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SUBPOPULATIONS OF PROLACTIN CELLS DISSOCIATED FROM DIFFERENT PARTS OF RAT ANTERIOR PITUITARY GLAND

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ABSTRACT

Two distinct subpopulations of prolactin (Prl) cells with respect to density, staining characteristics and response to secretagogues have been described. After Percoll separation, the low Prl secretors correspond to cells harvested at high density and high Prl secretors are recovered at low density. We previously observed heterogeneity in the Prl gene expression under basal conditions in the intact female pituitary gland : cells located in the periphery of the gland exhibited more Prl mRNA but less immunoreactive Prl protein than cells in the central area. To address the question whether the heterogeneity in the distribution of subpopulations of Prl cells obtained after Percoll gradient separation correspond to that observed in different parts of the rat pituitary, we cut the anterior pituitary of female rats into two parts - the outer peripheral part and the inner central part. Cells from these 2 parts were dissociated, separated by discontinuous Percoll gradient centrifugation and the number of cells containing Prl in each population was determined by the indirect immunoperoxidase technique. The results demonstrate that there is no difference in the number of immunoreactive Prl cells present in cells dissociated from the two parts of the rat anterior pituitary gland when cells harvested from the corresponding density interface are compared.

INTRODUCTION

Prolactin (Prl) is a protein hormone with a molecular mass of 23 kd.[1] It is thought to have evolved from a common ancestral gene together with growth hormone (GH) and placental lactogen (PL).[2-3] In mammals, Prl appears to be coded by a single gene made up of 5 exons and 4 large introns.[4-6] All exon-intron boundaries in the rat Prl gene contain more than one splice site,[7] raising the possibility that multiple Prl variants could arise by differential mRNA splicing, though most of the Prl variants are generated by post-translational modification such as cleavage, glycosylation, phosphorylation, deamidation, and polymerisation.[8-9] Several Prl variants with the same molecular weight but different net charges (isoforms) have been documented.[10-13] These isoforms differ markedly in their level of biological activity and are recognised to different degrees by Prl antibodies: the more acidic forms are the least antibody reactive and the most bioactive.[14] Although the major site of the Prl gene expression is the anterior pituitary, its expression has so far been reported in several extrapituitary tissues,[15] especially in the immune system.[16]

Rat prolactin (rPrl) is mainly produced in the anterior pituitary gland by lactotrophs or mammotrophs, hereafter Prl cells. Two types of Prl cells have been reported, they are characterised by the size of cytoplasmic secretory granules.[18-22] Prl cells can be separated by centrifugation on a discontinuous Percoll gradient.[23] Mammosomatotrophs (MS cells) have been reported to

secrete both Prl and GH.[24-27] These cells are localised more in the peripheral rim of the anterior pituitary[28] and appeared early in the neonatal development of rats as shown by reverse hemolytic plaque assays and immunocytochemistry.[29]

We previously observed a heterogeneous distribution of Prl cells in the intact female rat pituitary gland under basal conditions: cells located in the periphery of the gland exhibited more Prl mRNA and contained less immunoreactive Prl protein than cells in the central area.[30] In contrast to Prl cells, GH cells evenly distributed throughout the gland, either in basal conditions or after estradiol treatment[30] indicating a homogeneous population of pituitary GH cells. The differential expression of the Prl cells raises the possibility of a presence of Prl cell subpopulations with a regional heterogeneity in the intact pituitary. To test the hypothesis that both Prl cell populations observed *in vivo* reflect Prl cell subpopulations with different functional activities as seen after Percoll separation, we isolated cells from the peripheral and central area of the gland separately and subjected them to Percoll gradient centrifugation. The number of cells recovered from each part of the gland and the number of immunoreactive Prl and GH cells recovered at the different Percoll density interfaces were determined.

MATERIALS AND METHODS

Animals

Five virgin female Wistar rats (Proefdierencentrum, KUL, Leuven, Belgium) aged 14 to 16 weeks were housed in a controlled environment with illumination for 14 hours daily (6:00 to 20:00 h) at a temperature of 25±1 °C. with free access to food (standard rat chow) and tap water. After decapitation, the pituitary gland was carefully removed from the hypophyseal fossa with the neurointermediate lobe intact. It was immediately immersed in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Paisley, Scotland) supplemented with 12 mM $NaHCO_3$ (Merck, Darmstadt, Germany), 20 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (Merck, Darmstadt, Germany) and 0.3% bovine serum albumin (BSA; Serva, Heidelberg, Germany) pH 7.4, i.e. medium A. The gland was transferred to a sterile silicone-coated petri-dish (Rhodorsil RTV-2; Rhône-Poulenc, France) and was quickly cut into 2 parts using the neurointermediate lobe as a landmark (Figure 1). The central part, close to the neurointermediate lobe, will be referred to hereafter as the inner zone and the anterior peripheral part as the outer zone.

Chemicals

Unless specified, the chemicals used in this study were purchased from Sigma, St. Louis, MO, U.S.A.

Normal Sera, Antibodies, and Antigens for Immunocytochemistry

Normal sera used in the preincubation step were normal goat serum for detection of cytoplasmic rPrl and normal rabbit serum for detection of cytoplasmic rGH. The first antibodies were rabbit anti-rPrl at a 1:15,000 dilution and monkey anti-rGH at a 1:4,500 dilution (A gift from NIDDK, Bethesda, U.S.A.). The second antibodies were goat anti-rabbit immunoglobulin G conjugated to biotin (Vector, Cambridgeshire, U.K.) at a 1:250 dilution and rabbit anti-monkey immunoglobulin G conjugated to peroxidase (POD) at a 1:50 dilution. The third reagent for detection of rPrl is needed because the second antibody was not conjugated to POD. It was a complex of avidin conjugated to POD (streptavidin-POD-conjugate; Boehringer Mannheim Biochemica, Mannheim, Germany) at a 1:500 dilution. The purified hormones

used as antigens in the competition experiment to check the specificity of the antibodies were purified rPrl and rGH kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, U.S.A.).

Pituitary Cell Dissociation

Each of the two parts of the rat anterior pituitary was dissociated separately by both mechanical and chemical means as described by Velkeniers, *et al.*[23] Briefly, the anterior pituitaries were chopped, trypsinized (25 mg/ml trypsin in medium A) at 37°C for 15 min. in a shaking water bath, and further treated with deoxyribonuclease I (2 mg/ml) at room temperature (RT) for 1 min. Trypsin activity was terminated by an addition of soybean trypsin inhibitor (1 mg/ml) and cell suspensions were further incubated in Earle's balanced salt solution (EBSS) supplemented with 0.3% BSA and 2 mM EDTA at 37°C in the shaking water bath. After centrifugation (250 g, 10 min., RT), the pituitary cell sediment was resuspended in calcium-and-magnesium-free EBSS supplemented with 0.3% BSA, dispersed mechanically, filtered through a nylon gauze (50 mm mesh), collected and centrifuged. The cell pellet was resuspended in culture medium consisting of DMEM supplemented with 10% fetal calf serum (GibcoBRL, Paisley, Scotland) and a minimal amount of antibiotics (penicillin 35 mg/ml and streptomycin 50 mg/ml). Cell concentration was determined using a Bürker counting chamber and cell viability was determined by neutral red dye uptake. The pH of all solutions used in this experiment were adjusted to 7.4.

Separation of Dissociated Pituitary Cells

After dissociation, anterior pituitary cells were separated by isopycnic centrifugation using a discontinuous Percoll gradient (Percoll; Pharmacia, Uppsala, Sweden). The 8-ml Percoll gradient solution (density from the bottom to the top: 1.090, 1.080, 1.065, 1.045 g/ml) was made in a 17x100 mm polystyrene round-bottom tube (Becton Dickinson, Oxnard, CA, U.S.A.). Anterior pituitary cell suspension in 2 ml culture medium was layered on top of the gradients and centrifuged at 400 g at 20°C in a swing-out rotor (Hettich Rotixa/RP, Tuttlingen, Germany)

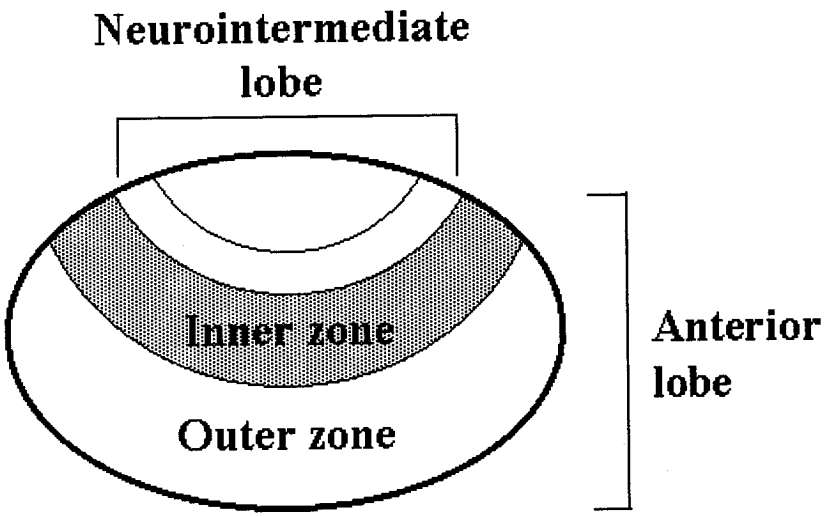

Fig. 1 The location of the outer and inner zone of the anterior pituitary used as the source for cells mentioned in this experiment.

for 20 min. (Figure 2). Colour marker beads of known densities (1.062, 1.075 and 1.085 g/ml) were centrifuged in the same run for calibration. Cells at the 1.045/1.065 (band 1), 1.065/1.080 (band 2) and 1.080/ 1.090 (band 3) density interface were collected, washed with DMEM, and resuspended in culture medium. Yield was determined and expressed as the actual number of cells recovered from each density interface. Cell recovery was expressed as percentage of cells recovered from each density interface relative to the total number of cells layered on top of the gradients.

Preparation of Separated Pituitary Cells

Pituitary cell suspensions recovered from each interface of the Percoll gradients after centrifugation were diluted in culture medium to a final concentration of 30,000 cells/100 ml. They were then applied to glass slides precoated with 3-Aminopropylethoxysilane (AEPES) and allowed to attach to the surface of the slides under 5% CO_2 environment at 37°C in a humidified incubator (Heraeus, Hanua, Germany) for 1 hour. Subsequently, the slides were rinsed with phosphate buffered saline solution (PBS) pH 7.4, cells were fixed with 4% paraformaldehyde at RT for 15 min., washed with PBS, successively dehydrated in ethanol/water (30%, 50%, 70%), and stored in 70% ethanol/water at 4°C until use.

Immunocytochemistry of Prepared Pituitary Cells

The indirect immunoperoxidase staining technique was used to detect immunoreactive Prl and GH cells. Pituitary cells were rehydrated and permeabilized with 0.3% Triton X-100 (E.Merck, Darmstadt, Germany) before the preincubation step. After rinsing with PBS, pituitary cells were preincubated with 10% (v/v) normal serum plus 0.8% (w/v) BSA in PBS pH 7.4 at RT for 30 min. Excess serum was aspirated and cells were subsequently incubated with the first antibody at the desired dilution at 4°C for 16 hours, and the second antibody at RT for 30 min. for rPrl and 1 hour for rGH. For detection of rPrl, the Streptavidin-POD-Conjugate was incubated with cells at RT for 30 min. Slides were rinsed with PBS between each step. The positive signal was visualised by adding 0.15 % (w/v) 3,3'-diaminobenzidine tetrachloride/0.3 % (v/v) H_2O_2 in PBS pH 7.8. The reaction was stopped by rinsing the slides with water. Finally, slides were counterstained with haematoxylin and mounted in Aquamount (BDH, England).

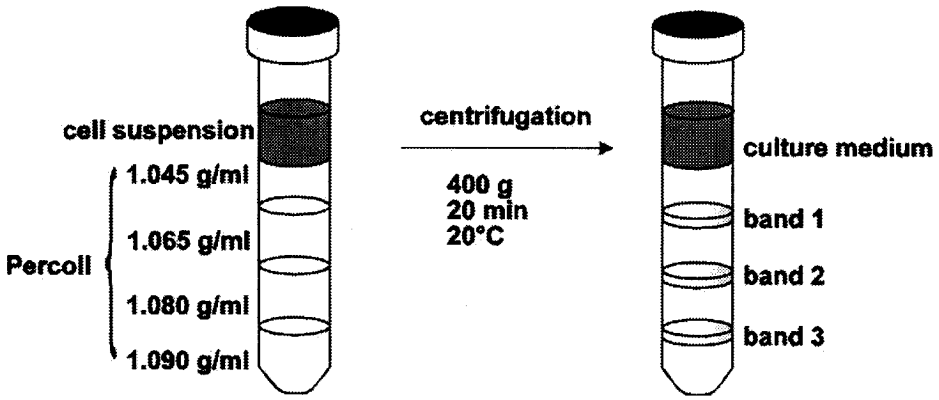

Fig. 2 Pituitary cell separation by isopycnic centrifugation. After dissociation, rat anterior ptuitary cell suspensions were layered on top of a 8 ml discontinuous Percoll gradient solution and centrifuged. After centrifugation, cells separated into 3 bands at each density interface according to their densities. Cells at density interfaces 1.045/1.065, 1.065/1.080, and 1.080/1.090 were defined as band 1, band 2, and band 3 cells, respectively. They were collected, washed and further processed as described in materials and methods.

The specificity of the positive reaction was checked by omission of certain steps in the procedure (the first antibody, the second antibody, or the third reagent) for the control slides and by using cells lacking the specific antigens. A competition experiment was also performed by mixing the first antibody with its specific purified hormone before the incubation step. None of the negative control slides carried any signal.

Data Analysis

Pituitary cell counts of unseparated cells and gradient fractions (bands) were made with a hemocytometer (Bürker counting chamber). The percentage of immunoreactive cells was determined by microscopic visualisation. At least 300 cells were counted in 5 randomly selected fields on each slide and 2-3 slides were analysed for cells obtained from each band. The actual number of positive cells in each band was calculated by multiplying the percentage of positive cells with the number of cells counted by hemocytometry. The resulting data were analysed and difference between the median value of each group was evaluated by the nonparametric Mann-Whitney two-tailed test.

RESULTS

In order to test the hypothesis that the pituitary cells which differentially express Prl mRNA and Prl protein previously observed in the intact rat pituitary correspond to the cells present in different bands after a Percoll gradient centrifugation, we isolated cells from the peripheral and central part of the rat anterior pituitary separately, subjected them to density gradient centrifugation and evaluated the cells from each band with anti-rPrl or anti-rGH specific antibodies.

Using the dissociation procedure described in materials and methods, we obtained an average of 1.016 million (M) cells from the outer zone and 0.635 M cells from the inner zone of the anterior pituitary of each adult female rat (Table 1). After Percoll gradient centrifugation, pituitary cells separated into 3 bands at each density interface. The total percentage of cells (harvested from those 3 bands together) recovered from the outer zone and the inner zone of the anterior pituitary was 63% and 54%, respectively. The cells retained their morphological integrity and their viability exceeded 95%. Cells collected at density interfaces 1.045/1.065, 1.065/1.080, and 1.080/1.090 were defined as band 1, band 2 and band 3 cells, respectively. The absolute number (yield) and relative number (recovery) of cells in each subpopulation are listed in Table 1. There is no statistical significant difference for the median values of cell numbers collected at the same density interface between the outer and inner zone when compared by Mann-Whitney two-tailed test.

The actual number of immunoreactive cells was calculated from the percentage of positive cells which was determined by counting at least 300 cells in each slide. Cells collected from band 1 contain the highest number of immunoreactive Prl cells [69 % (Figure 3a) or 0.210 M cells (Figure 4a) in the outer zone and 61 % (Figure 3a) or 0.085 M cells (Figure 4a) in the inner zone], but the lowest number of immunoreactive GH cells [11 % (Figure 3b) or 0.032 M cells (Figure 4b) in the outer zone and 12 % (Figure 3b) or 0.017 M cells (Figure 4b) in the inner zone]. On the contrary, cells collected from band 3 contain the lowest number of Prl cells [15 % (Figure 3a) or 0.015 M cells (Figure 4a) in the outer zone and 12 % (Figure 3a) or 0.008 M cells (Figure 4a) in the inner zone] but the highest number of GH cells [88 % (Figure 3b) or 0.085 M cells (Figure 4b) in the outer zone and 86 % (Figure 3b) or 0.057 M cells (Figure 4b) in the inner zone]. Cells collected from band 2 contain more Prl cells [65 % (Figure 3a) or 0.159 M cells (Figure 4a) in the outer zone and 56 % (Figure 3a) or 0.082 M cells (Figure 4a) in the

Table 1 Composition of pituitary cell populations before and after purification on Percoll gradient. The anterior pituitaries were removed, cells dissociated and separated according to density by Percoll gradient centrifugation. The number of cells obtained was determined using a Bürker counting chamber and actual cell number (mean ± standard error of mean, S.E.M.) are shown. Cells recovered from each band after Percoll separation were calculated relative to the number of cells loaded on Percoll gradients.

Cells	Density interface	Yield (x 10^6 cells)		Recovery (%)	
		Outer zone	Inner zone	Outer zone	Inner zone
Total cells*		1.016 ± 0.048	0.635 ± 0.076		
Band 1 cells	1.045/1.065	0.303 ± 0.069	0.136 ± 0.018	29.8 ± 6.1	21.4 ± 3.9
Band 2 cells	1.065/1.080	0.241 ± 0.033	0.147 ± 0.025	23.7 ± 2.5	23.1 ± 2.8
Band 3 cells	1.080/1.090	0.099 ± 0.019	0.061 ± 0.011	09.7 ± 1.6	09.6 ± 1.1

* Total number of dissociated anterior pituitary cells before Percoll separation.

Table 2 The ratio of immunoreactive Prl cells or GH cells over corresponding cell population and the ratio of immunoreactive Prl cells over GH cells in different anterior pituitary cell populations after Percoll separation. Cells were incubated with antibodies specific to rPrl or rGH and positive cells were visualised by the indirect immunoperoxidase method as described in materials and methods. At least 300 cells were counted and the actual number of positive cells was calculated.

Cells	Density interface	Prl cells/cells*		GH cells/cells*		Prl cells/GH cells	
		Outer zone	Inner zone	Outer zone	Inner zone	Outer zone	Inner zone
Band 1 cells	1.045/1.065	1/1.5	1/1.6	1/9.7	1/8.5	6.8 : 1	5.1 : 1
Band 2 cells	1.065/1.080	1/1.6	1/1.8	1/3.1	1/2.7	2.0 : 1	1.5 : 1
Band 3 cells	1.080/1.090	1/6.7	1/9.1	1/1.1	1/1.2	0.2 : 1	0.2 : 1

* band 1, band 2, or band 3 cells recovered after Percoll separation corresponding to each cell population compared.

inner zone] than GH cells [33 % (Figure 3b) or 0.079 M cells (Figure 4b) in the outer zone and 38 % (Figure 3b) or 0.058 M cells (Figure 4b) in the inner zone]. This means that neither the difference in percentage (Figure 3) nor the difference in the actual number (Figure 4) of Prl cells or GH cells is statistically significant when the median values of each cell subpopulation collected from the corresponding bands from different zones of the anterior pituitary were compared by Mann-Whitney two-tailed test. Therefore, Prl or GH cells from the different parts of the rat anterior pituitary do not correspond to the bands after Percoll gradient separation of unseparated cell populations.

The ratio of immunoreactive Prl cells or GH cells present in each band obtained from the two zones of the anterior pituitary over the total number of corresponding cells was calculated to demonstrate the relative abundance of Prl cells or GH cells in each zone (Table 2). The relative ratio of Prl cells over band 1 cells in both zones (1/1.5 and 1/1.6 in the outer and inner zone, respectively) is less than those over band 3 cells (1/6.7 and 1/9.1 in the outer and inner zone, respectively), indicating that Prl cells are more abundant in the band 1 cell population. In contrast, GH cells are more abundant in the band 3 cell population, and this is comparable for both zones of the gland.

DISCUSSION

Since we previously observed a differential expresssion of the Prl gene in the intact rat pituitary, we would like to test whether these cells possess different physical characteristics after isopycnic centrifugation (on their density basis). We isolated cells from the peripheral (outer zone) and central part (inner zone) of the rat anterior pituitary separately and subjected them to a Percoll density gradient centrifugation. Prl cells and GH cells present in cells harvested from each band were identified by incubation with anti-rPrl or anti-rGH specific antibodies, respectively. After centrifugation, cells sedimented at the density interfaces corresponding to their densities. We found that Prl cells were more abundant in band 1 cells (1.045/1.065 density interface) whereas GH cells were more abundant in band 3 cells (1.080/ 1.090 density interface) indicating that Prl cells are lighter than GH cells. However, neither the percentage nor the actual number of Prl cells was significantly different when cells collected from the corresponding bands from different zones of the gland were compared. Results obtained from GH cells were comparable to those from Prl cells.

There is strong evidence supporting heterogeneity in the responsiveness of anterior pituitary Prl cells to secretagogues and inhibitors.[31-34] Detection of Prl release from individual pituitary cells as determined by a reverse hemolytic plaque assay revealed that secretagogues such as thyroid stimulating hormone releasing hormone (TRH) could cause an increase in plaque number and size, and inhibitors such as dopamine (DA) caused the opposite result[31] and that this effect differred according to the type of Prl cells studied. Further analysis revealed that cells taken from the peripheral rim of the anterior pituitary of lactating rats were greatly responsive to stimulation by TRH, whereas those from the central area were little influenced by TRH but highly responsive to inhibition by DA.[29] Using discontinuous Percoll gradient centrifugation, Velkeniers and colleagues[23] have shown that cells harvested at high density are low Prl secretors, and those recovered at low density are high Prl secretors. Cells with low basal secretory and synthetic capacity are more sensitive to *in vitro* estradiol treatment in terms of Prl mRNA accumulation[35] but less sensitive to inhibition by DA.[36] We previously observed heterogeneity in Prl subpopulation in the intact female rat pituitary gland under basal conditions: cells located in the periphery of the gland contained more Prl mRNA but less immunoreactive

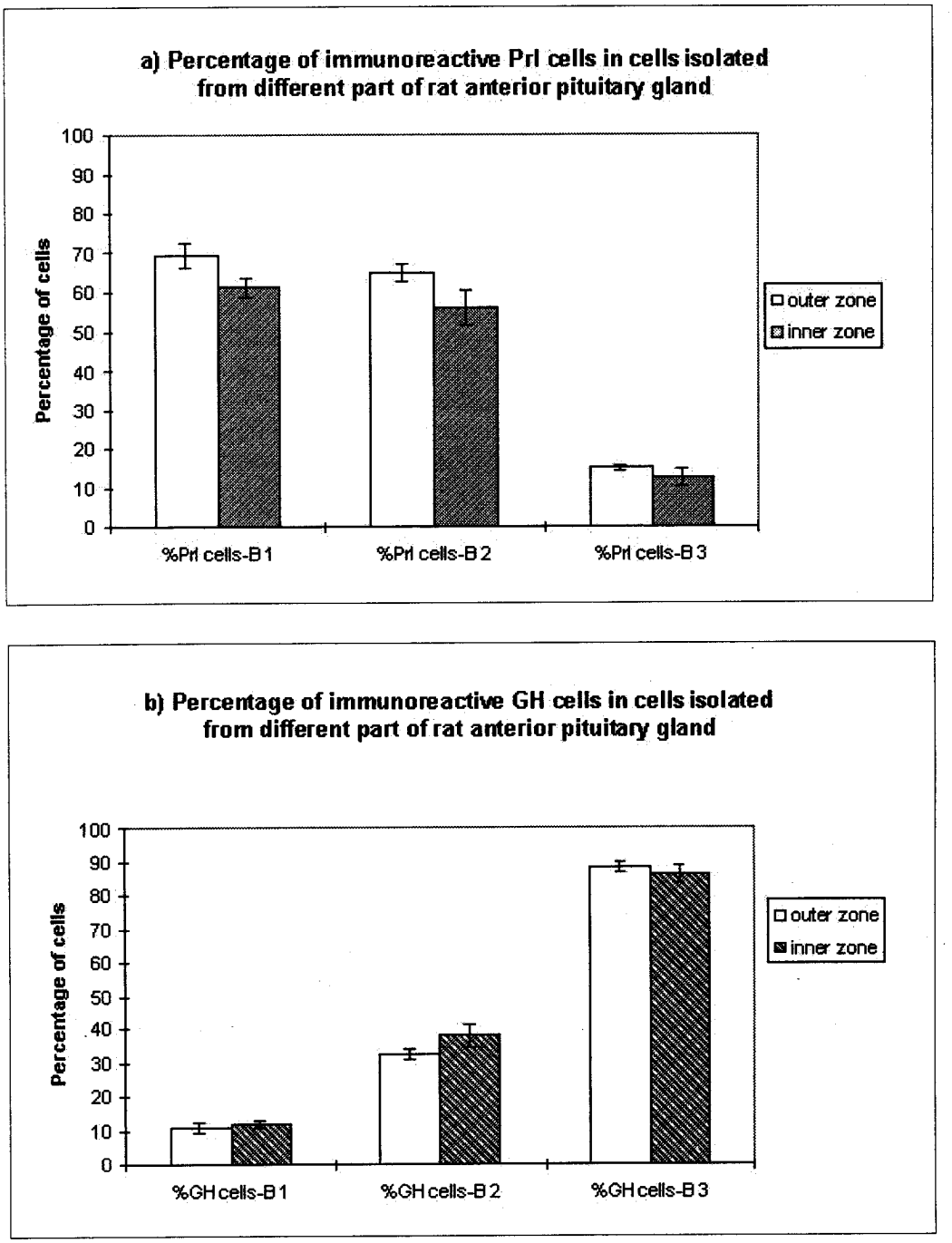

Fig. 3. Percentage of immunoreactive Prl cells (a) and GH cells (b) in cells of the inner and outer zone of the pituitary, dissociated, and further separated by Percoll gradient centrifugation. Cells collected at density interface 1.045/1.060, 1.060/1.080, and 1.080/1.090 were defined as band 1 (B1), band 2 (B2), and band 3 (B3) cells, respectively. Cells were incubated with antibodies specific to rPrl or rGH and positive cells were visualised by the indirect immunoperoxidase method as described in materials and methods.

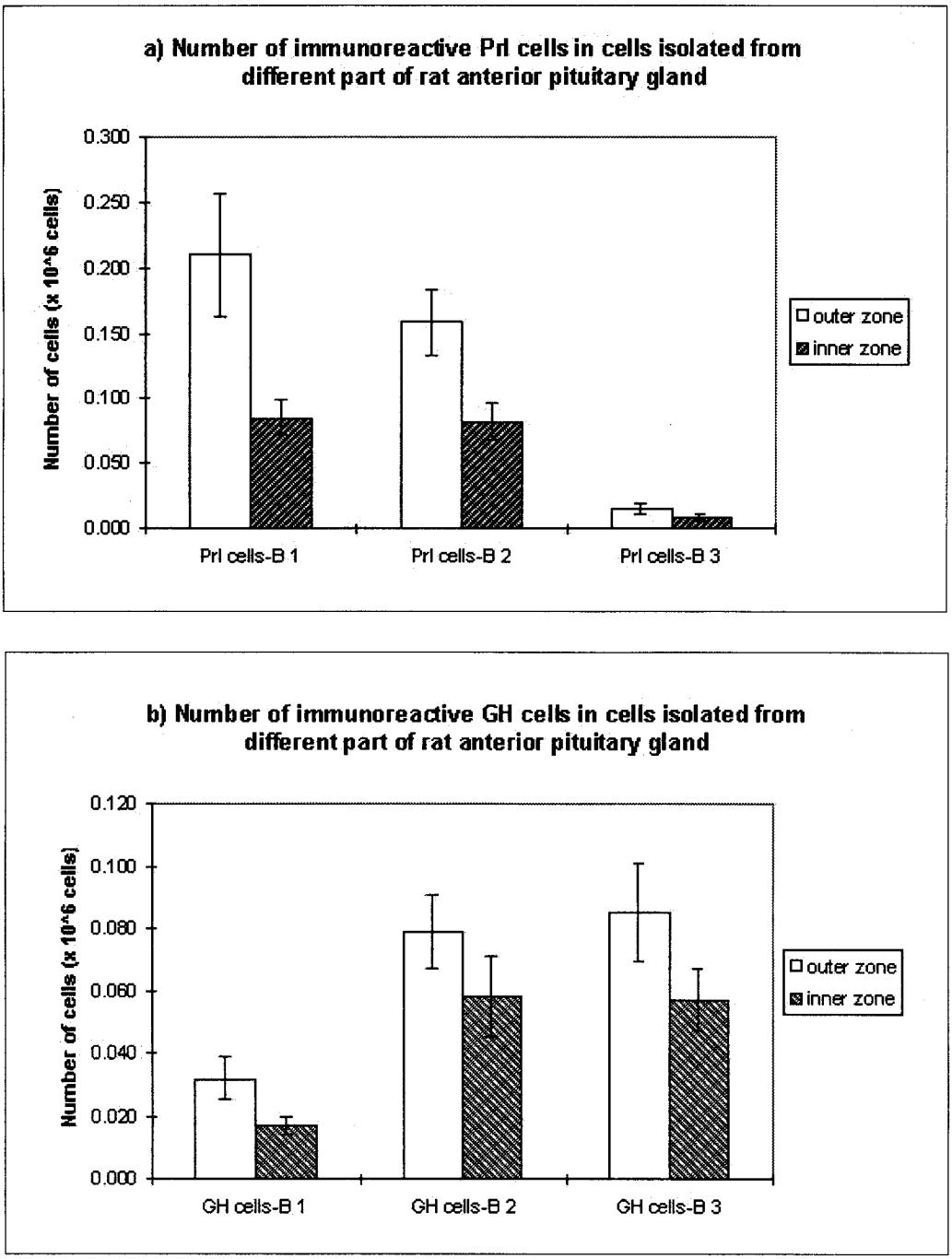

Fig. 4 The actual number of immunoreactive Prl cells (a) and GH cells (b) in freshly dissociated pituitary cells. These numbers were calculated by multiplying the actual number of cells harvested from each band after Percoll gradient centrifugation by the percentage shown in Figure 3.

Prl protein than cells in the central area. In contrast, cells located in the central area, close to the neurointermediate lobe, contained more Prl protein but less Prl mRNA than peripherally located cells.[30] The centrally located cells were more responsive to *in vivo* estradiol treatment in terms of Prl mRNA accumulation whereas cells in the periphery contained more immunoreactive Prl protein after the treatment, suggesting a preferential stimulatory effect of *in vivo* estradiol in Prl cells located in different regions of the rat anterior pituitary. This difference might indicate the influence of the different physiological state on Prl cell behaviour because different regions of the anterior pituitary receive different hypophysiotropic input. Reymond and colleaques[37] have demonstrated a regional variation in the release of hypothalamic DA into hypophysial portal blood: the central portal vessels which supply the inner zone of the gland contain higher level of DA than those supply the outer zone. The different pattern of blood supply within the pituitary may cause a regional variation in estradiol delivery as well. Estradiol has been shown to possess a potent antidopaminergic activity on Prl release.[38] Higher level of estradiol in portal blood supplied to the inner zone of the gland may abolish a tonic inhibition of hypothalamic DA and ameliorate the response of Prl cells to estradiol and/or other secretagogues. The increase in Prl mRNA accumulation in the centrally located cells may be due to complex interaction between estradiol and locally produced proteins as well. There is increasing evidence indicating that locally produced pituitary proteins mediate development, mature functions, and organization of the anterior pituitary.[39] Several investigators have shown that epidermal growth factor (EGF) stimulates Prl synthesis and growth of pituitary Prl cells.[40-46] Taken together, these observations suggest that there are either two distinct subpopulations of pituitary Prl cells, or cells that are under different physiological conditions, in the intact pituitary.

The results presented here show that cells dissociated from the outer and inner zone of the anterior pituitary exhibit the same physical characteristics. In population of cells collected at band 1, Prl cells constitute 69 % and 61 % of cells dissociated from the outer and inner zone, respectively. These numbers are higher than those reported by Hu and Lawson (45 % and 50 %) who studied ovariectomised rats[22] whereas we used normal young female rat at random cycle stages. Moreover, Zhang and colleagues found that in lactating rats, 75 - 80 % of cells collected at low density were Prl cells.[33] This difference suggests the influence of the different physiological state on the number of total pituitary Prl cells.

Nevertheless, our results show that these different cell types do not separate on a Percoll density gradient. This means that differential expression of the Prl gene does not result in the different physical characteristics (on their density basis) which would cause the cells to separate into different bands. Rather, the differences observed *in vivo* probably reflect a different physiological stage of Prl cells that relates to their topographical localisation within the intact pituitary. The change of Prl cells after isolation and evidenced as recovery of Prl cells over two bands after separation rather indicates that some cells switch behaviour after isolation from their natural environment. Therefore, the *in vivo* condition of Prl cell physiology has to be further analysed and manipulation of the *in vitro* conditions will allow us to determine the factors (cell-to-cell interactions and/or autocrine and paracrine effects) relevant to explain the observations.

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บทคัดย่อ

การทดลองนี้ต้องการพิสูจน์สมมุติฐานว่าเซลล์สร้างฮอร์โมนโปรแลคตินสองกลุ่มที่กระจายอยู่คนละบริเวณในต่อมใต้สมองส่วนหน้าของหนูจะแยกกันอยู่ที่ความหนาแน่นแตกต่างกัน หลังจากปั่นเซลล์ผ่าน Percoll gradients จึงตัดต่อมใต้สมองส่วนหน้าของหนูขาวเพศเมียออกเป็นสองส่วน คือ ส่วนนอก และส่วนในซึ่งอยู่ติดกับ neurointermediate lobe จากนั้นใช้วิธีเชิงกลและวิธีเคมีแยกเซลล์ให้เป็นอิสระ แล้วแยกเซลล์ตามความหนาแน่นโดยปั่นเซลล์ที่แยกจากแต่ละส่วนผ่าน Percoll gradients ที่ไม่ต่อเนื่อง เก็บเซลล์ที่แยกจากส่วนนอกหรือจากส่วนในดังกล่าวที่ผิวประจันระหว่างความหนาแน่น 1.045/1.065 ก./มล. (แถบ 1), 1.065/1.080 ก./มล. (แถบ 2), และ 1.080/1.090 ก./มล. (แถบ 3) ต่อจากนั้นเตรียมเซลล์ที่เก็บได้จากแต่ละแถบของแต่ละส่วนให้สามารถตรวจนับปริมาณเซลล์สร้างโปรแลคตินด้วยวิธีทางอิมมูโนได้ แล้วเปรียบเทียบปริมาณเซลล์สร้างโปรแลคตินในเซลล์ที่เก็บจากแถบเดียวกันของเซลล์ที่แยกได้จากส่วนนอกและของเซลล์ที่แยกได้จากส่วนในดังกล่าวโดยใช้ nonparametric Mann-Whitney two-tailed test ผลการทดลองแสดงว่า ปริมาณเซลล์สร้างโปรแลคตินในเซลล์ที่เก็บจากส่วนนอกและในเซลล์ที่เก็บจากส่วนในซึ่งตัดแยกจากต่อมใต้สมองส่วนหน้าของหนูขาวเพศเมีย ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ กล่าวคือ สมมุติฐานที่ตั้งไว้ไม่เป็นจริง