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Secretion of 23 kDa and glycosylated prolactin by rat pituitary cell culture in serum-free media: a comparative morphological, cyto- and immunochemical study

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The secretion of 23 kDa prolactin by rat pituitary cells has been thoroughly investigated, but secretion of glycosylated rat prolactin is not currently known. This is mainly due to the lack of an antiserum which is solely specific for glycosylated rat prolactin and therefore we studied the basal secretion of this variant by an indirect method. Rat pituitary cells were cultured in total culture medium and three different serum-free media (DMEM, keratinocyte-serum-free medium, protein-free hybridoma medium) and secretion of 23 kDa and glycosylated rat prolactin was recorded by radioactive techniques and immunoblotting. The pituitary cell quality was monitored by electron microscopy, cell activation-and cell death assessment. In short-range culture (2 days) the pituitary cell quality and behaviour was very good and comparable in total culture medium, DMEM and keratinocyte-serum-free medium, i.e. numerous secretory granules, moderate amount of ER, cristae well in place in the mitochondriae. In medium-range culture (8 days) only cells cultured in total culture medium and DMEM presented a parallel behaviour: migration of cells toward each other, marked degranulation, massive array of ER. The inner membrane of the mitochondria was no longer folded into cristae leaving an unoccupied central space. At day 2 of the culture span secretion of 23 kDa rat prolactin was very comparable in all media used; hereafter, secretion of 23 kDa rat prolactin in total culture medium and DMEM assumed the well known pattern of peaking and slowing down, whereas in the other serum-free media it steadily decreased over the culture span. Pertaining to the important novel point of glycosylated rat prolactin secretion, it was low in comparison to the one of 23 kDa rat prolactin and it assumed a near steady pattern in all media used. 26 kDa rat prolactin was identified as the preferentially secreted glycoform, and the 23 kDa isoform as the major secretory product of rat pituitary lactotroph cells.

Keywords: glycosylated prolactin; rat; pituitary cell

Introduction

Recently (Bollengier *et al.*, 1993) in a series of experiments we demonstrated the systematical occurrence of an array of near M_r glycosylated rat prolactin (rPRL) variants in secretory granules and microsomal vesicles, and we postulated that glycosylated rPRL is biosynthesized as a pool of proteins with a different degree of glycosylation. Our data also suggested that selection of definite molecular variants from this pool could perhaps target the glycosylated forms of rPRL to specific sites of action. Glycosylation might also control hormone storage, secretion and trafficking. In humans, glycosylated PRL is a circulating variant in serum, and under certain conditions such as pregnancy and lactation, more of the non-glycosylated PRL may be produced to fill special requirements (Markoff & Lee, 1987; Markoff *et al.*, 1988); however, there is no unanimity to whether glycosylated PRL is a major circulating variant (Brue *et al.*, 1992). Also, variable amounts of glycosylated PRL are secreted by human prolactinomas in culture (Pellegrini *et al.*, 1988), the non-glycosylated PRL isoforms being the main variant (Hoffmann *et al.*, 1992). The secretion of 23 kDa rPRL has been thoroughly investigated (Ben-Jonathan *et al.*, 1989), but to our knowledge almost nothing is known about the secretion of glycosylated rPRL. The major problem in assessing secretion of glycosylated and non-glycosylated rPRL is the lack of an antiserum solely specific for this variant, and consequently there is no RIA available which determines only this variant. In this report we tried to gain a first available insight in the secretion of glycosylated rPRL, by devising an indirect way: we investigated the basal secretion of glycosylated and non-glycosylated rPRL by rat pituitary cells in total culture medium (TCM) and serum-free media, using immunoblotting and radioimmunological methods. In parallel, the pituitary cell quality was monitored at every step by electron microscopy (EM) and cell activation-and death assessment.

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Results

Pituitary cells were incubated (10.10^6 cells/Petri dish) in TCM for 2 days and then further cultured either in TCM (control) or three different serum-free media, i.e. DMEM, keratinocyte-serum-free medium (keratinocyte-SFM) and protein-free hybridoma medium (PFHM) as described in section Materials and methods. Culture medium was harvested at regular intervals over 8 days.

At each harvest, on the one hand cells were collected by centrifugation in small clumps, which were processed for EM, and on the other hand their viability was assessed in terms of cellular activity by the MTT assay. In both TCM and DMEM cells at first harvest were markedly granulated and the areas of cytoplasm

which were not occupied by secretory granules were characterized by a moderate amount of ER (Figure 1a,b). At this restive state of the pituitary cell, the cristae in the mitochondria were well in position (Figure 1c). After 6 days in culture, the appearance of the cell was completely different: there was a marked degranulation, migration of cells towards each other (Figure 2a,b), massive arrays of parallel and occasionally concentric ER (Figure 3a,b). In the mitochondria the inner membrane was not folded into cristae, leaving an unoccupied central space (Figure 2c).

Also at first harvest, pituitary cells in keratinocyte-SFM were clearly comparable in EM to cells dispersed in TCM and DMEM. However, after 6 days of culture vacuolisation and dilatation in the ER was observed and the cells did not migrate towards each other, as was the case for cells in TCM and DMEM (Figure 4). The appearance of the mitochondria did not vary much in the course of the culture: i.e. a mixture of mitochondria with cristae well in place and with an unoccupied central space.

Pituitary cells in PFHM were characterized at first harvest by an already massive array of ER and marked dilatation; this appearance remained fairly constant throughout the culture schedule (not shown).

To assess the viability and activation of the pituitary cells, we used the reliable MTT assay which depends on the reduction by living cells of the tetrazolium salt MTT into a blue coloured product (formazan) by the mitochondrial enzyme succinate-dehydrogenase. The conversion takes place only in living cells. This method can therefore be used to measure cytotoxicity, and/or proliferation and activation of cells (Mosmann, 1983).

MTT-median values (each point represents the median) and significant differences with TCM, considered as control, are reported in Figure 5. Intra-assay variation per value of the triplicate was between 3% and 9% and the interassay variation for each triplicate ranged from 2% to 10%.

It is clear that MTT values of all the serum-free media differed from the control ones. For the first 48 h pituitary cells scored better in keratinocyte-SFM than in DMEM and PFHM. Thereafter, only cells in DMEM paralleled these in TCM in the sense that the mitochondrial activity steadily increased.

Viability of the cells was also measured with the trypan blue exclusion test (Shapiro, 1988) at stages 0 and 2 days of the culture span; at stages 4-6-8 days, obstinate clumping of the cells prevented correct measures. For all media concerned, at day 0 98.6% of viable cells, and at day 2 95% were counted, which corroborates MTT-tests at that level.

Figures 6 and 7 respectively represent secreted rPRL estimated by RIA, and the repartition of 23 kDa and glycosylated rPRL. The relative rPRL percentages were estimated on the basis of the major rPRL isoforms, i.e. 20, 23, 40–42 kDa and glycosylated rPRL by autoradiographic overlay (Bollengier *et al.*, 1993). Correction for possible competitive cross-reaction with 20 kDa growth hormone (GH) was made by immunoreacting blots with on the one hand anti-rGH, and on the other hand anti-rPRL absorbed with GH.

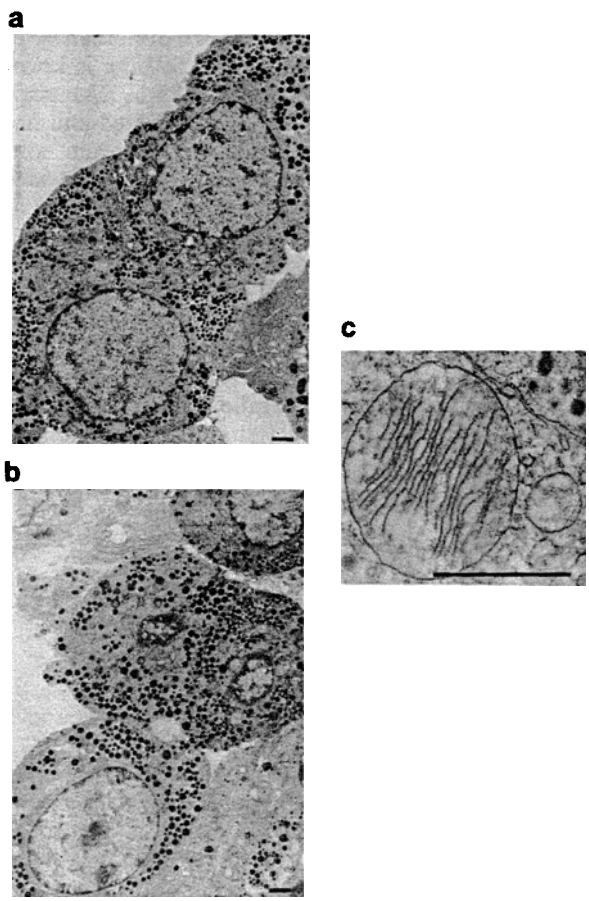

Figure 1 Electron microscopy of (**a**) pituitary cells cultured in TCM at first harvest (× 3 000). (**b**) pituitary cells cultured in DMEM at first harvest (× 3 000). (**c**) mitochondrion with cristae well in place (× 22 000). bar = 1 μm

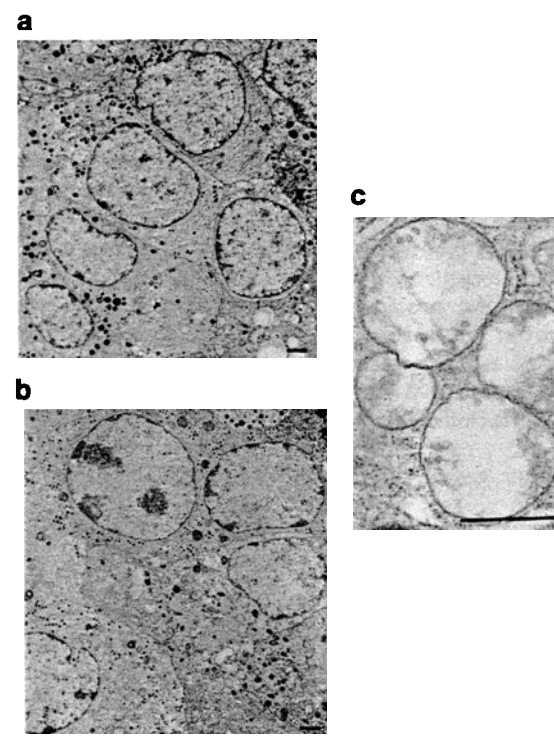

Figure 2 Electron microscopy of (**a**) pituitary cells cultured in TCM at third harvest (× 3 000). (**b**) pituitary cells cultured in DMEM at third harvest (× 3 000). (**c**) mitochondria with unoccupied central space (× 17 000). bar = 1 μm

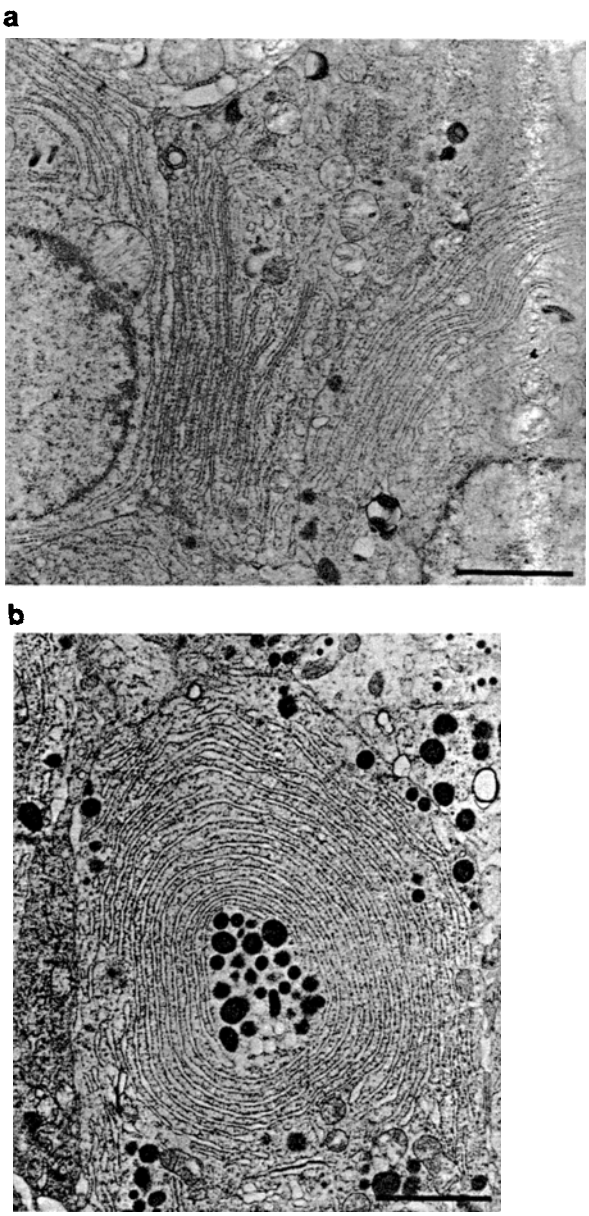

Figure 3 Electron microscopy of (**a**) pituitary cells cultured in TCM: massive arrays of parallel ER (× 13 000). (**b**) pituitary cells cultured in DMEM: concentric ER (× 13 000). bar = 1 μm

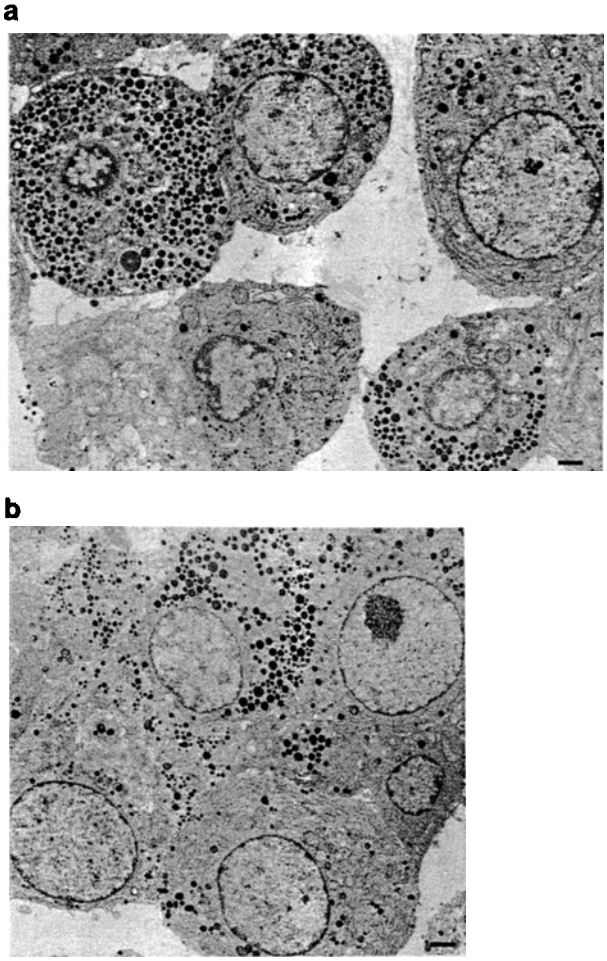

Figure 4 Electron microscopy of (**a**) pituitary cells cultured in Keratinocyte-SFM at first harvest (× 3 000). (**b**) pituitary cells cultured in Keratinocyte-SFM at third harvest (× 3 000). bar = 1 μm

Figure 8 represents a qualitative immunoblot of rPRL isoforms secreted by pituitary cells, clearly indicating 23 kDa and glycosylated rPRL. At the first harvest (day 2) total rPRL reached almost identical levels for all media under investigation, but then steadily decreased in keratinocyte-SFM and PFHM. In DMEM, maximal rPRL secretion was reached at the second harvest (day 4), whereas in TCM at third harvest (day 6). At the fourth harvest (day 8) both were declining.

From Figure 7 it is clear that glycosylated rPRL secretion is very low in comparison to the one of 23 kDa rPRL in the three media under investigation; one also notices that in both DMEM and keratinocyte-SFM secretion of glycosylated rPRL is relatively steady, this in contrast to 23 kDa rPRL secretion. Finally, after 8-days-culture in the different media, cells were extracted, the supernatants assayed for total protein content, for total rPRL in RIA and submitted to immunoblotting and autoradiography. In this experiment we were able to estimate the 23 kDa and glycosylated rPRL content and the relative distribution of both isoforms also for cells cultured in TCM, since the extraction buffer did not contain high concentrations of either FCS or BSA. Median values and significant differences for the assessed parameters are reported in Table 1.

For most of these parameters significant differences were observed between cells cultured in TCM and in serum-free media. However, it is clear that for all media under investigation, the dominant residual cellular isoform is 23 kDa rPRL. A striking fact was the high PRL content in regard to total protein content for pituitary cells cultured in TCM, keratinocyte-SFM, and especially in DMEM. From Table 1 it became also clear that pituitary cells cultured in PFHM scored badly at a global level.

Discussion

When addressing the phenomenon of rPRL secretion, particularly so of glycosylated rPRL, one should bear in mind two points: (1) there is no antiserum available that solely recognizes glycosylated rPRL and (2)

glycosylated rPRL is less precipitating in RIA than 23 kDa rPRL, presumably due to masking of epitopes by the oligosaccharide chain. Notwithstanding these considerations, we think that the approach we used, allowed us to gain a fairly reasonable first insight in the secretion of glycosylated PRL by rat pituitary cells in culture. As formerly described (Bollengier *et al.*, 1993), we measured total rPRL by RIA in all fractions under investigation, and taking advantage of the M_r difference between 23 kDa and glycosylated rPRL, subjected them to immunoblotting and autoradiographic overlay to estimate the relative distribution of 23 kDa

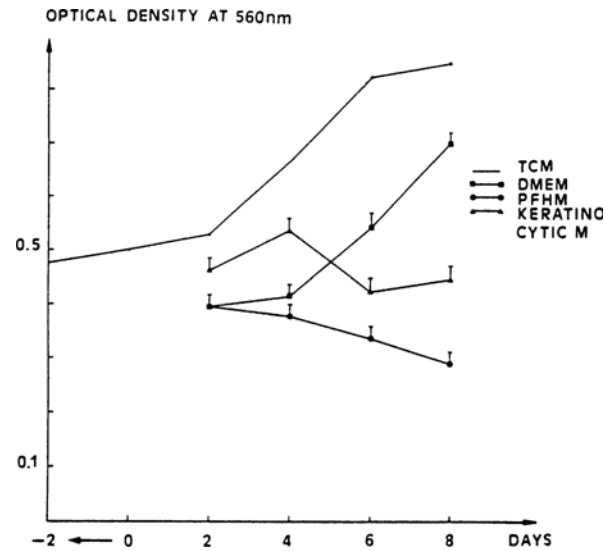

Figure 5 MTT median values of pituitary cells cultured in different media. Culture span: 8 days, four harvests. Day 0: start of culture in serum-free media. T: serum-free media differ from TCM for $2\alpha \leqslant 0.05$ ($n = 18$). −2←0: 48 h culture in TCM

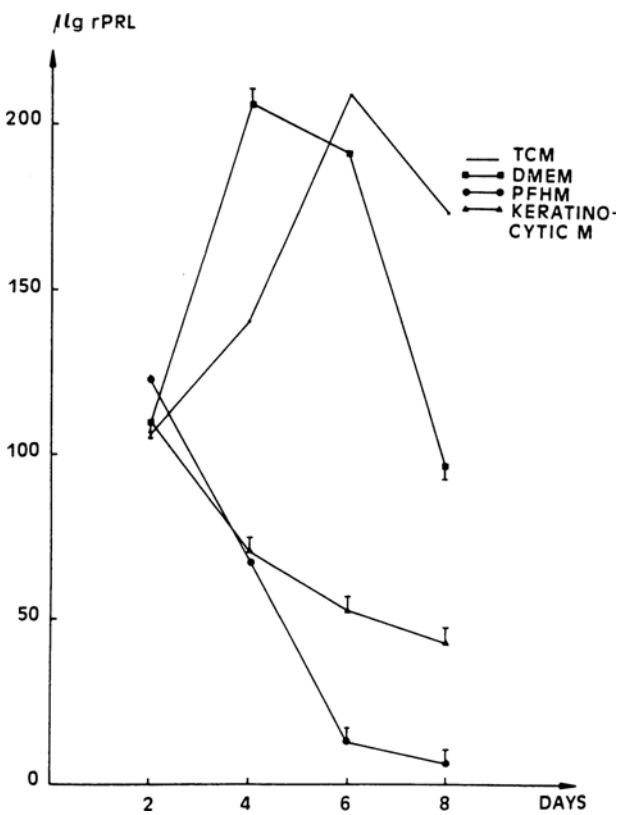

Figure 6 rPRL (median values) secreted by pituitary cells cultured in different media. Culture span: 8 days, four harvests. Day 0: start of culture in serum-free media. T: serum-free media differ from TCM for $2\alpha \leqslant 0.05$ ($n = 6$)

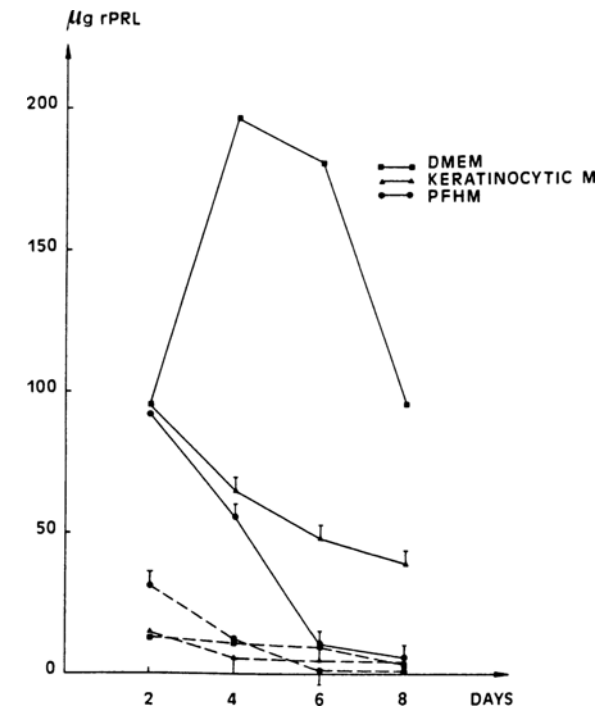

Figure 7 Repartition of 23 kDa and glycosylated rPRL secreted by pituitary cells cultured in different media. Culture span: 8 days, four harvests. Day 0: start of culture in serum-free media. Single line: 23 kDa rPRL. Dashed line: glycosylated rPRL. T: Keratinocyte-SFM and PFHM differ from DMEM for $2\alpha \leqslant 0.05$ ($n = 6$)

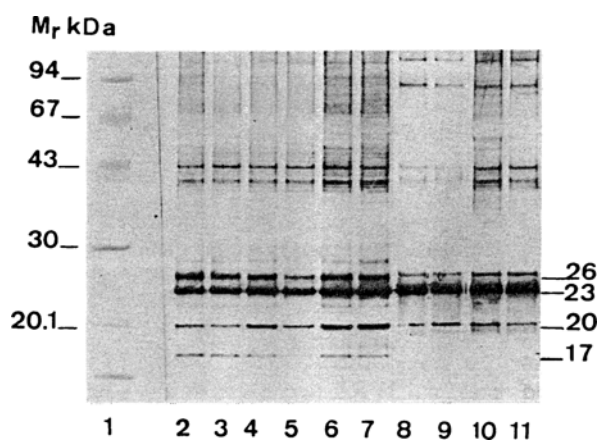

Figure 8 Qualitative immunoblot developed with anti-rPRL antiserum. Lane 1: molecular weight markers; lane 2: pituitary cell extract in TCM at day 2; lane 3: pituitary cell extract in TCM at day 8; lane 4: pituitary cell extract in DMEM at day 2; lane 5: pituitary cell extract in DMEM at day 8; lane 6: pituitary cell extract in keratinocyte-SFM at day 2; lane 7: pituitary cell extract in keratinocyte-SFM at day 8; lane 8: secretion DMEM at day 2; lane 9: secretion DMEM at day 8; lane 10: secretion keratinocyte-SFM at day 2; lane 11: secretion keratinocyte-SFM at day 8. rPRL molecular variants: glycosylated rPRL (26 kDa); monomeric rPRL (23 kDa); cleaved rPRL (17 kDa)

Table 1 Median values ($n = 6$) and significant differences for different parameters assayed in pituitary cell extracts after 8 days culture in different media

	µg rPRL[a]	*µg tot. prot.*	*% 23 kDa rPRL*	*% glyc. rPRL*	*µg rPRL/µg tot. prot.*
TCM	111.1	830.1	31.1	21.0	0.13
DMEM	93.7[b]	433.2[c]	29.3	13.8[c]	0.19[c]
Keratinocyte-SFM	44.7[c]	438.3[c]	22.7[c]	13.5[c]	0.11[b]
PFHM	12.1[d]	393.3[c]	33.8	14.8[c]	0.03[d]

[a]per 10.10^6 cells. [b]serum-free media differ from TCM (control) for $2a = 0.1$; [c]serum-free media differ from TCM (control) for $2a = 0.05$; [d]serum-free media differ from TCM (control) for $2\alpha = 0.02$

and glycosylated rPRL. However, immunoblotting of good and measurable quality of TCM is almost impossible to obtain, because the high albumin concentration in FCS causes considerable distortion and overloading, especially when the media have to be concentrated. Consequently, the rationale is to use serum-free medium. In addition, several serum-free media were selected, so as to allow a comparative appraisal of rPRL glycoform secretion.

The rationale of adding the supplements to the serum-free media, as reported in section Materials and methods, has been fully described by Romijn *et al.* (1984). Briefly resumed, albumin at low concentrations was chosen to stabilize pH, to serve as a buffer for keeping the concentrations of unbound/free fractions within physiological range, thus playing a detoxifying role; transferrin for its role of carrying iron to the cells, of binding and inactivating trace elements of toxic metal. Insulin is known to exert a general anabolic effect on cell metabolism and selenium plays a key-role in the cellular defence mechanism against free radical formation; it is generally agreed that oxygen-derived free radicals formed in the course of intracellular metabolisms are extremely harmful to the living cell (damage to cell membrane, mitochondria, ER, etc.).

The general ultrastructure of isolated rat pituitary cells cultured in respectively TCM and DMEM after 4–6 days culture is in good agreement with the cells actively synthesizing and secreting hormones (Farquhar, 1971, 1977; Thorpe & Wallis, 1991). In the majority of cells the cytoplasm becomes degranulated and filled with extensive ER. Degranulation is the privilege of very active cells, secreting their content as rapidly as they synthesize it (Pasteels, 1961, 1963).

As reported under Results, at the beginning of the culture, mitochondria were characterized by numerous cristae (Figure 1c). At 6 days culture, mitochondria were markedly characterized by an unoccupied central space (Figure 2c). MTT assays are in good agreement with these findings in the sense that MTT values steadily increased during the culture span, i.e. from resting to active state of the pituitary cells. The mitochondrial enzyme succinate-dehydrogenase is associated with the inner membrane of the mitochondria and mitochondria undergo dramatic changes when they switch from a resting state to a respiring state. In the respiring state, the inner membrane is not folded into cristae; it seems to leave a more voluminous space (Rawn, 1989).

In PFHM, pituitary cells behaved in a completely different way: there was practically no evolution in the EM appearance of the cells (few granulation, massive ER with marked dilatation) throughout the course of the culture experiment, and MTT values were not indicative neither of good viability nor passage from a resting state to a highly active state. Indeed, secretion of rPRL, as discussed below, residual rPRL and the ratio of PRL content versus total protein content in cellular extracts (Table 1) were very low in cells cultured in this medium.

In the investigation of total rPRL pituitary cell secretion, measured by RIA and consequently not hindered by high concentrations of serum components, we were able to compare the different serum-free media with TCM as control. *In vivo*, basal levels of PRL secretion are controlled by hypothalamic/dopaminergic inhibition; *in vitro*, withdrawal of this hypothalamic influence enhances PRL synthesis and release. For cells cultured in TCM and DMEM, the parallel behaviour already observed in EM and MTT assays was confirmed: hormone secretion steadily increased over approximately 5 days. This stood in marked contrast to the secretion behaviour in keratinocyte-SFM and PFHM; although starting from much the same level at day 2, secretion of rPRL reached no peak, but continuously decreased during the course of the culture. As far as PFHM is concerned, we were not surprised by the downward secretion pattern, since it confirmed the morphological and cytochemical findings (Table 1), i.e. decline of the cellular machinery after two days in culture. For pituitary cells cultured in keratinocyte-SFM, this secretion pattern does not entirely fit with the cellular findings; since the morphology and the MTT activity of cells cultured in this medium evolved without important changes (Figures 4–5), we expected a more constant secretion.

Interestingly, in TCM, DMEM and keratinocyte-SFM, we noticed relocation of the ER to the periphery of the cell, fewer granules adjacent to the cell membrane and granules entrapped in concentric ER (Figure 3a,b). The parallel stacks of ER adjacent to the cell membrane appear to form a barrier that perhaps could prevent granules from reading the cell membrane for release by exocytosis, and hence inhibition of secretion. The residual elevated PRL content in cells cultured for 8 days in TCM, DMEM and keratinocyte-SFM (up to 19% of total proteins, see Table 1) reinforces this finding: apparently biosynthesis of PRL still goes on in the lactotrophs while secretion is inhibited.

Pertaining to the principal aim of this investigation, i.e. the secretion of glycosylated rPRL by pituitary cells, it is low, not only in absolute values, but also in comparison with the one of 23 kDa rPRL.

In a recent report (Bollengier *et al.*, 1993), we showed that in purified secretory granules, taking into account the major rPRL isoforms, the repartition of 23 kDa rPRL versus glycosylated rPRL was largely in favour of 23 kDa rPRL, which means that already at the start there is much less glycosylated rPRL in store in secretory granules. We also showed by differential

centrifugation that the majority of glycosylated rPRL in the pituitary is stored in secretory granules, preferentially in the membrane-bound state.

Interesting observations are on the one hand the contrasting secretion pattern of 23 kDa and glycosylated rPRL, and on the other hand the nature of the secreted rPRL glycoforms. In function of the medium used, 23 kDa rPRL secretion considerably varied, whereas secretion of glycosylated rPRL assumed a much more stable pattern. In all media investigated, glycosylated rPRL of M_r ~26 kDa (Figure 8) was preferentially released out of the pool of available rPRL glycoforms, we recently observed in secretory granules and microsomes from pituitary cells (Bollengier *et al.*, 1993); earlier on, we also reported that 26 kDa rPRL possesses several sialyl residues (Bollengier *et al.*, 1991).

As yet we do not understand why 26 kDa rPRL is selectively secreted, but a comparable phenomenon has been observed *in vivo* for human (h) TSH by Papandreou *et al.* (1993): intrapituitary hTSH was more retained on Con A and less sialylated than circulating hormone. The suggestion arose that carbohydrate chains of intrapituitary molecules are less mature than these present in the circulation. The above reported findings reinforce this hypothesis and it could thus well be that regulation of terminal glycosylation has an effect on hormone secretion; in the case of PRL, glycosylation could act as a signal to the lactotrophs whether they secrete 23 kDa rPRL or not. If this is indeed the case, our data suggest that little glycosylated rPRL is required to fulfill this role.

In all fairness, one also has to consider the influence of buffer and medium supplements on the degree of glycosylation of proteins synthesized by cells cultured in serum-free medium. Megaw & Johnson (1979) have observed that the level of glycosylation of proteins *in vitro* is dependent on the medium composition, and recently in a review article Goochee & Monica (1990) have reported that acidotropic amines have disruptive effects on oligosaccharides. But since pituitary cells cultured in three basically different serum-free media, all preferentially secreted the 26 kDa rPRL glycoform, it is likely that this is a spontaneous biological phenomenon, rather than being induced by the experimental conditions.

Although this report focuses on the secretion of glycosylated rPRL, we should like to comment on another interesting feature of the obtained results: 17 kDa rPRL, formerly identified by Mittra (1980) as cleaved PRL, was systematically recorded intracellularly, but not secreted in the culture media used (Figure 8). According to Mittra (1980) cleaved rPRL was clearly detected in fresh pituitary glands and in unlabeled culture media. Thus our results do not confirm these findings; this could be due to technical reasons, i.e. the use of dissimilar culture media and different detection methods, fluorography (Mittra, 1980) *vs* immunoblotting. In any case, this means that 17 kDa rPRL is a fragile PRL isoform, since dependent on the culture medium, and secreted in extremely low amounts in basal conditions, since only detected in fluorography. Moreover, Mittra (1980) showed that cleaved rPRL significantly increased the rate of DNA synthesis and cell division in mammary epithelial cells. It is tempting to assign the extreme low secretion of 17 kDa rPRL to a normal division of mammary epithelial cells, and increased levels to a pathological one.

Taken together, our findings allow to conclude that culture of rat pituitary cells in serum-free media has allowed us (1) to gain a first reliable insight in the till now unraveled secretion of glycosylated rPRL (2) to identify respectively 26 kDa rPRL as the preferentially secreted glycoform and 23 kDa rPRL as the major secretory product of rat pituitary lactotroph cells, as is the case for human, ovine and porcine PRL (Ronin, 1992), (3) to recommend keratinocyte-SFM as the most satisfactory serum-free medium in terms of the assessed parameters, in short range secretion studies (up to 2 days). However, this does not mean that an antiserum that solely recognizes glycosylated rPRL and of good immunoprecipitating quality is no longer needed. Indeed, one must always bear in mind that the polyclonal anti-rPRL actually available, and classically used for determination of total rPRL does not equally recognize the different rPRL variants (Löhrke *et al.*, 1993).

Materials and methods

Anterior pituitaries from adult nulliparous Wistar rats (KUL Proefdierencentrum, Leuven, Belgium) at random cycle stages were used throughout the experiments. They were processed as formerly described (Bollengier *et al.*, 1989), i.e. minced and enzymatically and mechanically dissociated. The obtained cell suspension was filtered throughout nylon gauze, washed and centrifuged. Cells were then grown in uncoated Petri dishes for 48 h in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), TES, penicillin and streptomycin. Fungizone (125 ng/ml), gentamycin (50 μg/ml) and nystatin (25 U/ml) were further added. This medium was identified as total culture medium (TCM) because of the presence of 10% (w/v) foetal calf serum (FCS).

Then the cells were depleted of TCM and further cultured over 8 days in three different serum-free media: DMEM, Keratinocyte-SFM (Life Technologies Ltd, Oaisley, Scotland) and Protein-free Hybridoma Medium (PFHM II) (Life Technologies), all three supplemented with the following compounds: NaSe (Sigma Chemie, Bornem, Belgium) (5 ng/ml), transferrin (Calbiochem, San Diego, Ca, USA) (6 μg/ml), insulin (Sigma) (6 μg/ml), triiodo-L-thyronine (Sigma) (2 ng/ml), albumin (Serva, Heidelberg, Germany) (0.001% w/v) and the same antibiotics as for TCM. No attachment to the substratum was favoured. Each medium was assessed, in duplicate, three times. Culture medium was harvested and replenished with fresh medium every 2 days. After the final harvest of medium, cells were extracted as previously described (Bollengier *et al.*, 1989). Proteins in culture media were precipitated with ice-cold acetone, overnight at −20°C, and the precipitates taken up in the appropriate buffer. Cell extracts and culture media were either immediately assayed or stored at −80°C (for 2 weeks).

Preparation of the cells for electron microscopy (EM)

Pituitary cells were collected at different times of harvest and taken up and fixed in (sodium)cacodylate (Sigma) (0.1 M), pH 7.3–2.5% (v/v) glutaraldehyde (Merck, Darmstadt, Germany). The fixed cells were centrifuged and rinsed in cacodylate (0.1 M), pH 7.3–1% (w/v) $CaCl_2$. The cell pellet was postfixed in 1% (w/v) OsO_4 for 1 h, dehydrated in alcohol and embedded in Epon as routinely done. 2 μm sections were used for selecting lightmicroscopically the areas

for EM. Ultrathin sections (60 nm) were cut with a diamond knife and poststained with uranyl acetate and lead citrate. Representative micrographs were taken at different positions in the ultrathin sections.

MTT colorimetric assay

Was carried out according to Denizot & Lang (1966). Briefly resumed, the assay was carried out in Falcon 96-well micro test III flat bottomed microtitre trays (Becton-Dickinson, USA). To the cells in each well 50 µl of a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) was added. After a 3 h-incubation at 37°C, the plate was centrifuged and the untransformed MTT removed. Propranol was added to each well and the plate vigorously shaken in order to ensure the solubilization of the blue formazan. The optical density of each well was measured using an automatic plate reader (Molecular Dynamics, Ca, USA) at 560 nm wavelength. Each culture medium was done in triplicate, and each triplicate over six rows.

Trypan blue exclusion test for cell viability

Was determined according to Shapiro (1988).

Vertical SDS–PAGE and transfer electrophoresis

To either nitrocellulose or PVDF (Biorad, Nazareth, Belgium) followed by immunochemical staining (immunoblotting) were performed as previously described (Bollengier *et al.*, 1989). Briefly resumed, vertical SDS–PAGE was carried out in 10–20% gradient gels. The sample buffer was Tris (0.01 M)–EDTA (0.001 M)–1% SDS, pH 8, the gel buffer was Tris (0.33 M), pH 8.6. Immediately after gradient electrophoresis, proteins were transferred to either nitrocellulose or PVDF. After transfer, the sheets were immunochemically developed with the appropriate antisera, by using an alkaline phosphatase-conjugated antibody as secondary antibody.

Identification and estimation of proteins by autoradiography

Was accomplished as recently reported (Bollengier *et al.*, 1993). In short, after incubation with the appropriate antiserum the transfer membrane was incubated with [^{35}S]-protein A (Amersham–SJ444). The sheets were dried and exposed to autoradiographic film (Kodak film Ortho GX ray). ^{14}C-labelled Rainbow™ reference Molecular Weight Markers (Amersham CFA756) ranging from M_r 14.3–200 kDa were included. After development of the film, the bands of interest on the membrane were cut out, placed in vials, dissolved in scintillation solvent and counted. To calibrate the procedure, purified PRL standards were included in each blot. Additional areas of the blot, where no visible bands were present, were taken to determine the background radioactivity.

Hormone assay

PRL content was estimated by double antibody RIA, using NIADDK rPRL I-6 for iodination, NIADDK rPRL-RP 3 for cold standard and rabbit polyclonal rPRL antibody 6-10/90 (F. Bollengier); the assays were run according to the NIDDK protocol. rPRL antibody 6-10/90 was used at a final dilution of 1/57 500 and has higher specificity in terms of cross-reactivity with anterior pituitary hormones other than rPRL, compared to NIDDK anti-rPRL-S9. Samples were diluted to the optimal detectability level of 0.25–50 ng/ml. Intra- and interassay coefficients of variation were less than 6 and 8% respectively.

Protein determination

Protein was determined according to Lowry *et al.* (1951).

Statistics

The statistical method used to compare the parameters resulting from pituitary cell culture in the different media was the non-parametric U-test of Wilcoxon (Mann-Whitney) (Beyer, 1986). This form of analysis was used because hormonal secretion from a cell population is not normally distributed, nor is the population response to factors that modulate hormone secretion.

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