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SECRETION FROM THE BOVINE ADRENAL GLAND
RELEASE OF LYSOSOMAL ENZYMES

F. H. SCHNEIDER

Department of Pharmacology, University of Colorado School of Medicine, Denver, Colo. 80220, U.S.A.

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Abstract—Isolated bovine adrenal glands were perfused with a physiological salt solution and exposed to various secretogogues. Perfusates were assayed for catecholamines, protein, lactate dehydrogenase, acid ribonuclease, acid deoxyribonuclease, cathepsin, acid phosphatase and β-glucuronidase. Stimulation of the gland with carbachol induced secretion of catecholamines, protein and acid hydrolase activity. Perfusate LDH activity was unchanged after carbachol stimulation. Carbachol stimulation of the perfused isolated medulla evoked deoxyribonuclease secretion; the relationship between catecholamine secretion and secretion of deoxyribonuclease activity was the same for the perfused whole gland and isolated medulla.

Secretion of deoxyribonuclease activity was also evoked by acetylcholine, nicotine, potassium chloride and Triton X-100, agents that also cause catecholamine secretion. Carbachol-induced secretion of deoxyribonuclease activity was reduced in the presence of tetracaine or during perfusion with calcium-free fluid. The introduction of calcium chloride during perfusion with calcium-free fluid also caused secretion of deoxyribonuclease activity. It is concluded from these results that stimulation with carbachol of the isolated perfused bovine adrenal gland causes secretion of deoxyribonuclease activity from the adrenal medulla, and that the secretion process is dependent upon the presence of calcium.

RELEASE of typical lysosomal acid hydrolases from the isolated bovine adrenal gland in response to stimulation of the gland with acetylcholine, carbachol or dimethylphenylpiperazinium was reported earlier in a short communication.[1] Drug-induced acid hydrolase secretion was prevented in the presence of cocaine or hexamethonium, or during perfusion with calcium-free fluid. These observations suggested that the acid hydrolases may be secreted from the gland by a mechanism similar to that for secretion of catecholamines. This possibility, as well as other aspects of these observations, have been examined in more detail, and the results of these experiments are presented in this paper. These results are related to findings reported in the preceding paper.[2]

METHODS

Perfusion. Bovine adrenal glands, weighing between 10–25 g, were obtained approximately 15 min after the animals were killed, and kept in ice for 30–60 min until perfusion was begun. The glands were perfused as described previously.[3] Flow rates varied between 6·5 and 24·8 ml/min and were 10 to 15 ml/min in most experiments. The temperature of the perfusion fluid was 37° unless stated otherwise. Drugs were injected into the perfusion fluid immediately before the fluid entered the gland. In most

cases drugs were injected in a volume of 0·2 ml every 30 sec during a period of 120 sec. In some cases drugs were infused continuously into the perfusion fluid for a period of 2 min with a Harvard infusion pump. In the latter case, the drug was injected in a volume of 2 ml. All drugs used to stimulate the glands were dissolved in perfusion fluid.

Isolated medullae were prepared for perfusion by careful removal of the cortex with a pair of scissors. A small amount of cortical and aortic tissue was left surrounding the opening of the adrenal vein. A cannula was inserted into the adrenal vein and the medulla was perfused in a manner similar to that for perfusion of the whole gland.

Assays. Protein in perfusates was precipitated in 5% (w/v) trichloroacetic acid and measured by the microbiuret method of Goa.[4] Protein assays were standardized with bovine serum albumin. Perfusate catecholamines (CA) were analyzed by the colorimetric procedure of von Euler and Hamberg,[5] using citrate–phosphate buffer at pH 6·0.[6] Catecholamines were expressed as μmoles of epinephrine. A measure of the amount of hemoglobin in the perfusates was obtained from the optical density of the perfusates at 420 mμ after the addition of 1 vol. of 0·1 N HCl.

Acid phosphatase (APase) and β-glucuronidase (β-GLUCase) activities were measured by the methods of Gianetto and de Duve,[7] and cathepsin (CATH) activity was measured by the procedure of Anson.[8] Acid ribonuclease (RNase) and deoxyribonuclease (DNase) activities were measured according to Smith and Winkler,[9] except that ribonuclease was measured at pH 5·2 instead of at 5·5.[2] The pH optimal enzyme activity was found by using 0·68 M acetate buffer at pH values between 4·0 and 8·4 for acid deoxyribonuclease and 0·14 M acetate buffer at pH values between 3·5 and 6·5 for β-glucuronidase; sodium hydroxide was used to adjust the pH of the buffers. Acid phosphatase activity of perfusates was measured in 0·014 M Tris-acetate at pH 5·0 and 7·5 and cathepsin activity was measured at pH 3·8 and 6·0 in 0·07 M sodium–citrate buffer. Lactate dehydrogenase activity was measured by the procedure of Wroblewski and La Due.[10] Enzyme activities are presented as μmoles of substrate transformed per unit of time under the conditions of the assay.

Unconcentrated and concentrated perfusates were analyzed for enzyme activity. Perfusates were concentrated by dialysis against solid sucrose at 4° for 4–6 hr. This procedure reduced their volume by approximately two-thirds. The samples were then dialyzed at 4° for several hours against 3 changes of distilled water and then frozen and evaporated to dryness under reduced pressure. The freeze-dried samples were dissolved in a volume of distilled water equal to one-tenth to one-twentieth the original volume of the perfusate and dialyzed at 4° against Tris-sodium succinate buffer, pH 5·9 (I = 0·015) for 2–3 hr. Activities of acid phosphatase and cathepsin in unconcentrated perfusates were too low for accurate measurement.

Statistical analyses were performed on the Olivetti Underwood Programma 101 desk computer by procedures described in the Olivetti Underwood statistical analysis handbook.

Chemicals used in the enzyme assays are described in reference 2. Drugs used were carbachol (carbamylcholine chloride), acetylcholine bromide, nicotine sulfate, tetracaine hydrochloride and Triton X-100 (octyl phenoxy polyethoxyethanol).

RESULTS

Response to carbachol. The amount of protein in perfusates fromun stimulated glands

decreased rapidly during the first 30–60 min of perfusion and reached a slowly decreasing plateau after 60–90 min.[3] Figure 1 shows that the same washout phenomenon occurred for catecholamines, hemoglobin and acid deoxyribonuclease activity. Since these substances were present in the perfusates in relatively high concentrations during the initial period of perfusion, samples were not collected for analysis during the first hour.

Stimulation of the gland with carbachol caused the secretion of catecholamines and protein (Fig. 2). The ratio of the increase in catecholamines above control levels to

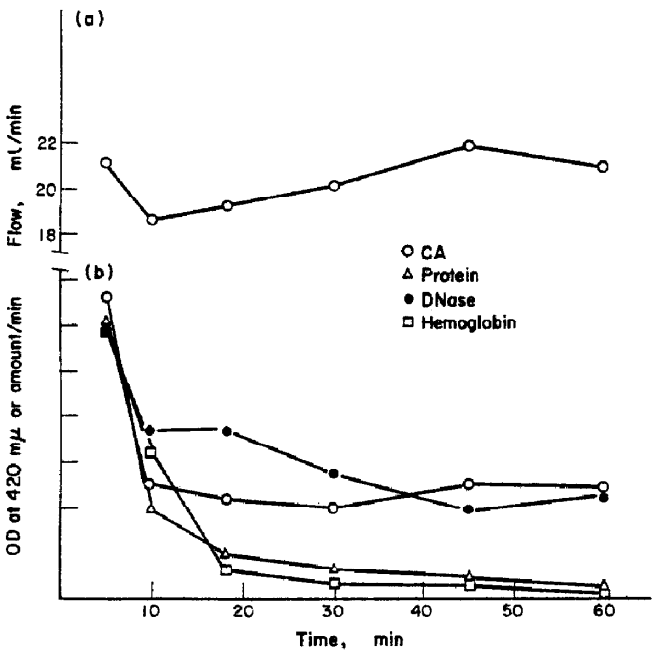

FIG. 1. a. Perfusion fluid flow rate (ml/min). b. Catecholamine, protein, deoxyribonuclease and hemoglobin content of perfusion fluid from an isolated nonstimulated adrenal gland. In "b" each division on the ordinate represents 0·1 μmole catecholamines, 2 mg protein, 0·05 μmoles/min for deoxyribonuclease activity and 0·05 OD_{420} units for hemoglobin content.

the corresponding increase in total protein for doses of 2·4 mg was 5·93 ± 0·49 ($n = 7$) and for 3·6 mg was 5·91 ± 0·30 ($n = 52$). The ratio of catecholamines to protein in soluble lysates of bovine adrenal medulla chromaffin granules is 4·8.[3]

Perfusates were also assayed for their activity of the lysosomal enzymes ribonuclease, deoxyribonuclease, cathepsin, acid phosphatase and β-glucuronidase. The activity (mμmoles of substrate converted/hr/g tissue) in control perfusates (concentrated and unconcentrated grouped together) collected during a 1-min period after approximately 90 min of perfusion was 10·38 ± 2·07 ($n = 23$) for ribonuclease, 2·64 ± 0·42 ($n = 25$) for deoxyribonuclease, 7·01 ± 0·17 ($n = 10$) for cathepsin, 0·93 ± 0·30 ($n = 6$) for acid phosphatase, and 0·25 ± 0·04 ($n = 10$) for β-glucuronidase.

Activity of each of the five acid hydrolases in the perfusates was increased after

carbachol stimulation (Fig. 2). The increases ranged between 50 per cent for acid phosphatase and ribonuclease and 300 per cent for deoxyribonuclease. The per cent increases for β-glucuronidase are similar for concentrated and unconcentrated samples; for ribonuclease and deoxyribonuclease the increase appears greater with unconcentrated samples, although the differences are not statistically significant in either case ($P > 0{\cdot}1$).

Quantitative relationships between the increases in perfusate acid hydrolase

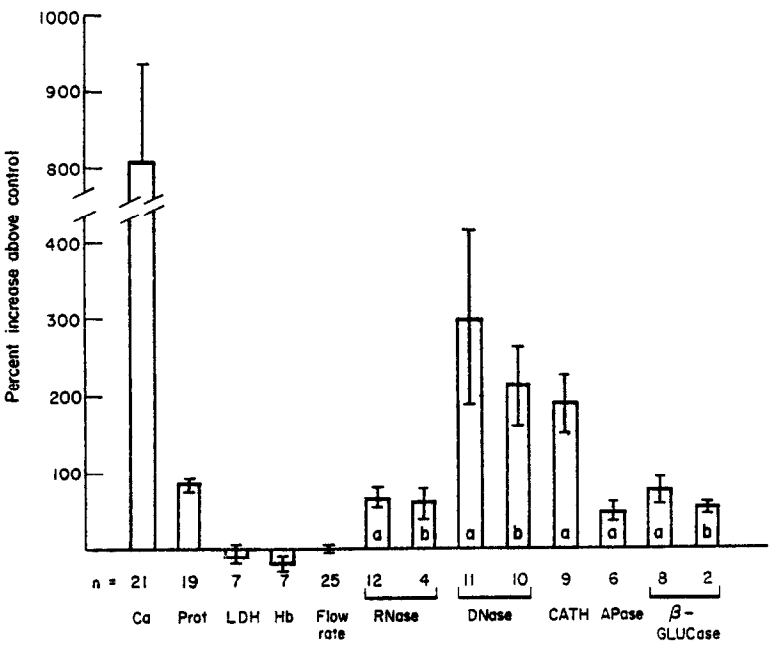

FIG. 2. Secretion of various tissue components from the isolated perfused bovine adrenal gland. Carbachol (3·6 mg) was injected over a 2-min period into the perfusion fluid before the fluid entered the gland. Total sample collection time was 6 min. The per cent increase was determined by the equation $(P_s - P_c/P_c) \times 100$, where P_c and P_s represent the content of a particular substance and stimulation perfusates respectively. For the acid hydrolase activities, the values marked "a" were obtained from perfusates that were reduced in volume by the technique described in the Methods section, and the values marked "b" were obtained from unconcentrated perfusates. The number of perfusates assayed is indicated by "n", and the brackets on each column represent the standard errors.

activities after carbachol stimulation are shown in Table 1. The ratios of the increase in ribonuclease activity to the increase in activity of the other enzymes are shown for both unconcentrated and concentrated perfusates. The corresponding ratios for bovine adrenal medulla and cortex, calculated from lysosome-rich fractions obtained by sucrose density gradient centrifugation,[2] are shown for comparison.

The relationship between the increase in the amount of catecholamines in the perfusates and the increase in ribonuclease and deoxyribonuclease activities after stimulation of the gland with carbachol was examined. The ratio of the increase in catecholamine secretion to the increment in ribonuclease activity (μmoles/min) in

TABLE 1. ACID HYDROLASE RATIOS FOR CONCENTRATED AND UNCONCENTRATED PERFUSATES AND FOR ADRENAL MEDULLA AND CORTEX*

Ratio	Concentrated	Unconcentrated	Medulla†	Cortex†
$\frac{\text{RNase}}{\text{DNase}}$	1·68 ± 0·13 (8)	2·15 ± 0·29 (9)	2·99 ± 0·12	4·01 ± 0·47
$\frac{\text{RNase}}{\text{CATH}}$	1·21 ± 0·23 (5)		2·19 ± 0·24	0·88 ± 0·16
$\frac{\text{RNase}}{\text{APase}}$	29·6 ± 11·0 (4)		25·27 ± 2·76	3·34 ± 1·00
$\frac{\text{RNase}}{\beta\text{-GLUCase}}$	82·0 ± 21·5 (5)	45·3 ± 10·2 (5)	47·49 ± 2·90	15·00 ± 2·32

* The figures for concentrated and unconcentrated perfusates are ratios of the increases in enzyme activities after stimulation with 3·6 mg of carbachol. The values represent the mean ± S.E.; the number of experiments is given in parentheses. Procedures used to concentrate perfusates are described in the Methods section.

† Ratios of enzyme activities for medulla and for cortex density gradient fractions taken from reference 2.

$P < 0{\cdot}001$: $\frac{\text{RNase}}{\text{DNase}}$ conc. vs. medulla; $\frac{\text{RNase}}{\text{DNase}}$ conc. vs. cortex.

$P < 0{\cdot}01$: $\frac{\text{RNase}}{\text{DNase}}$ unconc. vs. cortex.

$P < 0{\cdot}02$: $\frac{\text{RNase}}{\text{CATH}}$ vs. medulla; $\frac{\text{RNase}}{\beta\text{-GLUCase}}$ unconc. vs. cortex; $\frac{\text{RNase}}{\beta\text{-GLUCase}}$ conc. vs. cortex

$P < 0{\cdot}05$: $\frac{\text{RNase}}{\text{DNase}}$ unconc. vs. medulla; $\frac{\text{RNase}}{\text{APase}}$ vs. cortex.

response to stimulation with 3·6 mg of carbachol was 486·0 ± 76·6 ($n = 11$) for concentrated perfusates, and 662·1 ± 112·7 ($n = 5$) for unconcentrated perfusates. For deoxyribonuclease the corresponding ratios were 751·8 ± 119·8 ($n = 11$) and 917·7 ± 111·1 ($n = 28$) respectively. The correlation coefficient between catecholamine and ribonuclease secretion was 0·756 ($P > 0{\cdot}05$) for concentrated perfusates and 0·271 ($P > 0{\cdot}05$) for unconcentrated perfusates; for catecholamines and deoxyribonuclease the corresponding figures were 0·798 ($P < 0{\cdot}01$) for concentrated perfusates and 0·527 ($P < 0{\cdot}01$) for unconcentrated perfusates.

The relationship between secretion of catecholamines and secretion of deoxyribonuclease activity was also examined in unconcentrated perfusates after carbachol stimulation of the perfused isolated medulla. As with the whole gland, there was a significant relationship between secretion of catecholamines and secretion of deoxyribonuclease activity; the correlation coefficient was 0·690 ($n = 12$; $P < 0{\cdot}05$) for secretion induced by 2·4 mg of carbachol. The ratio of the amount of catecholamines secreted to the amount of deoxyribonuclease activity secreted was 918·6 ± 124·4 ($n = 12$). This value is not significantly different ($P > 0{\cdot}1$) from the corresponding value of the perfused whole gland.

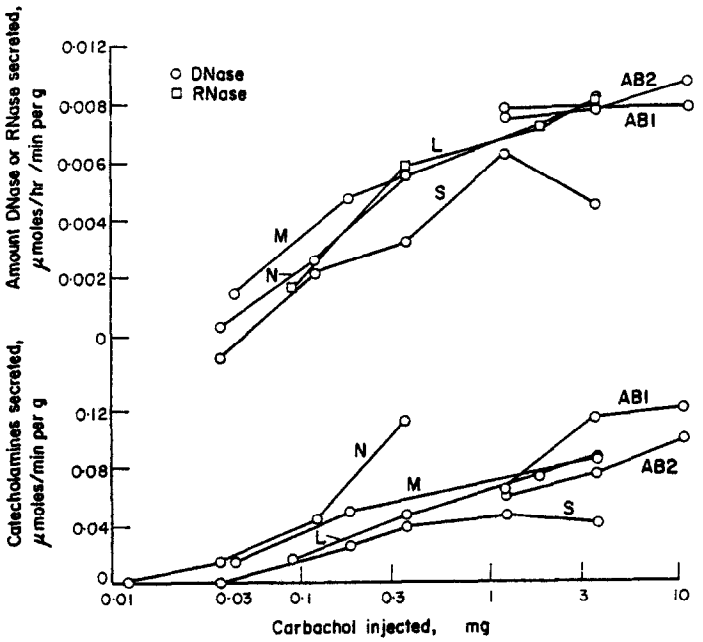

FIG. 3. Dose–response relationships between the amount of carbachol injected and the increase in perfusate catecholamines and ribonuclease and deoxyribonuclease activities. Carbachol was injected over a period of 2 min and total collection periods were 6 min. The letters corresponding to each curve represent a separate experiment.

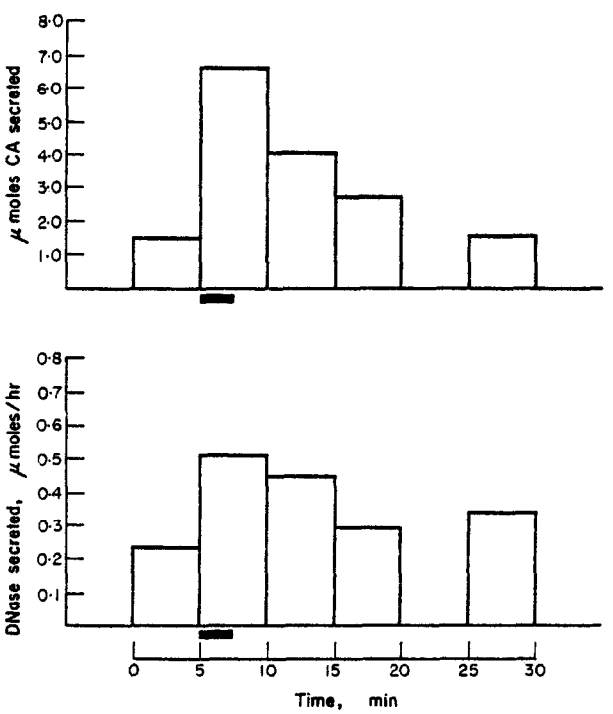

FIG. 4. Time course for carbachol-induced secretion of catecholamines and deoxyribonuclease activity from the isolated perfused bovine adrenal gland. The height of each column represents the total catecholamines or deoxyribonuclease activity secreted in a 5-min period. The solid bar represents the period during which the gland was stimulated with 2·4 mg carbachol.

Secretion of catecholamines, ribonuclease and deoxyribonuclease activity was also examined after injection of various amounts of carbachol. Figure 3 shows that secretion of each substance was related to the dose of carbachol, and that secretion of catecholamines and of the nucleases occurred within the same carbachol concentration range. The apparent similarity between secretion of catecholamines and deoxyribonuclease is also reflected in the time course for secretion after stimulation of the gland with an infusion of carbachol (Fig. 4). The time course for secretion is also shown for the isolated medulla (Fig. 5).

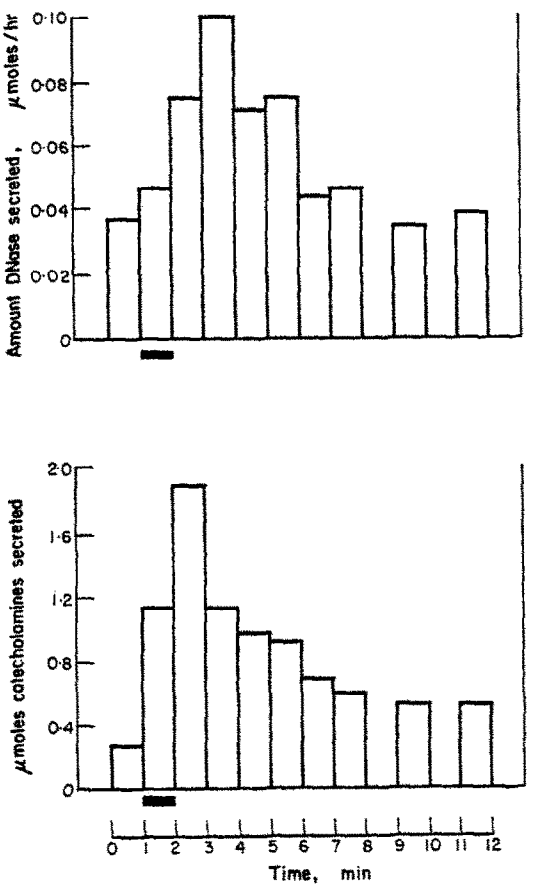

FIG. 5. Time course for carbachol-induced secretion of catecholamines and deoxyribonuclease activity from the isolated perfused bovine adrenal medulla. The height of each column represents the total catecholamines or deoxyribonuclease activity secreted in a 1-min period. The solid bar represents the period during which the medulla was stimulated with 2·4 mg carbachol.

Stimulation of the glands with carbachol did not cause an increase in perfusate LDH activity, or in the amount of hemoglobin in the perfusates (Fig. 2).

pH-Activity relationships of perfusate hydrolases. It was shown in a previous report that carbachol stimulation caused an increase in acid ribonuclease activity of perfusates but did not cause an increase in the alkaline ribonuclease activity that was also present in the perfusates.[1] In contrast to these findings, it was found that there was very

little alkaline deoxyribonuclease present in the perfusates. After stimulation the ratio of deoxyribonuclease activity at pH 4·6 to activity at pH 7·0 is 10·8, whereas the ratio of perfusate ribonuclease activity at pH 5·0 to activity at pH 7·0 is 1·3. The similarities between the pH optima of the five perfusate acid hydrolases and those of the corresponding enzymes of the adrenal medulla and cortex are apparent from Table 2.

Acid hydrolase secretion requirement for calcium. The presence of calcium is required

TABLE 2. pH–ACTIVITY RELATIONSHIP OF SEVERAL ACID HYDROLASES OF PERFUSATES FROM THE ISOLATED BOVINE ADRENAL GLAND AND IN BOVINE ADRENAL TISSUES

Acid hydrolases	Perfusate*	Medulla†	Cortex†
RNase	c‡ 6·0§ s	5·6	5·5–5·6
DNase	c 4·6 s 4·6	4·6–4·7	4·6–4·7
CATH	c, s } act. pH 3·9 > act. pH 6·0	3·2 (shoulder, 4·6)	3·2 (shoulder, 4·6)
APase	c, s } act. pH 5·7 > act. pH 7·5	3·5–4·5	3·5–4·5
β-GLUCase	c 5·0 s 5·0	4·8	4·8

* For ribonuclease, deoxyribonuclease and β-glucuronidase, the figures represent the pH values at which optimal enzyme activity was obtained. For cathepsin and acid phosphatase the activities are compared at two pH values.

† Values for medulla and cortex taken from reference 2.

§ Values for perfusate ribonuclease activity taken from reference 1.

‡ c and s refer to control and stimulation respectively.

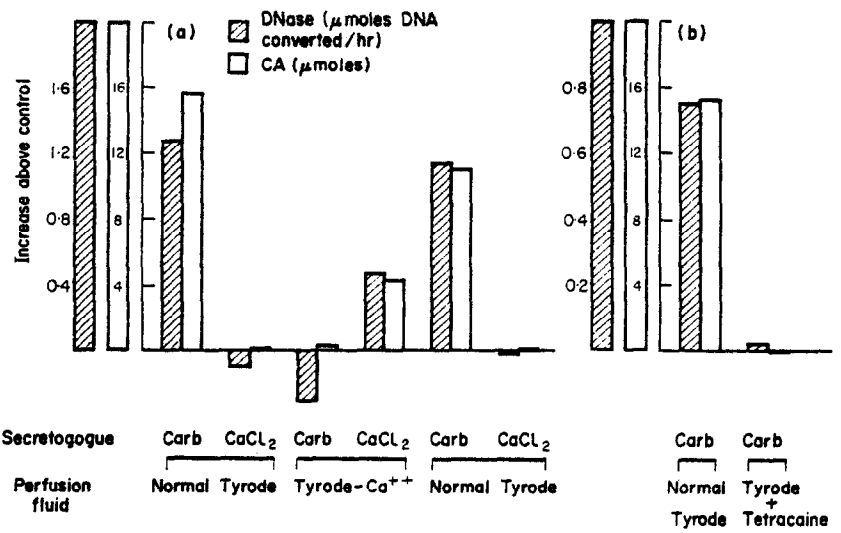

FIG. 6. Influence of calcium on carbachol-induced secretion of catecholamines and deoxyribonuclease activity from the isolated perfused bovine adrenal gland. Graphs "a" and "b" represent two separate experiments. The height of each column represents the total catecholamines or deoxyribonuclease activity secreted above background levels in a 6-min period. Stimulation was carried out by the injection of either 1·8 mg carbachol or 32·3 mg calcium chloride over a period of 2 min. Tetracaine was present in the perfusion fluid in a concentration of 4×10^{-4} M. The glands were perfused with the various perfusion fluids a minimum of 30 min before stimulation.

for acetylcholine-induced or carbachol-induced secretion of catecholamines and proteins from the adrenal gland.[3, 11, 12] Accordingly, the calcium requirement for secretion of lysosomal enzymes was examined. Deletion of calcium from the perfusion fluid abolished the usual increase in perfusate acid deoxyribonuclease activity after carbachol stimulation (Fig. 6). Perfusion with calcium-free perfusion fluid was associated with increased deoxyribonuclease activity in the absence of stimulation; in 3 experiments control deoxyribonuclease activity in the absence of calcium was 130·9 ± 31·1 per cent higher than deoxyribonuclease activity of perfusates collected during perfusion with normal perfusion fluid.

Exposure of the gland to calcium chloride (32·3 mg) during perfusion with calcium-deficient fluid resulted in increased catecholamine and deoxyribonuclease secretion. The total protein of perfusates was also increased after stimulation with calcium chloride; the ratio of increase in catecholamines to the increase in protein was 4·20 ($n = 2$). Stimulation with calcium chloride did not cause secretion of LDH ($n = 2$).

The relationship between calcium and carbachol-induced acid hydrolase secretion was examined further by assessing the effect of tetracaine on the response to carbachol. Tetracaine has been shown to block the acetylcholine-induced depolarization

TABLE 3. SECRETION FROM THE PERFUSED BOVINE ADRENAL GLAND: STIMULATION WITH VARIOUS SECRETOGOGUES*

	Per cent increase in CA	Per cent increase in protein	$\frac{\text{Protein}}{\text{CA}}$†	Per cent increase in DNase	$\frac{\text{DNase}}{\text{CA}}$† $\times 10^3$
Carbachol (10)	818·9 ± 113·0	121·9 ± 26·7	0·17 ± 0·01	329·7 ± 123·2	1·36 ± 0·22
Acetylcholine (6)	297·4 ± 55·0	60·0 ± 21·9	0·17 ± 0·03	62·8 ± 5·6	1·22 ± 0·20
Potassium chloride (7)	468·6 ± 89·7	103·8 ± 24·0	0·22 ± 0·02‡	104·2 ± 13·6	2·01 ± 0·41
Nicotine (5)	491·0 ± 79·4	131·5 ± 32·6	0·15 ± 0·02	166·2 ± 49·4	0·98 ± 0·09
Triton X-100 (5)	871·4 ± 288·0	989·0 ± 281·6	1·65 ± 0·25§	1132·7 ± 117·4	25·69 ± 4·81§

* Values represent the mean ± S.E. The figures in parentheses are the numbers of experiments. Drugs were injected in 1 ml of perfusion fluid over a 2-min period. Collection periods were 6 min. The dose of each drug given was: carbachol, 3·6 mg; acetylcholine bromide, 0·45 mg; nicotine sulfate, 0·22 mg; potassium chloride, 40 mg; Triton X-100, 10 mg.

† The ratios $\frac{\text{Protein}}{\text{CA}}$ and $\frac{\text{DNase}}{\text{CA}}$ represent the stimulation-induced increases in protein (mg) divided by the increases in catecholamines (μmoles), and the increases in deoxyribonuclease activity (μmoles substrate converted per min) divided by the increases in catecholamines (μmoles) respectively.

‡ $P < 0·05$ when compared with the corresponding ratio for carbachol.

§ $P < 0·001$ when compared with the corresponding ratio for carbachol.

of the chromaffin cell that is due to inward movements of calcium ions, and to prevent catecholamine secretion in response to stimulation with acetylcholine.[13] Release of catecholamines and deoxyribonuclease in response to stimulation with carbachol was prevented in the presence of tetracaine hydrochloride (4×10^{-4} M) (Fig. 6).

Secretion induced by other agents. Secretogogues other than carbachol were tested for their ability to release catecholamines, protein and lysosomal deoxyribonuclease activity (Table 3). The results of stimulation with carbachol in these same preparations are shown for comparison. The ratios of the increases in total protein of perfusates after stimulation to the increases in catecholamines and the ratios of the increases in

deoxyribonuclease activity to the increases in catecholamines are similar for carbachol, acetylcholine and nicotine. Potassium chloride appeared to have a greater tendency to release protein relative to the amount of catecholamines released than did carbachol. Exposure of the gland to the nonionic detergent Triton X-100 resulted in the loss of large amounts of protein and deoxyribonuclease activity from the gland. Triton was the only one among these drugs that produced an increase in perfusate lactate dehydrogenase activity (1072 per cent $\pm$ 465; $n = 3$).

The ability of catecholamines to evoke deoxyribonuclease secretion from the perfused gland was also tested. Figure 7 shows the response to different concentrations

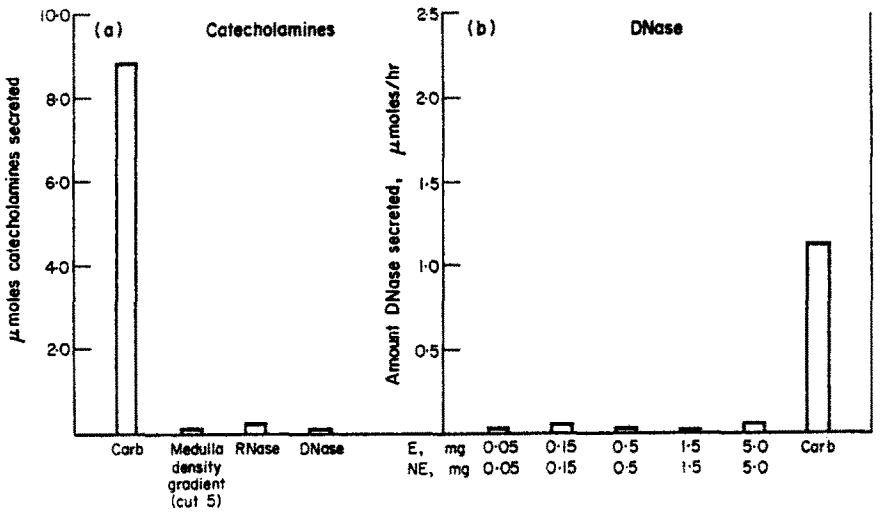

FIG. 7. Stimulation of the isolated perfused bovine adrenal gland with acid hydrolases, catecholamines and carbachol. Catecholamine secretion is shown in (a), and deoxyribonuclease secretion is shown in (b). Graphs (a) and (b) represent two separate experiments. Injections were made in 1 ml over a 1-min period and collection periods were 6 min. Carbachol was injected in a dose of 3·6 mg. Ribonuclease activity of the medulla density gradient fraction that was injected was 0·085 μmoles substrate converted per min per ml, and the activity of deoxyribonuclease was 0·038 μmoles substrate converted per min per ml.

of a mixture of norepinephrine and epinephrine; the response to carbachol (3·6 mg in 1 ml) is shown for comparison. This amount of carbachol will generally cause the secretion of 1–3 mg of catecholamines in a 6-min period. In contrast to carbachol, the catecholamines had little effect in this experiment. Perfusion of several glands with catecholamines showed that the effects on deoxyribonuclease secretion, although variable, were minimal (Table 4).

Perfused glands were also exposed to exogenous acid hydrolases. Figure 7 shows that injection of lysed bovine adrenal medulla lysosomes[2] or injection of pancreatic deoxyribonuclease or ribonuclease had no effect on catecholamine secretion. The amount of enzyme activity injected in 1 ml of the medulla lysosome preparation, as measured by ribonuclease activity, was similar to the total amount of activity secreted after stimulation of the gland with 3·6 mg of carbachol. The amounts of pancreatic deoxyribonuclease and ribonuclease injected were much higher. In two additional

preparations injections of lysed medulla density gradient fractions[2] and lysed medulla large-granule preparations[2] containing amounts of acid hydrolase activity similar to those found in perfusates after carbachol stimulation also failed to release catecholamines into the perfusates.

Extent of perfusion. Methylene blue (0·5 ml of a 1% solution) was injected into perfused whole glands in an effort to measure the relative perfusion of cortex and medulla. Six min after a 1-min injection the glands were rinsed, blotted dry and sliced at various angles to reveal the extent of dye penetration. The medulla was invariably well stained, whereas relatively little dye penetrated the cortex.

TABLE 4. EFFECT OF CATECHOLAMINE AND CARBACHOL STIMULATION ON DEOXYRIBONUCLEASE ACTIVITY IN PERFUSATES FROM ISOLATED BOVINE ADRENAL GLANDS*

NE† (1 mg)	NE (10 mg)	E† (1 mg)	E (10 mg)	NE + E (0·5 + 0·5 mg)	NE + E (5 + 5 mg)	Carbachol (3·6 mg)
−5·7 ± 32·8 (5)	13·0 ± 13·0 (3)	18·9 ± 17·5 (5)	0 (2)	2·2 ± 5·2 (4)	18·8 ± 45·2 (5)	195·8 ± 74·0 (7)

* Values represent the per cent increase above control levels and in each case represent the mean ± S.E. The figures in parentheses are the numbers of experiments. Deoxyribonuclease activities were determined in unconcentrated perfusates. In these experiments drugs were injected over a 1-min period.

† NE represents norepinephrine and E represents epinephrine.

DISCUSSION

These and earlier findings[1] show that carbachol stimulation of the isolated bovine adrenal gland causes the release of a group of acid hydrolases similar to those found associated with lysosomes of several tissues, including lysosomes of the bovine adrenal medulla.[9] It has already been shown that acid ribonuclease, acid deoxyribonuclease and β-glucuronidase are secreted from the gland after stimulation with acetylcholine and related drugs; in the present work it was shown that acid phosphatase and cathepsin are also released after stimulation. The increases in secretion of lysosomal enzymes after carbachol stimulation varied between 50–300 per cent, an observation that may reflect differences in the relative ease of which lysosomal enzymes are released from lysosomes.[2] The relatively small per cent increase for ribonuclease is due also to the high level of ribonuclease activity in prestimulation perfusates, which in part may be due to the presence of alkaline ribonuclease lost from the tissue.[1] Each of the enzymes secreted exhibited pH characteristics similar to those of the corresponding enzymes of lysosomes.

Phospholipase A_1 and phospholipase A_2, acid hydrolases that are associated with lysosomes of bovine adrenal medulla,[14] also are secreted from the perfused gland after stimulation with carbachol.* Smith found that the ratio of phospholipase A_1 activity in perfusates from stimulated glands to that in perfusates of unstimulated glands was 2·76 ± 0·33 ($n = 5$); the corresponding ratio for phospholipase A_2 was 2·57 ± 0·27 ($n = 5$). The pH curves of the same enzymes in perfusates from stimulated glands were similar to the pH curves of the same enzymes of lysosomes isolated from adrenal medulla by density gradient centrifugation.

* A. D. Smith, personal communication.

In view of the relationship between pH and the activity of these perfusate hydrolases, and of the association of these enzymes with lysosomes in the adrenal gland, there can be little doubt that these seven acid hydrolases are of lysosomal origin. Accordingly, these enzymes can be added to the list of products secreted from the adrenal gland in response to acetylcholine of carbachol stimulation. This list includes catecholamines,[15] adenine nucleotides and their metabolic derivatives,[16] dopamine-β-oxidase[17, 18] and the chromogranins.[3, 11, 12, 19, 20]

The increase in lysosomal enzyme activity of perfusates after carbachol stimulation varied between 50–300 per cent under the conditions of these experiments. Using the data in reference 2 it can be calculated that about 0·75 per cent of the medulla deoxyribonuclease activity, or about 0·35 per cent of the deoxyribonuclease activity in the whole gland, is secreted in response to an injection of 3·6 mg of carbachol. For ribonuclease the corresponding values are 0·30 and 0·11 per cent. These values, obtained by using a medulla weight of 4 g and a cortex weight of 10 g, are an order of magnitude less than the relative proportion of medulla catecholamines secreted during a similar collection period. Carbachol stimulation causes the release of 2–6 per cent of the medulla catecholamines.

The results of the experiments presented in this paper indicate that the enzymes secreted from the perfused gland come from the medulla. The ratios of perfusate ribonuclease activity to the other prefusate acid hydrolase activities, with the exception of the ribonuclease to cathepsin ratio, are closer to the corresponding ratios for medulla than to those for cortex. The value for cathepsin may be the least reliable, since it was necessary to work near the lower limit of the assay method when measuring cathepsin activity in perfusates. More conclusive, however, is the finding that the ratios of catecholamines to deoxyribonuclease activity of perfusates after stimulation are the same for the perfused whole gland and for the perfused isolated medulla. The apparent lack of substantial acid deoxyribonuclease secretion from the cortex may reflect a lack of perfusion of the cortex. The latter possibility is consistent with the observation that injected methylene blue stains little of the cortex, whereas essentially all the medulla is stained.

The characteristics of carbachol-induced lysosomal enzyme secretion indicate some degree of pharmacological specificity in the secretion process. This specificity is reflected in the dose–response relationship for the stimulation of ribonuclease and deoxyribonuclease secretion by carbachol; secretion of the nucleases is obtained with the range of doses of carbachol that also cause secretion of catecholamines. This similarity suggests that there may be a relationship between catecholamine secretion and lysosomal enzyme secretion, or that both processes share common cellular mechanisms. The time courses for secretion of catecholamines and deoxyribonuclease from the perfused gland were similar when collection periods were 5 min. If collection periods were only 1 min, as in the case of the experiment with the perfused isolated medulla (Fig. 5), the catecholamines appeared in the perfusate before the appearance of deoxyribonuclease. This lag in the appearance of deoxyribonuclease may reflect the longer time required for passage of the larger protein molecule across the basement membrane and through the vascular endothelium.

Several other experimental observations also point to the similarity between catecholamine and acid hydrolase secretion. For example, drugs or conditions that reduce or inhibit carbachol-induced catecholamine secretion likewise reduce lysosomal

enzyme secretion. Hexamethonium and cocaine,[1] and tetracaine and calcium deprivation all reduce carbachol-induced secretion of both catecholamines and deoxyribonuclease. A common link in the secretion processes is also reflected in the results of stimulation of the gland with acetylcholine, nicotine or potassium chloride, indicated by the similarity of the ratios of catecholamines to deoxyribonuclease activity in the perfusates. Potassium chloride, however, appeared to affect the tissue in a more generalized manner than the acetylcholine-like drugs did, since it provoked the loss of more protein from the gland relative to the amount of catecholamines released. Such an action may be related to the ability of potassium chloride to act as a general cell-depolarizing agent.

The apparent relationship between secretion of catecholamines and release of lysosomal enzymes might mean that secretion of either catecholamines or of lysosomal enzymes may in turn cause the release of the other. This possibility was tested by exposing the glands to exogenous catecholamines and lysosomal enzymes. Injection of norepinephrine or epinephrine, or mixtures of the two, in amounts similar to that secreted after carbachol stimulation, caused only a slight and highly variable increase in perfusate deoxyribonuclease activity. Secretion of deoxyribonuclease activity, however, on the average was only about 10% of that secreted after injection of 3·6 mg of carbachol. This dose of carbachol causes the secretion of about 1 mg of catecholamines in a 6-min period, which, at a flow rate of 10 ml/min, would provide a catecholamine concentration of about 0·015 mg/ml in the perfusate. This calculation, however, does not take into consideration the possibility that there might be a localized high concentration of amine at some specific site within the tissue during or immediately after release occurs. For this reason, the ability of exogenous catecholamines to induce lysosomal enzyme secretion was tested over a wide range of doses. An injection of 10 mg would provide an amine concentration in the fluid perfusing the gland about seventy times higher than that in perfusates after stimulation with a sufficient amount of carbachol to cause secretion of a similar amount of catecholamines.

Likewise, injection of lysates of bovine adrenal medulla lysosomes, or of pure pancreatic ribonuclease or deoxyribonuclease, was ineffective in causing catecholamine secretion. It does not appear likely from these findings that there is a functional relationship between release of catecholamine and lysosomal enzyme. This argument is not altogether conclusive, though, since exogenously administered lysosomal enzymes might not reach the intracellular sites of catecholamine storage.

An alternative possibility is that there exists a common cellular event leading to discharge of the contents of both chromaffin granules and lysosomes. There is considerable evidence indicating that the process of exocytosis is responsible for secretion of catecholamines from the bovine adrenal medulla.[21] Lysosomes at some point in their life cycle might also discharge their contents in a similar fashion. de Duve and Wattiaux have suggested exocytosis as a mechanism by which residual lysosome bodies empty their contents into the extracellular space.[22] Furthermore, there are in fact several reports of lysosomal enzyme secretion from a variety of tissues.[23–29] A likely candidate for a cellular process related to exocytosis of both chromaffin granules and of lysosomes arises from the observation that in both cases secretion is dependent upon the presence of calcium. This observation, and the finding that introduction of calcium into calcium-deficient perfusion fluid stimulates secretion of catecholamine and deoxyribonuclease from the gland, strongly implicates calcium as a link in the

secretion processes. If an influx of calcium promoted by acetylcholine or similar drugs is responsible for secretion of catecholamines, the ability of nicotine or potassium chloride to stimulate lysosomal enzyme secretion is not surprising since catecholamine secretion evoked by each of these agents is mediated through a stimulus-secretion step involving calcium.[30] The ability of the ganglionic blocking agent hexamethonium to abolish carbachol-induced secretion of catecholamines and lysosomal enzymes[1] is also compatible with a common mechanism of secretion. It is thought that hexamethonium blocks the interaction of carbachol with the chromaffin cell cholinergic receptors, an interaction that normally leads to catecholamine secretion through subsequent cellular events that may well involve intracellular redistribution of calcium ions.[30]

The exact role played by calcium in the secretion process is not yet known, although calcium has been shown to have marked effects on the physicochemical properties of bovine adrenal chromaffin granules.[31] Banks demonstrated that calcium in concentrations between 1–5 mM produced a dose-dependent reduction in the electrophoretic mobility of the granules. Calcium also caused agglutination of the granules, which was observed by phase contrast microscopy. It would be of interest to know if calcium causes a similar agglutination between chromaffin granules and the plasma membrane of the chromaffin cell, or if calcium causes lysosomal agglutination. It is interesting in this context that calcium is also required for the release of lysosomal enzymes from polymorphonuclear leukocytes in response to stimulation with staphylococcal leucocin,[32, 33] a phenomenon that appears to involve fusion of the enzyme-containing granules with the cell membrane.[32, 33]

Still remaining to be established is the way in which the soluble contents of chromaffin granules, and possibly of lysosomes as well, pass through the various membrane barriers during exocytosis, namely, the organelle membrane and the chromaffin plasma membrane. Both organelles contain potentially lytic substances. The chromaffin granule membrane is relatively high in lysolecithin,[34–37] and lysosomes of course contain a variety of lytic enzymes. It may be that these substances play a role in the actual secretion process, as suggested earlier for secretion of chromaffin granule constituents.[38]

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