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The calmodulin antagonist W-7 depletes intracellular calcium stores in FRTL-5 thyroid cells

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Incubating Fura 2 loaded thyroid FRTL-5 cells with the calmodulin inhibitor W-7 decreased the ATP-evoked increase in intracellular free calcium. In addition, pretreatment of the cells with W-7 decreased both the thapsigargin-evoked release of sequestered calcium and the entry of extracellular calcium. Studies with $^{45}Ca^{2+}$ showed that W-7 decreased the amount of sequestered calcium in the cells. Furthermore, after stimulating with ATP, the ability to sequester calcium was decreased in cells treated with W-7 compared with control cells. The results suggest that calmodulin is necessary for the signal-transduction system in FRTL-5 cells, and may be especially important in maintaining functional intracellular calcium stores in the cells.

Recent investigations in thyroid FRTL-5 cells suggest that stimulating the cells with the purinergic agonist ATP rapidly activates the release of sequestered calcium due to the production of inositol-1,4,5-trisphosphate (IP_3), and activates influx of extracellular calcium (1,2). Furthermore, FRTL-5 cells exerts a significant influx of calcium in response to the Ca^{2+}ATPase inhibitor thapsigargin (3). Recent studies in other cells have suggested that calmodulin may be of importance in the IP_3-induced release of sequestered calcium (4). In addition, calmodulin has also been suggested to be involved in the activation of putative ligand-activated calcium channels (5). The aim of the present study was to evaluate whether the calmodulin inhibitor N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W-7) could modify ATP- and thapsigargin-evoked calcium fluxes in FRTL-5 cells.

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Materials and Methods

Materials. Culture medium, serum and hormones needed for the cell culture were purchased from Gibco (Grand Island, NY) and Sigma (St. Louis, MO). Culture dishes were obtained from Falcon Plastics (Oxnard, CA). ATP and W-7 were purchased from Sigma. Thapsigargin was purchased from LC Services Corp (Woburn, MA). Fura 2-AM was purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals used were of reagent grade.

Cell culture. Rat thyroid FRTL-5 cells were a generous gift of Dr. Leonard D. Kohn (NIH, Bethesda, MD). The cells were grown in Coon's modified Ham's F 12 medium, supplemented with 5 % calf serum and six hormones (6) (insulin, 10 µg/ml; transferrin, 5 µg/ml; hydrocortisone, 10 nM; the tripeptide gly-L-his-L-lys, 10 ng/ml; TSH, 1 mU/ml; somatostatin, 10 ng/ml) as described previously (2). The cells were grown for 7-8 days before an experiment, with 2-3 changes of the culture medium. Fresh medium was always added 24 h prior to an experiment.

Measurement of $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$fluxes. The cells were prepared for measurements of $[Ca^{2+}]_i$ as described previously (2). Fura 2 fluorescence was measured with a Hitachi F2000 fluorimeter. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM $CaCl_2$ and digitonin to obtain F_{max}, and by chelating extracellular Ca^{2+} with 5 mM EGTA. To obtain F_{min} Tris-base was used to elevate pH above 8.3. $[Ca^{2+}]_i$ was calculated as described by Gryenkiewicz et al. (7), using a computer program designed for the fluorimeter with a K_d-value of 224 nM for Fura 2. $^{45}Ca^{2+}$-flux studies were performed exactly as described previously (2).

Results and Discussion

Recent studies have shown that calmodulin inhibitors, including W-7, may impair the release of sequestered calcium, without affecting the ability of the cells to sequester Ca^{2+} (4). However, in permeabilized pancreatic acini and in isolated endoplasmic vesicles (8), W-7 potentiated IP_3-induced release of Ca^{2+}. Stimulating FRTL-5 cells with 100 µM ATP rapidly increased $[Ca^{2+}]_i$ by 401 ± 10 nM. In cells pretreated with 100 µM W-7, the ATP-evoked transient was only 140 ± 51 nM ($p < 0.05$, Fig. 1). In cells incubated in nominally Ca^{2+}-free buffer containing 100 µM EGTA, the ATP-evoked increase in $[Ca^{2+}]_i$ was 263 ± 23 nM. In cells pretreated with W-7 , the increase in $[Ca^{2+}]_i$ was only 79 ± 23 nM ($p < 0.05$, Fig. 2).

To release sequestered Ca^{2+} from intracellular stores without activating the production of IP_3, the cells were stimulated with

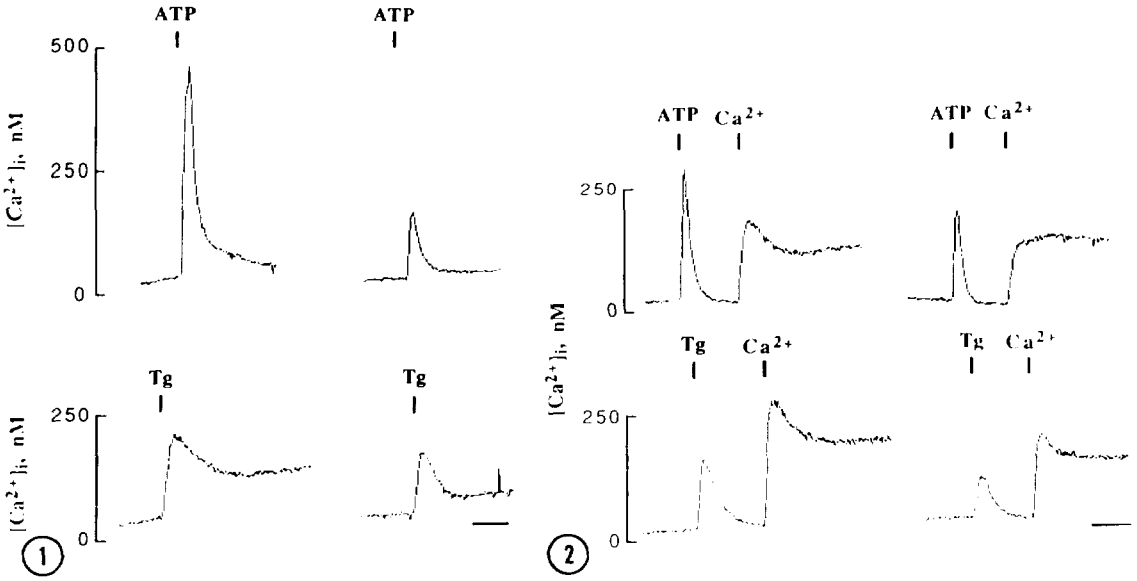

Figure 1. Action of W-7 on ATP- and thapsigargin-induced changes in $[Ca^{2+}]_i$. Control cells (left traces) and cells pretreated with 100 μM W-7 for 30 min (right traces) were stimulated with 100 μM ATP or 2 μM thapsigargin (Tg).

Figure 2. Action of W-7 on ATP- and thapsigargin-induced changes in $[Ca^{2+}]_i$ in cells incubated in Ca^{2+}-free buffer. Control cells (left traces) and cells pretreated with 100 μM W-7 for 30 min (right traces) were stimulated with 100 μM ATP or 2 μM thapsigargin (Tg). After termination of the response 1 mM Ca^{2+} was added to the cell suspension.

thapsigargin. This inhibitor of endoplasmic Ca^{2+}ATPase (9) releases sequestered calcium and activates influx of extracellular Ca^{2+} in FRTL-5 cells (3). In control cells, thapsigargin transiently increased $[Ca^{2+}]_i$ by 191 ± 9 nM which then stabilized at a new steady-state level 148 ± 17 nM above prestimulatory $[Ca^{2+}]_i$ levels (Fig. 1). In cells treated with W-7 the initial increase in $[Ca^{2+}]_i$ was not significantly altered (147 ± 24 nM) but the new elevated phase was decreased (55 ± 25 nM, $p < 0.05$, Fig. 1). In Ca^{2+}-free buffer the thapsigargin-induced transient in $[Ca^{2+}]_i$ was 143 ± 17 nM in control cells, and 66 ± 5 nM ($p < 0.05$) in cells pretreated with W-7.

Addition of Ca^{2+} to cells stimulated with ATP in Ca^{2+}-free buffer transiently increased $[Ca^{2+}]_i$ by 178 ± 15 nM which then stabilized at a new level 116 ± 8 nM above prestimulatory level. In W-7 pretreated cells the initial response in $[Ca^{2+}]_i$ was decreased (75 ± 18 nM, $p < 0.05$) but the plateau phase was not changed (74 ± 18 nM, Fig. 2). In cells stimulated with thapsigargin addition of Ca^{2+} increased $[Ca^{2+}]_i$ by 311 ± 38 nM which then stabilized at 192 ± 10 nM above prestimulatory level.

After pretreatment with W-7, both the initial increase in $[Ca^{2+}]_i$ (101 ± 20 nM) and the plateau level (80 ± 12 nM) were decreased ($p < 0.05$, Fig. 2).

In a recent study it was shown that addition of exogenous calmodulin inhibited the release of $^{45}Ca^{2+}$ from sarcoplasmic reticulum vesicles (10). To further investigate the effect of W-7 on the ability of FRTL-5 cells to sequester Ca^{2+}, the cells were loaded with $^{45}Ca^{2+}$, and the efflux of $^{45}Ca^{2+}$ was measured in the presence and absence of W-7. The result in Fig. 3 A show that in cells incubated with 100 μM W-7 the cell-associated $^{45}Ca^{2+}$ decreased more than in control cells. The effect of W-7 was dose dependent (not shown). The effect of W-7 on uptake of $^{45}Ca^{2+}$ was also investigated. No effect of W-7 was observed on basal uptake of $^{45}Ca^{2+}$ (not shown), but the ATP-induced uptake of $^{45}Ca^{2+}$ was decreased compared with control cells (Fig. 3 B).

The results in the present report show, that inhibiting calmodulin has a profound effect on calcium metabolism in FRTL-5 cells. Although a nonspecific effect of W-7 on FRTL-5 cells cannot be ruled out (11), our data suggest that calmodulin is of importance in maintaining sequestered

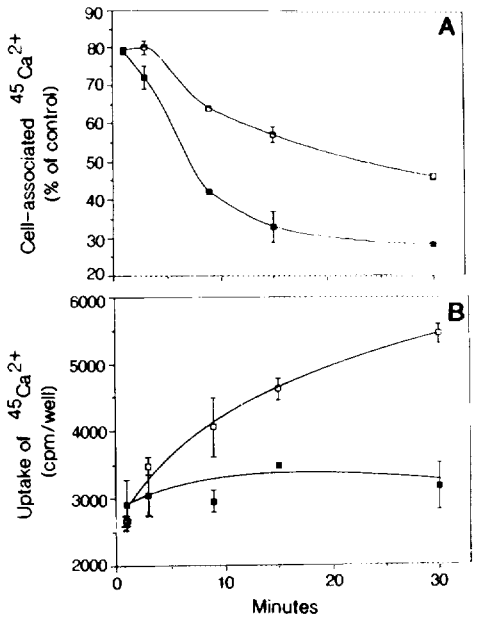

Figure 3. Action of W-7 on efflux of $^{45}Ca^{2+}$ and the ATP-induced uptake of $^{45}Ca^{2+}$. Upper panel. Cells loaded with $^{45}Ca^{2+}$ were stimulated with 100 μM W-7 (■) or buffer only (□) and the cell-associated $^{45}Ca^{2+}$ was measured. Lower panel. Control cells (□) or cells pretreated with 100 μM W-7 (■) were stimulated with 100 μM ATP and the cell-associated $^{45}Ca^{2+}$ was measured. Each point gives the mean ± SE of triplicate dishes.

Ca^{2+} at least in IP_3-sensitive intracellular organelles. This could be due to calmodulin being part of the IP_3-receptor/Ca^{2+} release channel (4), or that calmodulin is necessary for maintaining a functional Ca^{2+}ATPase in these organelles. We could not observe an acute effect (preincubations with W-7 2-3 min prior to agonist) of W-7 on ATP- or thapsigargin-evoked entry of extracellular Ca^{2+}, although calmodulin has been proposed to be an integral part of ligand-gated Ca^{2+} channels (5). Furthermore, the W-7-induced leakage of sequestered calcium made it impossible to distinguish an effect of W-7 on the entry of calcium after stimulation with ATP or thapsigargin. However, the results suggest that calmodulin is an important part of the signal-transduction system in FRTL-5 cells, and may be especially important in maintaining functional intracellular calcium stores in these cells.

Acknowledgments

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