0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 12
*Printed in U.S.A.*

# Endothelin Is an Autocrine Regulator of Prolactin Secretion*

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**ABSTRACT**

The aim of this study was to establish the cellular source of ET-like peptides affecting PRL secretion. Fluorescence double label immunocytochemistry and confocal laser scanning microscopy were used to demonstrate cellular colocalization for PRL and endothelin-1 (ET1)-like immunoreactivities in the anterior lobe of the pituitary gland of rats. An ET-specific reverse hemolytic plaque assay was applied to demonstrate that lactotrophs are capable of releasing ET-like peptides. A PRL-specific reverse hemolytic plaque assay was used to assess the influence of the released endogenous ETs on PRL secretion. $ET_A$-specific receptor antagonists BQ123 and BQ610, and endothelin convertase enzyme inhibitory peptide, [$^{22}$Val]big ET1-(16–38), increased PRL secretion, whereas the $ET_B$ receptor-specific antagonist BQ788 was ineffective. The $ET_A$ antagonist BQ123-induced increase in PRL secretion followed a bell-shaped dose-response curve in cells obtained from female rats, whereas it followed a sigmoid curve in males. Frequency distribution of PRL plaque sizes using logarithmically binned data revealed two subpopulations of lactotrophs with differential responsiveness to endogenous ETs.

These data demonstrate that a large proportion of lactotrophs is capable of expressing and secreting ET-like peptides in biologically significant quantities. As low pituitary cell density in reverse hemolytic plaque assay minimizes cell to cell communications, these findings constitute direct proof of autocrine regulation of PRL secretion by ET-like peptides. (*Endocrinology* **139:** 5164–5173, 1998)

ENDOTHELIN (ET)-LIKE peptides are best known for their strong vasoconstrictor activity (1–4). Indeed, the first prominent physiological role assigned to these peptides was to serve as paracrine regulatory signals emanating from the endothelium to affect vascular smooth muscle cells (5–7). The array of physiological and pathological processes where ETs play an important role has been expanded considerably (8–13), and their potential in modulating secretory functions in many endocrine tissues is now well recognized (11, 14, 15).

The evidence suggesting that ETs regulate pituitary hormone secretion is especially impressive. For instance, both ETs and their receptors are expressed in the pituitary gland and in the hypothalamic magnocellular nuclei (15–19). In addition, ET-like peptides have been detected in conditioned medium of long term pituitary cell culture (20). Moreover, it has been found that ET-like peptides are powerful modulators of hormone secretion from cultured pituitary cells, as they diminish PRL secretion and enhance LH, FSH, and TSH secretion (21–24).

The source of ETs affecting hormone secretion from the anterior lobe of the pituitary gland *in vivo* has not yet been identified unequivocally. The magnocellular neurons of hypothalamic supraoptic and paraventricular nuclei have been considered potential sources of ET-like peptides (16, 21). It seemed conceivable that these neurons might provide ETs to the anterior lobe in a way similar to their delivery of oxytocin and vasopressin (25–27), specifically through their axon collaterals to the median eminence or by releasing ET in the posterior lobe from where it can reach the anterior lobe by either the long or short portal system, respectively. There is diminished enthusiasm for these possibilities, however, because ETs released by the magnocellular neurons will probably influence the blood flow through the portal vascularizations affecting nonselectively the entire neurohumoral input to the anterior lobe. Indeed, several experiments on pituitary (28) and nonpituitary tissues (14) indicated that ETs are produced locally. It is now thought that ETs, under physiological circumstances, do not reach their target cells through the general circulation (14). Taken together, these studies indicate strongly that ETs subserve a role as intercellular paracrine and/or autocrine messengers in the pituitary gland. However, no direct evidence for such a physiological role of ETs has yet been provided.

In the present study we sought to establish a role for ETs as autocrine regulators of PRL secretion. First, we applied immunocytochemical and reverse hemolytic plaque assay methods to demonstrate that lactotrophs are capable of expressing and releasing ET-like peptides. Second, we used a pharmacological blockade of ET receptors to investigate whether ETs, released by lactotrophs, can influence PRL secretion in an autocrine fashion. Previous studies found relatively high ET3 immunoreactivity in rat as well as human pituitary tissue (29, 30). Moreover, the release of ET1 and ET3 from long term pituitary culture is regulated differentially by insulin-like growth factors (20). These data demonstrated the presence of both ET1 and ET3 in the pituitary and indicated that ET3 might have a physiological role distinct from that of ET1. As we have previously found that ET1 is about 4 orders of magnitude more potent on lactotrophs than ET3 (31), we focused on ET1-like peptides in this study.

Received July 28, 1998.
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* This work was supported by NIH Grants HD-11669 and DK-43200 (to M.E.F.).

## Materials and Methods

### *Animals and pituitary cell preparation*

Male and female Sprague-Dawley rats (200–250 g; Charles River Laboratories, Inc., Wilmington, MA) were used as pituitary donors. Pituitary glands were collected after rapid decapitation, intermediate and posterior lobes were removed, and anterior lobes were rinsed and diced into approximately 1-mm cubes. The anterior pituitary cells were enzymatically dissociated using collagenase and hyaluronidase as previously described (32). For immunocytochemistry (ICC), the animals were killed by an overdose of sodium pentobarbital and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The pituitary glands were removed, postfixed for 30 min in the same fixative at room temperature, and stored in 20% sucrose-PBS at −20 C until sectioning by cryostat.

### *Immunocytochemical detection of PRL and ET in sections of anterior pituitary glands*

An immunofluorescent double labeling technique was applied on fixed pituitary tissue to demonstrate cellular colocalization of PRL and ET1. Twenty-micron coronal cryostat sections were mounted on gelatin-subbed glass slides and incubated with 10% normal horse serum in PBS containing 0.4% (vol/vol) Triton X-100 and 0.1% (wt/vol) sodium azide for 30 min at room temperature to prevent nonspecific binding of antibodies. PRL immunoreactivity was visualized using a rabbit polyclonal antiserum (NIH antirat PRL IC-5; 1:20,000) followed by CY3-conjugated donkey antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). ET-like immunoreactivity was detected by applying monoclonal mouse anti-ET1 antibody (1:5,000; MCE-6901-01, Peninsula Laboratories, Inc., Belmont, CA) followed by CY2-conjugated donkey antimouse IgG (1:400; Jackson ImmunoResearch Laboratories). Images were obtained with a Zeiss LSM 410 confocal laser scanning microscope (Zeiss, New York, NY) equipped with two external (568 and 488 nm) argon-krypton lasers. Images were acquired with a Plan-Neofluar 63/1.4 objective lens in a dual scan mode with Z-sectioning (each optical section was 0.6 $\mu$m). The colocalization was analyzed on the overlayed red and green images using LSM 410 software (Zeiss) as previously described (33).

### *Measuring PRL secretion from individual lactrotrophs by reverse hemolytic plaque assay (RHPA)*

Cunningham chambers were constructed, and the hemolytic plaque assay was carried out as described previously (34, 35). Briefly, pituitary cells mixed with protein A-coupled ovine red blood cells were plated on poly-L-lysine-coated microscope slides (1 ml 2% ovine red blood cells contained approximately $5 \times 10^4$ pituitary cells). After 1-h equilibration in a humidified $CO_2$ incubator, the cells were washed with approximately 400 $\mu$l DMEM-BSA (Life Technologies, Gaithersburg, MD) containing 1 mg/ml BSA (fraction V, Sigma Chemical Co., St. Louis, MO). Subsequently, the chambers were filled with DMEM-BSA containing antirat PRL antiserum (1:50; provided by G. M. Nagy) and different concentrations of ET receptor antagonists or other test materials (listed below). Chambers filled with DMEM-BSA containing only the PRL antiserum served as controls. Each experiment contained three to five replicates of the same treatment. After 4 h of incubation, hemolytic plaques were developed by applying guinea pig complement for 30 min (1:50; Life Technologies). The complement's action was stopped by infusing PBS containing 4% formaldehyde and 0.1% glutaraldehyde into the chambers. After 1 h at room temperature, the chambers were kept at 4 C overnight. The following day, the fixative was removed with a wash of PBS, and the slides were stored in PBS at 4 C until ICC. Compounds tested in PRL RHPA were the selective $ET_A$ receptor antagonists BQ123, cyclo(D-Asp-Pro-D-Val-Leu-D-Trp), and BQ610, (*N*,*N*-hexamethylene)carbamoyl-Leu-D-Trp(CHO)-D-Trp (36, 37); the selective $ET_B$ receptor antagonist BQ788, *N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-1-methoxycarbonyl-Trp-D-Nle (38); and ET-converting enzyme inhibitory peptide (ECi), [D-Val$^{22}$]big ET1-(16–38), purchased from Peninsula Laboratories. ET1, the most potent $ET_A$ receptor agonist (31), was obtained from Sigma Chemical Co.

### *Characterization of ET-secreting lactotrophs by ET specific RHPA*

The hemolytic plaque assay for ET was performed essentially as described above, except that the anti-PRL antiserum was replaced by an anti-ET antiserum. During the development of the ET plaque assay, several anti-ET antisera from varying sources were tested in incubation for 3, 6, 12, 18, and 24 h in concentrations of 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800. We found two polyclonal rabbit anti-ET1 antisera (IHC-6901 and IHC-6910, both from Peninsula Laboratories) suitable for RHPA in 1:25 to 1:50 concentrations, whereas the applications of monoclonal anti-ET1 antibodies (MCE-6901-01 from Peninsula Laboratories and MET-1 from Accurate Chemical & Scientific Instruments Corp., Westbury, NY) did not result in hemolytic plaques (Table 1). The earliest plaque formation was detected after 6 h of incubation. The incidence of plaque-forming pituitary cells increased up to 18 h, but extending the incubation time to 24 h did not result in a further increase in the number of plaques.

### *Immunocytochemical identification of lactotrophs after ET-specific RHPA*

The Cunningham chambers were disassembled by carefully removing the coverslips, and the slides were processed for ICC as follows. After blocking with 10% normal horse serum in PBS containing 0.4% (vol/vol) Triton X-100 and 0.1% (wt/vol) sodium azide, slides were incubated with biotinylated antirat PRL antiserum (1:6000, IC-5, NIDDK) in a refrigerator overnight. The anti-PRL antibody was biotinylated using *N*-hydroxysuccinimidyl 6-(biotin amido) hexanoate (Vector Laboratories, Inc., Burlingame, CA). PRL-like immunoreactivity was visualized by streptavidin-conjugated CY3 (5 $\mu$g/ml in PBS, 3 h at room temperature). Images were acquired with a Microphot-FXA microscope (Nikon, Tokyo, Japan) equipped with an epifluorescent attachment and digitized by a remote head video camera (Optronics, Goleta, CA), reformatted by a frame grabber (Coreco, Inc., St. Laurent, Canada), and transmitted to an IBM-compatible personal computer. ImagePro Plus software (Cybernetics, Silver Spring, MD) was used to perform semiautomatic

**TABLE 1.** Comparison of antiendothelin antibodies and antisera used in immunocytochemistry and reverse hemolytic plaque assay

| Antibody or antiserum | Host species | Cross-reactivity (%) | | | ICC | | RHPA | |
|---|---|---|---|---|---|---|---|---|
| | | ET1 | ET2 | ET3 | Relative staining | Working dilution | Plaque formation | Working dilution |
| MCE-6901-01 | Mouse[a,b] | 100 | 100 | 3.5 | +++ | 1:5,000 | No | n/a |
| MET-1 | Mouse[a,c] | 100 | <5 | <5 | +++ | 1:2,000 | No | n/a |
| IHC-6901 | Rabbit[b] | 100 | 91 | 0.1 | ++ | 1:800 | Yes | 1:50 |
| IHC-6910 | Rabbit[b] | 100 | 100 | 100 | ++ | 1:800 | Yes | 1:50 |
| GAS-6901 | Guinea pig[b] | 100 | 100 | 0.1 | + | 1:200 | No | n/a |

n/a, Not applicable.
[a] Monoclonal antibody.
[b] Peninsula Laboratories.
[c] Accurate Chemical and Scientific Corp.

intensity measurements, and cells displaying at least 5 times higher intensity than the background level were considered immunopositive for PRL.

### *Data collection and analysis*

Plaques were examined using a Microphot-FXA microscope (Nikon) equipped with a ×4 phase contrast objective (used for measuring PRL plaques) and a ×20 DIC objective (used for ET1 plaque measurements). Plaques were always verified by visual inspection before measurements were made, and only plaques with a single pituitary cell in the center surrounded by red blood cell "ghosts" were accepted. Numerical values of each plaque area (square microns) were obtained individually by ImagePro Plus software and transported to a Microsoft Corp. (Redmond, WA) Excel spreadsheet. For statistical analysis, one-way ANOVA followed by Dunnett's multiple comparison test were applied using the Prism 2.01 program from GraphPad Software, Inc. (San Diego, CA), where $P < 0.05$ was considered the threshold of significance. For further analysis of cell populations, logarithmically binned data were analyzed by the Pstat program within the pClamp6 software package (version 6.03, Axon Instrument Co., Foster City, CA). Each dataset, representing different treatment groups, was fitted with Gaussian curves using the Levenberg-Marquart least squares method. The first, second, and third orders of fitted Gaussian curves within each groups were compared statistically using Pstat. The higher order was accepted only if it resulted in a significantly improved fit (signified by $F > 3.0$).

## Results

### *ET1-like immunoreactivity is expressed by lactotrophs*

Cellular colocalization of PRL- and ET1-immunoreactive substances was found on double labeled pituitary sections examined with confocal laser scanning microscopy (Fig. 1). All of the antisera capable of recognizing ET1 gave positive staining on pituitary sections (Table 1). The staining for ET-like immunoreactivity was completely eliminated when the antisera were preabsorbed with 10 $\mu$g/ml ET1 or when the primary antiserum was omitted from the staining protocol (Table 1). The monoclonal anti-ET1 antibodies (either MCE-6901-01 or MET-1) and the polyclonal antiserum specific for ET1 (IHC-6901) showed similar staining patterns and complete colocalization (data not shown).

In the present study, we did not pursue a quantitative assessment of the regional distribution of ET1-staining lactotrophs within the anterior lobe of the pituitary gland because the compact structure of the anterior lobe makes such quantitation difficult. We noticed, however, that the ET-immunopositive lactotrophs are not distributed evenly

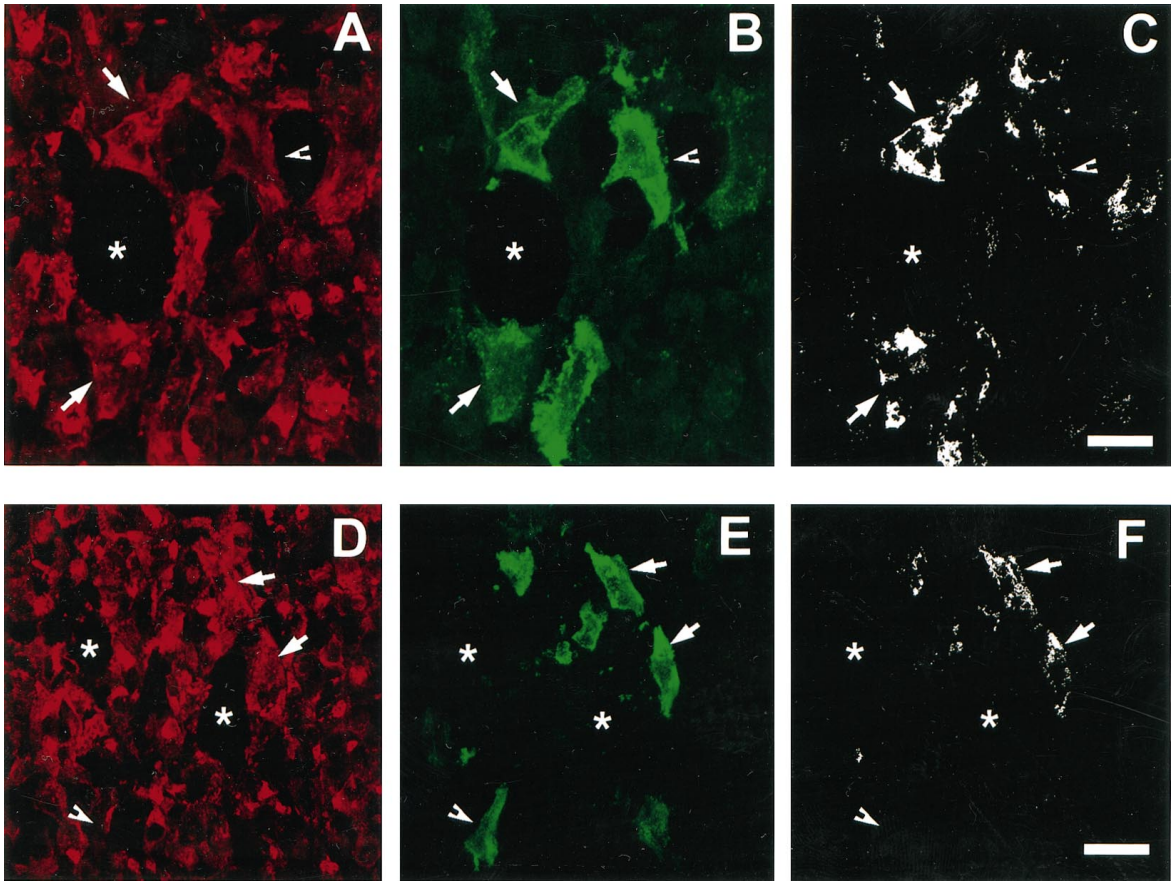


FIG. 1. Representative confocal images for cellular colocalization of ET and PRL immunoreactivities in the anterior lobe of the pituitary gland. Twenty-micron coronal sections of the anterior lobe of the pituitary gland were obtained from male (A–C) and female (D–F) rats. PRL (appears in red, A and D) and ET1 (appears in green, B and E) immunoreactivities were visualized by labeling with CY2- and CY3-conjugated second antibodies, respectively. The red and green images of the same section (A and B, and D and E) were acquired with a Plan-Neofluar 63/1.4 objective lens using a Zeiss LSM 410 laser scanning microscope equipped with an external argon/krypton laser (568 and 448 nm) in dual scan mode. Using the overlay of red and green images, colocalization of PRL and ET1 was visualized (C and F) by selecting only those pixels where the intensity in both red and green was high (for details, see *Materials and Methods*). *Solid arrows* indicate colocalization of PRL and ET1. *Arrowheads* point to cells immunoreactive only for ET1. *Asterisks* indicate sinusoid capillaries. *Scale bars* represent 10 $\mu$m in A–C and 20 $\mu$m in D–F.

throughout the anterior lobe, but they often formed a small cluster of 5–10 cells (Fig. 1). On fixed tissue sections, we estimated that approximately 5% of the lactotrophs were stained positive for ET1. This appraisal of the incidence of ET-expressing lactotrophs is comparable with data obtained previously from dispersed pituitary cells plated on microscope slides (39).

*Individual lactotrophs secrete an ET1-like substance(s)*

By using protein A-coupled sheep red blood cells in conjunction with rabbit anti-ET antisera that recognize ET1 (either IHC-6901 or IHC-6910, see Table 1), we were able to detect hemolytic plaque formations around PRL-immunopositive cells (Fig. 2), thus demonstrating that lactotrophs are capable of releasing an ET1-like immunoreactive substance(s). To compare ET release from lactotrophs of male and female origins, plaque assays were run in parallel sessions with cells obtained from male or female animals. After an incubation of 24 h in the presence of ET1-specific antiserum (IHC-6901), many ET plaque-forming lactotrophs were detected in both cases. After enumerating lactotrophs based on their PRL immunoreactivity, we found that 24.6% of lactotrophs from males (1221 of 4963) and 43.9% of lactotrophs from females (825 of 1879) released an ET1-like substance(s) in a quantity sufficient to initiate hemolytic plaque formation. The mean plaque area was significantly greater in the case of females: $1299 \pm 40$ *vs.* $758 \pm 16$ $\mu m^2$ (Fig. 3A). The relative frequency of logarithmically binned plaque sizes can be fitted best by a simple Gaussian function (Fig. 3B), suggesting a homogeneous population of ET-secreting lactotrophs. It is noteworthy that a sizable proportion of the ET plaque-forming cell population (56% in males and 34% in females) was not immunopositive for PRL. This observation indicates that ET-releasing capacity is not exclusive to lactotrophs, and ETs are probably used for intercellular communication by other pituitary cell types as well.

*Pharmacological blockade of $ET_A$ receptors enhances PRL*

Having established the ET-releasing capacity of the lactotrophs, we next examined whether the ET1-like material released from lactotrophs is sufficient to modulate PRL secretion. In other words, does the released ET regulate PRL secretion in an autocrine fashion? PRL secretion of individual lactotrophs obtained from diestrous female rats was assessed by RHPA throughout these experiments. As the predominant ET receptor in the pituitary is $ET_A$ (31, 40, 41), we first examined the effect of pharmacological blockade of $ET_A$ receptors on lactotrophs by using the well characterized se-

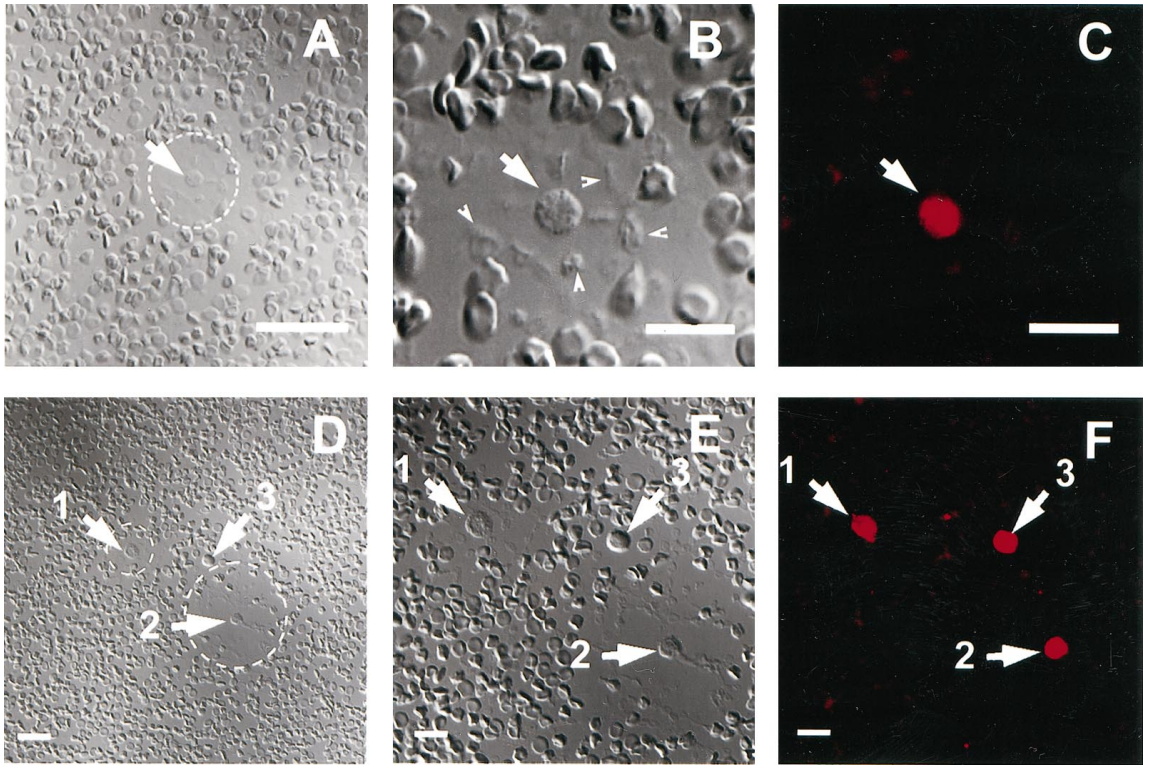


FIG. 2. Hemolytic plaques formed by anti ET1 antibody-coupled sheep red blood cells. Pituitary cells were obtained from either male (A–C) or female (D–F) rats. Endogenous ETs, released spontaneously by isolated pituitary cells, are bound to anti-ET1 antibodies on the surface of protein A-conjugated sheep red blood cells, thus making the red blood cells vulnerable to the complement system. Hemolytic plaques around pituitary cells were detected using DIC optics of a Zeiss LSM 410 laser scanning microscope (A and B, and D and E). To identify lactotrophs, cells were immunostained for PRL using CY3-conjugated second antibody, and fluorescent images were acquired with the 568-nm laser line. PRL-immunopositive cells (appear in red in fluorescent images, C and F) are indicated by *large solid arrows*. Hemolysed red blood cell ghosts around lactotrophs signify plaque formation (*small arrowheads*, B). Plaques surrounding PRL-immunopositive cells provide direct evidence for the release of ET1-like peptides from lactotrophs. Images of each field (A–C and D–F) were obtained using a ×63 objective (A) with ×2 zoom (B and C) and a ×40 objective (D) with ×2 zoom (E and F). *Scale bars* represent 30 $\mu$m (A), 15 $\mu$m (B and C), 20 $\mu$m (D), and 10 $\mu$m (E and F), respectively.

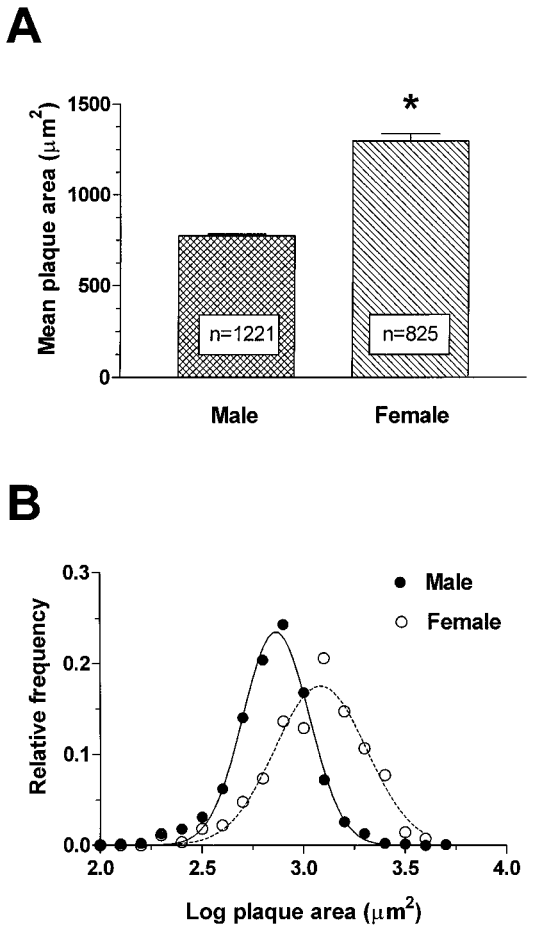


FIG. 3. Characteristics of ET plaques formed by lactotrophs: mean plaque size and frequency distribution. The lactotrophs were identified, and the plaque areas were estimated as described in *Materials and Methods*. The average plaque size was significantly larger when lactotrophs were obtained from female donor animals: 1299 ± 40 *vs.* 758 ± 16 $\mu m^2$ (A). The logarithmically binned values of individual plaque areas can be fitted best by a first order Gaussian function indicating a homogeneous population of the ET-secreting lactotrophs, detected in both sexes (B).

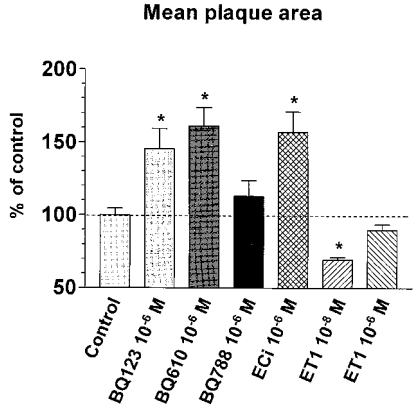


FIG. 4. Effects of ET receptor antagonists, ET-converting enzyme inhibitor, and ET1 on PRL plaque formation. Cells were obtained from diestrous female rats, and PRL RHPA was performed as described in *Materials and Methods*. Pituitary cells were incubated for 4 h in the presence of test materials at the concentrations indicated. Each *bar* represents an average of the mean plaque areas obtained from three to five individual experiments and expressed as a percentage of that in the controls (±SEM). The mean plaque area of the controls was 14,851 ± 664 $\mu m^2$ (n = 5). Coincubation with $ET_A$ receptor antagonists BQ123 and BQ610 as well as with the ET-converting enzyme inhibitor peptide significantly increased the mean plaque area, indicating that neutralizing the effects of endogenous ETs resulted in elevated PRL secretion from isolated lactotrophs. The $ET_B$ receptor antagonist BQ788 was ineffective. ET1 at a 100-nM concentration decreased the mean plaque area, whereas at a micromolar concentration the effect of ET1 vanished, indicating that lactotrophs under RHPA conditions have a responsiveness to ET1 similar to that of lactotrophs in monolayer cultures.

lective $ET_A$ receptor antagonists, BQ123 and BQ610 (36, 37). The presence of $ET_A$ receptor antagonists, either BQ123 or BQ610, in a $10^{-7}$ M concentration caused 45.5 ± 13.8% and 61.2 ± 12.5% increases, respectively, in average plaque size, indicating enhanced PRL secretion from isolated lactotrophs (Fig. 4). Thus endogenous ET-like peptides released by lactotrophs are indeed capable of effecting PRL secretion. Consistent with the previously established $ET_A$ receptor dominance in lactotrophs (31), the $ET_B$-specific antagonist, BQ788, was ineffective in changing PRL secretion (Fig. 4). When endogenous ET1 formation was impaired by ET convertase enzyme inhibitory peptide [D-Val$^{22}$]big ET1-(16–38) (42), the average PRL plaque size was increased by 56.9 ± 14.1% (Fig. 4). Exogenous ET1 at a concentration of $10^{-8}$ M inhibited PRL secretion by 30.5% (Fig. 4), indicating that the responsiveness of lactotrophs to ETs in a plaque assay preparation is comparable to that of lactotrophs in monolayer cultures (24, 31). The inhibitory effect of ET1 at a higher concentration ($10^{-6}$ M) was diminished (Fig. 4), suggesting that under RHPA conditions, lactotrophs possess similar biphasic biological responsiveness to the ETs described previously using different culture conditions (28, 43, 44). The average number of PRL plaques in the control group was 212 ± 27 plaques/slide (n = 5), constituting approximately 60% of the total number of lactrotrophs determined by ICC. Masking the effects of endogenous ETs by either $ET_A$ antagonists or an ET convertase inhibitory peptide did not affect the overall incidence of plaque-forming lactotrophs.

### *Comparison of autocrine regulation of PRL secretion by ETs in lactotrophs obtained from male and female rats*

Dispersed pituitary cells from both male and female rats were used in these experiments, and PRL secretion was assessed by hemolytic plaque assay as described. The overall response to $ET_A$ antagonism by BQ123 was stimulation of PRL secretion in both sexes (Fig. 5). However, there were salient differences between dose-response curves of BQ123 on lactotrophs obtained from female *vs.* male rats (Fig. 5). In lactotrophs obtained from diestrous females, the effect of BQ123 on PRL secretion followed a bell-shaped dose-response curve, reaching a maximum at $10^{-7}$ M (155.1 ± 14.5%), whereas at higher concentrations, the effect of BQ123 on PRL secretion gradually diminished (Fig. 5A). In lactotrophs obtained from males, the BQ123-induced increase in PRL secretion followed a sigmoid dose-response curve with an estimated half-maximum concentration of $1.34 \times 10^{-8}$ M BQ123. The maximum increase in PRL secretion was 242.0 ± 17.4% of that in the untreated groups (Fig. 5B).

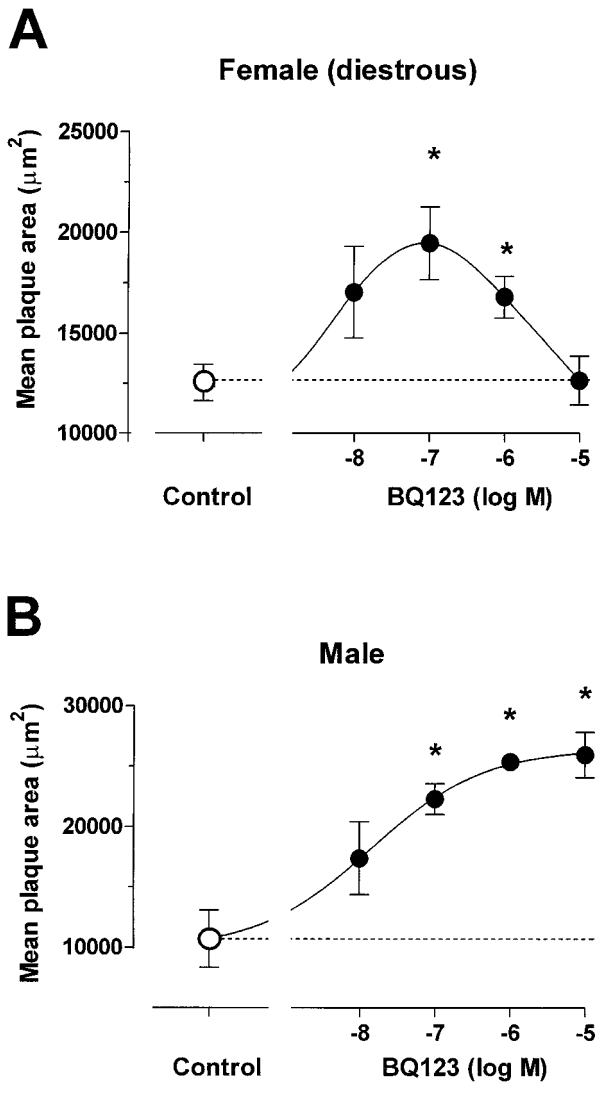


FIG. 5. Effects of the endothelin $ET_A$ receptor antagonist BQ123 on PRL secretion. The effect of $ET_A$ receptor antagonism on PRL plaque formation was assessed after 4 h of incubation in the presence or absence (control) of BQ123. Each value represents arithmetical averages of five (male) or four (female) individual experiments. When lactotrophs were obtained from diestrous female rats, the effects of the $ET_A$ antagonist on the size of PRL plaques resulted in a bell-shaped dose-response curve (A). The maximal effective concentration of BQ123 was $10^{-7}$ M, causing a 55% increase in PRL secretion. On lactotrophs obtained from male rats, the $ET_A$ antagonist increased the size of PRL plaques in a dose-dependent manner, resulting in a sigmoid dose-response curve (B). The effect of BQ123 reached a maximum at $10^{-6}$ M, causing about a 142% increase in PRL secretion.

To analyze the effects of the $ET_A$ antagonist BQ123 on lactotroph population dynamics, frequency distributions of logarithmically binned data were created and fitted with a Gaussian function as described in *Materials and Methods*. In control groups, our analysis revealed two lactotroph populations in both sexes, one with lower and another with higher secretory activity (Fig. 6). After retransformation of the maxima of the fitted Gaussian functions, estimates for the mean plaque area of the two populations were 1,180 and 6,531 $\mu m^2$ (female) and 946 and 4,852 $\mu m^2$ (male). In the presence of $10^{-7}$ M BQ123, the smaller plaque-forming cell population disappeared, and populations in both cases could be fitted best with a first order Gaussian function, where estimated maxima were 5,956 $\mu m^2$ (female) and 9,862 $\mu m^2$ (male). At higher BQ123 concentrations ($10^{-6}$ or $10^{-5}$ M), the smaller plaque-forming population (with a maximum at 1,762 $\mu m^2$) reappeared among female lactotrophs (Fig. 6), resulting in a decrease in the overall secretory activity as reflected by the descending phase of the bell-shaped dose-response curve presented in Fig. 5A. In males, treatment with higher concentrations of BQ123 also revealed two population of lactotrophs (Fig. 6). In this case, however, increased maxima of the large plaque-forming group (15,452 $\mu m^2$) offset the reappearance of the smaller plaque-forming group (4,045 $\mu m^2$), resulting in a small overall increase in PRL secretion, as reflected by the saturation phase of the dose-response curve shown in Fig. 5B.

## Discussion

It has long been postulated by us (24) and others (45) that ETs subserve a role as paracrine and/or autocrine regulators in the anterior lobe of the pituitary gland. Here, by using fluorescence double label ICC and ET-specific hemolytic plaque assay, we provide direct evidence that lactotrophs are capable of expressing and secreting ETs. In addition, by using ET receptor antagonists to block the effects of endogenous ET in a PRL-specific hemolytic plaque assay, we demonstrated that the amount of ETs released by a single lactotroph is sufficient to modulate PRL secretion, thus invoking a role for ETs in the autocrine regulation of lactotroph functions.

We have not identified the immunoreactive ETs unequivocally as ET1 in these studies. However, based upon the results with antisera having differential cross-reactivities for ET1, ET2, and ET3, we have reason to believe that the staining observed in pituitary sections is due to the presence of ET1 and/or its precursors. We can be less certain of the identity of the released ET-like material detected by the RHPA, although a similar assumption that the released substance is ET1 seems justified. The relatively low incidence of double labeled lactotrophs can be related to the fact that the steady state ET1 concentration in the pituitary gland is very low (29). The low tissue concentration of ETs is not surprising considering that the half-maximal concentration of ET1 affecting PRL secretion is around $10^{-15}$ M (31). Therefore, assuming an autocrine or paracrine mode of action, very little ET1 would be needed to act at such short distances from its source.

We concluded from the immunocytochemical data that lactotrophs are capable of producing ET-like peptides, although only a small proportion of the cells have the cellular level of ETs sufficiently high to be detectable by immunocytochemical methods. However, these observations should not detract from considering endogenous ETs as important regulatory peptides within the pituitary gland, as the number of immunocytochemically identified ET-positive cells probably underrepresents the true number of lactrotrophs that are capable of expressing and releasing ET-like peptides. Indeed, by using the ET-specific RHPA method, we found that the number of lactotrophs in which ET release was

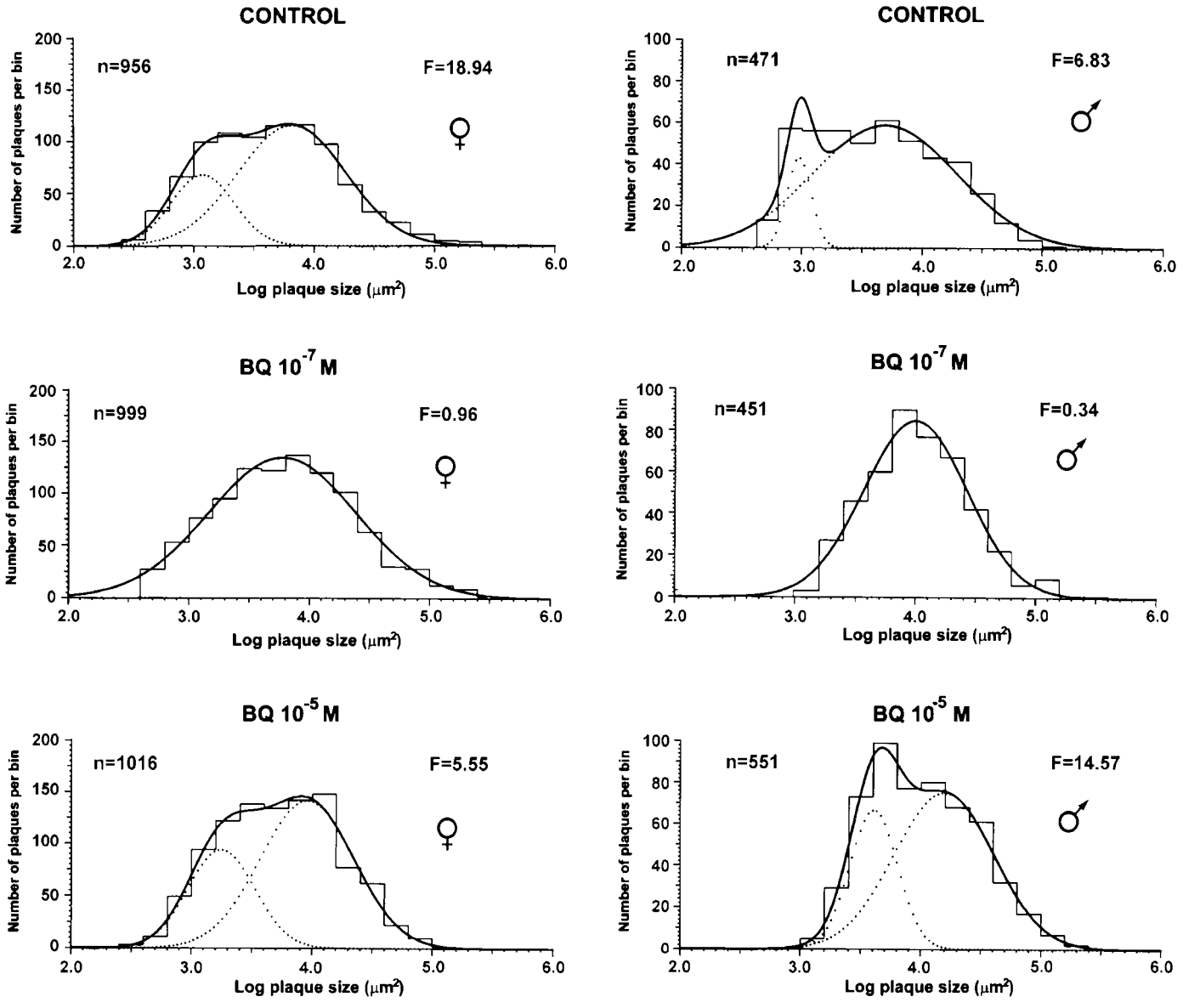


FIG. 6. Frequency distribution of PRL plaque sizes as affected by BQ123 treatment. Logarithmically binned data (bin size, 0.2) were fitted by Gaussian functions using the Levenberg-Marquart method. Second order Gaussian function was accepted if it resulted in a significant improvement of the fit compared with a first order Gaussian function ($F > 3.0$). N values represent the total number of plaques included in each treatment groups.

detectable by hemolytic plaque formation vastly outnumbered the number of ET-positive lactotrophs revealed by ICC. The discrepancy in the data obtained with the two methodological approaches can be resolved by considering that the incidence of ET-positive cells is determined by the intracellular ET contents at the moment of fixation, whereas in the plaque assay the incidence of plaque-forming cells depends on the rate of ET-like peptide secretion and the length of the incubation.

The RHPA was first developed to measure antigen secretion at the single cell level (46) and was adapted later for detection and measurement of hormone secretion from individual pituitary cells in culture (34, 47). This method has became indispensable in studying hormone secretion at the single cell level, and its applications provided important insights concerning heterogeneity and population dynamics of different hormone-producing phenotypes in the pituitary gland (48–54). However, attempts to develop a hemolytic plaque assay for small peptides (*e.g.* vasoactive intestinal polypeptide) have been unsuccessful to date. Rather serendipitously, we found two polyclonal anti-ET antisera that, in the presence of ET-like antigen and guinea pig complement, are capable of inducing hemolytic plaque formation. The observed ET plaques compared with PRL plaques are rather small, but easily detectable. As the presence of ET receptors has already been detected in lactotrophs (19, 31), demonstration of the release of ETs from individual lactotrophs immediately suggested the possibility of autocrine regulation of PRL secretion by ET-like peptides. It is not yet known how the synthesis, storage, and secretion of ETs are regulated

in the lactotrophs or whether the regulation of ET release from the lactotrophs parallels that of PRL secretion. Similarly to that of vascular endothelial cells (14), the regulation of ET synthesis and release is probably coupled in the lactotrophs as well; hence, ET would only be produced in substantial amounts if there is a demand for ETs, presented by a heretofore unidentified stimulus. This scenario could explain why only a small amount of ET1 is actually stored intracellularly.

Interestingly, lactotrophs obtained from cycling female rats showed signs of more vigorous ET secretion than lactotrophs obtained from males. The physiological significance of these observations is not clear at present. It is tempting to speculate, however, that ETs play a role in setting the responsiveness of lactotrophs to hypothalamic PRL-releasing factors. As the action of ETs on PRL secretion is predominantly inhibitory, a higher incidence of ET-secreting lactotrophs in cycling female rats seems compatible with the idea that the overall responsiveness to PRL secretagogues is lower in females than in males (except perhaps a short time window preceding the proestrous PRL surge).

Although our data strongly support a role for ET in autocrine regulation of PRL secretion, they do not rule out other possibilities. As lactotrophs constitute a majority of the cells in the anterior lobe of the pituitary gland, and many of the lactotrophs actively secrete ETs, our data imply that a paracrine type of interaction among lactotrophs through ETs may also exist. In addition, the fact that ET plaque formations were detected among nonlactotrophs leaves open the possibility of an ET-mediated paracrine type of communication between lactotrophs and other, heretofore unidentified, cellular phenotypes.

It has been recognized earlier that the RHPA offers an ideal approach to investigate autocrine regulation, because by applying this method, the secretory activity of a single cell can be assessed quantitatively and without interference from neighboring cells (55–57). In the present study, we have found that pharmacological blockade of $ET_A$ receptors increased PRL secretion. The plausible interpretation of the observed effects with the $ET_A$ receptor antagonists is that these compounds relieve the cells from inhibition exerted by their own secreted ETs. Similarly to $ET_A$ antagonism, inhibition of ET biosynthesis increased PRL secretion. The importance of the latter result is that it supports our interpretation that the effects of $ET_A$ antagonists on PRL secretion are indeed caused by antagonizing endogenously released ET, rather than result from their effects on $ET_A$ receptors as inverse agonists (58). Taken together, these experiments provided evidence that endogenous ETs modulate PRL secretion in an autocrine manner. In addition, as exogenous ET1 could significantly lower PRL secretion, the presumed autocrine inhibition of PRL secretion by endogenous ETs is probably not at its possible maximum. This latter observation could at least in part account for the relatively modest overall increases in PRL secretion induced by $ET_A$ antagonist applications.

The pharmacological data in this study, in agreement with those from previous reports (22, 23, 28), indicated that the effect of endogenous ETs on PRL secretion is predominantly inhibitory in nature. However, the bell-shaped PRL dose-response curve derived from administration of the $ET_A$ antagonist BQ-123 to lactotrophs obtained from female rats also suggests that the autoregulation of PRL secretion by endogenous ETs can be both stimulatory and inhibitory in nature. We propose that the observed bell-shaped dose-response curve with the $ET_A$ antagonist BQ123 resulted from differential sensitivities of the inhibitory and the stimulatory effects of the endogenous ETs toward $ET_A$ antagonism. Under different culture conditions, unusual dose-response curves of the effect of ET1 on PRL secretion have been observed, indicating the complexity of ET's action on PRL secretion (31, 41, 43). However, using monolayer cultures where cell to cell interactions probably persist, the observed biphasic effects of ETs on PRL secretion could not be interpreted unequivocally (31). Our present data indicate that the lactotrophs themselves can perceive the effect of ETs either as stimulatory or inhibitory; therefore, the potentially bidirectional responsiveness to ETs is inherent to lactotrophs and does not require cell to cell interactions.

Analysis of the frequency distribution of different PRL plaque sizes revealed that in terms of PRL-releasing activity, the population of lactotrophs is not homogeneous. In agreement with previous observations (34, 48–53, 57), under basal conditions, two populations of lactotrophs were apparent, one with relatively low and the other with higher PRL-releasing activity. The treatment with $ET_A$ receptor antagonist dose dependently modified the size distribution pattern of lactotrophs. At lower antagonist concentrations, the small plaque-forming population shifted toward the large plaque-forming group, and together they formed an apparently uniform population. At higher concentrations of the $ET_A$ antagonist, the relatively smaller plaque-forming population reappeared. The apparent redistribution of lactotrophs between different subpopulations can be interpreted by assuming that the inhibitory and stimulatory effects of ETs have differential sensitivities toward the $ET_A$ antagonist. At low antagonist concentrations inhibitory influences will be blocked, whereas stimulatory effects are still in place. At higher antagonist concentrations, at least in a certain proportion of lactotrophs, the stimulatory influence of the endogenous ETs will be blocked; hence, the smaller plaque-forming subpopulation reappears. A sequential application of PRL plaque assay under basal conditions as well as in the presence of low and high concentrations of an $ET_A$ antagonist would be necessary to establish whether the same individual lactotroph can respond in both ways to endogenous ETs or whether the stimulation and inhibition by ETs affect two different subpopulations. It seems likely that the cellular responsiveness to ETs and the size of the ET-responsive subpopulation of lactotrophs depend upon the physiological status of the animal. Preliminary data suggest that ovarian steroids might play a decisive role in that respect (39).

Taken together, the most important finding of these studies is that lactotrophs are capable of synthesizing and releasing ETs. By showing that PRL secretion, measured at the single cell level, increased upon $ET_A$ receptor antagonist application, we provided direct evidence that autocrine regulation by ETs is indeed operational in lactotrophs. In addition to vasoactive intestinal polypeptide, which was the first putative autocrine stimulator of PRL secretion described

(56, 59), it has been recently reported that two other peptides with established PRL-releasing capacity, angiotensin II (60) and galanin (61), perform similar regulatory functions. ET is the first regulatory peptide identified that is capable of strong autocrine inhibition of PRL secretion. However, further data derived from *in vivo* experiments are required to assess the relative contribution of the autocrine regulation to the overall control of PRL secretion under varying physiological conditions.

## Acknowledgments

We thank Dr. Albert Parlow for supplying the rat PRL antisera for ICC, and Dr. Gyorgy Nagy for supplying the rat PRL antisera for plaque assay. Kim Riddle is thanked for her assistance with confocal microscopy. The assistance of Jamie DeMaria in reviewing the manuscript is greatly appreciated.

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