Exocytotic protein components in rat pituitary gland after long-term estrogen administration

G Majó, M J Lorenzo¹, J Blasi and F Aguado

Laboratori de Neurobiologia Cel·lular i Molecular, Departament de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Universitat de Barcelona; Hospital de Bellvitge, Feixa Llarga s/n, L'Hospitalet de Llobregat 08907, Barcelona, Spain

¹Departamento de Bioquímica y Biología Molecular, Universidad de Alcalá, Alcalá de Henares, Spain

(Requests for offprints should be addressed to F Aguado, Departament de Biologia Cel·lular i Anatomia Patològica, Campus de Bellvitge, Facultat de Medicina, Universitat de Barcelona, c/Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat, Spain)

(F Aguado is now at Departament de Biologia Cel·lular Animal i Vegetal, Facultat de Biologia, Av. Diagonal 645, 08071 Barcelona, Spain)

Abstract

Recently, a set of proteins involved in the docking and fusion machinery of secretory organelles has been identified in anterior pituitary cells. In this study we analyzed, by Western blotting and immunocytochemistry, the expression of several proteins involved in exocytosis after long-term administration of 17 β -estradiol (E₂) in Fischer 344 rats. No differences were observed in the amount of synaptosomal-associated protein of 25 kDa, synaptobrevin 2, syntaxin 1, synaptotagmin I and Rab3a in total brain homogenates from treated rats after E₂ administration. In striking contrast, the levels of all of these exocytotic

proteins, including cellubrevin, were notably decreased in pituitary glands of E₂-treated rats. In addition, no differences were observed in the *in vitro* basal and 8-Br-cAMP-induced prolactin (PRL) release between pituitary cells from control and E₂-treated rats, whereas TRH-induced PRL release in anterior pituitary cells from E₂-treated animals was higher than in control donors. In conclusion, this study shows that protein components of the exocytotic machinery are specifically down-regulated in the pituitary gland of E₂-treated Fischer 344 rats.

Journal of Endocrinology (1999) **161**, 323–331

Introduction

In regulated exocytosis, secretory vesicles fuse with the plasma membrane in response to a physiological stimulus. Neuronal and endocrine cells are highly specialized cells that release their chemical signals for intercellular communication by regulated exocytosis. Membrane depolarization or the action of a secretagogue usually induces a transient rise in cytoplasmic calcium concentration, which triggers exocytosis (Burgoyne & Morgan 1993).

Insights have recently been made into the molecular mechanisms underlying exocytosis in neuronal cells. A set of proteins involved in the docking and fusion of the two neuronal secretory organelles (synaptic vesicles and large dense-cored vesicles) has been identified. Some of these synaptic proteins, like synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin, are located at the plasma membrane, whereas others, such as synaptobrevin, synaptophysin, Rab3A, synapsin and synaptotagmin, are located on synaptic and large dense-cored vesicle membranes (for review see Südhof 1995, Linial & Parnas 1996). In addition, the soluble proteins N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment protein (SNAPs) are essential for membrane trafficking (Rothman 1994). According to the SNAP receptors (SNARE)

hypothesis, a suitable interaction between plasma membrane proteins (t-SNAREs: SNAP-25 and syntaxin) and vesicular membrane proteins (v-SNAREs: synaptobrevin) with the soluble factors (NSF and SNAPs) is essential for the initiation of the fusion process (Söllner *et al.* 1993a,b, Rothman 1994). Moreover, the identification of isoform families of most synaptic proteins, including non-neuronal isoforms (e.g. cellubrevin as a synaptobrevin isoform), provides a further level of complexity (Linial 1997).

Many of the above proteins were originally identified as neuronal-specific components due to their abundant expression in nervous tissues. However, a functional role for these proteins and their non-neuronal isoforms has been proposed in different endocrine cell types (Lang *et al.* 1997, Morgan & Burgoyne 1997). These molecular components of the exocytotic machinery have also been identified in anterior pituitary cells (Wendland & Scheller 1994, Marquéze *et al.* 1995, Oho *et al.* 1995, Redecker *et al.* 1995, Aguado *et al.* 1996, Jacobsson & Meister 1996, Majó *et al.* 1998). In adenohypophyseal cells, t-SNAREs are located at the plasma membrane, and protein components of neuronal secretory organelle membranes are located on hormone-containing secretory granules and synaptic-like microvesicles (SLMV) (Wendland & Scheller 1994, Aguado *et al.* 1996, Jacobsson & Meister 1996, Majó

Table 1 Primary antibodies used in this study

Antibody to	Raised in	Code	Work dilution	Source and/or reference
PRL	Rabbit	AFP425-10-91	1:3000	A F Parlow, NIDDK
GH	Rabbit	AFP4115	1:3000	A F Parlow, NIDDK
βLH	Rabbit	AFP22238790	1:3000	A F Parlow, NIDDK
α-tubulin	Mouse	T9026	1:2000	Sigma
SNAP-25	Mouse	SMI-81	1:1000	Sternberger Monocl. Inc. (Baltimore, MA, USA)
Synaptobrevin 2	Rabbit	18.2	1:2000	R Jahn (Max-Plank Institute, Gottingen, Germany), Edelman <i>et al.</i> (1995)
Cellubrevin*	Rabbit	CB35	1:200	Majó <i>et al.</i> (1998)
Synaptotagmin I	Mouse	c1.41.1	1:3000	R Jahn, Brose <i>et al.</i> (1992)
Syntaxin 1	Mouse	HPC-1	1:2000	Barnstable <i>et al.</i> (1985)
Rab3A	Mouse	c1.42.2	1:1000	R Jahn, Matteoli <i>et al.</i> (1991)
Rab 5	Mouse	c1.621.2	1:500	R Jahn, Fischer-von-Mollard <i>et al.</i> (1994)
Na ⁺ /K ⁺ -ATPase	Rabbit	06-167	1:1000	Upstate Biotech. Inc. (Lake Placid, NY, USA)
Synaptophysin	Mouse	S5768	1:2000	Sigma

*The antibody raised against cellubrevin also recognizes its homolog synaptobrevin.

et al. 1998). It is thought that secretory granules are the counterpart of neuronal large dense-cored vesicles and SLMV of synaptic vesicles (Jahn & De Camilli 1991). Furthermore, the functional participation of some of these components in pituitary hormone exocytosis has been demonstrated. It has been shown that SNAP-25 is essential in the regulated release of adrenocorticotropin (ACTH) and prolactin (PRL) from AtT-20 and GH3 cell lines respectively (Aguado *et al.* 1997, Masumoto *et al.* 1997). In addition, different members of the Rab family are involved in secretory granule localization and calcium-induced exocytosis in anterior pituitary cells (Lledo *et al.* 1993, Nsee *et al.* 1993, Lledo *et al.* 1994, Perez *et al.* 1994).

It has been shown that SNAP-25 is overexpressed in human PRL cell adenomas (Majó *et al.* 1997). Since prolactinomas represent the most commonly occurring type of pituitary tumor in humans (Kovacs *et al.* 1977), it is important to study the possible regulation and functionality of the exocytotic molecular components in PRL-secreting tumor cells. The aim of this study was to analyze, by Western blotting and immunocytochemistry, the expression of several proteins involved in exocytosis during PRL-cell tumorigenesis induced by long-term 17β-estradiol (E₂) administration in Fischer 344 (F344) rats. In addition, we also compared the PRL secretion rates of control and E₂-treated pituitary cells.

Materials and Methods

Animals

Adult F344 female rats weighing 130–150 g (Criffa S.A., Barcelona, Spain) were housed in a controlled environment (22 °C; 12 h light/12 h darkness; 40–60% humidity) with food and water available *ad libitum*. Under deep

anesthesia (ketamine hydrochloride 20 mg/kg, Parke-Davis, Morris Plains, NJ, USA) 30 animals were ovariectomized bilaterally and implanted subcutaneously with 1-cm silastic capsules (Dow-Corning (Midland, MI, USA); outside diameter, 3·125 mm; inside diameter, 1·875 mm) containing 17β-estradiol (Sigma, St Louis, MO, USA). Thirty-five ovariectomized control rats were implanted with blank silastic capsules. Animal surgery and care were carried out in accordance with the European Community Council Directive (86/609/EEC). Rats were killed at different times from one week up to seven weeks after treatment.

Western blotting analysis

The rats were decapitated and the pituitaries were excised. After removal of the neurointermediate lobe, the anterior pituitaries were homogenized with 10 mM HEPES pH 7·4, 1 mM EGTA, and 0·5 mM phenylmethylsulfonyl fluoride (Sigma). Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (12·5% SDS-PAGE) was carried out as described by Laemmli (1970) using a mini-protean system (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were electrotransferred to nitrocellulose membranes (Amersham Iberica, Madrid, Spain) according to Towbin *et al.* (1979). The membranes were blocked in a solution consisting of 5% non-fatty milk powder in TBS (140 mM NaCl, 50 mM Tris-HCl pH 7·4 with 0·1% Tween-20) for 1 h at room temperature and then incubated at 4 °C overnight with the appropriate primary antibody diluted in blocking buffer. The primary antibodies used are shown in Table 1. After several washes with blocking solution, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody, and peroxidase activity was detected using the enhanced chemiluminescent method (ECL, Amersham).

Immunocytochemistry

Under deep diethyl ether anesthesia, the rats were perfused through the heart with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The pituitaries were removed, post-fixed for 4 h in the same fixative solution and cryoprotected with 30% sucrose solution in 0.1 M phosphate buffer at 4 °C overnight. Sections (40 µm) were obtained with a cryostat and collected in PBS. Pituitary sections were soaked for 1 h in PBS containing 10% methanol and 3% H₂O₂, washed in PBS and preincubated for 1 h in 5% fetal calf serum in PBS containing 0.3% Triton X-100 and 0.2% gelatin. Overnight incubations with the primary antibodies indicated in Table 1 were carried out at 4 °C in PBS containing 1% fetal calf serum, 0.3% Triton X-100 and 0.2% gelatin. The immunocytochemical reaction was developed with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The peroxidase complex was visualized by incubating the sections with 0.05% diaminobenzidine and 0.01% H₂O₂ in PBS. Sections were mounted, dehydrated and coverslipped in DPX. The specificity of the immunoreaction was tested by omitting the primary antibody or by replacing it with an equivalent concentration of non-specific rabbit or mouse immunoglobulin G. No immunostaining was observed in these sections. Double-labeling immunocytochemistry was carried out in sections to detect different proteins and pituitary hormones simultaneously by a combined light microscopic procedure according to Levey *et al.* (1986). The immunostaining of SNAP-25 was performed using the monoclonal antibody SMI-81, as described above for single labeling studies. After further rinsing, sections were processed to reveal the presence of growth hormone (GH) or PRL using polyclonal antibodies to rat hormones (1:1000). The immunocytochemical reaction, after peroxidase-anti-peroxidase procedures was developed in a medium containing 0.01% benzidine dihydrochloride (Sigma) and 0.025% sodium nitroprusside (Merck, Darmstadt, Germany) in PBS pH 6 for 10 min, and then in the same solution containing 0.05% H₂O₂ until the signal developed. The double labeling was done both ways round with equivalent results.

Primary cell cultures and RIA for PRL

Short-term pituitary cell cultures were prepared according to a modification of the method of Ben-Jonathan *et al.* (1983) as described previously (Aguado *et al.* 1996). Seven weeks after surgery, control and treated ovariectomized animals were decapitated, the pituitary glands were removed and the neurointermediate lobes discarded. After rinsing in Krebs-Ringer's bicarbonate buffer without Ca²⁺ and Mg²⁺ (KRBGA), the tissues were cut into small pieces. The fragments were incubated in KRBGA containing 0.2% trypsin (Sigma) for 1 h in a metabolic

incubator at 37 °C under CO₂ and dissociated mechanically by gentle passage through a Pasteur pipette. The tissue suspension was harvested by centrifugation and incubated in KRBGA with DNAase (1 mg/ml; Sigma) for 5 min. Dispersed individual cells were harvested by centrifugation and resuspended at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies, Gaithersburg, MD, USA). Viability, as evaluated by Trypan Blue exclusion, was greater than 90%. Finally, 10⁵ cells/well were seeded in 24-well tissue culture plates (Costar, Acton, MA, USA) coated with poly-D-lysine (Sigma) to minimize cell loss, and cultured in a metabolic incubator at 37 °C with 5% CO₂ and 95% air for two days. Before the experiments, the cells were rinsed twice with serum-free Dulbecco's modified Eagle's medium and then incubated at 37 °C in 5% CO₂ in 0.5 ml of the same medium containing the appropriate test substances. After two hours incubation, conditioned medium was collected from each well, centrifuged at 14 000 g for 5 min, and stored at -20 °C until assayed for PRL content. Attached cells were washed in PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min. Following further rinsing, immunocytochemical procedures to identify PRL-positive cells were carried out as described above for tissue sections. Incubation of the cells with 1 µM thyrotropin-releasing hormone (TRH) and 5 mM 8-Br-cAMP (Sigma) for 1 h was used to stimulate secretion. All tests were done at least in triplicate in three independent experiments. PRL was determined by RIA using a National Pituitary Hormone Distribution Program (NIAMDD, Bethesda, MD, USA) rat hormone kit. The initial dilution of the antibody was 1:20 000. The double antibody method for separating the bound from the free fraction was used. Assay sensitivity was 3 ng/ml, while the intra-assay and interassay variations were 3% and 10% respectively. Data were analyzed by the Mann-Whitney U test to identify which groups had significant differences.

Results

Exocytotic machinery in estrogen-induced tumoral pituitary cells

One week of treatment with E₂ resulted in a significant increase in anterior pituitary weight (30 ± 2.82 mg vs 12.25 ± 2.87 mg; *P* < 0.02) and pituitary PRL (Fig. 1). These differences were dependent on the time course of E₂ treatment (data not shown).

No differences were observed in the amount of t- and v-SNAREs in brain homogenates from E₂-treated rats 1, 3, and 6 weeks after surgery. Representative examples are shown in Fig. 2A. In striking contrast, the levels of all of these exocytotic proteins were notably decreased in total homogenates from pituitary glands of E₂-treated animals at

Figure 1 Western blotting analysis of PRL and GH in anterior pituitaries from control (C) and E₂-treated (T) animals one week after surgery. Each lane was loaded with 10 µg protein.

any time analyzed (Figs 2B and 3A). In addition, no changes were detected in the levels of the cytoskeletal component α -tubulin, the plasma membrane Na⁺/K⁺-ATPase, the SLMV marker synaptophysin, and the endosome marker linked to endocytotic pathway Rab5 (Gorvel *et al.* 1991) (Fig. 3B).

Immunocytochemical methods revealed that most of the pituitary cell population in the four-week E₂-treated rats consisted of PRL-positive cells (Fig. 4A,B), whereas immunoreactive cells for the rest of the hormones constituted a small percentage (data not shown). We found a lower immunostaining intensity for all the exocytotic protein components analyzed in most of the endocrine cells from pituitary glands of E₂-treated rats compared with controls. A representative immunostaining example is shown in Fig. 4C,D. Double-labeling immunocytochemistry showed a weak immunoreactivity for different exocytotic protein components in PRL-containing cells from E₂-treated animals compared with that found in

Figure 2 Western blotting analysis of α -tubulin, SNAP-25, synaptobrevin 2 and cellubrevin from (A) brain and (B) pituitary of control (C) and E₂-treated (T) rats 1, 3 and 6 weeks after surgery. In (A) a specific antibody against synaptobrevin 2 (18:2) was used; in (B) an antibody against cellubrevin (CB35), which also recognizes its homolog synaptobrevin was used. Note the specific decrease of SNAP-25 and synaptobrevin 2/cellubrevin in pituitary glands of E₂-treated rats.

Figure 3 Western blotting analysis of different proteins from pituitary gland homogenates of control (C) and E₂-treated (T) rats 4 weeks after treatment. Several exocytotic proteins are decreased in pituitaries of E₂-treated rats (A), whereas no changes are observed in the amount of other proteins (B).

PRL-containing cells from control animals (Fig. 5A–C). It is noteworthy that in addition to the common immunostaining pattern for SNAP-25, associated with the plasma membrane, luteinizing hormone (LH)-containing cells from ovariectomized control animals consistently exhibited immunoreaction products for SNAP-25 in ring-shaped structures within the cells (Fig. 5D–F).

PRL secretion in E₂-treated pituitary in culture

In an attempt to compare the PRL release rates between normal and E₂-treated cells, we analyzed the amount of PRL released per number of PRL immunostained cells in pituitary primary cell cultures from control and E₂-treated animals seven weeks after surgery (Table 2). No differences were observed in the basal PRL release from

Figure 4 Photomicrographs of anterior pituitary sections of control (A,C) and E_2 -treated (B,D) animals immunostained for PRL (A,B) and SNAP-25 (C,D). Note the increase in PRL-positive cells in pituitary glands of E_2 -treated rats, whereas SNAP-25 is decreased. Arrows, PRL-positive cells; arrowheads, SNAP-25-positive immunostaining; asterisks, blood vessels. Scale bar: 20 μ m.

Figure 5 Double labeling immunocytochemistry of PRL (A–C) or LH (E,F) and SNAP-25 in control (A,B,D–F) and E_2 -treated (C) pituitary sections 4 weeks after treatment. In all illustrations, SNAP-25 was visualized as a brown precipitate at the plasma membrane. PRL and LH immunoreactivity was detected as a granular blue precipitate located in cytoplasmic areas. A–C, PRL-positive cells (arrows) from control animals exhibit SNAP-25 immunoreactivity (arrowheads) (A,B). In E_2 -treated pituitary, SNAP-25 immunostaining is near background in PRL-positive cells (arrows) (C). D–F, In addition to the plasma membrane (big arrowheads), SNAP-25 immunostaining is also located in ring-shaped structures within certain cells (small arrowheads) in ovariectomized control pituitary sections. Double labeling with LH (E,F) reveals the gonadotrope identity. Scale bars: A–C, 30 μ m; D–F, 10 μ m.

Table 2 PRL secretion from pituitary primary cell cultures of control and E₂-treated rats. Basal (non stimulated, *n*=5) and 8-Br-cAMP (5 mM, *n*=4) or TRH (1 µM, *n*=4)-induced PRL secretion was measured by RIA

	PRL release (pg/lactotrope cell)	
	Median	Range
Basal		
Control	0.41	0.272
E ₂ -treated	0.488	0.192
8-Br-cAMP		
Control	0.595	1.254
E ₂ -treated	1.084	0.319
TRH		
Control	0.497	0.518
E ₂ -treated	1.712	1.821

8-Br-cAMP significantly stimulated PRL secretion from pituitary cells from both control and E₂-treated rats (*P*<0.02) whereas TRH significantly stimulated PRL secretion from pituitary cells from E₂-treated rats only (*P*<0.015).

pituitary cells of control and E₂-treated rats. Incubation with the cAMP analog 8-Br-cAMP resulted in a similar increase in PRL secretion in cultured pituitary cells from control and E₂-treated animals. The rate of TRH-induced PRL secretion in anterior pituitary cells from control rats was not increased. However, TRH had a notable effect on the rate of PRL secretion in pituitary cell cultures obtained from E₂-treated animals (more than 3 times the basal secretion).

Discussion

In recent years, a group of protein components of the exocytotic machinery has been shown to be present in the rat anterior pituitary gland (Wendland & Scheller 1994, Marquéze *et al.* 1995, Oho *et al.* 1995, Redecker *et al.* 1995, Aguado *et al.* 1996, Jacobsson & Meister 1996, Majó *et al.* 1997, 1998). The amount and immunocytochemical location of all of these proteins shown in the present study agrees with previous reports. Interestingly, SNAP-25 immunoreactivity is also located in ring-shaped structures within gonadotrope cells from ovariectomized rats. Although the identity of these structures is still unknown, these may be related to the ultrastructural changes described in rat gonadotrope cells after ovariectomy (Garner & Blake 1981). Future electron microscopic studies may shed light on this issue.

In this study we have shown that long-term E₂ administration in F344 rats causes a decrease in SNAP-25, synaptobrevin 2, cellubrevin, syntaxin 1, synaptotagmin I, and Rab3a levels in the anterior pituitary gland. Since total brain homogenates show no differences in the levels of the exocytotic machinery component proteins after E₂ treatment, the decrease observed in the pituitary gland of E₂-treated animals seems to be specific. It has been shown

that neonatal sex hormone manipulations can influence the expression of several synaptic proteins in the nervous system (Lustig *et al.* 1993). Administration of estrogen and estrogen inhibitor during the 'critical period' induces up- and down-regulation respectively of SNAP-25 mRNA expression (Lustig *et al.* 1993). The present results showing a lack of change in SNAP-25 protein levels after E₂ treatment in the adult brain may be related to the administration period. In addition, it is very likely that Western blotting analysis from whole brain homogenates could mask possible area-specific differences.

It is well established that long-term E₂ administration in rats produces PRL-cell adenomas and chronic hyperprolactinemia (McEuen *et al.* 1936, Meyer & Clifton 1956), with the F344 strain being particularly sensitive to developing E₂-induced pituitary tumors (Dunning *et al.* 1947, Lloyd 1983, Phelps & Hymer 1983). Ultrastructural examination of the E₂-induced pituitary tumor cells reveals that most of them are sparsely granulated, suggesting a low storage capacity for these cells (Lloyd 1983, Hymer & Motter 1988, G Majó, I Ferrer & F Aguado (personal observations)). Recent reports have shown that most exocytotic proteins located on synaptic vesicles are also present in secretory granules and SLMV in anterior pituitary endocrine cells (Wendland & Scheller 1994, Jacobsson & Meister 1996, Majó *et al.* 1998). Interestingly, the levels of most of the proteins located on secretory organelles are decreased in pituitary cells of E₂-treated rats, whereas no changes are observed for synaptophysin. Since synaptophysin is located exclusively on SLMV (Jahn & De Camilli 1991, Majó *et al.* 1998), the decrease in the levels of synaptobrevin 2, cellubrevin, synaptotagmin I, and Rab3a in E₂-treated pituitaries could reflect the low number of secretory granules in PRL cells of E₂-treated animals. In parallel with the levels of the exocytotic proteins present on secretory granules, the t-SNAREs, SNAP-25 and syntaxin 1 levels are also decreased in pituitary cells of E₂-treated rats. In agreement with this, a down-regulation of SNAP-25 expression in the rat pituitary has recently been shown after E₂ administration (Jacobsson *et al.* 1998). On the other hand, it has been reported that SNAP-25 immunoreactivity is increased in human prolactinomas (Majó *et al.* 1997).

Since several exocytotic proteins have been shown to be essential for adenohipophyseal hormone release (Lledo *et al.* 1994, Aguado *et al.* 1997, Masumoto *et al.* 1997), the observed low levels of these proteins in the pituitaries of E₂-treated rats may be correlated with the secretion rates. It has been established that the hyperprolactinemia observed in E₂-treated animals is due to the increased number of PRL cells produced by lactotrope proliferation (Lloyd 1983) and recruitment of nonsecreting cells into an active PRL secreting pool (Boockfor *et al.* 1986, Scarbrough *et al.* 1991), as well as the E₂-stimulated PRL synthesis (Scarbrough *et al.* 1991). Whether PRL release is increased or decreased in lactotropes from E₂-treated

animals is not well established yet. Our results show that neither the basal nor 8-Br-cAMP-induced PRL release in cultured cells from E₂-treated animals differs from that in control donors. These results agree with a previous report showing no differences in basal PRL secretion between E₂-induced adenoma and normal pituitaries, estimated by RIA (Yoshikawa *et al.* 1995). In addition, these authors found a smaller mean plaque area for E₂-treated cells from F344 rats using the reverse hemolytic plaque assay (RHPA), suggesting a lower PRL release rate in E₂-induced tumor cells (Yoshikawa *et al.* 1995). This observation could be in line with the low levels of exocytotic protein components detected in pituitary glands of E₂-treated rats. However, by using the same RHPA technique, other authors found that PRL release is increased in cultured lactotrophs from estrogen-primed rats (Kendall & Hymer 1987, Lloyd *et al.* 1987, Zhang *et al.* 1990, Scarbrough *et al.* 1991). Although the meaning of these differences is unknown, they may be related to the rat strains and experimental conditions used.

We observed that TRH induced higher PRL secretion in cultured lactotrophs from E₂-induced tumors than in any other condition tested. In like measure, it has been shown that pretreatment with E₂ enhances the TRH-stimulated PRL release in cultured rat lactotrophs (Zhang *et al.* 1990, Scarbrough *et al.* 1991). The increased response to TRH observed in E₂-treated animals could be related to an increase in the number of TRH receptors (Gershengorn *et al.* 1979) or alterations in the intracellular transduction signaling (Tang *et al.* 1982).

In conclusion, this study shows that protein components of the exocytotic machinery are down-regulated in the pituitary of F344 rats by E₂ administration. The functional significance of the low exocytotic protein levels found in pituitary glands of E₂-treated animals within the exocytotic process remains to be elucidated.

Acknowledgements

We thank Dr A F Parlow (Bethesda, MD, USA) and the National Hormone and Pituitary Distribution Program (NIDDK, Bethesda, MD, USA) for the supply of pituitary hormone antisera and the rat PRL RIA kit, Dr R Jahn for his generous gift of antibodies against Rab3a, Rab5, synaptobrevin 2 and synaptotagmin I, and Dr C J Barnstable (New Haven, CT, USA) for syntaxin 1 antibody. We wish to thank Dr I Ferrer for useful comments and suggestions, and I Gomez de Aranda and E Laforet for their technical assistance. We are grateful to R Abost for statistics and Drs I Ferrer, L Cacicedo and T Fernandez for helpful discussion. We thank T Yohannan for editorial assistance. G M has a fellowship from Generalitat de Catalunya (Spain). This study was supported by a grant from DGICYT (PM95-0116) and by the EU project BMH4-CT96-1586.

References

- Aguado F, Majó G, Ruiz-Montasell B, Canals JP, Casanova A, Marsal J & Blasi J 1996 Expression of synaptosomal-associated protein SNAP-25 in endocrine anterior pituitary cells. *European Journal of Cell Biology* **69** 351–359.
- Aguado F, Gombau L, Majó G, Marsal J, Blanco J & Blasi J 1997 Regulated secretion is impaired in AtT-20 endocrine cells stably transfected with botulinum neurotoxin type A light chain. *Journal of Biological Chemistry* **272** 26005–26008.
- Barnstable CJ, Hofstein R & Akagawa K 1985 A marker for amacrine cells development in rat retina. *Development Brain Research* **20** 286–290.
- Ben-Jonathan N, Peleg E & Hoefer MT 1983 Optimization of culture conditions for short-term pituitary cell culture. *Methods in Enzymology* **103** 249–257.
- Boockfor FR, Hoeffler JP & Frawley LS 1986 Estradiol induces a shift in cultured cells that release prolactin or growth hormone. *American Journal of Physiology* **250** E103–E105.
- Brose N, Petrenko AG, Südhof TC & Jahn R 1992 Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* **256** 1021–1025.
- Burgoyne HD & Morgan A 1993 Regulated exocytosis. *Biochemical Journal* **293** 305–316.
- Dunning WF, Curtis MR & Segaloff A 1947 Strain differences in response to diethylstilbestrol and the induction of mammary gland and bladder cancer in the rat. *Cancer Research* **7** 511.
- Edelmann L, Hanson PI, Chapman ER & Jahn R 1995 Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. *EMBO Journal* **14** 224–231.
- Fischer-von-Mollard G, Stahl B, Walch-Solimena C, Takei K, Daniels L, Khoklatchev A, DeCamilli P, Südhof TC & Jahn R 1994 Localization of Rab5 to synaptic vesicles identifies endosomal intermediate in synaptic vesicles recycling pathway. *European Journal of Cell Biology* **65** 319–326.
- Garner LL & Blake CA 1981 Ultrastructural, immunocytochemical study of the LH secreting cell of the rat anterior pituitary gland: changes occurring after ovariectomy. *Biology of Reproduction* **24** 461–474.
- Gershengorn MC, Marcus-Samuels BE & Geras E 1979 Estrogens increase the number of thyrotropin-releasing hormone receptors on mammothrophs in culture. *Endocrinology* **105** 171–176.
- Gorvel JP, Chavrier P, Zerial M & Gruenberg J 1991 rab5 controls early endosome fusion *in vitro*. *Cell* **64** 915–925.
- Hymer WC & Motter KA 1988 Heterogeneity in mammothrophs prepared from diethylstilbestrol-induced prolactinomas. *Endocrinology* **122** 2324–2338.
- Jacobsson G & Meister B 1996 Molecular components of the exocytotic machinery in the rat pituitary gland. *Endocrinology* **137** 5344–5356.
- Jacobsson G, Razani H, Ogren SO & Meister B 1998 Estrogen down-regulates mRNA encoding the exocytotic protein SNAP-25 in the rat pituitary gland. *Journal of Neuroendocrinology* **10** 157–163.
- Jahn R & De Camilli P 1991 Membrane proteins of synaptic vesicles: markers for neurons and endocrine cells; tools for the study of neurosecretion. In *Markers for Neural and Endocrine Cells*, vol 14, pp 25–92. Eds M Gratzl & K Langley. Weinheim: VCH.
- Kendall ME & Hymer WC 1987 Cell blotting: a new approach to quantify hormone secretion from individual rat pituitary cells. *Endocrinology* **121** 2260–2262.
- Kovacs K, Horvath E & Ezrin C 1977 Pituitary adenomas. *Pathology Annual* **12** 341–382.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Lang J, Regazzi R & Wollheim CB 1997 Clostridial toxins and endocrine secretion: their use in insulin-secreting cells. In *Bacterial Toxins: Tools in Cell Biology*, pp 217–240. Ed K Aktories. Weinheim: Chapman and Hall.

- Levey AI, Bolam JP, Rye DB, Hallanger AE, Demuth RM, Mesulam RM & Wainer BH 1986 A light and electron microscopic procedure for sequential double antigen localization using diaminobenzidine and benzidine dihydrochloride. *Journal of Histochemistry and Cytochemistry* **34** 1449–1457.
- Linial M 1997 SNARE proteins: why so many, why so few? *Journal of Neurochemistry* **69** 1781–1792.
- Linial M & Parnas D 1996 Deciphering neuronal secretion: tools of the trade. *Biochimica et Biophysica Acta* **1286** 117–152.
- Lledo PM, Vernier P, Vincent JD, Manson WT & Zorec R 1993 Inhibition of Rab3B expression attenuates Ca^{2+} -dependent exocytosis in rat anterior pituitary cells. *Nature* **364** 540–544.
- Lledo PM, Vernier P, Vincent JD, Manson WT & Zorec R 1994 New approaches in the study of stimulus-secretion coupling in anterior pituitary cells. *Annals of the New York Academy of Sciences* **710** 301–318.
- Lloyd RV 1983 Estrogen-induced hyperplasia and neoplasia in the rat anterior pituitary gland. An immunohistochemical study. *American Journal of Pathology* **113** 198–206.
- Lloyd RV, Coleman K, Fields K & Nath V 1987 Analysis of prolactin and growth hormone production in hyperplastic and neoplastic rat pituitary tissues by the hemolytic plaque assay. *Cancer Research* **47** 1087–1092.
- Lustig RH, Hua P, Wilson MC & Federoff HJ 1993 Ontogeny, sex dimorphism, and neonatal sex hormone determination of synapse-associated messenger RNAs in rat brain. *Molecular Brain Research* **20** 101–110.
- Majó G, Ferrer I, Marsal J, Blasi J & Aguado F 1997 Immunocytochemical analysis of the synaptic proteins SNAP-25 and Rab3A in the human pituitary adenomas. Overexpression of SNAP-25 in the mammosomatotroph lineages. *Journal of Pathology* **183** 440–446.
- Majó G, Aguado F, Blasi J & Marsal J 1998 Synaptobrevin isoforms in secretory granules and synaptic-like microvesicles in anterior pituitary cells. *Life Sciences* **62** 607–616.
- Marquze B, Boudier JA, Mizuta M, Inagaki N, Seino S & Seagar M 1995 Cellular localization of synaptotagmin I, II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. *Journal of Neuroscience* **15** 4906–4917.
- Masumoto N, Ikebuchi Y, Matsuoka T, Tasaka K, Miyake A & Murata Y 1997 Involvement of SNAP-25 in TRH-induced exocytosis in pituitary GH4C1 cells. *Journal of Endocrinology* **153** R5–R10.
- Matteoli M, Takei K, Cameron R, Hurlbut P, Johnston PA, Sdhof TC, Jahn R & DeCamilli P 1991 Association of Rab3A with synaptic vesicles at late stages of the secretory pathway. *Journal of Cell Biology* **115** 625–633.
- McEuen CS, Selye H & Collip JB 1936 Some effects of prolonged administration of oestrin in rats. *Lancet* **1** 775–776.
- Meyer RK & Clifton KH 1956 Effect of diethylstilbestrol-induced tumorigenesis on the secretory activity of the rat anterior pituitary gland. *Endocrinology* **58** 686–693.
- Morgan A & Burgoyne RD 1997 Common mechanism for regulated exocytosis in the chromaffin cell and the synapse. *Seminars in Cell and Development Biology* **8** 141–149.
- Nsee JK, Fleming AM & Scheller RH 1993 A rab protein regulates the localization of secretory granules in AtT-20 cells. *Molecular Biology of the Cell* **4** 747–756.
- Oho C, Seino S & Takahashi M 1995 Expression and complex formation of soluble N-ethyl-maleimide-sensitive factor attachment protein (SNAP) receptors in clonal endocrine cells. *Neuroscience Letters* **186** 208–210.
- Perez F, Lledo PM, Karagoeos D, Vincent JD, Prochiantz A & Ayala J 1994 Rab3A and Rab3B carboxy-terminal peptides are both potent and specific inhibitors of prolactin release by rat cultured anterior pituitary cells. *Molecular Endocrinology* **8** 1278–1287.
- PHELPS C & HYMER WC 1983 Characterization of estrogen-induced adenohypophyseal tumors in the Fischer 344 rat. *Neuroendocrinology* **37** 23–31.
- Redecker P, Cetin Y & Grube D 1995 Differential distribution of synaptotagmin I and rab3 in the anterior pituitary of four mammalian species. *Neuroendocrinology* **62** 101–110.
- Rothman JE 1994 Intracellular membrane fusion. *Advances in Second Messenger Phosphoprotein Research* **29** 81–96.
- Scarborough K, Weiland NG, Larson GH, Sortino MA, Chiu S, Hirshfield AN & Wise PM 1991 Measurement of peptide secretion and gene expression in the same cell. *Molecular Endocrinology* **5** 134–142.
- Sllner T, Bennet MK, Whiteheart SW, Scheller RH & Rothman JE 1993a A protein assembly–disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicles docking, activation, and fusion. *Cell* **75** 409–418.
- Sllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P & Rothman JE 1993b SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362** 318–324.
- Sdhof TC 1995 The synaptic vesicle cycle: a cascade of protein–protein interactions. *Nature* **375** 645–653.
- Tang LK, Martellock AC & Tang FY 1982 Estradiol stimulation of pituitary cAMP production and cAMP binding. *American Journal of Physiology* **243** E109–E113.
- Towbin H, Staehelin T & Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA* **76** 4350–4354.
- Wendland B & Scheller R 1994 Secretion in AtT-20 cells stably transfected with soluble synaptotagmins. *Molecular Endocrinology* **8** 1070–1082.
- Yoshikawa M, Uozumi T, Kawamoto K, Arita K, Ito A & Takahashi S 1995 Characteristics of prolactin secretion in normal and estrogen-treated pituitaries of rats at the single cell level: analysis by reverse hemolytic plaque assay. *Endocrine Journal* **42** 235–243.
- Zhang J, Chen C, Kukstas LA, Vincent JD & Israel JM 1990 Functional lactotroph heterogeneity in lactating rats and *in vitro* modification by 17β -estradiol. *Journal of Neuroendocrinology* **2** 815–823.

Received 24 June 1998

Accepted 20 November 1998