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HIGH AFFINITY BINDING SITES ON PLASMA MEMBRANE OBTAINED FROM THE LYMPHOBLASTOID CULTURED 1301 CELL LINE FOR HIGHLY RADIOACTIVE SERUM THYMIC FACTOR

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The interaction of the synthetic serum thymic factor (FTS, facteur thymique sérique) with a plasma membrane preparation of human T lymphocytes from the lymphoblastoid T cell line 1301 was studied using ^{3}H-labelled FTS (specific activity 120 Ci/mmol). The binding is temperature dependent and function of the concentration of both ^{3}H-labelled FTS and membrane proteins. At 37°C, using 1 nM of ^{3}H-labelled FTS a steady state is observed within 80 min. The binding is reversible, specific and saturable. Scatchard analysis reveals the existence of at least two binding sites with respective K_d of the order of 0.516±0.2 nM and 110±27.8 nM with concentrations of 0.186±0.045 pmol and 2.026±0.367 pmol per mg of membrane protein.

Introduction

The serum thymic factor FTS (facteur thymique sérique) is an immunologically active peptide which has been characterized by its capacity to induce the appearance of T cell markers on immature T cells [1]. The absence of FTS activity in the serum of thymectomized (Tx) or nude mice (athymic) and its reappearance after thymus grafting demonstrate its strict thymic dependence [2]. Its thymic origin has been furthermore demonstrated by its purification from thymic extracts [3] and by its localization in the thymus gland using immunofluorescence assays [4]. FTS has been isolated from pig serum [5], sequenced [6] (pyro Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn) and synthesized [7]. The synthetic material shows full biological activity and displays the same chromatographical characteristics as naturally derived FTS [6,7]. Formely, some of us [8] using a tritium-labelled derivative of FTS as a tracer, have characterized a specific cellular receptor on two lymphoblastoid T cell lines, 1301 and HSB2. The specific activity of this ^{3}H-labelled methylated FTS derivative was 5 Ci/mmol [8].

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Abbreviations: FTS (Facteur thymique sérique) for the nonapeptide

pyro Glu(1)-Ala(2)-Lys(3)-Ser(4)-Gln(5)-Gly(6)-Gly(7)-Ser(8)-Asn(9) OH

corresponding to the porcine serum thymic factor. The serum thymic factor analogs obtained by replacement, for instance the L-Asn in position 9 by L-Asp, or L-Ser in position 4 by D-Ser are designated [Asp^9]FTS and [D-Ser^4]FTS, respectively.
Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.
Tx mice, Thymectomized mice or mice with ablation of thymus.

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Now, a very highly radioactive FTS has been prepared (see Ref. 9) by catalytic tritiation of a triple bond containing FTS analogs obtained by replacing lysine by an acetylenic lysine residue [9]. This ^{3}H-labelled FTS thus prepared without any change of the chemical structure of the natural FTS, showed full biological activity and displayed the same immunoreactivity as the synthetic FTS.

In the present paper we report a procedure for the preparation of purified plasma membranes obtained from the 1301 cell line and the presence in these plasma membranes of highly specific binding sites for ^{3}H-labelled FTS.

Materials and Methods

Lymphoblastoid cell line. The 1301 cell line was obtained from S.M. Fu and M. Fellous. It was derived from a leukocyte culture of patients with acute lymphoblastic leukemia. Cells were maintained at 37°C in RPMI 1640 essential medium supplemented with 10% fetal calf serum, 1% glutamine, antibiotics and Fungizone. The cells were used for membrane preparation when they were in stationary phase of growth. Their viability was determined by their capacity to exclude Trypan blue dye.

Labelled FTS. The synthesis of triple bond containing FTS analogue [10] was performed by introducing a derivative of L-2,6-diamino-4-hexynoic acid (Dha, commonly acetylenic lysine) and the ^{3}H-labelled FTS was obtained after catalytic tritiation of this unsaturated FTS analogue [10].

After purification, thin layer chromatography was performed, biological activity in the rosette assay and immunoreactivity were assessed. No difference between unlabelled and labelled FTS was observed. Specific activity, determined by radioimmunoassay [11] and amino acid analysis was 120 Ci/mmol.

FTS, FTS analogues and biological activity determination. FTS and FTS analogues including [Asp^9]FTS, [D-Ser^4]FTS, [Pro^1]FTS and [Orn^3]FTS were synthesized by the homogeneous phase method [12,13]. Their biological activities were assessed by the rosette assay. This test has already been described in detail [14]. In brief, it consists in determining the ability of FTS or FTS analogues to confer azathioprine sensitivity on spleen rosette-forming cells from adult Tx mice. The lowest concentration of the peptide that induces this activity is considered as the minimal active concentration. The minimal active concentration for FTS is 10 fM and analogues which induce azathioprine sensitivity at concentrations higher than 10 nM are considered as inactive. Inactive analogues are tested in the rosette assay for their ability to inhibit the effect of FTS, as described elsewhere [15]. An analogue is considered as an FTS antagonist when its minimal active concentration inhibiting the effect of FTS (5 nM) is in the range of 0.05 to 10 nM.

Buffer used. The homogeneization buffer consisted of 10 mM Tris, 10 mM $CaCl_2$, and 0.25 M sucrose adjusted to pH 7.40. The homogeneization buffer was buffer A.

Binding studies were performed in a medium containing 135 mM NaCl, 50 mM glucose, 50 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Tris, and adjusted to pH 7.40 with Hepes. This binding buffer was buffer B.

Preparation of plasma membranes. All operations were performed at 0–4°C; $(100–200) \cdot 10^6$ 1301 cells were washed three times in Hanks solution to remove fetal calf serum and culture medium. The cells were suspended in 7 ml of buffer A. Only the cell suspensions with a viability of 95% or more were used. The presence of sucrose in buffer A, as described by Shiu and Friesen [16] allowed for the production of a cell suspension without aggregation and with less nuclear disruption. Homogenization was performed in a Dounce homogeneizer using a tight fitting pestle (B type). Fifteen strokes were sufficient to disrupt almost all the cells.

The whole homogenate was centrifuged at $1000 \times g$ for 20 min. The pellet was resuspended in 7 ml of buffer A, submitted to 10 strokes in the same Dounce homogeneizer and centrifuged as described above. The supernatants of the two $1000 \times g$ centrifugations were pooled and centrifuged again at $20000 \times g$ for 60 min. The $20000 \times g$ supernatant was centrifuged at $105000 \times g$ for 60 min. The pellets of these last three centrifugation phases were called fractions A, B and C, respectively. The fractions A, B and C were resuspended in buffer B (2 to 3 ml) and divided into 0.5 ml aliquots. The

fractions were immediately used or stored in liquid nitrogen or at −20°C. All fractions were submitted to protein, DNA determinations and enzymatic assays.

Protein and DNA determinations. Protein evaluation was made according to Lowry et al. [17] using bovine serum albumin as a standard. DNA was determined using the diphenylamine reaction according to Burton [18], modified by Giles and Myers with calf thymus DNA as a standard.

Enzymatic assays. 5′-Nucleotidase and alkaline phosphatase activities were used as markers for plasma membranes. 5′-Nucleotidase activity (EC 3.1.3.5) was determined by the method described by Emmelot et al. [19], inorganic phosphore production was determined as described by Chen et al. [20]. Alkaline phosphatase activity (EC 3.1.3.1) was measured according to Garen and Levinthal [21]. β-Glucuronidase activity (EC 3.2.1.31) was used as a marker for lysosomes; it was determined according to Fishman et al. [22]. Succinate dehydrogenase enzyme (EC 3.1.99.1) a mitochondrial marker, was measured according to Tisdale [23].

^{3}H-labelled FTS binding. The fraction containing the highest 5′-nucleotidase and alkaline phosphatase activities and the lowest DNA concentration were used for binding studies: 0.1 ml of membrane suspension (5 to 80 μg of protein) was mixed with an equal volume of buffer B containing ^{3}H-labelled FTS at a given concentration and adjusted to 0.3 ml with buffer B. Non specific binding was determined in the presence of a 1000-fold excess of unlabeled FTS. At the end of the incubation time, 2 ml of ice-cold buffer B were added and the samples were passed through Whatman GF/C filters, presoaked in buffer B before use. The filters were washed three times with 5 ml of ice-cold buffer B and dried. The radioactivity collected on the filters was counted by liquid scintillation spectrometry. Each determination was performed in triplicate.

Results

Characterization of plasma membranes

5′-Nucleotidase and alkaline phosphatase activities, plasma membrane markers were investigated in the pellet of these three centrifugation steps (A, B and C) (Table I). Maximal specific activities were present in fraction B but low specific activities were present in fraction C which was contaminated by plasma membrane fragments. In contrast, the specific activity of β-glucuronidase, a cytoplasmic enzyme, was concentrated in fraction C. DNA concentration was the highest in fraction A, due to the presence in this fraction of nuclei fragments and intact cells. The specific activity of succinate dehydrogenase enzyme, a mitochondrial marker, was very low or undetectable (0.5 $nmol \cdot min^{-1} \cdot mg^{-1}$ for fractions B and C) in fractions obtained in the membrane preparation. A low concentration of mitochondria was confirmed by microelectronic analysis of intact 1301 cells. Such a low concentration of mitochondria has been reported by other authors in tumoral and derived leukemic cell lines [24].

FTS binding studies

In order to determine in membrane preparations the fractions of 1301 cells binding specifically ^{3}H-labelled FTS, incubation of fractions A, B and C (100 μg/0.3 ml) with ^{3}H-labelled FTS

TABLE I

CHEMICAL COMPOSITION AND ENZYMATIC SPECIFIC ACTIVITIES OF DIFFERENT FRACTIONS A, B AND C OBTAINED DURING PLASMA MEMBRANE PREPARATION FROM THE 1301 CELL LINE

DNA was expressed in μg per mg of protein. Specific activities at 37°C were expressed as μmol/min per mg of protein for alkaline phosphatase and for β-glucuronidase and as nmol/min per mg of protein for 5′-nucleotidase and for succinate dehydrogenase.

Enzyme activities and DNA content	Fraction A	Fraction B	Fraction C
5′-Nucleotidase	0.2	25	2.5
Alkaline phosphatase	5	20	1
β-Glucuronidase	40	25	100
Succinate dehydrogenase	1.3	0.5	0.5
DNA	300	3	10

(2 nM) during 120 min at 37°C was performed. Binding capacities were 32 ± 11 fmol, 1250 ± 430 fmol, and 22 ± 15 fmol per mg of protein for A, B and C fractions, respectively. Only fraction B bound specifically ^{3}H-labelled FTS. Binding studies were performed in subsequent experiments using this fraction. ^{3}H-labelled FTS specific binding capacity of fraction B was stable for 1 or 2 weeks when it was stored in liquid nitrogen.

Characteristics of binding

Effects of temperature and duration of incubation. Binding of ^{3}H-labelled FTS to the 1301 membranes is time- and temperature-dependent. No binding was observed at 4°C. At 20°C and 30°C equilibrium was approached after 6 and 4 h of incubation, respectively. At 37°C, binding was more rapid and a steady state was obtained after 80 min of incubation. The stationary phase was stable for more than 2 h (Fig. 1). In the presence of a large excess (2 μM) of unlabeled FTS only a small percentage of ^{3}H-labelled FTS was bound (1 to 5% of the total binding).

Dissociation kinetics. After 2 h of incubation at 37°C of membranes with ^{3}H-labelled FTS (1 nM) the addition of unlabelled FTS (20 μM) induced a dissociation of the binding (Fig. 2). The dissociation curve replotted on a semi-logarithmic scale displayed apparently only one first order kinetic. 50% of specifically bound ^{3}H-labelled FTS were dissociated after 80 min. The k_d value was $2.09 \cdot 10^{-4} s^{-1}$ (Fig. 2 inset).

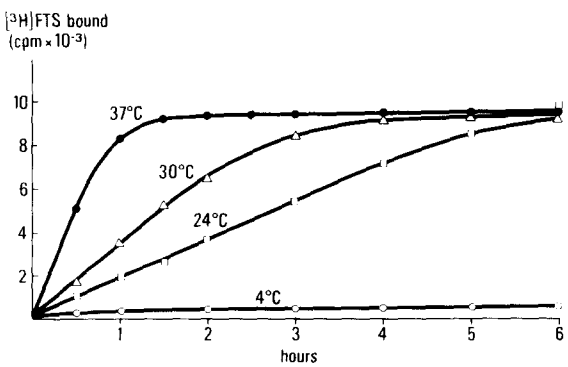

Fig. 1. Time and temperature dependence of ^{3}H-labelled FTS specific binding to the 1301 plasma membranes. 30 μg of membrane protein were incubated in 0.3 ml of buffer B containing 2 nM ^{3}H-labelled FTS at different temperatures. At various times, specific binding was determined as described in Experimental Procedures.

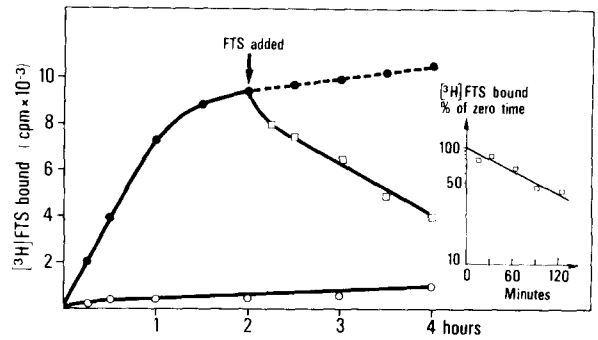

Fig. 2. Dissociation of ^{3}H-labelled FTS from its membrane receptors. 30 μg of protein membrane were incubated at 37°C with 2 nM ^{3}H-labelled FTS with (○) or without (●) 2 μM of unlabelled FTS. After 2 h of incubation unlabelled FTS (20 μg/ml, 20 μM) was added to the tubes containing ^{3}H-labelled FTS alone (□). Inset: Semi-logarithmic replot of the dissociation curve.

Effects of membrane concentration. The specific binding of ^{3}H-labelled FTS to 1301 membranes is a saturable process with respect to membrane protein concentration. At 37°C, the maximum ^{3}H-labelled FTS specific binding was obtained with about 500 μg of membrane protein per ml. In the range of 0 to 300 μg membrane protein per ml specific ^{3}H-labelled FTS binding was a linear function of membrane protein concentration. Therefore, subsequent binding studies were performed at 37°C using between 10 μg to 80 μg per volume of 0.3 ml.

Specificity of the binding. The ^{3}H-labelled FTS binding to 1301 membranes was competitively inhibited by low concentrations of unlabelled FTS. Increasing concentrations of unlabelled FTS gave a classical displacement curve, as shown in Fig. 3. Molecular analogues of FTS were also tested for their ability to interfere with specific binding of ^{3}H-labelled FTS to membranes. Biologically active FTS analogues, such as the agonist [Pro^1]FTS and the antagonist [Asp^9]FTS were effective competitors to ^{3}H-labelled FTS binding. Conversely, the biologically inactive analogues [D-Ser^4]FTS and [Orn^3]FTS did not alter ^{3}H-labelled FTS binding. Fig. 3 and Table II summarize these results.

Saturation data. Total ^{3}H-labelled FTS binding and non-specific binding (determined by an excess of at least 1000 of unlabeled FTS concentration) were studied as a function of total

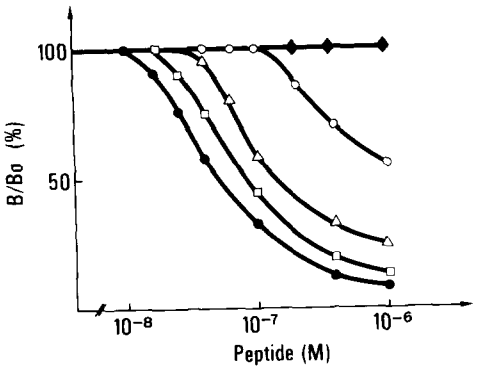

Fig. 3. Inhibition of ^{3}H-labelled FTS binding by unlabelled FTS and FTS analogs. 25 μg of protein membrane were incubated 80 min at 37°C in 0.3 ml of buffer B containing 1 nM ^{3}H-labelled FTS in the presence of peptides in the indicated range of concentration. FTS (●), [Asp^9]FTS (□), [Orn^3]FTS (○), [D-Ser^4]FTS (◆) and [Pro^1]FTS (△). Results are expressed in percentage of ^{3}H-labelled FTS bound in the presence (B) and in the absence (B_0) of unlabelled peptides.

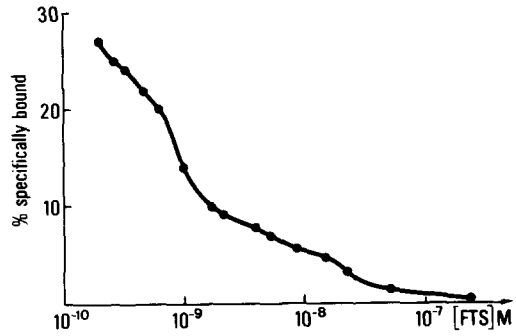

Fig. 4. Percentage of ^{3}H-labelled FTS specifically bound to 1301 plasma membranes as a function of total FTS concentration. Plasma membranes (80 μg/tube) were incubated for 120 min at 37°C with increasing concentrations of ^{3}H-labelled FTS in the indicated range. Membranes were then separated by filtration on GF/C filters and the membrane bound radioactivity was determined. Corrections for non-specific binding, which was less than 7% of total binding, have been made.

ligand concentration (Fig. 4). The percentage of specifically bound FTS was increased from 0.1% to approx. 25% of the total FTS concentration in the range of $3 \cdot 10^{-7}$ M to $2 \cdot 10^{-10}$ M, this increase being the expression of a multiple binding site saturation system. However, non-specific binding increased linearily. Data obtained in Fig. 4 were replotted according to Scatchard [25] and subjected to non-linear regression computer analysis, with a resulting curvilinear (concave upward) plot (Fig. 5). If the binding was considered to be heterogeneous, two saturable classes of binding sites appeared. Values for the dissociation constant K_d were of the order of 0.516 ± 0.215 nM and 110 ± 27.8 nM, and binding site capacities

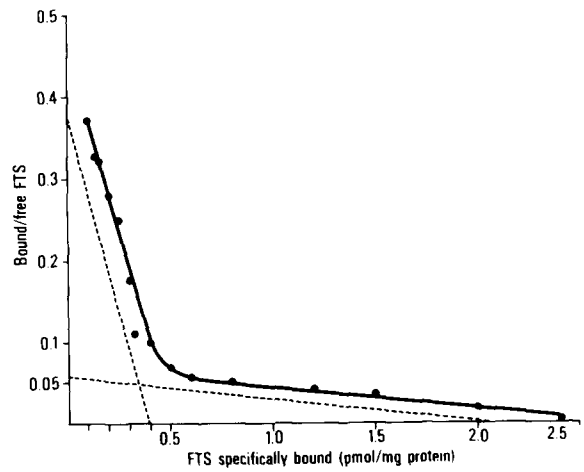

Fig. 5. Scatchard plot of the data in Fig. 4. The bound: free ratio was plotted as a function of ^{3}H-labelled FTS bound to plasma membrane (●). The intrinsic plot (- - - - -) is obtained by resolution of the experimental curve into high and low affinity independent linear components.

TABLE II

FTS AND FTS ANALOGS: RECEPTOR SPECIFICITY AND BIOLOGICAL ACTIVITIES

FTS and FTS analogs	Minimal active concentration in the rosette assay	Minimal active concentration inhibiting 5 nM of FTS in the rosette assay	Concentration providing 50% of [^{3}H]FTS inhibition of binding to plasma membrane receptor
FTS	10 fM	n.d.	80 nM
[Asp^9]FTS	>10 μM	0.1 nM	95 nM
[Pro^1]FTS	10 fM	n.d.	224 nM
[D-Ser^4]FTS	>10 μM	>10 μM	10 μM
[Orn^3]FTS	>10 μM	>10 μM	1 μM

n.d., not determined.

were respectively of the order of 0.186 ± 0.045 pmol and 2.026 ± 0.367 pmol per mg of membrane protein (mean ± S.D. of three independent determinations).

Negative cooperativity experiments. Dissociation of ^{3}H-labelled FTS specifically bound to plasma membranes induced by very large dilution occurred in two phases at 37 and 4°C, an early rapid phase followed by a long phase with slow rate of release (Fig. 6). Neither phase was accelerated by the excess of unlabelled FTS. This absence of 'dissociation enhancement by cold' phenomenon was consistent with the presence of two classes of independent FTS receptors on 1301 plasma membranes. The dissociation was temperature-dependent, greater at 37°C than at 4°C. The rebinding during dissociation experiments in infinite dilution (about 100-fold) was investigated. The absence of rebinding was proven by diluting the membrane receptor ^{3}H-labelled FTS complex into buffer B with addition membranes (2-fold excess of fresh membranes) in the absence or presence of 10^{-6} M unlabelled FTS. This excess of fresh membranes had no effect on the dissociation curves.

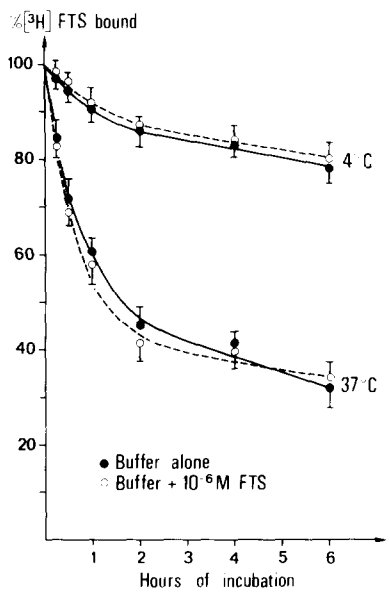

Fig. 6. Dissociation of specifically bound ^{3}H-labelled FTS from 1301 plasma membranes. Membrane fraction (60 μg of protein) were incubated with 0.5 nM ^{3}H-labelled FTS for 90 min at 37°C. The receptor ^{3}H-labelled FTS complex were separated from unbound FTS by centrifugation. After washing the pellet, membrane pellet was resuspended in 0.1 ml of buffer B and adjusted to 10 ml of same buffer in the absence or presence of an excess (10^{-6} M) of unlabelled FTS at 37°C or at 4°C. At the end of each incubation period, the mixture was filtered on GF/C membrane, washed and the radioactivity remained on the filter was measured. Each point is the mean and standard deviation of duplicate determinations.

Parameters of association and dissociation. At 37°C the association and dissociation kinetics of specific ^{3}H-labelled FTS binding were determined. Using different concentrations of ^{3}H-labelled FTS in the range of 0.4 to 3.1 nM, rate constants were determined from Fig. 7 by the equation [26]:

$$[HR] = [HR]_{eq} \left(1 - e^{-(k_a[H] + k_d)t}\right) \quad (1)$$

where $[HR]_{eq}$ is the specific binding at equilibrium, [HR] the specific binding at time t, [H] the free ligand concentration, k_d the dissociation rate constant and k_a the association rate constant. The equation is a pseudo first order kinetic equation in the case when [H] is almost constant. In these experiments, at the ^{3}H-labelled FTS concentrations studied, $[HR]_{eq}$ was much lower than [H] and therefore, the pseudo-first order kinetics was acceptable. The values obtained for the rate constants were $k_a = (1.8\text{–}2) \cdot 10^5$ $M^{-1} \cdot s^{-1}$ and $k_d = 2 \cdot 10^{-4}$ s^{-1}, given a K_d value (k_d/k_a) 1.11 to 1 nM (Fig. 7 inset). Independent confirmations for k_d obtained by dissociation experiments (Fig. 2) showed a k_d value of $2.09 \cdot 10^{-4}$ s^{-1} which was similar to that obtained in the association kinetics studies.

^{3}H-labelled FTS and FTS membrane-receptor integrities

The integrity of ^{3}H-labelled FTS present in the filtrate of prolonged incubations of ^{3}H-labelled FTS (1 nM) with plasma membrane (270 μg/ml) during 30, 60, 90, 120 and 300 min at 37°C was established in three control experiments.

(a) Rebinding to fresh membranes. No alteration of ^{3}H-labelled FTS binding to fresh membrane was produced by preincubation with membrane for 2 h at 37°C. However, rebinding was only about $75.7 \pm 3.1\%$ of control binding after preincubation of the tracer for 5 h, estimating a degradation of the tracer of the order of 25 to

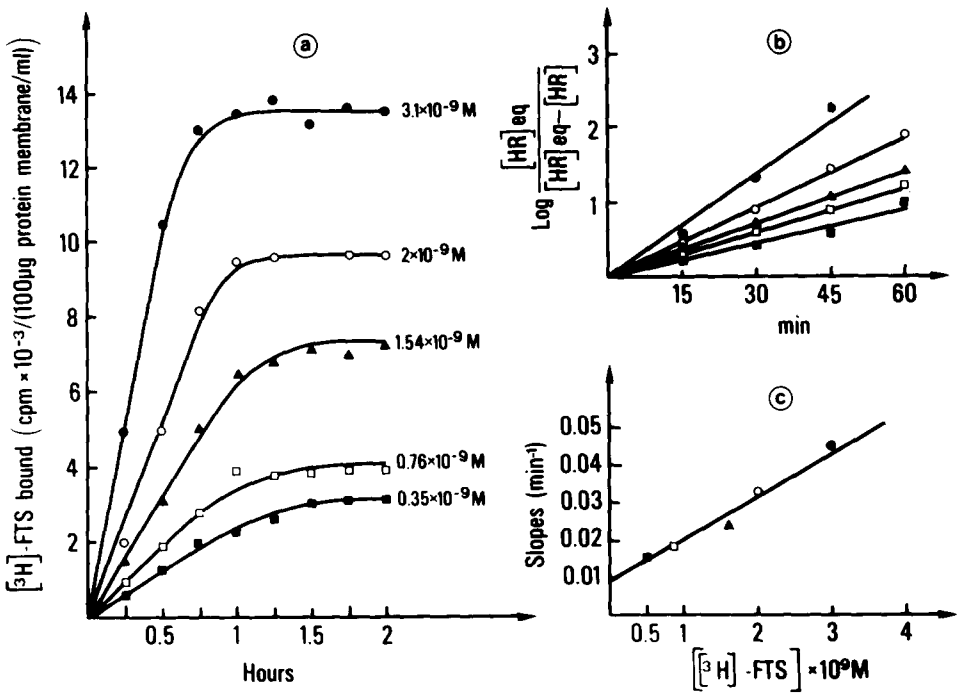

Fig. 7. Time course of ^{3}H-labelled FTS binding to 1301 plasma membranes. (a) ^{3}H-labelled FTS at the indicated concentrations was incubated with 10 µg of protein membrane in 0.3 ml of buffer B. At the indicated times, the samples were filtered through GF/C filters presoaked in buffer B and washed 3 times with 5 ml of ice-cold buffer B. The values were corrected for nonspecific binding. (b) Time course of ^{3}H-labelled FTS binding plotted according to the eqn. 1 with concentrations 0.35, 0.76, 1.54, 2.0, and 3.1 nM of ^{3}H-labelled FTS. (c) The slopes of these lines were plotted as a function of ^{3}H-labelled FTS concentration. The slope yields a k_a value of $(1.8–2.0)\cdot 10^5$ $M^{-1}\cdot s^{-1}$. The intercept yields a k_d value of $2.0\cdot 10^{-4}$ s^{-1}.

30%. Control binding was defined as the binding to fresh membrane of the ^{3}H-labelled FTS concentration (not preincubated with membrane) identical to free ^{3}H-labelled FTS concentration in the filtrate of membrane preincubation mixture at time t. However, all binding parameter determinations were obtained after 80 to 120 min incubation at 37°C, since our results were acceptable without correction.

(b) Immunoreactivity of the tracer. No significant differences in the immunoreactivity was induced by FTS preincubation. These results showed a dissociation between immunoreactivity and rebinding studies using ^{3}H-labelled FTS after 5 h of preincubation with membrane protein.

(c) Thin-layer chromatography. The integrity of ^{3}H-labelled FTS was finally established by thin-layer chromatography. One spot was obtained in three solvent systems after 120 min of preincubation.

In order to determine whether the receptors were degraded during the incubation time, the membranes were preincubated at 37°C for 0 to 180 min prior to the addition of labelled FTS for standard incubation. Neither specific nor non-specific binding was altered by this preincubation.

Discussion

Formerly, using a (^{3}H-labelled FTS)-labelled methylated derivative of FTS with low specific activity (5 Ci/mmol) as tracer, some of us characterized on the 1301 cell line a specific cellular receptor for FTS with at least two different binding sites, with K_d of the order of 3.5 and 110 nM, and maximum numbers of binding sites of the order of 48000 and 780000 per cell, respectively [8]. In this previous study, the low specific activity of the tracer and its structural modifications did not allow us to approach the binding sites with

affinity related with biological activity in the rosette assay. Furthermore, the subcellular localization of the receptors remains to be defined.

In the present study, using subcellular fractions from the 1301 cell line, and a ^{3}H-labelled FTS structurally identical to natural FTS, with very high specific activity (120 Ci/mmol) we have been able to find ^{3}H-labelled FTS specific binding sites on plasma membranes and to characterize their physico-chemical parameters. The method described for plasma membrane preparation of cultured cells was rapid, reproducible, providing a good yield of purified membrane (2 to 3 mg of membrane protein per $(100-150) \cdot 10^6$ 1301 cells), and did not alter dramatically FTS binding sites. The data in Table I show that the specific activities of plasma membrane markers are concentrated in fraction B. The ^{3}H-labelled FTS specific binding was also concentrated in fraction B. Conversely, fractions A and C, enriched in nuclei and in cytoplasmic enzymatic markers, did not show any significant specific binding. However, the possible existence of binding site in the supernatant of a $105000 \times g$ centrifugation was not investigated due to lack of a convenient method for separating free and bound ^{3}H-labelled FTS. Thus, the presence of specific cytoplasmic receptors for FTS cannot be excluded. Autoradiographic studies on FTS binding to 1301 cells are presently under way to define binding site distribution on these cells.

The interaction of ^{3}H-labelled FTS with 1301 membrane was stable, saturable and reversible as on intact cells. FTS binding (Fig. 1) is very temperature-dependent. This result is not unusual for hormone and receptor interactions like steroid hormones [27], FSH [28], angiotensine II [29]. In these systems low temperature can alter thermodynamically dependent parameters of association and dissociation, modify the conformation of ligands, or modify the membrane environment of receptors. In the FTS binding system the exact reasons for this high temperature sensitivity are not known and the hypothesis mentioned above are also valid; moreover a possible inside-outside formation of vesicles in the plasma membrane, at 4°C, including receptors, cannot be rejected. The fact that the association kinetics at 37°C, 30°C and 24°C show a similar stationary phase, but at different periods of the incubation time, could demonstrate an absence of receptor alteration at such temperatures. A possible association of ^{3}H-labelled FTS with the membrane at 4°C, detectable after 10 or 24 h or more, may occur, without, however, any biological significance.

Displacement of ^{3}H-labelled FTS binding was obtained with FTS analogues which were fully biologically active (agonist or antagonist). Conversely, inactive analogues [D-Ser^4]FTS and [Orn^3]FTS did not displace ^{3}H-labelled FTS binding (Fig. 3).

Saturation at low ligand concentration of ^{3}H-labelled FTS showed a curvilinear (concave upward) Scatchard plot. This could be explained by the presence of multiple classes of binding sites, such as were described for the nerve growth factor [30] and for histamine [31], or a single class of homogeneously binding sites with varying affinity [32]. The negative cooperativity model for hormonal action postulates that a non-linear Scatchard plot is the result of progressively lowered affinity when occupancy of a given population of receptors is increased. The presence of two classes of FTS receptors in 1301 plasma membrane was consistent with the results obtained from experiments on the dissociation of specifically bound FTS induced by infinite dilutions. In the insulin receptor model, which exhibits a non-linear Scatchard plot, the presence of negative cooperativity is suggested by the observation that the rate of dissociation induced by dilution without unlabeled peptide is increased in the presence of an excess of unlabeled peptide [33]. Moreover, this 'dissociation enhancement by cold' phenomenon has been demonstrated to be independent of the presence of a curvilinear Scatchard plot in the TSH binding system described by Powell-Jones et al. [34].

The hypothesis of two classes of independent FTS receptors in the 1301 plasma membrane has also been tested with theoretical arguments. We have used a personal computer programme to adjust experimental data to the theoretical Scatchard representation with two independent binding sites. We have used a hyperbolic, non-linear regression fitting of experimental data and calculated a fitting index to quantify its accuracy. This index is defined by the formula:

$$Index = \sum_{i=1}^{i=n} \left(Y(i) - \overline{Y(i)}\right)^2 / n$$

where $Y(i)$ is the experimental data, $\overline{Y(i)}$ the theoretical data for the ith point, and n the total number of experimental points. The best fitting index is not equal to 0 but equal to 10^{-7} in our precision test assessed with theoretical values. The index value for Scatchard analysis in Fig. 5 is $8.16 \cdot 10^{-6}$. Thus, theoretical and experimental results are in agreement, as far as the hypothesis of two independent binding sites for the FTS binding system is concerned.

Based on the evaluation of 'cellular equivalent unit' defined as the mean quantity of membrane protein obtained by the homogeneization of 'one' lymphoblastoid 1301 cell, the binding capacity was 13070 ± 530 sites per cellular equivalent for binding site, with K_d of 0.516 ± 0.2 nM. This site had not been characterized in the previous study [8] on intact 1301 cells, certainly because of the low specific activity of the tracer and its unnatural structure. The relation between this high affinity binding site and the rosette assay biological site remains to be established. According to the in vitro rosette assay, FTS is active until the low concentration of 10 fM and 50% of high affinity binding site saturation is about 0.5 nM. Then, a minimum occupancy of about 0.02% of highly binding sites could be detected in the rosette assay if such FTS receptor is present in the biological test. However, the rosette assay uses spleen rosette forming cells from ATx mice and not 1301 cells, and the activity in this biological assay is expressed in minimal active concentration of FTS inducing azathioprine sensitivity of rosette forming cells. This azathioprine sensitivity could be obtained with very low occupancy of binding sites.

The correlation between FTS binding and its biological activities at 1301 cellular level is presently under way, studying an activation of adenylate cyclase, activation of guanydyl cyclase or calmoduline interaction, and studying FTS-induced apparition of some T cell differentiation antigens on the surface of 1301 cells or on the surface of T cells with FTS receptors.

The binding site with K_d of 110 nM and binding capacity of the order of 142440 ± 24300 sites per cellular equivalent could be identical to the low affinity binding site found on 1301 intact cells. Its significance is not known. Kinetic studies considering both the approach to equilibrium at different ligand concentrations and the dissociation following addition of an excess of unlabeled FTS gave a K_d of the order of 1.08 ± 0.35 nM identical to that found by equilibrium techniques (0.516 ± 0.2 nM). The k_a value 1.8 to $2 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ was relatively low at 37°C and it was not typical of the association rate constant, especially at this temperature for most protein-ligand interactions. Koren and Hammes [35] gave a typical value for k_a between 10^6 and 10^7 $M^{-1} \cdot s^{-1}$. It is possible that the association of FTS to its membrane receptors is not limited by a diffusion process [36] but by a change in peptide conformation, or a peptide-peptide interaction in the medium. These hypotheses are speculative at the present time. In any case, these studies prove the existence of high affinity receptors on the 1301 plasma membrane, and provide interesting information on the binding characteristics. They also represent a good starting material for the isolation of FTS soluble receptors from human lymphoblastoid cells.

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References

1 Bach, J.F., Dardenne, M., Goldstein, A., Guha, A. and White, A. (1971) Proc. Natl. Acad. Sci. U.S.A. 64, 2734–2737
2 Bach, J.F. and Dardenne, M. (1973) Immunology 25, 353–366
3 Dardenne, M., Pleau, J.M., Blouquit, Y. and Bach, J.F. (1980) Clin. Exp. Immunol. 42, 477–482
4 Monier, J.C., Dardenne, M., Pleau, J.M., Schmitt, D., Deschaux, P. and Bach, J.F. (1980) Clin. Exp. Immunol. 42, 470–476
5 Dardenne, M., Pleau, J.M., Man, N.K. and Bach, J.F. (1977) J. Biol. Chem. 252, 8040–8044
6 Pleau, J.M., Dardenne, M., Blouquit, J.Y. and Bach, J.F. (1977) J. Biol. Chem. 252, 8045–8047
7 Bricas, E., Martinez, J., Blanot, D., Auger, G., Dardenne, M., Pleau, J.M. and Bach, J.F. (1977) Proc. 5th American Peptide Symposium, pp. 564–567, John Wiley, New York

8 Pleau, J.M., Fuentes, V., Morgat, J.L. and Bach, J.F. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2861–2865
9 Sasaki, A.N. and Bricas, E. (1980) Tetrahedron Lett. 21, 4263–4264
10 Morgat, J.L., Desmares, J. and Cornu, M. (1975) J. Labelled Compd. 11, 267
11 Pleau, J.M., Pasques, D., Bach, J.F., Gros, C. and Dray, F. (1977) Radioimmunoassay and Related Procedures in Medicine, vol. 2, pp. 505–510, I.A.E.A., Vienna
12 Blanot, D., Martinez, J., Auger, G. and Bricas, E. (1979) Int. J. Peptide Protein Res. 14, 41–56
13 Martinez, J., Blanot, D., Auger, G., Sasaki, A.N. and Bricas, E. (1980) Int. J. Peptide Protein Res. 16, 267–279
14 Dardenne, M. and Bach, J.F. (1975) The Biological Activity of Thymic Hormones, pp. 235–243, Kooyker Scientific Publications, Rotterdam
15 Pleau, J.M., Dardenne, M., Blanot, D., Bricas, E. and Bach, J.F. (1979) Immunol. Lett. 1, 179–182
16 Shiu, R.P.C. and Friesen, H.G. (1974) Biochem. J. 140, 301–311
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
18 Burton, K. (1968) Methods Enzymol. 12 B, 163–166
19 Emmelot, P., Bos, C.J., Benedetti, E.L. and Rümke, P. (1964) Biochim. Biophys. Acta 90, 126–145
20 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
21 Garen, A. and Levinthal, C. (1960) Biochim. Biophys. Acta 38, 470–474
22 Fishman, W.H., Springer, B. and Brunetti, R. (1948) J. Biol. Chem. 173, 449–452
23 Tisdale, H.D. (1967) Methods Enzymol. 10, 213–216
24 Warley, A. and Cook, G.M.W. (1973) Biochim. Biophys. Acta 323, 55–68
25 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
26 Bennet, J.P., Jr. (1978) Neurotransmitter Receptor Binding. (Yamamura, H.J., Enna, S.J. and Kukar, J.M., eds.), pp. 57–90, Raven Press New York
27 Clarck, J.H. and Peck, E.J., Jr. (1977) in Receptors and Hormone Action, 1st edn. (O'Malley, B.W. and Binbaumer, L., eds.), pp. 383–410, Academic Press, New York
28 Reichent, L.E., Jr. and Abou-Issa, H. (1978) in Receptors and Hormone Action, 3rd edn. (O'Malley, B.W. and Birnbaumer, L., eds.) pp. 341–361, Academic Press, New York
29 Catt, K.J. and Agrulera, G. (1980) in Cellular Receptors for Hormones and Neurotransmitters, (Schulster, D. and Levitzki, A., eds.), pp. 233–251, John Wiley and Sons, New York
30 Sutter, A., Riopelle, R.J., Harris-Warrick, R.M. and Shooter, E.M. (1979) J. Biol. Chem. 254, 5972–5982
31 Osband, M. and McCafrey, R. (1979) J. Biol. Chem. 254, 9970–9972
32 De Meytz, P., Bianco, A.R. and Roth, J. (1976) J. Biol. Chem. 251, 1877–1888
33 De Meyts, P., Roth, J., Neville, D.M., Gavin, J.R. and Lesniak, M.A. (1973) Biochem. Biophys. Res. Commun. 55, 154–161
34 Powell-Jones, C.H.J., Thomas, C.G., Jr. and Nayfeh, S.N. (1980) J. Biol. Chem. 255, 4001–4010
35 Koren, B. and Hammes, G.G. (1976) Biochemistry 15, 1165–1171
36 Marshall, A.G. (1978) Biophysical Chemistry Principles, Techniques and Applications, pp. 87–110, John Wiley and Sons, New York