Biochimica et Biophysica Acta 813 (1985) 87–95
Elsevier

BBA 72465

Specific binding of dexamethasone to plasma membranes from skeletal muscle

Michel Savart and Yvane Cabillic

Laboratoire de Biochimie et Technologie des Aliments, Département Alimentation et Nutrition, Université de Bordeaux I, Avenue des Facultés, 33405 Talence Cedex (France)

(Received July 16th, 1984)
(Revised manuscript received October 2nd, 1984)

Key words: Dexamethasone; Glucocorticoid receptor; Plasma membrane; (Rabbit skeletal muscle)

A procedure was developed to isolate plasma membranes from rabbit skeletal muscle. K^+-dependent phosphatase activity was used as marker enzyme for plasma membranes and was determined in the presence of CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate), a zwitterionic detergent. Ca^{2+}-ATPase and succinate dehydrogenase activities were used as marker enzymes for sarcoplasmic reticulum and mitochondria, respectively. Electron-microscopy revealed that plasma membranes were in the form of vesicles. Significant proteolysis of membrane proteins was observed during extraction, which was inhibited by EGTA and 20 mM molybdate. SDS-polyacrylamide gel electrophoresis revealed the disappearance of an intense 96 kDa protein band when membranes were purified in the absence of EGTA and molybdate. Specific binding sites for [^{3}H]dexamethasone were identified in plasma membranes after freezing and incubation with CHAPS. Dithiothreitol was essential for steroid binding and ATP increased it. Under standardized assay conditions, binding was complete with 50 min à 37°C. No binding occurred at 0°C, nor if EGTA and molybdate were absent from the extraction medium.

Introduction

It is generally considered that steroid hormones penetrate into cells by passive diffusion and form specific complexes with cytoplasmic proteins called cytosolic receptors. These complexes then enter the nucleus after activation and modify the expression of specific genes [1]. However, this classical theory is not sufficient to account for all the results obtained in the field of steroid receptors.

Firstly, the mechanism of steroid uptake by the cell remains controversial [2] and for glucocorticoids, a carrier mediated uptake has been reported [3,4]. The role of transcortin is also unclear, and in a previous paper we observed that this steroid carrier appeared as a possible effector involved in a complex system of regulation [5]. Latest developments in the study of cytosolic receptors raise the possibility that they could be native receptors released from damaged subcellular structures [6–9]. Welshons et al. [8] consider that cytosolic localization represents an extraction artefact. On the other hand, Grote et al. [10] reported an absence of correlation between binding of glucocorticoids to cytosolic receptors and enzyme induction. Finally, binding sites were reported in plasma membranes for estrogens [9], progesterone [11], aldosterone [12] and glucocorticoids [13,14]. From these considerations, we have studied the possibility of specific glucocorticoid binding by plasma membranes purified from rabbit skeletal muscle with particular attention to membrane protein integrity. Indeed, cyto-

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

solic receptors are particularly sensitive to Ca^{2+}-dependent proteolysis [15,16] and Ca^{2+}-dependent proteinases were purified from rabbit skeletal muscle in our laboratory [17,18]. Furthermore, by immunocytochemistry, Dayton and Schollmeyer [19] localized a Ca^{2+}-activated protease in plasma membranes from this same tissue. The present report also describes the dissociation of plasma muscle membranes by CHAPS, a detergent which was successfully used for the isolation of active opiate receptors from brain membrane preparations [20].

Materials and Methods

ATP, ionophore A23187, *p*-nitrophenyl phosphate (diTris salt), crystalline nonradioactive steroids and CHAPS were obtained from Sigma. *p*-Methylaminophenol sulfate used for inorganic phosphate determination was obtained from the Eastman Kodak Company. [1,2,4-^{3}H]Dexamethasone (40 Ci/mmol) was purchased from Amersham International. Other chemicals and reagents were of analytical grade.

Isolation and purification of the plasma membranes. All operations were conducted at 4°C and centrifugal forces are those at the bottom of the tube. Adult male rabbits killed by cervical dislocation, were used as the starting material. Psoas muscle, which contain a large percentage of fast-twitch white fibers [21], served as the source for isolation of membranes. Muscles were rapidly dissected and homogenized for 60 s in a Waring-Blendor with 5 vols. of buffer A (2 mM EGTA/20 mM Na_2 MoO_4/30 mM Tris-HCl (pH 7.4)). The homogenate was then treated with a Polytron PCU-2 (Professor P. Willems, Pat. Kinematica Gmbh, Luzern, Switzerland) at a setting 10 for 30 s. This last homogenate was centrifuged at $9000 \times g$ for 10 min. The supernatant was filtered through cheesecloth to remove free floating fat and centrifuged at $100000 \times g$ for 30 min. The resultant microsomal pellet was homogenized for 30 s in 6 vols. of buffer A. The resulting homogenate was layered by fractions of 5 ml over 20 ml of a 30% (w/v) solution of sucrose in buffer A and centrifuged at $75000 \times g$ for 30 min in a Beckman SW 28 swinging bucket rotor. The supernatant containing a resultant band at the buffer/30% sucrose interface was pelleted at $100000 \times g$ for 30 min. The pellet obtained was then resuspended in buffer B (2 mM EGTA/2 mM DTT/30 mM Tris-HCl (pH 7.4)) to a final concentration of 3 mg/ml, and stored at −20°C.

Electron microscopy. Pellets obtained after the last $100000 \times g$ centrifugation were fixed for 30 min at room temperature with 2.5% glutaraldehyde in 80 mM phosphate buffer (pH 7.0). Post-fixation was effected with 1% osmium tetroxide for 30 min. Preparations were then embedded in Araldite with propylene oxyde as intermediary solvent. Thin sections were contrasted using lead citrate and uranyl nitrate. Examination was performed with a Philips electron microscope EM 201.

Protein determination. Protein concentrations were assayed by the standard method described in Bio-Rad Technical Bulletin for protein assay (July, 1981) which was originally reported by Bradford [22]. Dye reagent concentrate for the assay was purchased from Bio-Rad laboratories. Bovine serum albumin was used as a standard protein.

K^+-dependent p-nitrophenylphosphatase assay. Microsomal and purified membranes were incubated for 30 min at 37°C at a protein concentration of 3 mg/ml with 3 mM $MgCl_2$/20 mM KCl/0.5 mM EGTA/2 mM DTT/3 mM *p*-nitrophenyl phosphate/30 mM Tris-HCl (pH 7.4). Different concentrations of a freshly prepared solution of CHAPS were added, varying from zero to 10 mM in the final volume assay. The reaction was started by the addition of *p*-nitrophenyl phosphate. After incubation, 500 µl of 0.1 M NaOH was added. Hydrolysis of *p*-nitrophenyl phosphate was estimated by measuring the concentration of *p*-nitrophenol by its absorbance at 405 nm. ε was assumed to be 18500 $M^{-1} \cdot cm^{-1}$. Activity in the presence of 0.1 mM ouabain was measured concurrently. This last activity was subtracted from the total *p*-nitrophenylphosphatase activity to give the K^+-dependent activity.

Ca^{2+}-ATPase assay. The Ca^{2+}-stimulated ATPase was assayed with slight modifications by the method of Seiler and Fleischer [23]. Microsomal and plasma membrane proteins (100 µg) were incubated for 30 min at 25°C in 30 mM Tris-HCl buffer (pH 7.4) containing 3 mM $MgCl_2$/3 mM ATP/100 mM KCl/ionophore A23187 (5 µg/ml) and either 50 µM $CaCl_2$ or 1

mM EGTA. The reaction was terminated by adding 1 ml of cold 20% trichloroacetic acid and the samples were centrifuged. Inorganic phosphate concentration was determined into the supernatants by the method of Lebel et al. [24].

Succinate dehydrogenase assay. Succinate dehydrogenase (EC 1.3.99.1) was assayed according to Singer et al. [25]. The reaction was started by addition of phenazine methosulfate and the kinetic of reduction of 2,6-dichloroindophenol was carried out at 578 nm. ε was assumed to be 21 000 $M^{-1} \cdot cm^{-1}$.

SDS-polyacrylamide gel electrophoresis. Samples and reference proteins were denatured at 100°C for 2 min in 30 mM Tris-HCl buffer (pH 7.4) containing 0.05% SDS and 3% β-mercaptoethanol. Electrophoresis was performed on a 7.5/22.5% (w/v) acrylamide gradient gel by the method of Laemmli [26]. The gels were subjected to electrophoresis at 110 V for 15 h.

Dexamethasone binding assays. After defining the characteristics of hormone binding, as described in detail in Results, the following standard procedure was adopted. Dexamethasone binding was carried out at 37°C for 60 min, in polyethylene tubes, with 3 mM $MgCl_2$/2 mM ATP/1 mM EGTA/2 mM DTT/2 nM [^{3}H]dexamethasone/30 mM Tris-HCl (pH 7.4) in the presence or absence of a 200-fold molar excess of unlabelled dexamethasone. Each incubation volume of 300 μl contained approx. 300 μg membrane protein. The reactions were started by the addition of [^{3}H]dexamethasone and were terminated by rapid cooling of the assay tubes in an ice bath and by the addition of 3 ml of iced buffer C (2 mM DTT/1 mM EGTA/30 mM Tris-HCl (pH 7.4)). Free dexamethasone was separated from dexamethasone bound to the plasma membranes by vacuum filtration on prewet glass microfibre filters (GF/C Whatman). After addition of the membranes, filters were immediately washed three times with 3 ml of iced buffer C. Then filters were dried and transferred to minivials containing 5 ml of scintillation medium (4 g PPO, 0.1 g POPOP per liter toluene). Radioactivity was determined in a Searle model delta 300 liquid scintillation spectrometer. All assays were carried out in triplicate. Specific [^{3}H]dexamethasone binding was defined as the difference in radioligand binding in the absence and presence of a displacing concentration (0.4 μM) of unlabeled dexamethasone. Non-specific binding constituted less than 30% of the total binding.

Results

Preparation of the membranes

In the method reported here, separation of plasma membranes from contractile elements and other sub-cellular fractions was obtained in a working day by differential centrifugation. After centrifugation of the microsomal membranes on 30% sucrose solution, a band at the buffer/30% sucrose interface and a pellet were recovered. Table I shows a protein yield of 0.8 mg for the fraction binding at the buffer/sucrose interface, from 1 g of ground muscle. If the membranes were extracted in the absence of molybdate, but always in the presence of EGTA, the protein yield of this fraction was in the range of 0.55 mg per g of fresh muscle. In the absence of EGTA and molybdate, the yield was in the range of 0.30–0.35 mg. So, it appeared that EGTA alone was not sufficient to completely inhibit this proteolysis, but that the presence of molybdate prevented this protein cleavage. Complementary experiments conducted in our laboratory with low and high Ca^{2+}-requir-

TABLE I

PROTEIN YIELDS AND SPECIFIC ACTIVITY OF MARKER ENZYMES DURING ISOLATION OF PLASMA MEMBRANE VESICLES

Yields are expressed as mg protein/g of skeletal muscle and specific activities as μmol/min per mg protein. K^+-dependent *p*-nitrophenylphosphatase activity was obtained by subtracting the values obtained in the presence of ouabain from the total *p*-nitrophenylphosphatase (PNPPase) activity. The results are averages of values from three separate experiments. n.a.: no activity.

Fraction	Microsomal membranes	Plasma membranes
Yield	3.40	0.80
Total PNPPase [a]	3.04	8.42
Total PNPPase [a] + 0.1 mM ouabain	2.44	6.54
K^+-dependent PNPPase	0.60	1.88
Ca^{2+}-ATPase	1.26	0.32
Succinate dehydrogenase	n.a.	n.a.

[a] Activity measured in the presence of CHAPS (Fig. 3).

ing proteinases from rabbit skeletal muscle [18] showed a complete inhibitory effect of 20 mM molybdate on the caseinolytic activity of these enzymes (unpublished results).

Morphology of the plasma membranes

Thin section electron microscopy of the fractions obtained at the buffer/30% sucrose interface indicated that all were constituted of vesicular elements and no contamination with mitochondrial or myofibrillar material was seen (Fig. 1). In contrast to the plasma vesicles obtained from skeletal muscle by Boegman et al. [27] and by Seiler and Fleischer [23] which present an irregular shape caused by the exposure to concentrated salt solutions, fractions prepared by our procedure consisted mainly of intact spherical vesicles (Fig. 1).

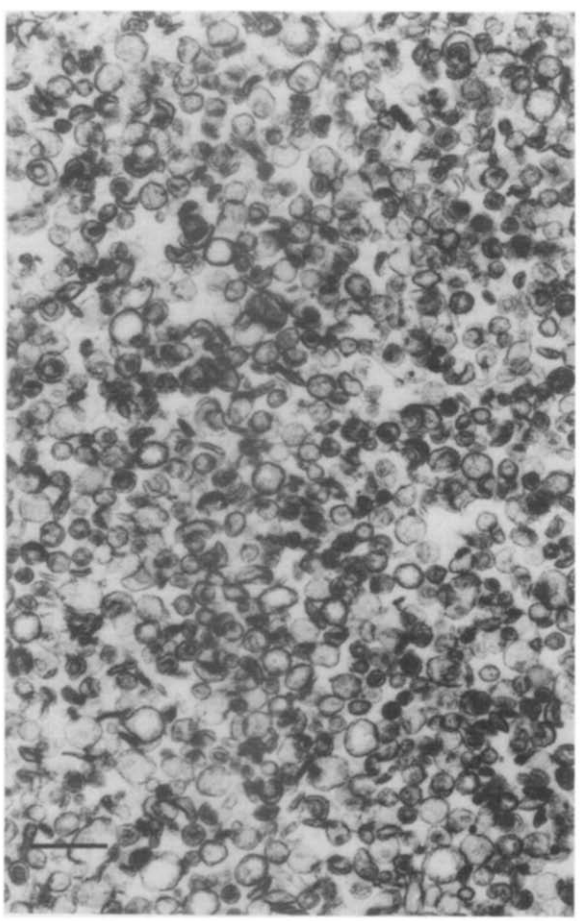

Fig. 1. Electron micrograph of the fractions obtained at the buffer/30% interface solution. The bar line represents 2μm.

Enzymatic activities of the membranes

K^+-dependent *p*-nitrophenylphosphatase activity associated with ($Na^+ + K^+$)-ATPase was studied in the membrane preparations obtained. In effect, the phosphatase activity is involved in the hydrolysis of the phosphate ester provided by the Na^+-dependent phosphorylation of the ($Na^+ + K^+$)-ATPase which is localized in the plasma membrane [28,29]. Table I shows that the highest K^+-dependent *p*-nitrophenylphosphatase activities were found in the band obtained at the buffer/30% sucrose interface, pointing to an enrichment of this fraction in plasma membranes. The quantity of CHAPS necessary for maximum activation of K^+-dependent *p*-nitrophenylphosphatase at a given protein concentration was determined after each membrane purification, and for different times of storage at −20°C (Fig. 2). A decrease of the enzymatic activity was observed when excess detergent was used in the incubation medium (Fig. 2). These results agree with those reported by Seiler and Fleischer [23] and by Jørgensen [30], who preincubated plasma membranes with SDS to unmask the activity of ($Na^+ + K^+$)-ATPase. On the other hand, no K^+-dependent *p*-nitrophenylphosphatase activity was revealed if EGTA and molybdate were omitted from the extraction medium.

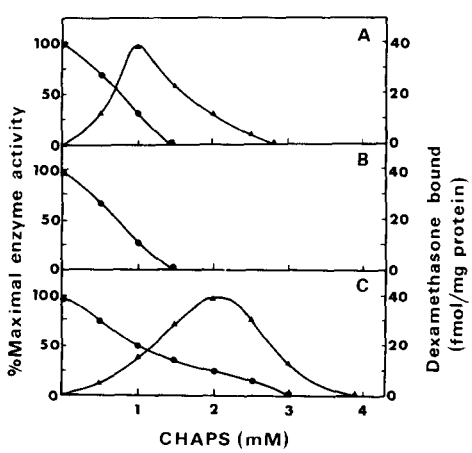

Fig. 2. K^+-dependent *p*-nitrophenylphosphatase activity and binding of [^{3}H]dexamethasone in plasma membranes from skeletal muscle as a function of CHAPS concentration, and for different times of storage at −20°C. Samples A, B, C correspond to three different animals. Storage time at −20°C (in days); ▲, 2; ●, 8.

The presence of a low residual Ca^{2+}-ATPase activity was observed in the fraction obtained at the buffer/30% sucrose interface (Table I). Although no contamination by sarcoplasmic reticulum was observable by electron microscopy, the possibility has been considered that this Ca^{2+}-ATPase activity was a contamination by these organelles. So, assays have been made to reduce this residual activity by decreasing the sucrose density of the cushion. However, the Ca^{2+}-ATPase activity was not modified and the yield of membrane obtained was very low. Another possibility to explain this fact was the presence of an endogenous (Mg^{2+}, Ca^{2+})-ATPase as reported by Sulakhe et al. [31] for plasma membranes isolated from rabbit skeletal muscle.

Succinate dehydrogenase, which is considered as a mitochondrial marker [32], was virtually undetectable in the microsomal and plasma membrane fractions (Table I).

SDS-polyacrylamide gel electrophoresis

By gel electrophoresis, a great variety of polypeptides of different molecular weights were found in the purified membranes (Fig. 3). Two protein bands in the range 96–98 kDa presented a high intensity of colouring, but the 96 kDa band masked partially the 98 kDa band (Fig. 3, lane A). When membranes were purified in the absence of EGTA and molybdate, the band of 96 kDa disappeared completely (Fig. 3, lane B). These results were in agreement with the data obtained during protein determination, which showed that a proteolysis of the membranes occurred when EGTA and molybdate were omitted from the extraction medium.

Optimal conditions for [^{3}H]dexamethasone binding

Fig. 4 shows the effects of time and temperature on the specific binding of [^{3}H]dexamethasone to plasma membranes purified from skeletal muscle. Specific binding occurred rapidly and was complete in 50 min at 37°C. At 4°C, no specific binding of dexamethasone was observable, contrary to the results of Suyemitsu et al. [13] and Koch et al. [14] who reported a rapid association of glucocorticoids with plasma membranes at 0°C. However, these authors observed specific binding at 0°C with natural glucocorticoids, but not with synthetic glucocorticoids like dexamethasone. Our results are in agreement with those of Omrani et al. [33] who related a dexamethasone binding to microsomes from rat liver within 30 min at 22–23°C. On the other hand, these data concern-

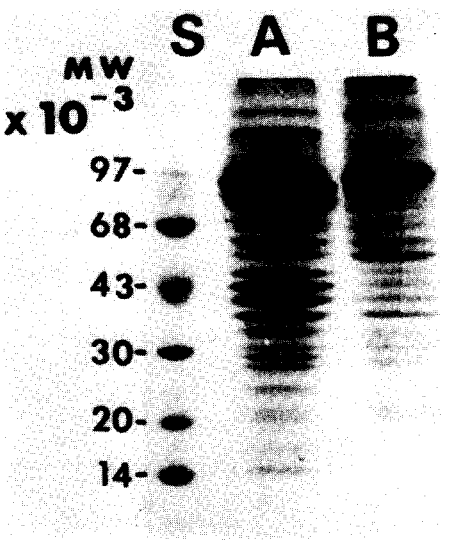

Fig. 3. SDS-polyacrylamide electrophoresis of plasma membranes from skeletal muscle. Membranes were purified in the presence of 2 mM EGTA and 20 mM molybdate in the buffer A (lane A) or in their absence (lane B). Lane S contained molecular mass markers (97 kDa, phosphorylase *b*; 68 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase. 20 kDa, soybean trypsin inhibitor; 14 kDa, lysosyme).

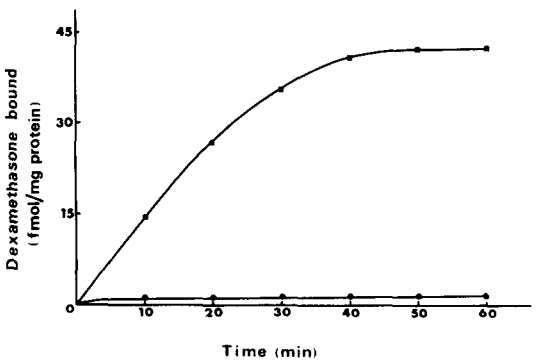

Fig. 4. Time-course of the association of dexamethasone with plasma membranes from skeletal muscle at 4°C (●) and at 37°C (■).

ing the effects of time and temperature were only observable after expression of latent binding by incubation of the plasma membranes with CHAPS. In each case, the concentrations of detergent required to obtain maximal K^+-dependent *p*-nitrophenylphosphatase activity and optimal [^{3}H]dexamethasone binding were exactly the same. The curves representing [^{3}H]dexamethasone binding and K^+-dependent *p*-nitrophenylphosphatase activity as a function of CHAPS concentration were strictly identical (Fig. 2). So, it was possible to obtain optimal [^{3}H]dexamethasone binding by determining the concentration of CHAPS which gave maximal K^+-dependent *p*-nitrophenylphosphatase activity. These results were consistent with a common action for detergent activation of K^+-dependent *p*-nitrophenylphosphatase activity and glucocorticoid binding. It appeared also that dexamethasone binding sites were tightly bound to plasma membranes, because after incubation with CHAPS and careful washing of the membranes on glass fiber filters, binding was always observable. This implied that dexamethasone binding on plasma membrane vesicles did not correspond to contamination with cytoplasmic glucocorticoid receptors or to physical entrapment of this last receptors into membrane vesicles. The binding of [^{3}H]dexamethasone was linear as a function of protein concentration between 50 and 750 μg.

Dexamethasone binding to plasma membranes was only observed in the presence of 2 mM DTT and was completely suppressed in its absence. This result was in agreement with those obtained by Housley et al. [34] on cytosolic receptors, who suggested that a sulphydryl group was required for steroid binding.

Without ATP, dexamethasone binding varied from 0 to 20 fmol per mg membrane protein from one animal to another. This probably reflected the concentrations of ATP in the muscle, which themselves, depend on the physiological state of the animals used. So, assays were effected with ATP concentrations from 1 to 10 mM, but above 2 mM, we observed artefactual precipitations of the membranes. With 2 mM ATP, an average binding of 40 fmol per mg protein was observed from one animal to another. These preliminary observations on ATP dependence of dexamethasone binding to plasma membranes are in agreement with the results of other authors [35–37] who showed a direct role for ATP in the binding of glucocorticoids to cytosolic receptors. More, Housley et al. [38] have recently demonstrated the phosphorylation of the cytosolic glucocorticoid in L-cells.

In the absence of EGTA and 20 mM molybdate during purification of the membranes, no binding of [^{3}H]dexamethasone was observable. On the other hand, the addition of 0.25 M sucrose into the extraction medium did not induce any change in dexamethasone binding.

Binding as a function of [^{3}H]dexamethasone concentration

The specific binding of [^{3}H]dexamethasone to plasma membranes from skeletal muscle was saturable with respect to the free concentration of ligand (Fig. 5). The saturation was nearly complete at about 1.8 nM dexamethasone. When binding data are rigorously submitted to Scatchard graphical analysis [39], the plot obtained is curvilinear at both extremities (Fig. 5, inset). Nevertheless, and in spite of the limits of the Scatchard representation which have been thoroughly emphasized by Braunsberg et al. [40] and Klotz [41], a rough estimate of the number of receptor sites and of the dissociation constant has been attempted by the least-square method. Thus, we obtained respective values of $0.6 \cdot 10^{-9}$ M for the dissociation constant and of $60 \cdot 10^{-12}$ mol/mg protein, for the concentration of receptors.

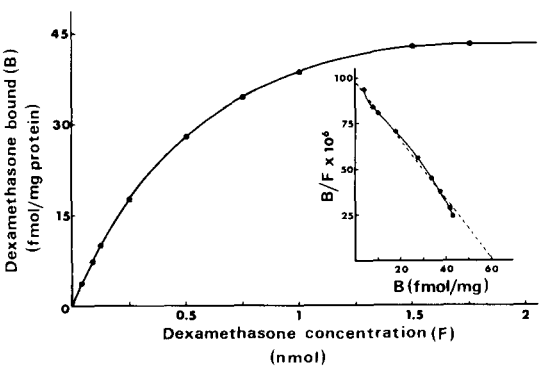

Fig. 5. Binding of [^{3}H]dexamethasone as a function of radioactive steroid concentration to plasma membranes from skeletal muscle at 37°C. Points indicate the means of triplicate determinations. Inset. Scatchard analysis of the same data. The dotted straight line was obtained by the least-square method and the continuous line by rigorously plotting all the points.

TABLE II

COMPETITION FOR [^{3}H]DEXAMETHASONE-BINDING SITES IN PLASMA MEMBRANES BY VARIOUS UNLABELED STEROIDS

Results are presented as the percentage of [^{3}H]dexamethasone displaced, relative to the displacement produced by unlabeled dexamethasone which was taken as 100%. Plasma membranes from skeletal muscle were incubated, with 2 nM [^{3}H]dexamethasone alone or in the presence of a 1000-fold excess of the unlabeled steroid, as indicated in Materials and Methods. The results are means of triplicate determinations.

Unlabeled competitor	% of displacement
Dexamethasone	100
Triamcinolone acetonide	96
Corticosterone	88
Cortisol	98
Progesterone	93
17β-Estradiol	0
Testosterone	8

Stereospecificity of dexamethasone binding

To assess the specificity of [^{3}H]dexamethasone binding to plasma membranes, the ability of various steroid hormones to displace dexamethasone was examined. Table II presents the values obtained for the inhibition of [^{3}H]dexamethasone binding. Unlabeled progesterone abolished specific binding of [^{3}H]dexamethasone with a great efficiency.

Neither estradiol nor testosterone affected [^{3}H]dexamethasone to the plasma membranes. In contrast, the natural glucocorticoids cortisol and corticosterone produced a marked displacement of dexamethasone bound to the plasma membranes.

Discussion

The results reported here describe the specific binding of dexamethasone to plasma membranes from skeletal muscle. Membrane receptors for glucocorticoids have been observed in liver [13] and pituitary gland [14], but only for natural steroids. In contrast to the present results, these receptors bound glucocorticoids at 0°C, in the absence of ATP and DTT, and without unmasking by detergent. In this report, two major arguments are in favour of the membrane location of dexamethasone binding sites. Firstly, a separation method permitting the isolation of well defined plasma membranes was developed. Secondly, a membrane treatment by freezing or by detergent was necessary to observe a specific binding.

The dexamethasone binding in plasma membrane could not be distinguished from cytosolic binding because both had similar steroid specificity, were affected by thiol reagents, were stabilized by molybdate and displayed similar dependence for ATP. However, a major difference exists for the optimal temperature of dexamethasone binding between the cytosolic receptors (4°C) and the membrane receptors (37°C). As explained by Nenci et al. [42], it may be that the binding of the cytosolic receptor towards a steroid be significantly modified if it is integrated into the hydrophobic microenvironment of plasma membranes. So, the interactions between receptors and steroids could be different in the presence of membrane phospholipids and, consequently, the temperature requirements could change. In addition, the strength of hydrophobic bonds increases when temperature increases [43]. With regard to physiological significance, the possibility of obtaining rapid binding at 37°C with plasma membrane receptors, and not at 4°C as with cytosolic receptors, is also more satisfactory. Moreover, Schulte et al. [44] have reported a phospholipid requirement in the specific binding of glucocorticoids to cytosolic receptors. This is compatible with a solubilization of native membrane receptors during cell homogenization and fractionation, as reported by other authors [6–9]. We have chosen skeletal muscle for our research on glucocorticoid membrane receptors because of its sensibility to this hormone [45], its cellular homogeneity and the experience of our laboratory for Ca^{2+}-dependent proteolysis in this tissue [17,18]. The inconveniences of muscle, namely the presence of a dense tissue network and the difficulties encountered in revealing plasma membrane marker activities, are compensated by the stability of the membranes during extraction, once the proteolysis has been controlled. This stability could explain that, in our experiment, the presence or absence of sucrose in the homogenization medium, which is currently used to preserve subcellular structures, had no effect on the membrane binding of dexametha-

sone. In the study of Pietras and Szego [9] estrogen receptors from uterine cells were localized in plasma membranes only if 0.25 M sucrose was used during extraction. In fact, these results are complementary to ours and show that the facility of obtaining cytosolic receptors could be directly related to the fragility of the plasma membranes in a particular tissue. In addition, cytosolic receptors for glucocorticoids were reported in skeletal muscle [45–48] and in myoblasts [49]. However, in each case these cytosolic receptors were prepared by centrifugation of a crude homogenate at $27\,000 \times g$ and by using the supernatant fraction for the binding assay. In view of our method of isolation and purification of the plasma membranes, and in the absence of data concerning an eventual contamination by cellular organelles, it is possible that the $27\,000 \times g$ fractions obtained by these authors contained membrane receptors.

In agreement with the results obtained for cytosolic receptors [50,51], we observed that the presence of molybdate and EGTA in the extraction medium was essential to observe a binding of dexamethasone to plasma membranes. In their absence, we observed a proteolytic activity, as shown by protein determination and by SDS gel electrophoresis. As reported in this paper, 20 mM molybdate completely inhibits caseinolytic activity of high and low Ca^{2+}-proteinases from skeletal muscle. So, we hypothesize that molybdate could stabilize membrane glucocorticoid binding by inhibiting Ca^{2+}-proteinase activities.

Acknowledgments

We are grateful to Professor P. Louvet for the fine electron micrographs, and to Doctor P. Cottin for stimulating discussions on Ca^{2+}-proteinases. We wish also to thank Mrs. C. Dubourg for typing the manuscript.

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