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ON THE MODE OF ACTION OF ACETYLCHOLINE IN EVOKING ADRENAL MEDULLARY SECRETION: INCREASED UPTAKE OF CALCIUM DURING THE SECRETORY RESPONSE

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Acetylcholine is generally believed to be the physiological transmitter of sympathetic nerve activity at the adrenal medulla. Recently Douglas & Rubin (1961*a*, *b*) described experiments indicating that the stimulant effect of acetylcholine involves some calcium-dependent process. They found that removal of calcium from the extracellular environment greatly depressed or even abolished the secretory response to acetylcholine; that the secretory response varied directly with the calcium concentration not only when this was low but also when it was high; and, finally, that calcium itself in certain conditions was a powerful stimulus to secretion. These findings, considered along with the known effects of acetylcholine at other sites in the body where it seems to act by causing some change in the permeability of the 'receptor membrane' to common species of ions, led them to suggest that acetylcholine evokes catecholamine secretion by causing calcium ions to penetrate the adrenal medullary cells.

The present experiments were designed to test the possibility that acetylcholine increases calcium uptake by the adrenal medulla. A preliminary account of our findings has appeared elsewhere (Douglas & Poisner, 1961).

METHODS

Preparation. All the experiments were carried out on cats' adrenal glands isolated from the animals and perfused *in vitro* in a retrograde fashion through the adrenal veins as described by Douglas & Rubin (1961*b*). The main modification in the technique was to use both glands from each animal, one serving as a test preparation and the other as a control; in the series of eight animals, right and left glands were used alternately for stimulation so that there were four left and four right glands in the stimulated group and also in the control group. Butterworth & Mann (1957) have shown that although adrenals from different cats vary greatly in their catecholamine content, the right and left glands in any one cat usually contain very similar amounts of catecholamines and, presumably, chromaffin cells. Thus

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they found that the ratio of amines in the left gland to amines in the right gland was $1{\cdot}053 \pm 0{\cdot}023$ (mean $\pm$ S.E.; 36 observations).

Perfusion fluids. Locke's solution was used with the following composition (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; glucose, 10. It was equilibrated with 100% O_2 and had a pH close to 7·0. In one experiment bicarbonate-buffered Locke's solution of the following composition was used (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $NaHCO_3$, 6·0; glucose, 10. This solution was equilibrated with 5% CO_2 in O_2 and also had a pH close to 7·0. When necessary the radio-isotope ^{45}Ca was added so that the activity in the perfusion fluid was about 0·5–1·0 μc/ml. This was done without appreciably increasing the total calcium content of the perfusion fluid by adding traces of ^{45}Ca of high specific activity (about 2×10^6 μc/mM). The ordinary Locke's solution will be referred to as ^{40}Ca Locke's solution; that labelled with the radio-isotope ^{45}Ca, as ^{45}Ca Locke's solution.

Procedure. The glands were placed in an apparatus which allowed them to be perfused separately with identical fluids under the same pressure. Since adrenal glands isolated from the body and perfused in the manner we have adopted tend to release catecholamines at an irregularly high rate in the first minutes after their removal from the animal, presumably because of the operative procedure (Douglas & Rubin, 1961*b*), the glands were first perfused for about 25 min with ^{40}Ca Locke's solution to allow them to reach a steady state. After this preliminary period, perfusion to both glands was switched to a common source of ^{45}Ca Locke's solution. Three minutes later perfusion to the test gland was switched to a second source of ^{45}Ca (a portion of the first) containing acetylcholine chloride 10^{-5} g/ml. for 15 sec, after which it was switched back to ^{45}Ca Locke's solution. This procedure was repeated each minute until 6–8 periods of ACh stimulation had been given. In the conditions of our experiment ACh has no significant effect on the flow of perfusion fluid (Douglas & Rubin, 1961*b*). No change in the flow of perfusion fluid was detected during these manipulations which involved simply the turning of a tap. Intermittent stimulation was adopted because the secretory response to a continuous exposure to ACh tends to fall off (Douglas & Rubin, 1961*b*). When the last period of ACh stimulation had been given, perfusion to both glands was switched to ^{40}Ca Locke's solution and the glands were washed out for 100 min during which time effluents were collected at 5 or 10 min intervals. Finally, perfusion to both glands was stopped and each gland was separated into cortex and medulla by careful dissection under a binocular microscope with $\times$ 6–12 magnification. The tissues were blotted gently and weighed on stainless-steel planchets. After drying overnight at 100° C, they were reweighed and finally ashed at 600° C for 2 hr. All the weights given are dry weights.

Radioactivity measurements. Portions (0·5 ml.) of the effluents were plated on concentric-ringed planchets and 2 drops of sodium oxalate (0·2M) added to precipitate calcium and ensure its uniform distribution. The planchets were then dried under a lamp and radioactivity counted. The ashed tissues were usually counted directly. It was found that the values so obtained closely agreed with those obtained by taking up the ash in acid and replating it. Counting was done with a low level β counter (Tracerlab Omniguard). This counter had a background of less than 0·5 counts/min so that it was relatively easy to obtain accurate measurement of radioactivity even in those samples (such as were sometimes obtained toward the end of the washout period) in which radioactivity was low (e.g. 5 counts/min.ml.). In each experiment control samples of the ^{45}Ca Locke's solution used for perfusion were plated (after dilution with ^{40}Ca Locke's solution to within the range of measurement of our β counter) and counted along with the effluents so that the observed counts/min could be converted to pmole *total* Ca (i.e. $^{40}Ca + ^{45}Ca$). In a typical experiment 1 count/min represented 8 pmole total Ca.

RESULTS

The experimental procedure we adopted was to perfuse both glands with ^{45}Ca Locke's solution for a period of time during which one gland was exposed intermittently to ACh, then to wash out the glands by perfusing with ^{40}Ca Locke's solution until such time as the radioactivity remaining in them might reasonably be supposed to be intracellular, and finally to compare this residual activity in the unstimulated (resting) and ACh-stimulated medullae. After the preliminary perfusion with ^{40}Ca Locke's solution perfusion to both glands was switched to ^{45}Ca Locke's solution and 3 min later one gland was exposed intermittently to ACh so that eight exposures totalling 2 min were given. After the 11th minute of exposure to ^{45}Ca, perfusion to both glands was switched back to ^{40}Ca Locke's solution for an additional 100 min. Finally, the radioactivity remaining in the glands at this time was determined. In a typical experiment the gland (cortex plus medulla) exposed to ACh was found to yield about twice as many counts as the control gland. In both glands radioactivity was particularly high in the medulla, each mg containing several times as many counts as the same weight of cortical tissue from the same gland. Of most immediate interest was the finding that the medulla stimulated with ACh yielded about three times as many counts as the control medulla. Eight such experiments were performed and in every instance the medulla exposed to ACh was found to have taken up more ^{45}Ca than the resting medulla (Table 1*a*). The difference between the ACh-stimulated and resting medullae proved on analysis to be significant at the level $P < 0{\cdot}005$. The experiments thus permit the conclusion that ACh increases the uptake of Ca by the adrenal medulla.

An estimate of the rate of Ca uptake by the resting medullae during the period of exposure to ^{45}Ca, arrived at by dividing the figure for total Ca uptake by the time of exposure, is shown in Table 1*b*: the mean rate of Ca uptake by the resting medullae was 0·50 pmole/mg.sec. An estimate of Ca uptake during exposure to ACh may be obtained by assuming that the excess Ca uptake in the ACh-stimulated medulla occurred only during the time ACh was present (ACh was present for only 1½–2 min of the exposure to ^{45}Ca), and that during the rest of the perfusion with ^{45}Ca the rate of uptake in the stimulated gland was the same as that in the control gland. This assumption seems reasonable since no systematic difference between the rate of resting Ca uptake in right and left medullae was found. Thus in a series of sixteen resting medullae (the present eight plus eight from another group of experiments) the ratio of the mean rate of Ca uptake in the right medullae to that in the left medullae was 1·06, which is not significantly different from 1·0 ($P = 0{\cdot}85$). The rate of Ca uptake

by the medulla during stimulation by ACh (R_s) was therefore calculated from the following relation:

$$R_s = \frac{S - T_2 . R_r}{T_1},$$

where S = Ca uptake by the stimulated medulla expressed in pmole/mg;

R_r = rate of Ca uptake by the resting medulla in pmole/mg.sec;

T_1 = time of exposure to ^{45}Ca in the presence of ACh, in sec;

T_2 = time of exposure to ^{45}Ca in the absence of ACh, in sec.

The mean rate of Ca uptake on exposure to ACh 10^{-5} g/ml. was 4·3 pmole/mg.sec, i.e. about eight times the resting rate (Table 1*b*).

TABLE 1. Uptake of calcium by resting and ACh-stimulated cats' adrenal medullae perfused with Locke's solution

The results were obtained from experiments on sixteen adrenal glands from eight cats. Each experiment was performed on a pair of glands from the same animal: one gland (Rest.) served as a control and the contralateral gland (Stim.) was exposed intermittently to ACh (10^{-5} g/ml.). The values indicated in (*a*) are based on the uptake of Ca in the paired medullae determined after 100 min washout. No correction has been made for loss of Ca from the cells during the washout period. These values (and those of (*b*) which are calculated from them) are thus minimal estimates (see Results). Analysis of the results in (*a*), using the *t* test on the mean of the differences between the paired glands, shows that the effect of ACh is significant at the level $P < 0{\cdot}005$.

	(*a*) pmole/mg			(*b*) pmole/mg.sec	
Expt. no.	Rest.	Stim.	Difference (Stim. − Rest.)	Rest.	Stim.
1	139	396	257	0·29	2·6
2	479	1151	672	0·95	7·2
3	381	1484	1103	0·60	7·5
4	202	290	88	0·41	1·3
5	243	627	384	0·51	2·9
6	211	484	273	0·39	3·4
7	213	673	460	0·40	5·5
8	247	757	510	0·46	4·2
Mean	264	732	468	0·50	4·3
S.E. (of mean)	69	142	110	0·07	0·8

The above interpretation of the results rests on the assumption that the residual radioactivity found in the glands derives from the cells and that no significant contribution is made by carry-over of ^{45}Ca present in the extracellular space when washing out was begun. In the light of work with other tissues this assumption would seem to be reasonable. For example, it is known that an effective washout of extracellular space occurs by diffusion alone in small muscles which are merely *bathed* in saline solution

for 90 min (Bianchi & Shanes, 1959) and *perfusion* must be considered a more rapid means of washing out this space. In each of the present experiments more direct evidence of the adequacy of the washout was sought by studying the time course of loss of radioactivity from the glands and constructing typical 'washout curves' (see Hodgkin & Keynes, 1957; Shanes, 1961). To construct such washout curves (during ^{40}Ca perfusion of the ^{45}Ca loaded glands), the radioactivity in serial samples of effluent—beginning with the last and working back towards time zero—was added (in cumulative fashion) to the radioactivity found in the gland at 100 min.

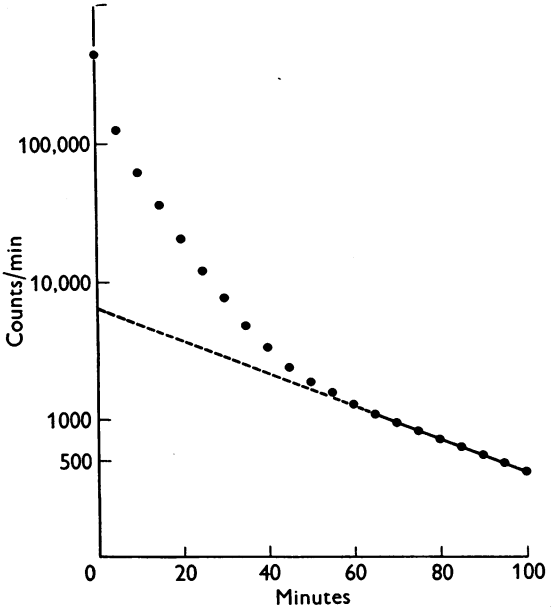

Fig. 1. Time course of decline of ^{45}Ca in a cat's adrenal gland previously perfused with ^{45}Ca Locke's solution for 11 min, and then, beginning at time 0, perfused for 100 min with ^{40}Ca Locke's solution. The curve was arrived at by adding the counts for the individual samples of effluent, in cumulative fashion, to the residual count for the whole gland (416 counts/min).

Each curve thus portrayed the radioactivity present in a given gland at any time during the washout period. One such washout curve is shown in Fig. 1. It shows an initial rapid phase which is followed at about 50 min by a slower phase reflecting a simple exponential decline of radioactivity. Similar curves were obtained in all experiments, whether on resting glands or on glands exposed to ACh. In each experiment the slow phase had become apparent within an hour of beginning the washout. The curves are very similar to those obtained by others in related studies on nerve and muscle in which the rapid phase is held to reflect loss from the extracellular

space, and the slow phase loss from the cells (Hodgkin & Keynes, 1957; Shanes, 1961). They thus indicate that the ^{45}Ca found in the adrenal glands at 100 min may be taken to be of cellular origin.

Estimation of absolute value of calcium uptake. By extrapolating the slow component of the washout curve back to the moment when washout was begun (as in Fig. 1) it should be possible to obtain an indication of the original intracellular ^{45}Ca content of the whole gland (see Hodgkin & Keynes, 1957; Shanes, 1961). This procedure indicates that the radioactivity in the cells at 100 min is only a small fraction of that present in the cells immediately after stopping perfusion with ^{45}Ca. The original intracellular ^{45}Ca content of the sixteen *whole glands* was estimated from the washout curves to be about fifteen times the value at 100 min. Due to the uncertainty of the precise effect of washout on the *medullae*, we have preferred to give (as in Table 1) only the uncorrected values of calcium uptake. These, however, must be recognized as underestimating the true values.

Calcium uptake by the adrenal cortex. Although we were interested mainly in the adrenal medulla in these experiments, information was also obtained about Ca uptake by the cortex. The mean Ca uptake in the resting cortices was 71 ± 27 pmole/mg and in the cortices exposed to ACh was 126 ± 17 pmole/mg. The mean of the differences between the paired cortices was 55 ± 11 pmole/mg. On applying the same method of statistical analysis as was used to analyse the results from the medullae (see legend to Table 1) this stimulant effect of ACh on Ca uptake in the cortex proved to be significant at the level $P < 0{\cdot}005$. The rates of Ca uptake by the cortices, calculated in the same way as for the medullae, were $0{\cdot}13 \pm 0{\cdot}02$ pmole/mg.sec for those resting and $0{\cdot}67 \pm 0{\cdot}14$ pmole/mg.sec for those exposed to ACh. This latter value is less than one sixth of the corresponding rate (4·3 pmole/mg.sec) for the medullae.

DISCUSSION

The most familiar action of acetylcholine in the adrenal gland is its stimulant action on the chromaffin cells leading to the release of the catecholamines, adrenaline and noradrenaline. This, indeed, forms part of the array of evidence that acetylcholine is the physiological transmitter of sympathetic nerve activity at the adrenal medulla. Under the same conditions as we have employed, Douglas & Rubin (1961*b*) found that acetylcholine causes a large increase in catecholamine output which may quite commonly reach a level fifty times greater than the level of spontaneous release. The most obvious interpretation of our present finding that acetylcholine increases calcium uptake by the adrenal medulla is, therefore, that it reflects a stimulant action of acetylcholine on the chro-

maffin cells. Such an interpretation is certainly in harmony with previous evidence that calcium is actively involved in the process of adrenal medullary secretion (Douglas & Rubin, 1961*b*), but it is not possible to say at present whether or not the other tissues in the medulla such as connective tissue or fine blood vessels also contribute significantly to the calcium movements we have observed, for nothing is known of the action of acetylcholine on calcium movement in these tissues. They appear however to make up only a small part of the medulla, which consists mainly of tightly packed chromaffin cells (Bennett, 1941).

In their experiments on the effect of calcium on adrenal medullary secretion Douglas & Rubin (1961*b*) demonstrated a close relation between extracellular calcium concentration and the effectiveness of acetylcholine in releasing catecholamines; they found, moreover, that calcium itself—in experimental circumstances known to increase membrane permeability—was a sufficient stimulus for catecholamine release. On such grounds they argued that acetylcholine evokes catecholamine release by promoting the entry of calcium into the chromaffin cells. While our present findings are certainly consonant with this hypothesis, they do not distinguish an uptake of calcium which might precede secretion from one which might accompany or follow it.

Douglas & Rubin (1961*b*) have previously commented on the parallelism between the evidence suggesting that calcium somehow links the stimulant action of ACh on medullary cells to the secretory response (the process they referred to as 'stimulus-secretion coupling') and the evidence which had already led others to propose a similar role for calcium in the process of 'excitation-contraction coupling' in muscles (Heilbrunn, 1943, 1956; Niedergerke, 1956; Sandow, 1952; Shanes, 1958). This parallelism is further extended by the present evidence that the adrenal medulla increases its calcium uptake on stimulation just as do various muscles (Niedergerke & Harris, 1957; Bianchi & Shanes, 1959; Shanes, 1961; Durbin & Jenkinson, 1961). The eightfold increase in the rate of calcium uptake observed in the medulla during ACh-induced secretion may be compared with the fourfold increase in the rate of calcium uptake found in frog's rectus abdominis muscle during sustained contraction (Shanes, 1961).

The previous experiments by Douglas & Rubin (1961*b*) and the present experiments together offer a considerable body of evidence indicating that calcium is critically involved in the process of secretion at the adrenal medulla and it is obviously pertinent to consider whether the adrenal medullary cell is unique in this respect or whether calcium may have a generalized role in the process of secretion. In the present experiments we have observed that although calcium movement in the cortex is much less brisk than in the medulla it too is augmented by acetylcholine and

we note that Rosenfeld (1955) has found that acetylcholine promotes the release of adrenal cortical hormones in perfused adrenal glands. It will be of interest to see whether calcium is involved in the process of cortical secretion induced by the more specific stimulant ACTH.

SUMMARY

1. Studies have been made of the uptake of ^{45}Ca by cats' adrenal glands perfused *in vitro* with Locke's solution.

2. Acetylcholine (10^{-5} g/ml.) caused about an eightfold increase in the rate of calcium uptake by the medulla.

3. This finding lends support to the previous suggestion that acetylcholine evokes catecholamine secretion from the medulla by promoting the uptake of calcium.

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