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AUTONOMIC NERVE STIMULATION, KALLIKREIN CONTENT AND ACINAR CELL GRANULES OF THE CAT'S SUBMANDIBULAR GLAND

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SUMMARY

The parasympathetic and sympathetic nerves to the submandibular gland of the anaesthetized cat were stimulated under specific conditions.

1. It was possible to decrease the kallikrein (kininogenase) content of the gland by as much as 90–95% by sympathetic nerve stimulation. In such a gland the appearance and concentration of secretory granules in the acinar cells were indistinguishable from an unstimulated gland.

2. Parasympathetic nerve stimulation, in contrast to sympathetic nerve stimulation, whilst having no significant effect on the kininogenase content of the gland, resulted in the disappearance of a great majority of the acinar granules.

3. These results demonstrate that the acinar granules in the submandibular gland of the cat are not a significant source of kallikrein.

4. Our experiments also failed to indicate any obvious correlation between the granules of the demilune cells and the kallikrein content of the gland.

5. The possibility is raised that kallikrein is located in the cells of the striated ducts.

INTRODUCTION

Although the serine protease kallikrein (or kininogenase) was described in saliva and salivary glands nearly forty years ago (Werle & von Roden, 1936), its physiological significance in these and other organs remains obscure (Schachter, 1969). Recent studies of its cellular and subcellular localization may prove productive, particularly if, as in the present experiments, structural detail and enzyme concentrations in the gland are correlated with various kinds of physiological stimulation. The only

previous work which attempted to do this was that of Emmelin & Henriksson (1953), who tried to correlate the light-microscopic appearance of the gland with the hypotensive activity (due to kallikrein) of the saliva. These authors concluded that the demilune cells of the cat's submandibular gland were altered in appearance after degenerative parasympathetic nerve section, and that there was also a marked decrease in the hypotensive activity of the saliva. They suggested, therefore, that the demilune cells were the source of salivary kallikrein. However, no quantitative data were presented and no micrographs of the gland were shown, so it is impossible to assess the significance of these early experiments.

Recently, experiments by Bhoola *et al.* (Bhoola & Ogle, 1966; Bhoola & Dorey, 1969; Bhoola & Heap, 1970) and by Erdös *et al.* (Chiang, Erdös, Miwa, Tague & Coalson, 1968; Erdös, Tague & Miwa, 1968; Geipert & Erdös, 1971) have shown that kallikrein is present in the secretory granules of the salivary glands of many mammals. These workers have isolated a population of granules by differential and density-gradient centrifugation which is rich in this enzyme. Bhoola and his co-workers, in general conclude or suggest that the kallikrein-containing granules are located in the acinar cells (guinea-pig, cat, mouse), in the junctional cells (rabbit), and possibly in the intercalated duct cells (mouse).

The aim of the present investigation was to develop further the above studies, by attempting to correlate changes in the ultrastructure of the cat's submandibular gland with changes in its kallikrein content after stimulation of the sympathetic and parasympathetic nerves respectively. We had some basis on which to proceed since we had previously shown (Beilenson, Schachter & Smaje, 1968) that whereas sympathetic nerve stimulation could almost deplete the gland of its kallikrein, parasympathetic nerve stimulation, which produced much more copious secretion, had little or no effect on the kallikrein content of the gland. The ultrastructure of the submandibular gland of the cat has been described by Shackelford & Wilborn (1970) and by Dorey & Bhoola (1972*a*, *b*).

METHODS

Cats of either sex weighing 2·6–4·1 kg, and starved overnight, were anaesthetized with chloralose (60 mg kg^{-1} I.V.) after induction with chloroform. Dissection of nerves, their preparation for stimulation and collection of saliva were carried out as described previously (Beilenson *et al.* 1968). The sympathetic nerve was stimulated at 20 Hz for 10 sec followed by intervals of 30 sec rest; totals of the actual stimulation times in two experiments were 10 and 25 min respectively. The parasympathetic chorda-lingual nerve was stimulated at 10 Hz (20 Hz in one experiment) in three experiments for 5 min consecutively followed by a 2–5 min rest interval. In four experiments the total times of actual stimulation of the parasympathetic nerve were

TABLE 1. Effect of sympathetic (Symp.) or parasympathetic (P.symp.) nerve stimulation on kallikrein content of cat submandibular gland

Cat	Nerve stimulation	Saliva (ml.)	Kallikrein:* stimulated gland / control gland (%)	Kallikrein:† stimulated gland + saliva / control gland (%)
1	Symp. 20 Hz, 10 min	3·9	5	280
2	Symp. 20 Hz, 25 min	2·3	7	190
3	P.symp. 10 Hz, 15 min	9·7	108	180
4	P.symp. 10 Hz, 37 min	12·0	90	150
5	P.symp. 10 Hz, 40 min	15·6	109	106
6	P.symp. 20 Hz, 46 min	11·7	50	150

N.B. In four of the six experiments kininogenase activity was assayed on dog or cat blood pressure. Results closely paralleled the esterase assay (see Methods).

* Ratio of units of esterase activity per milligram protein.

† Ratio of total units of esterase activity recovered.

15, 37, 40 and 46 min respectively (Table 1). The corresponding nerve to the contralateral gland was sectioned acutely in each experiment.

Samples of the stimulated submandibular gland and the contralateral control gland were removed whilst the blood flow was still intact and then prepared for electron microscopy. The rest of the gland was then excised, rinsed in 0·9 % saline, weighed, minced with scissors and freeze-dried. An aqueous extract of the freeze-dried glands was centrifuged and the supernatant solution freeze-dried. The esterase and kininogenase activities of the gland were determined from this powder. Esterase activity was measured by the method of Trautschold (1970) using benzoyl-L-arginine ethylester (BABEe) as substrate. Protein concentrations were determined by the spectrophotometric method of Lowry, Roseburgh, Farr & Randall (1951). The relative kininogenase concentrations of different extracts were determined by comparing their hypotensive actions on the arterial blood pressure of the anaesthetized dog or cat.

For electron microscopy small pieces of the gland were removed from different areas after gentle separation of the lobes and fixed immediately at room temperature in a mixture of 4 % formaldehyde, 2·5 % glutaraldehyde and 1 % acrolein in 0·5 M cacodylate buffer, pH 7·3 for 2 hr (Dorey & Bhoola, 1972*a*). Tissues were post-fixed in 1 % osmium tetroxide in 0·5 M cacodylate buffer, pH 7·3 for 1 hr and dehydrated in a graded series of alcohol concentrations and embedded in Epon. Thin sections were cut, stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

RESULTS

Effect of sympathetic and parasympathetic nerve stimulation on kallikrein content of the cat's submandibular gland

The purpose of these experiments was to alter the kallikrein content of the gland by selective nerve stimulation, and then to relate the kallikrein loss from the gland to the resulting ultrastructural changes. It has previously been shown that sympathetic nerve stimulation is much more

effective than parasympathetic in causing the secretion of kallikrein into the saliva (Beilenson *et al.* 1968). Table 1 shows the results of different periods of sympathetic or parasympathetic nerve stimulation on the kallikrein content of the cat's submandibular gland. They confirm the dramatic effect of sympathetic nerve stimulation on the mobilization of kallikrein. In the experiments involving sympathetic nerve stimulation at 20 Hz for 10 and 25 min, more than 90 % of kallikrein was lost from the gland. In contrast, stimulation of the parasympathetic nerve at 10 Hz for as long as 40 min did not have any significant effect on the kallikrein content although the volume of saliva secreted was much greater. Stimulation at 20 Hz for 46 min, however, did cause a fall in kallikrein content to about 50 %. The latter observation is consistent with the results of Beilenson *et al.* (1968), who found that although vasodilatation and secretory rate become maximal when the parasympathetic nerve is stimulated at 8–10 Hz, the kallikrein output is still minimal at these frequencies, but rises quickly at 15–20 Hz. It should also be noted that in all six experiments the amount of kallikrein remaining in the stimulated gland plus that secreted into the saliva exceeded that in the 'control' or unstimulated gland. This fact is evident in the figures of the last column of Table 1. The apparent quantitative discrepancy raises the possibility of resynthesis of kallikrein during, or due to, stimulation of either nerve.

Effect of nerve stimulation on acinar cell granules of the cat's submandibular gland

Pl. 1*A* shows a group of acinar cells in a submandibular gland which had been subjected to stimulation of the sympathetic nerve at 20 Hz for 10 min, excluding rest intervals (cat 1, Table 1). The appearance of these cells is indistinguishable from those in an unstimulated gland. The cells are full of granules which are so densely packed that it is difficult to identify other structures in the cell. The significant fact is that although the appearance of the acinar cells in such a sympathetically stimulated gland is indistinguishable from an unstimulated one, 95 % of the kallikrein has been lost from the gland. The microscopic findings were the same in a second experiment in which the sympathetic nerve was stimulated for 25 min and 93 % of the gland's kallikrein was lost compared to the unstimulated gland (cat 2, Table 1).

In the top right-hand corner of Pl. 1*A* there are two demilune cells. The granules in these cells are usually paler and less tightly packed than those in the acinar cells, and often contain a small, round, electron-dense subunit or inclusion body. Unlike the obvious depletion of acinar granules with parasympathetic nerve stimulation, stimulation of either nerve did

not correlate in any predictable way with the concentration of granules in the demilune cells.

Pl. 1*B* shows the characteristic appearance of acinar cells from a submandibular gland which had been subjected to parasympathetic nerve stimulation at 10 Hz for 40 min (cat 5, Table 1). In this case, in contrast to that following sympathetic nerve stimulation, the acinar cells are dramatically different from those of an unstimulated gland. Nearly all of the moderately dense granules have disappeared and only a few dark granules are present. However, although the acinar granules could disappear almost entirely after parasympathetic nerve stimulation at 10 Hz, there was no decrease at all in the kallikrein content of the gland when compared to the contralateral unstimulated one.

DISCUSSION

Our results indicate that the acinar cell granules in the submandibular gland of the cat are not a significant source of salivary kallikrein. On first consideration it appears difficult to reconcile this conclusion with that of Bhoola *et al.* (Bhoola & Dorey, 1969; Bhoola & Heap, 1970; Dorey & Bhoola, 1972*b*; Bhoola, Dorey & Jones, 1973) that kallikrein is located in the acinar granules. It is well known, however, that there is considerable variation in the microscopic structure of these glands in mammals. For example, salivary glands have been classified histologically as mucous or serous according to the reactivity of the secretory cells with certain substances such as periodic acid and alcian blue which react with cellular mucopolysaccharide. The submandibular acinar cells of the guinea-pig are classified as serous, but those of the cat as mucous (Shackleford & Klapper, 1962). It is of interest that most of the experiments of Bhoola *et al.* which locate kallikrein in acinar cell granules were carried out on the submandibular gland of the guinea-pig. It may well be, therefore, that there is variation in the cellular and subcellular location of this enzyme in the submandibular gland of different mammals, a view that has recently been expressed by Dorey & Bhoola (1972).

Unlike the marked effect of parasympathetic nerve stimulation on the acinar granules (Pl. 1*B*), stimulation of either nerve did not correlate in any way with the concentration of granules in the demilune cells. Many years ago, Rawlinson (1933) noted that sympathetic nerve stimulation and intravenous injections of adrenaline produced vacuolation of the demilune cells. This effect, however, was not uniform, the vacuolated cells appearing in scattered areas of the gland. Rawlinson also reported that parasympathetic nerve stimulation markedly affected the microscopic appearance of the acinar cells whereas the demilunes were unaffected. The observations

of Rawlinson that vacuolation of scattered demilune cells occurs after sympathetic stimulation, and those of Emmelin & Henriksson (1953), namely that after degenerative parasympathetic nerve section the kallikrein concentration of saliva (measured by hypotensive effects) was greatly reduced, and that this was accompanied by changes in the microscopic appearance of the demilune cells, indicate that the demilune cells cannot yet be excluded as a possible source of kallikrein. In view of our observations, however, we would regard the demilune cells as an unlikely source.

In our experiments in which kallikrein was largely depleted from the gland after sympathetic nerve stimulation, the granules in the cells of the striated ducts appeared to be greatly reduced in number. Similar changes occurred if the kallikrein content of the gland was reduced after other procedures, namely ligation of the duct for 3 days or after degenerative section of the parasympathetic nerve. We are currently extending these observations because the possibility exists that kallikrein is located in the granules of the striated ducts. Also, since these granules are approximately one quarter the size of those in the acinar cells, it may be possible to separate different granule populations by appropriate centrifugation techniques. Immunofluorescence and other immunochemical procedures should also help to establish the precise cellular and subcellular location of this enzyme in the cat and other animals.

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REFERENCES

Beilenson, S., Schachter, M. & Smaje, L. H. (1968). Secretion of kallikrein and its role in vasodilatation in the submaxillary gland. *J. Physiol.* **199**, 303–317.

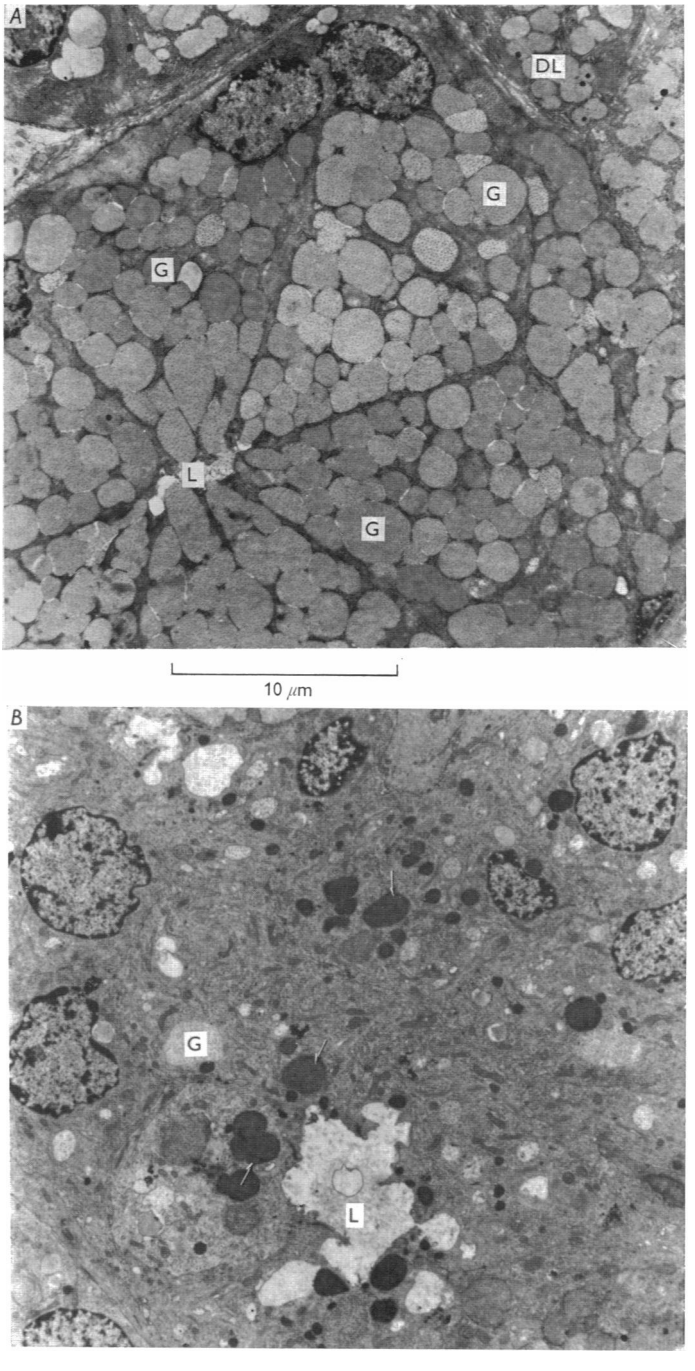
Bhoola, K. D. & Dorey, G. (1969). Isolation of kallikrein-containing granules from the pancreas and submaxillary gland of the cat. *J. Physiol.* **203**, 59–60*P*.

Bhoola, K. D., Dorey, G. & Jones, C. W. (1973). The influence of androgens on enzymes (chymotrypsin- and trypsin-like proteases, renin, kallikrein and amylase) and on cellular structure of the mouse submaxillary gland. *J. Physiol.* **235**, 503–522.

Bhoola, K. D. & Heap, P. F. (1970). Properties of kallikrein-containing granules isolated from the submaxillary gland of the guinea-pig. *J. Physiol.* **210**, 421–432.

Bhoola, K. D. & Ogle, C. W. (1966). The subcellular location of kallikrein, amylase and acetylcholine in the submaxillary gland of the guinea-pig. *J. Physiol.* **184**, 663–672.

Chiang, T. S., Erdös, E. G., Miwa, I., Tague, L. L. & Coalson, J. J. (1968). Isolation from a salivary gland of granules containing renin and kallikrein. *Circulation Res.* **23**, 507–517.



(*Facing p.* 369)

DOREY, G. & BHOOLA, K. D. (1972*a*). I. Ultrastructure of acinar cell granules in mammalian submaxillary glands. *Z. Zellforsch. mikrosk. Anat.* **126**, 320–334.

DOREY, G. & BHOOLA, K. D. (1972*b*). II. Ultrastructure of duct cell granules in mammalian submaxillary glands. *Z. Zellforsch. mikrosk. Anat.* **126**, 335–347.

EMMELIN, N. & HENRIKSSON, K. G. (1953). Depressor activity of saliva after section of the chorda tympani. *Acta physiol. scand.* **30**, 75–82.

ERDÖS, E. G., TAGUE, L. L. & MIWA, I. (1968). Kallikrein in granules of the submaxillary gland. *Biochem. Pharmac.* **17**, 667–674.

GEIPERT, F. & ERDÖS, E. G. (1971). Properties of granules that contain kallikrein and renin. *Experientia* **27**, 912–913.

LOWRY, O. H., ROSEBURGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

RAWLINSON, H. E. (1933). Cytological changes after autonomic and adrenalin stimulation of the cat's submaxillary gland. *Anat. Rec.* **57**, 289–296.

SCHACHTER, M. (1969). Kallikreins and kinins. *Physiol. Rev.* **49**, 509–547.

SHACKLEFORD, J. M. & KLAPPER, C. E. (1962). Structure and carbohydrate histochemistry of mammalian salivary glands. *Am. J. Anat.* **111**, 25–47.

SHACKLEFORD, J. M. & WILBORN, W. H. (1970). Ultrastructural aspects of cat submandibular glands. *J. Morph.* **131**, 253–276.

TRAUTSCHOLD, I. (1970). In *Handbook of Experimental Pharmacology*, ed. ERDÖS, E. G., vol. 25, p. 71. Berlin, Heidelberg, New York: Springer-Verlag.

WERLE, E. & VON RODEN, P. (1936). Über das Vorkommen von Kallikrein in den Speicheldrüsen und in Mundspeichel. *Biochem. Z.* **286**, 213–219.

EXPLANATION OF PLATE

A, electron micrograph showing a typical area of a submandibular gland after sympathetic nerve stimulation (Table 1, cat 1). The appearance of the acinar cells is indistinguishable from that in an unstimulated gland. However, the kallikrein concentration was reduced to 5 % of the unstimulated gland (Table 1). The demilune cells (DL) contain smaller, less closely packed, granules with dense inclusion bodies. G = granule, L = lumen × 5500.

B, electron micrograph showing a typical area of a submandibular gland after parasympathetic nerve stimulation (Table 1, cat 5). The acinar cells, in contrast to those in Pl. 1A, contain very few granules (G). However, the kallikrein concentration was not decreased at all (Table 1). Arrows indicate dark granules which are not seen in unstimulated glands. L = lumen × 5500.