The Role of Calmodulin in Rat Parotid Amylase Secretion: Effects of Calmodulin Antagonists on Secretion and Acinar Cell Structure

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Abstract—Using dispersed rat parotid cells, the effects of three calmodulin antagonists, trifluoperazine (TFP), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), and N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), on amylase release and acinar cell structure were examined. TFP and W-7 strongly inhibited both isoproterenol (ISO) and dibutyryl cyclic AMP-stimulated amylase release at a concentration of 50 or 100 \(\mu\)M, while W-5, a weak calmodulin antagonist, had only little effect. Cyclic AMP level was markedly elevated by ISO even in the presence of TFP or W-7. These results indicate that the calmodulin antagonists affect amylase release at steps distal to cyclic AMP metabolism. Electron micrographs demonstrated that treatment of parotid cells with either TFP or W-7 caused a loss of luminal microvilli and surface folds. When cells were stimulated by ISO in the presence of TFP or W-7, the enlarged lumina did not recover to their original size and the discharged secretory material was retained in the lumina. Numerous secretory granules remained in the acinar cytoplasm. W-5 affected the acinar cell structure only a little. These observations lead to the assumption that TFP and W-7 interfered with the normal functions of the cytoskeletal system. It is proposed that calmodulin may be involved in the exocytosis of parotid amylase through the regulation of the cytoskeletal system.

Amylase release from rat parotid glands is induced through the activation of neurotransmitter receptors such as \(\beta\)- and \(\alpha\)-adrenergic, muscarinic, and substance P receptors (1). The activation of \(\beta\)-adrenergic receptors promotes amylase release most strongly, while smaller amounts of amylase are induced by the activation of the other receptors. The amylase release by the \(\beta\)-adrenergic receptor stimulation is thought to be mediated through an increase in cellular cyclic AMP (cAMP) and not to directly require extracellular Ca\(^{2+}\) (1). However, intracellular Ca\(^{2+}\) may also play an important role in the amylase release, because depletion of intracellular Ca\(^{2+}\) by incubating parotid slices or cells in a Ca-free medium containing EGTA reduces the amylase release (2–4), and \(\beta\)-adrenergic receptor stimulation has been suggested to mobilize Ca\(^{2+}\) from intracellular pools (3, 5–8). On the other hand, the amylase release induced by \(\alpha\)-adrenergic, muscarinic, and substance P receptor stimulation appears to be directly dependent on intra- and extracellular Ca\(^{2+}\) (1).

Calmodulin has been accepted to play a central role in regulating a variety of Ca\(^{2+}\)-dependent cellular functions (9). Calmodulin antagonists such as phenothiazins and naphthalenesulfonamides are available for investigating the physiological role of calmodulin. Experiments with these antagonists have suggested that calmodulin is implicated in many exocrine (10–12) and endocrine (13–17) secretory functions. In the previous study (18), we reported that amylase secretion induced by \(\beta\)-adrenergic receptor stimulation was inhibited by trifluoperazine (TFP)
and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) in a dose-dependent manner. The IC50 values (concentration producing a 50% inhibition) were 22 μM with TFP and 42 μM with W-7. However, it has not been exactly determined which step in the secretory process is affected by the calmodulin antagonists.

Calmodulin has been shown to associate tightly with the cytoskeletal elements in a variety of mammalian cells (19–23). Since the cytoskeletal system is thought to play an essential role in maintaining cell structure, it is possible that inhibition of calmodulin by the antagonists may result in changes in cell structure through suppression of the normal function of the cytoskeletal system. In addition to the effects on amylase release and cAMP accumulation, this study examines the effects of TFP and W-7 on the ultrastructure of dispersed rat parotid acinar cells. N-(6-Aminohexyl)-1-naphthalenesulfonamide (W-5), a chlorine-deficient analogue of W-7 which has a lower affinity for calmodulin than W-7 (24), was used as the control substance for W-7.

**Materials and Methods**

**Reagents:** The ISO, dibutyryl cyclic AMP (DBcAMP), carbachol, TFP, bovine testis hyaluronidase (Type 1-S) and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). Collagenase (CLS II) was obtained from Cooper Biomedical (Malvern, PA, U.S.A.); Hanks’ balanced salt solution, from Gibco (Chagrin Falls, OH, U.S.A.); and W-7 and W-5, from Seikagaku Kogyo (Tokyo, Japan). All other reagents were from Wako Pure Chemical (Osaka, Japan).

**Cell preparation:** Male Wistar-strain rats, weighing 250–300 g, were anesthetized with ether and killed by cardiac puncture. Dispersed parotid acinar cells were prepared according to the method of Takuma and Ichida (25). Briefly, parotid glands from one rat were excised, minced finely and incubated for 60 min at 37°C in 10 ml of Hanks’ balanced salt solution buffered with 20 mM Hepes-NaOH, pH 7.4 (HBSS-H) containing collagenase (100–150 units/ml) and hyaluronidase (0.25 mg/ml) under 95% O2–5% CO2. At 20 min intervals, the minced tissue was gently pipetted and gassed. Thereafter, cells were filtered through two layers of gauze, washed twice with HBSS-H containing 0.1% bovine serum albumin and suspended in 30–50 ml of the same medium.

**Measurement of amylase release:** A 1.0 ml aliquot of cell suspension was pre-incubated for 5 min at 37°C in the presence or absence of one of the calmodulin antagonists and then stimulated for 20 min by secretagogues. After incubation, the medium was collected by filtration through filter paper. To determine the total amylase content of the cell before the beginning of the incubation, a portion of the cell suspension was homogenized with a glass-Teflon homogenizer. Amylase activity in media and homogenates was assayed by the method of Bernfeld (26). The amount of amylase release was expressed as % of total amylase.

**Measurement of cAMP content:** A 1.0 ml aliquot of cell suspension was pre-incubated for 5 min at 37°C in the presence or absence of a calmodulin antagonist, then stimulated for 5 min by 1 μM ISO, and immediately heated in a boiling water bath for 2 min. After centrifugation, cAMP in the supernatant fluid was measured by radioimmunoassay using a commercial assay kit (Yamasa Shoyu Co., Chiba, Japan). cAMP content was expressed as picomoles/mg cellular protein. Protein determinations were performed by the method of Lowry et al. (27).

**Electron microscopy:** Parotid cells were pre-incubated with a calmodulin antagonist and stimulated with 1 μM ISO in the same manner as in the case of the amylase release assay. After incubation, the cells were fixed for 60 min at room temperature with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.2% tannic acid and further post-fixed for 90 min with 1% OsO4 in distilled water. The cells were dehydrated in a graded sequence of acetone and then embedded in Spurr resin. Thin sections were doubly stained with uranyl acetate and lead citrate and examined with a Hitachi H-500 electron microscope.

**Statistical analysis:** The statistical significance of differences between values was determined by Student’s t-test.
Results

Effect of calmodulin antagonists on ISO- and DBcAMP-stimulated amylase release: Figure 1 shows the effects of the calmodulin antagonists TFP, W-7 and W-5 on amylase release from rat parotid cells stimulated with ISO. In control cells, ISO at 1 μM elicited a 5.3-fold increase in amylase release. When either 50 μM TFP or 100 μM W-7 was added to the incubation medium, the secretory response to ISO was markedly reduced, and the released amylase was only 1.3- or 1.2-fold the unstimulated basal level in the presence of TFP or W-7, respectively. In contrast, W-5 at 100 μM, a chlorine-deficient analogue of W-7, caused only a weak inhibition of ISO-stimulated release. Each calmodulin antagonist alone had little or no effect on amylase release.

Since it is possible that the inhibition of amylase release by calmodulin antagonists is due to the β-adrenergic receptor blockade and the decreased cAMP accumulation, we examined the effects of calmodulin antagonists on amylase release induced by DBcAMP which is supposed to bypass the β-receptor and mimic cAMP. As shown in Fig. 2, TFP and W-7 strongly reduced the amylase release by 1 mM DBcAMP, while W-5 had little effect. The inhibition rates were essentially similar to those observed in the case of ISO-stimulated amylase release.

Effect of calmodulin antagonists on cAMP accumulation: In order to directly rule out the possibility that the calmodulin antagonists inhibited amylase release by affecting the cAMP metabolism, we measured the accumulation of intracellular cAMP induced with ISO in the presence and absence of a calmodulin antagonist (Table 1). Neither W-7 nor W-5 alone had any effect on the basal level of cAMP at a concentration of 100 μM, while 50 μM TFP alone caused a measurable increase. The stimulation of control cells by

![Fig. 1](image1.png)

![Fig. 2](image2.png)
Table 1. Effect of TFP, W-7 and W-5 on cAMP levels induced by isoproterenol (mean±S.E., n=5).

| Calmodulin antagonists | Basal | + Isoproterenol (1 μM) | + Isoproterenol (0.1 μM) |
|------------------------|-------|------------------------|--------------------------|
| Control                | 1.9±0.2 (6.0±0.6%) | 544.7±82.3 (34.7±1.9%) | 176.9±32.6 (33.4±1.4%) |
| TFP (50 μM)           | 31.1±11.4 | 323.0±40.3 | — |
| W-7 (100 μM)          | 1.8±0.2 | 424.6±38.3 | — |
| W-5 (100 μM)          | 2.0±0.2 | 500.0±76.2 | — |

cAMP level was determined 5 min after the addition of isoproterenol. The calmodulin antagonists were added 5 min before the addition of isoproterenol. The % values in parentheses show the amylase levels released during the 20 min incubation.

1 μM ISO for 5 min markedly elevated the cAMP level, and the average value was about 300 times the basal level. Treatment with TFP significantly reduced cAMP accumulation induced by 1 μM ISO (P<0.05), but the cAMP level was still higher than that induced by 0.1 μM ISO which produced a sufficient amylase release (Table 1). The ISO-stimulated cAMP accumulation in the presence of W-7 or W-5 was not significantly different from the stimulated level in control cells.

Effect of calmodulin antagonists on carbachol-stimulated amylase release: Since amylase release is also induced through muscarinic receptor stimulation, we examined whether the amylase release stimulated with 10 μM carbachol, a muscarinic agonist, is inhibited by the calmodulin antagonists (Fig. 3). TFP and W-7 reduced the stimulated amylase release to 1.7 and 1.4 times the basal level, respectively. While in the control cells, the amylase release induced by carbachol was about 4 times the basal level. The stimulated amylase release was not significantly inhibited by W-5.

Effects of calmodulin antagonists on acinar cell structure: In control acinar cells (Fig. 4A) incubated in the absence of calmodulin antagonists, the lumen had an empty appearance and many microvilli lining the lumen were seen. Numerous secretory granules were localized in the apical cytoplasm. Incubation with either 50 μM TFP or 100 μM W-7 alone reduced the number and size of microvilli. Figure 4B shows a typical picture of the acinar cells treated with W-7. The lumen lost all microvilli and contained a small amount of secretory material, indicating that a few secretory granules were fused with the lumen. The lumen of acinar cells treated with 100 μM W-5 was essentially similar to that of the control cells (not shown). In addition, basal and lateral membranes of the acinar cells treated with TFP or W-7 usually showed a smooth surface (Fig. 4D), whereas in control or W-5-treated cells, the membranes were characterized by numerous surface
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Fig. 4 Electron micrographs of control and W-7-treated parotid acinar cells. A control incubated for 25 min without the calmodulin antagonists. The lumen (L) contained numerous microvilli (arrow) x 15,800. B cells incubated for 25 min with 100 μM W-7. The luminal membrane lost microvilli. x 15,800. C basal part of control cells. The basal membrane were characterized by numerous surface folds (arrow) x 12,500. D basal part of cells incubated with 100 μM W-7. The basal membrane showed a smooth surface x 12,500. SG, secretory granule. N, nucleus. Bars=1 μm.

folds (Fig. 4C)

When control cells were stimulated for 20 min with 1 μM ISO, the number of secretory granules in the cytoplasm decreased markedly as a result of induction of exocytosis (Fig. 5A). The lumen nearly recovered its original size.
Within 20 min and contained little secretory material. Numerous microvilli were seen on the luminal surface. Treatment of cells with either TFP or W-7 markedly blocked the secretory events induced with ISO stimulation. Figure 5B shows a typical micrograph of the acinar cells treated with W-7. The acinar lumen was enlarged by the addition of secretory granule membranes to the luminal membrane, but it did not return to the original size. The released secretory materials was retained in the lumen and numerous secretory granules were still present in the apical cytoplasm. The cells treated with W-5 showed almost similar structure to the control cells.

**Discussion**

It is well-known that the Ca\(^{2+}\)-calmodulin complex can activate adenylate cyclase and cyclic nucleotide phosphodiesterase in various tissues (9). Since parotid cells have been shown to contain the calmodulin-activated adenylate cyclase (28), it is possible that the calmodulin antagonists inhibit the amylase release by affecting the cAMP accumulation, as suggested by Arkle et al. (29). In our study, however, TFP and W-7 strongly inhibited both ISO- and DBcAMP-stimulated amylase release, and cAMP formation was sufficiently induced by ISO even in the presence of TFP or W-7. These results lead to the conclusion that the effect of TFP and W-7 on cAMP metabolism is not a major cause of amylase release inhibition.

Although W-7 has an inhibitory effect on cAMP-dependent protein kinase which is
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independent of the effect on calmodulin, the K_i value (170 \mu M) of W-7 for the protein kinase is much higher than that (18 \mu M) for myosin light chain kinase, a calmodulin-dependent enzyme (30). Takuma (31) has demonstrated that H-7 and H-8, which are more specific inhibitors for cAMP-dependent protein kinase than W-7 (32), have no effect on the ISO-stimulated amylase release from rat parotid cells. Accordingly, it is unlikely that inhibition of the amylase release by W-7 is due to a direct effect on cAMP-dependent protein kinase. It has also been shown that TFP has no effect on the protein kinase (33).

Since amylase release induced through muscarinic receptor stimulation is thought to be directly dependent on intra- and extracellular Ca^{2+} (1), it is possible that the Ca^{2+}-calmodulin system may play a central role in muscarinic-induced amylase release rather than in \beta-adrenergic-induced release. In this study, however, the inhibitory effect of TFP or W-7 on carbachol-stimulated amylase release was almost similar to the effect on ISO-stimulated release. This result does not support the view that the Ca^{2+}-calmodulin system is involved more directly in the muscarinic-induced amylase release. The calmodulin antagonists may affect a common step in the ISO- and carbachol-induced exocytosis.

In our morphological study, treatment of parotid acinar cells with TFP or W-7 caused a loss of microvilli and surface folds, while W-5, a weak antagonist, had little effect on the cell structure. Since the effects of TFP and W-7 on cell structure appear similar, the two antagonists are suggested to have acted on the same cellular components. It has previously been reported that after treatment with TFP, cultured mammalian cells show this same loss of microvilli and surface projections (34, 35) and that TFP and W-7 induce striking changes in erythrocyte (36) and platelet (37) shape. Calmodulin is thought to mediate the regulation of the organization of microfilaments and microtubules (9) which is essential for maintaining cell structure, and calmodulin-binding proteins such as spectrin or calspectin have been shown to be major components undercoating the plasma membrane (38). Therefore, it is possible that the calmodulin antagonists may alter the cell structure by inhibiting the normal function of calmodulin.

When secretion is stimulated by ISO, the acinar lumen in intact parotid cells becomes enlarged by a progressive fusion of the luminal membrane and the secretory granules, and it rapidly returns to its original size (39). Since the contractile protein system consisting of actin and myosin filaments are predominantly present beneath the luminal surface (40), the dynamic movement of the membrane observed during secretion is possibly controlled by the contractile protein system. In our study, the enlarged acinar lumen could not return to its original size in the presence of TFP or W-7 and was filled with the discharged secretory material. This suggests that the luminal membrane has lost its motile or contractile functions which are necessary for exocytosis. It has been shown that myosin light chain kinase, which regulates Ca^{2+}- and calmodulin-dependent myosin phosphorylation, is involved in several secretory functions (41–43), as well as in smooth muscle contraction. We have observed that amylase release from rat parotid cells is inhibited by ML-9 (Y. Tojo et al., unpublished data), which is a direct inhibitor for myosin light chain kinase (42). Therefore, it is likely that the protein kinase plays an important role in accelerating the secretory process of parotid amylase.

From the results reported here, we propose the possibility that the calmodulin antagonist affected amylase release from rat parotid cells by inhibiting the normal function of the cytoskeletal system. Calmodulin may regulate the later stages in amylase secretion, such as movement of secretory granules, membrane fusion and membrane uptake, through interaction with myosin light chain kinase and other cytoskeleton-related calmodulin-binding proteins. Further study is necessary to obtain more direct evidence for this assumption.

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