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PURIFICATION AND CHARACTERIZATION OF PLASMA MEMBRANE FRACTIONS FROM CULTURED PITUITARY CELLS

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Highly purified plasma membrane fractions have been prepared from GH_3 pituitary cells grown in suspension cultures. These membrane fractions have been obtained by differential and sucrose gradient centrifugation and were characterized in terms of their lipid content, marker enzyme analysis and the binding of ^{3}H-labelled thyrotropin-releasing hormone (TRH) to its receptor. Alkaline phosphatase and 5′-nucleotidase activities were enriched 12- to 15-fold in the plasma membrane fraction with somewhat greater enrichment (28-fold) of the specific binding component for [^{3}H]TRH, with a specific activity of 2286 fmol [^{3}H]TRH bound per mg protein. A single class of binding sites for TRH was observed with an apparent dissociation constant of 18 nM, a value similar to that observed for intact cells. No detectable TRH binding to the nuclear fraction was observed that could not be ascribed to residual plasma membrane contamination. By electron microscopy, these fragments appeared to be sealed vesicles with an average diameter of approximately 1800 Å. The binding of ^{125}I-labelled wheat germ agglutinin was used as a marker for plasma membrane purification. In addition to specific binding to this membrane fraction, specific binding was also observed in the nuclear fraction. Studies with fluorescein-labelled wheat germ agglutinin revealed that, in fixed cells, fluorescence was restricted to the plasma membrane. However, if the cells were treated with Triton before labelling, most of the fluorescence was then associated with the cell nucleus. Hence, the use of wheat germ agglutinin binding as a specific plasma membrane marker must be reevaluated.

The secretion of hormones from the pituitary gland is under regulation by various hypothalamic and circulating factors. These factors control such responses as the stimulation or inhibition of hormone secretion, rapid ion or nucleotide fluxes, alteration of levels of messenger RNA which code for various secretory products and the regulation of hormone receptor levels. Most of these responses are initiated or mediated by various macromolecular components or receptors in the plasma membrane of these cells. Some of these receptors in pituitary cells for hypothalamic hormones have been characterized by binding studies using radioactively labelled hormones. Specific receptors have been detected for thyrotropin-releasing hormone (TRH) [1,2] and for luteinizing hormone-releasing hormone [3–8]. Recently, specific receptors for somatostatin [9,10] and dopamine [11] have been partially characterized as well.

Many of the above studies were performed on uncharacterized membrane fractions isolated from intact pituitary glands. In addition to the relatively limited amount of material available for study, the pituitary gland contains a heterogeneous population of hormone-producing cells, each producing a single hormone and having its own specific complement of

Abbreviations: TRH, thyrotropin-releasing hormone; GH, growth hormone; PRL, prolactin; FITC, fluorescein isothiocyanate; Hepes; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulphate.

receptors and plasma membrane proteins. In some cases, two or more different cell types, each producing a different polypeptide hormone, respond to the same hypothalamic hormone. TRH, for example, stimulates both thyrotropin- and PRL-producing cells to secrete their respective hormone products. Similarly, somatostatin inhibits the release of both GH and thyrotropin in the pituitary as well as the release of other hormones in other organs. The question of whether the receptors for these hormones on the different cell types are the same molecular entities and operate by similar mechanisms can only be answered by the isolation and characterization of the specific receptors from each cell type.

One approach to circumvent some of the problems associated with the characterization of hormone receptors in the plasma membranes prepared from multiple cell types, as encountered in the pituitary, is the use of clonal cell lines as a source of relatively homogeneous plasma membranes. One such line is the pituitary tumor cell line, GH_3, first cultured by Tashjian and his colleagues [12]. These cells synthesize and secrete substantial quantities of both GH and PRL and show many of the same hormonal responses and differentiated functions as do their non-replicating normal counterparts. The GH_3 cell line and various subclones have been useful for delineating some of the pharmacological properties of GH- and PRL-producing cells and for studying the stimulus-secretion response of secretory cells. Identification and partial characterization of several different hormone receptors on these cells, including receptors for TRH [13] and somatostatin [9], have been reported.

We have undertaken to isolate, purify and characterize the plasma membrane fractions from GH_3 cells. This is the first step in the characterization of several polypeptide and neurotransmitter receptors found in the plasma membranes of these cells so as to understand at a molecular level the mechanisms involved in the binding of hormone to its receptor and the subsequent cellular responses. The cells can be grown in large quantities in suspension culture and relatively pure plasma membranes isolated after homogenization by differential centrifugation and sucrose density gradient steps. A 15- to 30-fold enrichment of membranes with specific receptors for TRH has been obtained with similar enrichments in the activities of plasma membrane marker enzymes 5′-nucleotidase and alkaline phosphatase. A partial characterization of some of the other subcellular fractions is also reported.

Experimental procedures

Cell culture. The GH_3 cell line was obtained from the American Type Culture Collection, Bethesda, MD and was maintained in monolayer culture under a slight modification of conditions described by Tashjian et al. [12]. The cells were routinely grown in the medium described by Higuchi [15] with the addition of 25 mM Hepes buffer. Horse and fetal bovine sera were obtained from Grand Island Biological Co. or Sterile Systems, Inc. Penicillin and streptomycin were included in the media at concentrations of 50 000 units/l and 50 mg/l, respectively. Monolayer cells were passaged by trypsin treatment (0.1% w/w in phosphate-buffered saline). Suspension cultures were readily initiated from trypsin-treated cells and maintained in the same media but with the addition of 0.05% methyl cellulose (Fisher Sci.) in spinner bottles or shaker flasks. Suspension cultures were maintained at cell densities below $(0.5–0.75) \cdot 10^6$ cells/ml by addition of fresh media.

Plasma membrane isolation and purification. GH_3 cells ($(2–4) \cdot 10^9$) from suspension cultures were harvested by centrifugation at $500 \times g$ for 5 min. The cell pellet (5 ml) was washed twice by gentle resuspension in 40 ml cold phosphate-buffered saline solutions followed by centrifugation as above. All subsequent procedures were performed at 4°C with buffers containing 0.02% sodium azide. The washed pellet was resuspended in 50 ml buffer A (10 mM Tris-HCl buffer, pH 8.0) and the cell suspension placed in a Parr Cell Disruption Bomb. With stirring of the cell suspension, the bomb was pressurized with nitrogen to 700 lb/inch^2 and allowed to equilibrate for 30 min. The disrupted cells were collected from the exit tubing upon release of the pressure. To this homogenate was immediately added 1/10 vol. of a solution of 30 mM $MgCl_2$/100 mM NaCl.

The homogenate was centrifuged in a Sorvall SS-34 rotor at $1000 \times g$ for 30 s. The nuclear pellet obtained from this step was resuspended in 10 ml buffer A and re-centrifuged as above. The supernatants from both centrifugations were combined and

centrifuged at 100 000 × *g* in a Beckman 35 rotor for 1 h. The washed nuclear pellet was resuspended in a small volume of buffer A for later analysis. After high speed centrifugation, the 100 000 × *g* supernatant was aspirated and the microsomal pellet was gently resuspended in 7 ml 0.25 M sucrose, in buffer A solution, with a Dounce homogenizer. 4 vol. 60% (w/w) sucrose in buffer A were added to give a final sucrose density of 48%. 5 ml of this solution were placed in the bottom of 1 × 3.5 inch polyallomer tubes and overlayed with successive 6-ml aliquots of 41, 37 and 33% sucrose solutions. The tubes were filled with 10 ml 8.5% sucrose and the gradients were centrifuged at 65 000 × *g* in a Beckman SW27 rotor for 15 h. After centrifugation, five distinct bands were observed at the interfaces between adjacent sucrose layers. The densities of the different bands were determined by refractometry with an Abbe Refractometer (American Optical). The individual membrane bands were harvested from the top by removal with a syringe and a narrow gauge needle. The samples were diluted 2–3-fold with cold buffer and immediately centrifuged at 100 000 × *g* for 1 h. The resultant pellets were resuspended in 0.5 to 1 ml at 2 to 5 mg protein/ml 10 mM phosphate buffer, pH 7.4, or 10 mM Tris buffer, pH 8.0, and stored under a nitrogen or argon atmosphere at 4°C or at −80°C.

Membrane marker assays. 5′-Nucleotidase activity was measured essentially as described by Avruch and Wallach [16] by following the release of [^{3}H]adenosine from tracer amounts of [^{3}H]AMP (5 Ci/mmol, New England Nuclear Corp.) as a function of protein concentration and time. Inclusion of 0.1% digitonin to the assay system was found to reduce background counts significantly without altering the specific activity of the enzyme. Alkaline and acid phosphatase activities were measured by the procedure of Luben et al. [17]. NADPH-cytochrome *c* reductase was measured by the procedure of Hodges and Leonard [18].

TRH receptor assay. TRH receptors in the various fractions were measured by quantitation of the binding of [^{3}H]TRH (115 Ci/mmol, New England Nuclear) by a procedure similar to that described by Hinkle and Tashjian [2]. To 80-μl aliquots of each fraction were added 10 μl of a stock 500 nM solution of [^{3}H]TRH and 10 μl buffer or 10 μl unlabelled TRH to a final concentration of $5 \cdot 10^{-6}$ M for measurements of nonspecific binding. After incubation at 0°C for 1 h, 800 μl cold phosphate-buffered saline was added to each tube and the total mixture quickly filtered under vacuum on Whatman GF/C filters with a Hoefer 10 place filter unit. The filters were quickly washed with 4 × 2.5-ml aliquots of ice-cold phosphate-buffered saline. The whole wash procedure was completed within 10 s. The filters were transferred to scintillation vials to which 10 ml Aquasol II (New England Nuclear) were added and the vials were shaken vigorously. The vials were kept at 10°C for 2–3 h with occasional shaking and counted in a Packard B2450 Liquid Scintillation counter at a tritium efficiency of about 50%. The amount of nonspecific radioactivity trapped on the filters in the presence of excess unlabelled TRH varied from fraction to fraction throughout the purification procedure but was generally not more than 2–4% of the total [^{3}H]TRH added to the samples. Identical receptor activities were obtained by a centrifugation assay in which the membrane fractions were pelleted in a Beckman Airfuge at 100 000 × *g* for 30 min with subsequent solubilization of the pellet for liquid scintillation counting of bound [^{3}H]-TRH.

Wheat germ agglutinin assay. Iodinated wheat germ agglutinin was prepared for use as a general plasma membrane marker as described by Cuatrecasas [19,20]. The iodinated product was purified by affinity chromatography on an ovomucoid-Sepharose column. Specific binding of ^{125}I-labelled wheat germ agglutinin to the various fractions was measured by incubating aliquots of membrane fractions with $5 \cdot 10^4$ cpm ^{125}I-labelled wheat germ agglutinin for 1 h at 25°C. Replicate samples contained 10 mM *N*-acetyl-D-glucosamine for determination of nonspecific binding. The samples were centrifuged at 100 000 × *g* for 30 min and the supernatants carefully removed. The membrane-bound ^{125}I-labelled wheat germ agglutinin in each sample was counted in a Packard Gamma Spectrometer.

Visualization of the subcellular organelles which specifically bind wheat germ agglutinin was accomplished by the use of fluorescein-labelled wheat germ agglutinin (FITC wheat germ agglutinin, Miles Laboratories, Elkart, IN). GH_3 cells, grown on glass cover slips, were rinsed three times with Dulbecco's phosphate-buffered saline solution at 4°C before fixation

with 2% formaldehyde in a Ca^{2+}-Mg^{2+}-free phosphate-buffered saline solution for 30 min at room temperature. The fixed cells were extensively washed with phosphate-buffered saline and permeabilized by a 2-min exposure to 0.1% Triton X-100. The cells were further washed and then incubated with a 40 μg/ml solution of FITC wheat germ agglutinin in phosphate-buffered saline for 30 to 60 min at room temperature. Some samples were also labelled in the presence of 10 mM *N*-acetyl-D-glucosamine. Subsequently, the cells were extensively washed and the cover slips were mounted on glass slides with 90% glycerol in phosphate-buffered saline. A Nikon Biophot Fluorescence Microscope with epi-illumination was used for phase contrast and fluorescence examination of the labelled cells.

Other assays. Protein was determined by the method of Lowry et al. [21] with crystalline bovine serum albumin as standard. Total phospholipids were determined by measuring the amount of phosphate released after ashing of samples as described by Chen et al. [22]. Membrane preparations to be used for phosphate determinations were prepared in phosphate-free buffers. Cholesterol was assayed by a fluorimetric enzymatic assay [23]. 50-μl aliquots of the various membrane fractions were added to 700 μl isopropanol and the solution sonicated for 10 s in a Branson sonicator at the lowest power output setting. The solutions were centrifuged to remove precipitated protein and 20-μl aliquots were withdrawn for total cholesterol analysis. The fluorescence of the samples were read in a Perkin Elmer MPF-44B spectrofluorometer at an emission wavelength of 415 nm after excitation at 325 nm. Stock solutions of cholesterol were prepared as standards. The assay was linear in the range of 0.1 to 3 nmol cholesterol per sample. DNA was determined by the method of Munro and Fleck [44] or by the procedure of Labarca and Paigen [45] with Hoechst dye 33258.

Electron microscopy. Membrane samples were prepared for transmission electron microscopy by fixation in 3% glutaraldehyde overnight at 0°C and rinsed by centrifugation and resuspension in 0.1 M sodium cacodylate. The membranes were centrifuged and the pellet was post-fixed in 1% osmium tetroxide, stained en bloc for 60 min in 1% uranyl acetate, dehydrated with increasing concentrations of ethanol and cleared in propylene oxide. The pellets were infiltrated with Poly/Bed 812 epoxy embedding medium and the polymerized pellets were ultra-thin-sectioned for transmission electron microscopy.

Gel electrophoresis. Polyacrylamide slab gels were prepared and electrophoresed in the presence of SDS according to the method of Laemmli [24] and stained with Coomassie Brilliant Blue R 250 in 25% isopropanol, 10% acetic acid solutions and destained in the same solvent system without stain. The gels were stored in 10% acetic acid.

Results

Plasma membrane purification

Several different homogenization and purification procedures were evaluated during the course of these studies. The most efficient and reproducible procedure of membrane purification involved the disruption of the GH_3 cells by nitrogen cavitation followed by differential and density gradient centrifugation. Among the various procedures, that described in Methods was found to produce routinely the best yields of plasma membranes. In the initial phases of this study, a Polytron PT-10 homogenizer was used; this resulted in substantial disruption of nuclei with a subsequent decrease in the purity of the plasma membrane fraction as determined by TRH receptor levels and by comparative analysis of the different subcellular fractions by SDS-polyacrylamide gel electrophoresis.

After removal of the nuclei by the low-speed centrifugation step and of the soluble cytoplasmic proteins by the high-speed centrifugation step, the crude microsomal pellet fraction was further fractionated on a five-step, discontinuous sucrose gradient. After centrifugation to equilibrium, five distinct bands were observed in the tube, four in the gradient and one pelleted at the bottom of the tube as shown in Fig. 1. A long syringe needle was used to obtain small samples of the gradient for refractive index measurements near the middle of each band. The median densities of the bands, designated F1 (top) to F5 (bottom) were 1.11, 1.15, 1.18, 1.20 and 1.22 g/ml, respectively. Occasionally, an additional membrane band was observed floating near the top of the gradient; this band consisted mainly of lipids and contained low protein and enzyme marker levels.

The lighest fraction, F1, was generally not completely separated from fraction F2. However, the rather flocculent appearance of F1 was sufficiently different from the smooth, uniform appearance of F2 to allow the two fractions to be manually separated by aspiration.

The purification of the plasma membrane fraction was monitored by measuring the activities of several marker enzymes in the cell homogenate, intermediate fractions and sucrose gradient fractions. Table I summarizes the results from a typical preparation which began with approx. $4 \cdot 10^9$ cells; comparable results have been obtained from over 30 different preparations using different cell numbers. Alkaline phosphatase and 5'-nucleotidase, which are generally thought to be plasma-membrane-bound enzymes, show the highest specific activity in the lightest fraction, F1, of the sucrose gradient and the lowest activities in the more dense fractions of the gradient and in the nuclear fraction. In general, the distribution and relative purity of these two enzymes was similar in all fractions analyzed. Similar results were obtained for the plasma membrane enzyme ($Na^+ + K^+$)-ATPase (data not shown). Approx. 16–17% of the total activities for 5'-nucleotidase and alkaline phosphatase were recovered in F1. Acid phosphatase, a presumptive lysosomal enzyme marker, was also enriched in fraction F1, although not to the extent of the other plasma membrane markers. Cytochrome *C* reductase, an endoplasmic reticulum marker, was not detectable in the supernatant, nuclear or sucrose gradient fractions F1 and F2 but was detected in fractions F3, F4

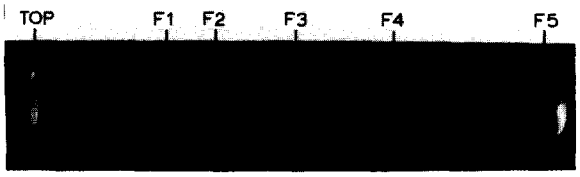

Fig. 1. Discontinuous sucrose gradient centrifugation of crude microsomal membranes from GH_3 cells. Approx. 44 mg membrane protein in a volume of 5 ml 48% sucrose were placed in the bottom of the centrifuge tube and covered with successive 5-ml layers of 41, 37, 33 and 8.5% sucrose. The membranes floated to their equilibrium densities during centrifugation at 65 000 × *g* for 15 h at 4°C.

TABLE I

DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS FROM GH_3 CELLS

Fraction	Protein (mg)	5'-Nucleotidase		Alkaline phosphatase		Acid phosphatase	
		(spec. act.) [a]	Purification [b] (-fold)	(spec. act.) [c]	Purification (-fold)	(spec. act.) [d]	Purification (-fold)
Homogenate	470	1.0	1.0	0.5	1.0	27	1.0
Nuclear peller	31	0.3	0.3	0.2	0.4	7	0.3
Microsomal pellet	88	2.2	2.2	0.8	1.6	34	1.3
100 000 × *g* supernatant	164	0.1	0.1	0.1	0.2	32	1.2
Sucrose gradient F1	5.1	15.3	15.3	7.5	15.0	171	6.3
F2	6.5	4.0	4.0	2.8	5.6	123	4.6
F3	3.3	3.4	3.4	1.3	2.6	65	2.4
F4	7.1	1.1	1.1	0.4	0.8	15	0.6
F5	19.2	0.2	0.2	0.2	0.4	5	0.2

[a] Specific activity reported as nmol AMP hydrolyzed/h per mg protein. A specific activity of 1.0 nmol/h per mg protein for the homogenate which leads to the same values in the specific activity column as in the purification column is fortuitous.

[b] Purification values are relative to the total homogenate.

[c] Alkaline phosphatase specific activity given as nmol *p*-nitrophenylphosphate hydrolyzed/h per mg protein at pH 10.3.

[d] Acid phosphatase specific activity given as pmoles *p*-nitrophenylphosphate hydrolyzed/h per mg protein at pH 4.8.

and F5 with specific activities relative to the homogenate for these fractions of 1.3-, 2.9- and 0.2-fold, respectively.

In preliminary experiments with Drs. Kaczorowski and Gimenez-Gallego, the distribution of several mitochondrial markers, including cytochromes, cytochrome *C* oxidase and succinate dehydrogenase has been determined. Undetectable levels of these activities or proteins were found in fraction F1; the highest levels were found in fractions F3 and F4 (unpublished results).

Total cholesterol and phospholipid content, as measured by the amount of inorganic phosphorus released upon ashing the samples, were used as measures of the lipid to protein ratio in the various fractions and, hence, of the relative density of each membrane fraction. Table II shows that the cholesterol to protein ratio is highest in the lightest membrane fraction, F1, isolated from the sucrose gradient and lowest in the most dense fraction, F5. The increase in the cholesterol/protein ratio from the homogenate to the F1 fraction (about 14-fold) is nearly identical to the degree of purification achieved for 5′-nucleotidase and alkaline phosphatase. In fact, the cholesterol/protein ratio throughout the gradient is a reasonable index of the degree of enrichment of these plasma membrane marker enzymes. On the other hand, the phospholipid/protein ratio is relatively constant throughout the purification scheme, with only a small relative increase seen in F1.

TRH receptor purification

Throughout the purification procedure, the specific binding of [^{3}H]TRH to its receptor was measured by a sensitive filter assay. Aliquots from the various fractions were incubated with 50 nM [^{3}H]-TRH for 60 min at 0°C. Particulate bound [^{3}H]TRH was separated from unbound hormone by filtration on glass fiber filters. Nonspecific binding was determined for each fraction by inclusion of 50 μM unlabeled TRH in replicate samples. Table III tabulates the specific binding and purification of the TRH receptor throughout the fractionation procedure. Although there is a small amount of TRH binding in the nuclear pellet, the specific activity of binding is considerably less than in other fractions and could easily represent residual plasma membrane contamination of the nuclear pellet. After removal of the cytoplasmic and other soluble proteins by high-speed centrifugation, there is typically a 3–4-fold increase in the specific activity for TRH binding in the crude microsomal fraction. Further purification of this fraction on discontinuous sucrose gradients leads to fractions, especially F1 and F2, which are substantially enriched in TRH receptor binding activity, with increases in specific activity as compared to the homogenate of 28.6- and 11.4-fold for these two fractions, respectively. Over 70% of the receptor activity of the microsomal pellet is localized in these two fractions. Negligible amounts of receptor are found in the other, more dense, fractions of the gradient.

Throughout the purification procedure, TRH

TABLE II

CHOLESTEROL AND PHOSPHORUS CONTENT OF SUBCELLULAR FRACTIONS

Values are expressed as nmol cholesterol or phosphorus per mg protein.

Fraction	Cholesterol	Lipid phosphorus	Cholesterol/phosphorus
Homogenate	32	770	0.04
Microsomal pellet	64	790	0.08
Sucrose gradient F1	464	1 380	0.34
F2	157	950	0.16
F3	85	910	0.09
F4	32	890	0.03
F5	8	950	0.01

TABLE III

DISTRIBUTION OF TRH RECEPTORS IN SUBCELLULAR FRACTIONS FROM GH_3 CELLS

Fraction	Total [^{3}H]TRH bound (fmol)	[^{3}H]TRH bound (fmol/mg)	Purification (-fold)	Recovery [a] (%)
Homogenate	37 600	80	1.0	100
Nuclear pellet	1 116	36	0.4	3
Microsomal pellet	25 168	286	3.6	67
100 000 × *g* supernatant	2 952	18	0.2	8
Sucrose gradient F1	11 567	2 286	28.6	31
F2	5 889	913	11.4	16
F3	1 630	494	6.2	4
F4	923	130	1.6	3
F5	211	11	0.1	1

[a] All values relative to the homogenate.

receptor levels were measured with several different sample concentrations at a fixed concentration (50 nM) of [^{3}H]TRH. To verify that the receptor in the most highly purified fraction has the same binding properties as in the cells or crude membrane preparations, equilibrium and kinetic binding properties were evaluated at several different TRH concentrations (from 1 to 100 nM) and compared to those for whole cell extracts. The binding was saturable and proceeded with rates similar to those of whole cells. A Scatchard analysis [43] of these data is shown in Fig. 2. Over the concentration range of [^{3}H]TRH used in this experiment, the Scatchard plot suggests that there is a homogeneous population of receptors with an affinity of about 18 nM, a value similar to that measured for intact cells or crude membranes (data not shown) and those reported by others [2,13].

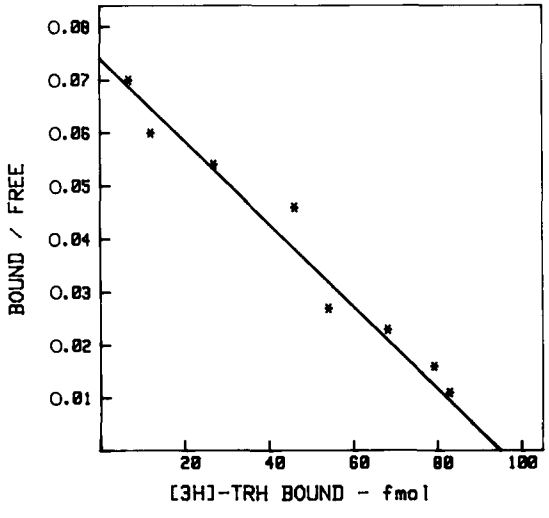

Fig. 2. Scatchard plot analysis of equilibrium [^{3}H]TRH binding to the F1 sucrose gradient membrane fraction. Approx. 40 μg membrane protein were incubated with various concentrations (from 1 to 100 nM) of [^{3}H]TRH in the absence or presence of 50 μM cold TRH. Specific [^{3}H]TRH binding was measured as described in Methods.

Wheat germ agglutinin binding

As a general marker for plasma membrane purification, the binding of ^{125}I-labelled wheat germ agglutinin was assessed in this system during the initial phase of these studies. Aliquots from several fractionation steps were incubated in the absence or presence of 10 mM *N*-acetyl-D-glucosamine with ^{125}I-labelled wheat germ agglutinin. Binding was complete after 30 min and the samples were centrifuged at 100 000 × *g* for 30 min and the supernatant carefully removed. The membrane pellets were counted and nonspecific binding, measured in the presence of cold *N*-acetyl-D-glucosamine, was subtracted from the total counts bound for each fraction. Table IV gives the specific and total binding of ^{125}I-labelled wheat germ agglutinin to four fractions. It is obvious that, on a protein basis, the nuclear fraction contained a considerable amount of specific binding sites for

TABLE IV

WHEAT GERM AGGLUTININ BINDING TO PARTICULATE FRACTIONS

Aliquots of each fraction were incubated with $5 \cdot 10^4$ cpm for 1 h at 25°C. Nonspecific binding was determined in the presence of 10 mM *N*-acetyl-D-glucosamine for each fraction and subtracted from the total counts bound. Nonspecific binding was generally about 8% of the total counts added to the incubation mixture. The usual membrane preparation was slightly altered in this study to yield a membrane fraction enriched in mitochondria. The cells were homogenized in 10 mM phosphate buffer, pH 7.4, with 10 mM KCl and 0.25 M sucrose. After centrifugation of the homogenate at 1 000 × *g* for 15 min to obtain the nuclear pellet, the resultant supernatant was centrifuged at 16 000 g for 15 min to obtain an intermediate membrane pellet, identified as the mitochondrial fraction. This fraction contained only small amounts of DNA (2% of the DNA concentration of the nuclear fraction) and showed a small relative increase (1.5-fold) in 5'-nucletidase specific activity compared to the homogenate.

Fraction	Specific binding (cpm/mg protein)
Homogenate	42 970
Nuclear pellet	25 730
Mitochondrial pellet	4 500
Microsomal membranes	50 020

wheat germ agglutinin; total binding to the nuclear fraction was considerably greater than for microsomal membranes. Only a small amount of specific binding to the mitochondrial fraction was observed.

Since the nuclear fraction was nearly devoid of several other plasma membrane markers, the binding of ^{125}I-labelled wheat germ agglutinin to this fraction was surprising. In order to confirm this observation, fluorescein-labelled wheat germ agglutinin was used to label cells and subcellular fractions. GH_3 cells grown on cover slips, fixed with formaldehyde and exposed to FITC-wheat germ agglutinin displayed specific fluorescence localized around the periphery of the cell (Fig. 3c). If, however, the cells were briefly treated with 0.1% Triton after fixation to allow access of wheat germ agglutinin to intracellular compartments, a dramatically different fluorescent pattern was observed (Fig. 3b). The nuclei of the cells were intensely labelled, as compared to the cellular membrane. It was apparent that the nucleoli, the dark spots seen in the phase-contrast microscope, are not labelled to a significant degree with FITC-wheat germ agglutinin. If an excess of unlabelled wheat germ agglutinin or 10 mM *N*-acetyl-D-glucosamine was included in the incubation mixture with FITC-wheat germ agglutinin little or no fluorescence

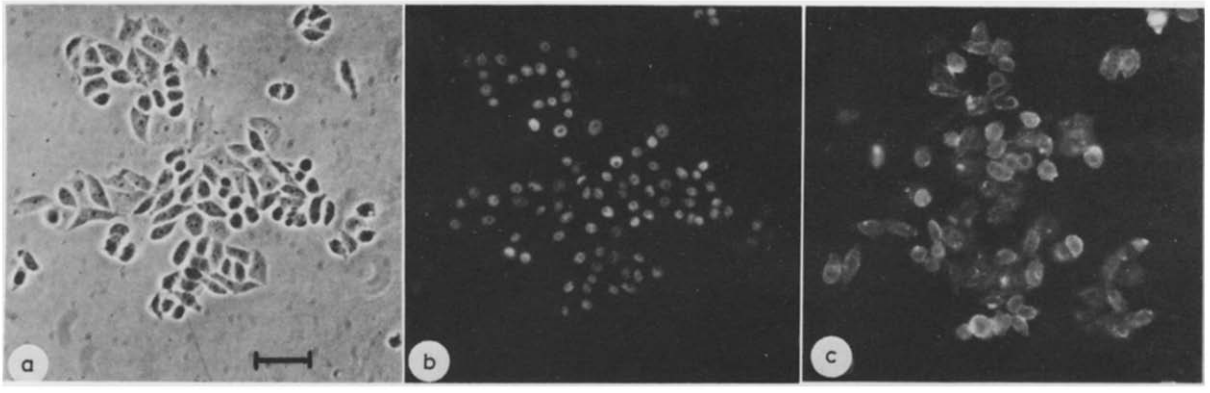

Fig. 3. Intracellular and extracellular labeling of GH_3 cells by fluorescein-conjugated wheat germ agglutinin. All photos were taken with Polaroid 665 film at identical magnification. The bar represents 5 μm. 3a. Phase contrast photomicrograph of formaldehyde-fixed, Triton-permeabilized cells labeled with FITC wheat germ agglutinin. 3b. Same cells as in 3a but illuminated and photographed with appropriate barrier filters for fluorescein emission. 3c. FITC wheat germ agglutinin fluorescence photomicrograph of fixed but not permeabilized cells to show external, plasma membrane-associated FITC wheat germ agglutinin binding. The slight fluorescence seen around the periphery of the nucleus in some cells is due to a slight permeabilization of the cell membrane caused by formaldehyde fixation.

was observed on either the cell surface or the nucleus. At higher magnification, other intracellular structures were also lightly stained with FITC-wheat germ agglutinin. Hence, the binding of FITC-wheat germ agglutinin parallels that observed with ^{125}I-wheat germ agglutinin and suggests that specific carbohydrate-containing wheat germ agglutinin-binding macromolecules are present in the nucleus or associated with the nuclear membrane. Other fluorescent lectins, including concanavalin A and ricin, were not observed to label the nucleus to a significant degree.

Electron microscopy of subcellular fractions

Transmission electron microscopy was performed on fixed and embedded fractions taken at various stages throughout the purification procedures. Micrographs of the microsomal fraction and of fractions 1–4 from the sucrose gradient are presented in Fig. 4. The microsomal pellet (Fig. 4a) consists of a heterodisperse mixture of small and large vesicles and membrane sheets. Fraction 1, the most enriched plasma membrane fraction, consisted of a relatively homogeneous population of small vesicles (Fig. 4b) with an average diameter of approximately 1800 Å. There was little evidence of contamination by large membrane sheets or ribosome-containing membranes. Fraction 2 (Fig. 4c) is comprised of a mixture of small (as in Fraction 1) and larger vesicles (4000–6000 Å diameter) and with some structures reminiscent of Golgi membranes. An occasional vesicle with

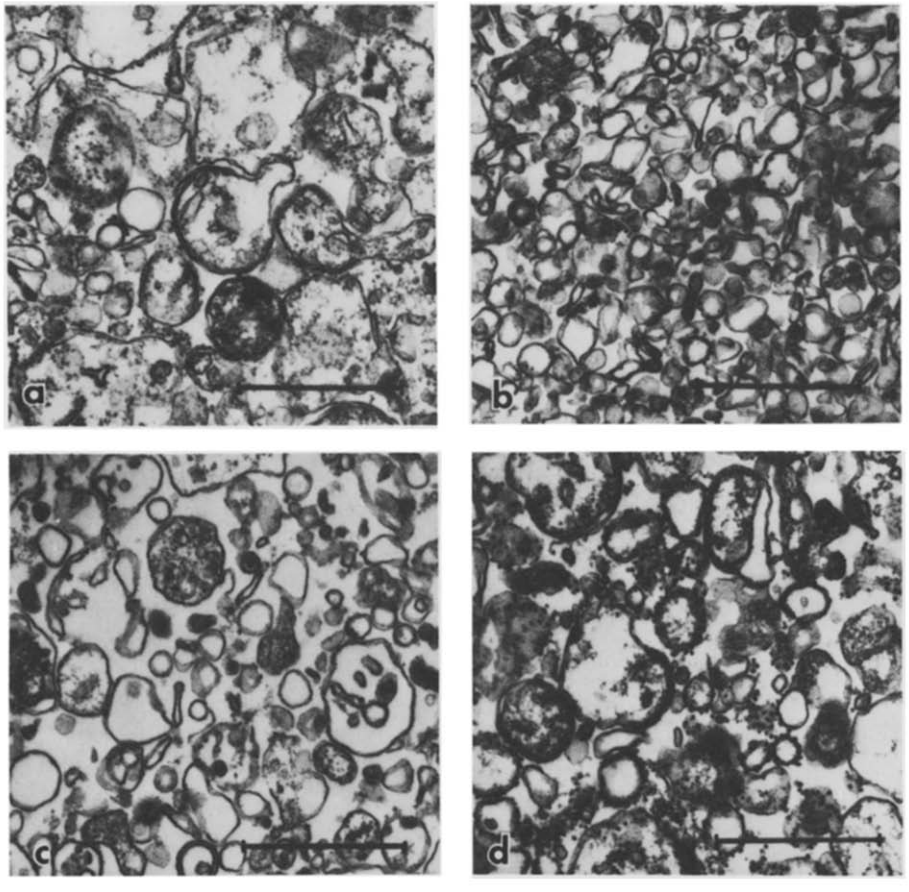

Fig. 4. Transmission electron microscopy of ultra-thin sections of fixed and embedded membrane fractions. (a) Microsomal membrane fraction; (b) purified plasma membrane fraction 1; (c) sucrose gradient fraction 2; (d) sucrose gradient fraction 4. The bar represents 1 μm.

a small density of ribosomal-like particles was observed.

Fraction 3 is composed of large membrane fragments with poorly defined structure, and mostly consists of a mixture of intracellular membrane units. On the other hand, Fraction 4 (Fig. 4d) is composed of a fairly uniform population of ribosome-studded vesicles and closed membrane sheets of rough endoplasmic membrane origin. The sucrose gradient pellet, Fraction 5, (not shown) is composed of mostly amorphous nondescript material with an occasional endoplasmic membrane.

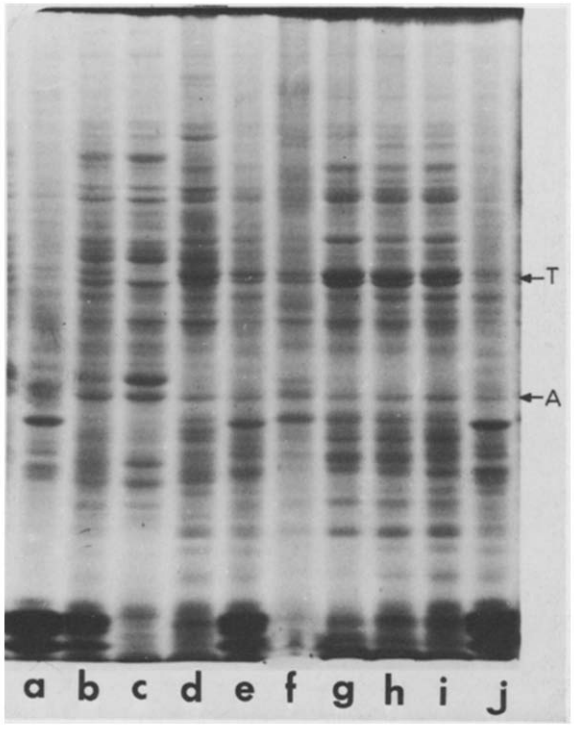

Fig. 5. SDS-polyacrylamide slab gel electrophoresis of samples from the various fractions obtained during fractionation of GH_3 cell homogenates. 40-μg samples were denatured and electrophoresed on a 10% polyacrylamide gel according to the procedures of Laemmli [24]. Lane (a) nuclear pellet; (b) cell homogenate; (c) 1000 × g supernatant; (d) 100000 × g supernatant; (e) microsomal pellet; (f) sucrose gradient fraction F1; (g) F2; (h) F3; (i) F4; and (j) F5. The arrows labelled A and T point to peptides which may be actin and tubulin, respectively, based on their known molecular weights.

SDS-polyacrylamide gel analysis of subcellular fractions

SDS-polyacrylamide gel electrophoresis was used to characterize the polypeptide patterns of the various fractions obtained during the purification procedure (Fig. 5). The most highly enriched plasma membrane fraction (lane f) is nearly devoid of the low molecular weight proteins which predominate in the nuclear and more dense sucrose gradient fractions. Fractions 2 to 4 have similar profiles even though they band at different, discrete sucrose densities and have marked differences (Table 1) in their relative content of the measured marker enzymes. The heaviest fraction, lane j, has a peptide pattern very similar to the nuclear fraction; this fraction also has the greatest DNA and RNA content of any of the gradient bands. The polypeptide profile obtained from the 100000 × g supernatant (lane d) was substantially different from all of the membrane-bound protein profiles.

One protein band which is prominent in several of the fractions may be actin, based on its co-migration with a rabbit muscle actin standard (arrow). Also prominent in the membrane fractions is a doublet which has an apparent molecular weight of approximately 55000 and comigrates with the α, β subunits of tubulin. Even though F1 has the highest level of TRH receptor and other plasma membrane marker enzymes, their concentrations (estimated to be up to 0.1% of the protein in F1) are so low as to be not observable in these gels.

Discussion

With the procedures reported here, an enriched plasma membrane fraction can be obtained in good yield with relatively high purity from cultured GH_3 pituitary cells grown in suspension cultures. These membranes show the highest specific activities and recoveries for several plasma membrane marker enzymes. This membrane fraction also had the highest cholesterol/protein and phospholipid/protein ratios, properties which are characteristic of other plasma membrane preparations [25–27]. Approx. 1% of the total cellular protein was recovered in the plasma membrane fraction. This fraction as revealed by electron microscopy is composed of a homogeneous population of small (1800 Å), closed vesicles

typical of plasma membrane vesicles.

During the purification of plasma membranes or of other subcellular organelles, it is common practice to assess the relative degree of enrichment or purity of these fractions by the specific activities of various marker enzymes. In general, it has been extremely difficult to show conclusively that these markers reside solely in the organelle of interest, even by ultrastructural localization of these marker enzymes at the electron microscope level [20,28]. The two marker enzymes used primarily in this study, 5′-nucleotidase and alkaline phosphatase, are generally accepted to be reasonably specific markers for the plasma membrane, as shown by many purification studies and also by ultrastructural localization studies [29,30], although a small percentage of the total activity of each may be localized in the Golgi apparatus or other subcellular membrane structures. Since the proteins which are present on the cell surface are synthesized on endoplasmic reticulum membranes and probably pass through the Golgi apparatus for glycosylation and other post-translational modifications [46,47], the low levels of these marker enzymes found in intracellular structures may be newly synthesized molecules destined for the plasma membrane.

The results from this study on the subcellular distribution, specific activities and yields of the enzymes 5′-nucleotidase and alkaline phosphatase strongly suggest that they both predominantly occur in the same cellular fractions in GH_3 cells. The 12- to 15-fold purification achieved for these enzymes is similar to the distribution and purity reported for highly purified plasma membrane fractions from fat cells [25] and from cultured HeLa cells [31]. The apparent density and relative content of cholesterol and phospholipids are also similar to the values reported for plasma membranes from bovine adrenal medulla [32] and from crustacean nerves [26,33]. Hence, the membranes which have been isolated in fraction F1 are, on a comparative basis with other well-characterized membrane systems, highly enriched plasma membranes.

From a consideration of the results tabulated in Table II of the specific binding of TRH to the various subcellular and sucrose gradient fractions, it is clear that the TRH receptor is primarily localized in the plasma membrane of GH_3 cells. Routinely, we have obtained increases in TRH receptor specific activities as compared to the homogenate of 15- to 30-fold with reasonable recoveries (approx. 30%) of the total binding activity. The TRH receptor in the purified membranes has a similar dissociation constant (18 nM) for [^{3}H]TRH as does the receptor on intact cells or broken cell homogenates. Only a single, high-affinity site is detected by our binding assay. The dissociation constant as measured here agrees well with the high-affinity, low-capacity site reported for cells and membrane fractions by Martin and Tashjian [34], Hinkle and Tashjian [35] and Grant et al. [36] for the receptor on murine thyrotropic pituitary tumor cells. A similar localization of the TRH receptor in plasma membrane fractions derived from whole anterior pituitary glands was observed [37,38].

The distribution of TRH receptor sites in the various subcellular fractions in this study is markedly different from that previously reported by Tixier-Vidal et al. [14,39,40]. In their studies, a considerable amount of [^{3}H]TRH binding (up to 25% of the total bound to intact cells) was observed in the nuclear fraction obtained from GH_3 cells, as detected both by binding measurements and by autoradiographic procedures. However, with the exception of electron microscopic examination of the nuclear fraction, no other analyses of the purity or composition of the various fractions were reported [40].

In the present studies, while no particular attempt was made to purify further the nuclear fraction, it is clear from Table II that only a small amount of specific [^{3}H]TRH binding, amounting to 3% of the total binding in the homogenate, was observed in the nuclear fraction. Furthermore, a comparison of the specific activities of the plasma membrane marker enzymes (Table I) in the nuclear fraction with the amount of specific TRH binding (Table II) suggests that the small amount of binding observed could very well be due to residual plasma membrane contamination of the nuclear fraction. Hence, the validity of the report of substantial [^{3}H]TRH binding specifically to the nuclear fraction [40] depends on comprehensive biochemical characterizations of their isolated nuclear fraction.

Chang et al. [20] have proposed the use of ^{125}I-labelled wheat germ agglutinin as a general marker for plasma membrane isolation procedures. Adapting their procedures for use in the present study, it was

surprising that the nuclear fraction bound a considerable amount of ^{125}I-wheat germ agglutinin. This led us to investigate the possibility that the nuclei of GH_3 cells contain wheat germ agglutinin sites. By fluorescent labelling of detergent-permeabilized cells, relatively high levels of FITC-labelled wheat germ agglutinin were associated with the nucleus of GH_3 cells. This observation has been extended to several other cell types (CHO, C6-glioma, LM cells and primary pituitary cultures, data not shown) and suggests that all cells may have nuclear binding sites for wheat germ agglutinin. Virtanen and Wartiovaara [41] have also observed the binding of FITC-wheat germ agglutinin to nuclei isolated from liver cells. Hence, the use of ^{125}I-wheat germ agglutinin as a specific probe of plasma membranes is not generally applicable. While Chang et al. [20] prelabelled their cells with ^{125}I-wheat germ agglutinin before homogenization, thereby, presumably, limiting the binding to the cell surface, it would be necessary to show that all of the cells were intact during labelling and that none of the label was lost from the plasma membrane during homogenization in order to use the binding of ^{125}I-wheat germ agglutinin as a general plasma membrane marker.

SDS-polyacrylamide gel analysis of the various subcellular fractions from GH_3 cells has revealed a complex array of polypeptides in all fractions. Several of the polypeptide bands have mobilities close to other known cytoskeleton proteins such as actin and tubulin which are present in relatively high concentration in most cells. At this time it is not possible to identify the TRH receptor polypeptide or peptides. However, a high-resolution analysis of purified plasma membrane fragments from clones of GH_3 cells which lack TRH receptors (Vandlen et al., unpublished results) and comparison with ones which have increased levels of TRH receptors relative to the parent GH_3 clone may be useful in this regard.

The similarity of the peptide patterns in fractions F2, F3 and F4 was somewhat surprising in view of their pronounced differences in equilibrium densities and marker enzyme activities. The only obvious differences are the relative amounts of the peptides with low molecular weights which run near the dye front. These proteins may be ribosomal subunits as proposed by Kreibich et al. [42]. In addition to decreased cholesterol concentrations, the sucrose density differences in these fractions may be due to discrete fragments of the endoplasmic reticulum which contain varying amounts of attached ribosomes. This is consistent with the increasing amount of ribosome-containing membrane structures as observed by electron microscopy. In addition, as shown by the marker enzyme analysis, some contamination of these fractions by plasma membrane fragements and other intracellular membranes may be present.

In summary, purified plasma membrane fragments have been prepared from cultured pituitary cells in good yield. These membranes have been enriched for several marker enzymes and for the TRH receptor 15- to 30-fold over whole cell homogenates and are relatively homogeneous in size and morphology by electron microscopy. Such preparations of purified membranes are a useful first step in the isolation, purification and characterization of the TRH receptor and of other plasma membrane receptors which are unique to pituitary cells. In addition, preliminary experiments have shown that these plasma membranes are sealed and are capable of maintaining electrical potentials; these purified fractions should be very useful for the study of the initial events in the mechanism by which TRH, acting through its receptor, may stimulate the secretion of pituitary hormones.

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