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Testosterone Activates Mitogen-Activated Protein Kinase via Src Kinase and the Epidermal Growth Factor Receptor in Sertoli Cells

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A new pathway of testosterone (T) action in Sertoli cells was recently identified that may be required to support spermatozoa production (spermatogenesis) and fertility. Specifically, T acts via the androgen receptor (AR) to rapidly activate the MAPK cascade and the cAMP response element-binding protein (CREB) transcription factor in Sertoli cells. In further characterizing the signaling pathway that transduces T actions, we now find that a population of AR is localized to the plasma membrane and that AR associates with Src kinase after T stimulation. In addition, we demonstrate that Src kinase is activated by T and that Src kinase activity is required for stimulation of the ERK MAPK and CREB. Furthermore, we determine that activation of the epidermal growth factor receptor downstream of Src contributes to the activation of the MAPK cascade and CREB. The elucidation of this nonclassical pathway of T action in the testis may provide new targets for the control of male fertility. (*Endocrinology* 148: 2066–2074, 2007)

TESTOSTERONE IS ESSENTIAL to maintain the production of spermatozoa in mammals. However, the molecular mechanisms by which testosterone (T) acts to support spermatogenesis are not well characterized. Developing germ cells are thought not to express receptors for T. Instead, T acts through an androgen receptor (AR) in the somatic Sertoli cells to cause the production of factors that are required to support the maturation of adjacent germ cells into spermatozoa (1). In the classical model of steroid action, steroid hormones such as T diffuse through the plasma membrane and bind to intracellular receptors. The hormone-receptor complex then translocates to the nucleus, where it binds to specific DNA elements and modulates gene transcription (2). However, steroids have been found to initiate additional nonclassical responses that are not mediated via their cognate nuclear receptors binding to DNA but rather by the direct stimulation of cell signaling pathways. For example, estradiol and T are each able to stimulate the MAPK cascade and activate downstream transcription factors in specific cell types (for review, see Ref. 3).

There is evidence that estradiol can interact with a pool of estrogen receptors (ERs) localized to the plasma membrane and activate intracellular signaling pathways and that the binding of estradiol causes additional ER translocation to the membrane (4–6). There is also support for the idea that a population of progesterone receptors is localized to the plasma membrane in some cells (7, 8). Localization of AR to the plasma membrane is less well characterized, although AR has been found to be associated with caveolin, an integral membrane protein that serves as a scaffolding protein for many signaling molecules including phosphatidylinositol 3-kinase (PI 3-kinase), Ras, and Src (9, 10). In LNCaP prostate cells, increased AR localization to caveolin-containing membrane fractions (lipid rafts) was ligand dependent and occurred within 10 min of dihydrotestosterone (DHT) stimulation (9). Recently, we demonstrated that AR is required in Sertoli cells for T-mediated stimulation of the ERK MAPK and the cAMP response element-binding protein (CREB) transcription factor (11).

Ligand-bound ERs and ARs have been found to activate numerous cell signaling pathways, and, in many cases, the nonreceptor tyrosine kinase Src is a major factor in the signal transduction (5, 6, 12–15). The essential role of Src kinase in the nongenomic action of steroid receptors was demonstrated in experiments with embryonic fibroblasts derived from $Src^{-/-}$ mice in which the cells from wild-type but not $Src^{-/-}$ mice showed rapid activation of the MAPK pathway in response to ligand binding to AR or ER (14). Src is able to activate the MAPK pathway by several mechanisms. Studies of osteoblasts, osteocytes, and embryonic fibroblasts demonstrated that the complex of androgen-AR-Src recruited the Shc adaptor protein to the complex (14). Src activation of Shc is known to rapidly induce the formation of Shc/Grb2/SOS complexes, leading to the activation of Ras or Ras-like GTPase proteins and the subsequent stimulation of a Raf MAPK kinase kinase, the initial member of the MAPK pathway (16). In response to steroid stimulation, Src can also activate membrane-associated metalloproteases to release heparin-bound (HB)-epidermal growth factor (EGF) from the cell surface, which in turn binds to the EGF receptor (EGFR) and results in the stimulation of the MAPK pathway (5, 6). Src is also capable of directly activating EGFR and subsequently the

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Abbreviations: AR, Androgen receptor; CREB, cAMP response element-binding protein; DHT, dihydrotestosterone; DN, dominant negative; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; ES, ectoplasmic specialization; EtOH, ethanol; FM, fluorescence microscopy; FSHR, FSH receptor; HB, heparin bound; MNAR, modulator of nongenomic action of ER; PI 3-kinase, phosphatidylinositol 3-kinase; T, testosterone; TIR, total internal reflection.

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MAPK pathway by phosphorylating tyrosine residues on the cytoplasmic face of EGFR (17, 18).

In the present study, we outline a signaling pathway used in Sertoli cells to mediate the nonclassical effects of T. We find that T stimulation results in AR migration to the plasma membrane, facilitates AR-Src interactions, and induces the phosphorylation and activation of the Src kinase. Subsequently, the MAPK pathway is stimulated through the EGFR via an intracellular mechanism. The simulation of the ERK MAPK and Src kinase may be important for the continued production of spermatozoa by regulating the structural proteins that maintain Sertoli cell-germ cell adhesion. Furthermore, the nonclassical T signaling pathway is capable of regulating numerous transcription factors including CREB, which has been shown to be essential for germ cell survival (19).

Materials and Methods

Reagents and antibodies

Chemicals were from Sigma (St. Louis, MO) unless stated otherwise. DHT was from Steraloids (Newport, RI). Antisera employed included a polyclonal against total Src; sc-18, a monoclonal antibody against total Src; sc-8056 and polyclonal antisera against the carboxy-terminal 19 amino acids of AR; sc-815 and EGFR phosphorylated on Tyr 1173; and sc-12351 (Santa Cruz Biotechnology, Santa Cruz, CA). Other antisera included those against phosphorylated Src (pSrc Tyr418, Invitrogen Biosource, Camarillo, CA), total ERK, total CREB, and phospho-CREB ser 133 (nos. 06-182, 06-863, and 06-519, Upstate Biotechnology Inc., Charlottesville, VA), vimentin (V 6630, Sigma), phospho-ERK-1/2 (Thr202/Tyr204, Cell Signaling Technology, Beverly, MA), total EGFR [M225, a gift from J. Siegfried (20)], and FSH receptor (FSHR) (amino acids 19–29, a gift from Michael Griswold, Washington State University, Pullman, WA).

Isolation of primary Sertoli cells and cell culture

Sertoli cells were isolated from 15-d-old Sprague Dawley rats as described previously (21). Decapsulated testes were digested with collagenase (0.5 mg/ml, 33 C, 12 min) in enriched Krebs-Ringer bicarbonate medium followed by three washes in enriched Krebs-Ringer bicarbonate medium to isolate seminiferous tubules. Tubules were digested with hyaluronidase (5 mg/ml) for 10 min, pelleted, and then digested in trypsin (0.5 mg/ml, 33 C, 12 min). An equal volume of DMEM containing 10% fetal calf serum was added to the Sertoli cells, which were then pelleted (100 × *g*, 5 min) and resuspended in serum-free medium containing 50% DMEM, 50% Ham's F12, 5 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml EGF, 3 μg/ml cytosine β-D-arabinofuranoside, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. Sertoli cells were cultured (33 C, 5% CO_2) on matrigel-coated dishes (Collaborative Research, Bedford, MA). Sertoli cells were routinely more than 95% pure as determined by immunofluorescence microscopy using antisera against Sertoli cell-specific vimentin and peritubular cell-specific smooth muscle actin. To reduce the basal levels of activated AR and ERK, the cells were maintained for 24 h in the absence of phenol red indicator and EGF before stimulation with vehicle or androgen. Animals used in these studies were maintained and killed according to the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. These studies were approved by the University of Pittsburgh IACUC committee.

Preparation of whole-cell extracts, membrane-associated protein extracts, Western blots, and immunoprecipitations

Three days after isolation, Sertoli cells were treated for various times with ethanol (EtOH) vehicle, T (10–250 nM), or R1881 (10 nM). In some cases, cells were pretreated for 2 h with the signaling pathway inhibitors PP2 (10 μM, Calbiochem, San Diego, CA), PD 98059 (50 μM, Sigma), wortmannin (100 nM, Sigma), GM6001 (20 μM, Calbiochem), or AG1478 (500 nM, Biosource). In some cases Sertoli cells were pretreated with an HB-EGF neutralizing antibody (25 ng/ml, PC319L, Calbiochem) or an EGFR blocking antibody (M225, 6 μg/ml) (20) (both gifts from J. Siegfried, University of Pittsburgh). To prepare whole-cell extracts for direct analysis by western immunoblot, cells were washed once with PBS and then lysed on the plates by using boiling Laemmli sample buffer to minimize phosphatase activity. To isolate purified membrane-associated proteins, cells were homogenized in 300 mM mannitol, 10 mM HEPES (pH 7.4), 1 mM EDTA, 10 nM DHT, and a cocktail of protease and phosphatase inhibitors. The homogenate was pelleted at 300 × *g* for 5 min, and the supernatant was rehomogenized and pelleted again. The supernatant was subjected to centrifugation for 1 h (48,000 × *g*), and the membrane pellet was resuspended in lysis buffer [1% Triton X-100, 10% glycerol, 25 mM HEPES (pH 7.2), 100 nM DHT, and a cocktail of protease and phosphatase inhibitors) and kept on ice for 20 min followed by centrifugation at 13,000 × *g* for 3 min to obtain the purified membrane-associated proteins in the supernatant. Cell lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with primary antibodies, followed by horseradish peroxidase-conjugated second antibody. The antigen-antibody complex was visualized with Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA). Digitized autoradiograms were quantified by using NIH Image 1.6 Software (National Institutes of Health, Bethesda, MD). The levels of p-Src, p-ERK, p-CREB, and p-EGFR levels were normalized to overall Src, ERK, CREB, and EGFR expression levels, respectively. Comparisons of androgen-stimulated levels of p-Src p-ERK, and p-CREB with that of vehicle-treated controls were performed by using ANOVA and Fisher's post hoc analysis with Statview 4.5 Software (Abacus Concepts, Berkeley, CA). For immunoprecipitations, Sertoli cells were lysed in extract lysis buffer [250 mM NaCl, 0.1% NP-40, 50 mM HEPES (pH 7.0), 5 mM EDTA, 0.5 mM DTT, and a cocktail of protease and phosphatase inhibitors] and then sonicated and subjected to centrifugation (12,000 × *g*, 15 min). The supernatants (5–20 μg) were added to 500 μl extract lysis buffer and incubated with 10 μl Src (sc-8056) or EGFR (M225) monoclonal antibodies followed by incubation with protein G Sepharose. The immunoprecipitate was subjected to western immunoblot analysis using p-Src or p-EGFR Tyr 1173 antisera. For coimmunoprecipitations, Sertoli cells extracts were precleared with 10 μl anti-Rabbit Ig (Trueblot, eBioscience, San Diego, CA) per 100 μl Sertoli cell extract. C-Src- and AR-interacting proteins were immunoprecipitated from Sertoli cells extracts with 5 μl c-Src (sc-18) or AR C-19 (sc-815) and 20 μl Trueblot anti-Rabbit IG beads. Precipitates were washed with lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% IGEPAL]. Bound material was eluted by boiling in 2× Laemmli sample buffer for 10 min and fractionated on 8% SDS-PAGE gels. Detection of antigen-bound antibody was carried out as described above. Trueblot rabbit IgG was used as secondary antibody.

Construction of AdDNSrc

Dominant negative (DN) Src containing the two mutations K296R and Y528F was excised from pUSEamp Src(K296R/Y528F) (Upstate Biotechnology) by *Hin*dIII digestion and inserted into pDC316 digested with *Hin*dIII to form pDC316 Src(K296R/Y528F). The pDC316 Src(K296R/Y528F) plasmid was cotransfected into 293T cells with pBHGloxdE1,3Cre to form AdDNSrc, a recombinant adenoviral genome expressing Src (K296R/Y528F). Packaged adenovirus was isolated from 293T cells as previously described (22), and adenoviral yield was calculated from OD_{280} readings (1OD $= 1 \times 10^{12}$ particles/ml).

Immunofluorescence and total internal reflection (TIR)-fluorescence microscopy (FM) imaging

Primary Sertoli cells were cultured in 35-mm^2 plates containing coverslips in serum-free media as described. Three days after initiating the cultures, the cells were treated with vehicle alone or T (100 nM) for 1–60 min. The cells were then washed twice in ice-cold PBS and fixed with 2% formaldehyde in saline for 20 min, washed three times with PBS, blocked with goat serum, and then incubated with a rabbit antiserum directed against the carboxyl-terminal 19 amino acids of AR. In some studies, the AR antiserum was preincubated for 2 h with peptides (50 μg/ml) corresponding to a region of the FSHR (control) or the region of

AR used to generate the antiserum. Staining was also performed in some cases with mouse antiserum against the Sertoli cell-specific vimentin protein. The cells were washed and then incubated with fluorescent secondary goat antirabbit Cy3 alone or also with goat antimouse Alexa 488. Images were obtained on a Nikon Provis II or confocal microscope (Nikon, Melville, NY). All image files were digitally processed for presentation in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). TIR-FM studies were performed as previously described (23). Briefly, cells were grown on glass coverslips, fixed, and stained as described above. Cells were imaged on a Nikon 2000E inverted microscope with a 1.45 numerical aperture oil immersion objective capable of both epifluorescence and TIR-FM illumination through the objective. Alexa 488 was excited with a 488-nm line of an argon laser, whereas Cy3 was excited with a 567-nm line of a krypton laser (laser bench provided by Prairie Technologies, Madison, WI). All laser lines were selectively blocked within the laser bench, and illumination (both intensity and blanking) was controlled by acoustooptical tunable filters. To image both green and red fluorophores simultaneously, an Optical Insights image splitter was used. Images were collected using a water-cooled Orca II ER (Hamamatsu, Tokyo, Japan). Data sets were acquired using SimplePCI (C-Imaging Systems, Cranberry, PA) software. The only postprocessing performed was to ensure appropriate registration (using fiduciary points) of two color images and image scaling such that labeled structures were clearly visible. No other filters or enhancements were applied to the data sets.

Results

T activates Src kinase in Sertoli cells

Thus far, the only Src family member that has been identified as a transducer of androgen signaling is c-Src (13–15). Therefore, we focused on the activation of c-Src by T. The phosphorylation of Src on Tyr 418 in the catalytic domain (p-Src) is an indication that Src has been released from its inhibited confirmation into an activated state, and measurements of p-Src are an established assay to assess the activation of Src (24). To investigate c-Src activation by T, a c-Src-specific antiserum was used to immunoprecipitate whole-cell extracts from vehicle (EtOH) or androgen-treated Sertoli cells. The immunoreactive protein was then immunoblotted with an antiserum that recognizes Src phosphorylated on position Tyr 418 (25). Both T (100 nM) and the nonhydrolyzable androgen agonist R1881 (10 nM) elevated the levels of phosphorylated Src (Fig. 1A). Reprobing the blot with antiserum against total c-Src indicated that similar levels of c-Src had been immunoprecipitated.

Further characterization of c-Src in the transduction of nonclassical T signaling determined that stimulation for 60 min with increasing concentrations of T caused a titratable increase in p-Src activity (Fig. 1B). A time course of stimulation using R1881 revealed that Src kinase stimulation occurred within 1 min and was sustained through at least 120 min of treatment (Fig. 1C). Reprobing of the blots with an antiserum specific for c-Src showed that c-Src comigrated with the p-Src immunoreactive proteins and that the overall expression of c-Src was not altered by androgen. Although it is possible that the activity of other Src family members may be regulated by T, these results indicate that androgen stimulation results in the rapid and sustained activation of c-Src in Sertoli cells.

Src kinase is a required mediator of T-induced signaling

To determine whether Src activation was required for T-mediated induction of MAPK, Sertoli cells were pretreated with PP2, a broad-spectrum inhibitor of Src. The inhibition of ERK phosphorylation by PP2 was comparable with that imposed by PD98059, a well-characterized inhibitor of MEK, the kinase that phosphorylates ERK (26) (Fig. 2A). In contrast, no inhibition of ERK phosphorylation was detected after preincubation with the PI 3-kinase inhibitor, wortmannin. These results suggest that T-dependent activation of ERK requires activated Src kinase. To confirm that Src kinase is required for T-mediated activation of MAPK, Sertoli cells were infected with adenoviral constructs (2×10^{10} viral particles/ml) expressing either a DN c-Src (SrcK296R/Y528F) or β-galactosidase. ERK could be phosphorylated after T stimulation in the presence of the control β-galactosidase but not after overexpression of DN Src (Fig. 2B). These studies dem-

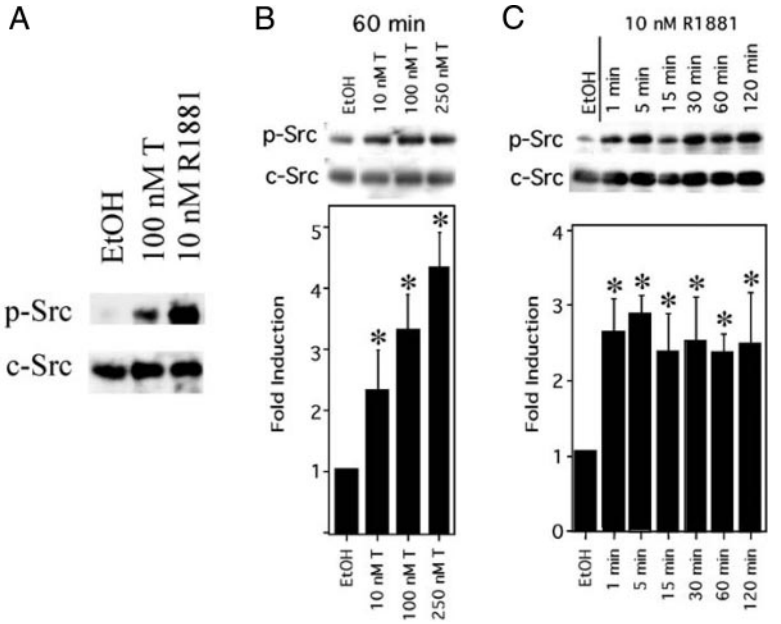

FIG. 1. T induces phosphorylation of Src in primary rat Sertoli cells. A, Whole-cell extracts from Sertoli cells treated for 15 min with EtOH vehicle, 100 nM T, or 10 nM R1881 were immunoprecipitated with c-Src antiserum followed by immunoblotting with an antiserum specifically recognizing Src phosphorylated on Tyr 418 (p-Src). The blot was then stripped and reprobed with an antiserum against all c-Src isoforms. The immunoreactive p-Src and c-Src are indicated. The data shown are representative of three independent experiments. B, Primary Sertoli cells were stimulated with EtOH and T at the concentrations shown for 60 min. C, Sertoli cells were stimulated with EtOH vehicle for 15 min or 10 nM R1881 for the times shown. Western immunoblotting of whole-cell extracts was performed with p-Src antiserum. The membranes were stripped and reprobed using an antiserum recognizing all c-Src isoforms. The intensities of the p-Src bands were normalized to those of c-Src, and the normalized values of the vehicle-treated cells were arbitrarily set equal to 1. The mean fold induction (±SE) of hormone-stimulated p-Src over vehicle-treated cells for three independent experiments is reported in the graphs. *, Values that are significantly different from vehicle-treated controls as determined by ANOVA ($P < 0.05$).

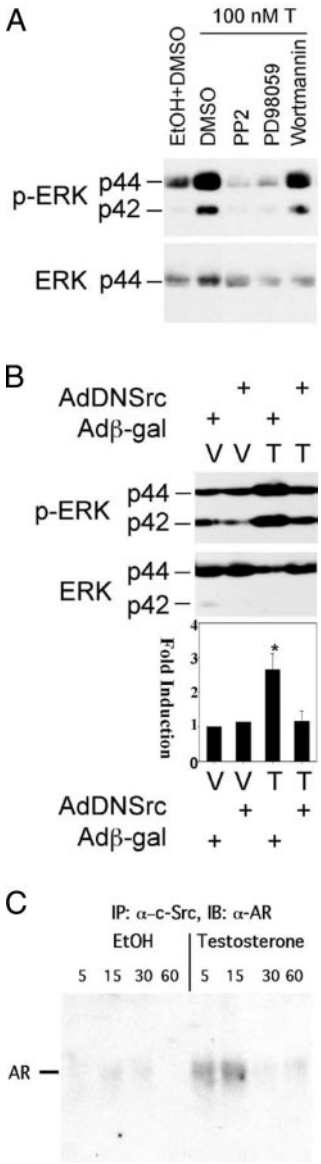

FIG. 2. T-mediated induction of ERK requires Src kinase. A, Primary Sertoli cells were pretreated for 2 h with DMSO (vehicle), PP2 (10 μM), PD98059 (50 μM), or wortmannin (100 nM) before stimulation for 2 h with EtOH (vehicle) or 100 nM T. Immunoblot analysis of whole-cell extracts was performed first using an antiserum against p-ERK followed by reprobing the blots with antiserum against total ERK. To obtain signals in the linear range, shorter exposures were required that did not permit visualization of the p42 form of ERK. B, Src kinase is required for T-mediated activation of ERK. Primary Sertoli cells were infected with adenoviral constructs expressing either β-galactosidase (Adβ-gal) or DN Src (AdDNSrc). Two days later, the cells were stimulated with EtOH-vehicle (V) or 100 nM T for 60 min. Whole-cell extracts were subjected to immunoblot analysis using an antiserum against p-ERK followed by a second antiserum against total ERK. The 42- and 44-kDa forms of ERK are indicated. The data shown are representative of two experiments. After normalizing to total ERK levels, the mean fold induction ($\pm$SD) of p-ERK over vehicle-treated, Adβ-gal-infected controls is reported in the graph below. *, Values that are significantly different from vehicle-treated Adβ-gal-infected controls as determined by Student's *t* test ($P < 0.05$). C, Whole-cell extracts (50 μg) prepared from Sertoli cells stimulated with EtOH (vehicle) or 10 nM T for 5–60 min were immunoprecipitated (IP) with c-Src antiserum and then subjected to western immunoblot (IB) analysis with antiserum against AR. The figure shown is representative of three experiments.

onstrate that T-induced phosphorylation of ERK requires Src activity.

T stimulates AR association with Src at the plasma membrane

To test whether AR-Src interactions were enhanced by T stimulation in Sertoli cells, c-Src protein was immunoprecipitated after stimulation of Sertoli cells with T (100 nM) for 5–60 min. Western blot analysis of the immunoprecipitated complexes with antiserum against AR revealed that T rapidly (within 5 min) induced the association of AR with c-Src in primary Sertoli cells (Fig. 2C).

The rapid T-mediated interactions of AR with Src suggested that a population of AR may be localized at or near the Sertoli cell plasma membrane. Using confocal microscopy and an antiserum against AR, a digital section of Sertoli cells corresponding to the cell surface identified AR immune complexes that were localized to the membrane region (Fig. 3A). The AR immunoreactivity was not present when the antiserum had been preincubated with competing AR peptide, thus demonstrating the specificity of antibody staining (Fig. 3B). TIR-FM was then used to detect immunoreactive AR near the cell membrane in cultures of primary Sertoli cells. In using TIR-FM, excitation of fluorophores is limited to within 70–100 nm of the cell surface (27). Sertoli cells were identified by the presence of immunoreactive Sertoli cell-specific vimentin intermediate filament proteins (*green* stain) (Fig. 3, C and D). The Sertoli cells were simultaneously probed with antiserum against AR. Punctate AR staining (*red* and *yellow* foci) was observed in the Sertoli cells treated for 5 min with vehicle or T. Due to the specificity of TIR-FM resolution, the observed AR foci must be localized to the vicinity of the plasma membrane.

Immunofluorescence studies of EtOH (control)-treated Sertoli cells detected AR in the nucleus and cytoplasm with some immunostaining present at the cell periphery. An increase in AR localized to the vicinity of the plasma membrane occurred within 5 min of T stimulation but by 60 min the membrane-localized AR decreased to basal levels (Fig. 4, A–C). Western immunoblot analysis of membrane fractions isolated from Sertoli cells confirmed that AR was associated with the membrane and that membrane-associated AR levels transiently increase for at least 5 min after T stimulation (Fig. 4D, *upper*). In contrast, the levels of the FSHR in the membrane fraction remained relatively constant (Fig. 4D, *lower*).

The EGFR is activated downstream of Src via an intracellular pathway

In some cell types, estradiol has been shown to activate the EGFR leading to stimulation of the MAPK cascade and the CREB transcription factor (6, 28). To determine whether T can activate the EGFR in Sertoli cells, whole-cell extracts from androgen-stimulated Sertoli cells were immunoprecipitated with a monoclonal antibody against total EGFR followed by immunoblotting with antiserum against EGFR phosphorylated at Tyr 1173. These studies determined that T and the nonhydrolizable androgen agonist R1881 could increase the levels of phosphorylated (activated) EGFR (Fig. 5A). To confirm that Src kinase is required for EGFR activation, Sertoli

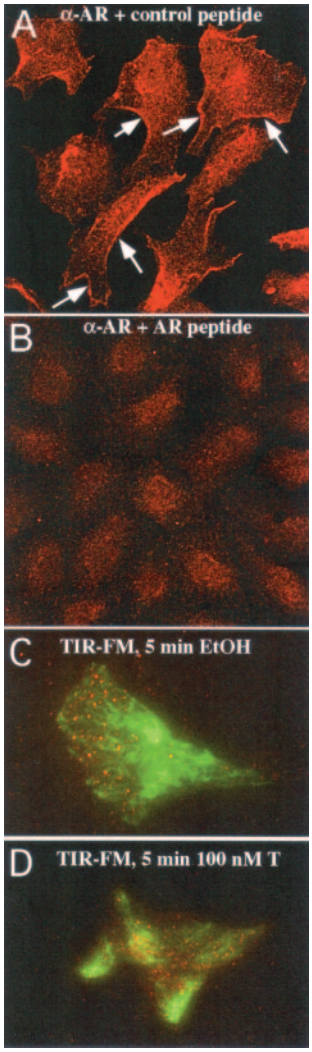

FIG. 3. A population of AR is localized to the plasma membrane of Sertoli cells. Primary Sertoli cells grown on glass coverslips were stimulated with 100 nM T for 15 min and then analyzed by confocal microscopy. Digital sections corresponding to the surface of Sertoli cells are shown. AR immunostaining (*red*) is shown for cells stained with AR antiserum preincubated with a control peptide (A) or competing AR peptide (B). *Arrows*, Regions at the plasma membrane that are immunoreactive for AR. The figures are representative of those from three experiments. C and D, TIR-FM images of individual Sertoli cells 5 min after stimulation with EtOH (vehicle, C) or 100 nM T (D). Cells were probed with Sertoli cell-specific vimentin antiserum (*green* staining) and AR antiserum (punctuate *red* and *yellow* staining). The figure shown is representative of images taken from two experiments.

cells were pretreated with PP2 and then stimulated with T or EGF. T-mediated phosphorylation of ERK and CREB was blocked by PP2; however, EGF treatment rescued the activation of ERK and CREB, thus demonstrating that EGF acts downstream of Src (Fig. 5B).

One mechanism by which steroid hormones can activate the EGFR is by causing Src kinase to stimulate a matrix metalloprotease in the plasma membrane to release HB-EGF from the cell surface. The liberated HB-EGF can bind to the EGFR, and the activated receptor then stimulates the MAPK cascade (6, 28). To test the possibility that the HB-EGF-dependent pathway is activated by T in Sertoli cells, the cells

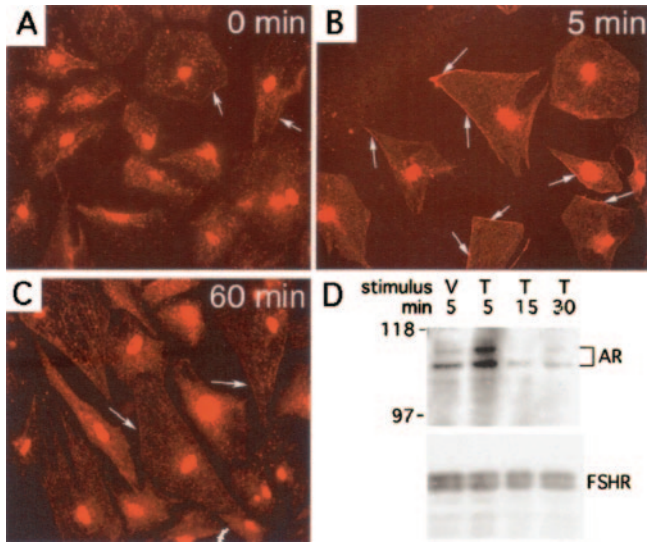

FIG. 4. T rapidly and transiently promotes AR localization to the plasma membrane. Primary Sertoli cells were untreated (0 min) or stimulated with T (100 nM) for 5 or 60 min (A–C), then fixed and probed with AR antiserum (*red*). *Arrows*, AR localized to the region of the Sertoli cell membrane. The figure shown is representative of more than three experiments. D, Purified membrane fractions from primary Sertoli cells stimulated with EtOH vehicle (V) or T for the times shown were subjected to western immunoblot analysis with AR antiserum, after which the blots were stripped and reprobed with antiserum against FSHR.

were pretreated with the broad-spectrum metalloprotease inhibitor GM6001 (10 μM), an antiserum against HB-EGF, or an antibody (m225) that blocks binding to the EGFR on the cell surface (20). Neither of the potential inhibitors of HB-EGF activity were effective in limiting androgen-induced increases in ERK or CREB phosphorylation (Fig. 6, A and B). In testing the efficacy of the inhibitory antisera against HB-EGF and EGFR, both of the antisera blocked HB-EGF induced phosphorylation of ERK (Fig. 6C). In contrast to the lack of regulation via HB-EGF, T-mediated phosphorylation of ERK and CREB was inhibited by AG1478 (500 nM) that selectively blocks the kinase activity of the EGFR by not allowing the trans-phosphorylation of the EGFR cytoplasmic tail (29, 30). Together, these studies indicate that T signaling occurs through activation of the EGFR kinase via an intracellular mechanism and that T-induced activation of MAPK is not dependent on the release of HB-EGF or other external stimuli of the EGFR.

Discussion

Our studies indicate that T-mediated induction of the MAPK pathway requires the activation of Src kinase. The rapid and sustained activation of Src (1–120 min) as well as the concentrations of T (10–250 nM) that are able to phosphorylate Src are similar to the data that we previously obtained for the T-mediated activation of ERK and the CREB transcription factor (11). The findings that T-mediated phosphorylation of ERK is blocked by the Src inhibitor PP2 as well as the expression of a DN Src confirms that Src is required for T signaling in Sertoli cells.

The rapid increase in Src phosphorylation correlates with

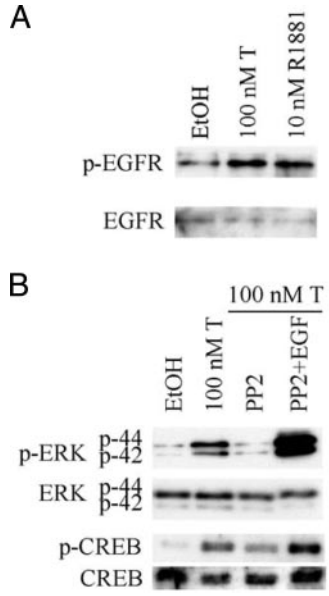

FIG. 5. T activates of the MAPK pathway via EGFR. A, Sertoli cells were stimulated with vehicle (EtOH), T, or R1881 for 15 min, and whole-cell extracts were immunoprecipitated with a monoclonal antibody against EGFR (M225) followed by immunoblotting with antiserum recognizing EGFR phosphorylated on Tyr 1173 (p-EGFR). The blot was then stripped and reprobed with an antiserum against all forms of the EGFR. The data shown are representative of three independent experiments. B, Sertoli cells were pretreated for 2 h with PP2 (10 μM), followed by stimulation with EtOH, T (100 nM), or T (100 nM) plus EGF (100 ng/ml) for 15 min. Whole-cell extracts were subjected to immunoblot analysis with p-ERK or p-CREB antiserum, and then the blots were stripped and probed with either total ERK or CREB antiserum. The data shown are representative of three (p-ERK) and two (p-CREB) independent experiments.

increased AR-Src interactions that occur within 5 min of T stimulation and are maintained for at least 15 min. The kinetics of increased AR-Src interactions in Sertoli cells are similar to those reported for LNCaP prostate cells. In LNCaP cells, treatment with R1881 (10 nM) triggered direct association of the proline-rich region of AR and the SH3 domain of Src, causing the activation of Src within 1 min and the subsequent stimulation of the MAPK cascade within 5 min (15). The AR-Src interactions that were detected in Sertoli cells are likely mediated via the same protein domains that were identified in LnCaP cells. Progesterone receptors and ER similarly activate ERK through interactions with c-Src via the SH3 and SH2 domains of c-Src, respectively (15, 31). It is possible that AR-Src interactions in Sertoli cells may be facilitated by other proteins such as the modulator of nongenomic action of ER (MNAR) (12). In androgen-dependent LNCaP cells, MNAR has been implicated in integrating the interaction of AR and Src and in the regulation of the Src/Ras/Raf/MAPK signaling pathway (13). Further studies will be required to determine whether MNAR contributes to T signaling in Sertoli cells.

The sustained activation of Src kinase by T is consistent with our earlier finding that T-mediated activation of the ERK MAPK and the CREB transcription factor is maintained for least 2 h (11). This sustained activation of the MAPK cascade and transcription factors such as CREB are significant for establishing elevated transcription activity. For example, CREB-mediated transcription requires that CREB be maintained in the phosphorylated state for at least 20 min (32). In our earlier study, we identified three CREB-regulated genes that are induced by nonclassical T signaling (11).

Confocal and TIR-FM microscopy analysis, as well as the detection of AR associated with membrane protein extracts, provides support for localization of a population of AR to the plasma membrane. The finding that AR accumulates at the plasma membrane after T stimulation is consistent with previous data demonstrating that a population of ER migrates to the plasma membrane in response to the addition of estrogen (33, 34). Furthermore, the kinetics of AR localization to the Sertoli cell membrane were similar to that found in LNCaP cells in which AR association with the integral membrane protein caveolin increased within 10 min of DHT addition but fell to undetectable levels after 60 min (9). AR-Src interactions may prolong AR localization to the membrane because much of Src is posttranslationally modified such that it is membrane bound (35). Thus, the perceived movement of AR to the membrane may be the result of the more efficient capture of ligand-bound AR by Src, EGFR, caveolin-1, or other members of the signaling machinery in close association with the plasma membrane.

The localization of a population of AR to the membrane would facilitate interactions not only with Src but would make the transduction of T signaling to downstream effectors more efficient. Members of the MAPK pathway have been shown to form complexes with AR on molecular scaffolds such as those that form on caveolin-1 in specialized membrane invaginations called caveolae (9, 10). AR interactions at the membrane with Src (15), caveolin-1 (9), PI 3-kinase (36), and EGFR (37) have been observed in other cell types. Western immunoblot and immunofluorescence studies indicated that after initially accumulating near the membrane, lower levels of membrane-associated AR decline within 60 min of T stimulation. Similarly, we found that the increased association of AR and Src identified by coimmunoprecipitation persists for less than 30 min. Nevertheless, the activation of the MAPK pathway was found to extend for at least 2 h in this study and a previous study (11). Currently, we do not understand the discrepancy between the timing of the transient increase in AR-Src interactions and the longer lasting activation of Src and ERK. It is possible that once the signaling pathway is activated and androgen is present, basal levels of AR-Src interaction are sufficient for maintenance. Alternatively, Src can act as both an activator and a target of EGFR (38); therefore, it is possible that once EGFR is activated, an AR-independent positive feedback loop is created to perpetuate the signaling. Another possibility is that the activation of Src and ERK in Sertoli cells may be mediated first by a rapid transient mechanism that requires steroid receptor-Src interactions and then by a delayed-sustained pathway that includes Src and EGFR but not steroid receptor as was recently described for progesterone actions in breast cancer cells (39). An alternative mechanism has been described in which ERK phosphorylation of steroid hormone receptors allows the potentiation of activity when initiating signals are limited (40). However, the phosphorylated AR would either have to initiate signaling more efficiently via

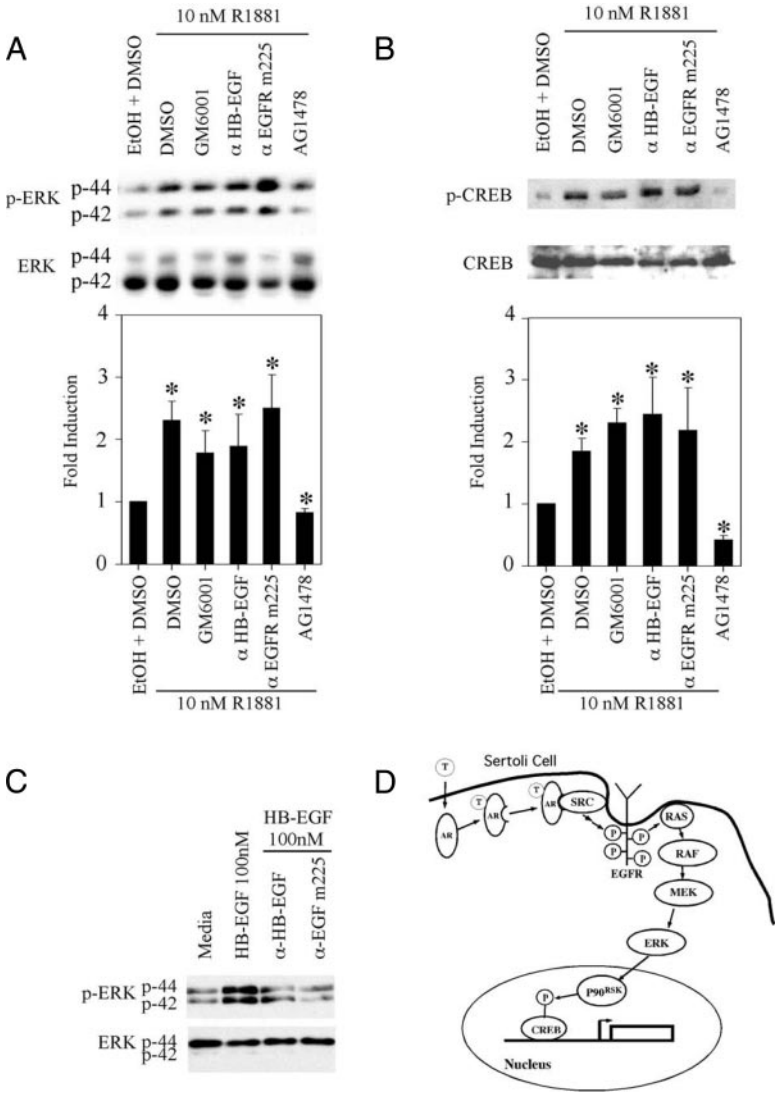

FIG. 6. T activates the EGFR via an intracellular mechanism. A and B, Sertoli cells were pretreated for 2 h with DMSO, a broad-spectrum metalloprotease inhibitor (GM6001), an antiserum against HB-EGF, an antibody against EGF that blocks ligand-EGFR interactions (αEGFR M225), or an inhibitor of EGFR intracellular phosphorylation (AG1478), followed by stimulation with EtOH or 10 nM R1881 for 15 min. Whole-cell extracts were subjected to immunoblot analysis with p-ERK or p-CREB antiserum, and then the blots were stripped and probed with total ERK or CREB antiserum. The mean fold induction (±SE) of R1881-stimulated p-ERK (A) or p-CREB (B) over that from cells treated with EtOH for four (p-ERK) or three (p-CREB) independent experiments is reported in the graphs below the western images. *, Values that are significantly different from vehicle-treated controls as determined by ANOVA ($P < 0.05$). C, Sertoli cells were treated for 2 h with antiserum against HB-EGF or EGFR (αEGFR M225) followed by stimulation with HB-EGF (100 ng/ml) for 15 min. Whole-cell extracts were subjected to immunoblot analysis with p-ERK antiserum, and then the blots were stripped and probed with total ERK antiserum. The data shown are representative of three independent experiments. D, Nonclassical T signaling pathway in Sertoli cells. T diffuses through the Sertoli cell membrane and binds to and alters AR such that it is better able to interact with and activate Src possibly located at caveolae invaginations in the plasma membrane or at ESs. Src then causes the phosphorylation and activation of the EGFR via an intracellular mechanism either directly or through intermediaries such as Shc and Grb. The activated EGFR then causes Ras to be stimulated, which then activates a RAF kinase that activates MEK that activates ERK. The activated ERK can then cause numerous proteins to be phosphorylated and can potentially stimulate transcription factors including SRF, Elk-1, AP1, and CREB. ERK causes the phosphorylation of CREB via the $p90^{RSK}$ kinase, and when phosphorylated, CREB recruits coactivators to facilitate the initiation of gene transcription.

basal AR-Src interactions or by a different mechanism that does not include increased AR-Src interactions.

The finding that the EGFR inhibitor AG1478 blocked T-mediated activation of ERK and CREB suggests that signaling downstream of AR-Src complexes passes through the EGFR. The rescue of ERK phosphorylation by EGF after inhibition with PP2 also confirmed that Src acts upstream of EGFR. Activation of the EGFR does not appear to occur via Src activation of membrane-associated metalloproteases or the release of HB-EGF from the Sertoli cell surface because inhibitors of metalloproteases, HB-EGF-neutralizing antibodies, and an antiserum blocking binding to the extracellular region of the EGFR did not block ERK phosphorylation. Therefore, Src is expected to act via an intracellular mechanism to activate the EGFR. Overexpression of c-Src induces phosphorylation of specific intracellular residues of the EGFR that are required for EGF-stimulated growth (17, 41). Presently, it is not known whether T-activated Src in Sertoli cells directly phosphorylates the EGFR or whether Src relies on intermediary proteins such as Shc and Grb to bind to and activate the EGFR.

It remains to be determined whether T can act through other pathways and transcription factors in addition to the Src-EGFR-ERK-CREB pathway. However, it is likely that other MAPK-inducible transcription factors such as SRF, Elk-1, and AP1 can be stimulated by T. In addition, T has been shown to activate PI 3-kinase in other cells, and studies are underway to determine whether the known T-mediated calcium influx into Sertoli cells results in the activation of downstream signaling pathways (42–46).

T and AR are required in Sertoli cells for three major functions related to spermatogenesis: 1) maintaining the blood-testis barrier that isolates developing germ cells, 2) survival of germ cells during the meiosis stage of development, and 3) the release of mature sperm from the Sertoli cell (47–49). Characterization of the nonclassical signaling pathway for T has important implications for understanding the molecular mechanisms by which T regulates the above three processes. For example, preliminary results from microarray studies suggest that nonclassical T signaling regulates the expression of some genes that contribute to maintaining Sertoli cell-germ cell junctions (Cheng, J., and W. H. Walker,

unpublished data). Thus far, there is little information regarding the factors that must be regulated by T to permit the survival of germ cells beyond meiosis. However, it is possible that some of the nonclassical T-stimulated signaling pathways and downstream-regulated genes will be found to be required to support germ cell survival. In regard to the release of mature sperm from Sertoli cells, phosphorylated Src and ERK have been localized in Sertoli cells to specialized adherens junctions called ectoplasmic specializations (ESs) that link Sertoli cells and maturing spermatids (50–52). Furthermore, Src- and ERK-mediated phosphorylation of junctional proteins is associated with the disruption of protein-protein interactions that result in the loss of adherens junction integrity and the release of sperm (53, 54). Studies are underway to determine whether the increase in Sertoli cell AR levels that occurs immediately before the release of sperm (55, 56) is responsible for an increase in T-mediated signaling as well as the increased levels of phosphorylated Src and ERK that are associated with breakdown of the ES. Such a finding would be consistent with earlier studies showing that activation of AR resulted in the rapid reorganization of the actin cytoskeleton in LNCaP cells via a nonclassical mechanism (43). With the identification of the components of the nonclassical T signaling pathway (Fig. 6D) and the characterization of their function, it will be possible to target specific factors in the pathway for inactivation as a contraceptive strategy to inhibit the release of sperm from the testis.

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