A role for src tyrosine kinase in regulating adrenal aldosterone production

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ABSTRACT

Adrenal aldosterone synthesis is influenced by a variety of factors. The major physiological regulators of aldosterone production are angiotensin II (Ang II) and potassium (K^+). Ang II stimulates aldosterone production through the activation of multiple intracellular signaling pathways. It has recently been demonstrated that Ang II activates src tyrosine kinases in vascular smooth muscle cells. The src family of tyrosine kinases are widely distributed non-receptor kinases that influence several signal transduction pathways. In the present study we evaluated the effect of a selective src family inhibitor, PP2, on aldosterone production using a human adrenocortical carcinoma-derived (H295R) cell line. Treatments for 6 or 48 h with PP2 (0·3 μM-10 μM) inhibited basal, Ang II, K^+ and dibutyryladenosine cyclic monophosphate (db-cAMP) stimulation of aldosterone production in a concentration-dependent manner. PP2 did not affect cell viability at any of the concentrations tested. Moreover, time course studies using PP2 (10 μM) for 6, 12, 24, and 48 h revealed a time-dependent inhibition of aldosterone production. Inhibition by PP2 (0·3–10 μM) was also observed for the metabolism of 22R-hydroxycholesterol (22R-OHChol) to aldosterone in H295R cells. Since 22R-OHChol is a substrate for cytochrome P450 side-chain cleavage enzyme (CYP11A) that does not require steroidogenic acute regulatory (StAR) protein for transport to the inner mitochondrial membrane, these results suggest that PP2 inhibition occurred beyond the rate-limiting step in aldosterone synthesis. Genistein, a non-specific tyrosine kinase inhibitor also blocked aldosterone production, but the inhibition was the result of a non-specific effect on 3β-hydroxysteroid dehydrogenase (3βHSD). In contrast, PP2 did not appear to act as a direct inhibitor of 3βHSD activity. To further investigate the site of PP2 action, we examined its effect on H295R cell metabolism of [^{14}C]progesterone using thin layer chromatography. PP2 treatment for 48 h caused an increase in the conversion of progesterone to 17α-hydroxyprogesterone. To determine if this apparent increase in 17α-hydroxylase activity was due to increased transcript, we examined the effect of PP2 on CYP17 mRNA. PP2 treatment caused an increase in CYP17 mRNA without an effect on 3βHSD mRNA levels. Inhibition of protein synthesis with cycloheximide increased basal levels of CYP17 mRNA levels and blocked the induction observed by PP2. This suggests that new protein synthesis is a necessary part of PP2 induction of CYP17. Taken together these data suggest that the src tyrosine kinase inhibitor, PP2, is a potent inhibitor of aldosterone production. One mechanism for the inhibition is through an induction of CYP17 mRNA and enzyme activity. Src tyrosine kinases, therefore, may be involved with the promotion of a glomerulosa phenotype through the inhibition of CYP17 expression.

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INTRODUCTION

Within the human adrenal, steroids are produced through the action of five forms of cytochrome P450 and 3β-hydroxysteroid dehydrogenase (3βHSD) (Simpson & Waterman 1992). It is the differential expression of these enzymes in the three adrenocortical zones that leads to the production of specific

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steroids within each zone (Suzuki *et al*. 2000). Like all steroidogenic cells, the glomerulosa cell uses pregnenolone as a precursor for steroidogenesis. Newly formed pregnenolone can be metabolized by either 3βHSD or 17α-hydroxylase,17,20-lyase (CYP17) and it is the relative expression of these enzymes that influences the synthesis of aldosterone versus cortisol or C_{19} steroids (Conley & Bird 1997). High 3βHSD expression combined with low CYP17 activity will favor aldosterone synthesis and oppose cortisol and adrenal androgen synthesis. In contrast, a high CYP17/3βHSD ratio supports C_{19} steroid biosynthesis but would inhibit aldosterone synthesis. The proposed importance of the ratio of these enzymes is supported by the lack of expression of CYP17 within the glomerulosa (Sasano *et al*. 1988, Suzuki *et al*. 2000). Therefore, understanding the mechanisms that positively and negatively regulate CYP17 is of primary importance.

We have previously demonstrated that angiotensin II (Ang II) promotes the production of aldosterone not only through acute actions (Clark *et al*. 1995) but also by increasing the expression of 3βHSD and inhibiting CYP17 expression (Bird *et al*. 1992, 1996*a*,*b*). Ang II signal transduction occurs through the action of calcium/calmodulin-dependent protein kinases, protein kinase C, and a number of tyrosine kinases (Bird *et al*. 1990, Quinn & Williams 1992, Berk & Corson 1997). Recent evidence suggests that Ang II can activate the src family of cytoplasmic tyrosine kinases (Ishida *et al*. 1995, Berk & Corson 1997, Sayeski *et al*. 1999). The availability of inhibitors of the src tyrosine kinases (Hanke *et al*. 1996) has allowed us to examine the role of src in adrenal cell production of aldosterone. We demonstrate that inhibition of src blocks the production of aldosterone. Part of the inhibition of aldosterone production appears to result from an increase in CYP17 activity and mRNA expression. This increase in CYP17 effectively removes substrate from the pathway leading to aldosterone. Experiments will be needed in the future to further define the role of the src kinases in adrenocortical function.

MATERIALS AND METHODS

Cell culture

H295R cells were cultured in Dulbecco's modified Eagle's and Ham's F-12 (DME/F12) medium (GIBCO BRL, Gaithersburg, MD, USA), supplemented with 2% Ultroser G (Biosepra SA, Villeneuve la Garenne Cedex, France), 1% ITS Plus (insulin, 6·25 μg/ml; transferrin, 6·25 μg/ml; selenium, 6·25 ng/ml; bovine serum albumin, 1·25 mg/ml; linoleic acid, 5·35 μg/ml) (Collaborative Research, Bedford, MT, USA) and antibiotics as previously described (Bird *et al*. 1993). Cells were subcultured onto 12-well culture dishes for steroid assay, 3βHSD activity assay and for thin layer chromatography experiments (5×10^5 cells/well). For experiments designed to study cellular RNA and pp60c-src activity, cells were plated onto 100 mm dishes (7×10^6 cells/plate) and used for experiments 48 h later.

Stimulation of steroid secretion and analysis of steroids

Prior to experiments, cells were maintained overnight in DME/F12 medium containing 0·1% Ultroser G and antibiotics (low serum medium). Where indicated, cells were then preincubated with PP2 (Calbiochem-Novabiochem Corporation, San Diego, CA, USA) for 30 min at 37 °C in fresh low serum medium. Ang II, K^+ and dibutyryladenosine cyclic monophosphate (dbcAMP) (Sigma-Aldrich, St Louis, MO, USA) were added to the cells and the incubation carried out at 37 °C for the indicated times. The aldosterone content of medium recovered from each well was determined against aldosterone standards prepared in low serum medium using an aldosterone radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX, USA). Results of aldosterone assay were normalized to the cellular protein content per well and expressed as pmol per mg cell protein.

Protein determination

Cells were solubilized in Tris–HCl (50 mM, pH 7·4) containing NaCl (150 mM), SDS (1%), EGTA (5 mM), $MgCl_2$ (0·5 mM), $MnCl_2$ (0·5 mM) and phenylmethylsulfonylfluoride (PMSF, 0·2 mM). The protein contents of samples were then determined by the bicinchoninic acid protein assay, using the BCA assay kit (Pierce, Rockford, IL, USA).

pp60c-src immune complex kinase assay

Src assay was carried out as previously described (Ishida *et al*. 1995). Cells were incubated with Ang II for 5 min. After a wash with phosphate-buffered saline, ice-cold cells were lysed in RIPA buffer (USBiological, Swampscott, MA, USA), scraped off the plate and centrifuged at 9000 r.p.m. in a microfuge (4 °C for 10 min); protein concentrations of the supernatants were determined as explained above. Lysates containing the same amount of soluble proteins were incubated overnight at 4 °C

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with the src antibody mAb327 (Oncogene Science Inc., Boston, MA, USA) and protein G-agarose (GIBCO BRL) to determine antibody complex precipitation. Precipitates were washed three times in buffer containing 50 mM Tris, pH 7·4; 150 mM NaCl; 0·1% Triton-X 100; 1 mM PMSF; 10 μg/ml leupeptin and 10 μg/ml aprotinin and twice in a buffer containing 20 mM PIPES, pH 7·0 and 10 mM $MnCl_2$. The precipitates were then suspended in the kinase reaction buffer (20 mM PIPES, pH 7·0; 10 mM $MnCl_2$ and 50 μM ATP) with 5 μg acid-denaturated (with 25 mM sodium acetate, pH 3·3, 30 °C, 5 min) rabbit muscle enolase (Sigma-Aldrich). The kinase reaction was started by the addition of 10 μCi [γ-^{32}P]ATP (3000 mCi/mmol) (Amersham) in 50 μl volume and run at 30 °C for 10 min. Reaction was finished by the addition of LDS-PAGE sample buffer (Novex, San Diego, CA, USA), samples were warmed 5 min at 65 °C and subjected to SDS-PAGE using a precast Novex gel electrophoresis system with 4–12% bis-tris NuPage gels (Novex). Dried gels were exposed to film and the amount of radioactivity was determined using phosphorimaging analysis (425E, Molecular Dynamics, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

Analysis of 3βHSD activity

3βHSD enzymatic activity was determined as previously described (Bird *et al*. 1996). Briefly, cells were rinsed with plain medium and incubated at 37 °C with 1·5 ml of a serum-free DME/F12 medium supplemented with 1 μM dehydroepiandrosterone (DHEA) and 100 000 d.p.m. [3α-^{3}H]DHEA. Radiolabel and kinase inhibitors were added simultaneously, and at the indicated times 100 μl of the medium were removed from each well and the volume was brought to 1 ml by adding 900 μl water. Radiolabeled steroids were then extracted by mixing with chloroform (3 ml), and phase separation achieved by brief centrifugation. An aliquot (750 μl) of the upper phase was recovered and mixed with an equal volume of charcoal/dextran (5%/0·5% w/v). Following centrifugation to pellet the charcoal, 1 ml of the aqueous phase was removed and radioactivity was determined in a β-counter. The 3βHSD enzymatic activity was then calculated after appropriate correction for volume, and expressed as pmol/mg cell protein.

Thin layer chromatographic identification of [^{14}C]progesterone metabolites

Cells treated for 48 h without (basal) or with PP2 (10 μM) were incubated for 6 h with serum-free medium containing [4-^{14}C]progesterone (150 000 d.p.m./well) (NEN Life Science Products Inc., Boston, MA, USA) and 0·5 μM unlabeled progesterone. At the end of the incubation, medium was extracted twice with dichloromethane (3 ml), dried and redissolved in 100 μl dichloromethane. The organic extracts were applied to silica gel plates (Keiselgel 60, F254, EM Industries, Hawthorne, NY, USA) and developed twice in chloroform-ethyl acetate, 90:10, vol/vol. Lanes containing radiolabeled samples were then scanned using a Bioscan detector (Bioscan Inc., Washington DC, USA). Peaks were identified by comparison to authenticated standards that were run on the same plate. Results are shown as percentage of total counts.

RNA extraction and Northern analysis

RNA was extracted from cells using Ultraspec RNA isolation system (Biotecx Laboratories Inc., Houston, TX, USA). Purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis prior to use. Samples of RNA (10 μg) were separated by electrophoresis on gels containing 1% agarose in the presence of formaldehyde. RNA was transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) by overnight blotting at 10 V and was cross-linked under UV light. Prehybridization was carried out at 42 °C for 6 h as previously described (Bird *et al*. 1995). Hybridizations were performed in the same composition buffer at 42 °C for 16 h, using antisense RNA probes. A 174 bp DNA fragment of the human 3βHSDII gene (GenBank accession no. M77144, nucleotides 7907–8080) and a 302 bp fragment of the human CYP17 gene (GenBank accession no. M14564, nucleotides 48–349) were cloned into pBluescript KS (Stratagene, La Jolla, CA, USA). To generate a radioactive riboprobe complementary to the cloned 3βHSD fragment, the plasmid was linearized with KpnI and used in a transcription reaction with [^{32}P]UTP (3000 Ci/mmol) (Amersham Pharmacia Biotech Inc.) and T7 RNA polymerase following the protocol from the MAXIscript T7/T3 kit (Ambion Inc., Austin, TX, USA). To generate a radioactive riboprobe complementary to the cloned CYP17 fragment, the plasmid was linearized with SacI and transcripted as above using T3 RNA polymerase. After hybridization, blots were washed in 2 × SSC containing 0·1% SDS at room temperature for 30 min, then in 0·1 × SSC containing 0·1% SDS at 37 °C for 30 min and at 42 °C for 15 min. Quantification of bound probe was determined using a PhosphorImager analysis (425E, Molecular Dynamics). Blots were subsequently exposed to film.

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Membranes were than stripped by repeated washing in 0·1 × SSC and 0·5% SDS at 80 °C and checked for lack of radioactivity before reprobing. Finally, membranes were probed with a 598 bp DNA segment from the human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene (GenBank accession no. M17851, nucleotides 185–782). Probe was generated with the Rediprime II DNA Labelling System (Amersham Pharmacia Biotech Inc.) and used in a hybridization reaction. Bound probe was quantified as described above. G3PDH transcript was used to normalize data for CYP17 and 3βHSD mRNA. For the cycloheximide (CX) experiments, CX (35 μM) was added to the plates 10 min before the addition of PP2. Because of the inhibitory effect of CX on G3PDH expression, loading was normalized by probing for 28S ribosomal RNA. 28S probe was prepared by incubation of the oligonucleotide 5′-AAA CGA TCA GAG TAG TGG TAT TTC ACC G-3′ with polynucleotide kinase in the presence of [γ-^{32}P]ATP (3000 mCi/mmol). Prehybridization and hybridization were performed exactly as described above.

RESULTS

Previous studies have shown that Ang II can activate the src signaling pathway (Hishida *et al*. 1995). Treatment of H295R cells for 5 min with Ang II stimulated the phosphorylation of the src substrate, rabbit muscle enolase, by 1·59 ± 0·13-fold (mean ± S.E. from three independent experiments). To determine if src tyrosine kinases play a role in aldosterone synthesis, the src kinase inhibitor PP2 (Hanke *et al*. 1996) was pre-incubated with H295R cells prior to stimulation with Ang II, K^+, or dbcAMP. As previously demonstrated, the H295R cell line responds to Ang II, K^+, and dbcAMP by increasing production of aldosterone (Bird *et al*. 1993) (Fig. 1). Treatment of cells for 6 h with Ang II (100 nM), K^+ (20 mM), or dbcAMP (1 mM) increased aldosterone by 2-fold, 2-fold, and 4-fold respectively (Fig. 1, upper panel). Chronic treatment (48 h) of the H295R cells increased the expression of enzymes involved in aldosterone and therefore had a greater fold increase in steroid production (Ang II, 6-fold; K^+, 4·7-fold; dbcAMP, 3-fold) (Fig. 1, lower panel). PP2 caused a concentration-dependent inhibition of aldosterone production under basal, Ang II, K^+, and dbcAMP stimulation. Inhibition was observed at both 6 and 48 h of treatment (Fig. 1). Half-maximal inhibition of aldosterone production was observed using between 1 and 3 μM PP2 for cell treatments. In H295R cells aldosterone production occurred in a

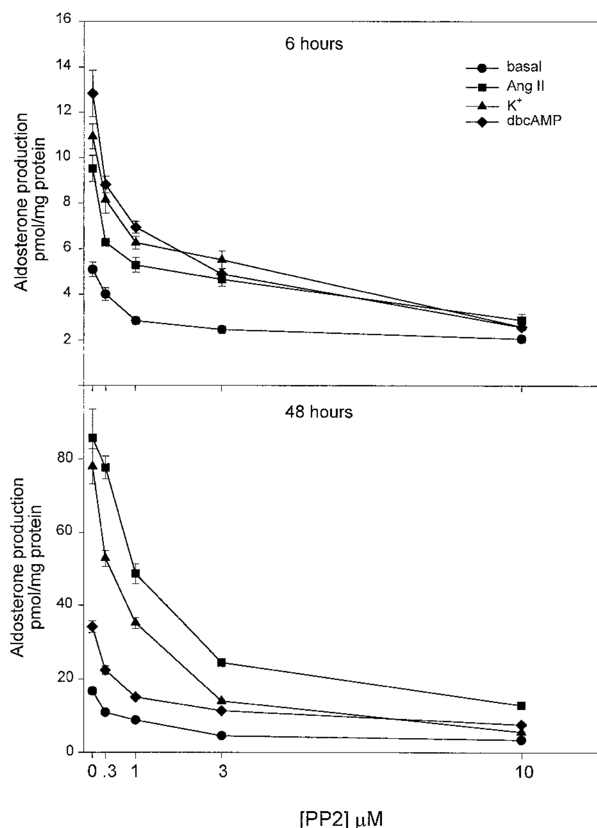

FIGURE 1. Effect of PP2 on agonist-stimulated aldosterone production in H295R cells. Cells were incubated for 6 h (upper panel) or 48 h (lower panel) with PP2 alone (0·3–10 μM) (basal) and with PP2 (0·3–10 μM) plus Ang II (100 nM), K^+ (20 mM) or dbcAMP (1 mM). The aldosterone content of the medium was determined by RIA and normalized to the tissue culture well protein content. Data points represent the mean ± S.E. of values from four separate culture wells expressed as pmol aldosterone/mg protein. Similar results were observed in two additional experiments.

time-dependent manner under basal and Ang II treatment (Fig. 2). Inclusion of PP2 significantly inhibited basal and Ang II-stimulated aldosterone production throughout the times examined (6, 12, 24, and 48 h).

The first step in the synthesis of aldosterone is the conversion of cholesterol to pregnenolone. This step occurs in the mitochondria and is rate-limiting. To determine if PP2 inhibited aldosterone production at the rate limiting step, we examined its effects on metabolism of 22R-hydroxycholesterol (22R-OHChol) (Fig. 3). 22R-OHChol enters the mitochondria of steroidogenic cells by-passing the normal transport mechanisms needed for

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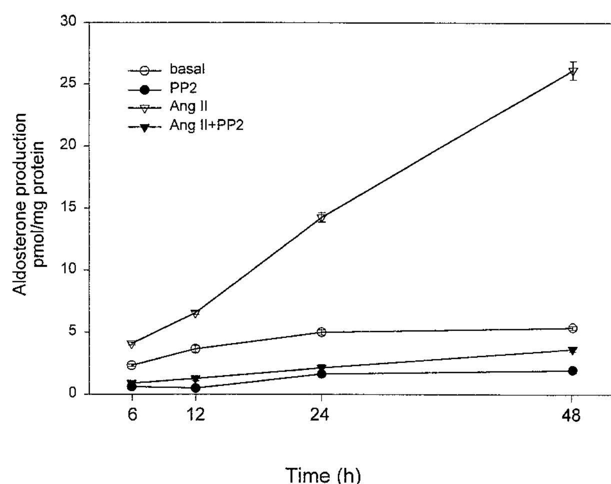

FIGURE 2. Effect of PP2 on the time-course of aldosterone production in response to Ang II. Cells were incubated for the times shown with Ang II (100 nM), PP2 (10 μM), Ang II (100 nM) plus PP2 (10 μM) or without any treatment (basal). The aldosterone content of the medium was determined by RIA and normalized to the tissue culture well protein content. Data points are the mean ± S.E. of determinations from three separate cell culture wells expressed as pmol aldosterone/mg protein. Similar results were observed in two additional experiments.

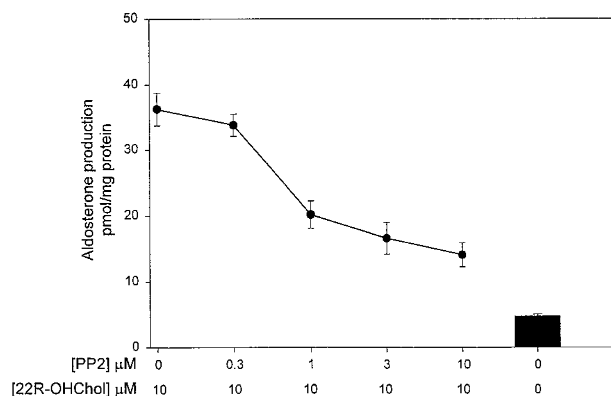

FIGURE 3. Effect of PP2 on 22R-hydroxycholesterol-stimulated aldosterone production in H295R cells. Cells were incubated for 6 h with 22R-OHChol (10 μM) alone or with increasing concentrations of PP2. The aldosterone content of the medium was determined by RIA and normalized to the tissue culture well protein content. Data points represent the mean ± S.E. of values from four separate culture wells. Similar results were observed in a second experiment.

cholesterol. Treatment with PP2 for 6 h inhibited the conversion of 22R-OHChol to aldosterone. The inhibition was concentration-dependent with a decrease of aldosterone by 61% observed at 10 μM PP2. These data suggested that PP2 inhibited aldosterone production in a manner beyond the regulation of cholesterol transport to the inner mitochondria membrane.

Treatments for 24 h with the general tyrosine kinase inhibitor, genistein, also inhibited aldosterone production when used alone (73%) or in association with Ang II (96%) (Fig. 4A). One report has suggested that genistein has a direct (albeit non-specific) effect on the activity of the enzyme 3βHSD (Wong & Keung 1999). To determine if either genistein or PP2 inhibited H295R 3βHSD activity, cells were incubated with PP2 or genistein and activity was determined. As shown in Fig. 4B, genistein completely inhibited 3βHSD activity while PP2 reduced the enzyme activity by no more than 25% at all times examined.

To determine if the overall metabolism of steroid precursor was modified by PP2, we examined the metabolism of radiolabeled progesterone (Fig. 5). H295R cells were incubated with PP2 (10 μM) for 36 h. Cells were then incubated with [^{14}C]progesterone (0·5 μM) for 6 h. Under basal conditions, very little progesterone was metabolized. However, treatment with PP2 caused a dramatic increase in progesterone metabolism to 17α-hydroxyprogesterone. These data suggest that PP2 increases the level of 17α-hydroxylase activity.

To determine the effect of PP2 on the expression of CYP17 mRNA, we carried out Northern analysis (Fig. 6). RNA was isolated from H295R cells under basal conditions as well as from cells treated with PP2 (10 μM), Ang II (100 nM), dbcAMP (1 mM) or Ang II (100 nM) plus PP2 (10 μM). As previously reported, Ang II and dbcAMP increased expression of 3βHSD. In addition, CYP17 transcript levels were increased by dbcAMP. However, PP2 caused a specific induction of CYP17 without an effect on 3βHSD mRNA. These data suggest that PP2 may inhibit aldosterone production, in part, by affecting the ratio of 3βHSD/CYP17 expression specifically by stimulating levels of CYP17 expression.

The transcription of some steroid-metabolizing enzymes is influenced by labile proteins and newly synthesized proteins. Therefore, the levels of steroidogenic enzyme transcripts can be affected by treatment with protein synthesis inhibitors such as cycloheximide (CX). We examined the action of CX on the PP2-induced expression of CYP17 (Fig. 7). As previously shown, CX increased mRNA levels of CYP17 compared with basal levels (Bird *et al.* 1998). Interestingly, CX blocked the PP2 induction of CYP17 mRNA suggesting that the PP2 effects rely on newly synthesized protein.

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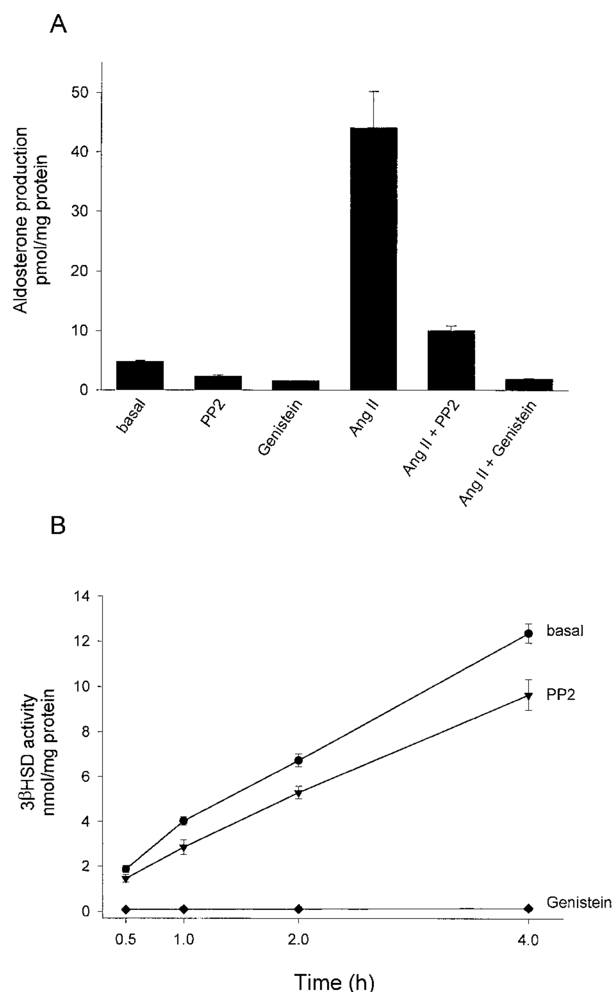

FIGURE 4. Effect of PP2, Ang II and genistein on (A) aldosterone production and (B) 3βHSD activity. (A) Cells were incubated for 24 h alone (basal) or with PP2 (10 μM), Ang II (100 nM) or genistein (30 μM). The aldosterone content of the medium was determined by RIA and normalized to the cellular protein per well. Data points are the mean ± S.E. of determinations from three separate cell culture wells expressed as pmol aldosterone/mg protein. Similar results were observed in two additional experiments. (B) Cells were incubated for 4 h in basal condition, or with PP2 or genestein, in the presence of 1 μM DHEA and 100 000 d.p.m. [3α-^{3}H]DHEA. At the times shown, 100 μl medium were collected and tested for radioactivity content as explained in the Methods section. Results represent the mean ± S.E. of data from one of two similar experiments performed in quadruplicate, and activity is expressed as nmol/mg cellular protein.

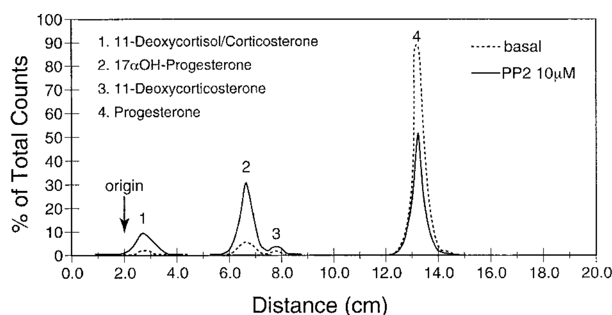

FIGURE 5. Effect of PP2 on H295R metabolism of progesterone. Cells treated for 48 h with (—) or without (······) PP2 (10 μM) were incubated with [4-^{14}C]progesterone (150 000 d.p.m./well) and unlabeled progesterone (0·5 μM) for 6 h. Steroid products recovered by dichloromethane extraction were separated by TLC in chloroform-ethyl acetate solvent system, as described in the Methods section. Peak identities were assigned by comparison to authenticated standards.

DISCUSSION

In this study, we demonstrate that inhibition of the src family of tyrosine kinases blocks aldosterone synthesis. The inhibition, in part, resulted from the induction of 17α-hydroxylase activity and mRNA levels, which effectively removed substrate away from the pathway leading to aldosterone. These data suggest an important role for src in the regulation of adrenal steroidogenesis and particularly in the regulation of CYP17 levels.

The regulation of CYP17 is a key determinant in defining the steroids produced in adrenocortical cells (Conley & Bird 1997). In the glomerulosa, this enzyme is not expressed therefore allowing substrate to move in the direction of aldosterone. In contrast, the fasciculata and reticularis express high levels of CYP17, as its activity is necessary for cortisol and adrenal androgen synthesis. The mechanisms that block expression of 17α-hydroxylase in the glomerulosa are not clearly understood. We and others, using several adrenal model systems, have shown that Ang II is a potent inhibitor of CYP17 expression (McAllister & Hornsby 1988, Rainey *et al*. 1991, Bird *et al*. 1992, 1996*a*,*b*, Mason *et al*. 1995). These data suggest that Ang II stimulation of aldosterone production occurs not only through the activation of aldosterone synthase expression, but also by the inhibition of CYP17 expression. In addition to Ang II, epidermal growth factor (EGF) has been shown to inhibit CYP17 levels and increase the expression of 3βHSD (Kim *et al*. 1998, Doi *et al*. 2000). Both Ang II and EGF have been shown to activate multiple signaling pathways that include the activation of src (Sayeski *et al*. 1999). Recently, an EGF-like protein (Pref-1) (Halder *et al*. 1998) was cloned that localizes to the glomerulosa of the adrenal. The role of Pref-1 in adrenal function has yet to be determined, however

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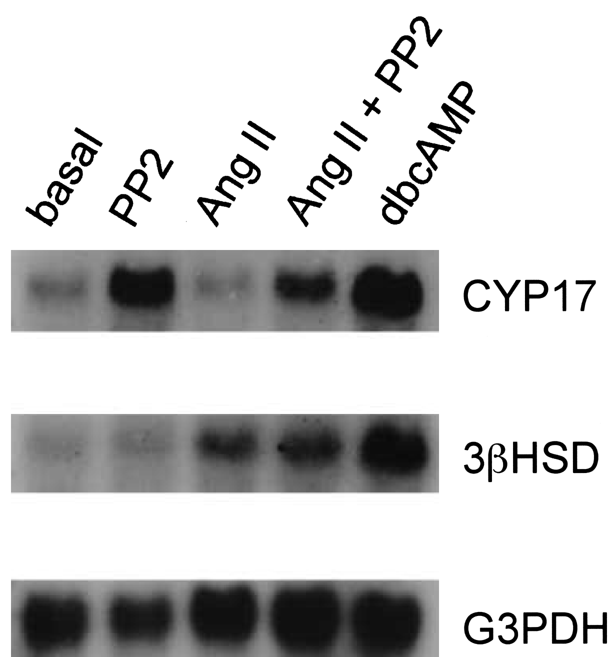

FIGURE 6. Effects of PP2 on basal and Ang II-induced levels of transcripts for CYP17 and 3βHSD. Cells were incubated as described for 24 h without (basal) or with PP2 (10 μM), Ang II (100 nM), PP2 (10 μM) plus Ang II (100 nM) or dbcAMP (1 mM). Medium was then removed and cellular RNA was recovered and subjected to Northern analysis, probing sequentially for 3βHSD and CYP17 mRNA. Results were quantified directly by PhosphorImager analysis and compared with levels of G3 PDH mRNA in the same lane. Results shown are from one of three similar experiments.

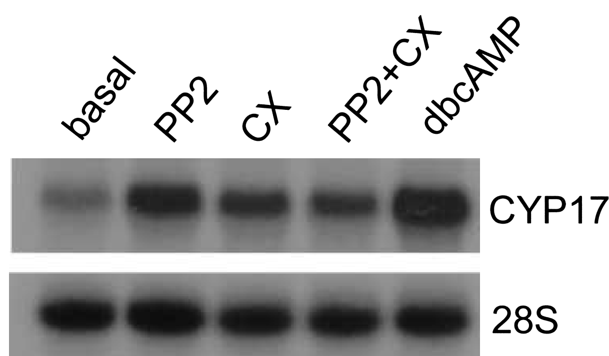

FIGURE 7. Effects of cycloheximide on CYP17 mRNA. Cells were incubated for 24 h alone (basal), or with PP2 (10 μM) or dbcAMP (1 mM) in the presence or absence of cycloheximide (CX, 35 μM). Medium was then removed and cellular RNA was recovered and subjected to Northern analysis, probing for CYP17 mRNA. Results were quantified directly by phosphorimaging analysis and compared with levels of 28S rRNA in the same lane. Results shown are from one of two experiments that gave similar data.

the similarity with EGF suggests that src could be part of its signal pathway. In addition, previous studies have shown that aldosterone production is inhibited by the non-specific tyrosine kinase inhibitor, genistein (Bodart *et al*. 1995, Aptel *et al*. 1999, Wong & Keung 1999). Recent reports have suggested that the effects of genistein are non-specific and may relate to a direct inhibition of the activity of 3βHSD (Wong & Keung 1999). Herein, we confirm that genistein is a potent inhibitor of aldosterone production. However, this inhibitor was also a potent inhibitor of 3βHSD activity in H295R cells. Thus, our results further question the use of genistein in the study of the role of tyrosine kinases in steroidogenic cells.

The development of more specific inhibitors of the tyrosine kinase families has allowed us to examine the role of src tyrosine kinases in adrenal aldosterone production. Src is the best understood member of a family of eight tyrosine kinases that regulate cellular responses to extracellular stimuli (Brown & Cooper 1996, Abram & Courtneidge 2000). In mammals, this family consists of Src, Fyn, and Yes that are ubiquitously expressed, whereas other members Lck, Hck, Fgr, Lyn, Blk and Yrk have more tissue restricted expression mainly in hematopoietic cells. Src is involved in the signaling of many receptors including receptor tyrosine kinases (i.e. EGF and platelet-derived growth factor), integrins, and some G-protein coupled receptors (i.e. AT1). PP1 and PP2 are recently developed inhibitors that show specificity for the src family of kinases (Hanke *et al*. 1996, Liu *et al*. 1999). Detailed studies of these inhibitors have shown that they rely on a single region revolving around Thr338 (Liu *et al*. 1999) that is found in all src family members. Detailed analysis of the inhibitors have confirmed that they are powerful tools to study the roles of src tyrosine kinases in differentiation and cell division.

There are currently no studies investigating the role of src in adrenocortical cells. Our study made use of PP2 to determine if src activity was important for aldosterone synthesis. PP2 potently inhibited basal, Ang II, K^+ and dbcAMP stimulation of aldosterone synthesis. The ability of PP2 to inhibit basal and agonist-stimulated aldosterone production suggests effects on either viability or expression of steroid metabolizing enzymes. We observed no effect on cell viability and the PP2-increased conversion of progesterone to 17α-hydroxyprogesterone suggests that cell integrity was maintained. As this conversion could occur as a

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result of increased expression of CYP17, we examined CYP17 mRNA by Northern analysis. These data confirmed that PP2 is an activator of CYP17 expression. Importantly, PP2 did not affect basal expression of 3βHSD or inhibit its activity. This shift in the ratio of CYP17 to 3βHSD effectively removed substrate from the pathway leading to aldosterone synthesis. Thus, one mechanism for PP2 inhibition of aldosterone production would be the induction of CYP17.

The exact role of the src family of tyrosine kinases in adrenal cell steroidogenesis will need further study. However, the ability of Ang II to increase src activity suggests that src signaling is yet another pathway that will influence steroid hormone production in adrenal cells. Indeed, the data presented herein support the idea that src signaling has an important role in the regulation of aldosterone production. Finally, because the inhibition of src increased CYP17 expression, it can be hypothesized that src tyrosine kinase may influence steroidogenesis by regulating the expression of steroid-metabolizing enzymes.

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