The role of tyrosine kinases in capacitative calcium influx-mediated aldosterone production in bovine adrenal zona glomerulosa cells

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Abstract

In adrenal glomerulosa cells, the stimulation of aldosterone biosynthesis by angiotensin II (Ang II) involves the activation of a capacitative Ca^{2+} influx through calcium release-activated calcium (CRAC) channels. In various mammalian cell systems, it has been shown that CRAC channel activation and Ca^{2+} entry require tyrosine kinase activity. We have therefore examined in this work whether similar mechanisms contribute to Ang II-induced mineralocorticoid biosynthesis. In fluo-3-loaded isolated bovine glomerulosa cells, two inhibitors of tyrosine kinases, genistein and methyl-2,5-dihydroxycinnamate (MDHC) (100 μM) prevented capacitative Ca^{2+} entry elicited by Ang II (by 54 and 62% respectively), while the inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase, lavendustin A, was without effect. Similar results were observed on Ca^{2+} influx triggered by thapsigargin, an inhibitor of microsomal Ca^{2+} pumps. The

inhibitors blocked Ang II-stimulated pregnenolone and aldosterone production in the same rank order. In addition to its specific effect on capacitative Ca^{2+} influx, genistein also affected the late steps of the steroidogenic pathway, as shown by experiments in which the rate-limiting step (intramitochondrial cholesterol transfer) was bypassed with 25-OH-cholesterol (25-OH-Chol), cytosolic calcium was clamped at stimulated levels or precursors of the late enzymatic steps were supplied. In contrast, genistin, a structural analogue of genistein devoid of tyrosine kinase inhibitory activity, was almost without effect on pregnenolone or 11-deoxycorticosterone (DOC) conversion to aldosterone. These results suggest that, in bovine adrenal glomerulosa cells, Ang II promotes capacitative Ca^{2+} influx and aldosterone biosynthesis through tyrosine kinase activation.

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Introduction

The involvement of tyrosine kinases in the signal transduction processes triggered by various growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor is now clearly established (Marshall 1995). In addition, it appears that protein tyrosine phosphorylation is also involved in a variety of responses not directly related to cell growth. For example, tyrosine kinase activity participates in the control of smooth muscle contractility (Yang *et al.* 1993). The mechanisms of cytosolic Ca^{2+} homeostasis are also known targets for tyrosine kinases: for instance, phospholipase $\text{C}\gamma_1$ interacts directly with tyrosine kinase receptors through its SH2 domains, an interaction which leads to phospholipase phosphorylation and activation, and therefore to the formation of inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) and to the release of Ca^{2+} from internal stores (Marrero *et al.* 1994, Rhee & Bae 1997). Furthermore, it has been shown that tyrosine kinase inhibitors, such as genistein and methyl-2,5-dihydroxycinnamate (MDHC), inhibit ADP- and

thapsigargin-stimulated Ca^{2+} entry in human platelets (Sargeant *et al.* 1993) and that bradykinin- and thapsigargin-induced Ca^{2+} influx into human foreskin fibroblasts can be blocked by tyrosine kinase inhibitors (Lee *et al.* 1993).

In adrenal zona glomerulosa cells, the octapeptide hormone, angiotensin II (Ang II) is, with extracellular potassium, the main regulator of aldosterone biosynthesis. Ang II elicits biphasic changes in cytosolic free calcium concentration, $[\text{Ca}^{2+}]_c$: an initial peak of $[\text{Ca}^{2+}]_c$ resulting from $\text{Ins}(1,4,5)\text{P}_3$ -induced emptying of intracellular stores is followed by a sustained calcium influx mainly due to capacitative Ca^{2+} entry through calcium release-activated calcium (CRAC) channels (Putney & Bird 1993, Burnay *et al.* 1994). This sustained Ca^{2+} influx is known to be essential for a full steroidogenic response (Burnay *et al.* 1994). It was therefore of interest to study whether, like in other cell types, tyrosine kinases can regulate CRAC channel activity and steroidogenic response in adrenal glomerulosa cells. Using freshly isolated bovine glomerulosa cells, we have examined the effect of two tyrosine

kinase inhibitors on the $[Ca^{2+}]_c$ signals and aldosterone production elicited by Ang II and thapsigargin, an inhibitor of microsomal Ca^{2+} pumps and an activator of the capacitative pathway.

Materials and Methods

Materials

Nicardipine, 25-hydroxycholesterol (25-OH-Chol), Hepes, insulin, transferrin, sodium selenite were purchased from Sigma (St Louis, MO, USA). Thapsigargin, genistein, genistin, MDHC and lavendustin A were obtained from Anawa (Zurich, Switzerland). Ionomycin was purchased from Calbiochem (Lucerne, Switzerland) and $[Ile^5]$ AngII from Bachem AG (Bubendorf, Switzerland). Dispace (grade II) was obtained from Boehringer Mannheim (Indianapolis, IN, USA) and Percoll from Pharmacia (Piscataway, NJ, USA). Horse serum, fetal calf serum, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY, USA). Fluo-3 acetoxymethylester (fluo-3/AM) and pluronic acid were purchased from Molecular Probes (Eugene, OR, USA) and metyrapone from Aldrich (Milwaukee, WI, USA). $[^3H]$ pregnenolone was obtained from DuPont NEN (Boston, MA, USA), rabbit polyclonal antibody directed against pregnenolone from Biogenesis (Poole, Dorset, UK), and WIN 19'758 (cyanoketone) was kindly donated by Sterling-Winthrop (Rensselaer, NY, USA).

Isolation and culture of bovine glomerulosa cells

Bovine adrenal glomerulosa cells were prepared by enzymatic dispersion with dispace and purified on a Percoll gradient, as described in detail elsewhere (Rossier *et al.* 1993). Cells were then either used the same day, for measurement of $[Ca^{2+}]_c$ and aldosterone or pregnenolone production, or cultured on 24-well tissue culture plates. In the latter case, cells were transferred into antibiotic-containing DMEM supplemented with 2 µg/ml insulin, 2 µg/ml transferrin, 2 ng/ml sodium selenite, 5 µM metyrapone, 2 mM glutamine, 6 units/ml nystatin, 2% (vol/vol) fetal calf serum and 10% (vol/vol) horse serum, plated in 24-well tissue culture dishes (500 000 cells/well) and incubated overnight at 37 °C in 5% CO₂. The next day, the medium was removed and replaced with serum-free DMEM and the cells were used 3–4 days after plating as described elsewhere (Rossier *et al.* 1993).

Measurement of $[Ca^{2+}]_c$

The determination of $[Ca^{2+}]_c$ in cell populations was performed with the fluorescent probe fluo-3. For this

purpose, cells purified on Percoll gradients were washed twice and resuspended in a modified Krebs–Ringer medium (containing 136 mM NaCl, 1.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5 mM NaHCO₃, 1.2 mM CaCl₂ and 5.5 mM D-glucose and buffered at pH 7.4 with 20 mM Hepes) at a concentration of 10⁷ cells/ml, then incubated at 37 °C for 60 min before being washed and incubated again for 45 min in the presence of 6 µM Fluo-3/AM and 12.5% (w/v) pluronic acid. Excess dye was washed out, and the cells were kept at ambient temperature in the same medium. Aliquots (3 × 10⁶ cells) were sedimented just before use and resuspended in 2 ml Krebs–Ringer medium in a thermostated cuvette at 37 °C. Fluo-3 fluorescence (excitation at 470 nm and emission at 540 nm) was recorded with a Jasco CAF-110 fluorescence spectrometer (Hachioji City, Japan) and $[Ca^{2+}]_c$ was calibrated as previously described for fura-2 (Gryniewicz *et al.* 1985), using a K_d of 390 nM.

Determination of steroid production

After dispersion and washing, freshly prepared glomerulosa cells were resuspended in 50 ml modified Krebs–Ringer medium, preincubated for 60 min at 37 °C before being washed twice and distributed at a final concentration of 500 000 cell/ml in test tubes containing various agonists. Cells were then incubated for 90 min at 37 °C. Experiments with clamped cytosolic calcium were performed as described elsewhere (Python *et al.* 1995). Briefly, cells were resuspended in Ca²⁺-free Krebs–Ringer buffer (containing 0.2 mM EGTA) and incubated for 120 min at 37 °C in the presence of 2 µM ionomycin, of various concentrations of total extracellular calcium, $[Ca^{2+}]_{ext}$, and of various tyrosine kinase inhibitors. At the end of the incubation period, cells were sedimented by centrifugation, and the aldosterone content in the medium was measured by direct radioimmunoassay using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX, USA).

When cells were used in primary culture, the serum-free medium was replaced with modified Krebs–Ringer medium at 37 °C at least 1 h before starting the experiment. Cells were then incubated in the same medium containing various agonists for 90 min. After 30 min incubation, media were replaced with fresh media of identical composition, and aldosterone was determined in the supernatant after 60 min of further incubation. Cellular protein content was measured in each well using the Coomassie blue method of Bradford (1976).

For pregnenolone production, Win 19'758 (5 µM) was included in the incubation medium to prevent conversion of pregnenolone into progesterone. The pregnenolone content in the medium was determined by direct radioimmunoassay using $[^3H]$ pregnenolone (Python *et al.* 1995).

Figure 1 Effect of genistein (Gen), MDHC and lavendustin A (Lav) on the capacitative Ca^{2+} influx induced by Ang II or thapsigargin (Thapsi). Fluo-3-loaded bovine glomerulosa cells were challenged with Ang II (A, C) or Thapsi (B, D), in the presence of nicardipine (Nic), and Gen was added either 3 min after (A, B) or before (C, D) stimulation. E, F: Effect of MDHC and Lav respectively, on the Ang II-induced $[Ca^{2+}]_c$ response. Each trace is representative of five independent experiments.

Statistics

Statistical significance of differences was assessed by the Student's *t*-test and is indicated with the two-tailed *P* value.

Results

Effect of tyrosine kinase inhibitors on capacitative Ca^{2+} influx in bovine glomerulosa cells

We first examined the effect of genistein, a known inhibitor of tyrosine kinases, on Ang II- and thapsigargin-induced capacitative Ca^{2+} influx. For this purpose, freshly

prepared, fluo-3-loaded bovine glomerulosa cells were challenged with Ang II or thapsigargin and genistein (100 μ M) was added either 3 min after or 3 min before stimulation. Nicardipine (2 μ M), a dihydropyridine blocker of voltage-gated Ca^{2+} channels, was present in all experiments, in order to isolate the capacitative component of the Ca^{2+} influx response (Burnay *et al.* 1994). Ang II induced the expected biphasic $[Ca^{2+}]_c$ response and genistein reduced the sustained phase by $54.1 \pm 16.2\%$ ($n=5$, $P<0.01$ versus controls) when added during Ca^{2+} influx (Fig. 1A and Table 1). Similarly, genistein significantly decreased the plateau $[Ca^{2+}]_c$ response elicited by thapsigargin by $62.4 \pm 16.1\%$ ($n=5$, $P<0.01$) (Fig. 1B and Table 1). When genistein was added before stimulation,

Table 1 Effect of genistein on Ang II- and thapsigargin-induced $[Ca^{2+}]_c$ changes in bovine adrenal glomerulosa cells. Fluo-3-loaded bovine glomerulosa cells were challenged with Ang II or thapsigargin in the presence of nicardipine and genistein was added 3 min after stimulation. The effect of genistein was calculated as the mean of the percent differences ($\Delta\%$) between plateau $[Ca^{2+}]_c$ measured before and after genistein addition in each experiment ($n=5$)

	Angiotensin II 10 nM ($n=5$)	Δ (%)	Thapsigargin 2 μ M ($n=5$)	Δ (%)
$[Ca^{2+}]_c$ (nM)				
Basal	242 $\pm$ 49		268 $\pm$ 42	
Basal+nicardipine	226 $\pm$ 48		251 $\pm$ 41	
Peak	431 $\pm$ 75			
Plateau	253 $\pm$ 40		295 $\pm$ 43	
Plateau+genistein	232 $\pm$ 39	- 54 $\pm$ 16 $P<0.01$	284 $\pm$ 43	- 62 $\pm$ 16 $P<0.01$

the plateau phase of the $[Ca^{2+}]_c$ response to Ang II was practically abolished (Fig. 1C) and the response to thapsigargin was no longer sustained (Fig. 1D). No significant effect of genistein was observed on the amplitude of the initial $[Ca^{2+}]_c$ peak elicited by Ang II (243 ± 78 nM above basal $[Ca^{2+}]_c$ in the absence of genistein and 166 ± 11 nM above basal with genistein pretreatment; $P=0.42$, $n=5$)

MDHC (100 μ M), another tyrosine kinase inhibitor, also exerted a significant blockade of Ang II-induced capacitative Ca^{2+} influx ($40.6 \pm 3\%$ inhibition, $n=5$, $P<0.01$ versus controls) (Fig. 1E). In contrast, the inhibitor of EGF receptor tyrosine kinase, lavendustin A, at the reported optimal concentration, was without effect on the $[Ca^{2+}]_c$ response to Ang II (Fig. 1F).

Effect of tyrosine kinase inhibitors on Ang II-induced steroidogenesis in bovine glomerulosa cells

Because an activation of CRAC channels is known to be crucial for a full steroidogenic response to Ang II, the inhibition of capacitative Ca^{2+} influx should be matched by a reduction in steroid biosynthesis. Indeed, in freshly isolated bovine glomerulosa cells, both genistein and MDHC inhibited Ang II-induced formation of pregnenolone, the first step of the steroidogenic cascade, in a concentration-dependent manner, with an IC_{50} of approximately 80 μ M (Fig. 2A). Interestingly, while MDHC prevented Ang II-stimulated aldosterone production with similar efficacy, the IC_{50} of genistein was much lower (11.0 ± 1.4 μ M, $n=3$) (Fig. 2B).

As it can be seen in Fig. 3A and B, genistein and MDHC inhibited Ang II-stimulated pregnenolone and aldosterone production, measured in the presence of nicardipine, with the same order of potency observed for inhibition of the capacitative Ca^{2+} influx. In contrast,

lavendustin, which was without effect on Ca^{2+} entry, did not affect steroid production.

Effect of tyrosine kinase inhibitors on 25-OH-cholesterol-induced steroidogenesis

The efficacy of genistein (Fig. 3B) suggested that this drug might inhibit aldosterone production by affecting not only the Ca^{2+} messenger system but also some steps of the steroidogenic cascade. To test this hypothesis, we measured pregnenolone and aldosterone production in bovine glomerulosa cells in response to 25-OH-Chol, a hydrophilic derivative of cholesterol that bypasses the rate-limiting step of intramitochondrial transport. As shown in Fig. 4A and B, while none of the three tested tyrosine kinase inhibitors affected 25-OH-Chol-supported pregnenolone production, genistein (100 μ M) markedly reduced 25-OH-Chol-stimulated aldosterone biosynthesis (by $50.5 \pm 6.2\%$, $n=5$, $P<0.05$). In contrast, MDHC (100 μ M) and lavendustin A (1 μ M) did not alter steroid production in response to 25-OH-Chol.

Effect of tyrosine kinase inhibitors on Ca^{2+} -induced steroidogenesis

That genistein might impact on steps located downstream of the generation of the Ca^{2+} signal was further confirmed in experiments in which $[Ca^{2+}]_c$ was clamped at various levels in bovine glomerulosa cells (Python *et al.* 1995). While Ca^{2+} elicited the expected increase in aldosterone output in a concentration-dependent manner, this response was markedly inhibited by genistein (Fig. 5).

Effect of genistein on the conversion of progesterone and deoxycorticosterone to aldosterone

To further localize the additional site of the inhibitory action of genistein, we examined its effect on the

Figure 2 Concentration dependency of the effect of genistein and MDHC on Ang II-induced steroidogenesis. A) Freshly isolated bovine glomerulosa cells were incubated for 90 min at 37 °C as described under Materials and Methods. Pregnenolone production was stimulated with 10nM Ang II, in the presence of increasing concentrations of genistein (■) and MDHC (●). Pregnenolone levels in the medium were expressed as percentage of control stimulations in the presence of Ang II alone (33.5 ± 4.9 ng/ 10^6 cell/90 min). Each point is the mean value $\pm$ S.E.M. of between three and four independent experiments. B) Aldosterone production was stimulated as above and expressed as percentage of control stimulations (11.7 ± 1.8 ng/ 10^6 cell/90 min). Each point is the mean value $\pm$ S.E.M. of between four and five independent experiments.

conversion of progesterone (10 μ M) or 11-deoxycorticosterone (DOC, 10 μ M) to aldosterone. Aldosterone formation from both precursors was inhibited in a concentration-dependent manner and with similar potency by genistein (Fig. 6). Interestingly, genistin (100 μ M), a structural analogue of genistein exhibiting little or no effect on tyrosine kinase activity, did not significantly alter either progesterone or DOC conversion to aldosterone (Table 2).

Figure 3 Effect of various tyrosine kinase inhibitors on steroidogenesis induced by capacitative calcium influx. Cultured bovine glomerulosa cells were prepared as described under Materials and Methods. Cells were stimulated for 60 min with Ang II (10 nM), in the presence of nifedipine (1 μ M) and either 100 μ M genistein, 100 μ M MDHC or 1 μ M lavendustin A and pregnenolone (A) or aldosterone (B) production was determined as described. Each point is the mean value $\pm$ S.E.M. of four independent experiments performed in triplicate. *Significantly different from controls in the presence of Ang II alone, with $P < 0.05$.

Discussion

The capacitative influx of calcium through CRAC channels plays a major role in the mechanism of activation of aldosterone biosynthesis by the octapeptide hormone, Ang II (Ely *et al.* 1991, Hajnoczky *et al.* 1991, Burnay *et al.* 1994, Rohacs *et al.* 1994). Although the cascade of events leading to the mobilization of Ca^{2+} from intracellular, $\text{Ins}(1,4,5)\text{P}_3$ -sensitive stores in adrenal glomerulosa cells

Figure 4 Effect of various tyrosine kinase inhibitors on 25-OH-Chol-supported steroidogenesis. Cultured bovine glomerulosa cells were prepared as described under Materials and Methods. Cells were stimulated for 60 min with 25-OH-Chol (10 µM), in the presence of nicardipine (1 µM) and either 100 µM genistein, 100 µM MDHC or 1 µM lavendustin A and pregnenolone (A) or aldosterone (B) production was determined as described. Each point is the mean value $\pm$ S.E.M. of four independent experiments performed in triplicate. Basal:steroid production in the absence of 25-OH-Chol. *Significantly different from controls in the presence of Ang II alone, with $P < 0.05$.

has been fairly well investigated (Barrett *et al.* 1989, Capponi & Rossier 1996), the link between Ca^{2+} pool emptying and activation of CRAC channels remains to be elucidated. Several mechanisms such as calcium influx factor (CIF), G-proteins, protein-protein interaction, etc. have been invoked (Berridge 1995, Parekh & Penner 1997). Among these potential modulators of capacitative Ca^{2+} influx, tyrosine kinases could play an important role. Indeed, various studies have shown that tyrosine kinase inhibitors prevent the $[\text{Ca}^{2+}]_c$ response induced by thapsigargin or a Ca^{2+} -mobilizing agonist (Lee *et al.* 1993, Sargeant *et al.* 1993, Pfeiffer *et al.* 1995, Flemming *et al.* 1996, Sharma & Davies 1996, Takemura *et al.* 1997). The present work was therefore designed to assess whether, in bovine glomerulosa cells, tyrosine kinases

Figure 5 Effect of genistein on aldosterone production in Ca^{2+} -clamped glomerulosa cells. Freshly isolated bovine glomerulosa cells were submitted to a cytosolic Ca^{2+} clamp for 2 h, as described under Materials and Methods, in the absence ($\square$) or in the presence ($\blacksquare$) of genistein (100 µM). Aldosterone levels in the medium were expressed as percentage of control values measured in the absence of added extracellular Ca^{2+} ($[\text{Ca}^{2+}]_{\text{out}}$). Each point is the mean value $\pm$ S.E.M. of between six and ten independent experiments.

contribute to the well characterized capacitative Ca^{2+} influx and to the subsequent biological response, aldosterone biosynthesis.

The major finding of the present work is that two known inhibitors of tyrosine kinases, genistein (Constantinou & Huberman 1995) and MDHC (Umezawa *et al.* 1990), blocked Ang II-induced capacitative Ca^{2+} influx in bovine adrenal glomerulosa cells. In these cells, Ca^{2+} influx through voltage-gated T- and L-type channels amounts to approximately 25% of the total influx under Ang II challenge (Burnay *et al.* 1994). Experiments were therefore performed in the presence of nicardipine, a dihydropyridine blocker, to ascertain that the observed Ca^{2+} entry could only occur through CRAC channels. Moreover, both tyrosine kinase inhibitors also blocked the sustained $[\text{Ca}^{2+}]_c$ response to thapsigargin, which is known to be exclusively due to capacitative influx (Takemura *et al.* 1989). A third inhibitor, lavendustin A, reportedly more specific of EGF receptor tyrosine kinase (Hsu *et al.* 1991), did not affect Ang II- or thapsigargin-induced Ca^{2+} influx. Importantly, $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} release remained unaffected by tyrosine kinase inhibition, a finding which puts tyrosine kinase activation by Ang II downstream of the mobilization of Ca^{2+} from intracellular stores, as suggested by others on the basis of purely pharmacological studies (Bodart *et al.* 1995).

Figure 6 Effect of genistein on the conversion of progesterone and DOC to aldosterone. Freshly isolated bovine glomerulosa cells were incubated for 90 min at 37 °C as described under Materials and Methods, in the presence of 10 µM progesterone (■) or 10 µM DOC (●) and various concentrations of genistein. Aldosterone content in the medium was expressed as percentage of controls in the absence of genistein (16.2 ± 1.3 ng/ 10^6 cell/90 min for progesterone and 66.3 ± 6.1 ng/ 10^6 cell/90 min for DOC). Each point is the mean value $\pm$ S.E.M. of between four and six independent experiments.

The biological response to Ang II in various target organs has already been shown to be altered by inhibitors of tyrosine kinase. Thus, for example, in longitudinal strips from guinea pig gastric smooth muscle, tyrosine kinase inhibitors selectively and completely blocked Ang II-mediated contraction (Yang *et al.* 1993). Tyrosine kinase inhibitors also decreased Ang II-stimulated aldosterone production in rat adrenal capsules and cultured bovine glomerulosa cells (Bodart *et al.* 1995, Kapas *et al.* 1995). In

fact, Ang II can activate tyrosine kinase activity in rat adrenal capsules (Kapas *et al.* 1995). However, in none of these studies, a functional link with Ca^{2+} influx was demonstrated.

Since the full aldosterone response to Ang II crucially depends upon capacitative Ca^{2+} influx (Burnay *et al.* 1994), agents that alter this influx should also affect mineralocorticoid biosynthesis. Indeed, both genistein and MDHC behaved as powerful anti-steroidogenic compounds, preventing both pregnenolone and aldosterone production. While the potency of these two agents was similar on the first step of the steroidogenic cascade (pregnenolone biosynthesis), genistein appeared to act also on downstream steps since it exerted additional inhibitory effects on Ang II-stimulated aldosterone production. Importantly, lavendustin A, which was without effect on the capacitative influx, did not affect the pregnenolone and aldosterone response to Ang II, thus confirming the causal link between CRAC channel activation by Ang II and the subsequent steroid output.

The hypothesis of additional impact sites for some tyrosine kinase inhibitors along the steroidogenic cascade was further confirmed by three different approaches. First, we performed experiments in which the rate-limiting step of intramitochondrial cholesterol supply to the P450_{sc} enzyme was bypassed by the addition of 25-OH-Chol. While none of the tested inhibitors affected the early steps of the steroidogenic cascade, genistein clearly inhibited 25-OH-Chol-supported aldosterone production. As expected, lavendustin A was without effect. Secondly, we stimulated steroidogenesis by mimicking a Ca^{2+} signal in bovine glomerulosa cells through a calcium clamp (Python *et al.* 1995), thus short-circuiting Ca^{2+} influx mechanisms. Under these conditions, genistein completely abolished Ca^{2+} -induced aldosterone production. Thirdly, we focused on the late steps of the steroidogenic pathway by measuring progesterone and DOC conversion to aldosterone. Once again, genistein inhibited in a concentration-dependent manner both processes, therefore suggesting that a genistein-sensitive tyrosine kinase also activates one

Table 2 Effect of genistein and genistin on conversion of progesterone and DOC to aldosterone in bovine adrenal glomerulosa cells. Bovine adrenal glomerulosa cells in primary culture were incubated in the presence of either no precursor (basal), progesterone (10 µM) or DOC (10 µM), with or without genistein (100 µM) or genistin (100 µM). At the end of a 2-h incubation period, aldosterone was determined in the medium and expressed as percentage of respective controls (112.6 ± 59.0 , 1024.6 ± 100.1 and 1246 ± 94.2 fmol/mg protein for basal, progesterone and DOC respectively). Data are the means $\pm$ S.D. of triplicate determinations from two independent experiments

	Basal	+ Progesterone (10 µM)	+ DOC (10 µM)
Control	100	100	100
Genistein (100 µM)	$2.5 \pm 2.9^{**}$	$37.3 \pm 12.9^{**}$	$58.6 \pm 24.4^*$
Genistin (100 µM)	67.1 ± 8.5	102.5 ± 5.1	81.1 ± 6.4

* $P < 0.05$, ** $P < 0.01$.

of the very late steps, the conversion of either DOC to corticosterone or of corticosterone to aldosterone. Importantly, genistein, an inactive structural analogue of genistein, was unable to reproduce the inhibitory effects of genistein. The different pharmacology of the two inhibitory effects of genistein on capacitative Ca^{2+} influx and on the late steps of aldosterone biosynthesis suggests that distinct tyrosine kinases may be involved in these processes.

In summary, we have shown that, in bovine adrenal glomerulosa cells, Ang II promotes capacitative Ca^{2+} influx and aldosterone biosynthesis through tyrosine kinase activation. The identity and the mechanisms of activation of tyrosine kinase(s) remain to be defined.

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References

- Barrett PQ, Bollag WB, Isales CM, McCarthy RT & Rasmussen H 1989 Role of calcium in angiotensin II-mediated aldosterone secretion. *Endocrine Reviews* **10** 496–518.
- Berridge MJ 1995 Capacitative calcium entry. *Biochemical Journal* **312** 1–11.
- Bodart V, Ong H & De Ljan A 1995 A role for protein tyrosine kinase in the steroidogenic pathway of angiotensin II in bovine zona glomerulosa cells. *Journal of Steroid Biochemistry and Molecular Biology* **54** 55–62.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Burnay MM, Python CP, Vallotton MB, Capponi AM & Rossier MF 1994 Role of the capacitative calcium influx in the activation of steroidogenesis by angiotensin II in adrenal glomerulosa cells. *Endocrinology* **135** 751–758.
- Capponi AM & Rossier MF 1996 Regulation of aldosterone secretion. *Current Opinion in Endocrinology and Diabetes* **3** 248–257.
- Constantinou A & Huberman E 1995 Genistein as an inducer of tumor cell differentiation: possible mechanisms of action. *Proceedings of the Society for Experimental Biology and Medicine* **208** 109–115.
- Ely JA, Ambroz C, Baukal AJ, Christensen SB, Balla T & Catt KJ 1991 Relationship between agonist- and thapsigargin-sensitive calcium pools in adrenal glomerulosa cells. Thapsigargin-induced Ca^{++} mobilization and entry. *Journal of Biological Chemistry* **266** 18635–18641.
- Flemming I, Fisslthaler B & Busse R 1996 Interdependence of calcium signalling and protein tyrosine phosphorylation in human endothelial cells. *Journal of Biological Chemistry* **271** 11009–11015.
- Grynkiewicz G, Poenie M & Tsien RY 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260** 3440–3450.
- Hajnoczky G, Varnai P, Hollo Z, Christensen SB, Balla T, Enyedi P & Spat A 1991 Thapsigargin-induced increase in cytoplasmic Ca^{++} concentration and aldosterone production in rat adrenal glomerulosa cells: interaction with potassium and angiotensin-II. *Endocrinology* **128** 2639–2644.
- Hsu CY, Persons PE, Spada AP, Bednar RA, Levitzki A & Zilberstein A 1991 Kinetic analysis of the inhibition of the epidermal growth factor receptor tyrosine kinase by lavendustin-A and its analogue. *Journal of Biological Chemistry* **266** 21105–21112.
- Kapas S, Purbrick A & Hinson JP 1995 Role of tyrosine kinase and protein kinase C in the steroidogenic actions of angiotensin II, alpha-melanocyte-stimulating hormone and corticotropin in the rat adrenal cortex. *Biochemical Journal* **305** 433–438.
- Lee KM, Toscas K & Villereal ML 1993 Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *Journal of Biological Chemistry* **268** 9945–9948.
- Marrero MB, Paxton WG, Duff JL, Berk BC & Bernstein KE 1994 Angiotensin II stimulates tyrosine phosphorylation of phospholipase C- γ in vascular smooth muscle cells. *Journal of Biological Chemistry* **269** 10935–10939.
- Marshall CJ 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80** 179–185.
- Parekh AB & Penner R 1997 Store depletion and calcium influx. *Physiological Reviews* **77** 901–930.
- Pfeiffer F, Schmid A & Schulz I 1995 Capacitative Ca^{2+} influx and a Ca^{2+} -dependent nonselective cation pathway are discriminated by genistein in mouse pancreatic acinar cells. *Pfluegers Arch: European Journal of Physiology* **430** 916–922.
- Putney JW & Bird GS 1993 The signal for capacitative calcium entry. *Cell* **75** 199–201.
- Python CP, Laban OP, Rossier MF, Vallotton MB & Capponi AM 1995 The site of action of Ca^{2+} in the activation of steroidogenesis: studies in Ca^{2+} -clamped bovine adrenal zona-glomerulosa cells. *Biochemical Journal* **305** 569–576.
- Rhee SG & Bae YS 1997 Regulation of phosphoinositide-specific phospholipase C isozymes. *Journal of Biological Chemistry* **272** 15045–15048.
- Rohacs T, Bago A, Deak F, Hunyady L & Spät A 1994 Capacitative calcium influx in adrenal glomerulosa cells: possible role in angiotensin II response. *American Journal of Physiology* **36** C1246–C1252.
- Rossier MF, Python CP, Capponi AM, Schlegel W, Kwan CY & Vallotton MB 1993 Blocking T-type calcium channels with tetrandrine inhibits steroidogenesis in bovine adrenal glomerulosa cells. *Endocrinology* **132** 1035–1043.
- Sargeant P, Farndale RW & Sage SO 1993 ADP- and thapsigargin-evoked Ca^{2+} entry and protein-tyrosine phosphorylation are inhibited by the tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate in fura-2-loaded human platelets. *Journal of Biological Chemistry* **268** 18151–18156.
- Sharma NT & Davies MJ 1996 Calcium entry activated by store depletion in coronary endothelium is promoted by tyrosine phosphorylation. *American Journal of Physiology* **270** H267–H274.
- Takemura H, Hughes AR, Thastrup O & Putney JW 1989 Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool, and not an inositol phosphate, regulates calcium fluxes at the plasma membrane. *Journal of Biological Chemistry* **264** 12266–12271.
- Takemura H, Imoto K, Sakano S, Kaneko M & Ohshika H 1997 Involvement of tyrosine kinase in capacitative Ca^{2+} entry pathway in rat glioma cells. *Research Communications in Molecular Pathology and Pharmacology* **98** 127–140.
- Umezawa K, Hori T, Tajima H, Imoto M, Isshiki K & Takeuchi T 1990 Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS Letters* **260** 198–200.
- Yang SG, Saifeddine M, Laniyonu A & Hollenberg MD 1993 Distinct signal transduction pathways for angiotensin II guinea pig gastric smooth muscle: differential blockade by indomethacin and tyrosine kinase inhibitors. *Journal of Pharmacology and Experimental Therapeutics* **264** 958–966.

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