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DRUG-INDUCED RELEASE OF CATECHOLAMINES, SOLUBLE PROTEIN AND CHROMOGRANIN A FROM THE ISOLATED BOVINE ADRENAL GLAND

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Abstract—Catecholamines, soluble protein, chromogranin A and lactate dehydrogenase (LDH) activity were measured in perfusates from isolated bovine adrenal glands after stimulation of the glands with carbachol, phenylethylamine and ethylenediamine. The ratio of catecholamine to chromogranin A secreted was similar in each case, whereas the amount of soluble protein secreted depended upon the stimulant used. LDH activity of perfusates was unchanged after stimulation with carbachol. The effect of phenylethylamine on LDH secretion was variable. Secretion by carbachol was completely abolished and secretion by phenylethylamine was partially inhibited in calcium-free solution. These results support the hypothesis that, as a result of stimulation with carbachol, catecholamines are released from the adrenal gland by a process of exocytosis.

EVIDENCE has recently been presented supporting the hypothesis that catecholamines of the adrenal medulla are secreted by a process of exocytosis.[1–3] Since secretion by this mechanism would result in the extrusion of the soluble contents of the granule into the extracellular spaces, the findings that chromaffin granule constituents other than catecholamines are released from the adrenal gland provide strong evidence for this type of secretion process. Thus, in addition to catecholamines and to adenine nucleotides and their metabolites,[4, 5] chromogranin A, the major soluble protein of chromaffin granules, is released upon stimulation of the gland both *in vivo*[6] and *in vitro*.[2, 3, 7–9] Furthermore, it has been shown that all of the soluble proteins of chromaffin granules are secreted upon stimulation.[2] The idea of catecholamine secretion by exocytosis is further strengthened by the observation that dopamine-β-oxidase, an enzyme associated with the insoluble portion of the chromaffin granule, is not secreted under conditions which lead to release of the soluble components of the granules.[3] Furthermore, lipids that are characteristic constituents of membranes are not secreted from the gland along with catecholamines.[1, 2]

In the present paper a number of drugs have been tested for their ability to release soluble protein as well as to release catecholamines. These experiments were undertaken in order to obtain more information about the mechanism by which catecholamines are released from the adrenal medulla.

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METHODS

Perfused adrenal gland. Bovine adrenal glands were prepared and perfused as described previously;[2, 10, 11] flow rates were maintained at 10–14 ml/min. Stimulation of the gland was initiated only after allowing a period of at least 60 min to elapse after beginning perfusion. Secretion of catecholamines and protein was induced by the injection of various chemical agents into the perfusion fluid immediately before the fluid entered the gland. Dose–response relationships were obtained with each drug, and only doses providing supramaximal secretion were employed in these experiments. The drugs, dissolved in Tyrode solution, were routinely injected in volumes of 0·4 ml or less in a series of 6 injections, each injection separated from the other by a period of 30 sec. The amounts of drugs given below are the total amounts injected into the gland. Collection periods were 6 min. Control values for secretion of catecholamines, total protein and chromogranin A, respectively, represent the amounts collected in the perfusate during the 6 min immediately preceding stimulation. The amounts of total protein and chromogranin A are expressed in mg, and the amounts of catecholamines are in μmoles. The amounts of catecholamines, chromogranin A and total soluble protein secreted from the gland as a result of stimulation are expressed as the amount of component secreted during the stimulation period minus the amount secreted during the control period.

In the experiments involving perfusion with Tyrode solution containing either cocaine or hexamethonium, or with calcium-free Tyrode solution, the glands were stimulated first in normal Tyrode solution and then perfused for a minimum of 30 min with the altered Tyrode solution before further stimulation. After responses were obtained in the altered Tyrode solution, the glands were perfused again with normal Tyrode solution and stimulated again. Secretion during perfusion with drug-containing or calcium-free solutions was compared with secretion obtained in normal Tyrode solution during the periods before and after perfusion with the altered solution. The effect on secretion of exposing the gland to cocaine or hexamethonium, or to calcium lack, was expressed as the quotient of the amount of the product secreted in response to stimulation during perfusion with altered Tyrode solution (average of a minimum of two stimulation periods) divided by the amount secreted in response to stimulation during perfusion with the normal Tyrode solution (average of a minimum of four stimulation periods).

Analyses. Perfusates were assayed for catecholamines by the method of von Euler and Hamberg,[12] employing a citrate–phosphate buffer,[13] and for total protein after its precipitation by trichloroacetic acid (5%, w/v) by the microbiuret method of Goa.[14] The amount of chromogranin A in the perfusates was measured by a complement fixation method previously described.[2] Pure chromogranin A, used as a standard in the complement fixation methods, was kindly provided by Drs. A. D. Smith and H. Winkler of this department. Lactate dehydrogenase (LDH) activity of the perfusates was assayed according to Wroblewski and LaDue[15] and expressed as the change in absorbancy in 1 min at 340 mμ $\times$ 1000 divided by 6·2 $\times$ 10^3 (molar absorbancy index for NADH). Drug-induced secretion of LDH was calculated by dividing the amount in the perfusate collected during the stimulation period divided by the amount in the control period perfusate. Statistical calculations were performed by the *t*-test according to Snedecor.[16]

RESULTS

Carbachol. The resting release of catecholamines and total protein from the adrenal gland decreased exponentially with time and after 40–60 min of perfusion was generally in the range of 0·01–0·1 μmole/min of catecholamines and 0·05–0·2 mg/min of protein. The amounts of chromogranin A appearing in the perfusates varied between undetectable amounts and 5 μg/min (sensitivity of the complement fixation assay for chromogranin A was 0·1 μg/ml, equivalent to the secretion of 1 to 1·4 μg/min). Carbamylcholine (carbachol, 5·5 mg) increased the secretion of catecholamines, total protein and chromogranin A. The magnitude of the increases in secretion differed among glands, but for catecholamines and chromogranin A the increase was generally 10- to 30-fold and for total protein 2- to 4-fold above control levels.

The ratios of catecholamines to protein and of catecholamines to chromogranin A secreted in response to carbachol are shown in Table 1. These ratios are similar to the

TABLE 1. RELATIONSHIPS BETWEEN CATECHOLAMINES (CA) AND PROTEINS IN PERFUSATES FROM THE ISOLATED ADRENAL GLAND AND IN SOLUBLE LYSATES OF CHROMAFFIN GRANULES*

Stimulant	Ratio	
	CA: total protein	CA: chromogranin A
Carbachol	4·81 ± 1·48 (n = 19)	14·9 ± 3·6 (n = 5)
Phenylethylamine	2·61 ± 0·50 (n = 8)†	13·5 ± 1·6 (n = 5)
Ethylenediamine	0·46 ± 0·04 (n = 4)†	11·2 ± 2·5 (n = 3)
Soluble lysate of chromaffin granules‡	4·8 ± 0·3 (n = 5)	10·1 ± 1·2 (n = 10)

* The amounts of CA and protein secreted during stimulation periods were calculated by subtracting the amounts of each constituent in the control period from the corresponding amounts in the stimulation period perfusates. CA are expressed in μmoles and protein in mg, and each figure represents the mean ratio (± S.E.) of n determinations.
† $P < 0.01$ (perfusate vs. soluble lysate of chromaffin granules).
‡ Values taken from ref. 2.

corresponding ratios in chromaffin granules from bovine adrenal medulla.

A slight delay was observed in the secretion of protein when compared with the appearance of catecholamines in the perfusate after stimulation by carbachol. This delay is evident when the ratios of catecholamines to total protein are calculated for each minute of a 5-min period after an injection of carbachol (0·23 mg). The respective ratios (average ratios ± S.E. from 4 experiments) were 6·6 ± 0·5. 4·6 ± 0·1, 4·5 ± 1·2, 3·3 ± 0·3 and 3·4 ± 0·8 for minutes 1 through 5.

LDH activity of the perfusates collected during control periods and periods of stimulation was also determined. The enzyme activity of the perfusates during control periods varied between 0·002 and 0·026 unit/min and changed only slightly in 5 of 6 experiments after injection of carbachol (0·80–1·22 of control). In one experiment, however, the enzyme activity of the perfusate was doubled (2·01 of control) after carbachol.

Phenylethylamine. An increase in secretion of total protein and chromogranin A, as well as of catecholamines, was produced by injection of phenylethylamine (as the hydrochloride; 150 mg)). Increases above the resting level of secretion were in the

ranges of 2- to 10-fold, 0·5- to 3-fold, and 2- to 10-fold for catecholamines, total protein and chromogranin A respectively. The mean of the ratios of catecholamine to total protein, however, is lower than the corresponding ratio for chromaffin granule (Table 1), although the ratio of catecholamines to chromogranin A is similar to the values for chromaffin granules and to that obtained with carbachol. Secretion of LDH upon injection of phenylethylamine was variable, being 0·23, 1·32 and 4·97 of control values, respectively, in three experiments.

Other drugs. The release of catecholamines, chromogranin A and total soluble protein was also examined after stimulation with an agent capable of producing nonspecific cell injury. The strongly basic substance, ethylenediamine, was used for this purpose. Although release of catecholamines and chromogranin A in response to ethylenediamine (1 ml of a 1 % solution; pH 11·4) was similar to that with carbachol, secretion of total protein was increased 10–40 times above the resting level. The pronounced effect of ethylenediamine on release of soluble protein is reflected in the low ratio of catecholamines to total protein of the perfusates (Table 1). Ethylenediamine also caused a marked increase in the release of LDH from the adrenal gland; in three experiments secretion of LDH was 4·91, 8·03 and 18·2 of the corresponding control values. Injection of an equimolar amount of ethylenediamine dihydrochloride (neutral pH) produced only a slight increase in secretion of catecholamines above the resting level (1·7 of control) and induced no change in secretion of soluble protein or LDH.

Injection of adrenaline (2 μmoles, 366 μg) in quantities similar to those found in perfusates after stimulation with carbachol did not cause an increase in secretion of total protein, chromogranin A or LDH.

Calcium depletion. Secretion of catecholamines, total protein and chromogranin A in response to carbachol was abolished in calcium-free perfusion fluid. Secretion induced by phenylethylamine, on the other hand, was only partially inhibited by calcium deprivation: secretion of total protein was reduced to $0{\cdot}38 \pm 0{\cdot}07$ ($n = 5$) and catecholamines were reduced to $0{\cdot}29 \pm 0{\cdot}07$ ($n = 5$) of the secretion obtained in response to phenylethylamine in normal Tyrode solution. Phenylethylamine-induced secretion of chromogranin A was abolished during perfusion with calcium-free medium in each of three experiments.

Cocaine. Secretion of catecholamines, total protein and chromogranin A in response to carbachol was markedly reduced when the gland was perfused with Tyrode solucontaining cocaine in a concentration of 30 μg/ml. Secretion of catecholamines was 0·12 and 0·06 of the amounts obtained in the absence of cocaine, respectively, in two experiments, and there was no secretion above control levels of total protein. Release of chromogranin A was 0·04 of control ($n = 1$).

Hexamethonium. The effects of perfusion with Tyrode solution containing hexamethonium bromide (250 μg/ml) on carbachol-induced secretion of catecholamines and proteins were similar to those of cocaine. In two experiments secretion of catecholamines by carbachol was 0·02 and 0·05 of control, respectively, and secretion of total protein above control levels was completely inhibited.

Hypothermia. Carbachol-induced secretion of catecholamines and protein was reduced when the temperature of the perfusion medium was lowered from 37° to 23°. Secretion of catecholamines was $0{\cdot}46 \pm 0{\cdot}07$ ($n = 6$) of the corresponding value obtained at 37° and secretion of total protein was $0{\cdot}38 \pm 0{\cdot}09$ ($n = 6$) of the secretion at 37°.

DISCUSSION

The results presented in this report confirm earlier findings of Banks and Helle,[7] of Kirshner *et al.*[3, 8, 9] and of Schneider *et al.*,[2] showing that the catecholamines and the major soluble protein component of chromaffin granules, chromogranin A, are released from the bovine adrenal gland in a ratio similar to that found in the adrenaline-containing storage granules of the adrenal medulla. In addition to chromogranin A, the other soluble proteins of chromaffin granules are also released upon stimulation, and they account quantitatively for the remaining soluble protein secreted in response to stimulation by carbachol.[2] In the latter work, the soluble proteins of the chromaffin granules were identified by starch gel electrophoresis of perfusates from adrenal glands stimulated with carbachol. Their identity was substantiated further by amino acid analysis. Immunochemical assay of chromogranin A revealed its presence in both the perfusate and in soluble lysates of chromaffin granules in the same proportion to the other soluble proteins.

The results of the present investigation, as well as the earlier findings of Banks and Helle[7] and of Schneider *et al.*,[2] suggest that the ratio of the amount of catecholamines to the amount of total soluble protein secreted from the gland in response to stimulation can be used as an indication of the specificity with which the secretogogues bring about the release of catecholamines. If catecholamine release occurs by exocytosis, the ratio of catecholamines to soluble protein secreted from the gland will be the same as the corresponding ratio for chromaffin granules. This appears to be the case for secretion induced by carbachol. On the other hand, another mechanism (or mechanisms) of release must operate for secretion induced by phenylethylamine and ethylenediamine. Secretion of catecholamines due to general tissue damage, as produced by ethylenediamine, is indicated by a ratio of catecholamines to protein secreted which is less than the corresponding ratio for chromaffin granules. The additional protein presumably derives from sources other than chromaffin granules. The presence of LDH in the perfusates after exposure of the gland to ethylenediamine, and in one case after phenylethylamine, indicates that proteins other than those deriving from chromaffin granules contribute to the protein secreted. The non-chromaffin granule protein could be coming from cells of the cortex as well as from the medulla.

The slight delay in the secretion of protein relative to that of catecholamines after stimulation with carbachol is in agreement with a similar delay in the secretion of chromogranin A in response to acetylcholine reported by Kirschner *et al.*[3] It is likely that this delay reflects a lower diffusion rate from the site of release into the perfusion fluid for the large protein molecule relative to the diffusion rate for the catecholamines.

The ability of the indirectly acting[17] sympathomimetic amine, phenylethylamine, to stimulate secretion of both catecholamines and protein was examined, since this amine had been shown to cause release of catecholamines[18–21] from the adrenal gland. Like carbachol, phenylethylamine also stimulated secretion of chromaffin granule constituents from the adrenal gland, although apparently in a less specific manner. The ratio of catecholamine to chromogranin A found in perfusates after stimulation with phenylethylamine was similar to that found with carbachol. The secretion of total protein, however, was relatively greater with phenylethylamine, as indicated by a lower ratio of catecholamine to soluble protein secreted. Further evidence for an action of phenylethylamine on the chromaffin cell different from that exerted by carbachol was obtained when the effects of calcium depletion were examined. Omission of calcium

from the Tyrode solution resulted in complete inhibition of chromogranin A secretion induced by phenylethylamine, although secretion of catecholamines and soluble protein was only partially inhibited. These results suggest that phenylethylamine may affect the chromaffin cell in more than one way, possibly by: 1) a calcium-dependent mechanism analogous to that operating in response to carbachol, whereby both catecholamines and chromaffin granule soluble protein are released; and 2) a process not affected by calcium depletion. A multiple action of phenylethylamine such as this might encompass both the direct displacement theory of release proposed by Schumann and Philippu[22] and the acetylcholine-like mode of action found by Rubin and Jaanus.[20] If catecholamines are displaced from chromaffin granules by phenylethylamine without a loss of chromaffin granule soluble proteins, the ratio of catecholamines to chromogranin A after stimulation with phenylethylamine should be higher than the corresponding ratio, both after stimulation with carbachol and in the soluble lysates of chromaffin granules. The limitations of the complement fixation technique used to measure the amount of chromogranin A, however, would not permit the demonstration of a difference of this magnitude.

The ratio of catecholamines to chromogranin A was similar to the corresponding ratio for chromaffin granules during the more generalized type of release caused by ethylenediamine. This agent also provoked the release of relatively large amounts of total soluble protein and released LDH in large quantities. It also caused the loss of the mitochondrial enzyme, fumarase, an enzyme not secreted in response to the injection of carbachol (Schneider, unpublished observations). Secretion of catecholamines and protein induced by ethylenediamine was undoubtedly due to the high pH of the solution injected, since an equimolar amount of ethylenediamine hydrochloride was much less effective in causing release.

The observed reduction in carbachol-induced secretion of catecholamines and soluble protein during perfusion at low temperature is in agreement with recent findings by Kirschner *et al.*[3] Reduction in secretion of chromaffin granule constituents during hypothermia may reflect an interference with an energy-dependent process associated with secretion, or with diffusion of the products from their site of release into the effluent. This latter point may be of importance if the structure of the various membranes through which the secretory products must pass is influenced by the environmental temperature. In this context, Schramm *et al.*[23] observed that the lipid structure of the zymogen granules from rat parotid glands is altered by exposure of the granules to low temperature.

The results presented in this paper demonstrate that a relatively constant relationship exists between catecholamines and chromogranin A released from the bovine adrenal gland in response to various secretogogues. This relationship persists even when loss of proteins not contained in chromaffin granules occurs, as happens in cell damage produced by ethylenediamine. Furthermore, secretion of catecholamines and soluble proteins of chromaffin granules is similarly altered under conditions that inhibit secretion (cocaine, hexamethonium, calcium-depletion, hypothermia). It appears, then that carbachol induces the release of catecholamines and chromogranin A through a common mechanism, and one that does not involve release either of constituents of the cytoplasm or of the insoluble portion of the chromaffin granule itself.[1-3] It seems unlikely that any mechanism of release other than exocytosis could account for these findings.

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REFERENCES

1. J. M. Trifaró, A. M. Poisner and W. W. Douglas, *Biochem. Pharmac.* **16**, 2095 (1967).
2. F. H. Schneider, A. D. Smith and H. Winkler, *Br. J. Pharmac. Chemother.* **31**, 94 (1967).
3. N. Kirshner, H. J. Sage and W. J. Smith, *Molec. Pharmac.* **3**, 254 (1967).
4. L. Stjärne, *Acta physiol. scand.* **62**, *suppl.* 228 (1964).
5. W. W. Douglas, A. M. Poisner and R. P. Rubin, *J. Physiol., Lond.* **179**, 130 (1965).
6. H. Blaschko, R. S. Comline, F. H. Schneider, M. Silver and A. D. Smith, *Nature, Lond.* **215**, 58 (1967).
7. P. Banks and K. Helle, *Biochem. J.* **97**, 40c (1965).
8. N. Kirschner, H. J. Sage, W. J. Smith and A. G. Kirshner, *Science* **154**, 529 (1966).
9. H. J. Sage, W. J. Smith and N. Kirshner, *Molec. Pharmac.* **3**, 81 (1967).
10. O. Hechter, R. P. Jacobsen, V. Schenker, H. Levy, R. W. Jeanloz, C. W. Marshall and G. Pincus, *Endocrinology* **52**, 679 (1953).
11. P. Banks, *Biochem. J.* **97**, 555 (1965).
12. U. S. von Euler and U. Hamberg, *Acta physiol. scand.* **19**, 74 (1949).
13. T. C. McIlvaine, *J. biol. Chem.* **49**, 183 (1921).
14. J. Goa, *Scand. J. clin. Lab. Invest.* **5**, 218 (1953).
15. F. Wroblewski and J. S. la Due, *Proc. Soc. exp. Biol. Med.* **90**, 210 (1955).
16. G. W. Snedecor, *Statistical Methods*, p. 45. Iowa State University Press, Ames, Iowa (1956).
17. J. H. Burn and M. J. Rand, *J. physiol., Lond.* **144**, 314 (1958).
18. H. W. Haag, A. Philippu and H. J. Schümann, *Experientia* **17**, 187 (1961).
19. A. Philippu and H. J. Schümann, *Experimentia* **18**, 138 (1962).
20. R. P. Rubin and S. D. Jaanus, *Arch. exp. Path. Pharmak.* **254**, 125 (1966).
21. R. P. Rubin and S. D. Jaanus, *Biochem. Pharmac.* **16**, 1007 (1967).
22. H. J. Schümann and A. Philippu, *Arch. exp. Path. Pharmak.* **244**, 466 (1963).
23. M. Schramm, R. Ben-Zvi and A. Bdolah, *Biochem. Biophys. Res. Commun.* **18**, 446 (1965).