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Total Salivary Calcium and Amylase Output of Rat Parotid with Electrical Stimulation of Autonomic Innervation (40374)

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A relationship between the kind of autonomic stimulation used to elicit salivary secretion and the concentration of amylase and calcium in the secretion has been demonstrated using an *in vivo* preparation (1–3). Administered autonomic agonists were compared with the effects of direct electrical stimulation of the autonomic nerve fibers (3). The results obtained using the more physiological condition of stimulation (i.e., the nerve) were not identical to those obtained using injected agonists (3). From recent work on the perfused main duct of submaxillary gland where effects of nerve stimulation were compared with effects of administered agonists, major differences between effects of drug administration and nerve stimulation were also observed (4, 5). These findings suggest that the effects observed with injected autonomic drugs may not be equated to effects observed under more physiological conditions of stimulation. When still another modification from the physiological state is introduced (such as use of an in vitro system), it is probable that additional discrepancies may become evident. Thus, although the in vitro parotid slice model has yielded important information regarding autonomic control of amylase and calcium secretion, it has become evident that the initial postulate of Schramm's group, i.e., that calcium and amylase are packaged together and secreted together across the luminal membrane (6, 7), probably is not true for all conditons of stimulation (3). In fact, recent work has implied that at least two routes for calcium secretion may exist, one involving packaging with amylase and the other may involve secretion of calcium in the saliva without packaging with amylase. To test this hypothesis further, in vivo systems that are more comparable to the *in vitro* ones were employed; these included analysis of gland depletion of calcium and amylase with stimulation and measurement of total salivary output of these two moieties. Disparities between gland depletion and salivary output would be indicative of the importance of other mechanisms. Finally, Schramm (8) also suggested that in the *in vitro* system, any apparent cholinergically induced release of amylase or calcium is actually the result of acetylcholine induced catecholamine release. The validity of this assumption was also examined in the present study, and appropriate adrenergic antagonists were used in conjunction with stimulation of the parasympathetic innervation to test this point.

Materials and methods. Female Long-Evans rats used in these experiments were 4–5 months of age, weighed approximately 200 g, and were maintained on lab chow and water *ad libitum*. After 18 hr of starvation, rats were anesthetized with 1% sodium pentobarbital in doses of 50 mg/kg of body wt. The trachea was cannulated to avoid respiratory complications. Collection of saliva was made by application of calibrated micropipettes to the cut orifice of the parotid duct (4). Electrical stimulation of either the auriculotemporal nerve or the superior cervical ganglion was used to elicit flow of saliva from the parotid; square wave pulses of 4 V at a frequency of 20 pulses/sec and 5 msec in duration were delivered to the nerves by a Grass stimulator, SD5. Flow rate was determined by measuring the time required for collection of a given volume of saliva and relating this to gland weight (9). Stimulation and collection of samples were continuous so that not only concentration but total salivary output of calcium and amylase were measured. Calcium concentration was determined on saliva samples by titration of the fluorescent calcium–calcein complex with Ethylene–glycol bis (2 amino

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ethyl ether)-NNN'N' tetra acetic acid (EGTA) (automatic calcium titrator, Fiske Associates, Inc.).

The stimulated parotid gland was removed immediately following termination of the stimulation period. The tissue was divided into two parts and weighed separately. One part was used for analysis of amylase activity; the other part was put in a crucible, dried overnight and reweighed to determine its water content. The dried residue was ashed at 500° for 12–14 hr. The ash was dissolved in 0.5 ml of 0.5 *N* HCl. The resultant solution was neutralized with 0.5 ml of 0.5 *N* NaOH. Calcium concentration was then determined by automatic calcium titrator (Fiske Associates, Inc.). Atomic absorption analysis of calcium was also done and the two methods gave essentially the same results.

The unstimulated contralateral control gland was also quickly removed and treated in exactly the same way as the experimental gland.

Amylase activity of appropriately diluted samples of saliva or gland homogenate was determined by methods previously described (9). Salivary amylase activity was expressed as milligrams of reducing substance formed per microliter of saliva in a 15 min digestion period at 37°, whereas glandular amylase activity was expressed as milligrams of reducing substance formed per milligram of gland weight in a 15 min digestion period at 37°. Samples of saliva were obtained continuously for a 60-min period of nerve stimulation. Total output of amylase in saliva was also measured.

To rule out the possibility that acetylcholine-mediated release of catecholamines was involved in any of the effects, the α-adrenergic blocking agent (phenoxybenzamine or Dibenzyline) and β-adrenergic blocking agent (propranolol or Inderal) were administered ip in doses of 5 and 3 mg/kg, respectively, 25 min before initiation of nerve stimulation.

Analysis of data. All data in the text, tables and figures are expressed as means ± SE. The control values were compared with the experimental values by unpaired Student's *t* test (13). Values were considered to be statistically significantly different if *P* values were less than 0.05.

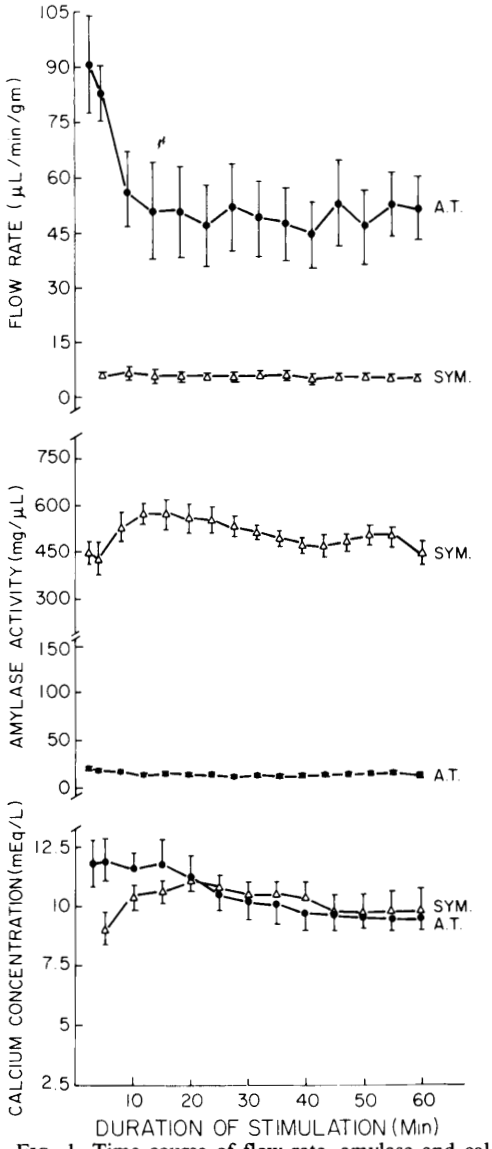

FIG. 1. Time course of flow rate, amylase and calcium concentration of rat parotid saliva during continuous stimulation of the auriculotemporal nerve (AT.) or superior cervical ganglion (SYM.).

Results. The data in Fig. 1 summarize the effects of direct nerve stimulation on calcium, amylase and flow rate of saliva from parotid of adult rats. No saliva flow could be observed during the prestimulation period. The calcium concentration of saliva evoked by supramaximal stimulation of the auriculotemporal nerve was initially high (11–12 mEq/l) and remained high (10 mEq/l) during a 60-min period of continuous stimula-

tion. Calcium concentrations of saliva evoked by stimulation of the superior cervical ganglion were initially somewhat lower (9–10 mEq/l), but reached levels similar to those induced by cholinergic stimulation within 20 min and remained at these levels thereafter.

Flow rate under the two conditions of stimulation differed markedly from each other. It was very high initially with cholinergic nerve stimulation (0.105 μl/min/mg of gland) but within 10 min fell to levels of about 0.06–0.05; these levels were then maintained for the 60 min of stimulation. On the other hand, flow rate with sympathetic nerve stimulation was initially very low (0.1 μl/min/mg) and remained at this level for the 60 min period of stimulation.

Amylase levels were also consistently (initially and thereafter) very low (20 mg/μl of saliva) with cholinergic nerve stimulation. However, with stimulation of the sympathetic nerve, while initial values were only 450–500 mg/μl, within 15–20 min, they attained a maximum of 600 mg/μl and remained at this plateau level thereafter.

Flow rate does not appear to be an important factor in regulating levels of amylase or calcium under these conditions of stimulation. However, since the total volume of fluid secreted under the two conditions of stimulation were so different (Table I), it was probable that the volume of fluid would affect total output of calcium and amylase. Thus, calculations of total volume of saliva secreted during the 60 min of nerve stimulation were made, and are presented by the data in Table I. With stimulation of the auriculotemporal nerve, 653 ± 103 μl of fluid were secreted over the 60-min period when collection of saliva was continuously made. During the same interval, only 82 ± 9 μl were secreted when the sympathetic nerve was stimulated. Thus, there is an eightfold difference in volume when comparison between effects of the two conditions of stimulation are made. (These data agree very well with those of Young *et al.* (10) who showed that electrolyte concentrations of precursor fluids were similar under the two conditions of stimulation but the total volume produced was eight times greater with cholinergic than with adrenergic stimulation.)

Calculations of total output of calcium and amylase were made. For example, since calcium concentrations under the two conditions of stimulation were generally similar in magnitude and in course of change whereas total volumes under the two conditions were markedly different, it was anticipated that total output of calcium with parasympathetic nerve stimulation would greatly exceed that obtained with stimulation of the sympathetic innervation. There was in fact about a ninefold difference in total calcium output when the two kinds of nerve stimulation were compared. This slight difference is attributable to the slight differences in calcium concentration observed between the two kinds of stimulation. Thus, a total of 6744 ± 867 nEq of calcium were secreted with cholinergic stimulation and only 762 ± 91 with sympathetic nerve stimulation. Similarly, amylase concentration of cholinergically-evoked saliva was very low (20 mg/μl) initially and throughout the period of collection, whereas the values with sympathetic nerve stimulation were 30–40 times greater. Again, the total output under the two conditions of nerve stimulation reflected these differences and the total amylase with stimulation of the sympathetic nerve was five times greater than that found with cholinergic stimulation, even though the total volume of cholinergically-evoked saliva was eight times greater.

Since flow rate was a modifying factor in assessment of total salivary output, it was necessary to relate the salivary output of these moieties to the levels remaining in the gland after stimulation was halted. From the data in Table II, it is clear that only a small (but

TABLE I. TOTAL OUTPUT OF AMYLASE, CALCIUM, AND FLUID IN RAT PAROTID SALIVA FOLLOWING STIMULATION OF THE AURICULOTEMPORAL NERVE (AT) OR SUPERIOR CERVICAL GANGLION (SYM) FOR 60 MIN.[a]

	Kind of stimulation	
	AT	SYM
Total volume (μl)	653 ± 103	82 ± 9
Total Ca output (nEq)	6744 ± 867	762 ± 91
Total amylase output (mg of reducing sub.)	8330 ± 906	41,076 ± 3337

[a] Values are means ±SE. The number of rats for each kind of stimulation was 7; output was continuously collected over 60 min. The differences between parasympathetic and sympathetic stimulation are statistically significant at a level of $P < 0.001$.

statistically significant ($P < 0.05$)) change in calcium concentration of the gland occurred when the auriculotemporal nerve was stimulated for 60 min. The change with sympathetic nerve stimulation was greater. Thus, a 35% reduction in gland calcium was found with stimulation of the sympathetic nerve, but only a 13% decrease was observed when stimulation of the auriculotemporal nerve was employed. These changes were quite unexpected since the total output of calcium in cholinergically-evoked saliva was nine times greater than that found with sympathetic nerve stimulation. The possibility that water changes in the gland could account for this apparent inconsistency was ruled out when comparisons of calcium were made using dry weight of the gland. The percent changes were not very different from those based on wet weight of the gland (Table II).

It also seemed possible that with cholinergic stimulation, calcium changes in the gland may have occurred earlier than 60 min and by 60 min, gland levels had been restored to normal. Accordingly, calcium levels of the glands were assessed 20 min after initiation of stimulation. These values were virtually the same as those of controls (Table III).

Since calcium levels of saliva evoked by cholinergic and adrenergic nerve stimulation were so similar, there was the possibility that catecholamines were indeed released by stimulation of the cholinergic nerve, and that high calcium levels with either kind of stimulation must be attributed to adrenergic influences. However, this assumption was not found to be the case. The calcium levels of saliva evoked by cholinergic stimulation initiated 25 min after injection of both α- and β-adrenergic blocking agents were not different from saliva levels of parasympathetically stimulated glands of rats that did not receive blocking agents (Table IV).

The depletion of gland levels of amylase paralleled the total output of the enzyme in the saliva. Thus, amylase levels of the gland were reduced by 23% after 60 min of stimulation of the cholinergic nerve; with sympathetic nerve stimulation there was a 46% reduction in gland levels.

Discussion. Present data strongly suggest that calcium and amylase secretion follow different routes with parasympathetic and sympathetic nerve stimulation. With auriculotemporal stimulation, calcium is transferred from plasma through the gland, with the

TABLE II. CHANGE IN WATER CONTENT, AMYLASE ACTIVITY AND CALCIUM CONCENTRATION OF RAT PAROTID GLAND WITH STIMULATION OF EITHER AURICULOTEMPORAL NERVE (AT) OR SUPERIOR CERVICAL GANGLION (SYM).[a]

Kind of stimulation*	Rat parotid gland		
	Water content (percent)	Amylase activity (mg/mg wet wt)	[Ca] (mEq/kg wet wt)
None	70.6 ± 0.8 (20)	537 ± 29 (11)	13.0 ± 0.3 (20)
AT.	74.3 ± 1.0 (8)	380 ± 24 (4)	11.3 ± 0.9 (8)
	($P < 0.05$)	($P < 0.001$)	($P < 0.05$)
SYM.	78.0 ± 2.0 (5)	199 ± 16 (5)	8.4 ± 0.2 (5)
	($P < 0.05$)	($P < 0.001$)	($P < 0.001$)

[a] Values are means ±SE; all values from experimental animals differ significantly from controls. * In each case, duration of period of stimulation was 60 min. The numbers in parentheses are number of rats.

TABLE III. CHANGE IN WATER CONTENT, AMYLASE ACTIVITY AND CALCIUM CONCENTRATION OF RAT PAROTID GLAND FOLLOWING 20-MIN PERIOD OF STIMULATION OF EITHER THE AURICULOTEMPORAL NERVE (AT) OR SUPERIOR CERVICAL GANGLION (SYM).[a]

Kind of stimulation	Rat parotid gland		
	Water content (percent)	Amylase activity (mg/mg wet wt)	[Ca] (mEq/kg wet wt)
None	70.6 ± 0.8 (20)	537 ± 29 (11)	13.0 ± 0.3 (20)
AT. (20 min)	72.2 ± 0.7 (6)	434 ± 21 (6)	13.0 ± 1.0 (6)
	NS	($P < 0.01$)	NS

[a] Values are means ± standard error. The numbers in parentheses are number of rats. NS = Not significantly different ($P > 0.05$).

TABLE IV. EFFECTS OF PRIOR ADMINISTRATION OF ADRENERGIC ANTAGONISTS ON CALCIUM OF SALIVA EVOKED BY STIMULATION OF THE AURICULOTEMPORAL NERVE (AT).[a]

Condition of stimulation	Ca concentration (mEq/l)
AT.	12.3 ± 0.76 (10)
AT. + IN.	12.8 ± 1.20 (6)
AT. + DI.	11.0 ± 0.58 (5)
AT. + DI. + IN.	11.3 ± 0.63 (4)

[a] Values are means ± SE. In no case did calcium values differ ($P > 0.05$) from each other or from levels found with nerve stimulation alone. Propranolol (IN) (3 mg/kg) or dibenzyline (DI) (5 mg/kg) was administered ip. singly or together 25–40 min before resumption of stimulation of auriculotemporal nerve. The numbers in parentheses are number of rats.

levels in the saliva mainly representative of the amounts transferred through the glandular cells; thus a greater proportion of the total calcium output is not packaged and secreted with the amylase. Since the levels of amylase are low, the amounts of calcium packaged with the amylase are also low, and a large excess of calcium may be transferred independently from plasma to saliva. This is not the case with adrenergic nerve stimulation. With such stimulation, the amylase levels are very high and virtually all of the calcium may be packaged and secreted with the amylase (3). A parallelism between secretion of these two moieties with adrenergic but not cholinergic stimulation would be expected consequences (3), and the present data also supported the previous finding (3).

The principal finding that remains inexplicable is that a large output of calcium was observed with cholinergically stimulated saliva but little depletion of calcium in the gland was found. On the other hand, a small output of calcium was obtained with sympathetically induced secretion and there was a parallel between depletion of gland calcium and total output in saliva. Thus, secretory mechanisms for calcium secretion are not the same for both kinds of stimulation. The only explanation presently tenable to account for these differences is that uptake of calcium into the gland proceeds as rapidly as it is released into the saliva when cholinergic stimulation is employed. Furthermore, this uptake must occur very early or continuously, since gland depletion was very insignificant in amount even as early as 20 min after stimulation was initiated.

Finally, the data show that the surprisingly high levels of calcium found with cholinergic nerve stimulation cannot be attributed to indirect effects of cholinergically-released catecholamines as postulated by Schramm's group (8) since the injection of both α- and β-adrenergic antagonists prior to stimulation of the auriculotemporal nerve did not cause any modification in calcium levels from those observed with nerve stimulation alone. Furthermore, other evidence refutes the postulate that cholinergic stimulation involves catecholamine release. Thus, amylase activity of cholinergically evoked saliva samples is very low and the inhibition of adrenergic activity does not modify these levels further (2, 9, 11, 14, 17). In addition, when isoproterenol in concentrations (2.5 μg/kg) too low to elicit secretion are injected during stimulation of the auriculotemporal nerve, a sharp increase in amylase, attributable to the isoproterenol, is observed (11). This clearly shows that the two different groups of receptors are involved in the amylase release and depend on kind of autonomic stimulation employed. Indeed, this view is further supported by work of several investigators (12, 14–16), since they have shown that cholinergic release of amylase is mediated through a pathway (cyclic GMP) separate from that of β-adrenergic release of amylase (cyclic AMP). Evidence is accumulating therefore that supports the thesis of separate pathways for the release of amylase and calcium, and this separation is determined by the kind of autonomic stimulation employed.

It is interesting that the total output of amylase induced by stimulation of the sympathetic nerve was fivefold greater than that of the parasympathetic nerve stimulation in spite of the finding that there was only a twofold difference in residual gland amylase. It is probable that stimulation of the sympathetic nerve enhances synthesis of amylase at a greater rate than that induced by stimulation of the parasympathetic nerve. However, the detailed mechanisms of enhancement of amylase synthesis evoked by the two kinds of autonomic nerve stimulation have not been clarified.

Summary. Calcium levels of rat parotid

saliva evoked by stimulation of the auriculotemporal nerve are high (11 mEq/l) and in fact, higher than those evoked by stimulation of the sympathetic innervation. Total calcium output in the cholinergically-evoked saliva is also very high but the depletion of gland levels is insignificant 20 or even 60 min after the initiation of stimulation. With sympathetic stimulation, there is a closer correlation between gland depletion and total output of calcium in the saliva. These findings suggest that the uptake mechanism for calcium with cholinergic stimulation is more rapid than that found with adrenergic stimulation. The high levels of calcium in the cholinergically evoked saliva are also not due to acetylcholine-induced release of catecholamines since calcium levels of cholinergically-evoked saliva are the same whether or not adrenergic blocking agents are present. The total output of amylase in the saliva when sympathetic stimulation is employed is about five times greater than that found with cholinergic stimulation, and the reduction in gland amylase under these two conditions of stimulation reflect these same relations. The data also show that there is a parallelism between depletion of gland amylase and calcium and concentration and total output of these two moieties in the saliva when adrenergic stimulation is used but that no parallelism between secretion of these substances is seen with cholinergic stimulation. It is suggested that with adrenergic stimulation all of the amylase is packaged together with calcium and the two are secreted together; however, with cholinergic stimulation, only a fraction of the total calcium is packaged with the amylase, and the remainder is transferred from blood through the gland to the saliva. Thus, two separate routes for secretion of calcium exist with cholinergic stimulation, and the pathways with the two kinds of nerve stimulation are different.

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