Capillaries of the Adrenal Cortex Possess Aminopeptidase A and Angiotensin-Converting-Enzyme Activities

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The enzymes required to convert the prohormone angiotensin I into angiotensins II and III, secretagogues of aldosterone, are enriched in association with capillary endothelium isolated from rat adrenal cortex. Thus the secretion of aldosterone may be controlled, in part, by processing of peptides occurring within the adrenal gland itself.

Published data suggest that the enzymes [angiotensin-converting enzyme, peptidyl dipeptidase (EC 3.4.15.1) and aminopeptidase A (EC 3.4.11.7)] necessary for the conversion of angiotensin I into des-Asp¹-angiotensin I, angiotensin II and angiotensin III (des-Asp¹-angiotensin II) occur within the adrenal gland. Hence, if the relevant enzymes are active and have access to angiotensin I *in vivo*, intraglandular formation of angiotensins II and III can be accomplished. Caldwell *et al.* (1976), using immunofluorescence techniques, have shown that adrenal-gland capillary endothelium and/or cells in juxtaposition to endothelium are reactive with monospecific antibody to rabbit lung angiotensin-converting enzyme. Similarly Campbell & Pettinger (1976) have shown that homogenates of rat adrenal glands contain aminopeptidase A-like activity. However, it is unclear whether the angiotensin-converting enzyme is active (Cushman & Cheung, 1971; Ackerly *et al.*, 1976, 1977), and there are conflicting data on the likely cellular disposition of the aminopeptidase A-like enzyme (Goodfriend & Peach, 1975; Ackerly *et al.*, 1977; Moore *et al.*, 1977). The present study attempted to define the activities and cellular disposition of angiotensin-converting enzyme and aminopeptidase A within the adrenal cortex. Indeed, both angiotensin-converting enzyme and aminopeptidase A were found to occur at high specific activities in association with the capillary endothelium of the adrenal cortex.

Materials and Methods

Materials

Bovine serum albumin, foetal bovine serum and medium 199 were from GIBCO, Grand Island, NY,

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U.S.A.; type 1 collagenase was from Worthington Biochemical Co., Freehold, NJ, U.S.A.; 35 mm-diam. × 10 mm tissue-culture dishes were from Lux Scientific Co., Newbury Park, CA, U.S.A.; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Calbiochem, La Jolla, CA, U.S.A. Bradykinin-potentiating peptide 9a (peptide BPP_{9a}) was a gift from Dr. M. Ondetti, Squibb Institute for Medical Research, Princeton, NJ, U.S.A. [³H]Benzoyl-Phe-Ala-Pro (25 Ci/mmol; ³H in the *para*-position of the phenyl ring of the benzoyl moiety) and α -Asp-[³H]benzylamide (24 Ci/mmol; ³H in the *meta*-position of the phenyl ring of the benzylamide) were prepared from their respective precursors, 4-iodobenzoyl-Phe-Ala-Pro and α -Asp-3-iodobenzylamide, by catalytic dehalogenation in ³H₂ gas as described previously (J. W. Ryan *et al.*, 1978). Labelling was performed by the Amersham Corp., Arlington Heights, IL, U.S.A. All amino acids were in their L-forms.

Isolation of adrenal-gland capillary segments and preparation of adrenal-gland suspensions

The adrenal-gland capillary segments were prepared by a modification of a method previously reported (Del Vecchio *et al.*, 1977). Adrenal glands from 24 female rats were dissected free of fat, and the capsules were removed by stripping with forceps. The procedure also removes a large proportion of the cells of the zona glomerulosa. The decapsulated adrenal glands were cut from superior to inferior poles into cross-sectional slices approx. 1 mm thick. Medullary tissue was removed by dissection with a scalpel blade while being viewed through a dissecting microscope. The resulting slices of cortex were kept for 1 h at room temperature (approx. 23°C) in 10 ml of a solution composed of bovine serum albumin (0.5%) and collagenase (0.75%) in medium 199, and the tissue was disrupted by

repeated pipetting. The cell dispersion was passed through a single layer of nylon bolting cloth (160 μ m openings; Tetko, Elmsford, NY, U.S.A.). The filtrate was diluted to 50 ml with medium 199, and a pellet was collected by centrifugation at 500 g for 10 min. The pellet was washed three times with 10 ml of medium 199 containing 20% (v/v) foetal bovine serum (complete medium). The pellet was resuspended in the complete medium (8 ml), and 2 ml of the cell suspension was incubated in each of four 35 mm-diam. $\times$ 10 mm tissue-culture dishes at 37°C in an atmosphere of CO₂/air (1:19) for 1 h. Capillary segments became adherent to the culture dishes within 1 h, and the cell debris and other cell types were removed with a gentle spray of complete medium from a 5 ml syringe equipped with an 18-gauge needle. The cells that remain on the culture dish are illustrated in Plate 1. The medium was changed to medium 199 previously conditioned by adrenal-gland parenchymal cells in culture and containing foetal bovine serum that had been heat-inactivated at 61°C for $\frac{1}{2}$ h. The heating step completely inactivates the angiotensin-converting-enzyme and aminopeptidase A-like activities of foetal bovine serum. The isolate was maintained in the latter medium for approx. 24 h, during which time the segments flattened to form a monolayer. Formed blood elements and plasma retained in the original isolate of capillary segments were released into the medium. The isolated endothelial cells, like adrenal-gland endothelium *in situ*, have caveolae and fenestrae.

On the day after isolation, the cells were assayed for angiotensin-converting enzyme and aminopeptidase A activities. The cells were washed in monolayer eight to 12 times with approx. 7 ml of Puck's saline without Ca²⁺ and Phenol Red (KCl, 0.40 g/l; NaCl, 8.00 g/l; NaHCO₃, 0.35 g/l; D-glucose, 1.00 g/l). The cells were suspended in 300 μ l of the Puck's saline by scraping with a rubber 'policeman' and were put into a uniform suspension by using a 1 ml tuberculin syringe equipped with a 30-gauge needle. Samples (50 μ l) of the cell suspension were assayed for protein (Lowry *et al.*, 1951), with bovine serum albumin as standard, and samples for the radioassays (200 μ l) were diluted with 800 μ l of 0.05 M-Hepes buffer, pH 8.0, containing 0.15 M-NaCl and 1% (v/v) Triton X-100. In addition, the buffer for the assay of aminopeptidase A activity contained 4 mM-CaCl₂ (Glenner *et al.*, 1962; Nagatsu *et al.*, 1965).

Dispersions of adrenal cortex were prepared as follows. The adrenal-cortical slices, obtained as described above, were chopped with a scalpel to yield a thick paste. The finely chopped material (from two glands) was suspended in 0.5 ml of the Puck's saline. The crude suspension was converted into a uniform dispersion by using a 1 ml tuberculin

syringe fitted with an 18-gauge needle. The suspension was passed successively through 22-gauge, 25-gauge and 30-gauge needles. The resulting dispersion was diluted to 10 ml with Puck's saline. The samples used for the assays of angiotensin-converting-enzyme and aminopeptidase A activities and proteins were treated exactly like those prepared from adrenal-gland endothelial cells.

Enzyme assays

Angiotensin-converting enzyme activity was measured by using a radioassay method developed in this laboratory (U. S. Ryan *et al.*, 1978; J. W. Ryan *et al.*, 1978) with [³H]benzoyl-Phe-Ala-Pro as substrate. Specificity of the reaction was assessed by adding the specific angiotensin-converting-enzyme inhibitor bradykinin-potentiating peptide 9a (peptide BPP_{9a}; cf. Bakhle, 1974). The substrate was added to a total of 10 μ Ci/ml (400 nM). The K_m of the reaction of [³H]benzoyl-Phe-Ala-Pro with angiotensin-converting enzyme of rat serum and lung homogenate ranged from 9 to 15 μ M as assessed by the method of Lineweaver & Burk (1934). At each time period tested, three 10 μ l samples were taken from each reaction mixture. The first was added directly to 10 ml of Hydromix (Yorktown Research, Hackensack, NJ, U.S.A.) and its radioactivity was counted as a measure of the total substrate present. Each of the second two 10 μ l samples was added to 1.0 ml of 0.1 M-HCl to inactivate the enzyme; and then the reaction product, [³H]benzoyl-Phe, was separated from unhydrolysed substrate by extraction with 1.0 ml of toluene. A 500 μ l sample of the toluene phase was added to 10 ml of Hydromix for liquid-scintillation counting of radioactivity.

The isolated endothelial cells and dispersions of adrenal cortex were assayed for aminopeptidase A activity with Asp-[³H]benzylamide (2 μ Ci/ml; 83 nM) as substrate. The K_m of the reaction Asp-[³H]-benzylamide with the aminopeptidase A of rat serum, lung homogenate and kidney homogenate ranged from 1.2 to 2.0 mM. The incubation was performed at 37°C and the buffer was identical with that used for angiotensin-converting enzyme with the addition of 4 mM-CaCl₂. At the appropriate time intervals, three 50 μ l samples were taken. The first was added to 10 ml of Hydromix and its radioactivity was counted as a measure of total substrate present. Each of the next two was added to 1.0 ml of 0.1 M-NaOH to stop the enzymic reaction. The reaction product, [³H]benzylamine, was separated from the unhydrolysed substrate by extraction with 1.0 ml of ethyl acetate. A 500 μ l sample of each ethyl acetate phase was added to 10 ml of Hydromix for liquid-scintillation counting of radioactivity.

Blanks for each assay (substrate incubated without enzyme) were prepared by making the

EXPLANATION OF PLATE 1

Low-magnification electron micrograph of a pellet from a freshly isolated preparation of adrenal capillary segments
 Notice the patent lumens and trapped blood cells in some of the segments. By light-microscopy the segments have an appearance of short tubes of one to four cells in length. The cytoplasm of the capillary segments has a lacy appearance. The insert in the lower right-hand corner is a higher magnification of the area enclosed by the square in the original micrograph. It reveals that the cytoplasm contains fenestrae (arrows), structures characteristic of adrenal endothelium *in situ*. After a short time in culture (about 18 h), the cells flatten and release any formed elements and plasma trapped within the lumen of the freshly isolated segments. The fenestrae are retained.

appropriate dilutions of the Puck's saline in assay buffer containing substrate. The blanks were incubated as were the reaction mixtures containing enzyme, and samples were extracted at timed intervals to assess the partitioning of substrate into toluene (for [^3H]benzoyl-Phe-Ala-Pro) or ethyl acetate (for Asp-[^3H]benzylamide).

In each assay, the amount of substrate used was well within the range of first-order enzyme kinetics. Thus enzyme activities were expressed in terms of percentage utilization/h per 100 μg of protein (e.g. see Ryan & McKenzie, 1968; J. W. Ryan *et al.*, 1968, 1978). Percentage substrate utilization was computed by the formula:

% utilization =

$$2 \times \frac{\text{test c.p.m.} - \text{blank c.p.m.}}{\text{substrate c.p.m.}} \times 100$$

The factor of 2 is to correct the radioactivity (c.p.m.) of 1.0 ml of the toluene or ethyl acetate phases.

Electron microscopy

Immediately after the isolation procedures, the segments were scraped from the culture dishes into a small volume of Puck's saline with a rubber 'policeman'. The segments were centrifuged at 800 g for 10 min, and the resulting pellet was resuspended and fixed overnight [2.5% (v/v) glutaraldehyde and 6% (w/v) sucrose in 0.05 M-sodium cacodylate buffer, pH 7.4] and prepared for electron microscopy by routine procedures (Ryan *et al.*, 1976). Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined in a Philips 301 electron microscope.

Results and Discussion

The amount of angiotensin-converting-enzyme activity in the endothelial cells (measured 60 min after beginning the incubation) was found to be approx. 6 times that of dispersions of adrenal cortex (in % of substrate converted/h per 100 μg of protein: 13.3 versus 2.2, Table 1). At a final concentration of 10 μM , peptide BPP_{9a} (added 3 min before addition of substrate) inhibited the reaction of endothelial cells with substrate by 98%, and peptide BPP_{9a} at 1.0 μM inhibited by 92% (inhibition assessed in terms of the decrease of percentage substrate utilization). The inhibitory effects of peptide BPP_{9a} persisted in incubations of up to 6 h. As shown in Table 1, the aminopeptidase A activity of the adrenal-gland endothelium (measured 60 min after beginning the incubation) exceeded that of adrenal homogenates by 11-fold (in % of substrate converted/h per 100 μg of protein: 5.5 versus 0.5). The properties of the enzyme reaction resemble those reported for aminopeptidase A (Glenner *et al.*, 1962; Nagatsu *et al.*, 1965). The reaction in assay buffer containing 4 mM-CaCl₂ proceeded at a rate 6 times that in buffer without added CaCl₂. EDTA (10 mM) lowered the reaction rate to 4% of that of the control. In either case, CaCl₂ or EDTA (disodium salt) was added to the Hepes buffer containing 1% Triton X-100, and the pH was adjusted to 8.0 if necessary. The cells were exposed to CaCl₂ or EDTA at room temperature for approx. 15 min before addition of Asp-[^3H]benzylamide.

The adrenal glands are major target organs in the overall functioning of the renin-angiotensin system. In addition to their vasoconstrictor effects,

Table 1. Association of angiotensin-converting enzyme and aminopeptidase A with capillary endothelial cells of the adrenal cortex

Assay procedures are described in the text. In each assay, the starting substrate concentration was well within the range of first-order enzyme kinetics, and the results are expressed in terms of percentage substrate utilization/100 μg of protein $\pm$ S.E.M. The substrate for angiotensin-converting enzyme, [^3H]benzoyl-Phe-Ala-Pro, was used at 400 nM, and an initial reaction rate of 10%/h per 100 μg of protein is equivalent to an absolute rate of 40 nM/h per 100 μg of protein $\pm$ S.E.M. The substrate for angiotensin-converting enzyme, [^3H]benzoyl-Phe-Ala-Pro, was used at reaction rate of 10%/h per 100 μg of protein is equivalent to an absolute rate of 8.3 nM/h per 100 μg of protein.

Time of incubation (min)	Angiotensin-converting-enzyme activity (% substrate utilization/100 μg of protein)		Aminopeptidase A activity (% substrate utilization/100 μg of protein)	
	Endothelial cells ($n = 5$)	Dispersed cortical cells ($n = 3$)	Endothelial cells ($n = 2$)	Dispersed cortical cells ($n = 2$)
0	0	0	0	0
15	4.20 $\pm$ 0.68	0.76 $\pm$ 0.37	1.25 $\pm$ 0	0*
30	7.00 $\pm$ 1.51	0.85 $\pm$ 0.22	2.40 $\pm$ 0	0*
60	13.30 $\pm$ 1.83	2.17 $\pm$ 1.20	5.50 $\pm$ 0.50	0.50 $\pm$ 0.10
120	22.80 $\pm$ 3.39	3.50 $\pm$ 1.04	10.50 $\pm$ 0.50	0.75 $\pm$ 0.25
180			15.0 $\pm$ 0.50	1.10 $\pm$ 0.10

* Percentage substrate utilization less than 0.1%.

angiotensins II and III are capable of increasing the rate of release of aldosterone (Freeman *et al.*, 1977). Furthermore, current understanding of the mechanism of the antihypertensive action of the experimental drug Captopril (compound SQ 14225, an inhibitor of angiotensin-converting enzyme) suggests that the ability of the drug to suppress aldosterone secretion may contribute to the longer-term blood-pressure response (Atlas *et al.*, 1978). The ability of the adrenal gland to process locally angiotensins I and II may well be important in determining the actual role of these peptides in the functioning of the adrenal cortex. Local conversion, binding and utilization of angiotensins II and III by the adrenal gland could mean that the angiotensins may be available within the gland in amounts far greater than would be expected from their concentrations in the systemic arterial circulation. It remains to be determined whether capillaries of other organs and glands, thought to be target tissues for angiotensins II and III, possess both angiotensin-converting enzyme and aminopeptidase A.

Our results demonstrate that the enzymes necessary for the conversion of angiotensin I into des-Asp¹-angiotensin I and angiotensins II and III are located prominently in association with capillary endothelium of the adrenal cortex. The enzymes may be located on the surface of the cells (as is the case for angiotensin-converting enzyme in pulmonary endothelium) (J. W. Ryan *et al.*, 1975; U. S. Ryan *et al.*, 1976) and may be able to act on the peptides within the vascular space as they pass through the adrenal-gland vascular bed. Alternatively, enzymes may be located intracellularly or on fenestrae and act on the peptides as they pass through the endothelium to the adrenal parenchymal cells.

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