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Role of Type IV Collagen in Prolactin Release from Anterior Pituitaries of Male Rats

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We previously demonstrated that laminin, a component of basement membranes, modulates pituitary hormone secretion. In the present study, we evaluated the effect of type IV collagen, another component of this membrane, on the release of prolactin (PRL) by anterior pituitary gland from adult male rats. Hemipituitaries were incubated for 3 h with type IV collagen or antibodies against it and PRL release was studied. Rabbit IgG to type IV collagen at concentrations of 10^{-7}–10^{-5} *M* had a significant stimulatory effect on PRL release, in comparison to normal rabbit serum IgG or medium alone used as controls. Type IV collagen induced a significant inhibitory effect on basal release of PRL at a concentration of 30 µg/mL. A slight decrease in PRL release was detected in thyrotropin-releasing hormone–stimulated hemipituitaries incubated with type IV collagen at all concentrations used. These results suggest that type IV collagen, similar to laminin-1, modulates PRL released from hemipituitaries, in vitro.

Key Words: Type IV collagen; prolactin; anterior pituitary.

Introduction

In multicellular organisms, cells interact with neighboring cells and with extracellular matrix (ECM) proteins, via specific receptors. These interactions are essential for cell survival, proliferation, differentiation, and migration. Basement membranes are thin, sheetlike ECM structures underlying epithelial and endothelial cells. Basement membranes are mainly composed of type IV collagen, laminin, proteoglycans, and entactin. Type IV collagen, the major component of basement membrane, was initially described as a single trimeric molecule composed of α_1(IV) and α_2(IV) chains. In some basement membranes, small amounts of α_3(IV), α_4(IV), α_5(IV), and α_6(IV) collagen chains have also been identified *(1)*. Cell-matrix interactions occur via transmembrane receptors such as integrins *(2)* that become associated with attachment domains on basement membrane proteins.

In the anterior pituitary gland, ECM has an important role in the maintenance of the complex structure of the gland, but little is known about the distribution of ECM's components in this tissue. Vila-Porcile et al. *(3)* described the immunohistochemical localization of type IV collagen in the basement membrane of anterior pituitary from adult male rats. They also reported *(4)* small amounts of this collagen in anterior pituitary endocrine cells cultured in vitro. By immunocytochemistry type IV collagen was localized in the cytoplasm of a rat pituitary tumor cell line (GH_3B_6) *(5)*. Farnoud et al. *(6)* have immunodetected type IV collagen on basement membranes surrounding normal and adenoma cells from human anterior pituitary. Horacek et al. *(7)* identified β_1 integrins (α_1, α_2, α_3, α_5) on adenohypophyseal cells from adult male rats. Murray et al. *(8)* also detected type IV collagen, laminin, and α_6 β_4 integrin in human adult normal pituitary gland and in epithelial and vascular basement membrane of pituitary adenomas.

Prolactin (PRL)-secreting cells have been widely studied for the analysis of the secretory process of the anterior pituitary gland. PRL secretion is known to be affected by a large variety of stimuli in the microenvironment and by external factors. Neuropeptides, thyroliberin (TRH), epidermal growth factor, fibroblast growth factor, and galanin, among others, are known to modulate in vivo PRL secretion. Moreover, thyrotropin-releasing hormone (TRH) is the most potent stimulator of PRL synthesis in vitro *(9)*.

ECM proteins can also influence PRL secretion. Brunet de Carvalho et al. *(10)* showed that laminin induces the formation of neurite-like processes in GH3B6 cells, a phenomenon that correlated with an increase in PRL secretion in both basal and TRH-stimulated conditions. We previously demonstrated that laminin has a biphasic effect on PRL release by anterior pituitary glands from adult normal male rats, in vitro *(11)*. The aim of the present work was to study the effect of type IV collagen on PRL release from the anterior pituitary gland of adult normal male rats with

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or without TRH stimulation. We studied this phenomenon in hemipituitaries in which the normal tissue architecture and cell-to-cell and cell-to-ECM interactions essential for hormone secretion are maintained.

Results

Similar cell and tissue architecture was observed in hemipituitaries incubated with type IV collagen or Krebs-Ringer medium used as control. Moreover, *in situ* end labeling (ISEL) technique revealed no apoptotic cells in tissue sections of hemipituitaries previously incubated with 30 μg/mL of type IV collagen (data not shown).

Immunofluorescence Assay

As shown in Fig. 1A, a linear immunofluorescence was detected around cells and in the vessel walls of tissue sections from hemipituitaries previously incubated with antibodies to type IV collagen (10^{-7}–10^{-5} *M*). By immunofluorescent techniques with fluorescein isothiocyanate (FITC) and rhodamine conjugates, some of the cells that express type IV collagen also express PRL in their cytoplasm (Fig. 1B). No fluorescence was observed in controls.

Effect of Anti–type IV Collagen IgG on PRL Release

Figure 2 shows PRL release by hemipituitaries incubated with antibodies to type IV collagen at concentrations of 10^{-9}–10^{-5} *M*. A significant increase in PRL release by hemipituitaries was observed at concentrations of 10^{-7}–10^{-5} *M*. For 10^{-7} *M*, $F(2,21) = 38.84$, $p < 0.001$; for 10^{-6} *M*, $F(2,21) = 80.43$, $p < 0.001$; for 10^{-5} *M*, $F(2,21) = 39.78$, $p < 0.001$ vs both controls: medium alone and normal IgG. No significant difference in PRL release was observed between medium alone and normal IgG.

Effect of Type IV Collagen on PRL Release

The effect of different concentrations of type IV collagen (0.003–30 μg/mL) on basal and TRH-stimulated hemipituitary PRL release is shown in Fig. 3. In basal conditions (Fig. 3A) the highest concentrations of type IV collagen (15 and 30 μg/mL) induced a significant decrease in PRL release ($F[6,49] = 1351,7$; $^{*}p < 0.05$, $^{***}p < 0.001$, respectively, vs medium alone). When hemipituitaries were incubated with the same concentrations of type IV collagen in the presence of exogenous TRH (Fig. 3B), a moderate decrease (24–43%) in PRL release was observed considering control values as 100%: $F(6,49) = 6.434$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs TRH-stimulated medium.

Discussion

Anterior pituitary glands from normal adult rats incubated with antibodies to type IV collagen released higher amounts of PRL into the medium than those incubated with normal rabbit serum IgG or medium alone. A similar effect was previously obtained with antibodies to laminin *(11)*. It is probable that antibodies interfere in ECM-integrin interactions by inducing changes in cell signaling.

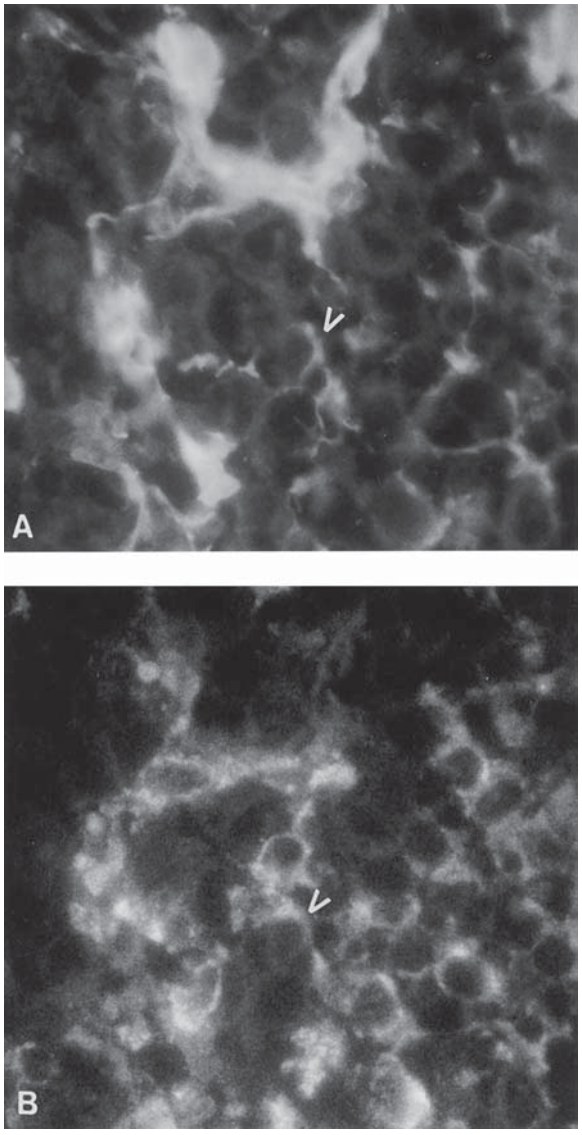

Fig. 1. Immunofluorescent technique using FITC (**A**) and Rhod (**B**) conjugates. Tissue sections are from hemipituitaries previously incubated with antibodies to type IV collagen. Type IV collagen is observed in blood vessel walls and around cells (A). Some of these cells also express PRL (B). Arrowheads show coexpression of type IV collagen and PRL. Magnification ×750.

We also observed an inhibitory effect of type IV collagen on PRL release by hemipituitaries. In basal conditions, strong inhibition was detected with type IV collagen at 30 μg/mL, while in TRH-stimulated hemipituitaries a moderate decrease in PRL release compared to controls was observed at all collagen concentrations used. We think that this inhibitory effect of collagen in basal conditions was not owing to a cytotoxic action, since hemipituitaries exhibit a good response to TRH. Moreover, we observed no apoptotic cells in tissue sections of these hemipituitaries. These results sug-

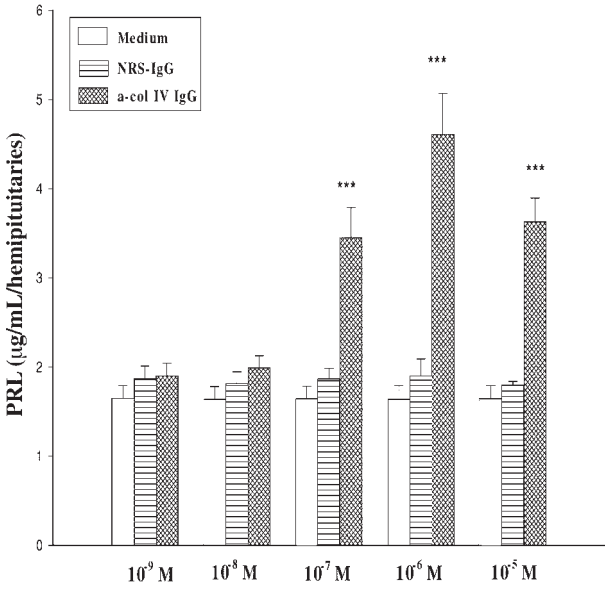

Fig. 2. Effect of anti–type IV collagen IgG (a-col IV IgG) at concentrations of 10^{-9}–10^{-5} *M* on PRL release from hemipituitaries of male rats compared with controls: medium alone or IgG from normal rabbit serum (NRS-IgG) at the same concentrations as a-col IV IgG. Results are the mean ± SEM ($n = 8$) of three separate experiments. ***$p < 0.001$ vs both, medium alone, or NRS-IgG.

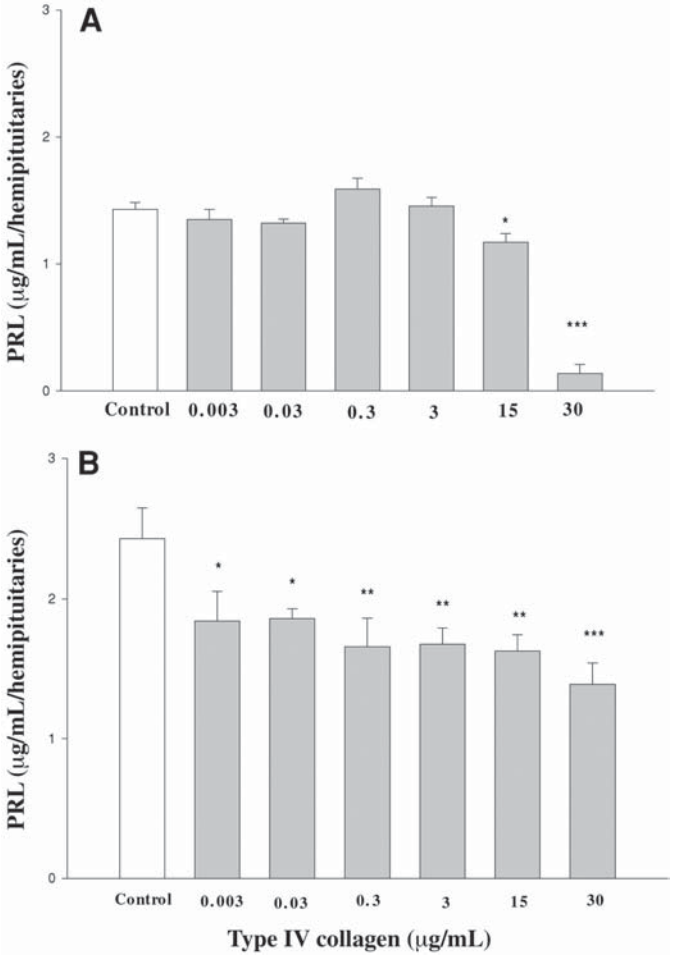

Fig. 3. (**A**) Effect of type IV collagen on PRL release from hemipituitaries of male rats compared to control (medium alone); (**B**) effect of type IV collagen on PRL release from TRH-stimulated hemipituitaries of male rats compared to control (TRH-stimulated medium). Results are the mean ± SEM ($n = 8$) of three separate experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control.

gest that type IV collagen is able to modulate PRL release from the pituitary gland. We previously demonstrated that laminin is also able to modulate PRL release by hemipituitaries *(11)*. Martinez-Campos and Dannies *(12)* reported that rat anterior pituitary cells cultured on type I collagen secreted more PRL into the medium than those cultured on plastic, but they described PRL stimulation or inhibition in a similar number of experiments. In addition, Horacek et al. *(13)* reported that adult or fetal pituitary cells from hamsters cultured on reconstituted basement membrane (Matrigel) secreted more PRL, luteinizing hormone, and follicle-stimulating hormone. Elias et al. *(14)* reported an increase in basal PRL secretion and mRNA synthesis by a rat pituitary tumor cell line (GH3) plated on ECM obtained from bovine corneal endothelial cells. Most of these studies showed a stimulatory effect of ECM proteins on PRL secretion by pituitary cell cultures. The difference with respect to our results could be owing to the fact that the other investigators used Matrigel composed of several ECM proteins while we used only type IV collagen. Another difference was the use of pituitary cell cultures or cell lines rather than pituitary tissue in which normal cell interactions are preserved. Moreover, we studied the effect of type IV collagen during a short period of incubation (3 h), whereas the other investigators *(12–14)* used 24- to 72-h cultures.

It has been demonstrated that ECM's components are essential for pituitary cell differentiation. Type IV collagen is present in basement membrane and has also been detected intracellularly in pituitary cells *(3,5,10)*. Horacek et al. *(15)* detected laminin, fibronectin, and type IV collagen in the basement membranes at the initial stages of pituitary cell development before the period of hormone storage, suggesting a possible role of ECM in the organization of pituitary epithelium. It has been demonstrated that cooperation between stroma and epithelial cells is required for the production of basement membrane components. These components could interact with cell receptors and trigger hormone secretion signals.

A relationship between hormone secretion and ECM proteins was also described in other tissues including endocrine glands. Tam et al. *(16)* studied the effect of cell-matrix interactions on parathyroid hormone (PTH) responsiveness in proximal renal tubular cells and osteoblast-like cells. In both cell types, type IV collagen induced an inhibitory

effect on PTH binding and PTH-mediated biologic response. Xie and Haslam *(17)* have demonstrated that fibronectin and type IV collagen might modulate responsiveness to progesterone by mouse mammary epithelial cells in vitro. They suggested that the interaction between ECM's components and hormones depends on the developmental stage of the mammary gland. Inui et al. *(18)* reported a positive correlation between serum levels of type IV collagen and thyroid disease. An increased level of type IV collagen peptide in serum was also observed in patients with hyperthyroidism whereas a relative decrease was detected in the hypothyroid state. We recently detected a significant decrease in testosterone production when basal human chorionic gonadotropin (hCG)–stimulated rat Leydig cells were cultured for 3 or 24 h on plates coated with type IV collagen or fibronectin, compared with uncoated plates *(19)*. Fujiwara et al. *(20)* reported that laminin, through its interaction with $\alpha_6\beta_1$, suppresses the luteinization of basal and hCG-stimulated human luteinizing granulosa cells by inhibition of progesterone production. Incubation of these cells with antibodies to α_6 integrin subunit reverted this inhibitory effect.

Substantial evidence supports the influence of ECM proteins on hormonal secretions. ECM appears to be able to change hormonal response either directly by altering the cell or by altering the availability of the hormone secretion or its spatial presentation *(21)*. The mechanisms of ECM action could involve the formation of spatial tracks for intracellular signaling pathways *(22)*. Our results showing the inhibitory effect of type IV collagen on PRL release by hemipituitaries could be explained by changes in intracellular signals depending on type IV collagen conformational changes through integrins.

In conclusion, our data show that type IV collagen induces an inhibitory effect on PRL release from the anterior pituitary of adult male rats in vitro, confirmed by the fact that antibodies to type IV collagen exert an increase in PRL release. Previous and present data suggest that laminin and type IV collagen, both components of basement membranes, are probably involved in the inhibitory control of PRL secretion, in vivo.

Materials and Methods

Antibodies to Type IV Collagen

Immunization Schedule

Type IV collagen obtained from the basement membrane of an Engelbreth-Holm-Swarm (EHS) murine sarcoma (Gibco-BRL, Grand Island, NY) was used as antigen. Rabbits were injected three times with an emulsion of type IV collagen and Freund's complete adjuvant (1:1). Immunizations were performed at 15-d intervals, with a total dose of 1.5 mg.

Purification of Antibodies

IgG was obtained from rabbit serum by ammonium sulfate precipitation and DEAE cellulose as previously described for antiserum to laminin *(23)*. Anti–type IV collagen IgG was further purified by affinity chromatography over a column of cyanogen bromide–activated Sepharose (Pharmacia, Uppsala, Sweden), coupled to type IV collagen. Antibodies to type IV collagen were eluted from the column and dialyzed against phosphate-buffered saline (PBS). A titer of 3.2×10^5 was detected by enzyme-linked immunosorbent assay, and no crossreaction with laminin was observed. IgG from normal rabbit serum was also obtained for use as control.

In Vitro Experiments

Adult male Wistar rats weighing 200–250 g were housed in controlled conditions of temperature (22–24ºC) and lighting (lights on 5:00 AM to 7:00 PM) and free access to food and tap water. The animals were treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were killed by decapitation and the anterior pituitaries were cut longitudinally into halves. Hemipituitaries were preincubated for 1 h in 1 mL of Krebs-Ringer bicarbonate medium (pH 7.4) at 37ºC in an atmosphere of 95% O_2/5% CO_2 in a Dubnoff metabolic shaker. After removal of preincubation medium, the hemipituitaries were incubated in the presence of 1 mL of antibodies to type IV collagen at a final concentration of 10^{-9}–10^{-5} *M* for 3 h. IgG from normal rabbit serum (NRS-IgG) at the same concentrations and medium alone were used as controls.

In another series of experiments, hemipituitaries preincubated with medium were resuspended in 1 mL of medium containing mouse type IV collagen EHS (Gibco) at a final concentration of 0.003–30 µg/mL (corresponding to approx 6×10^{-12} to 6×10^{-8} *M*, respectively). Type IV collagen solution was neutralized with NaOH to pH 7.2. Medium alone was used as control. At the end of the incubation and after the removal of a 100-µL aliquot for the determination of PRL basal release, the hemipituitaries were stimulated for 30 min with exogenous TRH (Trhelea, Buenos Aires, Argentina) at a final concentration of 5×10^{-7} *M*. Then, media were stored at –20ºC until PRL determination.

Morphology

For light microscopy, hemipituitaries were fixed in Bouin's solution, embedded in paraffin, and stained with Herlant's tetrachrome.

Indirect Immunofluorescence

Hemipituitaries were incubated for 3 h with anti–type IV collagen rabbit IgG (10^{-7}–10^{-5} *M*) or NRS-IgG used as control. Tissues were snap frozen, and cryostat sections (5 µm thick) were fixed in acetone. To detect coexpression of type IV collagen and PRL, tissue sections were incubated with anti-PRL guinea pig antibody 1/1500 (National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, MD) for 1 h at room temperature in a humidified chamber. As secondary antibodies, FITC-conjugated anti–rabbit IgG (1:50) (Vector, Burlingame, CA) and antirhoda-

mine-conjugated anti–guinea pig IgG (1:200) (Chemicon, Temecula) were used to reveal type IV collagen and PRL, respectively. Sections were observed under an Axiophot fluorescent microscope with epiillumination (Zeiss, Germany). As negative control, the first antibody was omitted.

ISEL of Apoptotic DNA

DNA fragmentation in individual cells was visualized in tissue sections by indirect immunoenzyme detection of digoxigenin-labeled genomic DNA by a technique previously described *(24)*. To facilitate antigen retrieval, deparaffinized and hydrated tissue sections (<5 μm thick) were heated in a microwave (5 min at 370 W) in 10 m*M* sodium citrate buffer, pH 6.0, and quickly cooled in PBS. Sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 4 min at 4ºC. Nonspecific labeling was prevented by preincubating the sections with 2% blocking reagent (Roche, Mannheim, Germany). For the negative control, TdT terminal transferase was replaced with the same volume of reaction buffer. Light counterstain was done with eosin.

Radioimmunoassay

PRL level was determined by double antibody radioimmunoassay as previously described *(25)*. Results are expressed in terms of the reference preparations (rat-PRL-RP3 supplied by NIDDK). Sensitivity for PRL assay was 0.05 ng/tube.

Statistical Analyses

Data were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls multiple comparisons test using the GraphPad program. Data were tested for normality of distribution by the Kolmogorov-Smirnov test and for homogeneity of variance by Bartlett test and log square root transformed as needed. A difference of $p < 0.05$ was considered significant.

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