as defined in Table I.

Derivative	Absorption maximum (nm)	Absorption coefficient $E(M^{-1}\cdot cm^{-1})$	Emission maximum (nm)	Quantum yield (Φ)
NBD-Trp^{25}-glucagon	496	$1.3\cdot10^{4}$	530	0.02
Fluorescein-Trp^{25}-glucagon	496	$2.6\cdot10^{4}$	520	0.18
Rhodamine-Trp^{25}-glucagon	553	$2.7\cdot10^{4}$	580	0.07
Difluorescein-(His^{1},Lys^{12})-glucagon	496	$5.2\cdot10^{4}$	520	0.27
Heterodimer	496	$5.2\cdot10^{4}$	520	0.27
Homodimer	496	$1.0\cdot10^{5}$	520	0.27

dimer derivatives were considerably more fluorescent than the sulfhydryl-linked derivatives with quantum yields of 0.27. The higher relative quantum yield probably results from their amide linkage to the fluorophore.

[^{125}I]Glucagon binding

A high receptor number is the most important criterion determining the suitability of a particular cell line for fluorescence microscopic receptor studies. Hepatocytes change morphologically to a high degree in primary culture and this is associated with alterations in hormone responsiveness and second messenger metabolism [14]. Previously, binding studies have been performed with freshly prepared cells in suspension [9,15,16]. However, the binding of glucagon to cells in suspension and in monolayer culture was very similar, and the Scatchard plots could be fitted by non-linear regression analysis for a two-site model. Hepatocytes possess, both in suspension (data not shown) and after 2 days in monolayer cultures (see Fig. 1, approx. 30000 high affinity binding sites per cell with a K_d of $1.5 \cdot 10^{-9}$ M and 200000 low affinity sites per cell with a K_d of $1 \cdot 10^{-8}$ M.

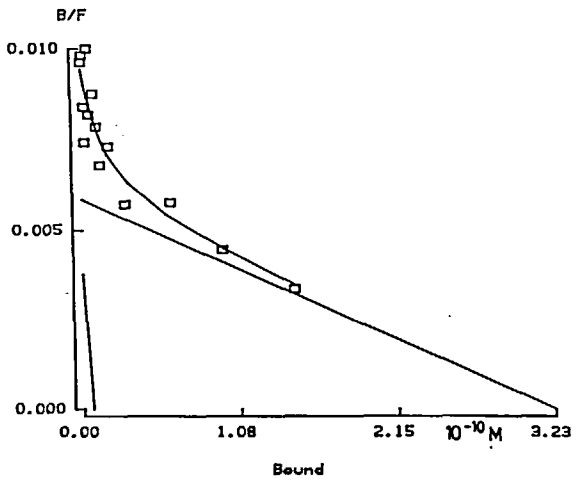

Fig. 1. Scatchard analysis of [^{125}I]glucagon binding to rat hepatocytes cultured in monolayers. Cells were incubated with 9 pm–18 nM [^{125}I]glucagon for 2 h at 20 °C. Non-specific binding was determined in the presence of 10^{-6} M native glucagon. The data were fitted by non-linear regression analysis [17,18] and the diagram shows the results of one such experiment and includes the lines of best fit.

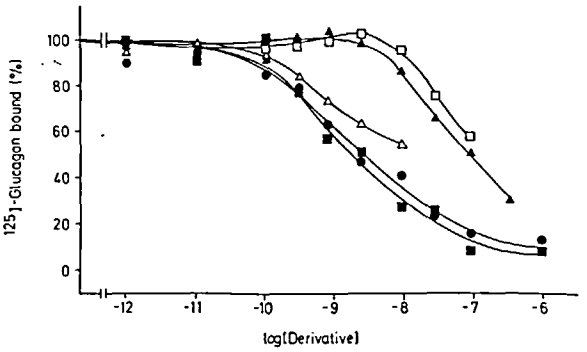

Fig. 2. Displacement of [^{125}I]glucagon by fluorescent glucagon derivatives. Native glucagon (●), fluorescein-Trp^{25}-glucagon (■), difluorescein(His^{1},Lys^{12})-glucagon (▲) and the difluorescein(2-thiolTrp^{25}-glucagon) containing heterodimer (△) and homodimer (□) (see text) were incubated with liver membranes as described under Materials and Methods.

Derivative binding properties and adenylate cyclase activation

The ability of the derivatives to bind to glucagon receptors was assessed by their ability to displace [^{125}I]glucagon from both isolated hepatocytes in primary culture and rat liver membranes. All derivatives were able to displace [^{125}I]glucagon from isolated hepatocytes and the rat liver membranes (Fig. 2). The dissociation constants were calculated [17,18] assuming a value of 2 nM for the dissociation constant of [^{125}I]glucagon [19]. The derivatives formed through Trp-25 bound to both membranes and cells with affinities similar to native glucagon (K_d $1.68 \cdot 10^{-9}$ M); NBD-Trp^{25}-glucagon, $2.2 \cdot 10^{-9}$ M; fluorescein-Trp^{25}-glucagon, $2.8 \cdot 10^{-9}$ M; rhodamine-Trp^{25}-glucagon, $2.9 \cdot 10^{-9}$ M. The EC_{50} values for adenylate cyclase activation were also similar and these derivatives showed full agonist activity (Fig. 3). Difluorescein-(His^{1},Lys^{12})-glucagon (K_d $8.25 \cdot 10^{-8}$ M) and the homodimer showed much lower binding affinity and poor agonist activity. In general, the dimeric glucagon derivatives appeared to have the binding properties of their respective components, although the problem of low solubility precluded measurements over the full concentration range. Over the concentration range permitted, the heterodimer displaced radiolabelled glucagon and stimulated adenylate cyclase as efficiently as fluorescein-Trp^{25}-glucagon, whereas the homodimer bound and activated adenylate cyclase

only weakly, thus resembling the difluorescein derivative.

Flow cytometric studies

Incubation of primary hepatocytes with $5 \cdot 10^{-8}$ and 10^{-7} M fluorescein-Trp^{25}-glucagon at 4° C for more than 40 min led to a dose-dependent increase in the mean fluorescence of the cells. This was shown as an increase in the observed channel number of maximum fluorescence. No difference in fluorescence intensity could be detected following further incubation for 60 or 120 min indicating that the system had reached equilibrium. The addition of 10^{-5} M glucagon caused only a slight reduction in total fluorescence, indicating that the observed increase in fluorescence was not all the result of the specific binding of the derivative to glucagon receptors. However, washing the cells prior to cytometric analysis led to a reduction in non-specifically bound fluorescence. When cells were incubated with 10^{-7} M fluorescein-Trp^{25}-glucagon for 120 min at 4° C and washed, the fluorescence was significantly higher than that in the presence of 10^{-5} M glucagon, where it was similar to the level of the autofluorescence of the hepatocytes (Fig. 4). The difference in fluorescence was considered to be specific receptor binding. Similar results were obtained with difluorescein-glucagon (data not shown). Incubation with fluorescent glucagon dimers at both 30° C and 4° C led to a large increase in the fluorescence intensity of the hepatocytes and a departure from the bimodal fluorescence distribution seen with fluorescein-Trp^{25}-glucagon. This could not be reduced by washing the cells or prior incubation with 10^{-5} M native glucagon and, as a result, no further experiments could be undertaken with these derivatives. The high non-specific binding may result from the high content of hydrophobic fluorescein groups.

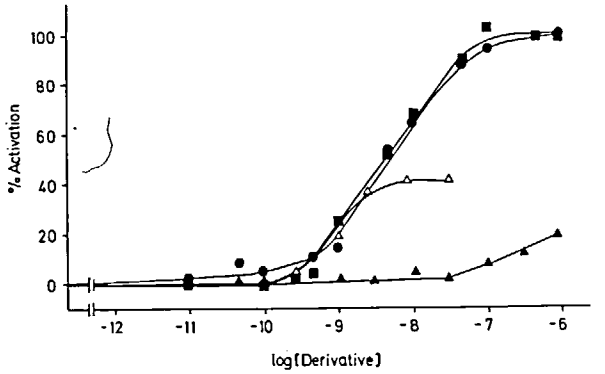

Fig. 3. Stimulation of adenylate cyclase by glucagon and glucagon derivatives. Native glucagon (●), fluorescein-Trp^{25}-glucagon (■), difluorescein-glucagon (▲) and heterodimer (△) were incubated with liver membranes as described under Materials and Methods.

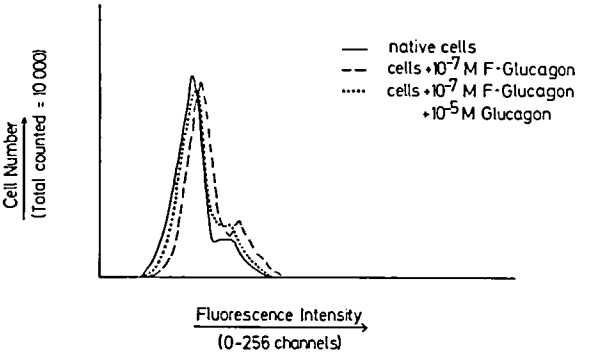

Fig. 4. Binding of fluorescein-Trp^{25}-glucagon (F-glucagon) to primary hepatocytes measured by FACS. Typical fluorescent histograms obtained by flow cytometry. Cells (1 million/ml) in PBS containing 0.1% BSA/0.1% bacitracin (pH 7.4) were incubated for 2 h at 4° C in the presence of fluorescein-Trp^{25}-glucagon.

Discussion

The fluorescent monomer derivatives of glucagon described above appear to be suitable probes of the glucagon receptor, since they retain the ability to stimulate adenylate cyclase and possess relatively high binding affinities. Their ability to activate adenylate cyclase is in agreement with reports using other glucagon analogues that have been derivatised in this region [20]. It appears that the carboxyl terminal region containing Trp-25 is involved in recognition of the receptor but not in the conformational transition in the course of activation, a function that has been assigned to the N-terminal region [20]. Indeed, glucagon derivatives that have been derivatised through Trp-25, namely 2-thiolTrp^{25}-glucagon, its monomeric and dimeric forms [2] and DNPS-glucagon and its dimeric form [21] have all been shown to retain the ability to activate adenylate cyclase. As would be predicted, the attachment of a large fluorescent

group near the carboxy terminal only slightly lowers the binding affinity. This relatively minor reduction in binding affinity does not preclude the use of these derivatives as sensitive fluorescent probes, since they still exhibit dissociation constants in the order of 2–3 nM. There was good correlation between the binding affinities of the fluorescent analogues and their EC_{50} values for activation of adenylate cyclase. This relationship is consistent with models in which the activation of the cyclase via the G-proteins is a function of the fractional occupation of the hormone receptor [22]. Treatment with fluorescein isothiocyanate resulted in the addition of fluorescein to the N-terminal His-1 and Lys-12. The binding affinity of both difluorescein-glucagon and the homodimer formed from it was reduced and their ability to activate adenylate cyclase was drastically reduced. This is consistent with modification of the free amino groups of glucagon [23,24]. However, the heterodimer, containing one molecule of 2-thiolTrp^{25}-glucagon, retained binding properties similar to native glucagon. Since a relatively high concentration of radiolabelled glucagon was used in the displacement studies, the ability of the derivatives to bind to high- and low-affinity receptors could not be assessed. Despite the high fluorescence of the dimers, their use was limited by their poor solubility and their high non-specific binding as seen by the generation of highly fluorescent anomolous cell populations in flow cytometry which could not be abolished by pretreatment with native glucagon. Cytometric analysis of hepatocytes from different regions of the liver show a spectrum of metabolic and morphological properties [25,26]. Thus it is possible that these derivatives bind selectively to only one population of hepatocytes, although the special circumstances for this association remain to be determined.

The mono- and difluorescein-glucagon derivatives were preferred to the more strongly binding NBD derivative for flow cytometric studies because of their higher quantum yields and relatively lower non-specific binding. The low fluorescence of the NBD-derivative was most likely the result of the fluorophore being linked to glucagon through a sulfhydryl group. NBD has been shown to be highly fluorescent when it is attached to proteins through an amide bond [27], although the derivative may become extremely environmentally sensitive and most fluorescent when in a hydrophobic environment [28].

The equilibrium binding studies, in agreement with previous studies using freshly prepared cells in suspension [14,15,29], confirm that hepatocytes in suspension possess a large heterogeneous population of glucagon receptors. Wakelam et al. [29] have described a functional classification in which high-affinity sites are linked to phosphatidylinositol metabolism and lower-affinity sites regulate adenylate cyclase. Freshly prepared rat hepatocytes exhibited a bimodal autofluorescence distribution in the flow cytometer. This does not seem to result from cells of different sizes, since essentially only a single population of cells could be detected by low-angle scatter. The high and heterogeneous autofluorescence may limit the usefulness of primary hepatocytes for a quantitative analysis of glucagon receptor mobility and distribution with the fluorescent glucagon derivatives in microscopic studies with primary hepatocytes. In contrast to flow cytometry in which a large number of cells are analysed and a statistical distribution of cellular fluorescence is generated, fluorescent microscopic studies rely on measurements made on single cells. Therefore, heterogeneity of the cellular fluorescence could make quantitation of specific binding to receptors difficult. In the following paper, the first results obtained with these new fluorescent glucagon derivatives are reported using video intensification microscopy and hepatocytes in monolayer culture.

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