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The Effect of Calmodulin Antagonists on Amylase Release from the Rat Parotid Gland in vitro*

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Abstract. The effect of several calmodulin antagonists on the release of α-amylase (EC 3.2.1.1) from rat parotid gland minces was investigated as an approach to determine whether calmodulin has a role in the stimulus-secretion coupling mechanism in this tissue. The phenothiazines, trifluoperazine, chlorpromazine, and thioridazine, failed to inhibit amylase release induced by N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate. All three phenothiazines increased basal amylase release at high concentrations. This release was independent of cellular energy, indicating that the release was probably due to the membrane perturbing properties of these compounds rather than their ability to antagonize calmodulin. R24571, a more potent calmodulin antagonist, also failed to inhibit amylase release induced by N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate but increased basal amylase release. A different calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, partially inhibited release while 8-(N,N-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) completely blocked the amylase release induced by the cyclic AMP derivative. However, concentrations of TMB-8 inhibiting amylase release drastically reduced the ATP concentration of rat parotid minces, suggesting that the inhibition of secretion was due to toxic effects of TMB-8 on parotid cells rather than the ability of the compound to antagonize calmodulin. The use of calmodulin antagonists has therefore failed to implicate calmodulin as an intermediate in the stimulus-secretion coupling mechanism of the rat parotid gland.

Key words: Parotid gland – Exocytosis – Calcium binding – Phenothiazines

Introduction

Exocytosis in the rat parotid gland is primarily regulated by β-adrenergic modulation of cyclic AMP levels (Butcher and Putney 1980). However, calcium may also function in the parotid exocytotic process or its regulation. Thus, depletion of intracellular calcium stores via incubation of parotid minces in media containing EGTA[1] reduces (Selinger and Naim 1970; Putney et al. 1977) or delays (Butcher 1978) the secretory response to β-adrenergic agonists and cyclic AMP derivatives. Furthermore, β-adrenergic stimulation and cyclic AMP derivatives increase $^{45}Ca^{2+}$ efflux from parotid minces or dissociated acinar cells preloaded with this nuclide (Putney et al. 1977; Butcher 1980). Indeed, cyclic AMP has been proposed to mediate exocytosis in this gland by mobilizing intracellular calcium pools, with the resulting increase in the cytoplasmic calcium ion concentration functioning as the final mediator for exocytosis (Putney et al. 1977).

Recent studies indicate that the calcium binding protein, calmodulin, mediates many of the intracellular actions of calcium (Cheung 1980; Means and Dedman 1980). The rat parotid gland has been shown to contain calmodulin (Ku and Butcher 1980). In order to determine whether calmodulin is involved in exocytosis or its regulation in the parotid, we have studied the effects of several calmodulin antagonists on the release of amylase from rat parotid gland minces in vitro.

Materials

Trifluoperazine dihydrochloride, chlorpromazine HCl and chlorpromazine sulfoxide HCl were provided by Smith Kline and French Laboratories, Philadelphia, PA, USA. Thioridazine HCl was from Sandoz Pharmaceuticals, E. Hanover, NJ, USA. R24571[2] was obtained from Janssen Pharmaceutica, Beerse, Belgium. Pr-MDI[3] and bu-MDI[4] were gifts from R. G. Rahwan, School of Pharmacy, Ohio State University, Columbus, OH, USA. TMB-8[5] was generously provided by E. H. Sypolt and M. R. Guthaus of The Upjohn Company, Kalamazoo, MI, USA. W-7[6] was a kind gift from H. Hidaka, Mie University School of Medicine, Japan. Other biochemicals were from the Sigma Chemical Co., St. Louis, MO, USA.

Methods

Tissue Preparation and Incubation. Male Sprague Dawley rats were obtained from Hilltop Lab Animals, Scottsdale, PA,

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1 EGTA, Ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetracetic acid

2 R24571, 1-[bis(p-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4 dichlorobenzyloxy)phenethyl] imidazolium chloride

3 pr-MDI, 2-n-propyl-3-dimethylamino-5,6-methylenedioxyindene hydrochloride

4 bu-MDI, 2-n-butyl-3-dimethylamino-5,6-methylenedioxyindene hydrochloride

5 TMB-8, 8-(N,N-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride

6 W-7, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide

7 LDH, lactate dehydrogenase (EC 1.1.1.27)

8 dB-cAMP, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate

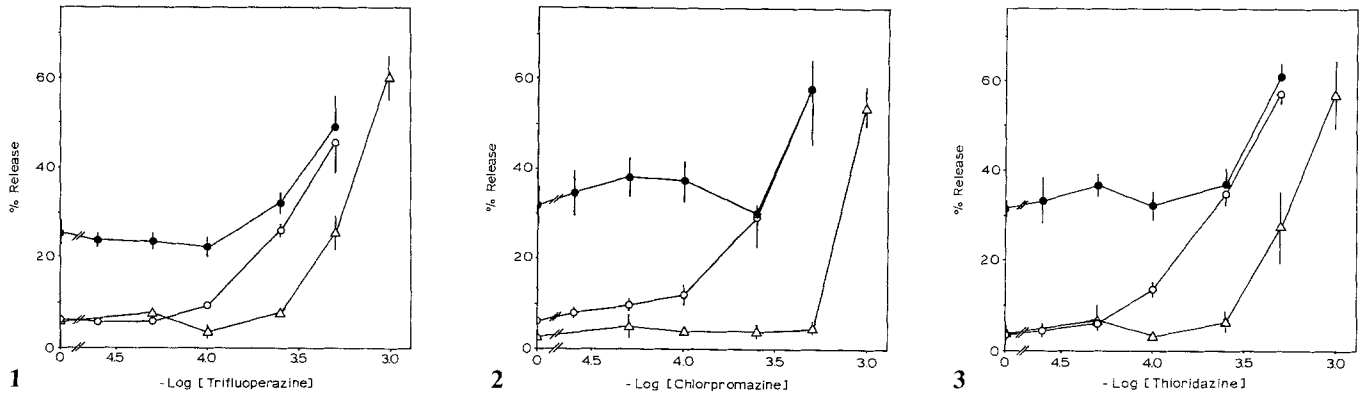

Fig. 1. The effect of trifluoperazine on the release of amylase and LDH from rat parotid minces. Conditions were as described under Methods. *Circles*, Amylase release; *triangles*, LDH release; *closed figures*, release in the presence of 1 mM dB-cAMP; *open figures*, release in the absence of dB-cAMP. Amylase release and LDH release were determined in separate experiments. Results are shown as the means ± standard error of three independent experiments

Fig. 2. The effect of chlorpromazine on the release of amylase and LDH from rat parotid minces. Conditions were as described under Methods. *Circles*, Amylase release; *triangles*, LDH release; *closed figures*, release in the presence of 1 mM dB-cAMP; *open figures*, release in the absence of dB-cAMP. Amylase release and LDH release were determined in separate experiments. Results are shown as the means ± standard error of three independent experiments

Fig. 3. The effect of thioridazine on the release of amylase and LDH from rat parotid minces. Conditions were as described under Methods. *Circles*, Amylase release; *triangles*, LDH release; *closed figures*, release in the presence of 1 mM dB-cAMP; *open figures*, release in the absence of dB-cAMP. Amylase release and LDH were determined in separate experiments. Results are shown as the means ± standard error of three independent experiments

USA, and were maintained on standard laboratory chow until the day of the experiment. They were anesthetized with Nembutal and killed by exsanguination. The parotid glands were quickly removed and placed in incubation media at 37° C (135 mM NaCl, 4.7 mM KCl, 2.54 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid-NaOH, pH 7.4, 6.1 mM glucose, 5 mM β-hydroxybutyrate). $CaCl_2$ was omitted from the media in experiments with bu-MDI, pr-MDI and TMB-8, since the effects of these compounds in other systems have been reported to be blocked by extracellular calcium (Rahwan et al. 1977; Smith and Iden 1979). The glands were trimmed of extraneous tissue, minced with two passes at right angles to each other on a McIlwain Tissue Chopper set at 0.5 mm (Brinkman Instruments, Westbury, CN, USA) and rinsed three times in incubation media.

Amylase Release. Minced tissue was blotted on filter paper and divided into approximately equal aliquots, each corresponding to one sixth of a gland. Each aliquot was placed in 1 ml of incubation media in a 15 ml glass scintillation vial. The tissue was preincubated 15 min at 37° C, a 50 µl aliquot of the media was withdrawn, test substances were added, and the incubation was continued as before for 30 min. A second 50 µl aliquot of the media was withdrawn, and 1 ml incubation media was added to the remaining sample, which was homogenized with a Polytron PT-10 (Brinkman Instruments). A 50 µl aliquot of the homogenate was withdrawn and, like the previously withdrawn aliquots, diluted to 500 µl with 20 mM phosphate buffer, pH 6.9, containing 6.7 mM NaCl and 1 mg/ml bovine serum albumin. Aliquots of the diluted samples were assayed for amylase activity by the method of Bernfeld (1955). The amylase released during the 30 min incubation is expressed as a percentage of the total amount of amylase present within the tissue immediately prior to the addition of the test substances.

LDH Release. LDH[7] release was determined by a protocol similar to that described above for amylase release except that undiluted samples were assayed for LDH by the method of Stolzenbach (1966).

ATP Levels. Parotid minces were prepared as described above. Each sample consisted of tissue equivalent to one sixth gland in 1 ml media. TMB-8 was added after a 15 min preincubation. The parotid minces were incubated with various concentrations of TMB-8 for 30 min, and the tissue was separated from the media on Whatman 3MM filter paper discs using suction. The tissue was then transferred to 1 ml boiling 50 mM Tris-EDTA pH 8.0. After boiling for 15 min the Tris-EDTA was removed and the volume measured. The tissue was homogenized in 1 ml 0.2 N NaOH with a Polytron PT-10 equipped with a microprobe. The protein content of the homogenate was determined by the method of Lowry et al. (1951). The ATP concentration of the tris-EDTA extract was determined using the luciferin-luciferase assay described by Addanki et al. (1966). Briefly, 5 µl of the extract was combined with 1.7 ml H_2O and 200 µl of a 10 mg/ml luciferin-luciferase (Sigma Chemical Co. no. L-0633) solution in 0.05 M sodium arsenate, 0.02 M $MgSO_4$ and, as quickly as possible, counted several times for 0.06 min in a Packard 3255 liquid scintillation spectrometer.

Results

Phenothiazines have been shown to function as calmodulin antagonists (Weiss and Levin 1978) and to inhibit exocytosis in a variety of secretory cells (Naccache et al. 1980; Heisler et al. 1981; Sugden et al. 1979; Schubart et al. 1980; Elferink 1979). Since phenothiazines have been shown to act as β-adrenergic antagonists in ligand binding studies (Weiss and Greenberg 1980), we used dB-cAMP[8] as the stimulus for secretion in all experiments instead of a β-adrenergic agonist.

Neither trifluoperazine (Fig. 1), chlorpromazine (Fig. 2), or thioridazine (Fig. 3) inhibited dB-cAMP-stimulated amylase release from rat parotid minces. In these experiments the phenothiazines were added to the tissue simultaneously with the dB-cAMP. Similarly, preincubation of tissue minces for up to 60 min with 0.1 mM trifluoperazine before the addition of dB-cAMP did not inhibit amylase release (not shown).

All three phenothiazines markedly increased unstimulated amylase release when used at concentrations in excess of 0.1 mM (Fig. 1 – 3). Phenothiazines are known to perturb cellular membranes and have been shown to lyse cells at high concentrations (Seeman 1966). We therefore determined the effect of the three phenothiazines on the release of the cytoplasmic marker enzyme, LDH. All three phenothiazines released LDH at high concentrations but the concentration required to increase LDH release was higher than that required to increase basal amylase release (Fig. 1 – 3).

Table 1. The effect of 2,4-dinitrophenol on amylase release induced by dB-cAMP, trifluoperazine, chlorpromazine, and thioridazine. Minced tissue was preincubated with or without 2,4-dinitrophenol for 30 min before the addition of dB-cAMP, trifluoperazine, chlorpromazine, and thioridazine. Other conditions were as described under Methods. Results are shown as the means ± standard error of three independent experiments in which each treatment was performed in duplicate

Additions	% Amylase released	
	–	+1 mM 2,4-dinitrophenol
–	6.7 ± 1.1	8.4 ± 1.3
1 mM dB-cAMP	22.2 ± 5.1	8.1 ± 0.7
0.25 mM trifluoperazine	35.0 ± 3.6	39.0 ± 4.0
0.25 mM chlorpromazine	21.9 ± 1.9	26.2 ± 9.9
0.25 mM thioridazine	45.8 ± 8.0	45.3 ± 4.3

Chlorpromazine sulfoxide, a poor calmodulin antagonist (Weiss and Levin 1978) did not induce amylase release at concentrations up to 0.5 mM (not shown). However this chlorpromazine metabolite is reported to be less hydrophobic than its parent compound (Zografi and Auslander 1965). This was confirmed by our finding that the sulfoxide did not release LDH from parotid minces at concentrations as high as 1 mM (not shown). This compound is therefore not useful in distinguishing between those effects of phenothiazine produced by calmodulin antagonism versus those due to membrane perturbation.

The energy dependence of phenothiazine-induced amylase release was determined, since neurotransmitter-induced amylase release requires metabolic energy (Bdolah et al. 1964), while release due to membrane perturbation might be expected to be energy independent. As shown in Table 1, amylase release induced by 0.25 mM trifluoperazine, chlorpromazine, or thioridazine was unaffected by 1 mM 2,4-dinitrophenol, whereas dB-cAMP-induced amylase release was completely inhibited.

The drug R24571 has been reported to be approximately 1000 times more effective in inhibiting calcium-calmodulin activation of phosphodiesterase activity than the phenothiazines (Van Belle 1981). This compound did not inhibit dB-cAMP-induced amylase release from parotid minces but increased unstimulated release at concentrations in excess of 5 μM (not shown). R24571, at a concentration of 0.5 mM, approximately doubled LDH release as compared to a control incubation (not shown).

Bu-MDI has been reported to act as a calmodulin antagonist (Piascik et al. 1981) and bu-MDI and pr-MDI have been reported to inhibit stimulus-secretion coupling in the bovine adrenal gland (Piascik et al. 1978) and to inhibit excitation-contraction coupling in a number of smooth muscle preparations (Rahwan et al. 1977). Neither bu-MDI (Fig. 4) nor pr-MDI (not shown) affected dB-cAMP-stimulated amylase release, but high concentrations of bu-MDI increased basal amylase release (Fig. 4).

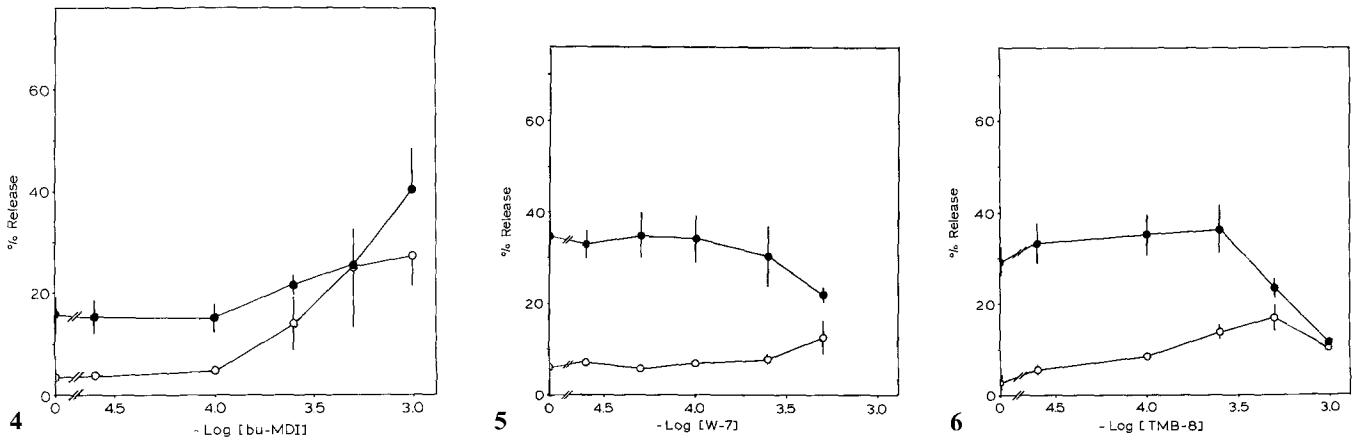

Fig. 4. The effect of bu-MDI on the release of amylase from rat parotid minces. Conditions were as described under Methods. *Closed circles*, Release in the presence of 1 mM dB-cAMP; *open circles*, release in the absence of dB-cAMP. Results are shown as the means ± standard error of three independent experiments

Fig. 5. The effect of W-7 on the release of amylase from rat parotid minces. Conditions were as described under Methods. *Closed circles*, Release in the presence of 1 mM dB-cAMP; *open circles*, release in the absence of dB-cAMP. Results are shown as the means ± standard error of three independent experiments in which each treatment was performed in duplicate

Fig. 6. The effect of TMB-8 on the release of amylase from rat parotid minces. Conditions were as described under Methods. *Closed circles*, Release in the presence of 1 mM dB-cAMP; *open circles*, release in the absence of dB-cAMP. Results are shown as the means ± standard error of three independent experiments in which each treatment was performed in triplicate

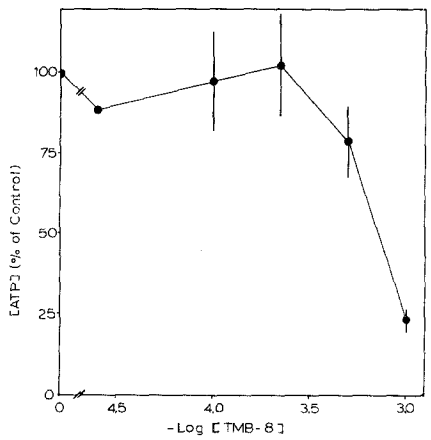

Fig. 7. The effect of TMB-8 on ATP levels in rat parotid minces. Conditions were as described under Methods. Results are shown as the means ± standard errors of three independent experiments. Basal ATP levels were 3.00 ± 0.77 μmoles/mg protein

The calmodulin antagonist W-7 (Hidaka et al. 1978) has been reported to inhibit exocytosis in neutrophils (Naccache et al. 1980; Smolen et al. 1981; Alobaidi et al. 1981). We have observed that this compound, at a concentration of 0.5 mM, partially inhibited dB-cAMP-stimulated amylase release from rat parotid minces (Fig. 5). Higher concentrations increased basal amylase release and resulted in measureable LDH release (not shown).

TMB-8 has been reported to act as an intracellular calcium antagonist (Malagodi and Chiou 1974) and to inhibit exocytosis in neutrophils (Smolen et al. 1981; Smith and Iden 1979). It was included in the present study because we have observed an interference with calmodulin activation of rat brain calmodulin-deficient phosphodiesterase with this compound (not shown). TMB-8 completely inhibited dB-cAMP-stimulated amylase release at a concentration of 1 mM (Fig. 6). This concentration is similar to that required to inhibit exocytosis in neutrophils (Smolen et al. 1981; Smith and Iden 1979). However TMB-8 decreased parotid ATP levels with a dose-dependence identical to that for inhibition of amylase release (Fig. 7).

Discussion

Of all the calmodulin antagonists tested in this study, only W-7 and TMB-8 inhibited dB-cAMP-stimulated amylase release from rat parotid minces. Phenothiazines, used to implicate calmodulin in the stimulus-secretion coupling mechanism of several other secretory systems (Naccache et al. 1980; Heisler et al. 1981; Sugden et al. 1979; Schubart et al. 1980; Elferink 1979) were ineffective at concentrations commonly used for this purpose. Higher concentrations increased basal amylase release but this was probably due to the ability of the phenothiazines to perturb biological membranes rather than their calmodulin antagonist properties. This conclusion is based on our observations that the amylase release seen at the higher phenothiazine concentrations was energy-independent and that high phenothiazine concentrations released LDH from parotid minces. It is not clear why, if phenothiazines release amylase by damaging cells, amylase release precedes LDH release. However it is interesting to note that we also found amylase preferentially released when parotid minces were exposed to the detergent triton X-100 (not shown).

R24571 has been reported to be a more potent calmodulin antagonist than the phenothiazines (Van Belle 1981). It was also ineffective in inhibiting dB-cAMP-induced amylase release and, like the phenothiazines, increased basal amylase release and released LDH at higher concentrations. TMB-8 inhibited dB-cAMP-stimulated amylase release at approximately the same concentration which inhibits secretion in neutrophils (Smolen et al. 1981; Smith and Iden 1979). However, levels of TMB-8 inhibitory to amylase release markedly depressed ATP levels in parotid minces. Therefore the inhibition of amylase release by TMB-8 is probably due to a toxic action of this drug on parotid acinar cells rather than inhibition of a calmodulin-dependent reaction in the parotid stimulus-secretion coupling mechanism.

The inhibition of amylase release produced by W-7 was of a small magnitude and was seen only at or near concentrations which increased basal amylase release. Since we did not test the effect of W-7 on parotid ATP levels, the possibility that the slight inhibition of amylase release seen with W-7 is due to toxic effects of this compound has not been eliminated. The small magnitude of the inhibition of amylase release seen with W-7, the observation that it occurs only at concentrations at which nonspecific effects begin to be apparent, plus the fact that it is not reproduced by the other calmodulin antagonists tested (one of which is a considerably more potent calmodulin antagonist) leads us to conclude that the present study has failed to indicate a role for calmodulin in the rat parotid stimulus-exocytosis coupling mechanism.

The results of this study also illustrate and emphasize some of the pitfalls inherent in the use of calmodulin antagonists as probes of calmodulin involvement in biological processes in intact cells. Our data clearly show that it is essential to assess the effect of these compounds on the integrity and viability of the cells in addition to considering their possible actions as neurotransmitter antagonists. Furthermore, our data demonstrate that LDH release must be used with caution as an indicator of nonspecific membrane perturbing actions of these drugs and suggest that the sulfoxide derivatives of phenothiazines are of only limited utility in determining whether any given effect of this class of drugs is due to calmodulin antagonism or to nonspecific effects.

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