Endocrine, *vol. 5, no. 1, 9–14, August 1996* 1046–3976/5/1:9–14/$6.50

Intrapituitary Distribution and Effects of Annexin 5 on Prolactin Release

Mitsumori Kawaminami,[1] Katsumi Okazaki,[1] Shohei Uchida,[1] Nobuyuki Marumoto,[1] Kazuaki Takehara,[2] Shiro Kurusu,[1] Inoru Hashimoto,[1] and Ameae M. Walker[3]

[1]Veterinary Physiology and [2]Poultry Diseases, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori, Japan; [3]Division of Biomedical Sciences, University of California, Riverside, CA

Annexin 5 is expressed by rat anterior pituitary cells and a depolarizing stimulus results in increased extracellular display and, depending on local calcium concentrations, potential release into the extracellular environment. In order to further investigate the role of annexin 5 in anterior pituitary function, we have examined the intracellular distribution by immunocytochemistry and the effects of annexin 5 on the release of a major secretory product, prolactin. Prolactin was chosen because we could easily monitor effects on basal release and effects on the immediate and sustained phases of thyroid stimulating hormone releasing hormone (TRH)-stimulated release. Immunocytochemical localization of annexin 5 showed staining of the majority of anterior pituitary cells. Labeling was predominantly on the nuclear envelope and plasma membrane. For the chosen secretory product, prolactin, annexin 5 was found in most, but not all prolactin positive cells. When recombinant annexin 5 (50 ng/mL) was added to a 3 h static culture incubation of rat anterior pituitary cells, prolactin release was inhibited by about 30% ($p < 0.05$). A lower dose had a reduced effect and higher doses had no further inhibitory effect, indicating that the effect was specific to annexin 5 and not a nonspecific toxic effect of some contaminant in the preparation. This interpretation was further strengthened in a time-course experiment demonstrating that when TRH and annexin 5 were added together, there was no effect of annexin 5 on the amount of prolactin released. After a 3 h preincubation in annexin 5, however, prolactin release, in response to TRH, was suppressed by about 30% in both the acute and sustained phases. These data suggest that annexin 5 may be a local regulator of release in the anterior pituitary, but a slow onset effect on both phases of TRH-stimulated release suggests that this is not an effect at the plasma membrane such as local extracellular calcium depletion by plasma membrane-bound annexin 5.

Key Words: Annexin 5; prolactin; anterior pituitary gland; immunocytochemistry.

Introduction

Annexins are a group of at least 13 structurally related proteins, which have the characteristic of calcium-dependent phospholipid binding. They are widely distributed among species from plants to mammals (Crompton et al., 1988; Flower, 1990; Smith and Dedman, 1990; Moss, 1992; Rainal and Pollard, 1994). The core structure of annexins is four repeats (eight for annexin 6) of a 70 amino acid conserved unit which is responsible for the binding to calcium and phospholipids (Crompton et al., 1988; Klee, 1988; Smith and Dedman, 1990; Moss, 1992). The structure of the calcium binding site is different from the "EF hand" of the classic calcium binding proteins (e.g., calmodulin and troponin). The N terminal segment is variable among annexins and gives a specific feature to each annexin (Moss, 1992; Rainal and Pollard, 1994). Annexins have been shown by in vitro experiments to inhibit blood coagulation (Funakoshi et al., 1987; Tait et al., 1988; Thiagaraja and Tait, 1990), protein kinase C (Toker et al., 1990; Schalaepfer et al., 1992), and phospholipase A_2 (Wallner et al., 1986; Pepinsky et al., 1988). Further, they have been postulated to be included in the process of exocytosis (Drust and Creutz, 1988; Nakata et al., 1990; Creutz et al., 1990; Sarafian et al., 1991), endocytosis (Lin et al., 1991), differentiation (Rainal and Pollard, 1994), and signal transduction (De et al., 1986; Pepinsky and Sinclair, 1986). Some of the annexins (1, 5, and 7) have also been shown to act as a transmembrane calcium channel (Pollard and Rojas, 1988;

Received February 8, 1996; Revised April 4, 1996; Accepted April 18, 1996.
Author to whom all correspondence and reprint requests should be addressed: Mitsumori Kawaminami, Veterinary Physiology, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori, 034, Japan. E-mail: mitsumor@vmas.kitasato-u.ac.jp

Rojas et al., 1990). Annexins are present in many mammalian tissues. Although annexins are expressed by a wide variety of tissues, they are not expressed in all organs and cells (Pepinsky et al., 1988; Rainal and Pollard, 1994). The biological function of annexins in tissues that do express them is largely unknown.

We have reported that annexin 5 is expressed by rat anterior pituitary cells and that a portion of the prolactin in pituitary homogenates comigrates with annexin 5 in native polyacrylamide gel electrophoresis (PAGE) gels (Kawaminami et al., 1992). Also, externalization of annexin 5 by pituitary cells, like exocytosis of prolactin, was increased by a membrane-depolarizing stimulus (Kawaminami et al., 1994). These findings led us to investigate the possibility that annexin 5 could play a role in the regulation of hormone secretion in pituitary cells, especially lactotrophs, by an autocrine or paracrine mechanism. To test this possibility, we have examined the localization of annexin 5 in relation to that of prolactin, i.e., is it in the correct locations to potentially play such a role. Also, because we have recently synthesized recombinant rat annexin 5 using a baculovirus expression system (Takehara et al., 1994), we were able to utilize this material to test the effects of annexin 5 on basal and stimulated prolactin secretion.

Results

The distribution of immunostainable annexin 5 in the anterior pituitary gland is presented in Fig. 1A. Annexin 5 was shown to distribute primarily on the nuclear envelope and plasma membranes of the majority, but not all, anterior pituitary cells (Fig. 1A). Sometimes a less intense staining was seen also in the cytoplasm. Annexin 5 associated with the nucleus was revealed only on the nuclear envelope. Substitution of normal rabbit serum for antiannexin 5 showed no positive staining (Fig. 1B). Also, addition of a crude annexin 5 preparation to the antiannexin, eliminated all positive staining (data not shown).

Double staining with antiprolactin and antiannexin 5 showed some cells to be positive for both (Fig. 1C). Most prolactin cells were positive for annexin 5, whereas not all annexin 5 cells contained prolactin. Cross reactions of the first antibodies and the different secondary antisera were examined. No crossreactivity was seen with different combinations of the first and the secondary antibodies (Figs. 1D,E). Substitution of mouse IgGκ for the monoclonal antiprolactin also showed no positive reaction (Fig. 1F).

When the effect of annexin 5 on prolactin secretion was tested, a three h static incubation with various doses of recombinant annexin 5 showed an inhibition of prolactin release (Fig. 2). A maximal inhibition (30% decrease from control) was attained at 50 ng/mL of recombinant annexin 5 (1.0 ± 0.05 μg/3h/10^6 cells vs 1.4 ± 0.12 in the control). The recombinant annexin 5 was purified by HPLC to a single peak and showed only a single band in SDS-PAGE. To exclude the possibility of interference by small contaminants, however, the medium containing annexin 5 was dialyzed against normal medium (20 vol, overnight with a 14 kDa cutoff) before cells were added. This procedure did not affect the observed inhibition of prolactin release by the recombinant annexin 5 (Fig. 2). Consistent with a specific effect of annexin 5 on secretion was no further inhibition at 100 or 200 ng/mL. Also consistent with an inhibitory effect of annexin on prolactin secretion was an apparent stimulation of prolactin release by the inclusion of antiannexin in a 3 h static incubation (Fig. 3). This apparent stimulation, however, was found not to be statistically significant because of the variability in the responses in the specific antibody samples, despite the fact that any effect seen was always stimulatory.

The effect of annexin 5 on prolactin release was further examined by sequential observation of prolactin release in step-wise infusion experiments where the step-wise infusion totaled 90 min. Addition of TRH ($10^{-7}M$) to culture media dramatically increased prolactin release with an expected two-phase pattern (Fig. 4). The concomitant administration of annexin 5 (50 ng/mL) did not alter prolactin release vs that seen with TRH alone (Fig. 4). Neither did annexin 5 affect nonstimulated prolactin release in this time frame. However, when annexin 5 was applied 3 h before TRH stimulation, the inhibitory effect of annexin 5 was again seen (maximally 33%) (Fig. 5). At 30 min of TRH treatment, prolactin release was 325 ± 32.3 ng/10 min. When annexin was present and had been for a total of 3 h, 30 min release in response to TRH was 217.5 ± 29 ng/10 min.

Discussion

In the present study, we confirmed our earlier observations concerning the distribution of annexin 5 in the anterior pituitary (Kawaminami et al., 1994). In addition, however, we were able to observe the distribution with the greater resolution of confocal microscopy and, by double immunolabeling, were able to determine the distribution relative to a major secretory product, prolactin. Annexin 5 is present on the plasma membrane and/or nuclear envelope of most anterior pituitary cells. With the improved resolution of confocal microscopy we saw no intranuclear labeling. We assume, therefore, that when this was observed in the previous study it represents fluorescence from underlying or overlying nuclear envelopes (Kawaminami et al., 1994). Most, if not all, prolactin-containing cells showed annexin 5 immunostaining, but immunostaining was not limited to prolactin cells.

Since annexin 5 is present on most cells, its function in the pituitary is likely to be common to all. In studying the effect of annexin 5 on secretion, we have used prolactin as an example because it has a high basal release in culture and also shows a two-phase response to TRH.

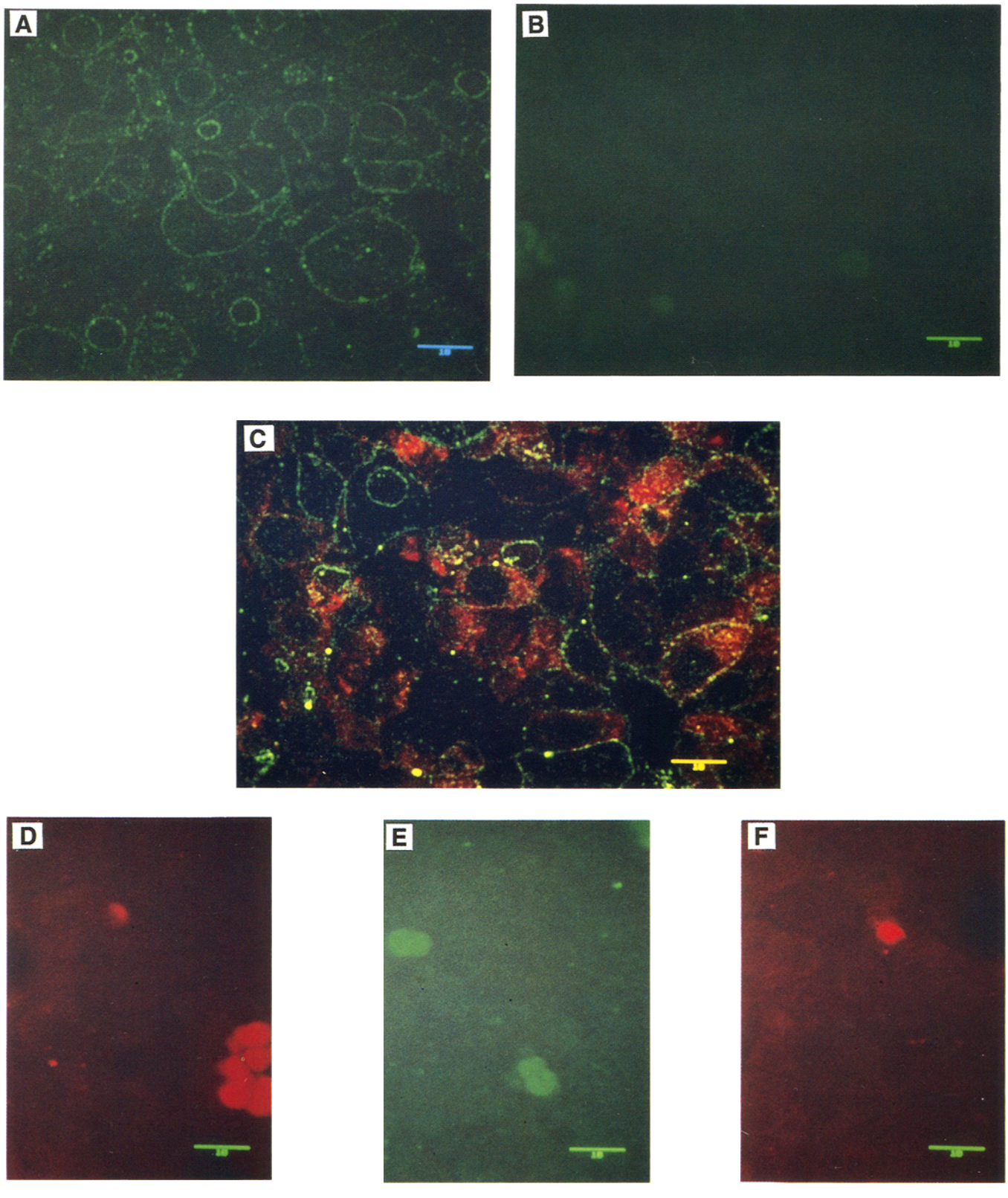

Fig. 1. Immunocytochemical staining of the anterior pituitary gland with antiannexin 5 and antiprolactin. Bars in the figures indicate 10 μm. **(A)** Immunocytochemistry with antiannexin 5. Note annexin 5 distribution predominantly on the nuclear envelope and the plasma membrane. **(B)** Control for staining with antiannexin 5. Normal rabbit serum was used instead of antiannexin 5 rabbit serum. **(C)** Double label immunocytochemistry with antiannexin 5 (fluorescein, shown by green) and antiprolactin (Texas Red, shown by red). **(D)** Control showing no crossreaction of antimouse IgG to antiannexin 5 rabbit serum. The photograph was overexposed by comparison to A and C hence autofluorescence of the red blood cells is seen. **(E)** Control showing no crossreaction of antirabbit IgG to monoclonal antiprolactin. **(F)** Control for staining with antiprolactin. Purified IgGκ was used instead of monoclonal antiprolactin.

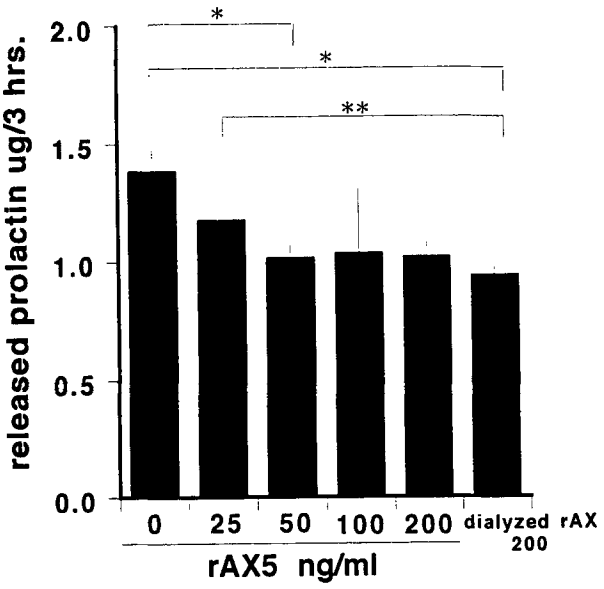

Fig. 2. Effect of annexin 5 on prolactin release. Primary cultures of anterior pituitary cells were prepared from adult female Wistar Imamichi rats. Culture medium was changed every 2 d. On the eighth day of culture, FCS was withdrawn by changing the medium. After a 3-h adjustment to a change of medium and serum removal, the medium was again changed to that containing various concentrations of recombinant annexin 5 (rAX5, 0, 25, 50, 100, 200 ng/mL) and maintained for another 3 h. Additionally, a group in which the medium containing recombinant annexin 5 (200 ng/mL) was dialyzed against normal medium before use was made (dialyzed rAX5 200). Three dishes of cells were prepared for each experimental group. Similar experiments were repeated and representative data are presented. $*p < 0.05$, $**p < 0.01$.

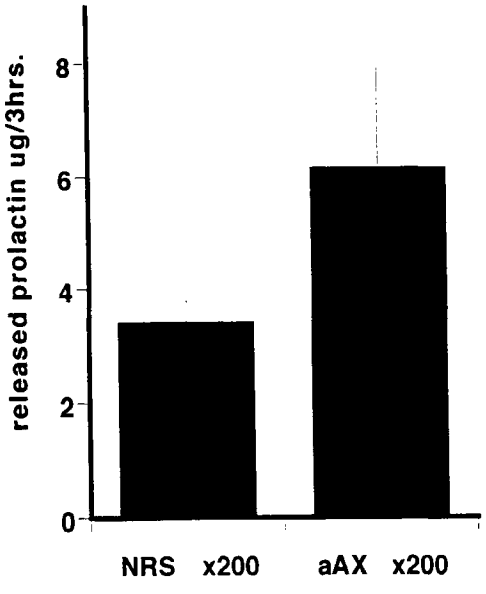

Fig. 3. Effect of antiannexin 5 on prolactin release. Cultures were treated as for Fig. 2 except that antiannexin was added to the second 3 h incubation and normal rabbit serum at the same dilution was added to the control dishes. Normal rabbit serum (NRS) and antiannexin 5 (aAX) were added at a dilution of 1:200 (×200) in serum-free culture medium. Triplicate dishes were used and the experiment was repeated three times with similar results.

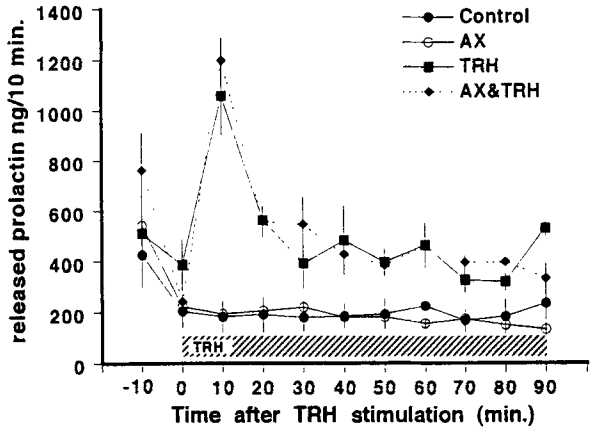

Fig. 4. Administration of recombinant annexin 5 with TRH in a rapid medium exchange system. Dissociated pituitary cells were seeded onto plastic disks placed in Petri dishes. Culture medium was changed every 2 d. On the eighth day of culture, medium was changed to DMEM without FCS by moving the plastic disk to a different dish. Again a 3-h preincubation was used before the pseudoperifusion experiment was started. Three plates per group were subjected to the experiment. Perifusion medium was collected by moving the disks every 10 min. TRH ($10^{-7}M$) and/or recombinant annexin 5 (50 ng/mL) were added at time zero. The duration of TRH stimulation is indicated by a shadowed bar near the bottom of the graph. Control; maintained in DMEM, AX; recombinant annexin 5 (50 ng/mL), TRH; TRH was added from time zero to 70 min, AX and TRH; TRH and recombinant annexin 5 were added.

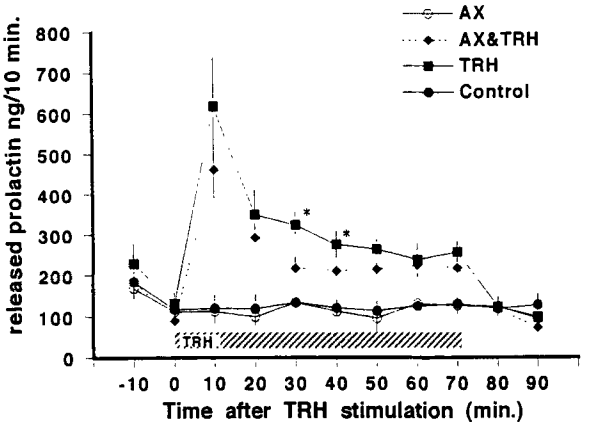

Fig. 5. Effect of recombinant annexin 5 pretreatment on TRH stimulated prolactin release. On the eighth day of culture, medium was changed to DMEM without FCS and with recombinant annexin 5 (50 ng/mL). Thus annexin 5 was present during the usual 3-h incubation. As before, perifusion medium was collected by moving the disks every 10 min. TRH ($10^{-7}M$) and/or fresh annexin 5 were added at time zero. Three disks of cells per group were subjected to the experiment. The duration of TRH stimulation is indicated by a shadowed bar at the base of the graph. Annexin 5 was present throughout the whole experiment in the appropriate samples. *Significantly different from AX and TRH. $p < 0.05$.

The two-phases of TRH-stimulated release have been particularly well characterized with regard to their dependence on extracellular calcium. The acute phase is not dependent on extracellular calcium, whereas the sustained phase is (Gershengorn, 1985). If annexin 5 were to affect prolactin release either by serving as a calcium channel (Rojas et al., 1990) or by binding calcium extracellularly, thereby reducing local free calcium levels, we should have seen an effect confined to the second phase of release. Instead we saw an inhibition in both phases of release.

While the inhibition was modest, it was statistically significant and highly reproducible. A modest response was not unexpected since we were adding to a complement of annexin 5 already on the cell surface. This degree of inhibition is about half that seen with dopamine under similar circumstances (BenJonathan, 1985). That the inhibition observed upon addition of annexin 5 was specific and not a result of small molecular contaminants in the preparation was demonstrated by the reproduction of the results after dialysis. That inhibition was not a result of other contaminants is supported by the lack of further inhibition with the preparation at 100 or 200 ng/mL and the lack of an acute effect on TRH-stimulated release. Even though antiannexin administration did not produce a statistically significant result, if there was an effect it was always stimulatory, consistent with an inhibitory effect of annexin 5 on prolactin release.

From the results demonstrating no inhibition up to 90 min by concomitantly added annexin 5 and TRH and inhibition of the TRH response after a 3 h preincubation in annexin 5, we conclude that it takes more than 90 min for the added annexin 5 to exert its effect. This time frame does not seem to be consistent with an effect at the plasma membrane either through the calcium mechanisms discussed earlier in this section, a change in membrane fluidity (Goosens et al., 1995), or through an effect on phospholipase A_2 (annexin 5 has been considered an inhibitor protein of phospholipase A_2 [Pepinsky et al., 1988]) or through an effect on the exocytosis of already formed secretory granules. We hypothesize, therefore, that annexin 5 is exerting its effect through some much more time-consuming event such as prolactin gene expression or prolactin synthesis or the production of new secretory granules. The intracellular localization of annexin 5 to the nuclear envelope gives us no real clue in this regard. While the nuclear envelope is a functional part of the endoplasmic reticulum, the rest of the endoplasmic reticulum was largely unstained. Further experimentation will, therefore, be necessary to determine where and how annexin exerts its effect. Nevertheless, we have succeeded in demonstrating the production of annexin 5 by pituitary cells and an effect on the release of a major secretory product. Furthermore, our data would suggest that the effect of annexin 5 on prolactin release is not via any of the previously proposed mechanisms of action of annexins.

Materials and Methods

Materials

The antiannexin 5 rabbit serum used for the immunocytochemical experiments was kindly donated by Dr. Pepinsky (Biogen Research Corp., Cambridge, MA). The monoclonal antiovine prolactin (6F11) was generously supplied by Dr. J. Scammell, University of South Alabama, Mobile, AL. The reactivity of the antibody to rat prolactin has been previously demonstrated (Scammel et al., 1990). Mouse IgGκ and TRH were obtained from Sigma Chemical Co. (St. Louis, MO). Antirabbit IgG fluorescein and antimouse IgG Texas Red were purchased from Cappel (Durham, NC). Recombinant rat annexin 5 was prepared using the baculovirus expression system (Takehara et al., 1994) and purified by reverse phase HPLC, Wakosil 5C18-200 (Wako Pure Chemicals LTD., Osaka, Japan) to a single peak fraction, which showed only one single band in SDS-PAGE. Some of the purified recombinant annexin 5 was dialyzed against culture medium (20 times of volume, overnight, cut off MW 14,000) before being added to cells.

Immunocytochemistry

Pituitary glands were obtained from adult female Sprague-Dawley rats (Bantin and Kingman, Fremont, CA). Each anterior pituitary gland was cut into pieces and fixed with neutralized formalin (10%) in an ice bath overnight. The tissue was dehydrated in an ethanol series to 95% and infiltrated with JB-4 resin (Polysciences, Inc., Warrington, PA), and embedded. Tissue blocks were sectioned with a glass knife to a 2-μm thickness.

Immunocytochemistry was performed on freshly cut sections using rabbit antirat annexin 5 serum (1:50 dilution) as a first antibody and antirabbit IgG fluorescein (1:50, Organon Teknika Corp., Durham, NC) as a second antibody. Antibody-binding buffer (50 m*M* Tris-HCl, 5 m*M* EDTA, 150 m*M* NaCl, 0.25% gelatin, 0.05% Nonidet P40; Sigma Chemical Co.) was used for the dilution of antisera. After each incubation with antibody, slides were washed by changing the antibody-binding buffer three times. The specimens were observed with a confocal microscope system (BioRad, Cambridge, MA and Nikon, Grand City, NY). When double staining was performed, incubation with the mixed solution of antiannexin 5 and antiprolactin (1:100) was followed by the second antibodies of antirabbit IgG fluorescein and antimouse IgG-Texas Red (1:600) (Organon Teknica Corp.). A control group for each staining was made by replacing specific antibody with normal rabbit serum or mouse IgGκ, respectively. Further, crossreactivity of each second antibody to the different primary antibodies was also checked.

Primary culture of pituitary cells: Dulbecco's Modified Eagle's Medium (DMEM, Gibco Lab., Grand Island, NY) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), nonessential amino acids (Difco Lab., Detroit, MI) and 10% FCS, was used for primary culture of pitu-

itary cells. Anterior pituitary cells were dissociated from adult female Wistar Imamichi rats as reported previously by Walker and Farquhar (1980). Briefly, anterior pituitary glands of rats were cut into about 1 mm^3 pieces and incubated in a spinner flask containing trypsin solution (0.25%, 1:250 trypsin, Difco Lab.) for 30 min at 37°C. Tissue pieces were washed and mechanically disrupted by passage through flame-polished Pasteur pipets. Cells were washed and resuspended in culture medium to 2.5×10^5/mL. Cells were cultured in a plastic dish (35 mm, Nunc Inc., Naperville, IL) or on a plastic disk (Sumilon, Sumitomo, Tokyo, Japan) placed in a dish, and were maintained in a 5% CO_2 atmosphere at 37°C. The medium was changed every 2 d and experiments were performed on the eighth day of culture.

On the eighth day of culture, the medium was changed to DMEM supplemented with 20 m*M* HEPES (pH 7.6) without FCS. After a 3-h preincubation, the first set of experiments tested the effect of recombinant annexin 5 or antiannexin on a subsequent 3-h static incubation. In other experiments, sequential changes in prolactin release were monitored by collecting medium every 10 min after moving cells on the plastic disk to the next dish containing fresh medium. The dishes containing fresh medium were prepared, warmed before the experiments and maintained in the CO_2 incubator (5% CO_2, 37°C). The administration of TRH and/or recombinant annexin 5 is described in the results section and figure legends.

Radioimmunoassay

The amount of prolactin in the media samples was measured by RIA using reagents obtained from the Pituitary and Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases. These included a standard preparation, RP-3, antiPRL serum, S-9, and highly purified PRL for iodination, I-5. Intra- and inter-assay variations were 9.6% and 15.9%, respectively. The presence of annexin 5 has no effect on the assayability of prolactin.

Statistics

Differences of the mean between experimental groups were analyzed by ANOVA and Student's *t*-test.

Acknowledgment

The authors appreciate the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, for the gift of the PRL assay kit.

References

BenJonathan, N. (1985). *Endocrin. Rev.* **6,** 564–589.

Creutz, C. E., Drust, D. S., Hamman, H. C., Junker, M., Kambouris, N. G., Klein, J. R., Nelson, M. R., and Snyder, S. L. (1990). In: *Stimulus Response Coupling. The Role of Intracellular Calcium-Binding Proteins.* Smith, V. L. and Dedman, J. R. (eds.). CRC: Boston, pp. 279–310.

Crompton, M. J., Moss, S. E., and Crumpton, M. J. (1988). *Cell* **55,** 1–3.

De, B. K., Misono, K. S., Lukas, T. J., Mroczkowski, B., and Cohen, S. (1986). *J. Biol. Chem.* **261,** 13,784–13,792.

Drust, D. S. and Creutz, C. D. (1988). *Nature* **331,** 88–91.

Flower, R. J. (1990). In: *Cytokines and Lipocortins in Inflammation and Differentiation.* Wiley-Liss, New York, pp. 11–25.

Funakoshi, T., Hendrickson, L. E., McMullen, B. A., and Fujikawa, K. (1987). *Biochemistry* **26,** 8087–8092.

Gershengorn, M. C. (1985). *Recent Prog. Hormone Res.* **41,** 607–653.

Goossens, E. L. J., Reutelingsperger, C. P. M., Jongsma, F. H. M., Kraayenhof, R., and Hermens, W. T. (1995). *FEBS Lett.* **359,** 155–158.

Kawaminami, M., Tanaka, L., Asawa, T., Osugi, S., Kawauchi, H., Kurusu, S., and Hashimoto, I. (1992). *Biochem. Biophys. Res. Commun.* **186,** 894–899.

Kawaminami, M., Uchida, S., Marumoto, N., Naito, K., Okazaki, K., Sato, Y., Kurusu, S., Hashimoto, I., Mutoh, K., and Walker, A. M. (1994). *Endocrine J.* **2,** 357–362.

Klee, C. B. (1988). *Biochemistry* **27,** 6645–6653.

Lin, H. C., Sudhof, T. C., and Anderson, G. W. (1992). *Cell* **70,** 283–291.

Moss, S. E. (1992). In: *The Annexins.* Moss, S. E. (ed.). Portland: London, pp. 1–9.

Nakata, T., Sobue, K., and Hirokawa, N. (1990). *J. Cell. Biol.* **110,** 13–25.

Pepinsky, R. B. and Sinclair, L. K. (1986). *Nature* **321,** 81–84.

Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K.-S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z., and Wallner, B. P. (1988). *J. Biol. Chem.* **263,** 10,799–10,811.

Pollard, H. B. and Rojas, E. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 2974–2978.

Rainal, P. and Pollard, H. B. (1994). *Biochim. Biophys. Acta* **1197,** 63–93.

Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C. and Burns, A. L. (1990). *J. Biol. Chem.* **265,** 21,207–21,215.

Sarafian, T., Pradel, L.-A., Henry, J.-P., Aunis, D. and Bader, M. F. (1991). *J. Cell. Biol.* **114,** 1135–1147.

Scammell, J. G., Scott, M. G., Outlaw, K. K., Thompson, M. E. and Belen, R. B. (1990). *J. Histochem. Cytochem.* **38,** 117–122.

Schalaepfer, D. D., Jones, J., and Haigler, H. T. (1992). *Biochemistry* **31,** 1886–1891.

Smith, V. L. and Dedman, J. R. (1990). In: *Stimulus Response Coupling. The role of Intracellular Calcium-Binding Proteins.* Smith, V. L. and Dedman, J. R. (eds.). CRC, Boston, pp. 1–19.

Tait, J. F., Sakata, M., McMullen, B. A., Miao, C. H., Funakoshi, T., Hendrickson, L. E., and Fujikawa, K. (1988). *Biochemistry* **27,** 6268–6276.

Takehara, K., Uchida, S., Marumoto, N., Asawa, T., Osugi, S., Kurusu, S., Hashimoto, I., and Kawaminami, M. (1994). *Biochem. Biophys. Res. Commun.* **200,** 1421–1427.

Thiagaraj, P. and Tait, J. F. (1990). *J. Biol. Chem.* **265,** 17,420–17,423.

Toker, A., Ellis, C. A., Sellers, L. A., and Aitken, A. (1990). *Eur. J. Biochem.* **191,** 421–429.

Walner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Pingchang Chow, E., Browning, J. L., Ramachandran, K. L., and Pepinsky, R. B. (1986). *Nature* **320,** 77–81.

Walker, A. M. and Farquhar, M. G. (1980). *Endocrinology* **107,** 1095–1104.