Effects of Stimulation by Carbachol on the Metabolism of the Bovine Adrenal Medulla

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(Received 10 May 1965)

1. A method is described that has made it possible to achieve a great decrease in the catecholamine and adenine nucleotide contents of the perfused bovine adrenal gland by the infusion of carbachol. 2. Although the catecholamines secreted were recovered in the perfusion medium, no evidence was obtained that the nucleotides are secreted by the gland. 3. It is concluded that the secretion of catecholamines is accompanied by extensive chemical alteration of the adenine nucleotides of the chromaffin granules. 4. The secretory response and the spontaneous release of catecholamines depends on the presence of Ca^{2+} in the perfusing Tyrode solution. 5. Anoxia does not have a significant effect on the carbachol-induced secretion of catecholamines. 6. Strips of bovine adrenal medullary tissue perfused with oxygenated Tyrode solution show an increased oxygen consumption when carbachol is added.

In recent years much has been learned about the storage of catecholamines in the adrenal medulla and about the ways in which the store can be depleted and replenished. However, little progress has been made towards investigating the metabolic changes that occur when the gland secretes.

The catecholamines are stored in specific sub-cellular particles together with an approximately equivalent amount of ATP (see Hagen & Barrnett, 1960). When the store of catecholamine is depleted as a result of insulin-induced hypoglycaemia, the ATP content of the gland also falls so that the molar ratio of catecholamines to ATP remains constant (Carlsson & Hillarp, 1956; Schümann, 1958; Burack, Weiner & Hagen, 1960). The fate of the ATP is unknown: Carlsson, Hillarp & Hökfelt (1957) were unable to detect any increase in the concentrations of ADP, AMP, adenosine or inorganic phosphate in the gland when the catecholamine and ATP concentrations had fallen by 72 and 66% respectively. It is, therefore, possible that the nucleotides are released from the gland together with the catecholamines. Holton (1959) has found that in the perfused rabbit ear the concentration of ATP present in the perfusion medium increases when the great auricular nerve is stimulated antidromically. Thus there is evidence that nerve stimulation can lead to the release of ATP.

The isolated and perfused bovine adrenal gland can be made to secrete by the injection of acetylcholine (Schümann & Philippu, 1962). This method has now been developed to allow the nucleotide and catecholamine compositions of the medulla and of its secretion to be studied. In previous experiments it had not been possible to stimulate either catecholamine secretion or oxygen consumption by adding cholinergic drugs to tissue slices of the adrenal medulla. The success obtained with the perfusion technique indicated that a continuous-flow method may provide conditions under which changes in oxygen consumption could be measured. This has been achieved by a perfusion method, and the results obtained are described in the present paper.

METHODS

Perfusion of the isolated bovine adrenal medulla. The left adrenal gland was removed as soon after slaughter as possible and the surrounding fat was dissected away in the laboratory. The gland was attached to a modified Langendorff heart-perfusion apparatus by inserting a polythene cannula into the adrenal vein. Tyrode solution (Ling & Smith, 1955) was allowed to enter the gland so that it became turgid, and the surface of the gland was then covered with incisions which passed through the cortical tissue but which did not penetrate the medulla. The perfusion rate was adjusted to about 20 ml./min., except in the experiment described in Fig. 2 where it was 6 ml./min. The Tyrode

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solution was gassed with O_2+CO_2 (95:5) and maintained at 37.5°.

This method of retrograde perfusion is similar to that used by Schümann & Philippu (1962) and is based on the technique described by Hector *et al.* (1953).

The gland was stimulated to secrete by the infusion of 10 mM-carbachol (carbamoylcholine chloride) at the rate of 1.7 ml./min. with a roller pump. Carbachol was obtained from British Drug Houses Ltd., Poole, Dorset.

Estimation of catecholamines and nucleotides in the adrenal glands. A small sample of the adrenal medulla (about 0.5 g.) was dissected from the gland and homogenized with a Potter-Elvehjem homogenizer having a Teflon pestle and glass tube in 5 ml. of 5% (w/v) $HClO_4$ at 4°; for the control glands this was done on arrival at the laboratory and for the perfused glands immediately after the period of perfusion. The homogenate was diluted to 25 ml. with more 5% $HClO_4$ and shaken at 4° for ½ hr. The acid homogenate was centrifuged and the resulting sediment extracted with a further 25 ml. of 5% $HClO_4$ and recentrifuged. The supernatants from the two centrifugations were pooled and adjusted to pH 7.0 with 20% (w/v) K_2CO_3 ; then the precipitated $KClO_4$ was spun down. The catecholamine content of the solution was estimated by the method of Euler & Hamberg (1949). Nucleotides were extracted from 20 ml. of the neutralized extracts by shaking with Dowex AG-1 ion-exchange resin (X4; Cl^- form; 200–400 mesh). The resin was then sedimented and washed four times by resuspension in water. The nucleotides were extracted from the resin by shaking with $N-HCl$ and the absorption spectrum was measured.

The absorption spectra of the supernatants, after removing the $KClO_4$, were also measured between 230 and 295 $m\mu$, with the pH adjusted to 2.0 with 0.1 $N-HCl$.

Catecholamine and nucleotide contents of the perfusion medium. As the perfusion medium was collected from the gland $HClO_4$ was added to give a final concentration of about 3% (w/v). The denatured protein was spun down and the deproteinized perfusion medium was neutralized to pH 7.0. The catecholamine content and absorption spectrum were then measured as described above. A 100 ml. sample of deproteinized and neutralized perfusion medium was extracted with 20 g. of Dowex AG-1 resin and the washed resin was extracted with the least possible volume of $N-HCl$. The absorption spectrum of the extract was measured and a 0.3 ml. sample was subjected to descending paper chromatography for 30 hr. on Whatman no. 1 paper, with a solvent system of butan-1-ol saturated with HCl .

Oxygen consumption of adrenal medulla. The O_2 consumption of a thin strip of bovine adrenal medulla, suspended in a glass capillary and bathed in a stream of oxygenated Tyrode solution, was measured with a recording oxygen electrode (see Gleichmann & Lübbers, 1960). The electrode was inserted through a hole in the capillary to make contact with the stream of Tyrode solution about 1 cm. below the tissue. The capillary, electrode and reservoirs containing the Tyrode solution were encased in a water jacket at 37°. The water in the jacket was saturated with O_2+CO_2 (95:5) and circulated by a pump so that there was neither an O_2 nor a temperature gradient between the solution in the capillary and the water in the jacket. Penicillin (20 000 units/l.) and streptomycin (7500 units/l.) were added to the Tyrode solution to prevent bacterial growth during the experiment.

The apparatus used was designed and made in this Laboratory by Dr K. Galenhofen; the oxygen electrode was supplied by L. Eschweiler G.m.b.H., Kiel, Germany.

RESULTS

Observations on the perfused bovine adrenal gland

Catecholamine secretion. Fig. 1 shows that the infusion of approx. 1 mM-carbachol for 6 min. caused a marked increase in the catecholamine output

Fig. 1. Secretion of catecholamines by the perfused bovine adrenal gland. The gland was perfused through the vein with Tyrode solution gassed with O_2+CO_2 (95:5) and maintained at 37.5°. The perfusion rate was approx. 20 ml./min. The black bars indicate 6 min. periods during each of which 17 μ moles of carbachol were infused/min.

from the perfused adrenal gland. The amount of amine appearing in the perfusion medium declined rapidly; however, after a 6 min. period of perfusion in the absence of carbachol, the response could be repeated by a further infusion of the drug. With a more prolonged period of carbachol infusion the decline in catecholamine output followed a roughly exponential course (Fig. 2). This decline is similar to that described by Douglas & Rubin (1961) during their studies on the adrenal gland of the cat perfused from the artery.

When Ca^{2+} was omitted from the perfusing Tyrode solution both the secretion of catecholamines in response to carbachol and the spontaneous release were abolished. This is in agreement with the results of Douglas & Rubin (1961) and of Schümann & Philippu (1962).

Catecholamine and nucleotide contents of the adrenal medulla after perfusion with carbachol. Perfusion of an adrenal gland for 3½ hr. with Tyrode solution caused only small decreases, 15.0 and 13.2% respectively, in its catecholamine and nucleotide contents (Table 1). The molar ratio of catecholamines lost to nucleotides was 3.84.

Fig. 2. Secretion of catecholamines by the perfused bovine adrenal gland during a prolonged infusion with carbachol. The gland was perfused through the vein with Tyrode solution gassed with O₂+CO₂ (95:5) and maintained at 37.5°. The perfusion rate was approx. 6 ml./min. The black bar indicates a 9 min. period during which 6.0 μmoles of carbachol were infused/min.

Table 1. *Effect of perfusion with Tyrode solution for 3½ hr. on the catecholamine and nucleotide contents of the bovine adrenal medulla*

The left gland was perfused and the right gland from the same animal was used as the unperfused control. The percentage depletions of catecholamines and nucleotides are given in parentheses.

	Catecholamines (μmoles/g. wet wt.)	Nucleotides (μmoles/g. wet wt.)	Molar ratio: Catecholamines Nucleotides
Control gland	81.8	24.2	3.38
Perfused gland	69.5 (15.0)	21.0 (13.2)	3.31

The perfused adrenal gland can be extensively depleted of catecholamine by the repeated infusion of carbachol. In the two experiments described in Table 2 there were, respectively, 17 and 14 stimulation periods of 3 min. duration, each being separated by a recovery period of 9 min. during which carbachol was not infused. From the stimulated glands 122.7 and 104.7 μmoles of catecholamines were released into the perfusion medium, representing depletions of 63.6 and 41.9% respectively. The nucleotide content of the adrenal medullae fell by 58.5 and 38.5% in the two experiments, and in both the molar ratio of catecholamines lost to nucleotides lost was 3.3.

The ultraviolet-absorption spectra of the nucleotides extracted from both the control and depleted glands indicated that adenine nucleotides accounted for almost all the nucleotides present in the glands.

Composition of the adrenal perfusion media. The absorption spectra of the perfusion media from the stimulated glands were similar to those of adrenaline or noradrenaline, although the base line was rather high (Fig. 3, curve 5). In contrast, the spectra of extracts of control and depleted glands (Fig. 3, curves 3 and 4) corresponded closely to that of a mixture of adrenaline and ATP having a 3:1 molar ratio (Fig. 3, curve 1). Thus the molar ratio of catecholamines to nucleotides in the perfusion media must be much greater than in the glands.

After the perfusion media from stimulated glands had been extracted with Dowex AG-1 (Cl⁻ form) ion-exchange resin no nucleotides could be detected in the acid extracts from the resin by ultraviolet spectroscopy or by paper chromatography. Recovery experiments showed that when ATP was injected into the perfusing Tyrode solution above the gland, throughout a 3 min. period of stimulation by carbachol and in amounts that would be required to give a 1:3 molar ratio with the secreted catecholamines, 26% of the ATP injected could be recovered as adenine nucleotide. If the injection of ATP took place below the gland then 77% was recovered as

Table 2. *Effect of stimulation by carbachol on the catecholamine and nucleotide contents of the perfused bovine adrenal medulla*

The left gland was perfused with Tyrode solution and stimulated by the repeated infusion of carbachol at 9 min. intervals. The perfusion rate was approx. 20 ml./min. and during the 3 min. stimulation period the concentration of carbachol in the Tyrode solution was 1 mM. In Expts. I and II there were 17 and 14 stimulation periods respectively. The right glands were used as the unperfused controls. The percentage depletions of catecholamines and nucleotides are given in parentheses.

Expt. no.....	Catecholamines (μmoles/g. wet wt.)		Nucleotides (μmoles/g. wet wt.)		Molar ratio: Catecholamines Nucleotides		Catecholamines in the perfusion medium (μmoles)	
	I	II	I	II	I	II	I	II
Control gland	75.8	76.6	24.8	24.9	3.06	3.09	—	—
Stimulated gland	27.6 (63.6)	44.5 (41.9)	10.3 (58.5)	15.3 (38.5)	2.7	2.9	122.7	104.7

Fig. 3. Absorption spectra of adrenal-gland extracts and perfusion media. Curve 1: absorption spectrum of a solution containing adrenaline and ATP in the molar ratio 3:1. Curve 2: absorption spectrum of a solution containing adrenaline and ATP in the molar ratio 1:1. Curve 3: absorption spectrum of an extract from an unperfused adrenal medulla; pH 2.0. Curve 4: absorption spectrum of an extract from a perfused gland that had been depleted of 63.6% of its catecholamines by the repeated infusion of carbachol; pH 2.0. Curve 5: absorption spectrum of deproteinized perfusion medium from a stimulated perfused adrenal gland; pH 2.0.

adenine nucleotide. The sensitivity of the chromatographic detection was 100-fold greater than would have been necessary to detect the nucleotides if the ratio of catecholamine to nucleotide in the perfusion medium from the stimulated glands had been 3:1, even allowing for a recovery of nucleotides of only 25%.

Effect of anoxia on catecholamine secretion by the perfused adrenal gland. When the gland was perfused with Tyrode solution gassed with nitrogen

Fig. 4. Effect of anoxia on the carbachol-induced secretion of catecholamines by the perfused bovine adrenal gland. The perfusion rate was approx. 20 ml./min. The black bar indicates 3 min. periods during which 17 μ moles of carbachol were infused/min. The white bar represents periods of perfusion with Tyrode solution gassed with O₂ + CO₂ (95:5). The hatched bar represents a period of perfusion with Tyrode solution gassed with N₂ + CO₂ (95:5).

+ carbon dioxide (95:5) for $\frac{1}{2}$ hr., neither the secretory response to the infusion of carbachol nor the spontaneous release of catecholamines was altered significantly (Fig. 4).

Observations on strips of bovine adrenal medulla

Measurement of oxygen consumption with and without carbachol. Tissue slices of bovine adrenal medulla 0.2 mm. thick were prepared with a Stadie-Riggs cutter as soon as possible after the gland had been obtained. Strips about 15 mm. long and 1 mm. wide were cut from the tissue slices; the fresh weight of the strips was about 12 mg.

A strip was tied into a loop of thread that passed through a ring of silver wire placed in the capillary above the oxygen electrode. The tissue strip was then hoisted up the capillary so that it was suspended about 1 cm. above the electrode.

These manipulations were carried out while a stream of Tyrode solution passed downwards through the capillary. When required, this solution could be changed to a Tyrode solution containing carbachol (1 mM) by turning a two-way tap above the capillary. The perfusion rate was kept constant at 0.15 ml./min.

Fig. 5 shows a record taken from such an experiment. By comparing the oxygen concentration of the Tyrode solution in the presence and absence of a strip of medulla, the resting oxygen consumption of the tissue was found to be 0.24 μ l. of oxygen/mg. wet wt. of tissue/hr. When the bathing fluid was changed to oxygenated Tyrode solution containing carbachol (1 mM), the oxygen consumption increased to 0.34 μ l./mg. wet wt. of tissue/hr. The peak was

Fig. 5. Oxygen consumption of a perfused strip of bovine adrenal medulla. A strip of medulla weighing about 12 mg. was perfused with oxygenated Tyrode solution at 37° and the oxygen concentration of the solution below the gland was recorded with a platinum oxygen electrode. The arrow (A) indicates the time at which the perfusing solution was changed to oxygenated Tyrode solution containing carbachol (1mm). The arrow (B) indicates the time at which the carbachol-free Tyrode solution was readmitted.

reached 3 min. after the oxygen consumption began to rise. The increased consumption was maintained fairly constant until 8 min. after the two-way tap had been turned to readmit carbachol-free Tyrode solution into the capillary. The oxygen consumption then returned slowly to the resting level. The response could be repeated several times during one experiment, although it decreased in size. Similar responses were obtained in the three experiments that have been carried out.

The lowest concentration of carbachol that will induce catecholamine release from the perfused adrenal gland is $10\mu\text{M}$; in the presence of that concentration of carbachol the oxygen consumption of the adrenal strip rose to 6% above the resting level.

DISCUSSION

When the perfused adrenal medulla is stimulated by the repeated infusion of carbachol, the catecholamine and adenine nucleotide contents of the gland fall. During secretion the molar ratio of catecholamines to nucleotides in the gland remains almost constant so that for every 3.3 mol. of catecholamine secreted 1 mol. of nucleotide disappears from the gland. Although the secreted amines can be measured in the perfusion medium, the loss of nucleotides by the gland cannot be accounted for by a corresponding rise in the nucleotide content of the perfusion medium. The nucleotides must therefore either be degraded to non-nucleotide compounds that remain in the cell or are secreted by it, or they must be converted into some complex acid-insoluble compounds that are not readily extracted from the gland by the methods used. In either case it is apparent that the nucleotides must undergo more extensive alteration during secretion than occurs in skeletal muscle during contraction. During the latter ATP is dephosphorylated to ADP, which is then rapidly rephosphorylated by the creatine

phosphate-creatine phosphokinase system (Cain, Infante & Davies, 1962).

In homogenates of the bovine adrenal gland roughly 75% of the catecholamines is contained in the chromaffin granules (Blaschko, Hagen & Welch, 1955); in the intact gland the percentage may be higher, as some granules will be disrupted during homogenization. The chromaffin granules also contain about 85% of the nucleotides of the adrenal medulla, which in the bovine gland consist almost entirely of ATP (Falk, Hillarp & Högberg, 1956; Hillarp, 1958; Blaschko, Born, D'Iorio & Eade, 1956; Blaschko, Hagen & Hagen, 1957). Thus, when the catecholamine and nucleotide contents of the adrenal medulla are extensively decreased after repeated stimulation, most of the catecholamines and nucleotides lost must have originated from the storage granules. This conclusion is supported by the observation that the molar ratio of catecholamines to nucleotides lost during secretion is very similar to that found in the chromaffin granules. It must be concluded that the loss of catecholamines from the chromaffin granules during secretion is associated with metabolic changes that cause a fall in the content of acid-soluble nucleotides of the cells. The nature of these changes is not understood.

The observation that anoxia does not affect the secretory response significantly indicates that the mechanism of secretion cannot be directly dependent on the oxidative metabolism of the cell. However, the experiments with tissue strips show that stimulation can lead to an increase in oxygen consumption. It is possible that this increased oxygen uptake is related to recovery processes rather than to the secretory mechanism. In this context it may be recalled that Hill (1940) found that the increase in oxygen consumption associated with a short tetanus of the frog sartorius muscle at 0° began after the tetanus had ended. If a similar situation existed in the adrenal medulla it would indicate why secretion can occur under anaerobic conditions but is associated with an increased oxygen consumption under aerobic conditions.

This research was supported by grants from the U.S. Air Force, European Officer of Aerospace Research, during the tenure of a Medical Research Council Scholarship. I thank Dr H. Blaschko, F.R.S., for his supervision and for help with the preparation of this paper.

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