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THE PREPARATION AND USE OF PURIFIED AND UNPURIFIED DISPERSED ADRENAL CELLS AND A STUDY OF THE RELATIONSHIP OF THEIR cAMP AND STEROID OUTPUT*

S. A. S. TAIT, J. F. TAIT, R. P. GOULD, B. L. BROWN and J. D. M. ALBANO

Department of Physics Applied to Medicine and Anatomy and Department of Nuclear Medicine, The Middlesex Hospital Medical School, London W1P 6DB, England.

SUMMARY

Zona glomerulosa cells purified from zona fasciculata contamination by unit gravity sedimentation have previously been characterized. Similarly, zona fasciculata cells have now been separated from zona glomerulosa and zona reticularis cells. Purified zona fasciculata cells respond to valine angiotensin II or ACTH. The maximal steroid response is equivalent for both stimuli (about one hundred fold) and also is about the same using purified zona fasciculata cells or unpurified cells from decapsulated tissue. Preparations consisting mainly of zona reticularis cells respond to a lesser extent than pure zona fasciculata cells to both stimuli. The cAMP output for purified glomerulosa cells is increased by high amounts of ACTH, angiotensin II, serotonin and K^+ (8·4 mM). Submaximal doses of these stimuli, which markedly increase corticosteroid output, do not detectably raise cAMP output. This could be due to kinetic or compartmental factors. However when a maximal steroidogenic dose of cAMP or serotonin ('pure' internal cAMP generator) was used to stimulate unpurified or purified glomerulosa cells, steroid output could still be altered by varying K^+ concentration. This suggests that alteration in cAMP output production is not the only mechanism for controlling steroidogenesis; alteration in cAMP activity by K^+ or a K^+ control mechanism independent of cAMP production are possible extra or alternative mechanisms. A formal treatment of the discussion of the role of cAMP in mediating steroidogenesis is presented.

INTRODUCTION

In previous papers[1, 2] the preparation of zona glomerulosa cells of rat adrenals by unit gravitational sedimentation of dispersed cells from capsule strippings of the gland, which originally contained 4% of the zona fasciculata contamination, was described. Electron and phase microscopic examination of the sedimented zona glomerulosa cells and their steroidogenic response to ACTH and cyclic AMP indicated that they were reasonably free of contamination from zona fasciculata cells. Electron microscopic examination of the purified glomerulosa cells indicated that most of them were reasonably normal in structure. The maximal response of corticosterone output to serotonin and potassium and the responses to all potassium concentrations were not significantly altered, indicating normal function for the cells producing steroids. Their maximal response to ACTH, valine angiotensin II and cyclic AMP was decreased after fractionation but at the doses used, steroidogenesis by the zona fasciculata contamination in an unpurified preparation was expected to be stimulated by these substances. The basal production of corticosterone per cell (on the basis of the number of zona glomerulosa cells counted by phase microscopy) decreased after sedimentation[1] and this is still largely unexplained.

Purified zona glomerulosa cells had about the same maximal response of corticosterone output (about two fold) to potassium concentration, valine and isoleucine angiotensin II, serotonin and ACTH. The maximal response of the purified zona glomerulosa cells to cyclic AMP is similar to that elicited by these stimuli.

Cyclic AMP output (from cells and media) by zona glomerulosa cells was significantly raised by large doses of the stimuli used to produce steroidogenesis[3–5]. Again the cAMP response was decreased after sedimentation of the cells following stimulation by valine or isoleucine angiotensin II and ACTH but not by potassium or serotonin. However, the cyclic AMP output of purified zona glomerulosa cells responded (about two fold) to all the stimuli. Some submaximal doses of serotonin, K^+ and angiotensin II, which markedly stimulated corticosterone did not significantly increase cAMP output[4, 5].

These results suggested that as cyclic AMP can increase steroidogenesis by purified zona glomerulosa

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cells to about the same extent as stimuli such as angiotensin II, serotonin, potassium and ACTH: and also cAMP can be increased by all these stimuli, then the cAMP second messenger hypothesis could be applied to steroidogenesis by glomerulosa cells. However, effective steroidogenic doses of serotonin, K^+ and angiotensin II did not increase cyclic AMP output significantly and this indicates that in a more physiological range, e.g. K^+ concentration from 3·6 to 5·9 mM, increase in cyclic AMP production may not play a dominant role in controlling steroidogenesis. Nevertheless, both kinetic[6] and compartmental effects[7] have been suggested to explain such phenomena and the data so far obtained is inconclusive in deciding the questions raised.

The aim of the present paper is to illustrate the application of the 1*g* sedimentation to the purification of other adrenocortical cells and the use of purified cells to investigate biological problems by (a) describing further experiments on the preparation of zona fasciculata cells and other adrenocortical cells from decapsulated tissue and the results of their responses to ACTH and angiotensin II and (b) by using both purified and unpurified zona glomerulosa cells to further examine the cAMP second messenger theory. Steroidogenesis was measured after addition of a maximal dose of cAMP and then varying the concentration of K^+ in the media.

EXPERIMENTAL AND RESULTS

Materials and methods

The materials and methods are as described in detail in previous publications[2] except where mentioned in the text or legends.

Zona glomerulosa cells were prepared from capsular tissue of rat adrenals as previously described[2]. They were essentially free from other adrenocortical cells.

Preparation and properties of zona fasciculata and zona reticularis cells

Zona fasciculata cells were purified by 1*g* sedimentation from dispersed cells prepared from decapsulated tissue. The system employed was identical to that used for the separation of zona glomerulosa cells from dispersed capsular tissue[2], Fig. 1a. The rate of sedimentation of the zona fasciculata cells from the decapsulated tissue, as determined by Coulter counter and phase microscopic examination, was the same as that of the small number ot zona fasciculata cells present in the capsular tissue preparations[2]. Their appearance on electron microscopic examination was also similar to that previously described[2]. There was a good correlation between the number of zona fasciculata cells counted by microscopic examination and by a

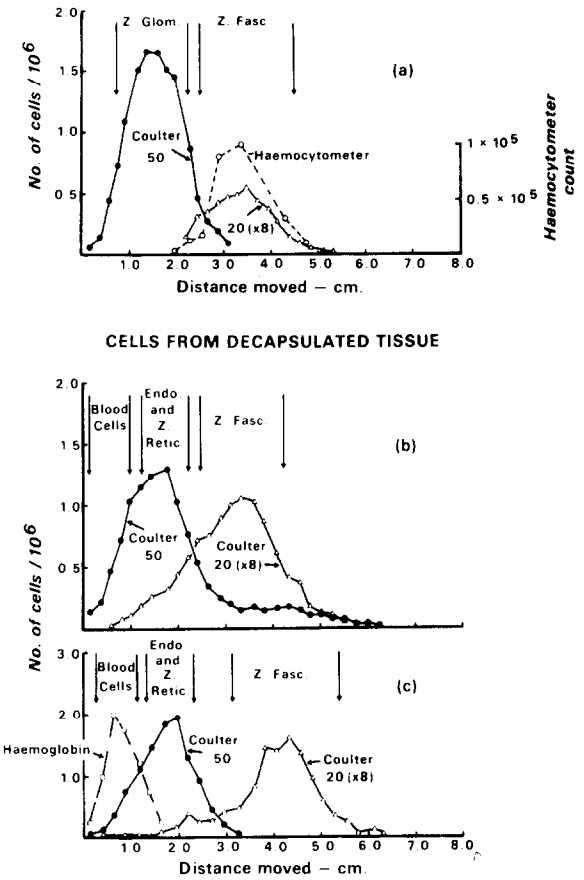

Fig. 1. Separation of zona glomerulosa, zona reticularis and zona fasciculata cells by unit gravity sedimentation. The pulse heights used for analysis (full lines) are at 50 amplification for zona glomerulosa (a) and reticularis (b and c) and 20 (× 8) amplification for zona fasciculata cells (a, b and c). Broken line shows haemocytameter count of zona fasciculata cells. The number of cells or pulses are from the same aliquots of the fraction but are shown on different scales for clarity.

Coulter counter (pulse size 20 × 8), Fig. 1b, c. Some zona glomerulosa cells tend to aggregate and then contaminate the zona fasciculata fractions. However the proportion of zona glomerulosa cells in the initial preparation of dispered cells from decapsulated tissue is small (<1%) as shown by microscopic examination and by the response of the unpurified cells to specific glomerulosa stimulators such as serotonin or increased K^+ concentrations. The final preparation of zona glomerulosa cells in the purified zona fasciculata fractions (Fig. 1b and c) is therefore satisfactorily low (<0·1%).

In addition to these fractions containing zona fasciculata cells (EM3, Fig. 2), there was another slower moving band of cells sedimenting at about the same rate as single zona glomerulosa cells from capsular tissue (Figs. 1 and 2).

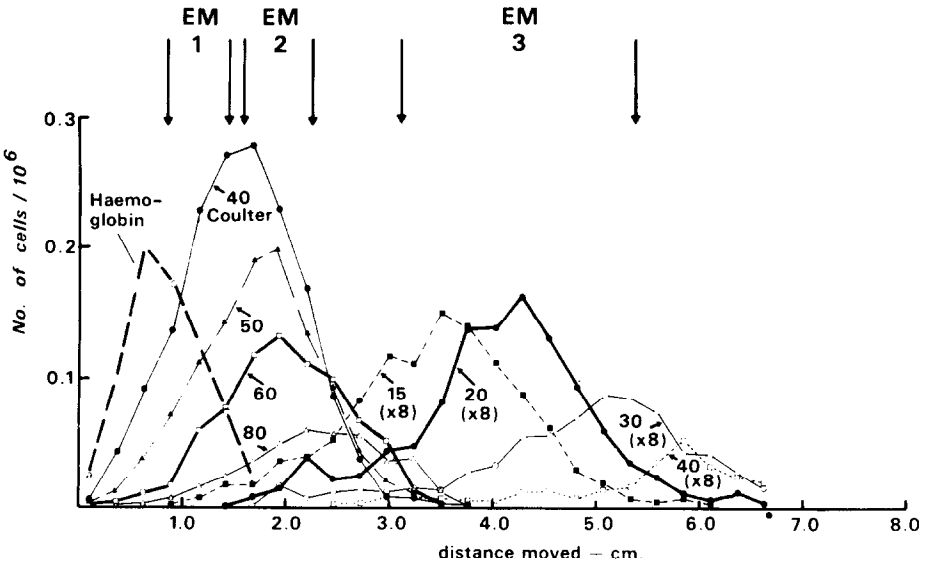

Fig. 2. Shows the details of 1*g* sedimentation of decapsulated cells (Fig. 1, c). Coulter counts were taken at different pulse heights (two amplifier positions were used, × 1 and × 8). The thick full lines at 50 and 20 (× 8) show counts at the pulse height to give the position of the zona reticularis and zona fasciculata cells. Haemoglobin assayed at 417 nm after lysis of the cells is also shown (in the full line). EM1, 2 and 3 are the positions of the pools taken for electron microscopic examination and incubation studies.

This band was divided into slow (EM1) and fast (EM2) fractions for electron microscopic examination (Fig. 2). The position of erythrocytes was also determined by haemolysis of an aliquot from the appropriate fractions and assay for haemoglobin at 417 nm (Fig. 2). The combined fractions (EM1 and EM2) contained examples of all the different cell types normally found in the adrenal cortex, namely cortical parenchymal cells, endothelial cells, red blood cells, macrophages and the various kinds of leucocytes. The proportion of cortical cells to the other cell types are listed in Table 1. The different dispersed cell types can easily be distinguished from one another. Thus the cortical cells have characteristic mitochondria and usually contain extensive areas of smooth endoplasmic reticulum; endothelial cells exhibit a complex, folded and reticulated plasma membrane; and the white blood cells are characterized by their specific content and nuclear form.

The dispersed cortical cells are of two main kinds, with a small number of indeterminate forms. We have identified the most numerous cortical cell in the combined EM1 and EM2 fractions as zona reticularis

Table 1. Cell types and viability.

Fraction	Cell type	%	% Viable	% Changed	% Non-viable
EM 1	Reticularis	6·0	59·0	23·0	18·0
1300 cells	Fasciculata	3·5	36·0	8·0	56·0
counted	Indeterminate cortical cells	1·0	75·0	25·0	—
	Rest of cells (endothelial, leucocytes, erythrocytes etc).	89·5	76·0	10·0	14·0
EM 2	Reticularis	26·0	65·0	14·0	21·0
500 cells	Fasciculata	10·0	63·0	10·0	27·0
counted	Indeterminate cortical cells	1·0	100·0	—	—
	Rest of cells (endothelial, leucocytes, erythrocytes etc).	63·0	70·0	18·0	12·0

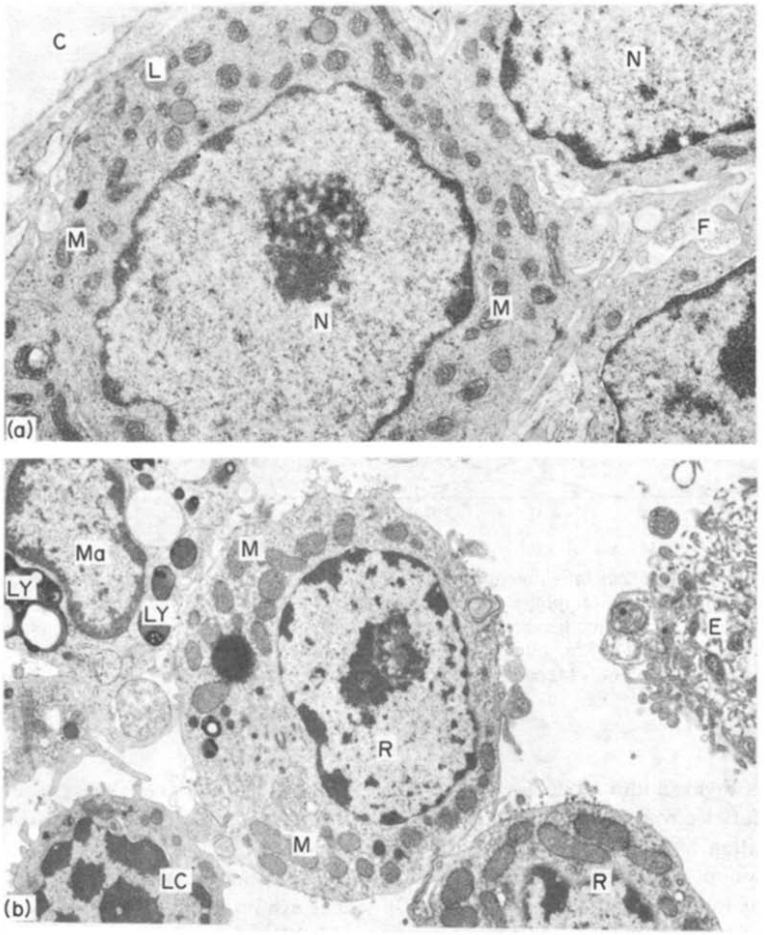

Fig. 3a. Several juxtamedullary zona reticularis cells of intact adrenal gland are illustrated. Nuclei (N) are variable in shape as are the mitochondria (M). Mitochondrial cristae are tubular or vesicular in profile. Lipid droplets (L) are few in number. Part of an adjoining capillary (C) can also be seen and intercellular collagen fibrils (F). × 10,500. Fig. 3b. This electronmicrograph illustrates some of the dispersed cell types found in pooled fractions (EM_2, Fig. 2) after sedimentation of decapsulated tissue. Two zona reticularis cells (R) can be identified by their typical mitochondria (M) which contain a mixture of tubular and vesicular cristae. Part of an endothelial cell (E) can be identified by its folded plasma membrane. Parts of a macrophage (Ma) containing several dense lysosome-like bodies (LY) and a lymphocyte (LC) can be seen × 12,500.

(Fig. 3b) by comparison with intact adrenal tissue (Fig. 3a).* They are spherical in shape with an average diameter of about 8 μm, slightly smaller than zona glomerulosa cells. Their nuclei are variable in form and much of the cell is occupied by smooth endoplasmic reticulum. A few lipid droplets and several lysosome-like dense bodies are often present. The mitochondria of the cells, which provide the main morphological criterion for distinguishing zona reticularis cells, are quite variable in shape and size but have characteristic cristae. In profile, these take the form of short tubes and vesicles (Fig. 3). However, it is likely that all the cristae are tubular in form but when sectioned appear as vesicles or tubes according to the plane of sectioning.

The second kind of cortical cell may be either about the same size as the zona reticularis or considerably larger (12–14 μm in diameter). A high proportion of these larger cells show signs of irreversible damage in the EM1 fractions. A loss of cytoplasmic density may account for their slow sedimentation speed. The characteristic feature of this type of cell is that in

* It is difficult to distinguish between zona reticularis and zona intermedia cells (situated between the zona glomerulosa and fasciculata). These conclusions are in agreement with those of Bennett *et al*[8] which were published during the course of this work. However the zona intermedia cells are likely to be low in number[9] especially when decapsulated tissue is used as starting material. Nevertheless whenever zona reticularis cells are mentioned in the text it must be borne in mind that some zona intermedia cells might be present in any experimental preparation.

addition to the usual steroidogenic features (smooth endoplasmic reticulum, lipid droplets etc.) their mitochondria tend to be round in shape and with a content of almost entirely vesicular cristae. They therefore identify by comparison with intact tissue with cells belonging to the inner fasciculata. They may represent zona fasciculata cells also present in EM3 as there could be some overlap of zona fasciculata and zona reticularis cells in neighbouring fractions. Similarly, the EM3 fractions contain some cells (up to 20%) identified as zona reticularis (or zona intermedia) cells. This may be due to aggregation of the smaller cells followed by sedimentation at a faster rate than the original single cells.

The electron microscopic examination showed that two pools of fractions containing adrenocortical cells could be usefully collected routinely. Firstly the faster sedimenting adrenocortical cells (EM3) contained mainly zona fasciculata cells, but also an appreciable proportion (up to 20%) of zona reticularis cells. The numbers of zona glomerulosa cells in this pool were insignificant. Secondly, the slower sedimenting adrenocortical cells (EM2) were mainly zona reticularis cells but also present were appreciable numbers of zona fasciculata cells (up to about 20% of the reticularis) and probably some zona glomerulosa cells.

Therefore the 1*g* sedimentation procedure, which yielded zona glomerulosa cells free from other adrenocortical cells when applied to the separation of dispersed cells from capsular tissue, gave enriched but not pure separate pools of zona fasciculata and zona reticularis cells when applied to cells from decapsulated tissue. There was some cross contamination. Nevertheless it was considered that some indications of the properties of the pure cells could be obtained by an examination of these enriched preparations.

The response (R = corticosterone output with stimulus/output at 3·6 mM K^+) of cells to ACTH and angiotensin II was examined. The doses used were found to be maximally effective in previous studies with zona glomerulosa and fasciculata cells. The results for the purified zona fasciculata and zona reticularis preparations and for unpurified cells are seen in Table 2. Full dose response curves for both stimuli applied to unpurified and purified zona fasciculata cells are shown in Fig. 4. It can be seen that the response of the purified zona fasciculata cells to ACTH is at least as great as that of the unpurified cells from decapsulated tissue. The maximal response of these unpurified cells to angiotensin II was about the same as for ACTH. There is an indication that the response of the purified zona fasciculata cells to angiotensin is greater than of the unpurified cells and this may be due to the diminution of the action of peptidases[8] but it is of the same order of magnitude.

The maximal response of the preparation of enriched but not pure zona reticularis to ACTH and angiotensin II is less than for zona fasciculata cells (R = 22 and 21 respectively compared with 260 and 340 for purified fasciculata cells). The purified zona fasciculata cells do not respond to 8·4 mM K (R = 1·19). However the purified zona reticularis cells showed a slight response (R = 1·70) which could have been accounted for by the zona glomerulosa contamination.

Steroidogenesis and cAMP output

Previous results have shown that the steroidogenic responses of unpurified and purified zona glomerulosa cells from capsular tissue differed when stimuli, such as angiotensin II and ACTH, which affect both zona glomerulosa and fasciculata cells were used[2]. However, the responses of the cells did not significantly alter

Table 2. Comparison of the response (R = stimulated/3·6 mM K control) of purified and unpurified zona fasciculata cells to maximal doses of ACTH and angiotensin II.

			R value	
			Unpurified zona fasciculata	Purified zona fasciculata
Experiment 1	100 mU/ml	ACTH	72	130
	200 μg/ml	Angiotensin	101	144
Experiment 2	10 mU/ml	ACTH	131	135
	1·6 mg/ml	Angiotensin	135	169
Experiment 3	10 mU/ml	ACTH	197	288
	800 μg/ml	Angiotensin	182	296
Experiment 4*	10 mU/ml	ACTH	207	259

*In experiment 4, the R value for zona reticularis cells was 22 for ACTH and 21 for Angiotensin II.

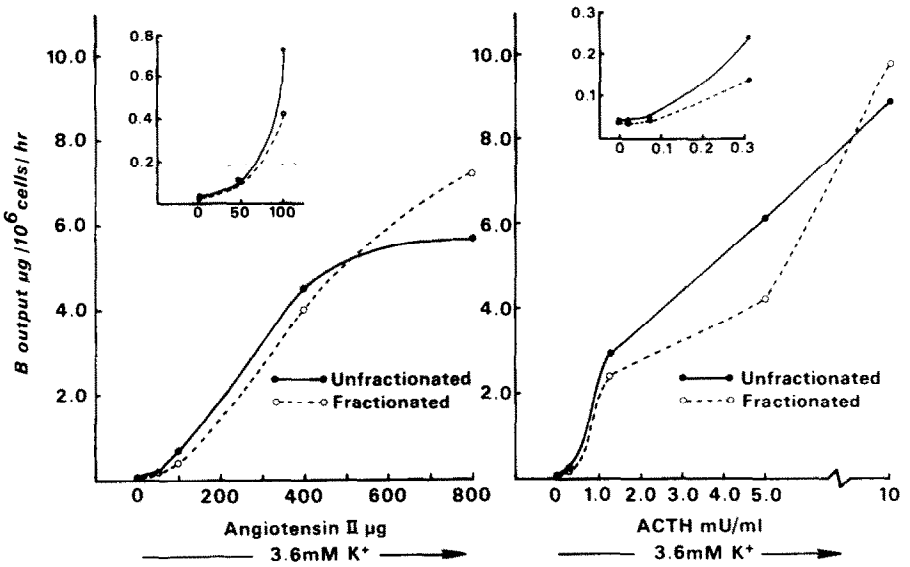

Fig. 4. Dose response curves for angiotensin II and ACTH for fractionated —○— and unfractionated —●— zona fasciculata cells from the same original cell suspensions. All incubations were for 2 h at 37°C under 95% O_2·5% CO_2 in a medium of Krebs Ringer Bicarbonate buffer, 3·6 mM K^+, with 0·2% glucose and 4% BSA added (KRBGA). Angiotensin II was added in KRBGA and ACTH in 0·05 ml pH4 saline. 0·05 ml pH4 saline was added to all remaining incubations.

after purification when specific zona glomerulosa stimuli, such as serotonin and increased K^+ concentration, were applied. Therefore, because of the expense and effort involved in preparing purified cells, unpurified capsular cells have usually been employed for experiments with K^+ or serotonin stimulation and purified zona glomerulosa cells with angiotensin II, ACTH or cAMP.

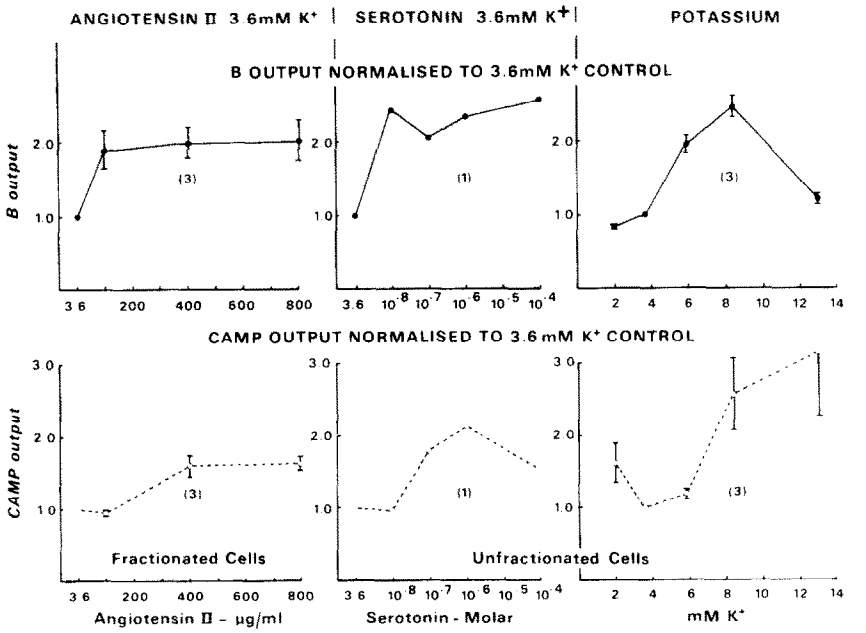

Fig. 5. The effect of angiotensin II (fractionate glomerulosa cells), serotonin and potassium (unfractionated cells) on corticosterone —●— and cAMP —○— outputs carried out simultaneously on the same cell suspensions. The outputs (mean ± S.E.) are normalized to those at 3·6 mM K^+ controls. The required K^+ concentrations were achieved by substituting special Krebs with appropriate K^+ concentrations for 3·79 mM K^+ KRBGA. Serotonin was added in 0·05 ml pH4 saline. Number of experiments are given in brackets. See legend to Fig. 4 for incubation conditions.

Combined results from previous studies including the correlation of corticosterone and cAMP output of zona glomerulosa cells under different conditions are shown in Fig. 5. Angiotensin II (with purified zona glomerulosa cells), K^+ and serotonin (unpurified capsular cells) were used as stimuli. For all three, it was possible to find a concentration of stimulator which could cause nearly maximal steroidogenic response with no corresponding significant increase in cAMP output. For K^+ stimulation, this phenomenon occurred in the physiological range of concentration (from 3·6 to 5·9 mM K^+), Fig. 5. At 8·4 mM K^+ concentration, the cells gave a highly significant increase in output of both corticosterone and cAMP.

Time course of cAMP output with K^+ stimulation

It has been suggested that the lack of correlation of steroid and cAMP output following stimulation could be due to a different time course in the increase of the steroid product and postulated messenger cAMP[6]. Therefore, in order to investigate the kinetics of the stimulation of cAMP output by increased K^+ concentration, two experiments were performed in which, with minimum disturbance of the conditions, the K^+ concentration was quickly increased from 3·6 to either 5·9 or 8·4 mM. By using several incubation vessels and stopping their production at various time intervals after stimulation, the time course of the altered cAMP output could be followed. The integrated steroid output was increased 2·83 and 2·27 fold (over 60 min, first experiment) and 1·88 and 1·69 fold (over 120 min, second experiment) by 5·9 and 8·4 mMK^+ respectively. These results roughly correspond to those of previous experiments (Fig. 5), which also gave similar steroid outputs at the two K^+ concentrations.

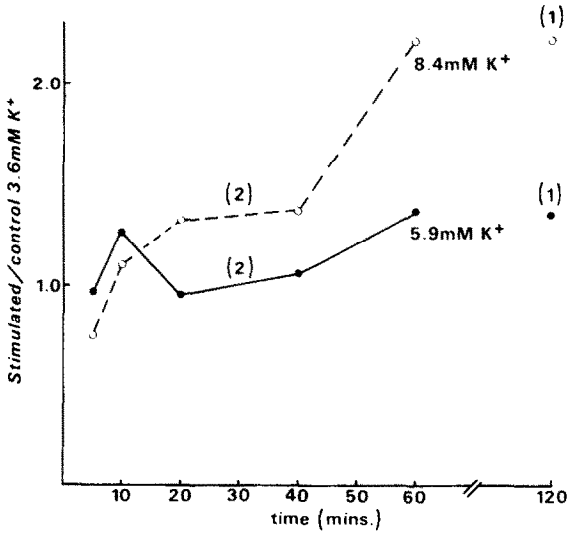

Fig. 6. Cyclic AMP output as a function of time of incubation. Outputs are expressed as a ratio (R) of the values at 5·9 and 8·4 mMK^+ to those of the appropriate 3·6 mMK^+ control. The corresponding *R* values for corticosterone outputs at 5·9 and 8·4 mMK^+ were 1·82 and 1·58 at 40 min, 2·28 and 1·64 at 60 min and 1·88 and 1·69 at 120 min.

The results of the kinetic experiments are shown in Fig. 6. The cAMP outputs at 5·9 mM K^+ show no consistent increase over the basal 3·6 mM K^+ outputs at any time after the initial stimulation. In contrast, the 8·4 mM K^+ outputs are consistently greater than those at 3·6 mM after about 40 min from the change in K^+ concentration. These results show that the lack of correlation of cAMP and steroid outputs at 5·9 mM K^+ was unlikely to be due to the kinetic characteristics proposed to explain similar problems in other situations [6].

Steroidogenesis with added external cAMP and different K^+ concentrations

It has also been suggested that the lack of correlation of steroid and cAMP output could be due to the cAMP output not being proportional to the effective cAMP concentration at the site of action[7]. In order to investigate whether this could be the whole explanation of the phenomenon, a concentration of cAMP (10 mM) which caused a maximal steroidogenic response at any K^+ concentration was added to the medium: the K^+ concentration was then varied and the corticosterone output measured.

It was confirmed that 10 mM cAMP was a maximally effective steroidogenic concentration by comparing the steroid output with addition of either 5 or 10 mM cAMP to the medium containing purified zona glomerulosa cells. It has already been found that the responses to these two concentrations of cAMP were equivalent at 3·6 mM K^+[2]. Recent results showed this to be also the case at 2 and 13 mM K^+. The *R* values with 5 and 10 mM cAMP stimulation at 2 mM K^+ were 1·51 and 1·59 respectively (1st experiment) and 1·17 and 1·28 (2nd experiment) and at 13 mM were 1·68 and 1·64 (1st experiment) and 1·23 and 1·24 (2nd experiment).

Unpurified capsular cells, including about 4% zona fasciculata cells, were used for the preliminary studies of steroid output with added 10 mM cAMP and different K^+ concentrations. It had previously been found that zona fasciculata cells are not stimulated by K^+[10]. Recent investigations of the steroid output of cells from decapsulated tissue (mainly zona fasciculata cells) with added 10 mM cAMP also showed no significant alteration in steroid output with different K^+ concentrations. The steroid output (y) as a function of K^+ concentration (K) could be described by the equation $y = 0{\cdot}867(\pm 0{\cdot}064\ \text{S.E.}) + 0{\cdot}013(\pm 0{\cdot}008\ \text{S.E.})$. K which shows no significant correlation of the two parameters. Therefore the effect of the stimulation of the zona

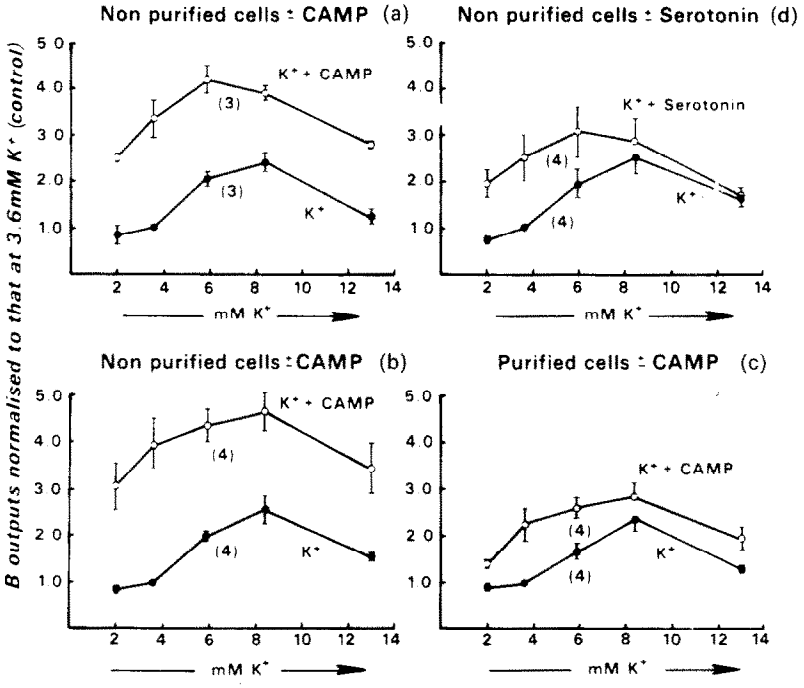

Fig. 7. Corticosterone outputs with, —○— and without, —●—, added 10 mM cAMP (a, c, d) or 10^{-4} M serotonin (b) as a function of K^+ concentration. Outputs (mean ± S.E.) are normalized to those at 3·6 mM K^+ controls without added cAMP or serotonin. Number of experiments are given in brackets. Experiments c and d were carried out on the same cell suspension before (c) and after (d) purification by 1*g* sedimentation.

fasciculata contamination by 10 mM cAMP could be simply to add a small constant steroid output at all K^+ concentrations.

Measured steroid outputs of the unpurified cells at different K^+ concentrations, with or without added cAMP, are shown in Fig. 7a and c. The constant stimulation of the zona fasciculata contamination will simply displace vertically the curves with added cAMP compared with those without cAMP. Nevertheless, the steroid outputs of the capsular cells both with and without added cAMP, vary markedly with K^+ concentration. The curves obtained without cAMP (steroid output vs K^+ concentration) have been remarkably constant over a period of 5 yr with a maximum steroid output at 8·4 mM K^+, Fig. 7a and c. The corresponding curve obtained with added 10 mM cAMP shows a horizontal displacement in all experiments with a tendency for higher steroid outputs at lower K^+ concentrations. In the earlier experiments, this displacement resulted in the position of the maximal steroid output being moved to 5·9 mM K^+, Fig. 7a. However, in later experiments the horizontal displacement of the whole curve is not sufficiently marked to shift the position of the maximum steroid output from 8·4 mM K^+. This is a consistent unexplained variation in the results of experiments conducted in different years and as will be shown occurs for both purified and unpurified cells. It is however not a major effect bearing in mind that the intervals of K^+ concentration used are large. The major consistent observation is that there is a marked dependence of steroid output on K^+ concentration even when a constant maximally effective concentration of cAMP was added to all the incubations.

The experiments were repeated using purified zona glomerulosa cells with essentially the same results as shown in Fig. 7a. However as expected the stimulation of the zona fasciculata contamination and the resultant vertical displacement of the curves obtained with and without added cAMP have been eliminated. There is again a noted dependence of the steroid output of K^+ concentration, with and without added cAMP. The curve with cAMP is displaced horizontally but not enough to result in the shift in the position of the maximum steroid output, Fig. 7d. At the time of these experiments, the unpurified cells also showed no significant shift of the maximum with cAMP added Fig. 7c.

Steroidogenesis with added serotonin and at different K^+ concentrations

Experiments, analogous to those with added cAMP but using maximally effective steroidogenic concentrations of serotonin instead of cAMP, were performed.

If serotonin were a 'pure' internal cAMP generator with no other effects but to increase cAMP production, its use to apply maximally effective amounts of cAMP to the cells would have advantages. It would affect zona glomerulosa cells only and hence unpurified cells could be used. Also the cAMP could be generated internally without the need for non-physiological high concentrations in the medium as occurs with added external cAMP.

Serotonin at a concentration of 10^{-4} M was used to obtain a maximally effective dose at all concentrations: 10^{-8} M being found to be the minimum concentration for this purpose, Fig. 5. In order to test whether at this concentration (10^{-4} M) could be regarded as a pure cAMP generator, the steroid output of capsular cells was measured with 10 mM cAMP added to all incubations of unpurified cells with and without 10^{-4} M serotonin. The R values found, without and with serotonin respectively, were 5·6 and 6·5 (1st experiment) 10·2 and 9·9 (2nd experiment) and 4·8 and 5·2 (3rd experiment). Therefore, there is no significant effect of serotonin additional to that of 10 mM cAMP and hence 10^{-4} M serotonin can be regarded as a pure cAMP generator in this system. It should be emphasized that this has been considered with this high dose of serotonin only.

Experiments were carried out on unpurified capsular cells measuring steroid output with and without 10^{-4} M serotonin and with different K^+ concentrations and the results are shown in Fig. 7b. These results had already been obtained with another aim in mind[11]. They show, as expected, no vertical displacement of the curves with and without added serotonin because of the effect of stimulation of zona fasciculata contamination, e.g. the steroid output at 13 mM K^+ (at which concentration the zona fasciculata output is not depressed) is the same with or without added serotonin. They also show a marked dependence of steroid output on K^+ concentration even with 10^{-4} M serotonin added to all incubations. There is a shift in the position of the maximum steroid output (as with the experiments with added cAMP at that time, Fig. 7a), from 8·4 to 5·9 mM K^+ concentration when serotonin is included.

Theoretical treatment of results

The data on the variation in cAMP output with K^+ concentration (Fig. 5) and the variation of steroid output with K^+ concentration, with and without added internal cAMP (serotonin), Fig. 7a, b, were used for the application of the theoretical treatment outlined in the Appendix and in Fig. 8.

DISCUSSION

As found by application of the method of 1g sedimentation, the major adrenocortical components of dispersed cells from the decapsulated tissue of rat adrenals were cells readily identifiable as zona fasciculata and slower moving cells of small diameter containing characteristic mixed tubular and vesicular cristae in their mitochondria (Fig. 3b). These small cells were identical in appearance with the juxtamedullary cells found in electron microscopic examination of adrenal tissue and usually classified as zona reticularis (Fig. 3a)[12], although the presence of some zona intermedia cells could not be excluded. The functions of the zona reticularis cells, as ascertained by direct incubation studies, is largely uninvestigated.

The purified zona fasciculata cells containing aggregated zona reticularis cells as a minor component showed about the same response to ACTH and angiotensin II as the unpurified cells from decapsulated tissue, Fig. 4. This indicates that previous investigations, such as those on the mode of action of ACTH, which have used decapsulated tissue or even whole adrenals have probably mainly studied effects on zona fasciculata cells. It is unlikely from these results, that the zona reticularis cells comprise a highly active component of unpurified cells, giving an enhanced response to ACTH. This was confirmed by stimulation of the purified zona reticularis cells, which contained about 5% zona fasciculata cells in the particular experiment as determined with ACTH and angiotensin II. The response was significant but much lower than with zona fasciculata cells and could be accounted for by the response of the contaminating zona fasciculata cells in the preparation. This confirms a previous preliminary result. It therefore seems that both the basal production and ACTH or angiotensin II stimulated corticosterone output of the zona reticularis cells must be quite low. The corticosterone output of this preparation also did not respond as much as did zona glomerulosa cells to stimulation by increased K^+ concentration and the slight increase in output observed could be accounted for by the response of the zona glomerulosa contamination. Nevertheless the function of the zona fasciculata cells is well preserved following the same separation procedure and the appearance of the mitochondria of the zona reticularis cell suggests an active steroidogenic function. It remains to be seen if and what steroids are produced by these cells and clearly the possibility of androgen production must be examined.

The purified zona fasciculata cells respond to angiotensin II and the maximal steroidogenic output is of the same order of magnitude for both ACTH and angiotensin II (about one hundred and fifty fold). Similarly the maximal corticosterone output of pure zona glomerulosa cells although must lower (about two fold) is the same for all stimuli investigated including angiotensin II, ACTH, K^+, serotonin and cAMP.

Peytremann *et al.*[13] reported that the steroid output of dispersed cells from the inner zone of calf

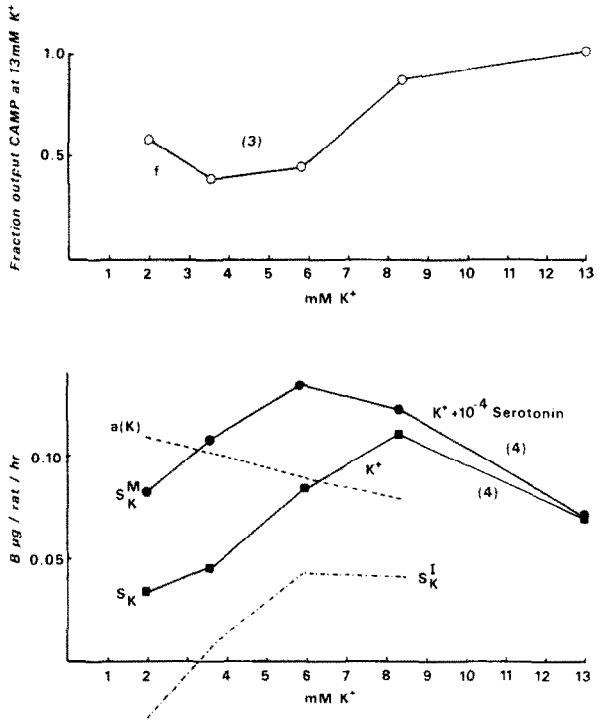

Fig. 8. Relationship between cAMP output, normalized to that at 13 mM K^+, upper Figure (cf. Fig. 5) and corticosterone outputs, lower Figure, without —●—S_K and with addition of 10^{-4} M serotonin (internal cAMP generator) W–■–S_K^M (cf. Fig. 7b) as a function of K^+ concentration. a(K) shows theoretical curve for activity of cAMP at constant cAMP concentration but dependent on K^+ concentration and S_K^I, the theoretical curve for steroid output due to a mechanism independent of the action of cAMP (cf. Particular solutions, section a, in Appendix for equations and assumptions used in calculating the theoretical curves).

adrenals increased in response to angiotensin II. The present study makes it likely that these investigators were examining the response of zona fasciculata cells.

The purified zona fasciculata cells did not respond to increased K^+ concentration and greater concentrations of angiotensin II were required to stimulate steroidogenesis compared with zona glomerulosa cells. This corresponds to the specificity expected from physiological considerations. The doses of ACTH and angiotensin II required were high but as it was not envisaged using the system for assay purposes, no inhibitors of proteases in the incubation medium were used as in the studies of Bennett *et al.*[8]. The sensitivity of the cells to K^+ concentration, cAMP and serotonin is as high as in corresponding *in vivo* experiments[14] or as in other *in vitro* preparations[15, 16].

A lack of correlation between hormone and cAMP production has been observed in several endocrine systems[17–19]. For ACTH stimulation of adrenal cells, it has been explained by statistical considerations [20]. From Fig. 5 and the associated detailed results[5], it can be seen that this type of explanation cannot apply to the data considered here. Other explanations in general postulate a lack of correlation between the measurement of cAMP and the effective steroidogenic concentration within the cells. This could be due to kinetic considerations as the increase in the effective concentration of cAMP could occur at a different time than the consequent rise in hormone production and integrated measurements would not take this into account. It could also be due to gross compartmental effects particularly if the extract of the medium is involved in the assay, e.g. variation in phosphodiesterase activity in the medium. Even more difficult to detect would be fine compartmental effects with translocation of cAMP concentration within the cell. If there were such lack of correlation between the cAMP measurement and the effective concentration within the cell then the hormone production might still be correlated with the effective concentration and cAMP might still play a role as second messenger in these systems.

The experiments previously described on the time

course of the increased cAMP output on stimulation by increased K^+ concentration preclude a kinetic explanation for the lack of correlation in cAMP output and steroidogenesis in changing from 3·6 to 5·9 mM K^+. However, fine compartmental effects cannot be directly investigated with present methods.

To study whether any of these explanations could be the whole solution of the phenomenon, a maximally steroidogenic effective amount of cAMP was added either externally to the medium or internally to the cells by the action of serotonin and the steroid output was then again measured after varying the K^+ concentration. If the explanations previously outlined were to account fully for the lack of correlation between steroid and cAMP output, then the output would have been expected to have been constant. However, with added cAMP, the steroid output varied markedly at different K^+ concentrations. It must be, therefore, that either the activity of cAMP varies with K^+ concentration or that there is a mechanism for controlling steroid output which is entirely independent of cAMP but is responsive to changes in K^+ concentration. In an analogous situation in studies on the blow fly salivary gland changes in calcium flux have been postulated as being involved in such a mechanism[20].

If there were no independent mechanism the curve obtained with added cAMP of steroid output against K^+ concentration (S_K^M in Fig. 8) could represent cAMP activity (i.e. steroid output at constant cAMP concentration) as a function of K^+ concentration. The product of this activity and the cAMP output at any K^+ concentration would give a parameter which could be proportional to steroid output. It is in this manner that the change in the position of the maximal steroid output from 8·4 to 5·9 mM K^+ on adding cAMP to all incubations, could be explained. However, the model and formal treatment outlined in the Addendum indicates that neither variation in activity of cAMP nor the existence of an independent mechanism can be the exclusive explanation of the present data. A complete solution is given in Fig. 8 showing both alterations in activity of cAMP and a significant independent mechanism but this depends, as outlined in the Addendum, on several assumptions being valid, in particular that the activity of cAMP is independent of cAMP concentrations at any particular K^+ concentration. This needs to be established.

It is still possible to postulate a fine compartmental effect to explain the phenomenon. However, it would have to involve a specialized transport mechanism which is limited in capacity for the movement of cAMP to the site of action. This capacity could be altered by changing the external K^+ concentration. This mechanism would be included as an aspect in the change in the activity of cAMP in the present treatment. However it is equally likely that the 2nd messenger theory must be altered from its simple form if it is to be applied to the control of steroidogenesis by zona glomerulosa cells at different K^+ concentrations. At the very least this would require that alterations in the activity rather than the production of cAMP would be the operative parameter.

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APPENDIX

Symbols

Let N ≡ cyclic AMP (output or concentration)
Let K ≡ potassium concentration of medium
Let O ≡ measured output from cells and medium (without additional cAMP)
Let C ≡ concentration within cell
Let S_K ≡ measured total steroid output (without additional cAMP)
Let S_K^I ≡ measured steroid output due to mechanisms independent of the action of cAMP (concentration or activity changes) but possibly dependent on K^+ concentration
Let S_K^D ≡ measured steroid output solely due to the action of cAMP (through changes in concentration and/or activity with either of these effects being dependent on K^+ concentration)
Let N^0 ≡ measured cAMP output (without additional cAMP)
Let N^C ≡ mean cAMP concentration within cell during time interval of effective initiation of action.
Let $e(N, K)$ ≡ cAMP transport factors relating cAMP output to cAMP concentration (gross compartmental and kinetic). These may be dependent on cAMP concentration at constant K^+ $[e(N)]$ or K^+ concentration at constant cAMP concentration $[e(K)]$
Let $a(N, K)$ ≡ activity of cAMP including fine compartmental effects of transport from mean concentration within cell to site of action. The activity may be dependent on cAMP concentration at constant K^+ concentration $[a(N)]$ or on K^+ concentration at constant cAMP concentration $[a(K)]$ N in brackets modifying a or e indicates concentration of cAMP within cell and no superscript is given.

General expressions

$$N^C = e(N, K) . N^0$$

and

$$S_K^D = a(N, K) . N^C$$

then

$$S_K^D = a(N, K) . e(N, K) . N^0$$

$$S_K = S_K^I + S_K^D$$

then

$$S_K = S_K^I + a(N, K) . N^C$$

and

$$S_K = S_K^I + a(N, K) . e(N, K) . N^0$$

At any K concentration—relevant expressions

Assume

$$e(N, K) = e(N) . e(K)$$

and

$$a(N, K) = a(N) . a(K)$$

then

$$S_k = S_K^I + a(N) . a(K) . N^C$$

and

$$S_k = S_K^I + a(N) . a(K) . e(N) . e(K) . N^0$$

If cAMP is added (either internally or externally) to give maximum steroidogenic response then there is a cAMP concentration which is just maximally effective i.e. N_M^C. Let us assume that this maximally effective concentration is the same for all K^+ concentrations (as found experimentally there is a maximum cAMP effect at all K concentrations with the added amount of cAMP used)
then

$$S_K^M = S_K^I + a(N_M) . a(K) . N_M^C$$

At 13 mM *K—relevant expressions*

Without cAMP.

$$S_{13} = S_{13}^I + a(N_{13}) . a(13) . N_{13}^C$$

With additional cAMP to give maximum output

$$S_{13}^M = S_{13}^I + a(N_M) . a(13) . N_M^C$$

Experimentally using internal cAMP generator (serotonin), it has been found that

$$S_{13}^M = S_{13}^I = a(13)[a(N_{13})N_{13}^C - a(N_M)N_M^C]$$

One solution of this equation is

$$N_{13}^C = N_M^C$$

and then

$$N_M^C = e(N_{13}) . e(13) . N_{13}^0$$

Solutions at any K concentration

$$S_K^M = S_K^I + a(N_M) . a(K) . e(N_{13})e(13) . N_{13}^0$$

$$a(K) = \frac{S_K^M - S_K^I}{a(N_M) . e(N_{13}) . e(13) . N_{13}^0}$$

then

$$\begin{aligned} S_K &= S_K^I + a(N) . a(K) . e(N) . e(K) . N^0 \\ &= S_K^I + \frac{a(N)}{a(N_{13})} . \frac{e(N)}{e(N_{13})} . \frac{e(K)}{e(13)} . \frac{N^0}{N_{13}^0} . \left[S_K^M - S_K^I \right] \\ &= S_K^I + r.f.(S_K^M - S_K^I) \end{aligned}$$

where

$$r = \frac{a(N)}{a(N_{13})} . \frac{e(N)}{e(N_{13})} . \frac{e(K)}{e(13)}$$

and

$$f = N^0/N_{13}^0$$

then

$$S_K^I = \frac{(S_K - r.f. S_K^M)}{(1 - r.f.)}$$

and

$$S_K^D = \frac{r.f. (S_K^M - S_K)}{(1 - r.f.)}$$

Particular solutions

(a) If $r = 1$ at all K concentrations i.e. $a . (N) . e(N)$ and $e(K)$ are constant then

$$S_K^I = \frac{S_K - f.S_K^M}{(1 - f)}$$

$$S_K^D = \frac{f(S_K^M - S_K^I)}{(1 - f)}$$

$$a(K) = \frac{(S_K^M - S_K^I)}{a(N_M) . e(N_{13}) . e(13) . N_{13}^0}$$

These are the equations, assumptions and solutions used in Fig. 8.

(b) If $r = 1$ at all K concentration and $S^1 = 0$ i.e. independent cAMP mechanisms are not significant
then

$$S_K = f.S_K^M$$

$$a(K) = \frac{S_K^M}{a(N_M) . e(N_{13}) . e(13) . N_{13}^0}$$

$$S_K^D = S_K$$

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DISCUSSION

Müller:

I admire your separation of pure zona glomerulosa cells but is it possible that by gaining this high cell specificity you are losing some of the steroidogenic enzymes? According to several of your papers, the aldosterone output of the zona glumerulosa cells is about ten times lower than the corticosterone output. In the complete capsular adrenals I use, the aldosterone and corticosterone outputs are almost equal. Perhaps this is due to differences in sex or strain, but is it possible that the proteolytic enzymes you use destroy some of the 18-hydroxylase?

Tait, S. A. S.:

It is certainly possible that loss of enzyme may occur during the preparation of dispersed cells by incubation of adrenal tissue with crude collagenase. However, we have no evidence that 18-hydroxylase is destroyed preferentially. The relative responses in corticosterone and aldosterone outputs to various stimuli are the same for dispersed capsular cells as for capsular tissue being of the order of 2 fold for corticosterone and 8 fold for aldosterone. The basal steroid output of both capsular and decapsulated cells is lowered compared with intact tissue but the output after stimulation does not appear to be affected. This is illustrated by the 150–200-fold increase in corticosterone output by dispersed fasciculata cells after maximal stimulation with ACTH compared with about a 10–15-fold increase using bisected or quartered adrenals.

Korenman:

I have a few comments; one of them is regarding the reduced output of steroids in the dispersed cells. In a number of other cell preparations there is evidence that the peptide hormone receptors declined in number in response to two features of this system. One of them is enzyme treatment of the surface to disperse the cells particularly if the collagenase is trypsin-contaminated and the second is incubation in the absence of proteins. Incubation in aqueous medium seems to allow loss of the cell surface receptors for peptide hormones and therefore it is possible that you may have lost a significant proportion of your cell surface hormone receptors.

Tait, S. A. S.:

It is possible that loss of receptors may occur during the preparation of dispersed cells by enzyme treatment. However, it should be pointed out that the preparation and subsequent incubations of the dispersed cells are carried out in Krebs bicarbonate buffer containing 4% BSA. None of the procedures used are carried out in aqueous media in the total absence of protein.

Korenman:

Another comment has to do with the dissociation between cAMP generation and hormone secretory response which has been noted in a number of systems and has been subjected to elaborate interpretation. There are a couple of observations of ours that may be of relevance. In studying the association reaction between cAMP and the receptor component of the protein kinase molecule in endometrium, we showed that there was a decline in the rate constant of association of cAMP with the purified receptor and with the receptor kinase complex in relation to concentration so that there may be less response to a tremendous increase in cAMP because the rate of association actually falls substantially with saturation of the receptor. Generation of excess cAMP may have relatively little importance in the amount of hormone produced, but may contribute to the duration for which this hormone is produced.

Tait, S. A. S.:

I think that these are very interesting observations and may be relevant to our results showing further examples of the dissociation of cAMP and steroid outputs using submaximal doses of stimulatory agents. However, I do not think they would explain the variation in steroid output with changes in external potassium concentration observed in incubations of zona glomerulosa cells with large amounts of cAMP added.