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Photolabeling of the human erythrocyte glucose carrier with androgenic steroids

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Androgenic steroids, which are potent inhibitors of facilitated hexose transport in human erythrocytes, were tested as possible natural photolabels of the hexose carrier protein. Androstenedione, which inhibited 3-*O*-methylglucose uptake half-maximally at 30–50 μM (EC_{50}), was the most potent inhibitor of the photolabile steroids tested. It appeared to interact directly with the carrier, since it (1) inhibited equilibrium [^{3}H]cytochalasin B binding to high affinity D-glucose-sensitive sites in both intact cells (EC_{50} = 63 μM) and protein-depleted ghosts (EC_{50} = 61 μM), (2) inhibited cytochalasin B photolabeling of the band 4.5 carrier region in electrophoretic gels of protein-depleted ghosts (EC_{50} = 50 μM), and (3) underwent photoincorporation into the same gel region in a D-glucose- and cytochalasin B-sensitive fashion. However, Dixon plots for inhibition of both cytochalasin B binding and transport were upward-curving, indicating the binding of more than one molecule of androstenedione to the carrier. The photoincorporation of androstenedione into band 4.5 protein was both time- and concentration-dependent, and not associated with damage to unlabeled carrier. It probably occured by activation of the α,β-unsaturated ketone on the steroid rather than indirectly by photoactivation of a group on the carrier protein, as occurs with cytochalasin B. Although androstenedione may bind to more than one region of the carrier, as well as to other non-carrier proteins, tryptic digestion of photolabeled ghosts produced a labeled M_r = 18000–20000 fragment, the labeling of which was inhibited by cytochalasin B, and which had an electrophoretic mobility similar to the major labeled tryptic fragment in cytochalasin B-labeled ghosts. These data suggest that androstenedione interacts directly with the hexose carrier and that it or other similar naturally photolabile steroids may serve as useful probes for structural dissection of the carrier protein.

Introduction

Androgenic steroids are potent inhibitors of hexose transport into human erythrocytes. Early studies by Lacko et al. [1] showed that a variety of steroids inhibited infinite *trans* glucose entry, with androgenic steroids having a greater potency than glucocorticosteroids. The kinetics of inhibition also differed between C-19 and C-21 steroids, the former showing either competitive or noncompetitive behavior, the latter only competitive inhibition. Krupka and Devés [2] clarified some of these kinetic observations by noting that whether substrate competed with the inhibitor depended on the location of steroid binding (to the endo- or exofacial carrier with substrate site facing inward or outward) and on the direction of transport (entry or exit). For example, androst-4-ene-3,17-

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dione (androstenedione), one of the most potent inhibitors of transport, was found to inhibit xylose efflux competitively and half-maximally at 20 μM [2], in contrast to the noncompetitive inhibition of glucose entry noted by Lacko et al. [1]. Krupka and Devés [2] took this to indicate that androstenedione binds endofacially either on or near the inward-facing substrate site of a carrier which cannot bind substrate and inhibitor simultaneously. Cytochalasin B has also been shown by Devés and Krupka [3] to bind to a site on the endofacial inward-facing carrier, however, the relationship between the binding sites of androstenedione and cytochalasin B is unknown.

Androgenic steroids having an α,β-unsaturated ketone configuration such as androstenedione can be excited by ultraviolet light of wavelength > 300 nm to a diradical state which can lead to reaction with nearby molecules [4,5]. This potential to act as a natural photoaffinity ligand has been utilized to photolabel the active site of Δ^5-3-ketosteroid isomerase [4], as well as the steroid binding site of the androgen-binding protein produced by the Sertoli cells of rat testes [6] and of testosterone-binding globulin present in the serum of rabbits [7]. Given the relatively high affinity of androstenedione for the hexose carrier and its potential for use as a covalent label, we reasoned that it should be possible to photolabel the hexose carrier protein in human erythrocytes with androstenedione. An additional advantage of this approach is that irradiation can be performed with wavelengths of light greater than 300 nm, thus minimizing possible photodestruction of the carrier or other membrane proteins. We found that androgenic steroids do indeed photolabel an erythrocyte band 4.5 protein in a D-glucose- and cytochalasin B-sensitive fashion. Although androstenedione probably binds to more than one site on the glucose carrier, tryptic digestion studies of labeled carrier protein suggest that the steroid does label the M_r = 18 000–20 000 tryptic fragment also labeled by cytochalasin B.

Experimental procedures

Materials. [4-^{3}H]Cytochalasin B (15.3 Ci/mmol), [1,2,6,7(n)-^{3}H]androst-4-ene-3,17-dione (90 Ci/mmol), and 17β-hydroxy[1α,2α-^{3}H]androsta-4,6-dien-3-one (60 Ci/mmol) were purchased from DuPont New England Nuclear. ICN supplied the 3-*O*-[*methyl*-^{14}C]methylglucose (57 Ci/mol). Unlabeled steroids were purchased from Sigma.

Preparation of aqueous solutions of steroids. All steroids were prepared from 5.36 mM stock ethanol solutions by a 30-fold or greater dilution in the appropriate aqueous buffer. In studies not shown, ethanol concentrations of 2% or less had no effect on either transport or cytochalasin B binding. At higher concentrations appropriate controls were performed.

Cell and membrane preparation. Blood was drawn from volunteers and anticoagulated with heparin (16.7 U/ml whole blood). Freshly drawn or stored erythrocytes were washed five times by centrifugation in equal volumes of 12.5 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.4). Following the first and second washes the cells were incubated 5 min at 37°C to allow exit of intracellular glucose [8]. After the washes the cells were brought to the appropriate hematocrit for subsequent studies. Erythrocytes were stored up to 3 days before use in the presence of 11 mM citrate, 22 mM D-glucose, 0.25 mM adenine, 12.8 mM phosphate, and 131.6 mM chloride with sodium as the cation (pH 7.0).

Leaky ghosts were prepared from erythrocytes by hypotonic lysis according to the method of Fairbanks et al. [9]. Protein-depleted membranes were prepared from leaky ghosts as described by Gorga and Lienhard [10] and resuspended in 50 mM Tris-HCl buffer (pH 6.8) before use.

Measurement of hexose transport. Zero-*trans* uptake of 3-*O*-methylglucose was measured as follows. Erythrocytes were suspended to a 20% hematocrit at 5°C in phosphate-buffered saline containing other agents as indicated. After a 10 min preincubation at the same temperature, uptake was initiated by pipetting 50 μl of swirled cells on to 20 μl of ice cold buffer containing 7 μM 3-*O*-[^{14}C]methylglucose. The cells were swirled and incubated for 30 s in an ice bath. The assay was terminated by addition of 1.2 ml of ice cold 'stop' solution containing 10 μM cytochalasin B in the phosphate buffer. The suspension was transfered to a microfuge tube and centrifuged for about 5 s in a Beckman Model B microfuge. The

supernatant was aspirated and the pellet was resuspended in another 1.2 ml of cold 'stop' solution followed by centrifugation. The supernatant of the second wash was aspirated, the pellet was resuspended in 0.2 ml of cold buffer, and 1 ml of 6% trichloroacetic acid was added with vortexing. The suspension was again centrifuged and 0.5 ml of the clear supernatant was removed for scintillation counting in 5 ml of ACS (Amersham). Correction for trapped extracellular label was made by subtraction of a 'zero time' value (cells plus 'stop' solution followed by labeled sugar). The transport rate was expressed as a percent of equilibrium values obtained by incubating a separate aliquot of cells for 30 min at 37°C. The concentration of steroid which inhibited transport of 3-*O*-methylglucose half-maximally (EC_{50}) was determined as described by Holman and Rees [11] from several steroid concentrations resulting in 25–75% inhibition of transport.

[^{3}H]Cytochalasin B binding to intact cells and protein-depleted membranes. Binding of cytochalasin B to intact cells was assessed by incubating 0.32 ml of washed erythrocytes at a 10% hematocrit in a microfuge tube with [^{3}H]cytochalasin B (final concentration = 10 nM) and the indicated steroid concentration in a final volume of 0.4 ml phosphate-buffered saline for 15 min at 37°C. The total radioactivity present in each incubation was determined by the following procedure. After completion of the incubation, the cell suspension was swirled vigorously, 50 μl of cells and buffer were removed and placed in a 7 ml scintillation vial, and the cells were lysed with 5 ml of ACS (Amersham). In order to decrease quenching, the vial was centrifuged at 3000 × *g* to precipitate the denatured protein. The radioactivity was then counted in a Packard 2000CA liquid scintillation counter with dpm calculation, and total radioactivity determined as eight times the radioactivity in each 50 μl aliquot. Preliminary studies showed that the decrease in counting efficiency of < 10% under these conditions could be corrected for by the dpm program. Furthermore, no significant loss of radioactive cytochalasin B in the precipitate could be detected in recovery studies.

The remaining cell suspension was centrifuged for 30 s in a Beckman Model E microfuge. The amount of free label was determined by removing 0.2 ml of the clear supernatant for scintillation counting, multiplying this value by the sampling factor of two, and adjusting for any radioactivity bound to cells in the presence of 50 μM unlabeled cytochalasin B (nonspecific binding) in that experiment. Bound cytochalasin B was calculated by subtracting the unbound radioactivity form the total radioactivity. In studies not shown it was confirmed that equilibrium binding was complete within 2 minutes.

Cytochalasin B binding to protein-depleted erythrocyte membranes (final concentration = 0.5–0.6 mg/ml) was determined in a fashion similar to that in cells except that the incubation temperature was 5°C, the incubation time was extended to 30 min, 50-μl aliquots for estimates of total radioactivity were counted directly, and membranes were pelleted at 15 000 rpm for 15 min in a Sorvall SM-24 rotor in 15 ml polycarbonate tubes.

Data from both intact cell and ghost experiments were analyzed by the method of Dixon [12] as modified by Gorga and Lienhard [10] for conditions in which there is a large excess of binding sites compared to total ligand.

Photolysis. Photolabeling of leaky or protein-depleted erythrocyte ghosts with [^{3}H]cytochalasin B was initiated by incubating 0.3 ml of ghosts (2–4 mg protein/ml) prepared in 5 mM sodium phosphate buffer, pH 8.0 (leaky ghosts), or Tris buffer (protein-depleted ghosts), with 0.4 μM [^{3}H]cytochalasin B, 0.1 mM cytochalasin E, and steroids as indicated, for 15 min at 5°C in plastic culture wells in the dark. Ethanolic solution of cytochalasins and steroids were dried under nitrogen before addition of ghosts. The ghost suspension were then irradiated on ice for 10 min with a USV-11 short-wave ultraviolet lamp (Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 1 cm from the ghost suspension. The membranes were diluted with 10 ml of the phosphate buffer, centrifuged for 15 min at 15 000 rpm in a Sorvall SM-24 rotor, and the supernatant aspirated. The washing procedure was repeated twice and the ghosts stored at −20°C until electrophoresis.

Photolysis of steroids was performed in a fashion similar to that of cytochalasin B, except that the preincubation period was 30 min at 5°C and

irradiation was performed for the indicated times using a 450-W Hanovia mercury lamp as described by Taylor et al. [6].

Gel electrophoresis. Electrophoresis of membrane protein (30 μg to each of three gel lanes) was performed by the method of Laemmli [13] as previously described [14], except that a mixture of C-12, C-14, and C-16 alkyl sulfates was used in the buffer ('lauryl' sulfate, Pierce Chemical Co.), samples were not boiled prior to electrophoresis, and 1.5 mm thick slab gels were used. Gels were cut into 1.9-mm sections, solubilized overnight in 0.5 ml of TS-1 solubilizer (Research Products International), and prepared for liquid scintillation counting in 5 ml of ACS. The radioactivity of the samples was determined after 48 h at room temperature with dpm calculations and correction for a small amount of residual chemiluminescence. Samples were counted until at least 1000 disintegrations had accumulated. Environmental backgrounds were subtracted from all samples. Prestained molecular weight markers (Bethesda Research Laboratories, Bethesda, MD) were run in lanes adjacent to the samples.

Results

Inhibition of hexose uptake by androgenic steroids

The EC_{50} values for hexose transport inhibition by several steroids with potential as photoaffinity labels were as follows (mean ± S.E. from N experiments): 17β-hydroxyandrosta-4,6-diene-3-one, 139 ± 22 μM (6); testosterone, 177 ± 28 μM (8); androstenedione, 38 ± 6 μM (4); and progesterone 71 ± 11 μM (6). The kinetics of transport inhibition by androstenedione, the most potent inhibitor of the steroids tested, were further evaluated in the experiment shown in Fig. 1. Under these conditions, the uptake of 3-*O*-methylglucose was essentially linear for 30–40 s, as shown in the inset. The inhibition of transport at two different concentrations of 3-*O*-methylglucose was a curvilinear function of the androstenedione concentration, when plotted according to Dixon [12].

Inhibition of cytochalasin B binding by androstenedione

The interaction of androstenedione with the carrier was further studied by measuring cytochalasin B binding in both erythrocytes and protein-depleted ghosts. When plotted according to Dixon [12], increasing concentrations of androstenedione inhibited [^{3}H]cytochalasin B binding to intact cells in an upward-curving fashion (Fig. 2) similar to that seen for transport (Fig. 1). The binding studies in intact cells were performed to mimic as closely as possible the state of the hexose carrier under transport conditions. Significantly, inhibition of cytochalasin B binding by D-glucose resulted in a linear Dixon plot (not shown). However, only 73% of labeled cytochalasin B binding could be prevented by 50 μM unlabeled cytochalasin B in intact cells, whereas 50 μM cytochalasin B plus 175 μM androstenedione inhibited total cytochalasin B binding by 81% ($P < 0.05$, paired 't'-test). There may well be some interaction of androstenedione with nonspecific cytochalasin B binding sites in intact cells. For this reason inhibition studies were also performed using

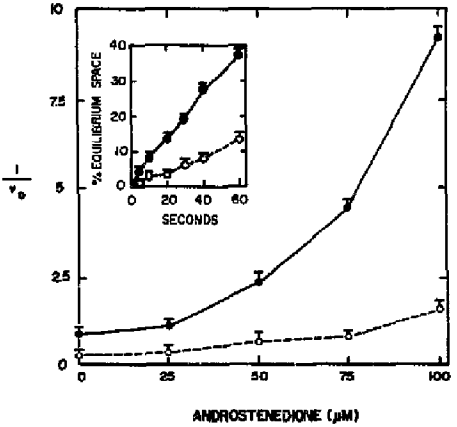

Fig. 1. Inhibition of hexose transport by androstenedione. The inhibition of hexose uptake by increasing concentrations of androstenedione in erythrocytes was measured at 0.25 (●) and 2.0 mM (○) 3-*O*-methylglucose. Erythrocytes were incubated for 10 min at 5°C with the indicated concentration of androstenedione and the transport assay performed as described under Experimental procedures. The duration of uptake was adjusted so that the observed uptake was 15% or less of the equilibrium 3-*O*-methylglucose space. Data are shown as mean ± S.D. of the inverse of the uptake rate (v_0 = μmol/l per s) of triplicate determinations from a representative experiment. The inset shows the time-dependence of 3-*O*-methylglucose uptake at 5.2 μM (●) and 2 mM (○) hexose from a representative experiment.

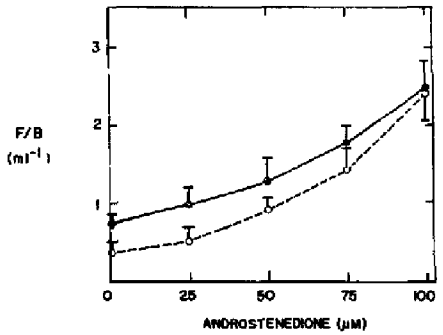

Fig. 2. Inhibition of cytochalasin B binding by androstenedione. The inhibition of [^{3}H]cytochalasin B binding by increasing concentrations of androstenedione in intact erythrocytes (●) and protein-depleted ghosts (○) was measured as described under Experimental procedures, and expressed according to the method of Gorga and Lienhard [10], in which F/B = ratio of free to bound cytochalasin B at equilibrium. In the experiments with cells, bound radioactivity was corrected for radioactivity which could not be displaced by 50 μM cytochalasin B. Data are mean ± S.E. of triplicate or duplicate determinations from five experiments for cells and two for ghosts.

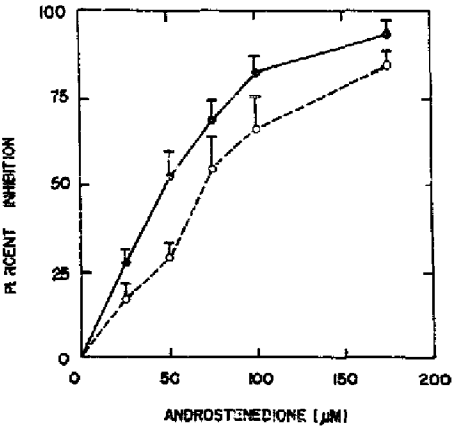

Fig. 3. Parallel inhibition of transport and cytochalasin B binding by androstenedione. The effectiveness of increasing concentrations of androstenedione in inhibiting erythrocyte hexose transport (●) and in inhibiting [^{3}H]cytochalasin B binding (○) was measured in intact erythrocytes. Hexose transport from five experiments is expressed as percent inhibition, derived by taking the percent of untreated control, minus 100, expressed as the absolute value. The percent inhibition of [^{3}H]cytochalasin B binding was calculated using data from five experiments according to the formula $(A-T)/((AG)-T)\times 100$, where A = unbound radioactivity in the presence of the indicated androstenedione concentration, T = unbound radioactivity with 10 nM labeled cytochalasin B alone, and (AG) = unbound radioactivity in the presence of 175 μM androstenedione and 150 mM D-glucose, all expressed as a percent.

protein-depleted ghosts, in which either 50 μM cytochalasin B or 500 mM D-glucose prevented essentially all label from binding (not shown). Once again upward-curving plots were obtained (Fig. 2), indicating a similar type of interaction in the two preparations, probably reflecting inhibition of cytochalasin B binding to more than one site on the hexose carrier.

Additional evidence that androstenedione inhibited cytochalasin B binding to sugar-sensitive sites in intact cells is derived from comparison of the EC_{50} values for inhibition of transport (50 μM) and cytochalasin B binding (62.5 μM) by increasing concentrations of androstenedione (Fig. 3). The EC_{50} for inhibition of [^{3}H]cytochalasin B binding by androstenedione to protein-depleted ghosts taken from the data in Fig. 2 was 61 μM. The similar effectiveness of androstenedione in inhibiting transport and D-glucose-sensitive cytochalasin B binding supports the notion of a stoichiometric albeit complex interaction of the steroid directly with the carrier protein.

Inhibition of [^{3}H]cytochalasin B photolabeling to band 4.5 by androstenedione

The location of the androstenedione-sensitive cytochalasin B binding sites was further evaluated with the use of [^{3}H]cytochalasin B photolabeling of erythrocyte ghosts. Androstenedione was effective in decreasing photolabeling of band 4.5 protein by cytochalasin B in leaky erythrocyte ghosts (Fig. 4). Additionally, androstenedione increased the radioactivity observed in regions corresponding to bands 1–2, 3 and 4.1–4.2, which are normally minimally labeled by cytochalasin B (Fig. 4, control gel). This increase in nonspecific labeling probably reflects the decreased numbers of high affinity sites on band 4.5 when modified by androstenedione, such that more cytochalasin B is

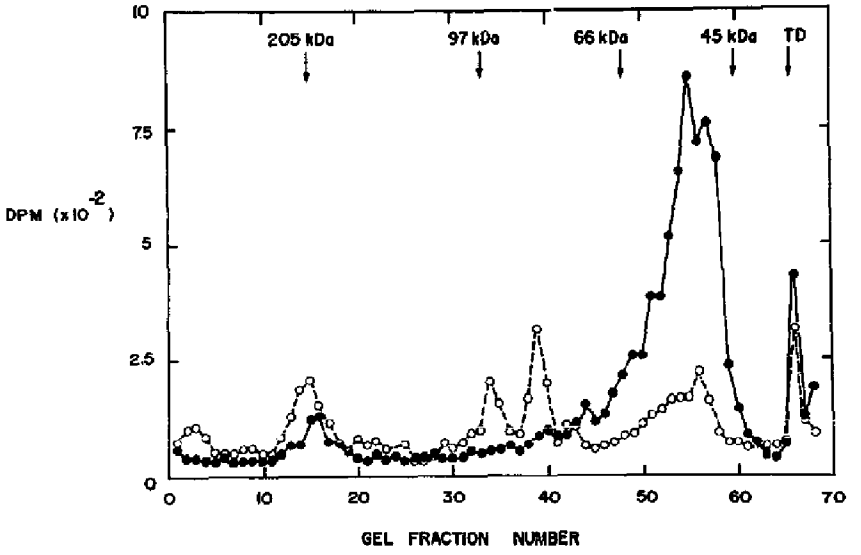

Fig. 4. Inhibition of [^{3}H]cytochalasin B photolabeling of band 4.5 by androstenedione. Photolabeling by [^{3}H]cytochalasin B of leaky erythrocyte ghosts incubated for 30 min at 5°C in the absence (●) or presence (○) of 200 μM androstenedione was carried out as described under Experimental procedures. Gel electrophoresis was performed as also described under Experimental procedures, with pre-stained molecular weight markers as noted.

available for attachment to other proteins. It is significant that this non-selective labeling by cytochalasin B occurred in the presence of 0.1 mM cytochalasin E, which was included to prevent labeling of actin (band 5). The inhibition of cytochalasin B photolabeling of band 4.5 by androstenedione was concentration-dependent and saturable (Fig. 5), with an EC_{50} of about 50 μM, similar to that for inhibition of transport and cytochalasin B binding (Fig. 3). The data presented thus far suggest that androstenedione in its inhibition of glucose transport binds to the band 4.5 hexose carrier, at a site or sites either overlapping with or having an allosteric effect on the cytochalasin B binding site.

Photolabeling of band 4.5 by [^{3}H]androstenedione

The interaction of androstenedione with the hexose carrier was next evaluated in experiments in which the steroid was used as a natural photolabel. In protein-depleted erythrocyte ghosts, irradiation with long-wave ultraviolet light in the presence of 0.27 μM [^{3}H]androstenedione resulted in the incorporation of radioactivity into the intrinsic membrane proteins, with prominent labeling of the broad band 4.5 carrier region (Fig. 6). Radioactivity incorporated into band 4.5 in this experiment represented about 150% of the total incorporated into bands 3 and 4.1–4.2. Band 4.5 labeling by androstenedione in the experiment shown in Fig. 6 was suppressed 68% by 50 μM cytochalasin B, and 57% by 300 mM D-glucose. This effect was also present but less dramatic at 30 μM labeled androstenedione (not shown). In other experiments neither 300 mM L-glucose ($n = 3$) nor 50 μM cytochalasin D ($n = 2$) had any effect on band 4.5 labeling by androstenedione compared to control. These results provide further

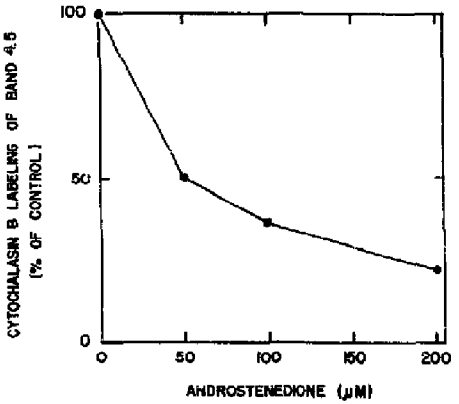

Fig. 5. Dose-response effect of androstenedione on inhibition of cytochalasin B photolabeling. Photolabeling of leaky erythrocyte ghosts by [^{3}H]cytochalasin B in the presence of increasing concentrations of androstenedione was performed as described in the legend to Fig. 4. Following electrophoresis, the total radioactivity in band 4.5 expressed as a percent of that found in band 4.5 in the absence of androstenedione was determined.

evidence that a substantial fraction of band 4.5 labeled by androstenedione corresponds to the hexose carrier. In leaky erythrocyte ghosts, 17β-hydroxyandrosta-4,6-diene-3-one also labeled a band 4.5 protein, with about 30% suppression by D-glucose (range = 16–41% suppression versus control, $N = 4$), although the labeling of other bands was much more prominent than with androstenedione (not shown).

The labeling of band 4.5 by androstenedione was time-dependent, with saturation beginning at about 1 h (Fig. 7), the time of irradiation chosen for most studies. Irradiation with > 300 nm light alone (no androstenedione) did not damage the hexose carrier, since even after 2 hours of irradiation, no loss of either total cytochalasin B binding or that sensitive to D-glucose could be detected in the protein-depleted ghosts (not shown).

A potential advantage of α,β-unsaturated ketones is a relatively high efficiency of incorporation at wavelengths of light > 300 nm. Photolysis of protein-depleted ghosts in the presence of increasing amounts of androstenedione resulted in in-

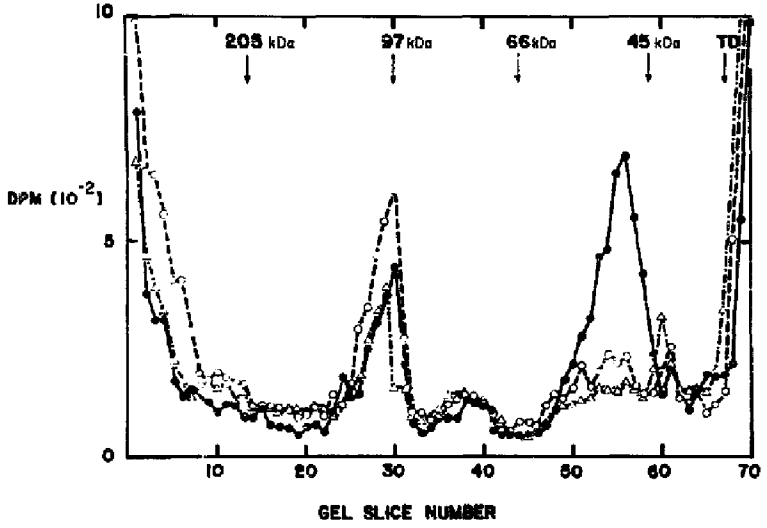

Fig. 6. Erythrocyte membrane photolabeling with androstenedione. Photolabeling of protein-depleted erythrocyte ghosts was performed at 0.27 μM [^{3}H]androstenedione without additions (●), or in the presence of 50 μM cytochalasin B (△), or 300 mM D-glucose (○) as described under Experimental procedures. The time of photolysis was 60 min.

creasing amounts of the steroid incorporated into band 4.5, expressed as efficiency of labeling in Fig. 8. The efficiency of labeling was calculated as a percent of the total amount of [^{3}H]cytochalasin B bound to separate ghost aliquots, the latter as determined by Scatchard analysis [15]. There was evidence of saturation over the range of concentrations of androstenedione employed, with the highest observed efficiency of about 5%. A maximal efficiency of 20% may be calculated using non-linear least-squares analysis [16], with a half-maximal effect occurring at 188 μM. This is to be contrasted with an efficiency of band 4.5 photolabeling by cytochalasin B of 0.2% in the studies of Fig. 4. However, even at 5% efficiency it was not possible to demonstrate irreversible inhibition of transport in dilute suspensions of whole cells by androstenedione following photolysis (not shown).

The mechanism of androstenedione incorporation into band 4.5 was probably through direct photolysis of the steroid, rather than by activation of a light-sensitive group on the carrier itself, since irradiation of protein-depleted ghosts in the presence of [^{3}H]cytochalasin B for 60 min with the 450-watt lamp through the pyrex filter resulted in only 0.02% efficiency of band 4.5 labeling, about 10-fold less than usually obtained at a shorter wavelength (see above).

In an attempt to determine whether androstenedione reacts near where cytochalasin B undergoes photoincorporation into band 4.5, protein-depleted ghosts which had been photolabeled either with [^{3}H]cytochalasin B or [^{3}H]androstenedione (top panel of Fig. 9) were submitted to tryptic digestion and electrophoresis on 12% acrylamide gels (bottom panel Fig. 9). Tryptic digestion of cytochalasin B-photolabeled membranes produced a single peak with an M_r = 18000–20000, while tryptic digestion of androstenedione-labeled membranes produced several

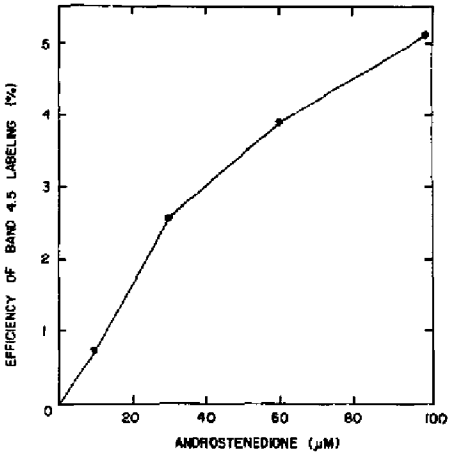

Fig. 8. Photoincorporation of increasing amounts of androstenedione into protein-depleted ghosts. The efficiency of photolabeling of band 4.5 in protein-depleted erythrocyte ghosts (2–2.5 mg/ml) was measured by incubating 0.3 ml of ghosts in the presence of the indicated androstenedione concentration and 10 μCi of [^{3}H]androstenedione with subsequent treatment and calculations as described under Experimental procedures and Results.

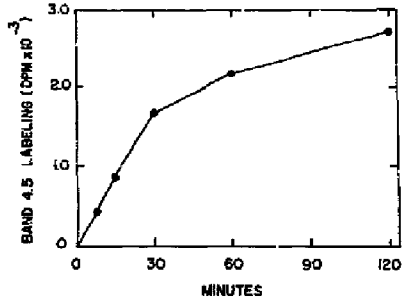

Fig. 7. Time course of androstenedione photolabeling of ghost membranes. The photoincorporation of [^{3}H]androstenedione into electrophoretic band 4.5 of protein-depleted ghosts was measured as a function of time of photolysis. A suspension of protein-depleted ghosts (2–2.5 mg/ml) in Tris buffer (pH 6.8) was added to a glass tube containing labeled and unlabeled androstenedione which had been dried under nitrogen. The membranes were allowed to warm briefly and swirled vigorously to aid in dissolution of the steroid (final concentration 60 μM, 0.5 Ci/mol). The suspension was irradiated with long-wave ultraviolet light as described under Experimental procedures and at the indicated times 0.3 ml aliquots were removed and placed on ice in the dark until the end of photolysis. Each of the samples was washed three times by centrifugation with 10 ml of Tris buffer and stored until electrophoresis.

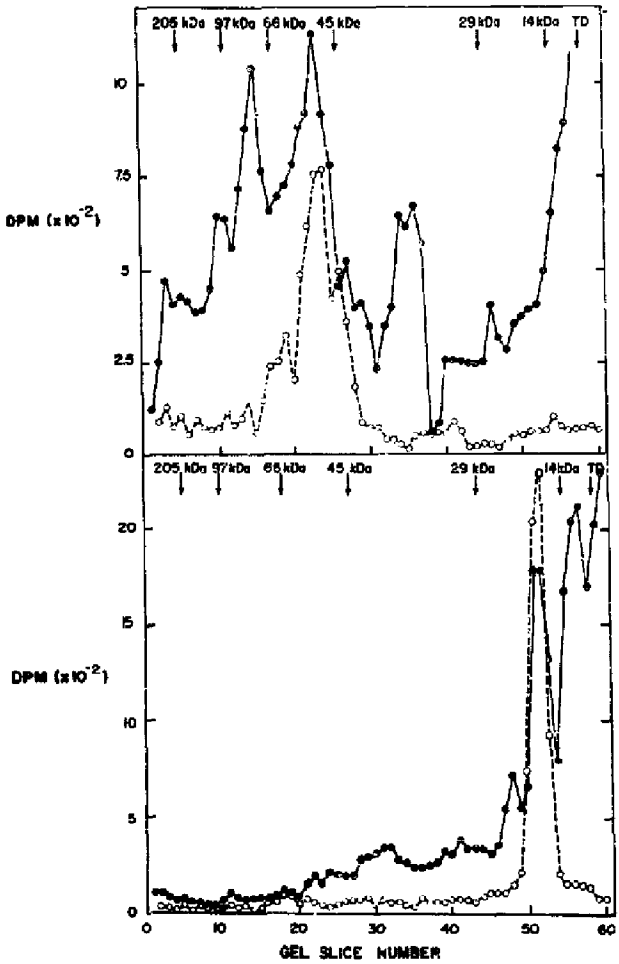

Fig. 9. Effects of trypsin on cytochalasin B- and androstenedione-photolabeled ghosts. Tryptic digestion of photolabeled protein-depleted ghosts was performed as follows. Protein-depleted ghosts (0.6 ml of 2 mg/ml protein) were photolabeled with [^{3}H]cytochalasin B (○) or 30 μM [^{3}H]androstenedione (●) as described under Experimental procedures, and each divided into a control (upper panel) and trypsin-treated (lower panel) aliquot. One-half of each photolabeled preparation was incubated for 30 min at 37°C, treated with 125 U/ml of ovalbumin trypsin inhibitor, washed three times by centrifugation at 22000×g in 10 ml of Tris buffer, and stored at −20°C. The other half of the photolysis suspension was incubated for 30 min at 37°C with 70 U/ml trypsin, followed by trypsin inhibitor, washes, and storage as above. Electrophoresis was performed as described under Experimental procedures except that 12% acrylamide gels were used.

low molecular weight peaks, one of which coincided with that labeled by cytochalasin B. When androstenedione photolabeling of protein-depleted ghosts was performed in the presence of 50 μM cytochalasin B or E, followed by tryptic digestion, there was a 60% suppression of labeling in the

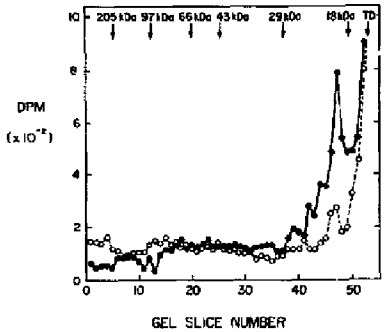

Fig. 10. Tryptic digestion of protein-depleted ghosts photolabeled with androstenedione in the presence of cytochalasin B or E. All conditions and procedures were the same as described in the legend to Fig. 9, except that protein-depleted ghosts were preincubated for 15 min on ice in the presence of 50 μM cytochalasin E (●) or cytochalasin B (○) prior to the addition of labeled androstenedione and photolysis.

M_r = 18000–20000 regions in membranes treated with cytochalasin B compared to membranes which had been treated with cytochalasin E (Fig. 10).

Discussion

The hexose carrier of human erythrocytes is quite sensitive to inhibition by androstenedione and other steroids. The extent of transport inhibition by steroids tends to parallel their lipid-buffer partition coefficients, which prompted Lacko et al. [1] to conclude that the inhibition of transport by steroids was related to strong hydrophobic interactions, possibly with membrane lipids. However, androstenedione and probably other steroids appear to interact directly with the carrier protein, based on the following observations. The results of kinetic studies of transport inhibition by androstenedione [1,2] are best explained if androstenedione binds to the cytosolic side of the carrier with the substrate site facing inward [2], a mechanism resembling that seen with the cytochalasin B [3]. In the present work further evidence for a direct interaction was obtained, since androstenedione decreased [^{3}H]cytochalasin B binding to high affinity and D-glucose-sensitive binding sites in erythrocytes (Fig. 2), inhibited [^{3}H]cytochalasin B photolabeling of the band 4.5 carrier region in electrophoretic gels (Figs. 4 and 5), and itself showed D-glucose- and cytochalasin B-sensitive photoincorporation into band 4.5 protein (Fig. 6).

However, it appears on the basis of transport and cytochalasin B binding data that more than one molecule of androstenedione bind to each carrier molecule. Lacko et al. [1] initially reported that androstenedione inhibited infinite-*trans* uptake noncompetitively, but when these data were further analyzed by the method of Dixon [12], upward-curving plots of $1/v$ versus inhibitor concentration were obtained. Similar curves for glucose exit have also been reported by Krupka [17]. Further kinetic analysis indicated that such a dramatic decrease in the inhibitor capacity was most compatible with binding of androstenedione with two sites on the carrier [1], although negative cooperative interactions between bound and unbound carriers could also explain the data. In the present work upward-curving Dixon plots were confirmed using zero-*trans* uptake of 3-*O*-methylglucose (Fig. 1). The inhibition of [^{3}H]cytochalasin B binding in whole cells and protein-depleted ghosts by increasing concentrations of androstenedione (Fig. 2) also showed the same pattern, again most compatible with more than one binding site for the inhibitor on the transport molecule. Although cytochalasin B is felt to interact with the carrier with a 1 : 1 stoichiometry [18], this does not appear to be the case for androstenedione.

Although photoincorporation of [^{3}H]androstenedione into protein-depleted erythrocyte ghosts (Fig. 6) was not as selective for the band 4.5 carrier region as was [^{3}H]cytochalasin B in leaky ghosts (Fig. 4), it nonetheless was comparable to that reported for [^{3}H]forskolin [19] or transported phenylazide sugar derivatives [20,21]. Moreover, at least one site of androstenedione photoincorporation into the glucose carrier may be similar to that observed with forskolin [19] and cytochalasin B [19,22,23]. This derives from studies of tryptic digestion of protein-depleted ghosts which had been photolabeled with either [^{3}H]cytochalasin B or [^{3}H]androstenedione (Fig. 9). It has been shown that such digestion of ghost membranes labeled with cytochalasin B results in exclusive labeling of a sharp 17–19 kDa band on electrophoresis, while

a broader glycosylated 30 kDa fragment is not labeled [19,22,23]. Given the lower initial specificity for band 4.5 labeling by androstenedione compared to cytochalasin B (Fig. 9, top panel), interpretation of electrophoretic patterns of tryptic digests of labeled membranes is problematic. Nonetheless, a sharply defined $M_r = 18000–20000$ fragment corresponding to that labeled by cytochalasin B was also prominent in tryptic digests of androstenedione-labeled ghosts (Fig. 9, bottom panel). Additionally, in ghosts photolabeled with androstenedione, cytochalasin B, but not cytochalasin E suppressed the labeling of this tryptic fragment (Fig. 10). This provides additional evidence that at least one of the androstenedione binding sites on the carrier protein lies on the same trypsin fragment as the binding site for cytochalasin B.

The photolabeling of band 4.5 by [^{3}H]androstenedione was time-dependent (Fig. 7), and a saturable function of the androstenedione concentration (Fig. 8). Considering the sensitivity of transport, cytochalasin B binding, and cytochalasin B photolabeling to inhibition by androstenedione (Figs. 3–5), one might have expected saturation of photolabeling to occur at concentrations of androstenedione less than 50 μM. Since irreversible photoincorporation did not saturate in the expected range (Fig. 8), it is possible that light-induced coupling is inefficient, or more likely that there are nonsaturable sites on band 4.5. The latter was difficult to estimate because concentrations of androstenedione over 200 μM required excessive amounts of ethanol for solubility.

The mechanism of photoincorporation of androstenedione into erythrocyte protein was very likely through direct activation of the α,β-unsaturated ketone on the steroid molecule, as demonstrated previously in the photolabeling of Δ^5-ketosteroid isomerase by various α,β-unsaturated ketosteroids [4]. This differs from the mode of covalent attachment of cytochalasin B to the hexose carrier, which is not the activated molecule in the photolabeling process. Convincing evidence ha been presented that an amino acid residue on the carrier, probably a tryptophan, is activated by the short-wave ultraviolet light (approximately 280 nm) required for cytochalasin B photolabeling, and it is this residue which reacts with cytochalasin B [24]. This type of endogenous activation probably also occurs in the photolabeling of forskolin, a potent inhibitor of transport which has recently been demonstrated by Lavis et al. [25] to bind to and by Shanahan et al. [19] to photolabel the hexose carrier protein. A major problem with the use of shortwave ultraviolet light in photoactivation is damage to the carrier protein [23,24]. Deziel et al. [24] reported that cytochalasin B binding was decreased 88% following irradiation with an unfiltered xenon-arc lamp, while only 2.4% of available carriers were labeled. By restricting the wavelength to 280 nm, they were able to achieve an efficiency of 8%, with inactivation of only half the carriers. The use of photolabels which can be activated at wavelengths of light > 300 nm such as α,β-unsaturated steroids or azidosalicyloyl sugar derivatives [26] allows labeling of some carriers without damaging those which are not labeled. This would facilitate identification of the carrier through subsequent purification, reconstitution, or antibody mapping studies.

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