ISOLATION OF ACTH RESPONSIVE CELLS FROM RAT ADRENAL CORTEX AND THE DETERMINATION OF THE DENSITY OF MALE AND FEMALE CELLS

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1. Introduction

While density gradient centrifugation in gradients of varying shape and density has long been used in the fractionation of subcellular particles, such procedures have not been widely employed in preparing whole cell populations. This is due largely to the fact that most gradient materials exhibit undesirable properties such as high toxicity, high ionic strength or high viscosity at the concentrations suitable for cell separations. Metrizamide was developed because of this lack of appropriate gradient materials and has now been used in the separation of a number of different cell types from a variety of tissues [1].

The purification of specific cells from heterogenous populations after tissue dispersion is often essential to the study of cellular biochemistry. In the case of specific endocrine cells such a preparation of isolated cell populations is critical in the determination of mechanisms of hormone action [2,3]. The separation and steroidogenic activity of intact adrenal cortical rat cells after density gradient centrifugation in metrizamide is reported here together with an analysis of the density of male and female rat adrenal cortical cells.

2. Methods and materials

ACTH, adrenocorticotropic hormone (corticotropin, Park-Davis, Detroit, MI), collagenase (Worthington Biochemical, Freehold, NJ), metrizamide (Nygaard, Oslo) and bovine serum albumin (BSA) and deoxyribonuclease (DNase) were obtained from Sigma Chemical, St Louis, MO. The centrifuges used for cell isolation and cell fractionation were Beckmann J-21, Beckmann L2-65B, and International Clinical, table model. Sprague Dawley albino rats from Bio-Lab, St Paul, MN were housed singly, fed lab chow and water ad libitum, and maintained in 12 h light–dark 7 a.m.–7 p.m./day for 1 week before use.

Buffers used for tissue preparation were as follows. Krebs-Ringer bicarbonate (KRB) was made up in the following order of addition: 100 vol. 0.154 M NaCl; 4 vol. 0.154 M KCl; 1 vol. 0.154 M KH_2PO_4; 1 vol. 0.154 M $MgSO_4$ $7H_2O$; 21 vol. 0.154 M $NaHCO_3$; 3 vol. 0.110 M $CaCl_2$. The solution was bubbled with 95% O_2–5% CO_2 to give pH 7.4. Glucose was added to 200 mg/100 ml to give KRBG. Bovine serum albumin (Sigma, lyophilized) was added to KRBG at 1% concentration to give KRBGA. All glassware, pipettes, tubes and containers were treated with silicone, washed in distilled water and dried prior to use.

Lipids were extracted in chloroform : methanol (2:1) according to [4] and were separated by thin-layer chromatography on silica gel G using *n*-hexane: diethyl ether:acetic acid (80:20:1) as solvent. Separated lipids were detected in iodine tanks and the outlined spots were removed and quantitated as in [5]. Cholesterol was determined by gas–liquid chromatography on a 3% OV 1 column operating in a dual flame ionisation detector instrument. Protein was determined with the Lowry procedure [6].

Preparation of tissue digest: The procedure in [7] was modified using a collagenase digestion procedure. Adrenal glands were cleaned of fat, weighed and quartered in groups of 20 glands. The quarters were incubated in KRBG buffer in a Dubnoff incubator with shaking at 37°C for 1 h. The media was aspirated with a Pasteur pipette and discarded. This tissue suspension was bubbled with 95% O_2:5% CO_2 while being digested for 45 min at 37°C with enzyme in the following proportions: collagenase, 25 mg; DNase, 2.5 mg; bovine serum albumin, 200 mg; 20 quartered adrenals; 5.0 ml KRBG buffer; all in a 25 ml Erlenmeyer flask. After incubation the cells were dispersed by repeated aspirations with a Pasteur pipette, strained through a 100 μm wire mesh screen into 15 ml centrifuge tubes, and centrifuged for 10 min at 1000 × g. The cells were twice resuspended in 5.0 ml KRBGA and spun down at 1000 × g. Where it was desirable to remove erythrocytes the second wash contained 0.87% NH_4Cl in KRBGA which specifically lysed the erythrocytes. The final cell-pellet was resuspended in KRBGA to 2.5 adrenals/ml.

Cell count: A small quantity of the adrenal cell suspension was stained with methylene blue and counted in a hemocytometer (AO Instr. Co., Buffalo).

Density gradient centrifugation: Metrizamide solutions were prepared in KRBGA; 15 ml linear gradients were generated by a conventional linear gradient maker and ranged from 10–40% (w/v) metrizamide. A 2 ml aliquot of the cell suspension was layered on top of the gradient which was subjected to centrifugation at 7000 rev./min at 15°C for 15 min. in a SW 27 rotor. The tube contents were fractionated by aspiration using a pasteur pipette.

Refractive indices were determined using a Bausch and Lomb refractometer. Densities were determined from the refractive indices by the procedure in [8]. Corticosterone was measured by the fluorescence procedure in [9].

3. Results

The appearance of the tube contents after centrifugation is shown diagrammatically in fig.1. Three bands are readily observed, the major active band being located in the middle of the tube. The position of the bands in the centrifuge tube versus metrizamide concentration is also shown in fig.1. After fractionation of the gradient the fractions which made up the various regions of the gradient were diluted with an equal volume of KRBGA and centrifuged at 1000 × g for 10 min. The resulting pellets were resuspended in 1 ml KRBGA. These fractions were incubated for 2 h with two concentrations of ACTH and corticosterone production was measured. The results obtained are shown in fig.2. Only the middle region of the gradient was responsive to ACTH. As shown in fig.2 there was a 2–3-fold increase in the quantity of corticosterone produced/10^6 cells in the case of the gradient fractionated cells when compared to a control population which had not been subjected to density gradient centrifugation.

The preparation of intact adrenal cells in media other than KRBG was generally not successful. The

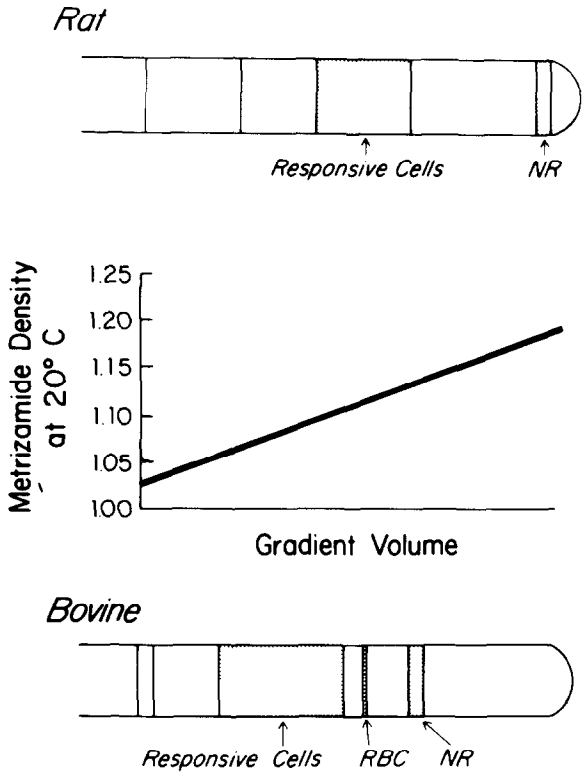

Fig.1. Concentration of ACTH responsive adrenal cells by isopycnic density gradient centrifugation in metrizamide. NR, lower layer of cells which do not respond to ACTH. The density range of cells can be estimated by comparing band width to metrizamide density scale.

assay for ACTH-stimulatable corticosterone production by cells was always carried out in standard 1% KRBGA. When the cells were prepared and separated in either 0.32 M sucrose, 3% Ficoll or 0.1 M potassium phosphate buffer (pH 7.4), the subsequent response to ACTH was not satisfactory (fig.3). This indicated considerable variation in cell fragility and viability between different media. Separation of cell

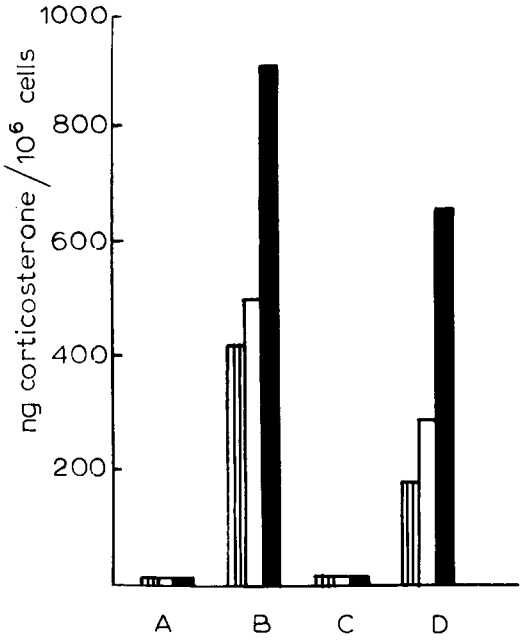

Fig.2. ACTH stimulation of intact adrenal cells obtained from the middle layer following density gradient centrifugation in metrizamide. Control value was determined on cells prior to exposure to metrizamide. Upper layer (debris and broken cells) and lower layer (NR) did not respond to ACTH. About 10^6 cells/flask per incubation. Assay of cells as corticosterone equivalents by fluorescence. A, B and C refer to upper, middle and lower layers of gradient fractionated material, respectively, while D refers to the cells not subjected to centrifugation. Cells were treated with 20 μUnits ACTH (▨) and 100 μUnits ACTH (■). Cells were also incubated in the absence of ACTH (□).

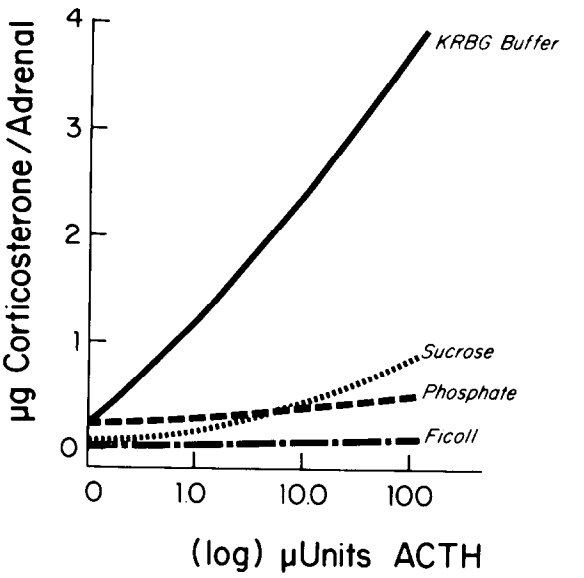

Fig.3. Response of cells to ACTH after centrifugation and isolation procedures for cells were carried out in different media. Standard KRBG buffer media (——), 0.32 M sucrose (. . . . . .), 3% Ficoll (-.-.-) and 0.1 M sodium phosphate buffer (- - - -). In all cases final incubation of cells with ACTH was carried out in KRBGA buffer containing 1.5–3 × 10^5 cells equivalent to 1 adrenal.

preparations from male and female rat adrenal cortical tissue was then carried out using the above procedure. The distribution of the cells in the gradient was analysed by measuring the following parameters of the gradient fractions: corticosterone production in response to ACTH; protein; cholesterol; cholesterol ester; total neutral lipid. The data was plotted as frequency histograms versus metrizamide density and the median densities were determined according to [10].

The results are summarised in table 1. For all constituents measured it is evident that male adrenal cortical cells have a higher density in metrizamide gradients than do female cells.

Table 1
Median density of constituents of male and female rat adrenocortical cells in metrizamide gradients

Cells	Protein	Cholesterol	Cholesterol ester	Corticosterone	Neutral lipid
Male	1.0893 ± 0.003	1.0965 ± 0.002	1.0925 ± 0.002	1.0930 ± 0.0034	1.0931 ± 0.004
Female	1.0860 ± 0.0028	1.0869 ± 0.003	1.0858 ± 0.003	1.0850 ± 0.0022	1.0829 ± 0.002

4. Discussion

Cells which respond to ACTH stimulation can be obtained by isopyonic centrifugation in a buffered medium using a continuous gradient with 10–40% metrizamide. A sharp narrow band of red blood cells can be separated from the main band of active adrenal cells in the middle region of such a gradient. It is also possible to remove the erythrocytes by washing with 0.87% NH_4Cl which does not affect the viability of the adrenal cells.

The rat adrenal tissue was preincubated in a Dubnoff metabolic shaker for 1 h prior to dispersion of the cells by protease activity. The preincubation was essential because it lowered the basal secretion values and enhanced the subsequent response of cells to ACTH [11]. Such a procedure does not alter the inherent circadian periodicity of tissue response [12]. The cells isolated by such a density gradient procedure showed a 2–3-fold increase in steroidogenic response to ACTH than unfractionated material. The number of cells in this region of the gradient corresponded to ~40% of those applied to the gradient.

Cells from bovine adrenal cortex were also subjected to density gradient centrifugation in metrizamide following dispersion by the trypsin–trypsin inhibitor method [7]. This dispersion method was also used to disperse rat adrenal cortical cells with equally good results as those obtained by collagenase treatment. Bovine cells gave a considerably lower steroidogenic response to ACTH than did rat cells.

The measurement of cell density in male and female rat adrenal cortical cell preparations indicates that male cells have a greater density than female cells with median density values of 1.0929 and 1.0853, respectively.

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