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A Role for Protein Tyrosine Kinase in the Steroidogenic Pathway of Angiotensin II in Bovine Zona Glomerulosa Cells

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Stimulation of aldosterone synthesis in bovine adrenal zona glomerulosa (ZGB) cells by angiotensin II (AngII) is believed to be mediated by the phospholipase C (PLC) pathway that results in the increase of cytosolic free calcium concentration and in the activation of protein kinase C (PKC). However, the cell proliferation and contraction associated with AngII action are known to be mediated in part by protein tyrosine kinases (PTK). To assess the potential role of PTK in the stimulatory effect of AngII on adrenal steroidogenesis, the actions of a series of PTK inhibitors on this metabolic pathway were examined in isolated ZGB cells. Tyrphostin 23 (TP23) caused a dose-dependent inhibition of AngII-stimulated aldosterone production with an IC_{50} of 15 μM and reached complete inhibition at 100 μM. Genistein (GS) was more potent with an IC_{50} of 35 nM and complete inhibition at 10 μM. The stimulation of aldosterone production by the calcium-mobilizing agent thapsigargin (Thaps) was also dose-dependently inhibited by TP and GS with the same potency. A specific PKC inhibitor, calphostin C (0.1 μM) caused only a 51.7% inhibition of AngII-stimulated aldosterone production. In the same way, a specific Ca^{2+}/calmodulin-dependent protein kinase inhibitor, KN-62 (1 μM), reduced aldosterone production stimulated by AngII by 64%. As expected, thapsigargin-stimulated aldosterone biosynthesis was not affected by calphostin C, but was completely inhibited by KN-62. These results demonstrate for the first time that protein tyrosine kinase activity is part of the angiotensin II signalling pathway in bovine zona glomerulosa cells. The activation of this PTK occurs subsequently to the mobilization of intracellular calcium. This calcium-dependent protein tyrosine kinase pathway is essential for the steroidogenic response to AngII in bovine zona glomerulosa cells.

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INTRODUCTION

In bovine zona glomerulosa (ZGB) cells, angiotensin II (AngII) stimulates aldosterone biosynthesis. Activation of the AngII receptor is typically associated with the generation of Ca^{2+}-mobilizing inositol-1, 4,5-triphosphate and protein kinase C (PKC)-activating diacylglycerol from phosphatidylinositides. The cellular responses to these second messengers are thought to be mediated by the activation of serine/threonine protein kinases, e.g. PKC and Ca^{2+}/calmodulin (CaM)-dependent protein kinase [1]. The later steps of this activation up to the conversion of cholesterol to pregnenolone are still unknown.

Recently AngII, which acts through G-protein-coupled receptor, also appears to involve a tyrosine kinase pathway in various cell systems, e.g. vascular smooth muscle cells [2], glomerular mesangial cells [3] and the rat liver epithelial cell lines WB and GN4 [4, 5]. In vascular smooth muscle cells in culture, AngII has been shown to stimulate the tyrosine phosphorylation of many proteins including PLC-γ1 [6]. As the AngII receptor itself has no tyrosine kinase activity, it would appear that one or more intracellular tyrosine kinases are stimulated by AngII and are responsible for early signal transduction events in these cells. In guinea-pig gastric smooth muscle preparation, inhibition of AngII-stimulated tyrosine phosphorylation by two specific inhibitors of tyrosine kinases, tyrphostin and genistein, was directly associated with inhibition of AngII-elicited muscle contraction [7].

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The present study was designed to assess whether the stimulation of aldosterone biosynthesis by AngII in bovine zona glomerulosa cells also requires activation of protein tyrosine kinases. For this purpose, we tested the effects of specific protein tyrosine kinase inhibitors, e.g. tyrphostins and genistein, on AngII-stimulated aldosterone biosynthesis.

In order to localize the potential protein tyrosine kinases involved in the signalling cascade generated by AngII, we used thapsigargin (Thaps), a sesquiterpene lactone that inhibits the microsomal calcium ATPase pump [8]. Consequently, thapsigargin increases cytosolic Ca^{2+} concentration, depletes intracellular Ca^{2+} stores and promotes increased flux of Ca^{2+} from the extracellular medium into the cytoplasm [9]. In this way, thapsigargin mimics the effects of Ca^{2+}-mobilizing hormones, but unlike them, does not affect inositol phosphate metabolism [10].

We report here on the effects of tyrphostin (TP) and genistein (GS) on the stimulation of aldosterone production by either AngII or Thaps. As AngII is also known to activate the serine/threonine kinases PKC and Ca^{2+}/CaM-dependent kinase in ZGB cells, we will compare the effects of these inhibitors to those of calphostin C, a specific PKC inhibitor [11] and KN-62, a specific Ca^{2+}/CaM-dependent PK inhibitor [12]. We have also included in this study the cardiac hormone atrial natriuretic peptide (ANP) since it is a physiological inhibitor of aldosterone biosynthesis. The mode of action of this hormone is still unclear [13] and comparison of its effects with those of the other inhibitors might help to understand its mode of action better. The results suggest that one or more protein tyrosine kinases might be involved in the signalling pathway of angiotensin II in bovine adrenal zona glomerulosa cells. These tyrosine kinases are likely to be calcium-dependent and play a major role in the stimulation of aldosterone production.

MATERIALS AND METHODS

Materials

Ham's F12 medium, horse serum and fetal bovine serum were purchased from Gibco Labs Inc. (Burlington, Ontario). Collagenase type 2 was from Worthington Biochemical Corporation (Freehold, NJ). DNAse type I, thapsigargin, ionomycin, calphostin C, aminoglutethimide and cytochrome c were from Sigma Chemical Co. (St Louis, MO). Tyrphostins and genistein were from Calbiochem (San Diego, CA). KN-62 was purchased from Research Biochemicals Inc. (Natick, MA). Human ANP was from IAF Biochem (Montreal, Canada). Angiotensin II was from Peninsula (Belmont, CA). Aldosterone-3-(*O*-carboxymethyl)oximino-(2-[^{125}I]iodohistamine) was purchased from Diagnostic Products Corporation (Markham, Ontario). Anti-aldosterone-3-BSA antibody was from ICN Biomedicals Inc. (Costa Mesa, CA). [7-^{3}H(N)]pregnenolone was from Du Pont (Mississauga, Ontario). Anti-pregnenolone antibody was a gift from Dr A. Bélanger (Centre hospitalier de l'université Laval, Ste-Foy, Québec).

Cell culture

Primary culture of bovine adrenal zona glomerulosa cells was performed as described [14]. Briefly, bovine adrenal glands were obtained from a local slaughterhouse. The glands were cleaned of fat and a 0.5 mm layer containing the capsule and the zona glomerulosa was dissected with a scalpel. The cells were dispersed in Ham's F12 medium with 0.2% collagenase type 2, 0.025% DNAse type I and 0.25% BSA. Washed cells were resuspended in Ham's F12 medium supplemented with 10% horse serum, 2% fetal bovine serum, 1% streptomycin, 1% penicillin and 2.5 μg/ml fungizone. The cell suspension (10^6 cells/ml) was distributed in 1 ml fractions in 24-well cluster plates. Cell viability, as monitored by Trypan blue exclusion, was generally greater than 90%. The viability of the cells was not affected by 48 h of culture nor by the various cell treatments.

Cell stimulation

After 24 h in culture, the culture medium was replaced by the same medium as above without serum. On the next day, the cells were washed with the same medium without serum and quadruplicate cell culture wells were stimulated for 2 h at 37°C with various agents added to fresh serum—free Ham's F12 medium containing 0.02% lysozyme. At the end of the incubation, the medium was rapidly removed and frozen at −20°C until assayed for aldosterone determination.

Mitochondrial cholesterol side-chain cleavage activity

The assay measures the rate of pregnenolone formation from endogenous cholesterol when mitochondria are provided with means of regenerating reducing equivalents. Mitochondria were isolated from ZGB cells incubated in the presence or the absence of 10 nM AngII and 30 μM TP23 as above. Cell cultures were washed three times and scraped off into buffer A (0.25 M sucrose, 1 mM EDTA, 25 mM HEPES, 1% (w/v) BSA, 0.76 mM aminoglutethimide, pH 7.0), and disrupted by 40 strokes of a Potter–Elvehjem homogenizer. The resulting homogenate was centrifuged at 200 *g* for 10 min, the pellet discarded and the mitochondrial fraction obtained by centrifuging the supernatant at 6720 *g* for 10 min. The mitochondrial fraction was washed in buffer A without BSA and resuspended in incubation buffer (123 mM KCl, 0.5 mM $MgCl_2$, 10 mM KH_2PO_4, 25 mM HEPES, 0.2 mM EDTA, pH 7.0). Cholesterol side-chain cleavage activity was measured in triplicate in the presence of 10 μM trilostane, which blocks the conversion of pregnenolone to progesterone. The incubation conditions consisted of 0.2 ml of mitochondrial suspension, 10 μM

trilostane and 0.760 ml incubation buffer in a final volume of 1 ml. The tubes were preconditioned at 37°C for 5 min and the reaction was started by adding 20 μl of sodium isocitrate (20 mM final concentration). The side-chain cleavage activity was stopped after 5 min by plunging the tubes into liquid nitrogen. Two freeze–thaw cycles were carried out and the tubes were centrifuged at 27,000 g for 30 min. Pregnenolone was assayed by RIA of the supernatant.

Cytochrome c oxidase activity

Cytochrome c oxidase (complex IV, EC 1.9.3.1) activity was assayed by the method of Wharton and Tzagoloff which measures the oxidation of reduced cytochrome c at 550 nm [15]. Cell treatments and mitochondrial preparation were as above except that the final mitochondrial pellet was resuspended in distilled water. Absorbance measurements were carried out using a Beckman DU 640 spectrophotometer in a final volume of 3 ml, in a cuvette with a 1 cm light path, at room temperature. The reactions were initiated by addition of the enzyme. Enzyme activity was expressed as the first-order reaction constant (k/min/mg of mitochondrial protein).

Steroid determination

Aldosterone and pregnenolone were directly measured in cell culture medium by specific radioimmunoassays as previously reported [16, 17]. The lowest detectable levels of the RIAs were 5 fmol/ml for aldosterone and 300 fmol/ml for pregnenolone. The production of pregnenolone was determined in the presence of trilostane (WIN 24,540 from Sterling-Winthrop Research Institute, NY), an inhibitor of 3β-hydroxysteroid dehydrogenase. At a concentration of 1 μM, this compound quantitatively inhibits the formation of progesterone and thus allows accumulation of pregnenolone in the incubation medium.

Data analysis

Dose–response curves for aldosterone production were analyzed with the program ALLFIT for Windows* based on a four-parameter logistic equation [18] to obtain estimates of the ED_{50}. Results are expressed as mean ± SEM of quadruplicate cell culture wells and are compared by the two-tailed Bonferroni t-test. A level of $P < 0.05$ was considered as statistically significant and reported as such.

RESULTS

Effects of tyrphostins and genistein on AngII—stimulated aldosterone production

Genistein is a potent protein tyrosine kinase inhibitor competing for the ATP binding site on the kinase. Tyrphostins are more specific protein tyrosine kinase inhibitors as they act as competitive substrates. To date, more than 100 tyrphostin derivatives have been synthesized and shown to display different specificity towards the various classes of PTK. Therefore, besides genistein, we have selected for testing in our model four types of tyrphostin based on their structure and their relative EGF receptor kinase inhibitory activity [19]. As seen in Fig. 1, GS was the most potent inhibitor of AngII-stimulated aldosterone production in ZGB cells, with 74.9% of inhibition at a concentration of 1 μM. TP 23, 24, 47 and 51 at 30 μM inhibited aldosterone production by 74, 33, 40 and 22% respectively. The order of potency of these PTK inhibitors was thus as follows: GS > TP23 > TP47 > TP25 > TP51.

Of the tyrphostins tested, TP23 was the most potent in inhibiting AngII-stimulated aldosterone production. The complete dose–response curve for this compound is shown in Fig. 2. Aldosterone production stimulated by 10 nM AngII was dose-dependently inhibited by TP23 with an IC_{50} of 15 μM and maximal effect at 100 μM. None of the inhibitors inhibited basal aldosterone production even at the highest dose tested, indicating that these compounds do not directly affect the steroid synthetic machinery but rather inhibit the AngII-stimulated signal transduction pathway.

These results demonstrate that the stimulation of aldosterone synthesis by AngII in bovine zona glomerulosa cells involves one or more protein tyrosine kinase activities. The marked inhibition of aldosterone production obtained with GS and TP23 suggests that these protein tyrosine kinases are essential components of the signal transduction pathway of

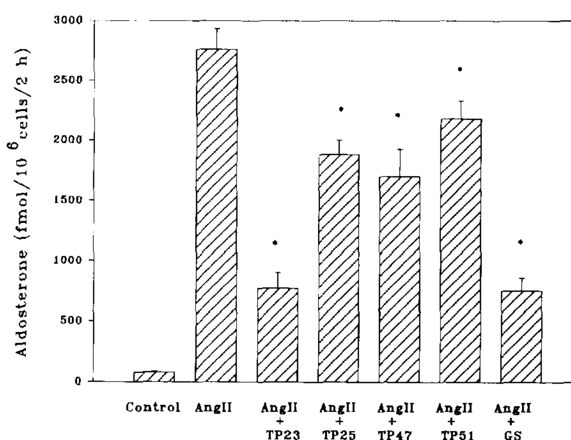

Fig. 1. Inhibition of AngII-stimulated aldosterone production by various protein-tyrosine kinase inhibitors. ZGB cells were pre-incubated (10^6 cells/ml, 37°C, 30 min) with either tyrphostin (TP, 30 μM) or genistein (GS, 1 μM) and then incubated (37°C, 2 h) with AngII (10 nM) and TP (30 μM) or GS (1 μM). Also shown are the levels of aldosterone production in the presence or the absence of AngII (10 nM) alone. Plotted are the means ± SEM of quadruplicate incubations of three different experiments. *$P < 0.05$ vs AngII.

*Requests for the program ALLFIT for Windows can be addressed by e-mail to: DELEAN@ERE.UMontreal.CA.

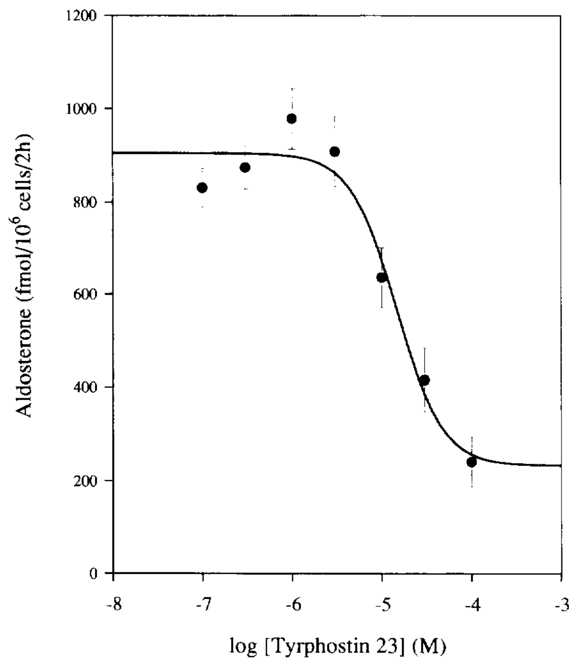

Fig. 2. Dose-dependent inhibition by TP23 of AngII-stimulated aldosterone production by ZGB cells in culture. ZGB cells were incubated (10^6 cells/ml, 37°C, 2 h) with AngII (10 nM) and increasing doses of tyrphostin 23 (10 nM–1 mM). The data are the means ± SEM of quadruplicate incubations and are representative of at least three different experiments.

AngII leading to increased aldosterone synthesis in ZGB cells.

Effect of tyrphostin on mitochondrial pregnenolone synthesis

Transfer of cholesterol to cytochrome $P450_{SCC}$ is generally regarded as the rate-limiting step in aldosterone synthesis. AngII stimulates this cholesterol transfer both through increasing the availability of cytosolic free cholesterol and through enhanced cholesterol transfer between the outer and the inner mitochondrial membranes by an unknown mechanism. We thus have examined the effect of tyrphostin 23 treatment of AngII-stimulated ZGB cells on *in vitro* cholesterol side-chain cleavage activity of isolated mitochondria. As shown in Fig. 3, 2 h incubation of ZGB cells with 30 μM TP23 did not affect the basal pregnenolone synthesis by mitochondria isolated from these cells. This shows that TP23 does not directly affect the steroid synthetic machinery of ZGB cells and in addition does not alter mitochondrial viability. Stimulation of ZGB cells with 10 nM AngII produced a 2-fold increase in pregnenolone synthesis, and simultaneous treatment with 30 μM TP23 inhibited this increase by 50%. The results demonstrate that the tyrosine kinases involved in the AngII signalling pathway play a key role in increasing cholesterol substrate availability at the site of cytochrome $P450_{SCC}$ in the mitochondria.

Effect of tyrphostin on the mitochondrial cytochrome c oxidase activity

To ensure that inhibition of AngII-stimulated aldosterone production by TP23 is due to specific inhibition of protein tyrosine kinases and not to cytotoxic effects, we looked at the nonspecific effect of TP23 on mitochondrial cytochrome c oxidase activity. Cytochrome c oxidase is a component of the mitochondrial respiratory chain that catalyzes electron transfer from ferrocytochrome c to molecular oxygen. It has been shown to act as a proton pump driven by the free energy of the electrochemical membrane potential promoting oxidative ATP-production.

ZGB cells were stimulated with 10 nM AngII in the presence or in the absence of 30 μM TP23 for 2 h at 37°C. Mitochondria from these cells were isolated and cytochrome c oxidase activity was assayed as described in Materials and Methods. For AngII-treated cells, the k value obtained was 1.1403 ± 0.0635 (min^{-1} mg^{-1}). However, for the combined treatment of AngII and TP23, a k value of 0.9556 ± 0.0009 (min^{-1} mg^{-1}) was found. After a 2 h incubation, TP23 inhibited cytochrome c oxidase activity by 16% with aldosterone production inhibited by 74%, suggesting that the inhibition of AngII-stimulated aldosterone production reflects a relatively specific effect of TP23 on tyrosine kinase activity.

Effect of thapsigargin on aldosterone production

In adrenal ZGB cells, AngII-induced aldosterone synthesis is highly dependent on intra- and extracellular calcium mobilization. We have used thapsigargin to selectively activate the calcium-signalling pathway of AngII without generating inositol

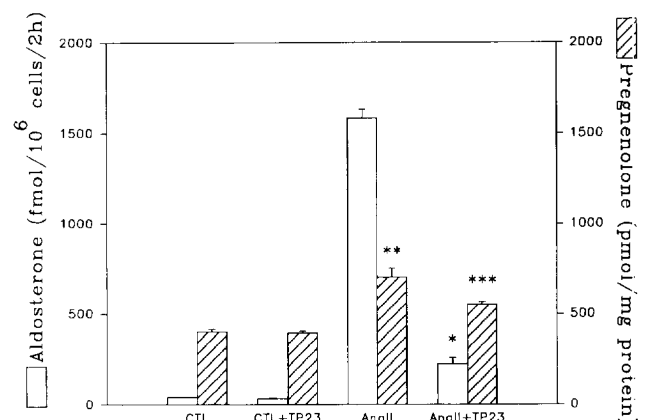

Fig. 3. Inhibition of AngII-stimulated aldosterone production and cholesterol side-chain cleavage activity by TP23. Aldosterone production was measured as in Fig. 1. Pregnenolone synthesis by mitochondria isolated from cells incubated (2×10^7 cells/15 ml/37°C/2 h) in the presence or the absence of 10 nM AngII and 30 μM TP23 was measured as described in Materials and Methods. The data are the means ± SEM of triplicate incubations and are representative of three different experiments. *$P < 0.05$ vs AngII, **$P < 0.05$ vs CTL, ***$P < 0.05$ vs AngII.

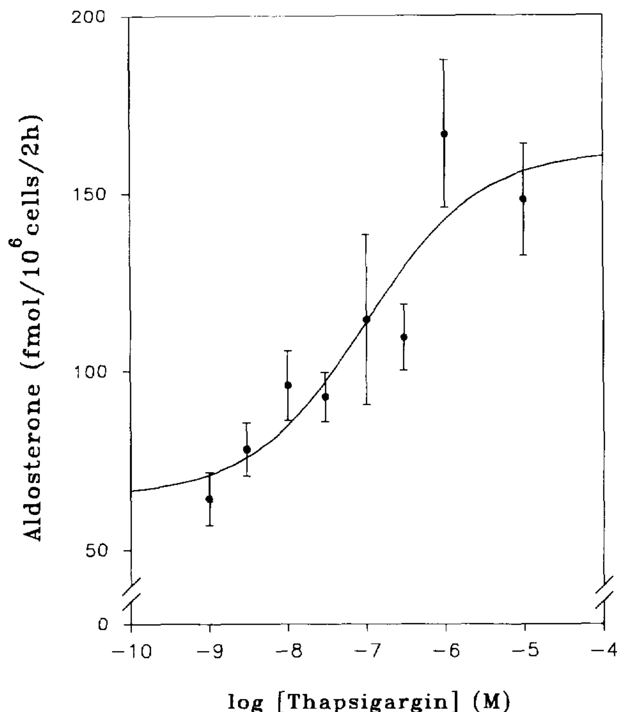

Fig. 4. Dose-dependent stimulation of aldosterone production by thapsigargin in ZGB cells in culture. ZGB cells were incubated (10^6 cells/ml, 37°C, 2 h) with increasing concentrations of thapsigargin (0.1 nM–100 μM). These data are the means ± SEM of quadruplicate incubations and are representative of at least three different experiments.

phosphates and diacylglycerol. The presumed increase in cytosolic calcium concentration caused by Thaps is sufficient to stimulate aldosterone production by ZGB cells, although it was less efficient than AngII. Thaps caused a 2–3-fold increase in aldosterone production by these cells (control, 64 ± 7; Thaps, 148 ± 16 fmol/10^6 cells/2 h). This increase was dose-dependent, with an ED_{50} of 0.1 μM and maximal stimulation was reached at about 3 μM (Fig. 4). In the same way, the calcium ionophore ionomycin which promotes an increased influx of calcium from the extracellular medium into the cytosol also stimulated aldosterone production in ZGB cells. At a dose of 1 μM, ionomycin caused a 4-fold increase in aldosterone production (control, 52 ± 11; 1 μM ionomycin, 209 ± 21 fmol/10^6 cells/2 h).

Effect of tyrphostins and genistein on Thaps-stimulated aldosterone production

To localize the site of action of the protein tyrosine kinases involved in the AngII signalling pathway in ZGB cells, we have documented the effects of the protein tyrosine kinase inhibitors on Thaps-stimulated aldosterone biosynthesis. The various PTK inhibitors used in this study also inhibited the production of aldosterone stimulated by 1 μM Thaps, and this inhibition was even more profound that that observed with AngII-stimulated production, in that 1 μM GS and 30 μM TP23 completely inhibited Thaps-stimulated

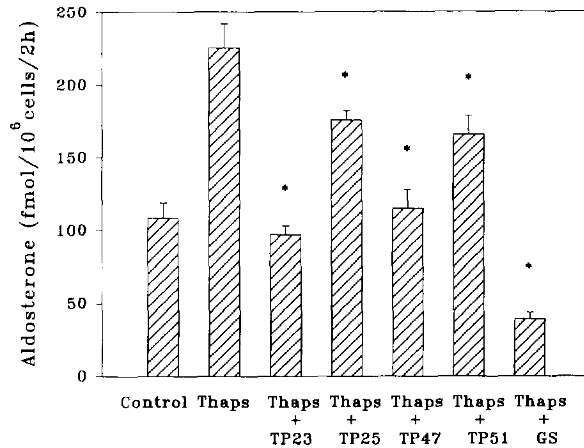

Fig. 5. Inhibition of Thaps-stimulated aldosterone production by various protein-tyrosine kinase inhibitors. ZGB cells were incubated (10^6 cells/ml, 37°C, 2 h) with Thaps (1 μM) and either TP (30 μM) or GS (1 μM). Also shown are the levels of aldosterone production in the presence or the absence of Thaps (1 μM) alone. Plotted are the means ± SEM of quadruplicate incubations of three different experiments. *$P < 0.05$ vs Thaps.

aldosterone production. Inhibition of 42, 94 and 51% of the Thaps effect was observed with TP 25, 47 and 51, respectively (Fig. 5). These PTK inhibitors exhibited the same order of potency against Thaps as that seen for AngII: GS > TP23 > TP47 > TP25 > TP51. Of these inhibitors, GS showed the best dose–inhibition curve, as seen in Fig. 6, with a dose-dependent inhibition of the Thaps-stimulated

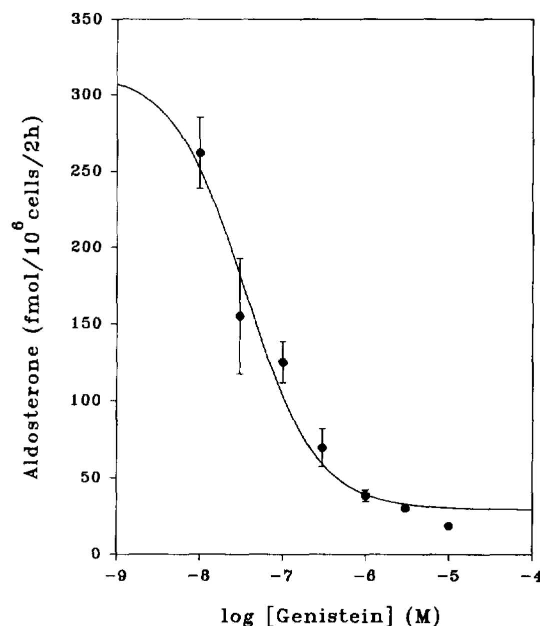

Fig. 6. Dose-dependent inhibition by genistein of Thaps-stimulated aldosterone production by ZGB cells in culture. ZGB cells were incubated (10^6 cells/ml, 37°C, 2 h) with Thaps (1 μM) and increasing doses of genistein (1 nM–10 μM). The data are the means ± SEM of quadruplicate incubations and are representative of three different experiments.

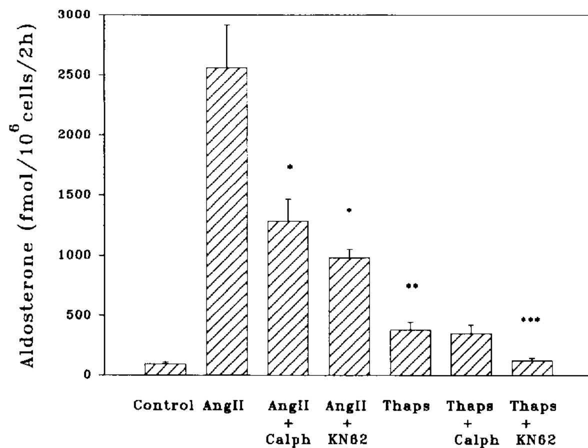

Fig. 7. Effects of the PKC inhibitor, calphostin C, and the Ca^{2+}/CaM-dependent PK inhibitor, KN-62, on aldosterone production stimulated either by AngII or Thaps. ZGB cells were pre-incubated (10^6 cells/ml, 37°C, 30 min) with either Calph (0.1 μM) or KN-62 (1 μM). Then the cells were incubated (10^6 cells, 37°C, 2 h) either with AngII (10 nM) or Thaps (1 μM) in the presence of the inhibitors. Plotted are the means ± SEM of quadruplicate incubations of three different experiments. *$P < 0.05$ vs AngII, **$P < 0.05$ vs control, ***$P < 0.05$ vs Thaps.

aldosterone production with an IC_{50} of 35 nM and complete inhibition at 10 μM. In addition, 30 μM TP23 completely inhibited aldosterone production stimulated by 1 μM ionomycin (control, 58 ± 3; 1 μM ionomycin, 221 ± 13; 1 μM ionomycin + 30 μM TP23, 61 ± 3 fmol/10^6 cells/2 h).

Treatment of ZGB cells with thapsigargin presumably activates one or more protein tyrosine kinases involved in the stimulation of aldosterone synthesis. As thapsigargin raises cytosolic calcium concentration, this result suggests that the PTK(s) involved in aldosterone synthesis are calcium-dependent and so act on a step following calcium mobilization in the AngII signalling pathway.

Effects of PKC and Ca^{2+}/CaM-PK inhibitors on aldosterone production

The stimulation of aldosterone biosynthesis by AngII in ZGB cells seems to require the activation of at least three types of protein kinases, e.g. PKC, Ca^{2+}/CaM-PK and PTK. To compare the relative importance of each of these kinases, we tested the effects of specific inhibitors of PKC and Ca^{2+}/CaM-PK on AngII- and Thaps-stimulated aldosterone biosynthesis. Calphostin C is a potent and specific inhibitor of PKC with an IC_{50} of 0.05 μM and a 1000-fold selectivity for PKC compared with PKA or PTK [11]. 0.1 μM calphostin C partially inhibited AngII-stimulated aldosterone production with a maximal inhibition of only 52% (Fig. 7). As expected, calphostin C did not affect Thaps-stimulated aldosterone production, since Thaps is known not to activate PKC (Fig. 7). KN-62 is a specific inhibitor of Ca^{2+}/CaM-dependent protein kinase with an IC_{50} of 0.9 μM and more than 100-fold selectivity compared with PKC or PKA [12]. At a dose of 1 μM, KN-62 decreases aldosterone production stimulated by AngII by 64%. It was more efficacious against Thaps, producing 90% inhibition of Thaps stimulation (Fig. 7).

Effect of ANP on aldosterone production

ANP is a well-known physiological inhibitor of aldosterone biosynthesis in ZGB cells, although the mechanism of this inhibition remains unknown. 1 nM ANP caused an 80% inhibition of AngII-stimulated aldosterone production by ZGB cells: Control, 87 ± 9; 10 nM AngII, 2980 ± 380; 10 nM AngII + 1 nM ANP, 659 ± 127 fmol/10^6 cells/2 h. In addition, ANP also inhibited by 90% aldosterone production stimulated by Thaps: control, 87 ± 9; 1 μM Thaps, 232 ± 29; 1 μM Thaps ± 1 nM ANP, 102 ± 11 fmol/10^6 cells/2 h. The data are the means ± SEM of quadruplicate incubations of three different experiments. These results showed that ANP almost completely inhibited aldosterone production stimulated by the calcium-mobilizing agent thapsigargin, suggesting that ANP acts on a step following the mobilization of intracellular calcium in the signal transduction pathway of AngII.

DISCUSSION

By using specific inhibitors of protein tyrosine kinase, the present work demonstrates that protein tyrosine kinase activity is involved in the signal transduction pathway of angiotensin II in bovine adrenal zona glomerulosa cells. To our knowledge, this is the first report of a role for protein tyrosine kinases in the physiological function of AngII in bovine zona glomerulosa cells, i.e. stimulation of aldosterone biosynthesis.

In vitro pregnenolone synthesis by mitochondria isolated from untreated ZGB cells was not inhibited by tyrphostin, and cytochrome c oxidase activity, which is essential for mitochondrial and cellular viability and thus for adequate functioning of cytochrome $P450_{SCC}$, was only very slightly inhibited by tyrphostin. Tyrphostin inhibition of stimulated aldosterone synthesis is thus probably not due to nonspecific effects of this inhibitor. Cholesterol side-chain cleavage activity of mitochondria isolated from AngII-stimulated cells was inhibited by 50% by tyrphostin, suggesting that the protein tyrosine kinases involved in AngII-stimulated aldosterone synthesis are associated with the process leading to increased cholesterol availability in the mitochondria. However, additional regulatory effects of protein tyrosine kinases on later steps of steroidogenesis cannot be excluded by the present studies.

Various calcium ionophores have been used to increase aldosterone production by adrenal zona glomerulosa cells from different species. In contrast to these ionophores, thapsigargin increases cytoplasmic

free calcium concentration by depleting intracellular calcium stores. In this way thapsigargin more closely mimics the calcium mobilizing effect of AngII without stimulating the PKC pathway. We have shown that protein tyrosine kinase inhibitors also inhibit aldosterone production stimulated by thapsigargin. This suggests that the protein tyrosine kinases involved in aldosterone production are calcium-dependent and thus are located downstream to the calcium-mobilizing step in the AngII signalling pathway. The key role of calcium in the activation of these protein tyrosine kinases is further supported by the fact that TP23 only partially inhibited AngII-stimulated aldosterone production, while completely inhibiting steroidogenesis when cells were challenged with calcium-mobilizing agents, e.g. thapsigargin, ionomycin.

Angiotensin II has been shown to stimulate protein tyrosine phosphorylation in a calcium-dependent manner in WB and GN4 liver epithelial cell lines [4, 5]. However, the mechanism of activation of protein tyrosine kinases following the increase of cytosolic free calcium concentration is as yet unclear. The PTK could be directly regulated in a positive manner by a Ca^{2+}-binding regulatory protein; alternatively, Ca^{2+}-dependent enzymes could activate PTK by phosphorylation. Another possibility is that Ca^{2+} might maintain PTK in an activated state by inhibiting its dephosphorylation. A Ca^{2+}-inhibited tyrosine phosphatase has been isolated from bovine brain [20], and Ca^{2+}-dependent inhibition of tyrosine phosphatase CD45 has been reported [21].

As shown, calcium ionophores and thapsigargin by themselves can stimulate aldosterone production without simultaneous activation of PKC. In contrast, in our hands, well-known activators of PKC such as phorbol esters were not able to stimulate aldosterone synthesis. Furthermore, simultaneous treatment of ZGB cells with phorbol esters and calcium ionophores did not reproduce the stimulation obtained with AngII (unpublished observations). On the other hand, we observed that the inhibition of AngII-stimulated aldosterone production by KN-62 was more pronounced than that obtained with calphostin C. These results agree with a dominant role of the calcium mobilization pathway in the stimulation of aldosterone production by ZGB cells as previously reported in the literature [22].

At a concentration corresponding to twice their IC_{50} TP23, KN-62 and calphostin C inhibited AngII-stimulated aldosterone production by 74, 64 and 52%, respectively. This result suggests that the protein tyrosine kinases activated by AngII are absolutely crucial for the stimulation of aldosterone synthesis, whereas PKC activation may be less crucial.

The inhibition of Thaps-stimulated aldosterone production by ANP suggests that ANP might also interfere with the calcium signalling pathway of AngII in ZGB cells. ANP has been shown to inhibit T-type Ca^{2+}-channels, which are activated at the resting membrane potential of the bovine glomerulosa cells [23]. So, ANP could inhibit aldosterone synthesis by reducing Ca^{2+} influx into the cell, a step which has been shown to be essential for sustained response to AngII. Whether ANP acts directly on Ca^{2+}-channels via a G-protein or indirectly through cyclic GMP as demonstrated in vascular smooth muscle cell [24] is still unknown. ANP and PTK inhibitors might thus act on two different but complementary signalling pathways of AngII leading to aldosterone synthesis in ZGB cells.

Considering the specific inhibitory effect of tyrphostin on protein tyrosine kinase activity, its micromolar potency and its marked inhibitory action on aldosterone biosynthesis, the involvement of protein tyrosine kinases in the regulatory pathway of aldosterone biosynthesis appears established. The identification of these kinases and their substrates will, however, be required for proper understanding of their role as regulators of adrenal steroidogenesis.

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