Calmodulin inhibitors block prolactin secretion and accumulate secretory granules in agranular clonal cells *in vitro*

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ABSTRACT

1G4 Clonal cells derived from Rathke's epithelium of the fetal rat are essentially agranular when grown *in vitro*, in spite of their active secretion of prolactin. They may secrete prolactin from exocytotic pits originating from vesicles (90 nm in diameter). When the cells are incubated in growth medium supplemented with calmodulin inhibitors, W7 or W5, for 3 h, prolactin release is inhibited and a number of secretory granules (160–200 nm in diameter) appear.

Prolactin (PRL) cells in pituitary gland usually contain secretory granules and release them by exocytosis. However, the 1G4 clonal cells are essentially agranular in spite of their active secretion of prolactin (8). Landolt and Rothenbühler (9) hypothesize that the size of secretory granules is determined by functional factors such as the speed of hormone synthesis and release, but not by the type of hormone secreted.

A number of neuroleptic drugs have been found to bind to the Ca^{2+} -calmodulin complex and inhibit its action (10). Trifluoroperazine and prochlorperazine inhibit prolactin secretion from isolated pituitary cells (12), and pimozide also inhibits the gonadotropin-releasing hormone (GnRH)-stimulated LH release from pituitary cell culture (2).

W7[N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] (5) is a specific inhibitor of Ca^{2+} -calmodulin complex, and also inhibits the secretion of insulin (22). W5[N-(6-aminohexyl)-1-naphthalenesulfonamide], an analog of W7, decreases the affinity for calmodulin (6). Both W7 and W5 easily penetrate the intercellular space *in vitro* (6), making W7 and its derivatives useful tools for research on calmodulin in cell biology.

In this study, the agranular PRL-secreting 1G4 clonal cells, were used to reveal 1) what type of secretions could occur and 2) whether secre-

tory granule formation is related to the rate of hormone release that may be regulated by calmodulin.

The PRL-secreting agranular cells, 1G4, were cultured in growth medium (Ham's F10, 82.5%; 5% fetal calf serum, 10% newborn calf serum, and 2.5% donor horse serum) supplemented with Streptomycin (50 $\mu\text{g}/\text{ml}$) and Penicillin (50 U/ml). All sera were obtained from Flow Laboratories (Rockville, MD). The calmodulin inhibitors, W7 and W5 (both obtained from Rikaken Co., Kyoto, Japan), were dissolved first in 100% ethanol, while the final concentration of ethanol in the growth medium was below 0.5%. Then 4×10^6 cells/2ml/dish were cultured in growth medium supplemented with 50 μM of W7 or W5 for 3 h, respectively. After incubation, cells were removed by a rubber policeman and precipitated by centrifugation for electron microscopic observations, and the conditioned media were stored at -70°C until radioimmunoassay.

Electron micrography revealed that 1G4 clonal cells have a number of polysomes, several lysosomes, oval or spherical mitochondria containing few cristae, and a well developed Golgi apparatus; however, the rough-endoplasmic reticulum (r-ER) was poorly developed (Fig. 1). The cells were essentially agranular except for small vesicles surrounding mostly the Golgi apparatus and were similar to 2B8 clonal cells

Fig. 1 Electron micrograph of IG4 agranular prolactin (PRL) cells. A well-developed Golgi apparatus (G), and a number of polysomes are observed, but the rough-endoplasmic reticulum (ER) is not well developed. However, many exocytotic pits (arrows; 90 nm in diameter), vesicles (90 nm), subsurface vesicles (90 nm) and apparently nonconcentrated vesicles (90 nm; arrowheads) were observed. A few electron low-density spherical structures (90 nm in diameter) are also observed in the control culture. L: lysosome, N: nucleus. $\times 13,200$. IG4 agranular clonal cells, derived from rat Rathke's epithelium, were cultured in the growth medium specified in the text. They were removed from the bottom-surface of the Petri dish (60×15 mm, Petray, Japan) by using a rubber policeman designed for electron microscopic observations. The cells were fixed with 1% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.4 for 30 min. Then, they were rinsed several times with 2% sucrose in phosphate buffer solution, and were postfixed with 1% OsO_4 in Millonig's buffer (13) at pH 7.4 for 30 min, followed by dehydration with ethanol, propylene oxide, and embedded in Epon-Araldite resin (14). Polymerization of the resin was carried out at 40°C for 10 days. The sections were stained with uranyl acetate and lead citrate according to specific directions.

(7) derived from the same NRR5 cell line (8). Even though the cells were agranular, a number of exocytotic pits and subsurface vesicles about 90 nm in size were frequently observed. When the clonal cells were cultured with the calmodulin inhibitor, a number of secretory granules, about 160–200 nm in diameter, appeared (Fig. 2) which were localized to one side in the cells. Morphological characteristics of these granulated cells were very similar to those of Nogami's PRL cells which had small spherical granules about 130–200 nm in diameter. Nogami's cells contained poorly developed r-ER, and secretory granules were also located to one side of the

cells (18). Agranular cells or cells containing few secretory granules were observed among Nogami's cells. When calmodulin inhibitors were added to the growth medium, the number of lysosomes increased which were probably required for degrading the excessive amount of PRL. Lipid droplets were also present which may have originated from the lysosomes (21). When 2×10^6 IG4 cells were cultured in the absence of calmodulin inhibitors, prolactin secretion during 3 h amounted to 3,471 ng. W7 (50 μM) incorporated in the culture medium inhibited the prolactin secretion to 36.5% of the control level (Fig. 3). W5 (50 μM) also inhibited

Fig. 2 Immuno-electron micrograph of 1G4 PRL cells incubated with $50 \mu\text{M}$ of W7 in growth medium for 3 h. A number of secretory granules and a few lipid droplets (L) were observed. The number of lysosomes (arrowheads) increased in cells cultured with the calmodulin inhibitor. Higher magnification of the enclosed square area (inset) reveals many immunoreactive products on the secretory granules. Electron immunocytochemical staining was carried out according to the post-embedding method of Nogami and Yoshimura (19). Specificity of the rat PRL antiserum had been confirmed by the absorption test and radioimmunoassay system (19). M: mitochondria, N: nucleus. $\times 9,200$. Inset, $\times 53,000$

the secretion, though to a lesser extent compared with W7 (Fig. 3).

Hormone release is stimulated when manipulations increase cytosolic calcium (15). However, the precise role of intracellular calcium in the secretory process is still unknown. Calmodulin, a protein that has a high affinity and specificity for calcium, has been structurally and functionally preserved throughout the animal and plant kingdom. There is an increasing body of evidence demonstrating a role for calmodulin in many Ca^{2+} -regulated functions (1). W7 and W5 are specific calmodulin inhibitors, but affect neither the rate of glucose oxidation in the pancreatic islets nor the total incorporation of [^3H]leucine into proinsulin and insulin (17). Meanwhile, neuroleptic drugs usually inhibit not only calmodulin but also the enzyme activity (17, 20, 16).

In this study, W7 and W5 also inhibit PRL

secretion, decrease the number of exocytotic pits and vesicles, and cause an accumulation of a number of secretory granules *in vitro*. These results strongly suggest that the prolactin release from agranular cells may be accomplished by the exocytotic pits of vesicles, as postulated by Gumbiner and Kelly (4), and others (3, 11). It is also suggested that calmodulin regulates hormone granule formation and secretion. Therefore, in the presence of calmodulin, granule formation and secretion rates of 1G4 agranular cells are too rapid to produce electron dense and large secretory granules.

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Fig. 3 Amount of released prolactin by control IG4 clonal cells and by clonal cells cultured with calmodulin inhibitors, W7 or W5. The cells were incubated in growth medium supplemented with or without calmodulin inhibitors, W7 or W5 (50 µM each), for 3 h. Prolactin released into the medium was measured in duplicate by radioimmunoassay using the assay system, National Pituitary Agency, NIAMDD, National Institutes of Health (Bethesda, MD). The results were expressed in terms of NIAMDD rPRL-RP-1, and the significance was assessed by analysis of variance.

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