

# Aldosterone secretion by the rat adrenal cortex is stimulated by the activation of protease-activated receptor 1

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## Abstract

Stimulation of aldosterone by a serine protease, trypsin, was first reported in 1982, although the mechanism of this effect was unclear. Recently, a family of protease-activated receptors (PARs) has been described and four members of the family characterised and cloned, including the previously recognised thrombin receptor. This study investigated whether PARs mediate the action of trypsin on aldosterone secretion. Using intact rat adrenal capsular tissue, thrombin was found to increase aldosterone secretion, and the effects of trypsin on aldosterone secretion were confirmed. Both trypsin and thrombin were shown to activate phospholipase C, as measured by an increase in inositol triphosphate turnover by adrenal capsular tissue. It

was also shown that U73122, a phospholipase C inhibitor, attenuated the aldosterone response to trypsin. These effects were consistent with the activation of a PAR. Northern blot analysis revealed the presence of mRNA encoding PAR-1, but not PARs-2, -3 or -4 in the adrenal capsule/zona glomerulosa. Messenger RNA encoding PAR-1 was increased by dietary sodium depletion, consistent with previous reports of an increased response to trypsin after sodium depletion. These data suggest that the actions of trypsin on aldosterone secretion are mediated by PAR-1.

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## Introduction

The development of enzymatically dispersed cell suspensions in 1968 was a major step in the study of adrenocortical endocrinology, allowing steroidogenic responses to low concentrations of ACTH to be seen for the first time (Kloppenborg *et al.* 1968). However, in general these cell preparations did not respond well to angiotensin II, and it appeared that an element of the calcium signalling system was disrupted during the process of cell dispersal (Vinson *et al.* 1989). In the early days of work with adrenal cell suspensions, the dispersal methodology used either trypsin (Swallow & Sayers 1969) or collagenase (Kloppenborg *et al.* 1968) as the digestive enzyme, but this was later replaced exclusively with collagenase, and adaptations of the method enabled the detection of considerably more sensitive responses to angiotensin II (Enyedi *et al.* 1981).

Studies from this laboratory demonstrated that proteolytic enzymes, including trypsin, were able to stimulate aldosterone secretion directly from intact rat adrenal capsular tissue (Raven *et al.* 1982, 1983a,b). This discovery

challenged many assumptions that had previously been made about methods of tissue preparation, and the interpretation of physiologically relevant results (for review see Vinson *et al.* 1985). However, the mechanism of the effect of trypsin was unclear: it was believed either to break down possible steroid–protein complexes sequestered in the cell membrane, or to cause the transmembrane activation of steroidogenesis by a novel mechanism (Raven *et al.* 1982, Vinson *et al.* 1985). More recent studies revealed that trypsin activated protein kinase C in the adrenal zona glomerulosa (Vinson *et al.* 1990), although the possibility that trypsin may directly activate a phospholipase C-linked receptor was not considered a likely explanation of the effects seen.

Recently, a new family of G-protein coupled receptors has been described, termed protease-activated receptors (PARs), which include members of the thrombin receptor family (for review see Kawabata & Kuroda 2000). To date, four members of this receptor family have been described, and the genes encoding them cloned. These receptors are activated by trypsin and by thrombin (Kawabata & Kuroda

2000). In the light of the previous findings it was deemed worthwhile to determine whether activation of a PAR may explain the effects of trypsin on aldosterone secretion.

In order to establish that trypsin stimulation of aldosterone secretion is mediated by a member of this receptor family, it is necessary to establish that: a) the adrenal cortex expresses the gene encoding the receptor, b) thrombin mimics the effects of trypsin, and c) the effect is associated with an increase in inositol triphosphate (IP<sub>3</sub>), as all members of the PAR family are G<sub>q</sub> linked. In addition, as the actions of trypsin are greatly enhanced by dietary sodium restriction (McAuley *et al.* 1985b), this procedure should also be shown to increase receptor expression. The present study was designed to address these questions and determine whether a member of the PAR family mediates the actions of trypsin on the rat adrenal zona glomerulosa.

## Materials and Methods

All chemicals were of analytical grade and obtained from Sigma Aldrich or Merck-BDH, Poole, Dorset, UK, with the following exceptions. All radiolabels were obtained from Amersham International plc, Amersham, Bucks, UK. AG1-X8 resin anion-exchange columns were obtained from Bio-Rad Laboratories, Hemel Hempstead, Herts, UK. ACTH(1–24) (Synacthen) was from Ciba-Geigy, Horsham, W Sussex, UK. The phospholipase C inhibitor, U73122, was from Calbiochem, Nottingham, Notts, UK.

Male and female Wistar rats (250–400 g body weight) were obtained either from A Tuck and Sons, Battlesbridge, Essex, UK or from the colony maintained at Queen Mary and Westfield College. Animals were maintained on normal rat chow, except for the dietary sodium restriction experiments, for which groups of animals were maintained for 21 days on a diet with fixed low (4 mmol/kg) or normal (174 mmol/kg) sodium concentrations (Hinson & Kapas 1995).

The rats were stunned and then killed by cervical dislocation, in accordance with Home Office regulations. Adrenals were rapidly removed and cleaned of adhering fat. Capsule fractions (with zona glomerulosa cells attached) were separated from inner adrenocortical tissue by pressure between glass plates. Capsules were preincubated in Krebs Ringer bicarbonate containing glucose (2 mg/ml) (KRBG) for 1 h at 37 °C under an atmosphere of 95%O<sub>2</sub>–5%CO<sub>2</sub>. After preincubation, capsules were incubated in fresh KRBG for 1 h under an atmosphere of 95%O<sub>2</sub>–5%CO<sub>2</sub> in the absence or presence of stimulants or inhibitors. The maximal concentrations of protease enzyme used were chosen to approximate those used in previous studies (1 mg/ml; Raven *et al.* 1982).

After incubation the capsules were discarded and the incubation media were placed into clean glass test tubes and heated at 100 °C for 20 min to destroy the proteolytic activity of the enzymes used. Aliquots of the media were

cooled and transferred to microfuge tubes, which were stored at –20 °C until the media were assayed for aldosterone. Aldosterone was measured in an aliquot of unextracted incubation medium by direct radioimmunoassay (Kapas *et al.* 1992).

Adenylyl cyclase activity was measured by competitive radioligand binding, using a binding protein prepared from bovine adrenal glands, as described previously (Kapas *et al.* 1994). IP<sub>3</sub> turnover was assessed using an adaptation of existing methods (Kapas *et al.* 1994), as follows. Briefly, rat adrenal capsules were obtained as described above. After 1 h of preincubation, the capsules were incubated in 3 ml KRBG with 10 µCi myo-[2-<sup>3</sup>H]inositol for 4 h. After incubation, capsules were washed twice with KRBG containing 10 mmol/l inositol and 10 mmol/l LiCl. Capsules were then individually placed in tubes containing 400 µl KRBG and preincubated for 10 min, after which time the stimulants were added in 100 µl and the content of the tubes mixed rapidly. After 3 min the reaction was terminated by the addition of ice-cold perchloric acid (15% v/v), the capsular tissue was removed, and the supernatant neutralised by the addition of 1.5 ml Freon: trioctylamine (1:1 v/v). The [<sup>3</sup>H]IP<sub>3</sub> was separated by anion-exchange chromatography, following established methods (Downes & Michell 1981, Enyedi *et al.* 1985).

## mRNA analysis

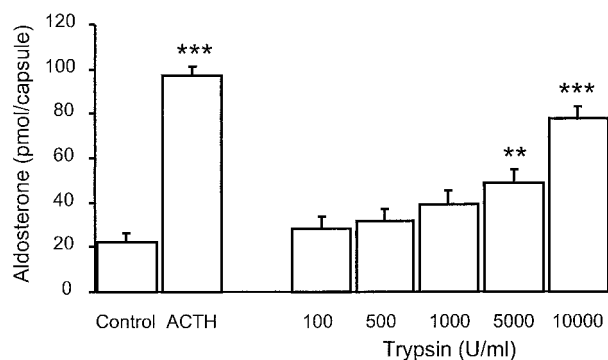
Total tissue RNA was isolated by acid guanidium thiocyanate–phenol–chloroform extraction using RNazol solution (Biogenesis, Poole, Dorset, UK) following the manufacturer's instructions. The purity and concentration of RNA were measured spectrophotometrically at 260/280 nm. Five micrograms of total RNA were primed with 1.5 µg oligo-d(T)<sub>12–18</sub> and reverse transcribed by avian myeloblastosis reverse transcriptase using the First-Strand cDNA synthesis kit (Life Technologies, Paisley, Renfrewshire, UK). PCR was carried out as described previously (Martinez *et al.* 2000). For mRNA analysis, 10 µg total RNA was electrophoresed in a formaldehyde–1% agarose gel and transferred to Hybond-N nylon membrane (Amersham). After fixation by u.v. crosslinking, the membrane was hybridised overnight at 42 °C with [ $\alpha$ -<sup>32</sup>P]-dCTP-labelled full-length probes for PARs-1 to -4.

## Statistical analysis

Arithmetic means and s.e.m. values were calculated. One-way ANOVA was used to test whether trypsin or thrombin had a significant effect on basal (control) levels of aldosterone or IP<sub>3</sub> release.

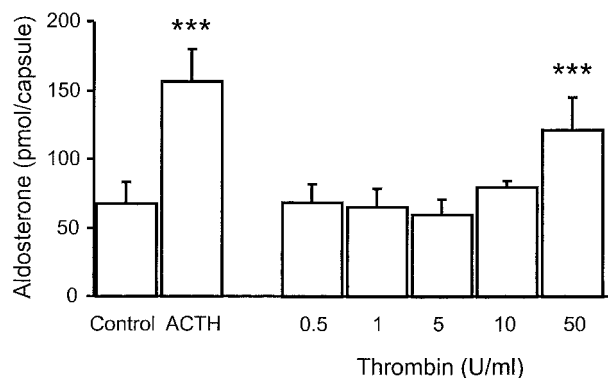
## Results

Both trypsin and thrombin were found to stimulate aldosterone secretion by rat adrenal capsular tissue (Figs 1

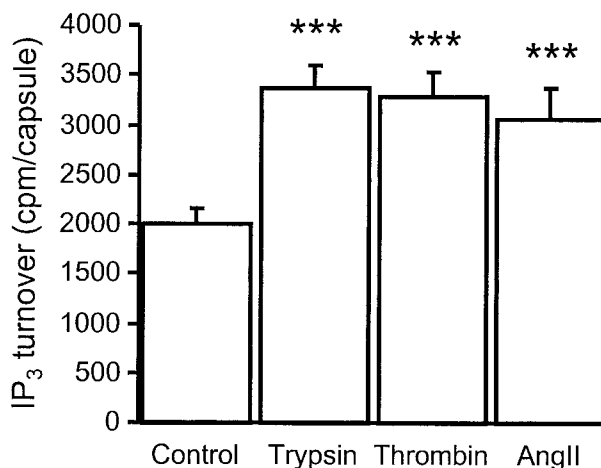


**Figure 1** Effects of increasing concentrations of trypsin on release of aldosterone from intact adrenal capsular tissue (containing glomerulosa cells). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with control (ANOVA). ACTH (1 nmol/l) was included for comparison.

and 2). In the case of trypsin, a dose-related effect was seen, with a threshold of 5000 IU/ml and a maximal effect seen at 10 000 IU/ml. With thrombin a significant effect was only seen with the greatest concentration used, 50 IU/ml. Neither thrombin nor trypsin had any effect on release of cAMP by rat adrenal capsular tissue (data not shown). Both thrombin and trypsin stimulated  $IP_3$  turnover, to levels comparable to those seen with angiotensin II stimulation (Fig. 3). The phospholipase C inhibitor attenuated the aldosterone response to trypsin (Fig. 4), but did not affect basal secretion. Northern blot analysis of rat capsular/zona glomerulosa mRNA revealed the presence of mRNA encoding PAR-1, but not PARs-2, -3 or -4 (Fig. 5). Analysis of rat adrenal mRNA obtained from animals maintained on a low-, normal- or high-sodium diet revealed that the expression of PAR-1 was greatly increased in response to the low-sodium diet, and diminished in the group fed a high-sodium diet (Fig. 6).



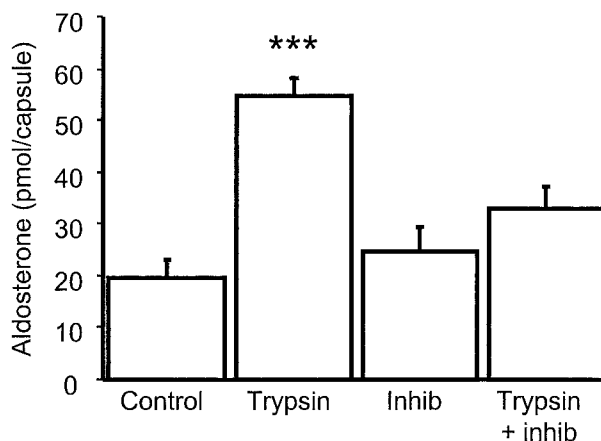
**Figure 2** Effects of increasing concentrations of thrombin on release of aldosterone from intact adrenal capsular tissue (containing glomerulosa cells). \*\*\* $P < 0.001$  compared with control (ANOVA). ACTH (1 nmol/l) was included for comparison.



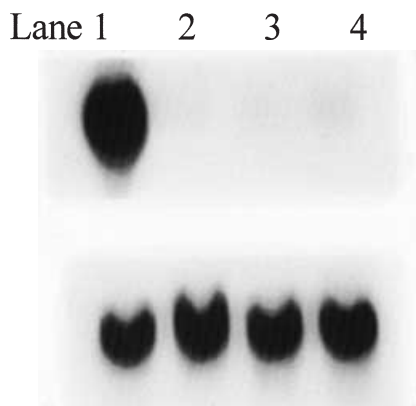
**Figure 3** Effects of trypsin (10 000 IU/l), thrombin (50 IU/l) and angiotensin II (AngII) (100 nmol/l) on production of [ $^3H$ ]IP $_3$  by [ $^3H$ ]inositol-loaded adrenal capsular tissue. \*\*\* $P < 0.001$  compared with control (ANOVA).

## Discussion

This study demonstrates for the first time the expression of PAR-1 in the rat adrenal zona glomerulosa, and provides data that suggest that both thrombin and trypsin may activate this receptor and thereby stimulate aldosterone secretion. Previous studies in the early 1980s described the stimulatory effect of trypsin on aldosterone secretion, although no attempt was made to determine whether the effects were dose dependent. The present study confirms these findings and provides limited evidence for a dose dependency of the effect. A clear dose dependency may not be expected, however, as the activation of PARs by trypsin and thrombin is quite distinct from that of other G-protein coupled receptors, and is dependent upon their



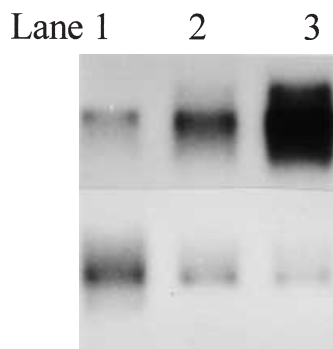
**Figure 4** Effects of the phospholipase C inhibitor, U73122 (10  $\mu$ mol/l), on basal and trypsin-stimulated release of aldosterone. \*\*\* $P < 0.001$  compared with control (ANOVA). In the presence of inhibitor, trypsin did not significantly increase aldosterone secretion.



**Figure 5** Northern blot analysis of rat adrenal zona glomerulosa mRNA reveals the presence of mRNA encoding PAR-1 (lane 1, top), but not PARs-2, -3 or -4 (lanes 2–4, top). The bottom lanes show GAPDH for comparison.

proteolytic action on the extracellular domain of the receptor (Kawabata & Kuroda 2000), which may not be subject to the same kinetics as simple ligand–receptor interactions. In addition, the findings of the present study suggest that thrombin is also able to stimulate the release of aldosterone from rat adrenal capsular tissue. The finding of mRNA for PAR-1 in the zona glomerulosa is consistent with the possibility that PAR-1 mediates the actions of proteolytic agents on aldosterone secretion. This member of the PAR family of receptors is activated by both trypsin and thrombin (Altrogge & Monard 2000) – an effect consistent with the present finding that both these enzymes stimulated the release of aldosterone. This is supported by the observation that  $IP_3$  turnover was enhanced by both trypsin and thrombin, as PAR-1 is known to be linked to  $G_q$  activation and polyphosphoinositide turnover (Dery *et al.* 1998).

There are, at present, no specific antagonists available for the rat PAR-1 receptor. It is not possible to use a



**Figure 6** Northern blot analysis of mRNA to show the effects of dietary sodium restriction on the expression of PAR-1 by rat adrenal zona glomerulosa (top lanes). Lane 1: High-sodium diet; lane 2: normal diet; lane 3: low-sodium diet. Bottom lanes show GAPDH for comparison.

trypsin inhibitor to check the specificity of the action of trypsin as these agents themselves have direct actions on adrenocortical steroid secretion (Pedersen & Brownie 1983, McAuley *et al.* 1985a).

These studies also confirm a role for the calcium/polyphosphoinositide signalling system in the adrenal response to trypsin and thrombin. Previous studies demonstrated a translocation of protein kinase C from cytosol to membrane fraction in the adrenal zona glomerulosa in response to trypsin, suggesting that trypsin may activate this intracellular pathway (Vinson *et al.* 1990). At that time it was not clear how trypsin achieved this effect, although it was postulated that trypsin may somehow directly activate phospholipase C (Vinson *et al.* 1990). The present findings confirm that trypsin increases  $IP_3$  turnover in the adrenal and, furthermore, present evidence that this effect may be mediated by the activation of a trypsin-sensitive receptor, as these receptors are  $G_q$  linked (Dery *et al.* 1998). This suggestion is further supported by the finding that a phospholipase C inhibitor attenuated the aldosterone response to trypsin.

The finding of increased PAR-1 expression in response to dietary sodium restriction is consistent with a role for this receptor in the regulation of aldosterone secretion, and particularly the response of the adrenal to changes in sodium balance. Previous studies have shown that the aldosterone response to trypsin is greatly enhanced by prior sodium depletion (McAuley *et al.* 1985b). The results of the present study suggest that this effect may be mediated by increased expression of PAR-1 in response to dietary sodium depletion.

The question of the physiological ligand for this receptor remains unclear, although several studies have demonstrated the production of both a trypsin-like protease (Evangelista *et al.* 1982, Lindberg *et al.* 1982) and other proteases (Roberts *et al.* 1992) by the adrenal gland. More recently, findings by Lowry's group have implicated a novel adrenal protease in the control of compensatory adrenal growth (Bicknell *et al.* 1998). It remains to be seen whether any endogenous adrenal protease can activate PAR-1 in the zona glomerulosa and thereby stimulate aldosterone secretion.

In conclusion, these data demonstrate that: a) mRNA encoding PAR-1 is present in the rat adrenal zona glomerulosa, suggesting that the PAR-1 receptor is present in this tissue; b) thrombin, like trypsin, is able to stimulate aldosterone secretion; c) the effects of both trypsin and thrombin are associated with an increase in  $IP_3$  turnover; d) dietary sodium restriction increases PAR-1 expression. Taken together, these findings make it clear that PAR-1 is likely to mediate the effects of trypsin on the rat adrenal cortex.

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