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# The Hormone Exocytosis in Prolactinoma and Normal Adenohypophysis Cell Cultures by the Effects of Hypocalcaemia

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## Abstract

The biological systems are opened, complex objects, which can regularly exchange feedbacks with their environment. The calcium ion is a universal messenger, which can regulate several cellular functions e.g. exocytosis machinery. The primary aim of this study was to investigate the response mechanisms of normal adenohypophysis and adenohypophyseal prolactinoma cell populations at different extracellular $Ca^{2+}$ levels with an otherwise isoionic milieu of all other essential ions. We focused on prolactin (PRL) and adrenocorticotrophic hormone (ACTH) release.

In our experimental study, female Wistar rats (n=10) were treated with estrone-acetate (150 μg/kg b.w/week) for 6 months to induce prolactinomas in the adenohypophysis. Primary, monolayer cell cultures were prepared by enzymatic and mechanical digestion. PRL and ACTH hormone presence was measured by radioimmunoassay or immuno-chemiluminescence assay. Repeated measurements of ACTH and PRL hormone release in different treatment groups on cell cultures during 80 minutes were compared using marginal models.

Differences between the effects of hypocalcaemia on normal adenohypophysis cultures and prolactinoma cell populations were investigated. Significant alteration (p<0.001, n=12) in hormone exocytosis was detected in $Ca^{2+}$ treated adenohypophyseal and prolactinoma cell cultures, compared to untreated groups.

Diminution of $Ca^{2+}$ may inhibit the SNARE mediated fusion of hormone containing vesicles to plasma membrane. In conclusion, the main finding of this study is that a strict correlation exists among certain biophysical properties, especially extracellular $Ca^{2+}$ milieu and hormone vesicle exocytosis.

## Introduction

The biological systems are opened, thus they regularly exchange feedbacks with their environment. In the adaptation processes the hypophyseal hormones, primarily adrenocorticotrophic (ACTH) [1] and prolactin (PRL) [2] play major role. Firstly the hypothalamo-pituitary-adrenal (HPA) axis activity is essential in the adaptability of living organisms and their capacity to construct new operating conditions via ACTH release of corticotroph cells [3]. The HPA axis regulates the circadian rhythm, activates in response to stress and activates the release of adrenal corticosteroids. Secondly PRL is a common mediator of the immune-neuroendocrine system and affects the different reproductive states. PRL secretion is also modified by environmental inputs such as light, sound and stress [2].

To examine these hormone effects in the level of organism it is important to investigate the mechanisms of endocrine cells. A complex dynamical system can be described with its internal structure and the relationships among the internal structure and its external environment, therefore in the level of cells the extracellular homeostasis is determined by the ion regulation, e.g. $Ca^{2+}$ regulation. In cellular level the calcium ion ($Ca^{2+}$) is a universal messenger, which can act an extracellular and intracellular messenger to regulate a diverse array of cellular functions [4,5]. It has been known, that changes in $[Ca^{2+}]$ interfere with gating properties of plasma membrane channels, which in turn, affect spontaneous and receptor-controlled changes in the intracellular $[Ca^{2+}]$ [6,7].

Upon entering the cytoplasm, most $Ca^{2+}$ is trapped by $Ca^{2+}$ binding proteins [4,8]. Intracellular $Ca^{2+}$ sensors are e.g. calmodulin, Ras, Raf, MEK, MAPK [8].

$Ca^{2+}$-channels in many different cell types activate on membrane depolarization and mediate $Ca^{2+}$ influx in response to action potentials [9]. $Ca^{2+}$ entering the cells through voltage-gated $Ca^{2+}$-channels serves (CaV) as the second messenger of electrical signaling, initiating many different cellular events [10,11].

The β subunit of the $Ca^{2+}$-channels bounds to calmodulin via their carboxy-terminal domain. Calmodulin is essential for exocytosis machinery. C1 domain of calmodulin bounds to diacylglycerol (DAG), meanwhile C2 domain interacts with 2 or 3 $Ca^{2+}$, than activates phospholipases and protein-kinases [5,12].

The exocytosis machinery is triggered by membrane depolarization followed by $Ca^{2+}$ entry (Figure 1). In resting cells the intracellular $[Ca^{2+}]$ is low. By the effects of extracellular stimuli, alterations of the electrical properties of cell membrane induce the voltage-gated $Ca^{2+}$ channels and mediate G-protein utilization. Gα subunit of Gq/G11 protein activates the phospholipase-C that cleaves phosphatydil-inositol 4,5-bisphosphate into inositol [1,4,5] trisphophate (IP3) and DAG [7,13]. These intracellular messengers induce higher intracellular $[Ca^{2+}]$ that trigger the fusion of hormone containing vesicles to plasma membrane.

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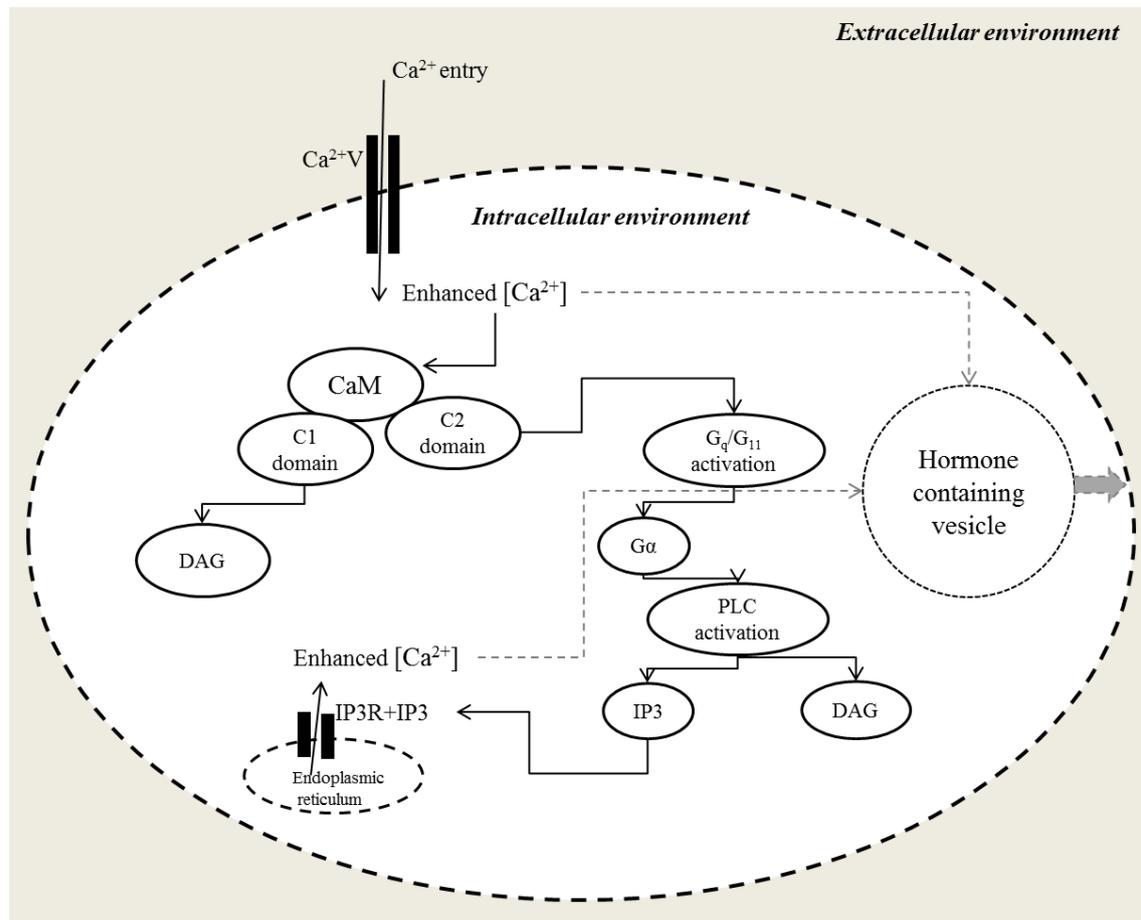
**Figure 1:** The possible mechanism of hormone exocytosis.
$Ca^{2+}$ enters the cells via voltage-gated $Ca^{2+}$-channels ($Ca^{2+}$V). $Ca^{2+}$ bounds to calmodulin (CaM) than the C1-domain of the active CaM activates diacyl-glycerol (DAG); meanwhile the C2 domain mediates $G_q/G_{11}$ proteins. The Gα-subunit of G proteins enhance the activation of phospholipase-C (PLC), that cleaves phosphyatydil-inositol 4,5 bisphosphate into inositol (1,4,5)-trisphosphate and DAG. IP3 bounds to IP3 receptors, which release $Ca^{2+}$ from the endoplasmic reticulum. The higher intracellular $[Ca^{2+}]$ supports the fusion of hormone containing vesicles to plasma membrane

$Ca^{2+}$-channels interaction with proteins such as syntaxin, SNAP25 has begun to increase. Synaptotagmin plays major role in membrane fusion and transmitter release as well as in hormone exocytosis [14,15]. Then the excitosome can fuse to the plasma membrane [16].

To investigate the hormone secretion mechanisms of endocrine cells is essential to understand disorders, which are manifested in overexpression of these hormones. In these days it has been recognized that pituitary adenomas are frequently in general human population. PRL secreting prolactinomas are the most common pituitary adenomas and account for up to 45% of these tumors. In young adults, prolactinomas occur more frequently in women than men, while this sex imbalance is not apparent in the middle aged population [17,18].

This study examines the extracellular environment of cells and their adaptation mechanisms as a complex system. We wanted to investigate the cell membrane function and/or mechanisms under hypocalcaemic effects, because the effect of extracellular hypoionic conditions on cellular (exocytosis) functions is intriguing and likely important factor in a number of endocrine disorders, and since most of the published work, investigates the release of neurotransmitter vesicles in neurons and this present work is potentially interesting due to its focus on hormone release.

## Materials and Methods

### Experimental model

**Experimental protocol:** Female Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for hypophysis cell culture model systems. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary. During the research period, rats were kept under controlled relative air humidity of 55-65% and 22 ± 2°C ambient temperature. Experimental animals lived under automated diurnal conditions (12 h dark and 12 h light system) in groups of 10 animals for 6 months. Standard pellet food and tap water were available *ad libitum*. Female Wistar rats (n=10) were treated subcutaneously with estrone-acetate (CAS registry number: 901-93-9, Sigma, Germany; 150 μg/kg b.w./week) for 6 months to induce adenohypophyseal prolactinomas.

After pentobarbital anaesthesia (4.5 mg/kg b.w. Nembutal, Abbott, USA) the animals were killed and decapitated. Tissues were separated under a preparative microscope. Primary, monolayer cell cultures were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2%/Sigma, Germany/for 30 min; collagenase/Sigma, Germany/:30 μg/ml for 40 min; dispase/

Sigma, Germany/:50 µg/ml for 40 min in phosphate-buffered saline/ PBS-A/; temperature: 37°C). Mechanical dispersion was achieved with nylon blutex sieves (∅: 83 and 48 µm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2 \times 10^5/cm^3$. The dissociated cells were placed onto 24 well-plastic plates (5% collagen coated /Nunc., Geramany/; Dulbecco's Modified Essential Medium/DMEM/+20% Fetal Calf Serum/FCS/+antibiotics/Penicillin+Streptomycin: 1.0 µg/ml/). The cells were cultured at 37°C in a $CO_2$ incubator that provided a humidified environment of 95% air and 5% $CO_2$. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking for PRL and ACTH protein release. After functional standardization, the basal ACTH and PRL levels were determined in both normal Adh and PRLoma (Tyrode's medium/Sigma, Germany/) [19]. In the medium, only the $[Ca^{2+}]$ was modified; all other essential anions and cations were under homeostatic (e.g. isoionic) conditions. The hormone release of primary cell cultures was detected under hypocalcaemic conditions of varying degrees ($[Ca^{2+}]$: 0; 0.5; 1.0; 1.5 mM; n=12 in each group). Samples were taken at 10, 20, 30, 60 and 90 minutes after treatments to measure hormone kinetics.

The PRL and the ACTH content were detected in the supernatant media. From the supernatant media, 500 µL samples were removed by Gilson pipette at appropriate times and stored at -80°C until peptide radioimmunoassay (RIA) [20] and immuno-chemiluminescence assay (LIA) were performed.

A rat PRL RIA KIT (Institute of Isotopes Ltd., Budapest, Hungary) was used to determine the supernatant PRL content; all components were stored at 2-8°C, where they were stable. Non-specific binding, defined as the proportion of tracer bound in the absence of antibody, was determined to be <5%. The sensitivity of the RIA procedure was 0.07 ng/tube. The intra-assay precision obtained was 0.92 ± 0.03 ng. PRL data are given in ng PRL/mg protein.

The ACTH levels of supernatant media were measured by LIA with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL) and DPC kit (L2KAC-02; Euro/DPC Ltd, Glyn Rhonwy, UK). ACTH data are given in pg ACTH/mg protein.

A modified Lowry Method [21] and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

**Statistical analysis:** Repeated measurements of ACTH and PRL hormone release in different treatment groups on cell cultures during 80 min (time points at 10, 20, 30, 60 and 90 min; n=12 in each group) were compared using marginal models (a.k.a. population average models) [22,23]. PRLoma with 1 group and treatment with 5 groups were used as between-subject fixed factors and time with 5 time points as within-subject fixed factor for the analysis. Restricted maximum likelihood estimation and Kenward-Roger method for adjusting the degrees of freedom were applied. In case of ACTH, unstructured covariance matrix, for PRL data, the heterogeneous first order autoregressive covariance matrix resulted the best fit among different structures (variance components, compound symmetry, first order autoregressive, toeplitz, unstructured and their heterogeneous versions), based on Akaike's information criterion (AIC) statistic [24]. Pairwise comparisons were estimated by least squares means using Sidak p-value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions. In case of extreme values, winsorization technique was applied by shifting the strongly outlying data toward the center to protect parameter estimation against the emergence of unexpectedly large errors [25,26].

Statistical analyses were performed in SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA), where p-values of <0.05 were considered to indicate statistical significance (SAS, 2011).

## Results

Cell culture standardization resulted that the ACTH positive cells were accounted for approximately 15.81% in Adh and 18.43% in the *in vivo* estron-acetate pretreated, than cultured adenohypophysis tissues (the monolayer contained prolactinoma cells and the adjoining adenohypophysial tissue). The percentage of PRL positive cells was 24.0% in normal Adh and 52.2% in PRLoma. Significant interaction was found between time, PRLoma and treatment groups (p<0.0001) for ACTH and PRL as well. Pairwise comparisons revealed that all treatment groups differ significantly (p<0.0001) from the control at every time points.

### The effects of hypocalcaemia on the release of ACTH and PRL in normal adenohypophysis

Figure 2 shows the ACTH release of both the normal AdH control systems and the treated groups.

The basal PRL release of the control AdH systems and the treated primary cell cultures are represented in Figure 3.

Our experiments revealed statistical differences between treated and untreated groups. In Figure 2 the ACTH content of absolute control groups in supernatant media were compared with that of the groups treated with 0 and 0.5 mM $[Ca^{2+}]$. During the experiment the ACTH secretion of treated groups decreased appreciably (in groups treated with 0 mM $[Ca^{2+}]$: 293.92 ± 1.97, 291.4 ± 2.45, 314.08 ± 1.72, 408.75 ± 1.21, 440.08 ± 1.07 and in groups treated with 0.5 mM $[Ca^{2+}]$: 370.75 ± 1.37, 323.08 ± 1.18, 304.92 ± 1.45, 330.42 ± 1.04, 383.58 ± 1.0; pg hormone/mg protein; means ± S.E.M, p<0.0001) compared with controls (531.75 ± 3.57, 570.83 ± 5.02, 757.25 ± 2.78, 1476.50 ± 4.30, 1853.42 ± 82.72; pg hormone/mg protein; means ± S.E.M). It was observed that at 60 and 90 min of the experiment the hormone content was higher in 0 mM groups than in 0.5 mM groups.

The hormone levels of cell cultures treated with 1.0 mM $[Ca^{2+}]$ (Figure 3) were reduced significantly (392.25 ± 0.76, 370.0 ± 1.55, 387.92 ± 6.32, 1010.0 ± 2.18, 1194.25 ± 1.53 pg hormone/mg protein; means ± S.E.M, p<0.0001) depending upon the duration of exposure, correlating with the control groups. The hormone levels of supernatant media in 1.5 mM $[Ca^{2+}]$ treated groups were decreased significantly (444.50 ± 1.25, 427.67 ± 0.93, 439.25 ± 1.07, 1014.42 ± 1.49, 1194.25 ± 1.53 pg hormone/mg protein; means ± S.E.M, p<0.0001).

As shown in Figure 3 the PRL secretion was reduced significantly by the effects of 0 and 0.5 mM $[Ca^{2+}]$ depending upon the duration of exposure (in groups of treated with 0 mM $[Ca^{2+}]$: 4.52 ± 0.02, 4.11 ± 0.02, 3.82 ± 0.02, 3.89 ± 0.02, 3.88 ± 0.01 and in groups treated with 0.5 mM $[Ca^{2+}]$: 5.25 ± 0.01, 3.77 ± 0.01, 2.96 ± 0.02, 3.03 ± 0.01, 6.78 ± 0.02 ng hormone/mg protein; means ± S.E.M, p<0.0001) compared with control groups (7.19 ± 0.01, 10.16 ± 0.01, 13.21 ± 0.01, 17.88 ± 0.02, 19.88 ± 0.02 ng hormone/mg protein; means ± S.E.M).

Figure 3 shows that the levels of PRL were reduced significantly by the effects of 1.0 mM $[Ca^{2+}]$ (6.13 ± 0.02, 5.86 ± 0.02, 5.29 ± 0.02, 8.78 ± 0.01, 12.79 ± 0.01 ng hormone/mg protein; means ± S.E.M, p<0.0001) compared with the controls. The PRL secretion in the cardinal points of
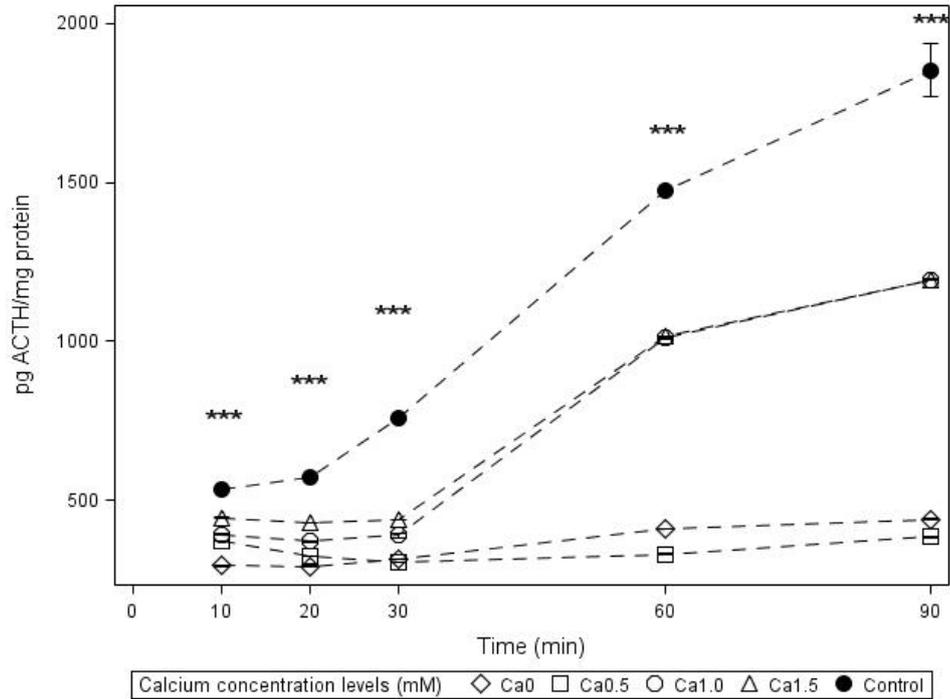
**Figure 2:** The effects of hypocalcaemia on the ACTH release in adenohypohysis cell cultures.
Cell cultures were treated as follows [Ca²⁺]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated groups versus the ACTH release of normal Adh as the control group. The level of significance was chosen as p<0.0001
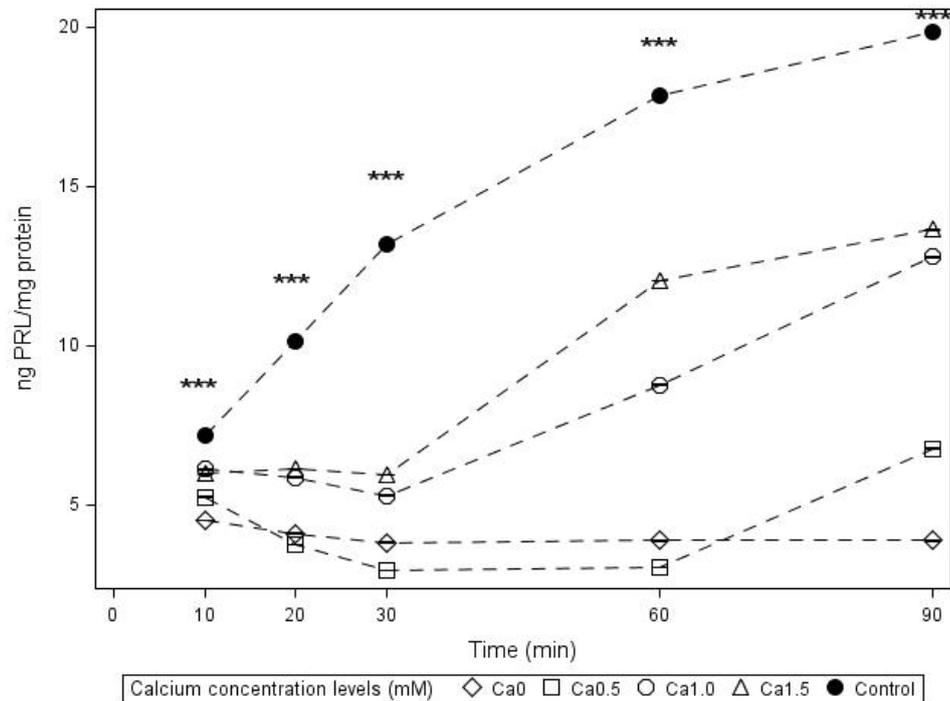


**Figure 3:** The effects of hypocalcaemia on the prolactin release in adenohypophysis monolayer cell cultures.
Cell cultures were treated as follows [Ca²⁺]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated groups versus the PRL release of normal Adh as the control group. The level of significance was chosen as p<0.0001

the research protocol decreased significantly (5.98 ± 0.01, 6.13 ± 0.01, 5.96 ± 0.02, 12.04 ± 0.02, 13.64 ± 0.02 ng hormone/mg protein; means ± S.E.M, p<0.0001) by the effects of 1.5 mM $[Ca^{2+}]$.

### The effects of hypocalcaemia on the release of ACTH and PRL in the prolactinoma cell cultures

Figure 4 shows the ACTH release of both the PRLoma cell culture control systems and the $Ca^{2+}$ treated PRLoma groups.

The basal PRL release of the control PRLoma systems and the treated primary PRLoma cell cultures are represented in Figure 5.

As shown in Figure 4, at 10 min of the experiment the ACTH of PRLoma cell cultures treated with 0 mM $[Ca^{2+}]$ was enhanced (598.75 ± 2.63 ng hormone/mg protein; means ± S.E.M, p<0.0001) and also enhanced in 1.5 mM $[Ca^{2+}]$ treated groups (469.83 ± 1.16 ng hormone/mg protein; means ± S.E.M, p<0.0001) compared with the control groups (441.0 ± 1.5 ng hormone/mg protein; means ± S.E.M). In the supernatant media the hormone levels were decreased significantly in both treated groups compared with the controls depending upon the duration of exposure. It was observed that the hormone release of PRLoma cell cultures treated with 0 mM $[Ca^{2+}]$ was increased significantly at 90 min of the experiment (2113.17 ± 7,8 ng hormone/mg protein; means ± S.E.M, p<0.0001) correlating with the control group (1946.5 ± 1946.5 ng hormone/mg protein; means ± S.E.M).

In Figure 5, it can be observed that the PRL release of PRLoma cell cultures, under different hypocalcaemic conditions, was decreased significantly depending upon the duration of exposure compared with the control group. Interestingly notable enhancement was detected in the PRL secretion in groups treated with 1.5 mM $[Ca^{2+}]$ (5.6 ± 0.02

ng hormone/mg protein; means ± S.E.M, p<0.0001) in contrast to control (3.82 ± 0.01 ng hormone/mg protein; means ± S.E.M). At 90 min of the experiment the PRL content was increased significantly as a consequence of 0 mM $[Ca^{2+}]$ (49.05 ± 0.03 ng hormone/mg protein; means ± S.E.M, p<0.0001), correlating with the control. (48.63 ± 0.04 ng hormone/mg protein; means ± S.E.M, p<0.0001).

## Discussion

To investigate cellular phenomena, *in vitro* model systems, namely primary monolayer cell cultures from normal, healthy AdH and PRLoma adenohypophysis were used and their cellular functions were standardized. In this paper the alteration of the kinetics of ACTH and PRL release in AdH and PRLoma cell cultures were studied under hypocalcaemic conditions. ACTH is essential for the HPA axis regulation [27,28]. At the surface of adrenal cortex cells the ACTH receptors are highly expressed, thus ACTH-signalling pathway is crucial for the growth and proliferation of adrenocortical cells [29]. The association of hormone molecules to the receptors can initiate G-protein than adenylyl cyclase stimulation [30,31]. This process induces generic alterations in the HPA axis, which is important to stress response mechanisms [30]. PRL is synthesized and secreted by the anterior lobe of the pituitary gland. PRL has several biological functions, it plays role in the reproduction, osmoregulation, growth, synergism with steroids, immunoregulation and protection [32,33]. PRL has also been suggested to be involved in parental behaviour [33]. The adaptability of living organisms is maintained by the HPA axis and PRL release systems [34,35]. Plasma PRL levels were also studied due to the large amount of evidence showing that this hormone and ACTH are activated by stress to the organism [1,36,37]. Therefore these hormones
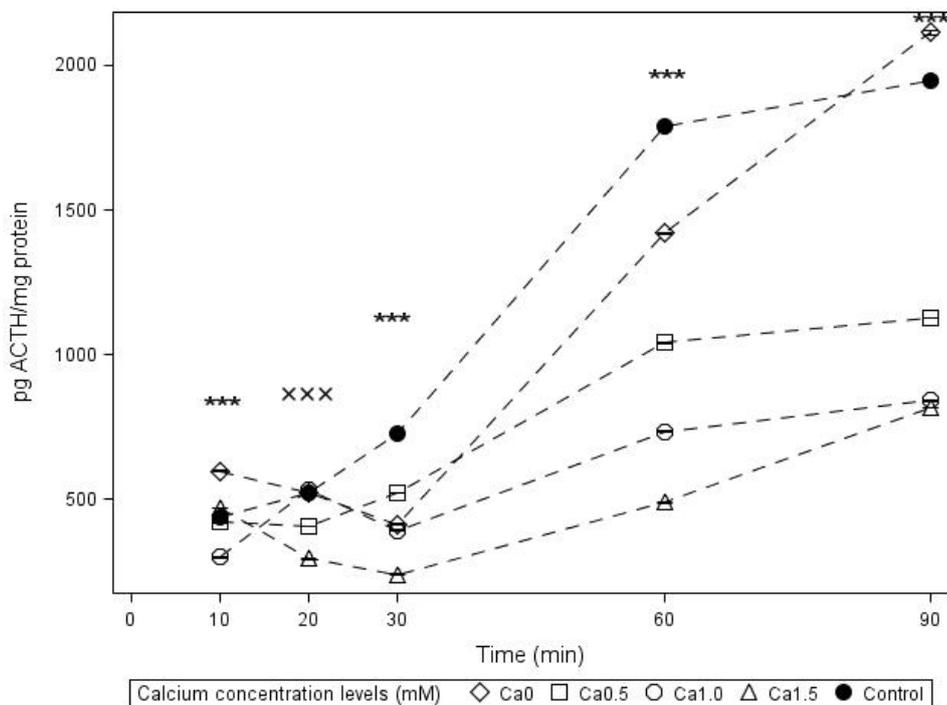


**Figure 4:** The effects of different calcium ion concentrations on the release of ACTH in prolactinoma cell cultures.
Cell cultures were treated as follows $[Ca^{2+}]$:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated PRLoma groups versus the ACTH release of PRLoma control group. The level of significance was chosen as p<0.0001
XXX indicates that the control is not significantly different from the data of hormone release by the effects of 0 mM $[Ca^{2+}]$
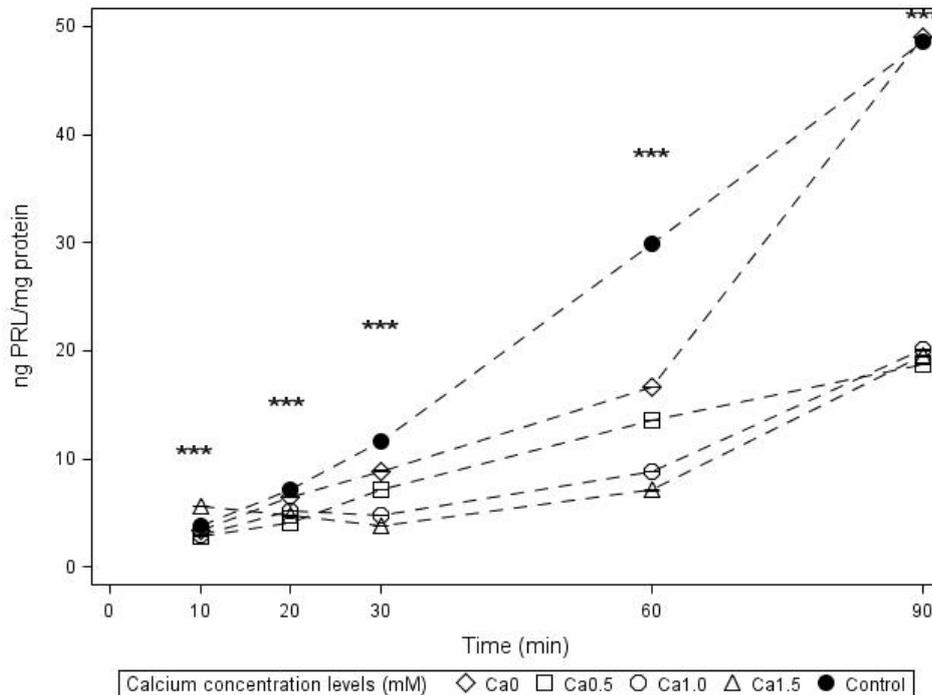
**Figure 5:** The effects of different calcium ion concentrations on the PRL hormone release in PRLoma cell cultures.
Cell cultures were treated as follows [Ca$^{2+}$]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated PRLoma groups versus the PRL release of PRLoma control group. The level of significance was chosen as p<0.0001

are essential for the vertical homeostatic regulation, which is related to extracellular Ca$^{2+}$ concentrations. PRL secreting tumors are the most frequently occurring pituitary tumors. Abnormal, tumorous cells are more active than normal cells; however our results showed that their behaviour was similar under hypocalcaemia.

Significant differences between the control and the treated primary cell cultures were investigated in both groups. The role of changes to the extracellular milieu in cell function can be defined by hormone exocytosis, sensitivity of intracellular receptors, or the discrete alteration of intracellular messenger molecules.

Since the extracellular environment is constantly fluctuating, cells must adapt to it [1]. A reduced rate of hormone exocytosis was observed as a result of hypocalcaemia. The extracellular hypocalcaemia induced generic alterations in the end-differentiated cell functions. The homeostatic [Ca$^{2+}$] is essential for the normal exocytotic processes. For the altered extracellular conditions the AdH and PRLoma cells responded with the reduction of hormone release. The results showed that the intracellular Ca$^{2+}$ allocation derived from Ca$^{2+}$ pools (endoplasmic reticulum and mitochondria) resulted hormone release by the exposition of 0 mM [Ca$^{2+}$]. At the endpoint of the experiment the PRL and ACTH release were higher in PRLoma cultures by the effects of 0 mM [Ca$^{2+}$]. Our earlier studies suggest that apoptosis mechanisms may play role in this phenomenon [17].

The results showed reduced exocytotic activity by the effects of higher, but still hypocalcaemic extracellular ion milieu. The Ca$^{2+}$ influx via CaV is seemed not sufficient to the Ca$^{2+}$ message. The decreased intracellular Ca$^{2+}$ concentration may block the PLC activation. This mechanism leads to reduced IP3 and DAG conversion and then blocks the release of Ca$^{2+}$ from intracellular pools. Diminution of Ca$^{2+}$ may

inhibit the SNARE mediated fusion of hormone containing vesicles to plasma membrane.

## Conclusion

In conclusion, the main finding of this study is that a strict correlation exists among certain biophysical properties, especially extracellular Ca$^{2+}$ milieu and hormone vesicle exocytosis.

To better understand the relationship between thy hypocalcaemia and hormone release of PRLoma and normal AdH is essential, because PRL and ACTH hormones play crucial role in the adaptation processes of living organisms. Our results provide strong evidence that sustained lack of Ca$^{2+}$ inhibits the hormone release in both cell types.

**Declaration of Interest**

The authors report no declarations of interest.

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