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THE PLASMA MEMBRANE OF EPIDIDYMAL EPITHELIAL CELLS HAS A SPECIFIC RECEPTOR WHICH BINDS TO ANDROGEN-BINDING PROTEIN AND SEX STEROID-BINDING PROTEIN

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Summary—The binding of [^{3}H]Δ_6-testosterone photoaffinity-labelled rat androgen-binding protein (rABP) has been studied in an enriched fraction of plasma membranes of epithelial epididymal cells in immature (15 days) and adult rats (40 days). The binding was maximal in <30 min and more rapid at 4°C than at 34°C. It was calcium and pH dependent. Scatchard plots of the binding data gave curvilinear plots with two types of binding sites corresponding to a K_{ass_1} of 18.2 nM^{-1} and K_{ass_2} of 1.6 nM^{-1} (2.2×10^{11} sites/mg protein and 5.4×10^{11} sites/mg protein, respectively). In adult rats, only one type of binding site was found, with a K_{ass} of 3.7 nM^{-1} (4.5×10^{11} sites/mg protein). The number of receptors was 5-fold lower in the cauda than in the caput of the epididymis. The pretreatment of the isolated intact cells with streptozotocin induced a 45% reduction of the binding. Only unlabelled rABP and hSBP (human sex steroid-binding protein) but not other proteins (lactotransferrin, serotransferrin, asialofetuine, fetuine and bovine serum albumin) competed with the labelled ligand to bind plasma membranes. The membrane fraction was solubilized by triton X-100. Its incubation with labelled rABP and hSBP provoked the elution of the tracer as an aggregate into the void volume fraction of superose 6B mini-gel filtration columns. Structural homology between hSBP and rABP could be responsible for the common behaviour of the steroid-carrier molecules for the ABP receptor of rat epididymal epithelial cells.

INTRODUCTION

Rat androgen-binding protein (rABP) is synthesized in the testis by Sertoli cells. A transient synthesis has also been demonstrated in fetal liver [1]. ABP production by the testis proceeded from undetectable levels in 10-day-old rats to reach 80–90 μg/testis in 30-day-old rats [2]. The extracellular ABP may play a role in the intraluminal transport of androgens in the male reproductive tract and in the sexual differentiation [3]. A single gene encodes ABP and SBP (sex steroid-binding protein) and the coding region of the gene has been cloned and characterized [4–6]. Recent evidence suggests that rABP and human SBP (hSBP) interact with target cells via membrane receptors [7, 8]. For example, ABP binding to a receptor has been demonstrated in rat epididymal epithelial cells [9]. The receptor-mediated endocytosis of labelled rat ABP and labelled human SBP has been observed by autoradiography respectively in epididymal epithelial cells of the rat [10] and monkey [11].

In a first study, we have demonstrated *in vitro* that internalization of ABP is receptor-mediated in isolated epithelial cells of rat epididymis [9]. The present work confirms this hypothesis by studying the binding of labelled rABP and hSBP to an enriched fraction of plasma membranes from epithelial epididymal cells of immature and adult rats.

EXPERIMENTAL

Chemicals and reagents

Mannitol, phenyl methyl sulphonyl fluoride (PMSF) and soya-bean trypsin inhibitor (SBTI) were all purchased from Sigma (St Louis, MO, U.S.A.). Collagenase dispase, DNase (Type I) were supplied by Boehringer Mannheim

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(Indianapolis, IN, U.S.A.). All other reagents were obtained as described previously [9, 12].

Purification and photoaffinity labelling of rABP

The testes were removed from 200–250 g adult Wistar rats; rABP was purified by HPLC as described in detail previously [12, 13]. The photoaffinity-labelled rABP was prepared as described previously [12] according to Taylor *et al.* [14]. Testicular extract (10 ml) was incubated with 1 nmolΔ_6 [^{3}H]testosterone ([1.2-^{3}H]17β-hydroxy-4,6-androstadien-3-one: 48.6 Ci/mmol, New England Nuclear Corp., Boston, MA, U.S.A.) overnight at 4°C, under rotary agitation. The sample was photolysed in a reactor using a u.v. light (345 nm), for four 15 min periods under controlled temperature and was injected in preparative HPLC (column Diol Lichroprep 200, 25 × 2.5 cm) to separate the covalently bound Δ_6 [^{3}H]testosterone from the remaining free. The peak of photoaffinity-labelled rABP was eluted with a retention time of 7 min and was stored in aliquots at −80°C. Each aliquot was filtered by fast protein liquid chromatography superose 6 gel filtration (Pharmacia, Uppsala, Sweden) prior to use. The peak of dimeric photoaffinity-labelled ABP was eluted with a retention time of 32 min corresponding to a M_r of 90 kDa. It was collected and stored at −80°C, in aliquots, before use.

Purification and photoaffinity labelling of hSBP

The hSBP has been purified from human late-pregnancy serum using a modification of the method described previously [15]. Four steps were performed, including ammonium sulphate precipitation, carboxymethyl cellulose chromatography, DEAE-cellulose chromatography and gel filtration. Purity of hSBP was checked by polyacrylamide gel electrophoresis. Quantitative estimation of hSBP was performed by electroimmunodiffusion using a polyclonal rabbit anti-hSBP serum [11]. The photoaffinity labelling of hSBP was performed as described above for rABP [11].

Iodination of hSBP and of rABP was prepared as described previously for rABP [13] according to Marckwell [16]. Two iodobeads were washed in Tris–HCl buffer (20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4), and incubated for 5 min with 0.1 mCi ^{125}I in 0.2 ml Tris–HCl buffer. Then, 20 μg of purified protein was added. After 15 min, the iodinated molecule was poured onto a Sephadex G-25 column (2.5 × 10 cm, Pharmacia) and eluted in the presence of dextran blue 2000.

Isolation of epididymal epithelial cells and preparation of membrane fractions

Epididymal cells were isolated from testes of 2- and 6-week-old Wistar rats as described in detail previously [9], using collagenase dispase (500 mg/l) and DNase (type I) for cellular dissociation.

The plasma membrane-enriched preparations were obtained from isolated epididymal epithelial cells. About 10×10^6 cells/ml were disrupted in ice-cold buffer (20 mM Tris–HCl/l, pH 7.4 containing 0.4 mM PMSF, 0.1% aprotinin, 0.01% bacitracin, 1 mM $CaCl_2$ and 1 mM $MgCl_2$), with a mixer (ultraturax IKA®), three times for 5 min at 4°C. The homogenate was centrifuged for 10 min at 1000 *g* and the pellet was discarded. The supernatant was further centrifuged for 30 min at 27,000 *g* for 30 min at 4°C. The pellet was resuspended in the Tris–HCl buffer described above in the presence of 300 mM mannitol and centrifuged for a second period in the same conditions. The pellet was homogenized in the Tris–HCl buffer described above and was stored at −80°C, in aliquots before use. Protein concentration was measured according to the method of Lowry [17] using bovine serum albumin as standard. Compared to the initial homogenized tissue, the specific activity of 5'nucleotidase (a standard marker for plasma membranes) vs protein concentration was increased 5 times in the plasma membrane fraction. The enriched fractions were also controlled by electron microscopy.

Binding of labelled rABP and of labelled hSBP to the membrane fractions

General protocol. Photoaffinity-labelled rABP and hSBP binding to membrane fractions was studied by incubating increasing amounts (0.05 to 2.5 pmol) of labelled rABP or hSBP with membrane fraction aliquots corresponding to 10^6 cells per test tube, in duplicate and in the presence or absence of a 100-fold excess of unlabelled rABP. After 1 h incubation at 4°C while shaking, separation of bound from free ligand was achieved by centrifugation at 20,000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet washed 3 times in 20 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% bacitracin and 1 nM benzamidine/l. The radioactivity of the supernatant and of the pellet

was measured by addition of 3 ml of scintillation liquid (Scintran®) to 50 μl aliquots. The data were analysed using Scatchard plots of the specific binding (difference between total binding and non-specific binding).

Kinetics of association–dissociation

The time-course of the uptake of labelled rABP by plasma enriched membrane preparations was studied by incubating 2.5 mg protein material (aliquots corresponding to 10×10^6 cells) with 100 fmol of photoaffinity-labelled rABP, in triplicate, in a final volume of 10 ml. 1 ml aliquots of the suspension were collected after shaking at incubation times ranging from 2 to 120 min, at 4 and at 34°C. The bound ABP was separated from free form by centrifugation and washing of the pellet as described above. A second experiment was performed in the same way except that a 100-fold excess of unlabelled rABP was added 15 min after incubating the membrane fractions with the tracer at 4°C, in order to study the dissociation of labelled rABP.

Inhibition of rABP binding to plasma membrane fractions

The binding of rABP to plasma cell membranes was studied in the presence of either 15 mM EDTA or of trypsin (10 mg/ml). Specificity of the binding was studied by incubating the membrane fraction for 1 h at 4°C with 0.1 nmol of human lactotransferrin, serotransferrin, asialofetuin, fetuin and bovine serum albumin prior to the addition of 1 pmol of labelled rABP. The binding was also studied as a function of pH. The membrane fraction aliquots were incubated with labelled rABP for 2 h in the presence of various buffer solutions (0.02 M Tris–HCl buffer pH 8.0; 0.1 M sodium phosphate buffer pH 7; 0.1 M sodium phosphate buffer pH 6; 0.05 M sodium acetate buffer pH 5; 0.05 M acetate buffer pH 4; 0.02 M citrate–HCl buffer pH 3; 0.02 M citrate–HCl buffer pH 2). The pH of the incubation mixture was adjusted to the pH of the corresponding buffer.

Effect of streptozotocin

Intact epididymal epithelial cells (from 15-day-old rats) were exposed for 2 h at 4°C with 10 μg/ml of streptozotocin (Sigma). rABP labelled binding was then performed at different concentrations (0.5 to 1.4 pmol).

Gel filtration of Triton X-100 solubilized rABP and hSBP receptor

Membrane fractions of epididymal epithelial cells were extracted with 20 ml Tris–HCl buffer (pH 7.4) containing 1% Triton X-100, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.4 mM PMSF and 0.1% aprotinin. 0.5 ml of membrane fractions was incubated 1 h with 1.5 pmol of labelled rABP or hSBP. The same protocol was repeated at pH 5 instead of pH 7.5 and also in the presence of 15 mM EDTA instead of $CaCl_2$. The samples were poured onto a superose 6B mini-gel filtration column 0.46 × 20 cm (Merck, Germany). The eluate was collected in 0.2 ml fractions using a Frac-100 fraction collector (Pharmacia). The non-specific binding was studied by preincubating the membrane extract with a 100-fold excess of unlabelled rABP.

RESULTS

The kinetics of specific binding reached a maximum after 20–30 min of incubation of labelled rABP with the plasma membrane-enriched fraction of epididymal epithelial cells from 2-week-old rats, at 4°C (Fig. 1). Bound labelled rABP was displaced by an excess of radioinert rABP, demonstrating that the uptake was reversible (Fig. 1). The kinetics of the binding of labelled rABP was more rapid at 4 than at 34°C (Fig. 2). All subsequent incubations were therefore performed at 4°C for 1 h.

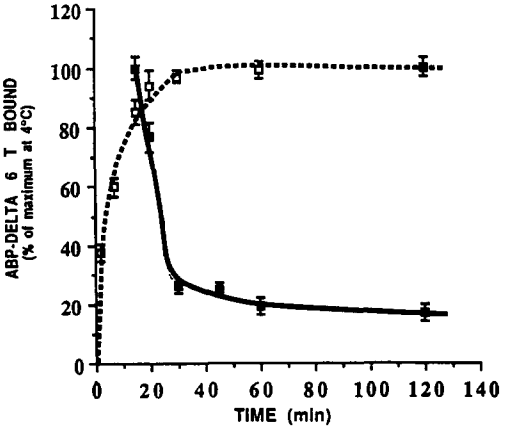

Fig. 1. Time course of association (□) and dissociation (■) of labelled rABP to membrane fractions of epididymal epithelial cells (15 days) at 4°C. Maximum binding was observed after 30 min of incubation. The dissociation was studied by adding a 100-fold excess of non-labelled rABP after 15 min incubation of the tracer with the membrane fractions. The results are expressed as the percentage of maximum binding of labelled rABP (mean ± SD of triplicate incubation).

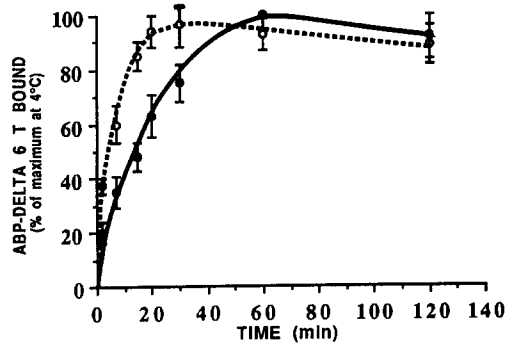

Fig. 2. Effect of time and temperature on specific binding. Membrane fractions of epididymal epithelial cells from 15-day-old rats were incubated with 100 fmol of labelled rABP, in triplicate, at 4 (○) and 34°C (●). The kinetics of the binding was lower at 34 than at 4°C. The specific binding was determined as described in Experimental (each value represents the mean ± SD of different experiments).

The binding of labelled rABP was pH-dependent and was maximal of pH 6–8 with a sharp decline at a more acidic pH (Fig. 3).

Scatchard analysis [18] of the binding to membrane fractions was performed with rABP and hSBP at 15 and 40 days old. We found curvilinear plots for immature rats, indicating a double class of binding sites, with $K_{ass_1} = 18.2\,nM^{-1}$ and $K_{ass_2} = 1.6\,nM^{-1}$ and a number of binding sites of 2.2×10^{11} and 5.4×10^{11} sites/mg protein, respectively [Fig. 4(A)]. With ageing, labelled rABP binding sites in 40-day-old rats showed a single class of binding sites with a K_{ass} of $3.7\,nM^{-1}$ and 4.5×10^{11} sites/mg protein [Fig. 4(B)]. The level of non-specific binding ranged between 10 and 20%. Under the same experimental conditions, the binding of hSBP was plotted with a K_{ass} of $2.6\,nM^{-1}$ and 3.8×10^{11} sites/mg [Fig. 4(C)]. The number or rABP binding sites of membrane fractions from immature rats (15 days) was higher than that obtained with fractions from mature rats (40 days).

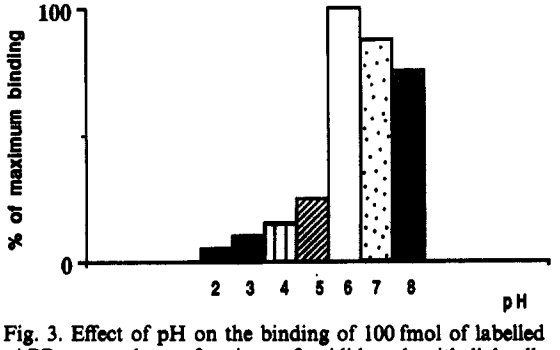

Fig. 3. Effect of pH on the binding of 100 fmol of labelled rABP to membrane fractions of epididymal epithelial cells from 15-day-old rats. Specific binding was pH dependent and maximal at pH 6–8.

EDTA and trypsin pretreatment [Fig. 5(A)] abolished the specific binding (65 and 42%, respectively), indicating that labelled rABP bound to a membrane protein and that the binding was calcium-dependent.

We noted a $38.7 \pm 2.4\%$ inhibition of the binding after preincubation of the intact epithelial cells with streptozotocin [Fig. 5(B)]. The

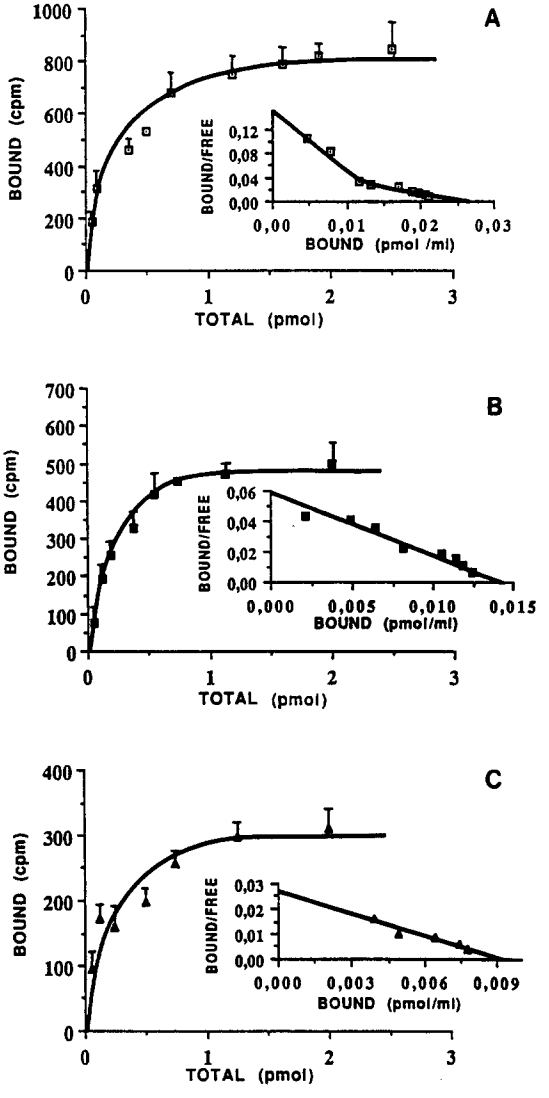

Fig. 4. Saturation curves of photoaffinity-labelled rABP to epididymal cell membrane fractions from (A) 15-day-old rats, (B) 40-day-old rats and of photoaffinity-labelled hSBP (C) to membrane fractions of immature rats (15 days). Binding was studied by incubating 0.05 to 2.5 pmol of labelled rABP or hSBP with membrane fraction aliquots (corresponding to 10^6 cells per test tube) for 1 h at 4°C. Affinity constants (K_{ass}) estimated from Scatchard plots were: 18.2 and $1.6\,nM^{-1}$ for plot A, $3.7\,nM^{-1}$ for plot B and $2.6\,nM^{-1}$ for plot C. The curvilinear scatchard plot A was calculated using the graphic analysis of Rosenthal [23]. Non-specific binding was determined to be approx. 15% by duplicating experiments in the presence of a 100-fold excess of cold rABP. The data are shown as the mean of triplicated separate experiments, using a different membrane extract.

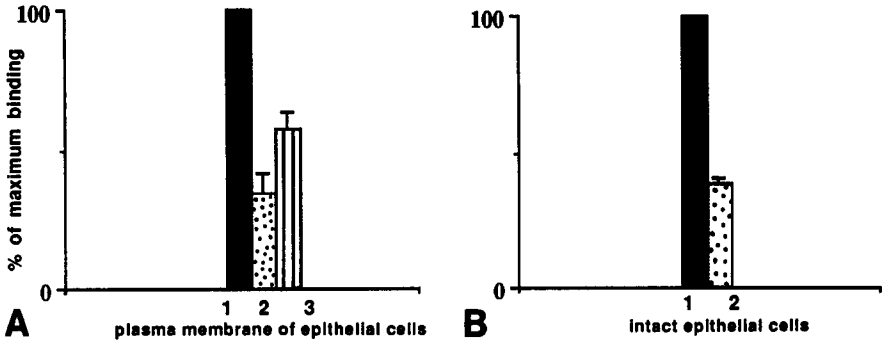

Fig. 5. Inhibiting effect of binding of labelled rABP to membrane fractions of epithelial cells (15 days) by either trypsin (A2) or EDTA (A3) and to intact epithelial cells (15 days) by streptozotocin (B2). Specific binding was evaluated as described in Experimental. Untreated samples represented 100% specific binding (A1, B1). Each value is the mean of triplicate experiments (bars indicate the standard deviation).

binding of rABP to membrane fractions was 5-fold lower with enriched preparation of plasma membranes from the cauda than with plasma membranes from the caput of immature rat epididymis (data not shown).

No competitive inhibition was observed in excess of lactotransferrin, serotransferrin, fetuin, asialofetuin and bovine serum albumin. On the contrary, hSBP was as efficient as cold rABP in inhibiting the binding of labelled rABP to the membrane fraction of epididymal epithelial cells (Fig. 6).

Labelled rABP incubated with Triton X-100 solubilized membranes was eluted as a high molecular mass peak (retention time of 8 min) in Superose 6B mini-gel filtration. The same elution profile was obtained when labelled hSBP was incubated with the solubilized membrane extract (Fig. 7). The 8 min peak was largely abolished in the presence of a 100-fold excess of unlabelled rABP (Fig. 7) or in the presence of EDTA instead of calcium (data not shown). This peak was not observed when the tracer and the extract were incubated and filtered at pH 5 (data not shown).

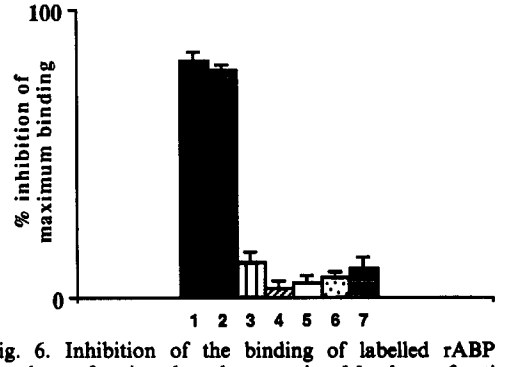

Fig. 6. Inhibition of the binding of labelled rABP to membrane fractions by other proteins. Membrane fractions of epididymal epithelial cells (15 days) were preincubated for 60 min at 4°C in the presence of 0.1 nmol of either hSBP (2), human lactotransferrin (3), serotransferrin (4), fetuin (5), asialofetuin (6) or bovine serum albumin (7) before adding 1 pmol of labelled rABP. Untreated samples (1) represented 100% of specific binding: each determination is the mean of triplicate experiments (bars indicate the standard deviation).

DISCUSSION

We have previously demonstrated the presence of a receptor for rABP in epididymal homogenates and in isolated epididymal cells from immature rats [9]. In the present work, we studied the binding of labelled rABP to an enriched fraction of membranes from epididymal epithelial cells of mature and immature rats. The kinetics of binding and dissociation of the tracer in excess of cold rABP were similar to those observed for isolated cells. The binding between the ligand and the receptor was found to be more rapid at 4 than at 34°C. This result

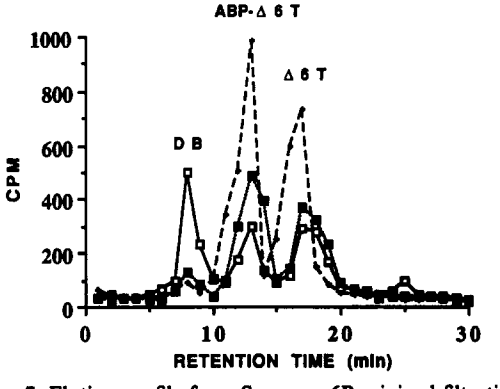

Fig. 7. Elution profile from Superose 6B mini-gel filtration of [^{3}H]Δ_6-testosterone–rABP (ABP–Δ_6T) incubated with the triton X-100 solubilized membrane fractions of rat epididymal epithelial cells (15 days). The ABP–receptor complex was eluted with a retention time of 8 min (□). This peak was largely abolished when the membrane extract was preincubated for 1 h with a 100-fold excess of radioinert rABP (■). ABP–Δ_6T and Δ_6T were eluted in a retention time of 12 and 17 min, respectively (---). Dextran blue 2000 (DB) was used as a marker eluted into the void volume position.

was not explained but the same difference has also been observed for the binding of 1-25 dihydroxyvitamin D_3 to its receptor, in human testis [19]. The specific capacity of rABP receptor was estimated to be 7.6×10^{11} sites per mg protein. Since the isolated fraction of membranes was prepared from aliquots of 10^7 cells, it was therefore calculated that about 12,000 receptor sites were present per epididymal cell from the immature rat. This capacity was very close to that previously determined with isolated cells in suspension [9]. In addition we also observed two affinity types of binding sites with respective K_{ass} of 1.6 and 18.2 nM^{-1} and a lower receptor activity in the cauda than in the caput epididymis. The binding of labelled hSBP to the plasma membranes was similar to that of labelled rABP. In addition, a 100-fold excess of cold hSBP was able to inhibit the binding of labelled rABP to the membrane fractions. This showed that the receptor binding site binds both molecules. This is not surprising since the two molecules have a high percentage of homology for their aminoacid sequence [20]. The binding site of the membrane fraction is a protein since we observed that trypsin treatment of the membrane and streptozotocin treatment of the cells decreased the receptor activity by 42 and 38%, respectively. The receptor activity of membrane fractions from mature rats was about 1.7-fold lower than that obtained with fractions from immature rats. It has been shown that the concentration of rABP in testis is higher in mature rats (40 days) than in immature rats (15 days) [21]. In addition, Danzo *et al.* [2] observed that all hormonal treatments that increased testicular content of rABP also increased its transport into the epididymis. One may therefore assume that the decrease of specific activity of rABP epididymal receptor which was observed as a function of age corresponded to a higher *in situ* saturation of the receptor by rABP. The binding of rABP to the receptor could be associated with a down regulation [22]. The fact that only one single affinity type of receptor was found in 40-day-old rats was in favour of such an hypothesis, since a high level of rABP in the epididymis of 40-day-old rats could be responsible for a high level of recycling of the receptor. On the contrary, the low concentration of rABP in the epididymis of 15-day-old rats can explain that two affinity types of rABP receptor were observed at this age.

In conclusion, our data demonstrated the presence of a receptor for rABP and hSBP in an enriched fraction of plasma membranes from rat epididymal cells. The receptor activity was higher in immature rats than in 40-day-old rats.

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