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Calcium, Amylase, and Flow Rate of Rat Parotid Saliva with Diverse Frequencies of Parasympathetic Nerve Stimulation (40692)[1]

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Calcium concentrations of parasympathetically evoked saliva are generally reported to be lower than those of sympathetically evoked saliva (1). However, recent evidence shows that when electrical stimuli of supramaximal intensity are used to stimulate the parasympathetic innervation to rat parotid, the evoked saliva has a high concentration of calcium, as well as a high rate of flow (2). Since sympathetically evoked saliva (especially with isoproterenol) from rat parotid also has a similarly high concentration of calcium but a much lower flow rate than parasympathetically evoked saliva (3), it became important to determine if calcium concentration of saliva is in fact independent of flow rate, especially since some reports indicate a dependence of calcium concentration on flow rate, while other reports show the opposite relation (4, 5). It is believed that those controversial differences have arisen because comparisons were made without regard to kind of stimulation employed (6). With a particular kind of autonomic stimulation, concentration of calcium may be dependent on flow rate. Therefore, the present work was undertaken to determine the relationship between calcium concentration and flow rate with a single kind of autonomic stimulation. The postganglionic parasympathetic innervation to rat parotid can be stimulated electrically, and intensity of stimulation can be altered by varying the frequency of electrical impulses to the nerve. By this procedure, flow rate also is readily varied and increases with increases in frequency (6).

Establishment of the relationship between calcium and flow rate is important for a number of reasons. First, if, like Na^+, it is found to be dependent on flow rate, or like K^+, independent of flow rate, inferences regarding transport of this ion can be made (5, 7, 13). Second, it has been suggested that secretion of calcium is closely bound to secretion of the enzyme amylase (8). However, while this appears to be true under some conditions of stimulation (1, 9), a second pathway for calcium secretion that is not linked to amylase secretion may occur under conditions of stimulation of the parasympathetic nerve (2). Thus, determination of the influence of flow rate on amylase as well as calcium concentration of saliva may provide further evidence for existence of a second pathway for calcium secretion that is not linked to secretion of amylase.

Materials and methods. Long–Evans adult female rats, used in these experiments, were maintained on standard lab chow and water *ad libitum* until 18 hr before experimentation, when food but not water was withdrawn. Rats were anesthetized with 1% Nembutal and the parotid duct and auriculotemporal nerve were then exposed. The duct was cut at its anterior end, and micropipets, applied to the cut surface, were used to collect measured volumes of fluid during a timed interval. This provided a measurement of flow rate. Since gland weight could not be determined at each successive point of stimulation, flow rate was more conveniently expressed as μl/min.

Flow of saliva was elicited by continuous stimulation of the parasympathetic nerve (auriculotemporal) to the parotid for at least 80 min. A Grass stimulator was used to deliver square wave pulses (4 V, 4-msec duration) at frequencies varying from 5 to 20 Hz (7).

Since it had previously been shown that 20 Hz represented an intensity that was supramaximal (7), frequencies above this were not investigated. The previous work (7) also showed that with 60 min of continuous stimulation at the single supramaximal frequency of 20 Hz, flow rate remained consistently high for about 30 min but gradually decreased thereafter. It was, therefore, necessary to eliminate changes that appeared with in-

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creasing time so that the influence of frequency of stimulation on flow rate could be more precisely assessed. The present kinds of experiments were thus performed so that this could be accomplished. Thus, frequency of stimulation was changed sequentially in the same rat (total rats used, 12) from low (5 Hz) to high (20 Hz) and then from 20 to 5 Hz. While these changes in frequency were in each case done in the same animal, the order of change of frequency was varied from animal to animal. Duration of stimulation in these experiments at each frequency was usually about 10 min; before a sample of saliva was collected at a new frequency, an interval of at least 5 min was allowed to elapse. This was more than ample (3 min in fact was adequate) to establish a steady state (accounting for gland and duct lag in clearance of saliva) and the new values obtained were, therefore, representative of the effect of the new frequency.

Twelve animals were examined and for three of these, the flow rate, calcium concentration and amylase activity of saliva elicited at each frequency of stimulation were examined over a period of 80 min of continuous nerve stimulation. In one additional rat, stimulation was maintained for 120 min and since gland levels of amylase change within this time, and the saliva concentrations reflect these changes, amylase of saliva was not determined in this experimental animal (1D). With eight additional rats, the parameters mentioned were examined at particular frequencies and during a specific period (initial 40 min after initiation of nerve stimulation). These provided mean values for each parameter with each frequency of stimulation.

Amylase activity was determined by the method of Myers *et al.* (10) and expressed as milligrams of reducing substance formed per microliter of fresh saliva in 15 min at 37°C.

Calcium concentration of saliva or serum samples was determined using a Fiske titrator. Comparison with values obtained using atomic absorption method for calcium determination showed that the values from the two methods agreed well.

Results. Table I includes data from eight rats and shows the mean values for flow rate, calcium concentration and amylase activity of the saliva during stimulation of the auriculotemporal nerve at frequencies of 5, 10, 16, and 20 Hz; furthermore, these values are those obtained during the early period following initiation of continuous nerve stimulation of 80-min duration. Duration of stimulation in these experiments at each frequency was usually about 10 min; the details for stimulation and collection of saliva have already been described. The data show that flow rate and calcium concentration rise with sequential increases in frequency of stimulation, with mean increments in flow rate of approximately 6 μl/min (eight rats) and mean increments in calcium concentration of 3–4 meq/liter evident when frequency was increased from 5 to 10 Hz, and then from 10 to 16 Hz. These represented increases of at least 50% in flow rate and calcium when frequency was changed from low to high. In fact in a specific instance (when frequency of stimulation was increased from 5 to 10 Hz), there was a 1.5-fold increase in flow rate. Conversely, when frequency was decreased from high to low, flow rate and calcium concentration were decreased.

Amylase concentration on the other hand was independent of flow rate and the values of approximately 16 mg/μl of saliva represent the activity of this enzyme at all flow rates

TABLE I. EFFECTS OF VARYING FREQUENCY OF STIMULATION OF AURICULOTEMPORAL NERVE ON FLOW RATE AND CALCIUM AND AMYLASE CONCENTRATIONS OF RAT PAROTID SALIVA[a]

Frequency (Hz)	Flow rate (μl/min)	Calcium conc. (meq/liter)	Amylase activity (mg/μl of saliva)
5	4.5 ± 0.38	5.7 ± 0.31	15.6 ± 1.81
10	11.2 ± 1.70	9.1 ± 0.25	16.4 ± 1.56
16	17.1 ± 2.21	13.8 ± 0.26	17.2 ± 1.61
20	16.8 ± 2.10	12.7 ± 0.23	16.0 ± 0.98

[a] Values are means ± SE; total number of rats was eight. Flow rate is expressed as volume/min (μl/min), and amylase concentration as milligrams of reducing substance formed in 15 min at 37°C. Frequency of stimulation is expressed as Hz. Values for flow rate and calcium concentration (but not amylase) at 5 and 10 Hz are statistically different from those at 16 and 20 Hz ($P < 0.001$); those at 5 differ from those at 10 Hz, and those at 10 differ from those at 16 Hz ($P < 0.001$), but again amylase values are the same at all frequencies. Values for calcium concentration and flow rate at 16 and 20 Hz are virtually identical. Mean calcium concentrations of serum from unstimulated rats were 4.9 ± 0.4 meq/liter (eight rats), and those of serum obtained after 80 min of nerve stimulation were 4.6 ± 0.5 meq/liter (eight rats).

and frequencies of stimulation examined (Table I and Fig. 1).

The data in Fig. 1 confirm and extend this observation. Data from 4 of the 12 rats used in these experiments are presented separately so that individual values at each flow rate for a particular animal can be examined during the entire 80 min of continuous nerve stimulation. In one of these, stimulation was continued for as long as 120 min but no amylase values were determined on saliva from this animal (1 D) since the concurrent gland depletion of this enzyme over this extended period of time would modify amylase levels of saliva and yet not be related to changes in flow rate. Other additional points are also illustrated by data in Fig. 1.

It is clear, e.g., that the changes in calcium and flow rate are very sensitive to changes in frequency of stimulation not only when frequency is sequentially increased but also when it is sequentially decreased. Furthermore, these changes are induced rapidly in the same animal and this alteration can be continued for a long period of time. The advantage of altering frequency sequentially in the same animal over a long period of time has already been alluded to in Materials and Methods. Thus, when effects due to time supervene (within 30 min after initiation of stimulation), the effects of frequency on flow rate or calcium concentration become obscured by these time-induced changes. In these present experiments, even if absolute levels of calcium or flow have decreased as a consequence of time to a new lower level, and at 30 min, for example, a frequency of 16 Hz no longer causes as high a calcium concentration and flow rate as initially induced by this frequency, from this new, lowered-base line, a change in frequency still brings about a change in calcium or flow and it is usually similar in magnitude to the original changes (Fig. 1).

Calcium levels of serum obtained from the control and experimental animals were 4.6 ± 0.5 meq/liter and 4.9 ± 0.3 meq/liter respectively (after 8 rats) and did not differ from each other ($P < .001$). For control values, serum was obtained from unstimulated rats; for the experimentals, serum was obtained following the 80 min of stimulation.

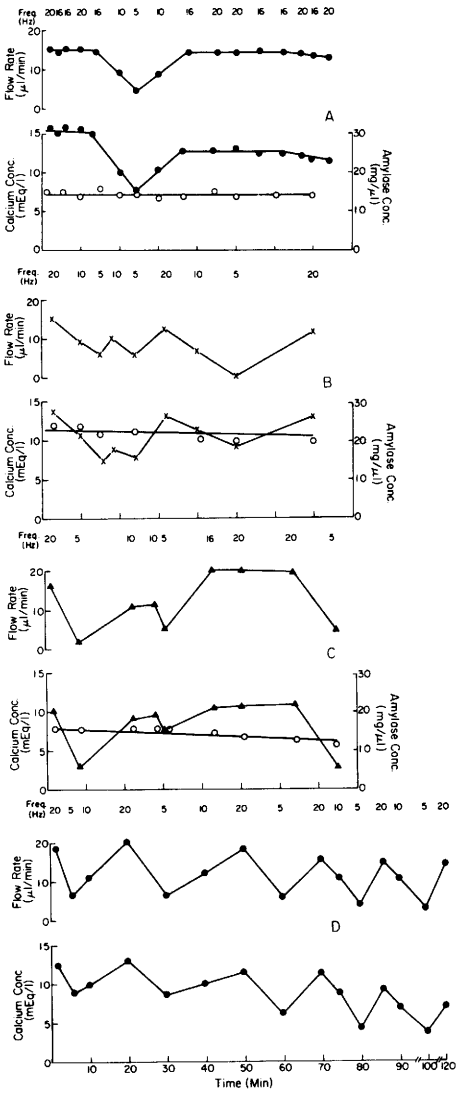

FIG. 1. The data in Fig. 1 show the relationship between frequency of stimulation, flow rate, calcium concentration, and amylase concentration. Each of the four graphs (A–D) represent individual values from four separate rats. Data were collected over a period of at least 80 min (120 min for 1 D) when continuous electrical stimulation of the auriculotemporal nerve was employed. Flow is expressed only as vol/min since the same gland was being stimulated in each case. Black signs are used to represent calcium concentration and flow rate; open circles are used to show amylase activity (expressed as mg reducing substance/µl of saliva). Frequency variation is presented horizontally by appropriate numbers at the top of each of the four experiments, and time (in min) at the bottom. Amylase activity was not determined for animal 1 D.

The calcium concentration of saliva was not statistically different from that of serum at a frequency of 5 Hz, but double that of serum at 10 Hz, and three times that of serum at 16 or 20 Hz.

Discussion. The frequency of stimulation of the parasympathetic nerve to parotid gland of rat is an important factor in regulating flow rate and calcium concentration of the nerve-evoked saliva but has no influence in regulation of amylase concentration of the saliva. Both calcium concentration and flow rate increase with increases in frequency of stimulation, until a plateau is reached at 16 Hz. From these observations it may be concluded that flow rate has a regulating influence on calcium concentration of parasympathetically evoked saliva but the occurrence of a plateau suggests that the ability of gland cells to absorb or secrete calcium has a fixed limit. Thus, salivary calcium levels are in part also regulated by this fixed capability of the cells as well as by flow of the saliva. However, these conclusions must be considered valid only when a particular kind of autonomic stimulation is employed. When flow rates and calcium concentrations of saliva evoked by two different kinds of autonomic stimulation are compared, flow rate has no role in accounting for differences in calcium concentration. It has been shown, for example, that isoproterenol (3) or stimulation of the sympathetic nerve (6) can elicit saliva of very low flow rates (4 μl/min). Yet the calcium concentrations of such salivas are usually as high (11–13 meq/liter) (3) or nearly as high (11 meq/liter) (6) as that observed with high rates of flow induced by high frequency of stimulation of the parasympathetic nerve (11–13 meq/liter), and are not at all comparable to the low calcium concentrations (5 meq/liter) observed at low, parasympathetically induced, flow rates (4 μl/min at 5 Hz).

From the present data some inferences can be made concerning the mechanism by which salivary concentration of calcium is regulated and what bearing flow rate has on this. For example, at the highest flow rates, the final saliva does exhibit a calcium concentration that is three times higher than that of serum (11). One possibility to account for this would assume that at the acinar cell level a precursor fluid rich in calcium is produced, and that modification in calcium concentration does not occur as this fluid rapidly passes through the ducts (12). The contact time of the calcium-rich precursor fluid with duct cells is too brief at the highest flow rates to permit any reabsorption of calcium; thus concentrations in the final saliva are high (12). Conversely, at lower flow rates, where contact time between duct cells and calcium-rich precursor fluid is prolonged, more calcium is reabsorbed, and the calcium concentration of the final saliva is much lower (11, 12). While flow rate thus has an important effect in regulating calcium concentration of saliva for a particular kind of autonomic stimulation, it does not have exclusive control over it.

It must be made clear that the above arguments assume that a calcium-rich precursor fluid is formed at the acinar level and that a flow-related reabsorption of calcium occurs at the ductal level. This assumption seems most logical on the basis of present data for several reasons: (i) calcium concentration in the final saliva is three times that of serum; (ii) since calcium concentration increases with increased flow rate, rather than decreasing with increasing flow, secretion of calcium from duct cells into a final saliva is not likely; furthermore, recent unpublished data on duct perfusion show that calcium is reabsorbed (not secreted) by duct cells of submaxillary. However, definitive resolution depends on examination of calcium concentration of the precursor fluid. Until that is accomplished other possibilities must be considered. For example, the calcium concentration of the precursor fluid may be similar to that of serum, but increases as the precursor fluid passes through the duct system, where calcium is secreted by duct cells and calcium of the final saliva thereby is increased. Such a mechanism could thus also result in calcium levels in the final saliva that are three times higher than that of serum. (iii) Water reabsorption by ductal cells could also account for this threefold increase in calcium of saliva (when compared with serum levels). On the basis of other evidence, this does not appear likely, however. Thus, it has been shown in perfused main duct of rat submaxillary gland that the duct cells are relatively impermeable to water (5). In general, the behavior of calcium with flow rate makes the first possibility more tenable.

Amylase is independent of changes in flow

rate and remains the same at all levels of stimulation. Since changes in flow rate produce changes in calcium concentration, it must be concluded that at least some fraction of the total secreted calcium must not be bound to amylase. This is not in contradiction to the generally held view that when amylase is secreted, it is secreted with calcium (14). However, calcium may be secreted into saliva without amylase bound to it under certain conditions of stimulation (11).

Summary. Calcium concentration of saliva, when electrical stimulation of the parasympathetic postganglionic fibers to rat parotid is used to evoke secretion, increases with increases in flow rate, but both calcium concentration and flow rate reach maximal levels at frequencies of 16 Hz. The concentration of calcium at highest flow rate is three times that of serum. Amylase activity of the saliva remains consistently low at all flow rates. Therefore, these data show that while calcium and amylase are secreted together, some calcium may be secreted without amylase. By inference it is also possible to suggest that the concentrating mechanism for calcium is at the acinar level, and that as the precursor fluid, rich in calcium, flows through the duct system, very little absorption of calcium by ductal cells occurs at high flow rates, but as contact time is increased with decreasing flow rate, more calcium is reabsorbed, resulting in a final saliva low in calcium. It is not likely that the increased calcium concentration is the result of reabsorption of water by ductal cells since in perfused main submaxillary duct at least, the duct cells are relatively impermeable to water. The present data suggest this interpretation, but micropuncture analysis of calcium concentration of the precursor fluid is required to establish this as a fact. Flow rate differences found with different kinds of autonomic stimulation cannot be used to assess relationships between calcium concentration and flow rate of saliva, and only when the flow rate and calcium concentration are compared with a particular kind of autonomic stimulation are such comparisons meaningful.

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