Effect of Isoproterenol and Forskolin on Amylase Release from Parotid Tissue after Chronic Pilocarpine Administration in Rats Following Ligation Removal

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Abstract—The influence of chronic pilocarpine administration on the recovery of the parotid gland from an obstruction was investigated by studying the amylase release induced by secretagogues in rats. Treatment with pilocarpine (5 mg/kg, daily for 7 days) increased amylase release induced by isoproterenol (10^{-5} M) or forskolin (10^{-5} M) in the parotid tissue of rats after the removal of a 7-day-duct ligation. However, there was no significant difference in the amylase activity of the parotid tissue between pilocarpine-treated rats and control rats. These results suggest that the accelerated recovery of amylase release from parotid tissue in rats chronically treated with pilocarpine may be due to the increased response of amylase release, rather than the increased accumulation of secretory materials in the cells; and furthermore, cyclic AMP-mediated events may be involved in the increased response of amylase release.

There are many reports concerning the alteration of the parotid function by duct ligation, but little is known about the functional recovery after the removal of ligation in the parotid gland. Our previous studies demonstrated that isoproterenol-induced amylase release from the parotid tissue *in vitro* decreased progressively after duct ligation and that the decreased amylase release recovered gradually after the removal of the ligation (1, 2).

Isoproterenol stimulates amylase release from the parotid tissue via the action of cyclic AMP (3). Cyclic AMP accumulation in tissue occurs via interaction of β -adrenergic agonists binding to receptors and activation of adenylate cyclase activity. The importance of cyclic AMP in the response of amylase release has been accepted. Forskolin as well as isoproterenol stimulate cyclic AMP accumulation and amylase release from parotid slices, but forskolin specifically activates adenylate cyclase (4). Forskolin is a valuable tool for investigating the role of cyclic AMP on the secretory response.

In the histochemical studies, Sonobe (5)

reported that chronic pilocarpine administration after the removal of duct ligation accelerated the parotid gland recovery from an obstruction in the rabbit. The present study was undertaken to investigate the influence of chronic pilocarpine treatment after the removal of ligation on the secretory response induced by isoproterenol in rat parotid gland. In addition, and in order to explore the functional significance of the adenylate cyclase system for the amylase secretion, we also studied the effect of forskolin.

Materials and Methods

Animals and pretreatment: Male Wistar rats weighing 180 to 200 g were deprived of food for 18 hr before experiments, but water was available *ad libitum*. The excretory duct of the parotid gland was carefully isolated from the nerve and ligated unilaterally. After seven days, the ligation was removed and a polyethylene tube (inside diameter: 0.50 mm, outside: 0.80 mm) was inserted to open the duct and to eliminate the influence of the ligation. The duct ligation and the removal of ligature were done under anesthesia with

sodium pentobarbital (40 mg/kg, i.p.) and the unoperated side was used as the paired control. Then, pilocarpine (5 mg/kg) was administered intraperitoneally daily for 7 days to rats, and the animals were used 24 hr after the last injection. Saline was given to the control animals. Amylase release from parotid slices: Amylase release from parotid slices was carried out by the procedure of Leslie et al. (6). Briefly, the parotid glands removed from the rat under sodium pentobarbital anesthesia were cut into small pieces, and about 20 mg of parotid slices were incubated in 5 ml Krebs-Ringer-Tris (KRT) solution, pH 7.4, with the following composition (mM): NaCl, 120.0; MgCl₂, 1.2; CaCl₂, 3.0; β -hydroxybutyrate Na, 5.0; Tris(hydroxymethyl)-aminomethane, 20.0; KCl, 5.0; bubbled with pure oxygen at 37°C. Parotid slices were preincubated for 25 min, and then the slices were incubated for 30 min in fresh KRT solution with or without drugs. To measure the amount of amylase released into the incubation medium, 0.10 ml aliquots were used. The amount of amylase release in the absence of drugs was expressed as the basal release. After the incubation, the slices were weighed and homogenized in 5 ml of fresh buffer for 30 sec, and 0.10 ml of the homogenate was used for an assay of the enzyme activity.

Assay of amylase activity: Amylase activities in the medium and homogenate were assayed photometrically using blue insoluble starch substrate (Neo-amylase test, Daiichi Pure Chem. Co., Ltd., Japan) (7). A value of 1.0 for the absorbance at 620 nm per medium or homogenate incubated at 37°C

for 30 min was defined as 1 unit. The amount of released amylase was expressed as both units of amylase activity released into the medium per 20 mg (wet weight) per 30 min (released amylase activity) and the percentage of the total amount of the enzyme initially contained in the tissue slices (percentage of amylase release). The amount of released amylase represents the difference between the basal and the stimulated amylase release. Amylase activity in the parotid tissue was defined as the total amount of amylase activity in the medium plus homogenate.

Drugs: L-Isoproterenol hydrochloride and forskolin were purchased from Sigma. Isoproterenol was used as a solution dissolved with KRT solution. Forskolin was made up in 95% (v/v) ethanol to give a stock solution of 30 mM and diluted with KRT solution as required.

Statistical analysis: The data obtained in this study were statistically analyzed by Student's *t*-test.

Results

Effects of isoproterenol and forskolin on the released amylase activity: Table 1 shows the released amylase activities induced by isoproterenol (10^{-5} M) and forskolin (10^{-5} M) in the parotid tissue from pilocarpine-treated rats after the removal of duct ligation. In the parotid tissue from control rats, the released amylase activity induced by isoproterenol in the operated gland was about 60 percent of that in the unoperated gland. This showed that secretory response to isoproterenol in the parotid gland had not recovered completely on day 7 after the

Table 1. The released amylase activity in chronically pilocarpine-treated rats after the removal of ligation

Stimulator	Treatment	Amylase activity (units/20 mg/30 min)		Rate of recovery (%) (Operated gland/ Unoperated gland $\times 100$)
		Operated gland	Unoperated gland	
Isoproterenol	Saline (Control)	2768 $\pm$ 299	4477 $\pm$ 407	62 $\pm$ 7
	Pilocarpine	4085 $\pm$ 309	4335 $\pm$ 252	94 $\pm$ 5**
Forskolin	Saline (Control)	2764 $\pm$ 420	4223 $\pm$ 439	67 $\pm$ 12
	Pilocarpine	4017 $\pm$ 324	4130 $\pm$ 457	98 $\pm$ 7*

Pilocarpine (5 mg/kg) was intraperitoneally injected into the rats once a day for 7 days. Amylase release was induced by isoproterenol (10^{-5} M) or forskolin (10^{-5} M). Each value represents the mean $\pm$ S.E. of 5–6 experiments. **P* < 0.05, ***P* < 0.005, compared with saline-treated rats.

removal of ligation following 7 days of obstruction. On the other hand, the activity of the enzyme in the operated gland of pilocarpine-treated rats was about 95 percent of that in the unoperated gland. Chronic pilocarpine treatment significantly increased the recovery of the released amylase activity induced by isoproterenol in the parotid gland after the removal of the duct ligation.

The released amylase activity induced by forskolin in the operated gland was about 65 percent of that in the unoperated gland in the control rats. However, treatment with pilocarpine induced almost complete recovery of the enzyme activity in the parotid gland after removing the ligation.

Changes on the percentage of amylase release induced by isoproterenol and forskolin: The percentage of amylase release in the tissue from chronically pilocarpine-treated rats after the removal of duct ligation are shown in Table 2. In the control rats, there was no significant difference in the percentage of amylase release induced by isoproterenol between the operated and the unoperated glands. However, in the tissue from rats treated with

pilocarpine, the percentage of amylase release increased significantly as compared with the unoperated gland.

On the other hand, the percentage of amylase release induced by forskolin was similar to that induced by isoproterenol: no significant difference was observed in the control rats, but chronic pilocarpine treatment increased that induced by forskolin in the tissue from rats after the removal of the ligation.

Amylase activity in the parotid tissue: Table 3 shows amylase activities in the parotid tissue of rats after the removal of the ligation. The activity of the enzyme in the unoperated gland of pilocarpine-treated rats was similar to that of the control. In the operated gland, pilocarpine treatment slightly increased the enzyme activity, but there was no significant difference in amylase activity between the control and pilocarpine-treated rats.

Discussion

In our previous studies, it was demonstrated that isoproterenol-induced amylase release from rat parotid slices decreased pro-

Table 2. The percentage of amylase release in chronically pilocarpine-treated rats after the removal of ligation

Stimulator	Treatment	Amylase release (% of total)	
		Operated gland	Unoperated gland
Isoproterenol	Saline (Control)	18.9±1.4	16.0±2.1
	Pilocarpine	24.1±1.0**	16.0±1.1
Forskolin	Saline (Control)	17.3±2.1	14.8±1.9
	Pilocarpine	22.8±2.0*	14.4±1.3

Pilocarpine (5 mg/kg) was intraperitoneally injected into the rats once a day for 7 days. Amylase release was induced by isoproterenol (10^{-6} M) or forskolin (10^{-6} M). Each value represents the mean±S.E. of 5–6 experiments. * $P<0.05$, ** $P<0.01$, compared with saline-treated rats.

Table 3. Amylase activity in the parotid tissue

Treatment	Amylase activity (units/20 mg/30 min)		Rate of recovery (%) (Operated gland/ Unoperated gland ×100)
	Operated gland	Unoperated gland	
Saline (Control)	16284±1962	29859±2456	55±6
Pilocarpine	20456±2577	30784±2470	66±5

Pilocarpine (5 mg/kg) was intraperitoneally injected into the rats once a day for 7 days. Each value represents the mean±S.E. of 5–6 experiments.

gressively after duct ligation, and the decreased amylase release recovered reversibly after removing the ligation (1, 2). Based on these results, the durations of duct ligation and the timing for its removal were determined. The released amylase activity induced by isoproterenol in operated gland of control rats, as shown in Table 1, was similar to that induced by forskolin. Accordingly, amylase release induced by isoproterenol and forskolin in the parotid gland after removing the duct ligation may be inhibited by a common mechanism: the degeneration of the cell membrane by duct ligation inhibits the sequence of receptor reactions initiated by isoproterenol or the activation of adenylate cyclase in the case of forskolin. However, it was demonstrated that secretory granules and amylase activity in the parotid tissue decreased remarkably after duct ligation (1, 8), and the recovery of amylase activity in the tissue after the removal of the ligation was about 60 percent, as shown in Table 3, and was similar to that of released amylase activity. These results suggest that the decreased amylase release after the removal of duct ligation is due to the incomplete recovery of secretory granules and not due to the impairment of the stimulus-secretion coupling mechanism in the parotid gland.

Chronic pilocarpine administration increased the released amylase activities which were induced by isoproterenol and forskolin in the parotid gland following ligation removal. The enzyme activities recovered almost completely (Table 1). However, the recovery of amylase activities in the tissue of rats treated with pilocarpine was only about 65 percent, which was not significantly different from the control (Table 3). On the other hand, as shown in Table 2, the percentage of amylase release in operated glands of rats administered pilocarpine increased significantly compared with that of the control. Therefore, the acceleration of the recovery of released amylase activity with pilocarpine treatment after removing the ligation is due to the increased response of amylase release, rather than to the enhanced accumulation of secretory materials in the parotid cells.

A β -adrenergic agonist such as isopro-

terenol stimulates amylase release from parotid tissue, and cyclic AMP mediates the effects of β -adrenergic stimulation of protein secretion (3). However, it was shown that a β -adrenergic agent increased cyclic GMP levels as well as cyclic AMP in the parotid acini (9). Both of these cyclic nucleotides appear to regulate amylase release in the parotid gland (10). On the other hand, forskolin as well as isoproterenol stimulates amylase release from parotid tissue as a result of an increase in the formation of intracellular cyclic AMP (11); however, forskolin has been shown to activate adenylate cyclase specifically and appears to act directly on the catalytic subunit of adenylate cyclase (4, 12). Forskolin provides the means for selectively determining the contributing role of cyclic AMP in salivary gland secretion. The concentration of forskolin used in this study, 10^{-5} M, is suitable for studying amylase release from parotid tissue (11). In present study, as shown in Table 2, both of the responses of amylase release induced by isoproterenol and forskolin were increased significantly in the parotid gland treated with pilocarpine after the removal of ligation, and the response induced by isoproterenol was similar to that induced by forskolin. Therefore, it is suggested that the adenylate cyclase system may be involved in the accelerated recovery of amylase release from parotid tissue in pilocarpine treated-rats after removing the ligation. This may be supported by the study of Mednieks et al. (13): the treatment of parotid acinar tissue with forskolin resulted in a secretory response biochemically identical with that induced by isoproterenol.

The action of pilocarpine is performed via muscarinic cholinergic receptor. Accordingly, the accelerated recovery of amylase release by chronic pilocarpine treatment in the present study is based on membrane events. If not, the stimulative action of isoproterenol and forskolin may not appear.

Pilocarpine induces an increase in the concentration of cyclic GMP (14). Increase of cyclic GMP levels appears to require calcium. So chronic pilocarpine administration may induce the increases of cyclic GMP accumulation and calcium concentration in the

parotid gland after removing the duct ligation. Muller et al. (15) showed that chronic treatment with pilocarpine increased the concentration of calcium in acinar cells of the salivary gland. Calcium plays important roles in amylase release from parotid tissue stimulated by cyclic AMP: an intracellular pool of calcium is required for β -adrenergic agonists to increase amylase release (16). However, in the present study, the relationship between the calcium concentration and the accelerated recovery of amylase release after removing the duct ligation are not clear. It may be interesting to investigate whether or not calcium participates in the increased responses of amylase release in the parotid gland treated with pilocarpine after removing the duct ligation.

In conclusion, the present results showed that chronic pilocarpine treatment increased amylase release in the parotid tissue of rats after the removal of duct ligation. The increased response of amylase release was induced by not only isoproterenol but also forskolin. It seems that cyclic AMP-mediated events may be concerned in the enhanced response of amylase release caused by pilocarpine treatment after the removal of the parotid duct.

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