Stimulation of Amylase and Sialic Acid Releases from Dog Submandibular Gland Slices by Pilocarpine or High K^+ Medium: A Possible Role of Calmodulin for Their Releases

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Abstract The mechanism of amylase and sialic acid releases stimulated by pilocarpine or high K^+ medium was investigated in the slices of dog submandibular glands. The release of both amylase and sialic acid was dose-dependently increased by pilocarpine and a considerable release was observed at pilocarpine concentrations of more than $1 \mu M$. Similar effects were observed when K^+ concentration in the medium was increased and the maximal response was observed at $75 \text{ mM } K^+$. The release of amylase and sialic acid by pilocarpine or K^+ considerably decreased by removing Ca^{2+} from the medium and the slices. The release of amylase in the Ca^{2+} -deficient slices was nearly recovered by the addition of 2.5 and $5.0 \text{ mM } Ca^{2+}$, whereas that of sialic acid was recovered by only $60\text{--}75\%$. Ca^{2+} inhibitors, La^{3+} and verapamil, and calmodulin inhibitors, trifluoperazine, prenylamine, and W-7, significantly inhibited the release of amylase and sialic acid induced by the stimulants. These results suggest that the release of amylase and sialic acid stimulated by pilocarpine or K^+ is dependent on the presence of Ca^{2+} , and that the activation of calmodulin is involved in the process of the release.

Key Words: amylase release, sialic acid release, calmodulin, dog submandibular slice.

The release of amylase and K^+ from the parotid and submandibular glands can be evoked by adrenergic and cholinergic stimulants. Stimulation of β -adrenergic and cholinergic receptors causes the release of amylase, whereas the release of K^+ is activated by α -adrenergic and cholinergic receptors (BATZRI *et al.*,

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1971, 1973; MARTINEZ and QUISSELL, 1976; PUTNEY, 1976; WATSON *et al.*, 1979; CARLSÖÖ *et al.*, 1981). Secretion *via* the cholinergic or α -adrenergic mechanism requires extracellular Ca^{2+} , but the β -adrenergic mechanism does not. In addition, the β -adrenergic mechanism requires the increase of cyclic AMP level in the tissue, but the α -adrenergic or cholinergic mechanism does not (SCHRAMM and SELINGER, 1975; VREUGDENHIL and ROUKEMA, 1975; LESLIE *et al.*, 1976). Thus, the mechanism of amylase and K^+ releases has been elucidated to a certain extent.

Submandibular glands are composed of both the serous and mucous cells. In particular, the mucous cells of dog submandibular glands contain a higher percentage of glycoproteins (*e.g.* sialic acid) compared to other animals (SHACKLEFORD and KLAPPER, 1962). The glycoproteins in saliva have a lubricating property, and help the tongue, oral mucosa, and teeth to function smoothly in speaking and swallowing. It is thought that some differences exist in the secretory process of the serous and mucous cells. For example, the fusion process of the granule membranes is different between the serous and the mucous cells. During secretion of mucus, the membranes of mucous granules are discharged as evidenced by the presence of membrane-bound granules and/or aggregates of membranous materials in the lumen. In contrast, the membranes of the serous granules are retained in the cell as part of the cell membrane (KIM *et al.*, 1972). Therefore, the intracellular reactions leading to the release of the secretory products are probably not the same for the serous and the mucous cells. However, the reports in which the secretory process of the mucous cells is investigated are few. Some evidence exists that isoproterenol stimulates the release of sialic acid from rat submandibular and sublingual glands, but not parotid glands (BYRT and GLANVILL, 1967; BOGART and PICARELLI, 1978).

Following the discovery of calmodulin by KAKIUCHI *et al.* (1970) and CHEUNG (1970), a wide distribution of this Ca^{2+} -binding protein has been demonstrated in several animals. Calmodulin has been found to mediate many of Ca^{2+} effects in cellular functions (WOLFF and BROSTROM, 1979; CHEUNG, 1980). A possibility is suggested that calmodulin plays an important role in the release of prolactin from pituitary gland and that of insulin and glucagon from pancreas (SUGDEN *et al.*, 1979; MERRITT *et al.*, 1981; YAGA *et al.*, 1981). The presence of calmodulin in the salivary glands was demonstrated by YOKOYAMA *et al.* (1983). However, it is not known whether calmodulin is involved in the releasing process of amylase and sialic acid from submandibular glands.

It is well known that high K^+ concentrations in the medium produce membrane depolarization. In muscles, where it is accepted that K^+ stimulates depolarizing, much the same sort of relation has been observed between K^+ concentration and the contracture response. DOUGLAS and RUBIN (1961) have suggested that high K^+ concentrations act, similarly to the electrical stimuli, by depolarizing the neurosecretory terminals of the neurohypophysis, and dose-dependently cause the release of vasopressin. This finding shows that high K^+ concen-

trations may be mimicking the natural stimulus for the release.

In the present report, we have studied the mechanism of the release of amylase and sialic acid induced by pilocarpine or K^+ in dog submandibular gland slices with particular interest in a possible role of calmodulin in this mechanism.

MATERIALS AND METHODS

Mongrel dogs (both sexes, 7–15 kg) were killed by thiopental sodium (60 mg/kg, i.v.) and both the submandibular glands were removed. The glands were sliced with a Stadie-Riggs slicer (slice thickness *ca.* 0.5–0.8 mm). At all the subsequent stages the slices were maintained in Krebs-Ringer phosphate buffer (pH 7.4) containing enough oxygen. Each slice (approx. 80–120 mg) was immersed in 4 ml medium and incubated for 60 min at 37.5°C. The agents were added before incubation. When increased K^+ concentrations were used, isotonicity was maintained by reducing the concentration of Na^+ . In the experiments of Ca^{2+} addition, the phosphate buffer in the medium was replaced with Tris-HCl buffer. After filtration of the incubation solution containing the samples, the activity of amylase and the content of sialic acid in the medium were determined by the colorimetric methods of BERNFELD (1955) and SVENNERHOLM (1957), respectively. The amylase activity was expressed as Somogyi units.

The following agents were used: pilocarpine (Sanko Pharmaceutical Co., Ltd.), thiopental sodium (Tanabe Pharmaceutical Co., Ltd.), verapamil (Eizai Co., Ltd.), trifluoperazine (Yoshitomi Pharmaceutical Co., Ltd.), prenylamine (Kaken Pharmaceutical Co., Ltd.), and W-7 (Rikaken Co., Ltd.). All other chemicals used were of analytical grade.

RESULTS

Time course of the release of amylase and sialic acid

Figure 1 shows the time course of the release of amylase and sialic acid after addition of 10 μM pilocarpine and the elevation of medium K^+ to 75 mM. The release of sialic acid increased with time and tended to reach a steady level. The pattern of amylase release was similar to that of sialic acid release.

Dose-dependent effects of pilocarpine or K^+ on amylase and sialic acid releases

Submandibular gland slices were exposed to varying concentrations of pilocarpine or K^+ for 60 min. The dose-response curves for the effects of pilocarpine are shown in Fig. 2 A. Pilocarpine dose-dependently stimulated the release of amylase and sialic acid from submandibular gland slices. A considerable increase was observed at the concentrations of 1 μM and above. On the other hand, as shown in Fig. 2 B, the release of amylase and sialic acid induced by K^+ dose-dependently increased. The K^+ effect on each release reached a peak at 75 mM. Also, 100 mM K^+ slightly stimulated their releases. On the basis of these results,

Fig. 1. Time course of the release of amylase and sialic acid from submandibular gland slices. The agents were added immediately before the incubation. (A) The release of amylase and sialic acid induced by $10 \mu\text{M}$ pilocarpine. (B) The release of amylase and sialic acid induced by 75 mM K^+ . Open symbols, the response of $10 \mu\text{M}$ pilocarpine or 75 mM K^+ ; filled symbols, basal responses. Each point represents the mean $\pm$ S.E. for seven experiments.

Fig. 2. Dose-response curves for the effects of pilocarpine or K^+ on the release of amylase and sialic acid from submandibular gland slices. (A) Pilocarpine, (B) K^+ . Each point represents the mean $\pm$ S.E. for seven experiments. * $p < 0.01$ compared with pilocarpine ($0 \mu\text{M}$) or K^+ (4.75 mM) responses.

subsequent experiments for the release of amylase and sialic acid were made in the presence of $10 \mu\text{M}$ pilocarpine or 75 mM K^+ .

Effects of the Ca^{2+} -free medium on the release of amylase and sialic acid stimulated by pilocarpine or K^+ in the Ca^{2+} -deficient slices

The Ca^{2+} -deficient slices were prepared by immersing them in the medium containing 2 mM EGTA for 60 min at 4°C . In the presence of $10 \mu\text{M}$ pilocarpine

Table 1. Effects of the Ca^{2+} -free medium on the release of amylase and sialic acid of the Ca^{2+} -deficient slices induced by $10\ \mu\text{M}$ pilocarpine or $75\ \text{mM}$ K^+ .

	Conditions	Amylase release (units/(100 mg·hr))	Sialic acid release ($\mu\text{g}/(100\ \text{mg}\cdot\text{hr})$)
$10\ \mu\text{M}$ pilocarpine	Normal Ringer+normal slice	0.127 ± 0.007	50.9 ± 2.1
	Ca^{2+} -free Ringer+ Ca^{2+} -deficient slice	$0.025\pm0.006^*$	$25.5\pm4.2^*$
$75\ \text{mM}$ K^+	Normal Ringer+normal slice	0.171 ± 0.003	56.8 ± 1.4
	Ca^{2+} -free Ringer+ Ca^{2+} -deficient slice	$0.064\pm0.004^*$	$2.8\pm0.2^*$

The Ca^{2+} -deficient slices were prepared in the medium containing $2\ \text{mM}$ EGTA for $1\ \text{hr}$ at 4°C . Each value represents the mean $\pm$ S.E. for seven experiments. $^*p<0.01$.

Fig. 3. Effects of various concentrations of Ca^{2+} on the release of amylase and sialic acid from the Ca^{2+} -deficient slices. Recovery rate determined from the corresponding value with normal slices. Each point represents the mean for seven experiments.

or $75\ \text{mM}$ K^+ , the release of amylase and sialic acid was considerably decreased by removing Ca^{2+} from the medium and the slices (Table 1). Amylase release was inhibited more strongly than sialic acid release in the presence of $10\ \mu\text{M}$ pilocarpine, whereas the release of sialic acid in the presence of $75\ \text{mM}$ K^+ was inhibited more strongly than amylase release.

Effects of various concentrations of Ca^{2+} on the release of amylase and sialic acid in the Ca^{2+} -deficient slices

Figure 3 shows the effects of various concentrations of Ca^{2+} on the release of amylase and sialic acid induced by $10\ \mu\text{M}$ pilocarpine or $75\ \text{mM}$ K^+ in the Ca^{2+} -deficient slices. Amylase release by $10\ \mu\text{M}$ pilocarpine or $75\ \text{mM}$ K^+ did not recover by the addition of $0.25\ \text{mM}$ Ca^{2+} , but nearly recovered by increasing Ca^{2+} concentrations to 2.5 and $5.0\ \text{mM}$. On the other hand, the recovery rate of sialic acid release was far less compared with that of amylase release and was by only $60\text{--}75\%$ at $2.5\ \text{mM}$ Ca^{2+} .

Effects of Ca^{2+} inhibitors on the release of amylase and sialic acid by pilocarpine or K^+

Table 2 shows the effects of Ca^{2+} inhibitors on the release of amylase and sialic acid induced by 10 μM pilocarpine or 75 mM K^+ . The release of amylase and sialic acid by 10 μM pilocarpine or 75 mM K^+ was markedly inhibited by 2.5 mM La^{3+} . In addition, 10 μM verapamil significantly inhibited the release of amylase and sialic acid induced by pilocarpine or K^+ .

Table 2. Effects of La^{3+} and verapamil on the release of amylase and sialic acid induced by 10 μM pilocarpine or 75 mM K^+ .

Conditions	Amylase release (units/(100 mg·hr))	Sialic acid release (μg /(100 mg·hr))
10 μM pilocarpine	0.110 ± 0.008	61.3 ± 5.9
+0.25 mM La^{3+}	$0.082 \pm 0.007^{**}$	68.5 ± 4.9 N.S.
+2.5 mM La^{3+}	$0.024 \pm 0.004^*$	$26.8 \pm 1.7^*$
+1 μM verapamil	0.097 ± 0.005 N.S.	$42.9 \pm 1.6^{**}$
+10 μM verapamil	$0.049 \pm 0.006^*$	$40.0 \pm 1.8^{**}$
75 mM K^+	0.115 ± 0.007	53.7 ± 1.9
+0.25 mM La^{3+}	$0.080 \pm 0.004^{**}$	$25.7 \pm 2.9^*$
+2.5 mM La^{3+}	$0.038 \pm 0.003^*$	$24.8 \pm 0.5^*$
+1 μM verapamil	$0.097 \pm 0.005^{**}$	52.6 ± 1.7 N.S.
+10 μM verapamil	$0.078 \pm 0.006^*$	$37.6 \pm 1.8^*$

Each value represents the mean $\pm$ S.E. for seven experiments.

* $p < 0.01$, ** $p < 0.05$. N.S.: not significant.

Table 3. Effects of trifluoperazine, prenylamine, and W-7 on the release of amylase and sialic acid induced by 10 μM pilocarpine or 75 mM K^+ .

Conditions	Amylase release (units/(100 mg·hr))	Sialic acid release (μg /(100 mg·hr))
10 μM pilocarpine	0.118 ± 0.007	53.1 ± 4.0
+10 μM trifluoperazine	0.097 ± 0.005 N.S.	$32.2 \pm 5.6^*$
+100 μM trifluoperazine	$0.084 \pm 0.003^{**}$	$37.7 \pm 2.9^*$
+10 μM prenylamine	0.129 ± 0.013 N.S.	$43.3 \pm 2.7^{**}$
+100 μM prenylamine	$0.030 \pm 0.005^*$	$34.7 \pm 3.5^*$
+8.3 μM W-7	0.096 ± 0.008 N.S.	$33.6 \pm 0.7^*$
+83 μM W-7	$0.029 \pm 0.010^*$	$30.6 \pm 0.6^*$
75 mM K^+	0.154 ± 0.006	60.4 ± 1.7
+10 μM trifluoperazine	$0.064 \pm 0.003^*$	$41.9 \pm 4.7^*$
+100 μM trifluoperazine	$0.031 \pm 0.003^*$	$38.2 \pm 3.9^*$
+10 μM prenylamine	$0.116 \pm 0.007^*$	$41.7 \pm 3.4^*$
+100 μM prenylamine	$0.060 \pm 0.007^*$	$37.2 \pm 2.5^*$
+8.3 μM W-7	0.138 ± 0.006 N.S.	56.4 ± 2.6 N.S.
+83 μM W-7	$0.100 \pm 0.008^*$	$36.7 \pm 3.4^*$

Each value represents the mean $\pm$ S.E. for seven experiments.

* $p < 0.01$, ** $p < 0.05$. N.S.: not significant.

Effects of calmodulin inhibitors on the release of amylase and sialic acid by pilocarpine or K⁺

It has been investigated whether calmodulin is involved in the releasing process of amylase and sialic acid from submandibular gland slices. The results are shown in Table 3. The release of amylase and sialic acid by 10 μ M pilocarpine or 75 mM K⁺ was significantly inhibited by the calmodulin inhibitors, trifluoperazine, prenylamine, and W-7.

DISCUSSION

Although there have been many investigations on the effects of adrenergic and cholinergic agonists on the release of amylase and K⁺ from the salivary glands of several species (VREUGDENHIL and ROUKEMA, 1975; BOGART and PICARELLI, 1978; KAUFMAN, 1978; KAUFMAN and WONG, 1983), the reports in which the release of sialic acid from the salivary gland is studied are few. DOUGLAS and POISNER (1964) and SAMLI and GESCHWIND (1968) reported that high K⁺ concentrations in the medium provoked the release of vasopressin and luteinizing hormone from pituitary gland. Using dog submandibular gland slices, we showed that the release of sialic acid was dose-dependently activated like that of amylase by pilocarpine or K⁺ (Fig. 2), and that the release was increased according to the incubation time (Fig. 1). The effects of high K⁺ concentrations were mediated by two factors; its direct stimulation of plasma membranes and its indirect stimulation by the transmitters released from autonomic terminals. Unfortunately we did not use the autonomic blockades in the present experiment. Therefore, it does not reveal whether the effects of K⁺ are due to the direct stimulation for plasma membranes or the release of transmitters from autonomic terminals.

The relationship between Ca²⁺ and many secretory processes has been extensively studied (DOUGLAS and RUBIN, 1961; DOUGLAS and POISNER, 1963; LESLIE *et al.*, 1976; KANNO *et al.*, 1977; KOELZ *et al.*, 1977; MILLER and NELSON, 1977; WATSON *et al.*, 1979; RUBIN, 1982). Ca²⁺ antagonists, La³⁺ and verapamil, block the permeability of cell membranes to Ca²⁺. La³⁺ and verapamil inhibited the catecholamine release from adrenal medulla (BOROWITZ, 1972; ARQUEROS and DANIELS, 1978; COHEN and GUTMAN, 1979) and histamine release from mast cells (FOREMAN and MONGAR, 1972). With isolated bovine adrenals, BOROWITZ (1972) found that La³⁺ inhibited the increased catecholamine release obtained with acetylcholine or high K⁺ concentrations. KERYER and ROSSIGNOL (1978) also reported that La³⁺ blocked Ca²⁺ uptake, protein secretion, and K⁺ efflux induced by carbachol and K⁺ efflux activated by norepinephrine. Our previous studies (KOMABAYASHI *et al.*, 1977; SAKAMOTO *et al.*, 1981) showed that the oxygen uptake stimulated by pilocarpine, acetylcholine, or high K⁺ concentrations was significantly inhibited by removing Ca²⁺ from the medium and the slices and by the addition of La³⁺ in rat and dog submandibular slices. In the present ex-

periment, the release of amylase and sialic acid stimulated by pilocarpine or high K^+ concentrations was significantly inhibited by removing Ca^{2+} from the medium and the slices (Table 1). In addition, amylase release from the Ca^{2+} -deficient slices in the presence of these agents was nearly recovered by the addition of 2.5 and 5.0 mM Ca^{2+} , whereas sialic acid release was recovered by only 60–75% by 2.5 mM Ca^{2+} (Fig. 3). This difference in reversibility may be presumably attributable to the distinction of the required amount of Ca^{2+} . Also, Ca^{2+} inhibitors, La^{3+} and verapamil, significantly inhibited the release of amylase and sialic acid activated by pilocarpine and high K^+ concentrations (Table 2). These results suggest that the release of amylase and sialic acid strongly depends on the presence and permeability of Ca^{2+} . Thus, although it is apparent that the release of amylase and sialic acid depends on Ca^{2+} , the regulatory mechanism of Ca^{2+} in the cells can not be clarified from these results. Therefore, further studies are necessary.

In the present study calmodulin inhibitors, trifluoperazine, prenylamine, and W-7, were used to investigate the possible involvement of calmodulin in the activation of the Ca^{2+} -dependent amylase and sialic acid releases from dog submandibular gland slices. MERRITT *et al.* (1981) reported that phenothiazine derivatives, trifluoperazine and prochlorperazine, dose-dependently inhibited the secretion of prolactin by thyrotropin-releasing hormone or 35 mM K^+ in the anterior pituitary gland. YAGA *et al.* (1981) and HEISLER *et al.* (1981) also reported that chlorpromazine, trifluoperazine, and W-7 markedly inhibited the release of amylase, insulin, and glucagon stimulated by carbachol and glucose in rat pancreas. In addition, KANAMORI *et al.* (1981) and ASANO *et al.* (1982) showed that W-7 and prenylamine dose-dependently blocked the contraction of rabbit aortic strips induced by norepinephrine, serotonin, histamine, or high K^+ concentrations. These findings indicate that calmodulin participates in the cellular functions of several organs. In the present experiment, we confirmed that the release of amylase and sialic acid activated by pilocarpine or high K^+ concentrations was significantly inhibited by trifluoperazine, prenylamine, or W-7 (Table 3). These results suggest that the activation of calmodulin is involved in the releasing process of amylase and sialic acid induced by pilocarpine or high K^+ concentrations.

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