Inhibition of endothelin- and phorbol ester-stimulated tyrosine kinase activity by corticotrophin in the rat adrenal zona glomerulosa

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1. The experiments described in this study were carried out to investigate the role of tyrosine kinase in the acute adrenal response to peptide hormone stimulation, and to determine whether the activity of this kinase may be subject to regulation by other intracellular signalling mechanisms in the adrenal zona glomerulosa. 2. Previous studies from this laboratory have shown that angiotensin II stimulates tyrosine kinase activity in the rat adrenal cortex. This study has shown, for the first time, that endothelin-1 also stimulates tyrosine kinase activity in this tissue. 3. Using the specific inhibitor of protein kinase C (PKC) activity, Ro 31-8220, we have shown that stimulation of tyrosine kinase

activity, in response to endothelin-1, angiotensin II or the phorbol ester phorbol 12-myristate 13-acetate, is at least partly dependent on increased PKC activity. 4. The data presented also provide further evidence of cross-talk between signalling systems in the adrenal cortex. Corticotrophin and its intracellular second messenger, cyclic AMP, significantly attenuate the increment in tyrosine kinase activity seen in response to each of the effectors used. 5. The results of this study provide important new evidence for the regulation of protein kinases by other intracellular second messenger systems.

INTRODUCTION

Endothelin-1 (ET), a 21-amino-acid peptide with two disulphide bonds, is known chiefly as a hormone with vasoconstrictor activity secreted from endothelial cells. ET has also been shown to stimulate a number of other responses, including inotropic responses in atria [1], release of atrial natriuretic peptide from cardiac myocytes [2] and mitogenesis in a number of different cell types [3,4]. However, ET can also stimulate steroidogenesis in adrenocortical cells, a property it shares with another vasoconstrictor, angiotensin II (AngII) [5–7].

AngII and corticotrophin (ACTH), the two major stimuli of aldosterone secretion, act via distinct signal-transduction pathways. ACTH stimulates cyclic AMP production by binding to a receptor which is coupled to a stimulatory guanine nucleotide-binding (G-protein) regulator of adenylate cyclase (G_s) [8,9]. AngII inhibits adenylate cyclase and stimulates phospholipase C and protein kinase C (PKC) by binding to the AngII AT_1 receptor which is coupled to both an inhibitory G-protein regulating adenylate cyclase (G_i) and a stimulatory G-protein regulating phospholipase C (G_q) [10–12]. Studies have shown that ET mediates its steroidogenic effects in a similar manner to AngII [7]. However, these hormones also appear to exert growth regulatory effects [13–16]. Cyclic AMP, the second messenger of ACTH, is known to inhibit adrenocortical cell proliferation *in vitro*, whereas AngII stimulates the proliferation of bovine adrenocortical cells [17]. Although the effects of ET on adrenocortical cell mitogenesis have not been investigated, this peptide is known to stimulate division of rat mesangial cells [18]. Clearly these effects require the existence of cross-talk mechanisms at the level of second messengers or protein kinases in adrenocortical cells.

Tyrosine kinase activity, and phosphorylation of tyrosine residues, are normally considered to be fundamental elements of the mitogenic response to various growth factors, particularly epidermal growth factor [19–21]. It has been demonstrated that

these kinases are activated by extracellular signals which either act directly on tyrosine kinase-coupled receptors or indirectly by the activation of G-protein-coupled receptors [22]. When activated, tyrosine kinases lead to the phosphorylation of a variety of proteins, including nuclear transcription factors such as *c-fos* and *c-jun* [23].

The chain of events linking G-protein-coupled receptors to the activation of tyrosine kinase is unclear. Activation of G_i - or G_q -coupled receptors has been reported to stimulate tyrosine kinase activity in many cell lines, whereas G_s -coupled receptors and the cyclic AMP pathway appear to have different effects on tyrosine kinase depending on the cell type [24–26]. Previous studies from this laboratory have demonstrated that tyrosine kinase activity is stimulated by AngII by both a PKC-dependent pathway and a PKC-independent pathway [27].

These experiments were designed to examine the roles of tyrosine kinase and cyclic AMP in ET- and phorbol ester [phorbol 12-myristate 13-acetate (PMA)]-stimulated aldosterone secretion using the antagonists Ro 31-8220, a potent and selective PKC inhibitor [28], and tyrphostin 23, an inhibitor of tyrosine kinase [29].

EXPERIMENTAL

Materials

All chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K., with the following exceptions: ACTH(1–24) (Synacthen) and angiotensin II amide (Hypertensin) were supplied by Ciba-Geigy, Horsham, Sussex, U.K.; collagenase (Worthington type I) was purchased from Lorne Diagnostics, Reading, Berks., U.K.; the PKC inhibitor Ro 31-8220 was obtained from Roche Laboratories, Welwyn Garden City, Herts., U.K.; Raytide (peptide substrate for tyrosine kinase) was from Cambridge Bioscience, Cambridge, U.K.; tyrphostin 23 was obtained from LC Laboratories, Calbiochem-Novabiochem

Abbreviations used: ACTH, corticotrophin; AngII, angiotensin II; ET, endothelin-1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; dbcAMP, dibutyryl cyclic AMP; KRBG, Krebs bicarbonate Ringer solution containing glucose.

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Ltd., Nottingham, U.K. ET-1 was purchased from Peninsula Laboratories Europe Ltd., St. Helens, Merseyside, U.K. All radiolabels were purchased from Amersham International plc, Amersham, Bucks., U.K.

Male and female Wistar rats, body weight 350–450 g, were obtained from A. Tuck and Sons, Battlesbridge, Essex, U.K. and were maintained at Queen Mary and Westfield College for a minimum of 5 days prior to use. They were fed standard rat chow and water was available *ad libitum*.

Preparation of whole rat adrenal glomerulosa tissue

Rats were stunned, then killed by cervical dislocation. Adrenals were rapidly removed and cleaned of adhering fat. Capsule fractions (containing mainly glomerulosa cells) were separated from inner adrenocortical tissue by pressure between glass plates. Adrenal capsules were incubated in 5 ml of Krebs bicarbonate Ringer solution containing glucose (200 mg/100 ml) (KRBG) for 60 min at 37 °C under an atmosphere of 95% O₂ and 5% CO₂. After 60 min preincubation medium was discarded and fresh KRBG was added. Capsules were then incubated in 1 ml of KRBG for 2 h under an atmosphere of 95% O₂ and 5% CO₂ in the absence or presence of stimulants $\pm$ inhibitors.

Effect of protein kinase inhibitors on peptide-stimulated steroidogenesis

All peptides were dissolved in KRBG to the required concentrations. A range of concentrations of ET or PMA from 10⁻¹³ to 10⁻⁷ M were used in the presence or absence of Ro 31-8220 (150 nM), or tyrphostin 23 (50 nM). This concentration of tyrphostin 23 was chosen as the optimum concentration after carrying out a series of experiments to determine the effects of different doses of the compound on maximally stimulated tyrosine kinase activity [27]. The IC₅₀ of tyrphostin 23 in this preparation was found to be 0.5 nM. After a 60 min incubation period, capsules were discarded and media decanted into Eppendorf tubes. Tubes were stored at -20 °C until the media were ready to be assayed. Aldosterone was measured in an aliquot of unextracted incubation medium by direct radioimmunoassay [30].

Preparation of adrenal subcellular fractions

Adrenal capsules were obtained and treated as described above. Adrenal subcellular fractions were obtained by differential centrifugation of capsular homogenates [27]. Protein content was estimated by the method of Lowry [32].

Assay of kinase activity

Tyrosine kinase and PKC activities were assayed as previously described [27].

Statistical analysis

Arithmetic means and S.E.M. values were calculated, usually for $n = 4$, that is the results from four separate experiments were combined. One-way analysis of variance was used to test whether ET or PMA had a significant effect on basal (control) levels of aldosterone, PKC or tyrosine kinase activity. Student's *t*-tests were used to test whether the protein kinase inhibitors had any effect on agonist-stimulated events compared with agonist alone.

RESULTS

Effect of PKC inhibitors on steroidogenesis

ET caused dose-dependent increases in aldosterone production from the rat adrenal glomerulosa (Figure 1a). Threshold stimulation of aldosterone secretion was 10⁻¹² M. PMA also caused significant increases in aldosterone secretion; threshold stimulation occurred at 10⁻⁹ M and was maximal at 10⁻⁷ M PMA (Figure 1b). Figure 1(a) shows that the action of ET on aldosterone production was significantly attenuated by the PKC inhibitor Ro 31-8220 ($P < 0.001$), which caused up to 75% inhibition. The protein tyrosine kinase inhibitor tyrphostin 23, at a concentration of 50 nM, caused significant attenuation of ET-induced steroid output from rat zona glomerulosa *in vitro* (Figure 1), lowering the maximum aldosterone response to ET by 30%. Similar observations were made with these two protein kinase

Figure 1 The effect of Ro 31-8220 and tyrphostin on ET- or PMA-stimulated aldosterone secretion

Effect of increasing concentrations of (a) ET or (b) PMA on aldosterone secretion from whole rat capsules in the absence (○) and presence of 50 nM tyrphostin 23 (■) or 150 nM Ro 31-8220 (●). C = control. Values are means $\pm$ S.E.M., $n = 4$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with basal levels of aldosterone (analysis of variance). + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ compared with agonist alone (Student's *t*-test).

Table 1 Subcellular distribution of Ca^{2+} /phospholipid-sensitive PKC in the rat adrenal zona glomerulosa

The effect of Ro 31-8220 and tyrphostin on ET- or PMA-stimulated PKC activity. Concentrations of additions: Ro, 150 nM; Ro 31-8220, Tyr, 50 nM; tyrphostin 23. Values (expressed as means $\pm$ S.E.M., $n = 3$) show ^{32}P incorporation into type-III histone substrate from [^{32}P]ATP. $**P < 0.01$, $***P < 0.001$ compared with control (analysis of variance). $+P < 0.05$, $+++P < 0.001$ compared with agonist alone (Student's t -test).

Pretreatment	PKC activity (% of total)		
	Cytosol	Membrane	Nucleus
None (control)	80 $\pm$ 9	11 $\pm$ 2.2	9 $\pm$ 0.1
ET (10^{-7} M)	19 $\pm$ 1.5***	65 $\pm$ 5.5***	16 $\pm$ 1.1**
ET + Ro	70 $\pm$ 6.1+++	20 $\pm$ 3.0+++	10 $\pm$ 1.2
ET + Tyr	20 $\pm$ 2.3	68 $\pm$ 5.6	12 $\pm$ 1.1+++
PMA (10^{-7} M)	38 $\pm$ 2.5***	48 $\pm$ 4.5***	14 $\pm$ 2.6
PMA + Ro	70 $\pm$ 8.0+++	19 $\pm$ 2.1+++	11 $\pm$ 0.8
PMA + Tyr	55 $\pm$ 4.5+	25 $\pm$ 2.4+++	20 $\pm$ 3.1+

Figure 2 Time-course study of agonist-stimulated tyrosine kinase activity

The effect of increasing lengths of time on tyrosine kinase activity stimulated in rat adrenal zona glomerulosa tissue in the absence (○) or presence of 10^{-7} M of either ET (■) or PMA (▼).

inhibitors and PMA-stimulated aldosterone secretion (Figure 1b).

Effects of inhibitors on PKC activity

In control (unstimulated) tissue PKC activity was mainly (80%) found in the cytosolic fraction, with 12% and 8% in the membrane and nuclear fractions respectively (Table 1). ET stimulation significantly altered the subcellular distribution of PKC activity in whole glomerulosa tissue (Table 1). After incubation with 10^{-7} M ET, PKC activity associated with both membrane and nuclear fractions was significantly increased and cytosolic activity was decreased compared with control.

To study the effects of inhibitors on PKC activation, tissue was exposed to 150 nM Ro 31-8220 or 50 nM tyrphostin 23 prior to incubation with various stimulants. The PKC inhibitor, Ro 31-8220, abolished the increase in both membrane and nuclear PKC activity in response to ET. Ro 31-8220 thus prevented trans-

Figure 3 The effect of Ro 31-8220 and tyrphostin on ET- or PMA-stimulated tyrosine kinase activity

Effect of increasing concentrations of (a) ET or (b) PMA on aldosterone secretion from whole rat capsules in the absence (○) and presence of 50 nM tyrphostin 23 (■) or 150 nM Ro 31-8220 (●). C = control. Values are means $\pm$ S.E.M., $n = 4$. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with basal levels of aldosterone (analysis of variance). $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$ compared with agonist alone (Student's t -test).

location of PKC activity in response to ET. Table 1 also illustrates the effect of the tyrosine kinase inhibitor tyrphostin 23 on the subcellular distribution of PKC in rat glomerulosa tissue stimulated with ET. The inhibitor caused a significant reduction (50%) in ET-stimulated nuclear PKC activity, but it did not prevent the translocation of PKC activity from the cytosol to the membrane fraction.

Similar observations were made with PMA-stimulated PKC activity. PMA caused translocation of PKC from the cytosol to both membrane and nuclear fractions. As with ET-stimulated PKC redistribution, this effect was reversed by Ro 31-8220. However, tyrphostin 23 also inhibited the PMA-stimulated translocation of PKC from the cytosol to the membrane, but paradoxically enhanced nuclear PKC activity in response to PMA (Table 1).

Figure 4 Effect of ACTH on agonist-stimulated tyrosine kinase activity

Effect of increasing concentrations of ACTH on 10^{-7} M ET- (■), AngII- (●) or PMA (▼)-induced activation of tyrosine kinase in whole rat capsule preparations. C = control, A = agonist. Values are means $\pm$ S.E.M., $n = 4$. ** $P < 0.01$, *** $P < 0.001$ compared with agonist alone (Student's t -test).

Effects of inhibitors on tyrosine kinase activity

The ability of ET or PMA to activate tyrosine kinase was studied using Raytide as a substrate for the kinase assays. All kinase assays were carried out with a substrate concentration of 0.25 mg/ml which was found to give optimal activity (results not shown). Figure 2 shows a time-course study from which the optimum incubation period for the assay was determined. From this Figure it can be seen that a peak of activity was observed within 5 min for both ET and PMA.

Figure 3 shows the levels of ^{32}P incorporation by tyrosine kinase upon activation. ET dose-dependently stimulated tyrosine kinase activity. Threshold stimulation of tyrosine kinase was 10^{-12} M ET (Figure 3a). Figure 3(a) also shows that the actions of ET on tyrosine kinase activity were significantly ($P < 0.001$) inhibited by the protein tyrosine kinase inhibitor tyrphostin 23. The PKC inhibitor Ro 31-8220 also caused significant attenuation of ET-induced tyrosine kinase activity from rat glomerulosa *in vitro*.

PMA also induced a dose-dependent increase in tyrosine kinase activity; threshold stimulation occurred at 10^{-9} M and was maximal at 10^{-7} M PMA. This response was significantly

inhibited by Ro 31-8220, but almost totally abolished by tyrphostin (Figure 3b).

We have previously shown that ACTH does not stimulate tyrosine kinase activity in rat adrenal glomerulosa tissue [27]. Figure 4 clearly illustrates the effect of incubating ET, PMA or AngII in the presence of ACTH. ACTH significantly and dose-dependently inhibited the tyrosine kinase response to all of these agonists, with an IC_{50} of 10^{-10} M. In order to study whether this inhibitory effect was mediated via cyclic AMP, zona glomerulosa tissue was incubated with 10^{-5} M dibutyryl cyclic AMP (dbcAMP) and then stimulated with ET, PMA or AngII. Table 2 shows that dbcAMP caused a significant ($P < 0.001$) attenuation of tyrosine kinase activity in response to the three agonists.

DISCUSSION

The present studies provide new insights into the role of protein kinases in aldosterone secretion stimulated from the rat adrenal zona glomerulosa. In a previous study from this laboratory we showed that AngII stimulates tyrosine kinase activity [27]. The present study is the first report of ET-stimulated tyrosine kinase activity in the rat adrenal gland. ET, AngII and PMA were all found to stimulate tyrosine kinase activity within 5 min of incubation. Previous studies using mesangial cells have shown that ET-induced tyrosine kinase activity is mediated by ET receptors of the ET_A subtype [18,34], and this is the ET receptor subtype which predominates in the rat adrenal zona glomerulosa. It has been suggested that ET acts via phospholipase C and PKC [3,7,35], but it is clear from the data presented here that ET stimulation of aldosterone secretion is, in addition, at least partly dependent on increased tyrosine kinase activity. ET was shown to stimulate tyrosine kinase activity in a concentration-dependent manner, and the tyrosine kinase inhibitor, tyrphostin 23, significantly inhibited steroidogenesis at a concentration which caused almost complete inhibition of ET-stimulated tyrosine kinase activity. It has been reported that ET can stimulate tyrosine kinase in other cell types such as vascular smooth muscle cells [36], fibroblasts [37] and glomerular mesangial cells [18]. In these cells, ET is known to have mitogenic effects which appear to be mediated by tyrosine phosphorylation. This mitogenic activity has been shown to be associated with the stimulated expression of the proto-oncogenes *c-fos* and *c-myc* [38].

The rat adrenal zona glomerulosa tissue used in this study expresses the AngII AT_1 -type receptor [11,12,39–41]. The receptor has been cloned [10] and does not appear to have intrinsic tyrosine kinase activity. Despite this, a recent study by Chabre et al. [17] shows that AngII-mediated activation of tyrosine kinases occurs via its specific AT_1 receptor. A very recent study [42]

Table 2 Effect of ACTH or dbcAMP on agonist-stimulated tyrosine kinase activity in whole rat capsule preparations

All agonists were used at a concentration of 100 nM. Values are means $\pm$ S.E.M., $n = 3$. *** $P < 0.001$ compared with agonist alone (Student's t -test).

Treatment	Tyrosine kinase activity (pmol of ^{32}P incorporated/min per mg of protein)			
	Control	ET	AngII	PMA
Control	2.0 ± 0.1	35.2 ± 2.1	25.1 ± 2.1	30.0 ± 3.1
ACTH (10 nM)	2.5 ± 0.2	$15.1 \pm 1.1^{***}$	$16.3 \pm 2.0^{***}$	$15.2 \pm 3.2^{***}$
DbcAMP (10 μM)	2.1 ± 0.1	$9.3 \pm 1.2^{***}$	$12.2 \pm 1.5^{***}$	$13.3 \pm 1.9^{***}$

suggests that some phospholipase C-linked receptors, particularly AngII, act in a similar manner to cytokine receptors in so much as these receptors can activate the Jak-STAT signalling pathway which involves the rapid phosphorylation of tyrosine residues of intracellular kinases [43,44]. In other cells, the Jak-STAT pathway has been proposed as the signalling pathway by which transcriptional activation of early growth response genes occurs [43,45]. It is possible to speculate that this tyrosine kinase pathway may also play a role in the control of ET- and AngII-mediated events in the rat adrenal gland.

The precise mechanisms responsible for functional coupling of these receptors and tyrosine kinase activity are unclear, but the present studies provide evidence for the involvement of PKC. The results show that when PKC was inhibited by Ro 31-8220, ET-stimulated aldosterone production was significantly decreased (Figure 1), confirming the importance of the PKC-signalling pathway in the aldosterone response to ET. The present studies also clearly demonstrate that PKC is involved in the activation of tyrosine kinase in response to ET stimulation, as Ro 31-8220 also had a significant inhibitory effect on ET-induced tyrosine kinase activity (Figure 3). Despite the blocking of the PKC-dependent pathway by Ro 31-8220, however, agonist-stimulated tyrosine kinase activity was not totally inhibited, suggesting that there may be a PKC-independent pathway of tyrosine kinase activation in the adrenal zona glomerulosa. We also found that the phorbol ester, PMA, stimulated tyrosine kinase activity in the rat adrenal zona glomerulosa, which lends further support to the theory that PKC has a role upstream of tyrosine kinase. It has been suggested that activation of PKC may be an important intermediate step prior to tyrosine kinase activation [38]. Experiments with PKC inhibitors, together with depletion studies, demonstrated that, although agonists may directly stimulate tyrosine kinase, higher levels of tyrosine kinase activity are seen when PKC is also activated [34,36,37]. Sequential activation of intracellular protein kinases has been observed in a number of tissues [36,37,46,47].

Previous work carried out by this laboratory has shown that AngII-stimulated aldosterone secretion is also inhibited by the PKC inhibitor Ro 31-8220 [27]. When PKC activity was studied directly, it was interesting to discover enzyme activity present in the nucleus. We, and others, have shown that there is endogenous PKC present in the nucleus and that enzyme activity is translocated from the cytosol to the nucleus upon stimulation [27,48]. However, there has been little characterization of protein kinases that act at the nuclear level, even though a number of serine/threonine kinases are reported to be present in the nucleus [38,49]. Upon stimulation with either ET or the phorbol ester, PMA, the intracellular distribution of PKC changed dramatically, resulting in significant increases in membrane and nuclear PKC, associated with a decrease in cytosolic PKC. In this respect ET had a greater effect than PMA.

The present data reveal a previously unrecognized effect of cyclic AMP on the tyrosine kinase pathway in the rat adrenal cortex. Integrated control of any cell cannot be achieved by a number of linear signal transduction pathways alone, and interaction between pathways is clearly required. Tyrosine kinase is activated by a diverse range of effectors, but notably not by stimulants that increase intracellular cyclic AMP levels [50,51]. We have previously shown that ACTH has no effect on tyrosine kinase activity [27]. In the present study, however, ACTH was found to completely block ET-, AngII- and PMA-stimulated tyrosine kinase activity. This suggests that cyclic AMP, the second messenger of ACTH, is involved in this inhibition. When this question was addressed directly it was clear that cyclic AMP itself was able to attenuate tyrosine kinase. Such inhibition of

kinase activation by the cyclic AMP pathway has been reported in smooth muscle cells [52], adipocytes [53] and other cell lines [37,50]. The mechanisms of this inhibition remain to be elucidated, but it has been suggested that phosphorylation of Raf-1 on Ser⁴³ may be one mechanism by which cyclic AMP (presumably via cyclic AMP-dependent protein kinase) inhibits signalling from tyrosine kinases [52].

In summary, in the present study we took advantage of the fact that steroidogenesis in the rat adrenal zona glomerulosa is influenced by three different pathways: PKC, cyclic AMP and tyrosine kinase pathways. Regardless of the precise target of cyclic AMP, the ability of ACTH to selectively attenuate the activity of the tyrosine kinase pathway advances our understanding of the inhibitory action of cyclic AMP in zona glomerulosa tissue. Furthermore it reveals a novel example of cross-talk between different G-protein-coupled receptor-linked signalling pathways and tyrosine kinase-linked receptors, and provides a convenient model for use towards complete understanding of the integrated cellular events following hormone action on the rat adrenal cortex.

We wish to thank Ciba-Geigy for the generous donation of Synacthen and Hypertensin used in this study and Dr. G. Lawton of Roche Products for the gift of Ro 31-8220. We are most grateful to the MRC and the Wellcome Trust for project grant support.

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