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Expression, Purification and Characterization of the Rat Luteal 20α-Hydroxysteroid Dehydrogenase*

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ABSTRACT

The enzyme, rat ovarian 20α-hydroxysteroid dehydrogenase (20αHSD), plays a central role in luteolysis and parturition. It catalyzes the reduction of progesterone, leading to the formation of progestationally inactive steroid, 20α-hydroxypregn-4-ene-3-one (20α-hydroxyprogesterone). Recently, we reported the cloning, sequencing, and deduced amino acid sequence of the rat luteal 20αHSD. To further investigate whether phosphorylation and/or glycosylation affect the activity of 20αHSD and to study its kinetic and biochemical properties, we established both bacterial and insect expression systems for obtaining large quantities of enzyme. The recombinant (rec) 20αHSD expressed as glutathione-*S*-transferase-20αHSD fusion protein was purified from bacterial lysates by affinity binding to glutathione-Sepharose beads followed by thrombin digestion, whereas the rec enzyme expressed in baculovirus-insect cell system was purified to apparent homogeneity by ion exchange chromatography, followed by dye affinity chromatographies. Both rec preparations of 20αHSD demonstrated a single polypeptide chain of 37 kDa with similar K_m values for 20α-hydroxyprogesterone and NADP, although the corresponding maximum velocity values were slightly lower for the rec 20αHSD expressed in the insect cells. The rec 20αHSD showed preference for progesterone/20α-hydroxyprogesterone. 17α-Hydroxyprogesterone was only 30% as effective. The enzyme also used various substrates specific for aldo-keto reductases, although with much less efficiency. The rec enzyme preparations showed an absolute requirement for NADP(H). *In vitro* phosphorylation of rec bacterial enzyme with either protein kinase A or protein kinase C had no demonstrable effect on its activity. Finally, no differences in enzyme activity were noted between glycosylated (expressed in insect cells) and nonglycosylated (expressed in bacteria) forms of the enzyme.

In conclusion, these studies demonstrate that rat luteal 20αHSD can be prepared in large amounts from either bacterial or insect expression systems in a catalytically active form. Indirect evidence also suggests that the catalytic activity of 20αHSD may be independent of phosphorylation and glycosylation states of the enzyme protein, *i.e.* posttranslational modification of 20αHSD may not be required for the maximal expression of enzyme activity. (*Endocrinology* **138:** 182–190, 1997)

THE 20α-HYDROXYSTEROID dehydrogenase (20αHSD) enzyme plays a central role in the termination of pregnancy (1–4). The enzyme catalyzes the NADPH-dependent reduction of progesterone to 20α-hydroxypregn-4-ene-3-one (20α-hydroxyprogesterone) (5, 6), which is biologically inactive and cannot maintain pregnancy (2, 7–9). It is the increase in luteal 20αHSD activity rather than decreased progesterone synthesis that contributes to the reduced systemic progesterone levels associated with the termination of pregnancy. Indeed, 20αHSD activity in the rat corpus luteum remains repressed throughout pregnancy, but it is rapidly induced to a high level before parturition (2, 3).

Ovarian 20αHSD activity is thought to be under hormonal regulation. Hormones such as PRL, which signal through the tyrosine kinase system, have been shown to down-regulate 20αHSD activity (10–13), whereas hormones that activate protein kinase A (PKA; LH) and protein kinase C (PKC; GnRH and $PGF_{2\alpha}$) simulate the activity of this enzyme (4, 14–18). However, in general, it is not clear whether the hormone-induced changes in 20αHSD activity are due to allosteric modulation, covalent modification, or changes in enzyme concentration. Recently, we demonstrated that at least the PRL-mediated inhibition of 20αHSD activity is accompanied by down-regulation of both its 20αHSD gene expression and enzyme synthesis (19, 20).

Efforts to characterize the structural/functional aspects of the ovarian 20αHSD enzyme have been hampered by the limited availability of purified native enzyme. Additionally, such preparations are unsuitable for studies designed to evaluate the role of posttranslational events (*e.g.* glycosylation and phosphorylation) in enzyme catalysis. Recently, we and others have reported the cloning of a gene encoding rat luteal 20αHSD (20, 21). The deduced amino acid sequence contains potential sites for phosphorylation by PKC, PKA, and tyrosine kinase(s) and putative sites for *N*-glycosylation (20). Also, the complementary DNA (cDNA) and deduced amino acid sequences show putative sites for NADH/NADPH binding and exhibit a high degree of homology with the members of the aldo-keto reductase family, including, aldehyde/aldose reductases, and bovine $PGF_{2\alpha}$ synthase (20, 21). Enzymes with weak 20αHSD activity have recently been cloned from bovine testes and human placenta (22–24); they differ markedly from the ovarian enzyme by their amino acid structure and the substrate they use (20, 24, 25). Therefore,

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the current studies were initiated to further explore the kinetic/functional properties of ovarian 20αHSD and to examine the effects of glycosylation and phosphorylation on the enzyme activity. To accomplish these goals, efforts were made to express 20αHSD in both bacterial and insect cell systems. The heterologous proteins produced in a bacterial system are generally biologically active. However, these proteins may not adopt structural conformations of native proteins (*i.e.* similar to those found in mammalian cells), are nonglycosylated, and are poorly phosphorylated. The advantages of the baculovirus expression system are the abundant expression of recombinant protein combined with the co- and posttranslational modifications, including *N*-glycosylation and phosphorylation (26–29). Thus, the use of these two expression systems provides an opportunity to evaluate the roles of co- and posttranslational modifications, such as glycosylation and phosphorylation.

Here we describe the high level expression of rat luteal 20αHSD using bacterial and baculoviral expression systems and the purification and characterization of the rec enzyme. Overall, purified rec enzyme preparations from the two systems exhibit the antigenic and kinetic properties and substrate specificity of native enzyme purified from ovarian sources (6, 7, 9). In addition, rec 20αHSD can use substrates specific for aldose/aldehyde reductases. Both bacterial and baculoviral expressed enzymes, however, lack the intrinsic PG synthase activity. Finally, we have also shown that phosphorylation and/or glycosylation of enzyme protein are not necessary for enzyme catalysis.

Materials and Methods

Materials

pGEx-4T-2 vector and glutathione-Sepharose 4B were purchased from Pharmacia (Piscataway, NJ). pBlueBacIII vector and liposome-mediated transfection kit with *Autographa californica* nuclear polyhidrosis virus (AcNPV) and *Spodoptera frugiperda* 9 (Sf9) insect cell line were obtained from Invitrogen Corp. (San Diego, CA). Grace's insect culture medium was supplied by Life Technologies (Gaithersburg, MD). The DNA sequencing kit was the product of U.S. Biochemical Corp. (Cleveland, OH). 20α-[1,2-N-^{3}H]Hydroxypregn-4-ene-3-one (SA, 51.2 Ci/mmol; 1.9 tetrabecquerels/mmol) and [5,6,8,9,11,12,14,15-N-^{3}H]arachidonic acid (SA, 60–100 Ci/mmol; 2.22–2.370 tetrabecquerels/mmol) were obtained from DuPont-New England Nuclear Research Products (Boston, MA). Various restriction enzymes and T4 ligase were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Polyclonal antibodies against the 20αHSD were prepared by us. All others reagents used were of analytical grade.

Expression of 20αHSD in Escherichia coli and its purification

Plasmid construction and expression. Standard procedures of DNA manipulation and transfection were followed (30). A DNA fragment of 1.2 kilobases containing the entire coding region of 20αHSD was removed from pBluescript vector by *Eco*RI and *Xho*I digestion, purified, and ligated into the *Eco*RI and *Xho*I cloning sites of the pGEX-4T-2 vector, a glutathione-*S*-transferase (GST) fusion protein expression vector. Correct orientation of the translational reading frame for the GST-20αHSD fusion protein was confirmed by DNA sequencing.

Purification of expressed 20αHSD. Two hundred microliters of overnight culture of *E. coli* DH5α transfected with GST-20αHSD cDNA was inoculated into 200 ml fresh, prewarmed, 2 × YTG medium (16 g/liter tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl) containing 100 mg/ml ampicillin. The culture was incubated at 37 C with shaking to achieve an absorption reading between 1.0–2.0 at 600 nm. Subsequently, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The incubation was continued for an additional 4 h. After centrifugation, the pelleted bacteria were resuspended in PBS, sonicated, made 1% with respect to Triton X-100, and mixed gently at room temperature for 30 min. The suspension was then centrifuged at 1200 × *g* for 10 min. The supernatant containing recombinant (rec) 20αHSD GST was further processed to obtain pure 20αHSD using standard protocols (31). In brief, a 50% slurry of glutathione-Sepharose 4B beads was mixed with supernatant (~2 ml of the 50% slurry of beads to each 100 ml supernatant) and agitated gently at room temperature. At the end of incubation, the beads were collected by centrifugation and washed several times with 10 vol PBS. After final centrifugation, the glutathione beads (~2 ml) with bound 20αHSD-GST fusion protein were resuspended in 1 bed volume PBS and digested with 500 cleavage units of bovine plasma thrombin at room temperature for 16 h. After thrombin cleavage of the fusion protein, the beads with bound GST were separated from the rec 20αHSD protein by centrifugation at 10,000 × *g* for 10 min at 4 C. The supernatant was adjusted to 50% (vol/vol) glycerol, aliquoted, frozen in liquid N_2, and stored at −70 C. Usually, 500 μg rec 20αHSD was obtained from 200 ml bacterial cell culture.

Expression of 20αHSD in insect cells using the baculovirus system

Construction of a 20αHSD cDNA-containing baculovirus transfer vector. A 1.2-kilobase *Bam*HI fragment consisting of the entire coding region of rat luteal 20αHSD was ligated into the pBlueBacIII vector immediately downstream of the polyhedrin promotor to create pBlueBacIII-20αHSD. Correct orientation was confirmed by DNA sequencing.

Transfection and isolation of recombinant baculovirus. Recombinant baculovirus, AcNPV, containing the 20αHSD sequence under the transcriptional control of the polyhedrin promotor was produced by *in vivo* homologous recombination according to Webb and Summers (32) as described in Invitrogen's instruction manual. In brief, 2 μg of the pBlueBacIII-20αHSD were mixed with 1 mg linearized wild-type AcNPV viral DNA and cotransfected into Sf-9 insect cells by cationic liposome-mediated gene transfer according to the protocol suggested by the manufacturer (Invitrogen). One hundred and twenty hours postinfection, the medium was collected, centrifuged, diluted 10- to 10,000-fold, and used to infect a fresh monolayer of Sf-9 cells. To facilitate the identification of viral plaques, a layer of 0.625% agarose containing 75 μg/ml X-Gal (5-bromo-4-chloro-3-inolyl-β-D-galactopyranoside) was applied to the transfected cells. After 6–8 days, recombinant viruses, designated AcNPV-20αHSD, were detected by the formation of blue plaques. Several blue plaques were picked and subjected to three cycles of plaque purification until cells with inclusion bodies were not detected. After purification, several strains of recombinant AcNPVs were obtained, and four strains (designated as AcNPV-20αHSD 1, 2, 3, and 4) were used in further studies.

Insect cell culture. Sf-9 insect cells were maintained in Grace's medium supplemented with 10% FBS, supplemented with yeastolate (3.3 g/liter), lactalbumin hydrolysate (3.3 g/liter), gentamicin (50 μg/ml), and fungizone (2.5 μg/ml) in monolayer or suspension culture. Cells were transfected or infected in log phase of growth at 2 × 10^6 cells/ml.

Expression and purification of recombinant 20αHSD. Sf-9 cells were seeded at a density of 2–2.5 × 10^6/60-mm dish or 9 × 10^6 cells/75-cm^2 flask. After the cells were attached, the medium was removed, and a volume of virus inoculum, sufficient to just cover the cells at a multiplicity of infection of 3 or 4 to 1, was added. After incubation at 27 C for 1.5 h, the inoculum was replaced with fresh Grace's medium and incubated at 27 C for up to 5 days; infected insect cells were collected and used for purification of recombinant 20αHSD (9, 33).

Immunoblot analysis of recombinant proteins

Immunoblotting of bacterial and insect cell-expressed 20αHSD was performed using polyclonal antirat 20αHSD antibody (34). The fusion protein expressed in either *E. coli* or Sf9 cells were subjected to SDS-PAGE under reducing conditions on a gel containing 7.5% polyacrylamide and then transferred to a nitrocellulose membrane. After transfer,

the blots were blocked with 3% BSA and then probed with the anti 20αHSD antibody or preimmune serum. The immunoreactive proteins were visualized using alkaline phosphatase-conjugated antirabbit IgG as secondary antibody.

Enzyme assays

Measurement of 20αHSD activity. Enzyme activity was determined as the rate of conversion of [1,2-^{3}H]-20α-hydroxy-pregn-4-ene-3-one (20α-hydroxyprogesterone) to [1,2-^{3}H]progesterone in the presence of NADPH as described by Jones and Hsueh (15). One unit of enzyme activity is defined as that amount of enzyme catalyzing the formation of 1 nmol progesterone/min. Specific activity is expressed as units per min/mg protein. The protein concentration was determined by the Bradford method, using BSA as the standard (35). In some cases, enzyme activity was also measured spectrophotometrically either by following the reduction of NADP (6, 9) or by oxidation of NADPH (7, 9).

Measurement of PG endoperoxide synthase activity. To test for the PG endoperoxide synthase type catalytic activity in rec 20αHSD, both cyclooxygenase activity (that converts arachidonic acid into the hydroperoxide, PGG_2) and peroxidase activity (that reduces PGG_2 and other hydroperoxides to the corresponding alcohols, such as PGH_2) were measured. Cyclooxygenase assay was performed by following the conversion of [^{3}H]arachidonic acid to [^{3}H]PGs (PGH_2 and PGE_2) as described by Mitchell *et al.* (36). Peroxidase activity was measured by monitoring the oxidation of *N,N′,N′*-tetramethyl-*p*-phenylenediamine at 611 nm (37).

Aldo-keto reductase activity. Aldo-keto reductase activity of rec 20αHSD was assayed spectrophotometrically by measuring the rate of oxidation of NADPH at 340 nm with a Gilford DU model spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) at 37 C using the saturating concentration of substrate (benzaldehyde, 4-nitrobenzaldehyde, D,L-glyceraldehyde methylglyoxal, 9,10-phenanthrequinone, or D-galactose) (38). One unit of enzyme activity is defined as that amount of enzyme that catalyzes the formation of 1 nmol NADP/min.

In vitro phosphorylation of rec 20αHSD. Purified bacterially expressed rec 20αHSD was first incubated in 500 ml 20 nM Tris-HCl (pH 8), 1 mM $MgCl_2$, 1 mg/ml BSA, 0.5 mM dithiothreitol, and 60 U alkaline phosphatase coupled to agarose beads for 30 min at 30 C. After centrifugation, the supernatant containing 20αHSD was used as a substrate in various *in vitro* kinase assays. The PKA, PKC, and insulin receptor-associated tyrosine kinase activities were measured as described previously (39, 40). Src kinase ($p60^{src}$) activity was assayed as described by Simonson and Herman (41). The concentration of rec dephosphorylated 20αHSD used in these assays was between 2–3 μg.

To test the relationship between phosphorylation and alteration of 20αHSD activity, aliquots of 20αHSD (1–2 μg) were incubated with 25 mM PIPES, pH 6.8, containing 10 mM Mg acetate, 100 μM ATP (or [γ-^{32}P]ATP to monitor phosphorylation), and EGTA (0.5 mM) plus purified rat liver PKC (4 μg; 10 U) or PKC, phosphatidylserine (250 μg/ml), diolein (10 μg/ml), plus $CaCl_2$ (0.25 mM) in a final volume of 50 ml for 60 min at 30 C. Similarly, the PKA-mediated phosphorylation of rec 20αHSD was carried out in a final volume of 50 ml containing 50 mM MOPS (pH 7.0), 10 mM $MgCl_2$, and 250 μM ATP (or [γ-^{32}P]ATP) to monitor phosphorylation and the catalytic subunit of PKA (10 U) for 60 min at 30 C. Reactions without ATP were performed under identical conditions. The catalytic activities of phosphorylated and nonphosphorylated forms of 20αHSD were determined radiochemically as described above. The phosphorylated ^{32}P-labeled 20αHSD preparations were analyzed by SDS-PAGE followed by autoradiography (42).

Results

Expression and purification of rec 20αHSD in E. coli

20αHSD was expressed as GST fusion in bacteria using pGEX expression plasmids. After induction with isopropyl-β-D-thiogalactopyranoside, a 63-kDa protein corresponding to GST-20αHSD was the most prominent band within the crude bacterial lysates, as judged by the SDS-PAGE (data not shown). The GST-20αHSD was purified by binding to glutathione-Sepharose beads (Fig. 1, lane 2) followed by thrombin cleavage (Fig. 1, lanes 3 and 4). Incubation of purified GST-20αHSD with thrombin released a 37-kDa protein (the predicted size of 20αHSD) as determined by SDS-PAGE and immunoblot analysis (Fig. 1, lanes 3 and 4). The purified preparation of 20αHSD showed high levels of enzymatic activity (Fig. 2). The average specific activity of the purified preparations of 20αHSD was 332 ± 47 nmol/min·mg protein ± SE), and activity was concentration (enzyme protein) and time dependent. No such enzyme activity was detected using the GST protein alone.

Expression and purification of 20αHSD in insect (Sf-9) tissue culture cells using the baculovirus system

Insect cells infected with recombinant 20αHSD viral construct AcNPV/20αHSD 1, 2, 3, or 4 were harvested 5 days after infection, lysed, and analyzed by SDS-PAGE and immunoblotting. All four recombinants showed a prominent band in the 37-kDa region of the Coomassie blue-stained gel (Fig. 3, *left panel*); the recombinant virus AcNPV/20αHSD no. 4, however, exhibited the highest expression of 37-kDa protein. Immunoblots of these same samples using a rat luteal 20αHSD specific antibody are shown in Fig. 3 (*right panel*). The antibody immunoreacted with the 37-kDa protein, confirming that the protein is indeed 20αHSD.

To assess the functional activity of recombinant 20αHSD, Sf-9 cells were harvested 5 days after infection with AcNPV/20αHSD 1, 2, 3, or 4 (or wild-type virus as a control), and cellular lysates were tested for 20αHSD activity. As shown in Fig. 4, only one of the four recombinant viral clones (no. 4) showed strong expression of 20αHSD activity and was used for further study. Uninfected control cells or wild-type virus exhibited cells infected with no activity (data not shown). Next, Sf-9 cells infected with the recombinant baculovirus

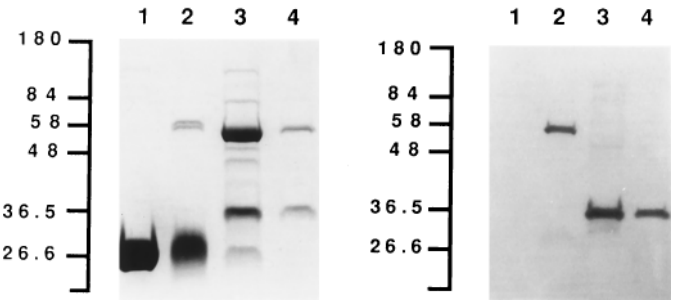

FIG. 1. SDS-PAGE and Immunoblotting of purified rec GST-20αHSD and purified rec 20αHSD. *E. coli* transfected with pGEX-4T-3-20αHSD was grown in 2 × YTG medium. 1PTG was added to induce the expression of fusion protein. Twenty micrograms of protein were used as a starting material. GST and rec GST-20αHSD were purified with glutathione beads. To release 20αHSD, the bound GST-20αHSD proteins were digested with thrombin at room temperature for 6 h. The purified rec GST-20αHSD and purified rec 20αHSD proteins (3.2 μg each) were subjected to SDS-PAGE, transferred to cellulose membranes, and immunodetected with the anti-20αHSD antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. *Left panel*, Gel stained for proteins with Coomassie blue. *Right panel*, Immunoblots. Lane 1, Purified GST; lane 2, purified GST-20αHSD; lane 3, thrombin cleavage for fusion protein bound to glutathione-Sepharose 4B; lane 4, proteins remained absorbed to column after thrombin digestion.

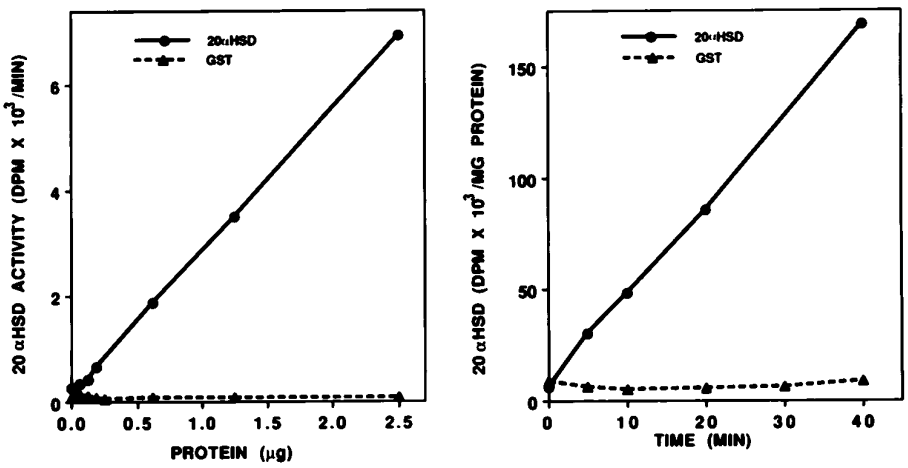

FIG. 2. Catalytic activity of bacterially expressed rec 20αHSD as a function of enzyme concentration and incubation time. *Left panel*, Various amounts of purified 20αHSD (0.025–2.5 μg 20αHSD expressed on the *x*-axis) were incubated with 100 μM [^{3}H]20α-hydroxyprogesterone and 300 μM NADP in buffer for 30 min. *Right panel*, 0.625 μg purified 20αHSD was incubated with 100 μM [^{3}H]20α-hydroxyprogesterone and 300 μM NADP for 5–40 min. In each case, the enzyme activity was quantified by the formation of [^{3}H]progesterone. The purified preparation of rec 20αHSD was obtained as described in Fig. 1 and *Materials and Methods*. Essentially the same results were obtained for three separate experiments.

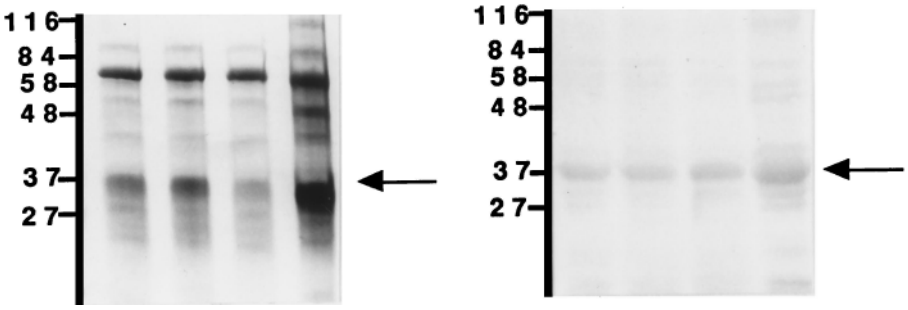

FIG. 3. SDS-PAGE and immunoblot analysis of insect cells infected with rat 20αHSD recombinant baculoviruses. Sf-9 cells were infected with recombinant viruses, and after 5 days, cell lysates (obtained from 1×10^7 cells) were subjected to SDS-PAGE. *Left panel*, The 10% gel was stained for proteins with Coomassie blue. *Right panel*, After gel electrophoresis, the proteins were transferred to nitrocellulose membrane. The blot was visualized with rabbit anti-20αHSD followed by alkaline phosphatase-conjugated goat antirabbit IgG. Lane 1, AcNPV/20αHSD 1; lane 2, AcNPV/20αHSD 2; lane 3, AcNPV/20αHSD 3; lane 4, AcNPV/20αHSD 4.

containing 20αHSD cDNA (AcNPV/20αHSD 4) were assayed for 20αHSD activity as a function of days after infection (Fig. 5). Enzyme activity in the total cell lysates was maximal (range, 750–893 pmol/min·mg protein) at 2 days postinfection. Between days 3–6 there was a steady and substantive decline in 20αHSD activity.

To purify 20αHSD to homogeneity, 5.3 g insect cell extract were subjected to several steps of purification, as shown in Table 1. The purification procedure sequentially used ion exchange chromatography on diethylaminoethyl cellulose and affinity chromatographies on Matrex Gel Green dye and Red-Sepharose (9, 33). The protocol resulted in 238-fold purification of rec 20αHSD (Table 1). On SDS-PAGE, the purified rec 20αHSD showed a single protein band with a mol wt of 37 kDa (data not shown).

Properties of rec 20αHSD

The purified preparations of recombinant 20αHSD expressed in *E. coli* and insect cells were employed to examine the enzyme characteristics.

To determine the substrate specificity of 20αHSD, we examined the oxidation/reduction of several steroid substrates (Table 2). In addition to its capacity for 20α-hydroxyprogesterone oxidation, 20αHSD reduced progesterone and 17α-hydroxyprogesterone. However, compared to 20α-hydroxyprogesterone, 20αHSD activity toward 17α-hydroxyprogesterone was weaker, and its activity toward progesterone was relatively stronger. In contrast, corticosterone and 5α-dihydrotestosterone were not used by the enzyme. Recombinant 20αHSD showed an absolute requirement for NADP when using 20α-hydroxyprogesterone as a steroid substrate; 20αHSD activity with NAD as a cofactor was less than 1% of that with NADP.

Steady state kinetic analysis of rec 20αHSDs

As the structure of 20αHSD contains a putative *N*-glycosylation site, we sought to determine whether glycosylation was required for catalytic activity. To accomplish this, we compared the catalytic parameters of rec enzyme preparations expressed in bacteria (nonglycosylated) and insect cells (presumably glycosylated protein). Kinetic constants were determined with 20α-hydroxyprogesterone as a substrate

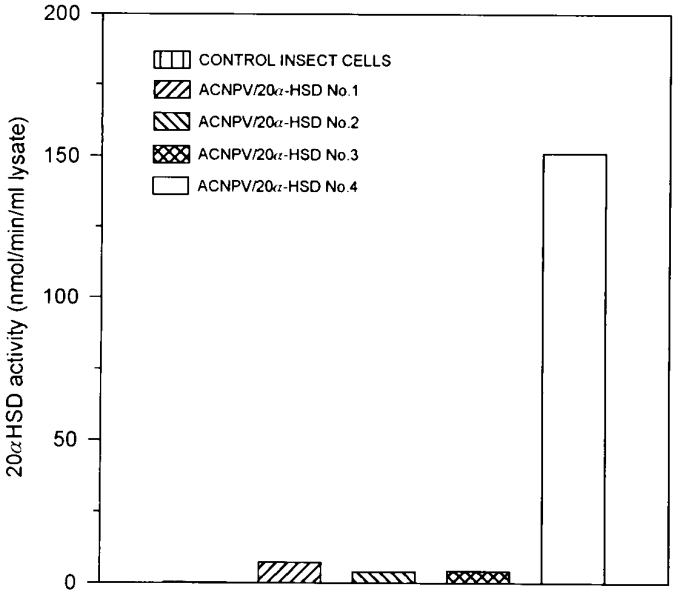

FIG. 4. Demonstration of catalytic function of 20αHSD in Sf-9 cells infected with recombinant viruses. Sf-9 insect cells were infected with recombinant viruses AcNPV/20αHSD 1, 2, 3, and 4 as described in Fig. 3. After 5 days, the cells were harvested, and total cell extracts were assayed for 20αHSD activity radiochemically. The data are expressed as the mean of duplicate determinations from a single experiment.

and NADP as a cofactor. The results obtained with different amount of 20α-hydroxyprogesterone (1–100 μM) and a fixed concentration of NADP (1 mM) using bacterially expressed rec enzyme shown in Fig. 6A indicate that enzyme activity reached a saturation level around 10 μM 20α-hydroxyprogesterone. The data were transformed into a reciprocal plot [Lineweaver and Burk plot to calculate the K_m and maximum velocity (V_{max}) values; Fig. 6A, *inset*]. The initial velocity experiments were also performed under conditions where NADP concentrations varied (1–1000 μM) at a fixed saturating concentration of 20α-hydroxyprogesterone (100 μM). Such data using bacterially expressed enzyme are shown in Fig. 6B. The maximal 20αHSD activity was around 100 μM NADP. Similar data were obtained using the purified preparations of 20αHSD expressed in insect cells, and the kinetic parameters obtained from bacteria and insect cell-derived 20αHSD are summarized in Table 3. The calculated K_m values of bacterially expressed enzyme for 20α-hydroxyprogesterone and NADP were 5.9 and 9.5 μM, respectively. The corresponding values for baculovirus-expressed 20αHSD were 5.8 and 9.6 μM, respectively. Thus, the enzyme preparations from two different sources had similar values. However, the rec 20αHSD expressed in *E. coli* had V_{max} values that were 20–30% higher than those of the rec 20αHSD expressed in insect cells when measured using similar assay conditions. These results suggest that glycosylation of 20αHSD may not be required for the expression of catalytic activity.

Aldose/aldehyde reductase and PG synthase activities of rec 20αHSD

As the cDNA and predicted amino acid sequence of rat luteal 20αHSD are related to bovine lung $PGF_{2\alpha}$ synthase and aldose/aldehyde reductase (20, 21, 43), we sought to determine whether 20αHSD was active as an aldose/aldehyde reductase and/or PG synthase. As shown in Table 4, the purified rec enzyme preparations were quite active against the general substrate of aldose/aldehyde reductase. The enzyme reduced benzaldehyde, 4-nitrobenzaldehyde, and D,L-glyceraldehyde very efficiently, whereas it was only weakly reactive toward methylglyoxal, 9,10-phenanthrenquinone, and D-galactose. Maximal activity was observed with either benzaldehyde or D,L-glyceraldehyde, which was roughly one third of that observed with 20α-hydroxyprogesterone (Table 4). Also, the rec bacteria- and baculovirus-expressed proteins showed similar reactivities. In contrast, no PG synthase type of activity (measured by following the conversion of [^{3}H]arachidonic acid to PGs or the production of H_2O_2) was detected in the purified preparations of rec bacterial and baculoviral 20αHSDs.

Phosphorylation and catalytic function of 20αHSD

To test the possibility that phosphorylation modulates 20αHSD activity, the purified rec bacterial 20αHSD was incubated with various kinases after pretreatment with alkaline phosphatase to ensure the protein was in a nonphosphorylated state. After treatment with various kinases the rec enzyme was assayed for 20αHSD activity. As shown in Table 5 and Fig. 7, A and B, rec 20αHSD was readily phosphorylated by *in vitro* incubation with purified PKA or PKC in the presence of [γ-^{32}P]ATP. Phosphoamino acid analysis of phosphorylated 20αHSD confirmed that [^{32}P]phosphorylation was restricted to serine and threonine residues, and label was predominantly on serine residues (data not shown). In contrast, members of the tyrosine kinase family, including Src kinase and insulin receptor-associated tyrosine kinase, failed to phosphorylate 20αHSD (Table 5). All kinases tested, however, rapidly phosphorylated their respective artificial substrates (Table 5). As shown in Fig. 7C, when rec 20αHSD was phosphorylated with PKC in the presence of ATP-Mg^{2+}, DAG, and phosphatidylserine, 20αHSD activity was unaffected. Similarly, the enzyme activity was not significantly affected by phosphorylation with PKA. These results suggest that phosphorylation of 20αHSD is not essential for catalytic activity.

Discussion

Although 20αHSD plays a central role in luteolysis and parturition (1–4), the limited availability of the purified form of enzyme has restricted its biochemical and functional studies. The recent cloning of 20αHSD gene (20, 21) revealed a high degree of homology to members of the NADP aldo-keto reductase family such as aldose/aldehyde reductase and $PGF_{2\alpha}$ synthase and has helped to identify several important structural features that may be critical to enzyme function. These included potential sites for phosphorylation by serine/threonine and tyrosine kinases and sites for adenine nucleotide binding and glycosylation (20). To evaluate the functional significance of these features, we first directed our efforts at establishing conditions that would allow for high level 20αHSD protein expression through the use of bacterial

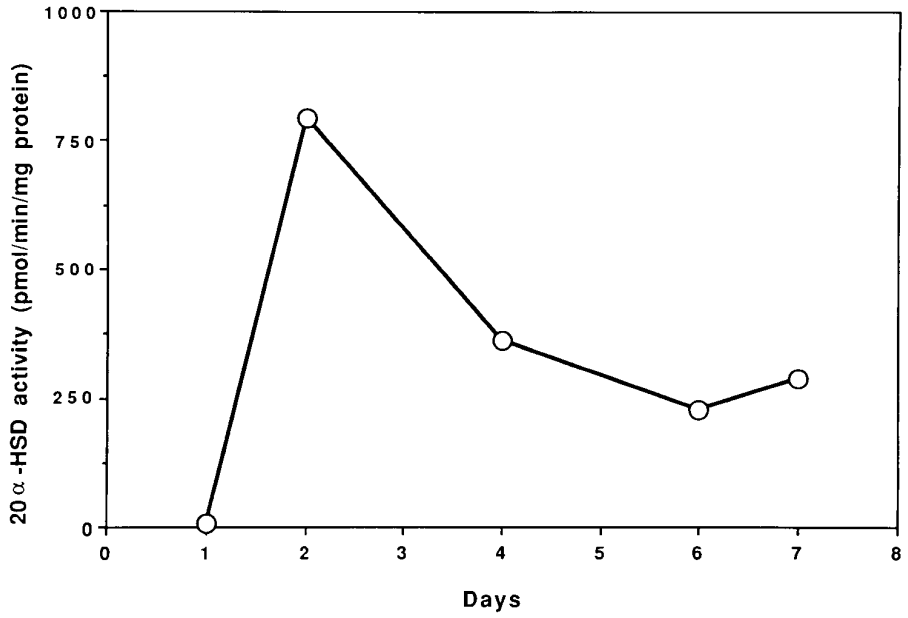

FIG. 5. Time course of expression of 20αHSD activity in insect cells infected with 20αHSD recombinant baculovirus. Sf-9 cells were infected with a recombinant baculovirus vector (AcNPV/20αHSD 4) and then harvested 1, 2, 4, 6, and 7 days postinfection, as indicated. Total cell lysates were prepared and assayed for 20αHSD activity.

TABLE 1. Purification of recombinant 20αHSD from insect tissue culture cells

Purification step	Total units (nmol/min)[a]	Total protein (mg)	SA (U/mg protein)	Yield (%)	Purification (fold)
Cell lysate	252	231	1.09	100	1.0
100,000 × *g* supernatant	225	124	1.80	89	2.0
DEAE-cellulose	187	7.3	25.6	74	23.0
Matrex Gel Green dye	152	2.8	54.1	60	50.0
Red-Sepharose	108	0.42	259.6	43	238.0

The cell pellet (5.3 g wet weight) was resuspended in 30 ml homogenization buffer containing 10 mM potassium phosphate buffer (pH 8.0) 1 mM EDTA, and 10 mM dithiothreitol (buffer A) and homogenized in a Potter Elvehjem homogenizer at 4 C. The homogenate was sonicated and centrifuged for 10 min at 1,000 × *g* to remove nuclei, unbroken cells, and cell debris. The resulting supernatant was centrifuged for 1 h at 100,000 × *g*, and the resultant supernatant was loaded onto a DEAE-cellulose column (1.6 × 20 cm) that had been previously equilibrated with buffer A. The column was eluted with a 120-ml linear gradient of NaCl (0–500 mM) in the equilibration buffer. The fractions containing the enzyme activity (detected by spectrophotometric assay method) were pooled, concentrated, and dialyzed against buffer A. After dialysis, the sample was applied to a Matrex Gel Green dye affinity column (1.2 × 12 cm) (7, 33) preequilibrated with buffer A. The enzyme activity was eluted with a linear gradient of KCl (0–2 M) in buffer A. The active fractions were combined, concentrated by ultrafiltration, dialyzed against buffer A, and further purified by affinity chromatography on a Red-Sepharose (1.2 × 10 cm). The enzyme activity was eluted from column in buffer A containing 1 mM NADPH. Fractions containing the enzyme were combined, concentrated, and dialyzed against buffer A containing 20% glycerol before storage at −80 C.

[a] One unit is defined as the amount of enzyme that will produce 1 nmol progesterone/min. Results are the means of duplicate determinations from a single experiment.

TABLE 2. Substrate specificity of recombinant 20αHSD

Substrate (100 μM)	*rec* 20αHSD activity (nmol/min · mg protein) expressed in	
	E. coli	Sf-9 cells
Progesterone	459	352
20α-Hydroxyprogesterone	321	250
17α-Hydroxyprogesterone	97	85
Corticosterone	ND	ND

The enzyme activity was measured spectrophotometrically. The data are expressed as the mean of duplicate determinations from three separate experiments. ND, Not detected.

and baculoviral expression systems (31, 32). This was then followed by purification of rec enzyme to apparent homogeneity and the detailed characterization of the kinetic, biochemical, and functional properties of the enzyme.

Our results show that these two expression systems provide large quantities of rec 20αHSD for biochemical studies and for examination of the various catalytic functions performed by the enzyme. Also, these results may provide insights into the roles of glycosylation and phosphorylation in enzyme catalysis. In general, rec 20αHSD had catalytic properties similar to those reported for 20αHSD purified from rat ovarian tissues (6, 7, 9). Immunoblotting experiments using purified rec 20αHSD preparations from bacterial and insect cells and specific 20αHSD antibody resulted in the detection of a 37-kDa protein. This agrees closely with the calculated molecular mass of the encoded protein (20) and with that of the native ovarian enzyme found in the corpus luteum (34).

Data presented in this report also demonstrate that rec 20αHSD was able to use (reduce) substrates specific for aldose/aldehyde reductases, although these substrates were 3–5 times less active relative to progesterone/20α-hydroxyprogesterone. Although these findings could be interpreted as indicating that rec enzyme might be an aldose/

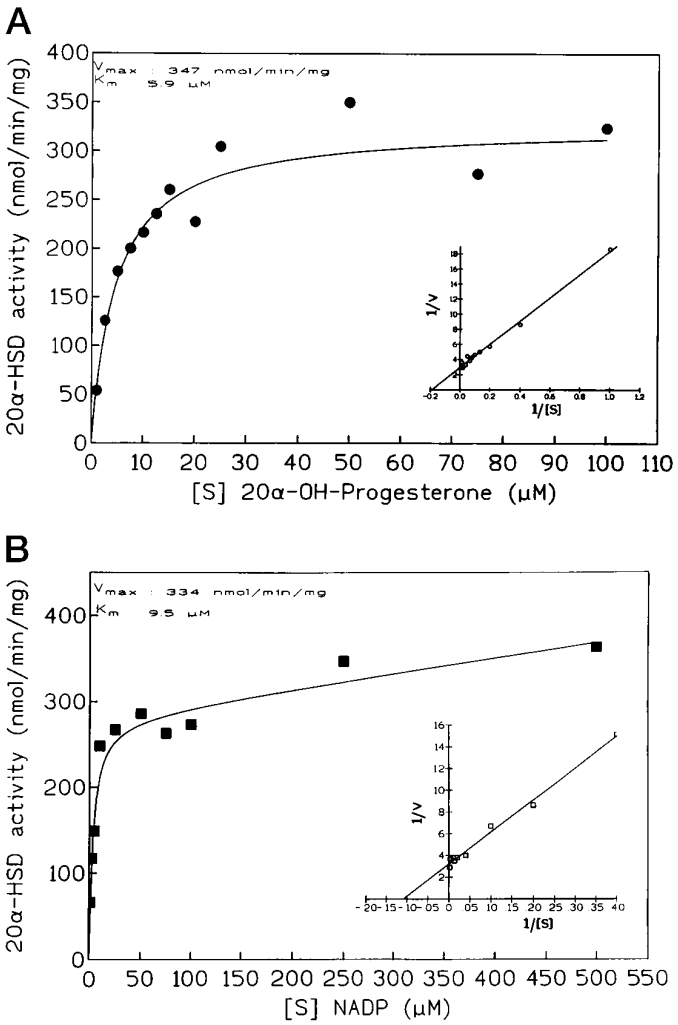

FIG. 6. 20α-Hydroxyprogesterone and NADP dependence of purified rec 20αHSD expressed in bacteria. A, Initial velocities *vs.* 20α-hydroxyprogesterone concentrations. Each reaction mixture contained 200 ng rec 20αHSD, the indicated concentrations of 20α-hydroxyprogesterone, and 1 mM NADP. The assays were conducted at 37 C for 10 min. B, Initial velocities *vs.* NADP concentrations. Each assay mixture contained 200 ng rec 20αHSD, the indicated concentration of NADP, and a saturating concentration of 20α-hydroxyprogesterone. The assays were carried out at 37 C for 7.5 min. The double reciprocal plot for each substrate is shown in the *inset*. Essentially the same results were obtained for three separate experiments.

aldehyde reductase, the cDNA sequence information does not support this possibility (20). The cDNA encoding rat luteal 20αHSD shows less than 50% nucleotide sequence homology with that of rat aldose/aldehyde reductase (20, 21). We believe that the observed aldose/aldehyde reductase activity for the rec 20αHSD represents an additional intrinsic catalytic function of the enzyme protein. Recently, 20αHSD activity in bovine testis has been shown to be due to aldose reductase (22). In this case, however, the cDNA encoding bovine testicular 20αHSD is 100% identical with that encoding bovine lens aldose reductase, and that deduced amino acid is identical with that of bovine aldose reductase (22). In addition, the testicular enzyme, unlike the ovarian 20αHSD, favors 17α-hydroxyprogesterone over progesterone (24).

TABLE 3. Steady state kinetic parameters of recombinant 20αHSD

Parameters	*rec* 20αHSD expressed in	
	E. coli	Sf-9 cells
Mean SA ± SE (nmol/min · mg protein)	332 ± 47	259 ± 36
K_m, 20α-hydroxyprogesterone (μM)	5.9	5.8
K_m, NADP (μM)	9.5	9.6
V_{max}, 20α-hydroxyprogesterone (nmol/min · mg protein)	347	265
V_{max}, NADP (nmol/min · mg protein)	334	259

The K_m and V_{max} values of *rec* bacterial enzymes for 20α-hydroxyprogesterone and NADP were derived from the data shown in Fig. 7. The kinetic parameters of *rec* baculoviral 20αHSD for 20α-hydroxyprogesterone were determined by incubating 250 ng 20αHSD, 300 μM NADP, and 1–100 μM 20α-[^{3}H]progesterone for 10 min at 37 C in a buffer containing 0.1 M Tris-HCl (pH 8.0), 5 mM dithiothreitol, 1 mM EDTA and 10 mM nicotinamide. The kinetic parameters for NADP were determined by incubating 250 ng 20αHSD, 1–1000 μM NADP, and 100 μM 20α-hydroxyprogesterone for 7.5 min at 37 C in a buffer containing 0.1 M Tris HCl (pH 8.0), 5 mM dithiothreitol, 1 mM EDTA, and 10 mM nicotinamide. In each case, the formation of [^{3}H]progesterone was quantified by TLC followed by scintillation spectrometry. The results are expressed as the mean of duplicate determinations from two separate experiments.

TABLE 4. Aldo-Keto reductase activity of recombinant 20αHSD

Substrate	*rec* 20αHSD activity (nmol/min · mg protein) expressed in	
	E. coli	Sf-9 cells
20α-Hydroxyprogesterone (100 μM)	327	258
Benzaldehyde (250 μM)	122	81
4-Nitrobenzaldehyde (1 mM)	103	89
D,L-Glyceraldehyde (250 μM)	115	96
Methylglyoxal (1 mM)	67	50
9,10-Phenanthrenquinone (100 μM)	48	32
D-Galactose (400 mM)	65	45

The enzyme activity was measured spectrophotometrically. Results are the means of duplicate determinations from three separate experiments. Other details are described in *Materials and Methods.*

Also, the purified enzyme can reduce glucose, benzaldehyde, and glyceraldehyde (the classic aldose reductase substrates) with the same high efficiency as that of 17α-hydroxyprogesterone (22).

Despite extensive efforts, we were unable to demonstrate PG synthase activity using rec 20αHSD preparations purified from bacterial and baculoviral expression systems. This lack of PG synthase activity is surprising, as the cDNA deduced amino acid sequence of 20αHSD shows a great degree of sequence homology (~67%) with bovine lung PG synthase (20). The reason for this unexpected finding is not clear, but one could consider several possibilities. One of the simplest possibilities could be that despite a close sequence homology, the rec 20αHSD does not possesses PG synthase-type activity. Other possibilities might include that rec 20αHSD lacks proper protein folding and configuration, is not properly glycosylated, or is not adequately processed. Indeed, glycosylation *per se* is necessary for the catalytic function of both the constitutive and inducible forms of the cyclooxygenase component of PG synthases (44, 45). Clearly, more experi-

TABLE 5. Effects of various serine/threonine and tyrosine kinases on the phosphorylation of recombinant 20αHSD

	Histone IIIs (nmol/min · mg protein)	*rec* 20αHSD (nmol/min · mg protein)
PKC		
Basal (EGTA)	279	0.034
Stimulated (Ca^{2+} + PS + DO)	2496	0.593
	Histone V (nmol/min · mg protein)	*rec* 20αHSD (nmol/min · mg protein)
PKA		
Free catalytic subunit	415	0.593
	PolyGlu:Tyr (4:1) (pmol/min · mg protein)	*rec* 20αHSD (pmol/min · mg protein)
Insulin receptor TK		
Basal	38	ND
Stimulated (insulin)	206	ND
	cdc2 (6–20) peptide (nmol/min · mg protein)	*rec* 20αHSD (nmol/min · mg protein)
src kinase (p^{60src})	782	ND

The experimental details are described in *Materials and Methods*. Results are expressed as the means of duplicate determinations from two separate experiments. ND, not detected; TK, tyrosine kinase.

mental work is needed to determine whether rec 20αHSD has any intrinsic PG synthase activity.

Because the activity of ovarian 20αHSD is regulated by hormones that activate tyrosine kinase(s), PKC and PKA, we considered the possibility that phosphorylation may affect enzyme activity. We first compared the catalytic activities of rec bacterial and insect enzymes, which should differ in their phosphate content. In general, the proteins expressed in bacteria are presumed to be nonphosphorylated, whereas the rec phosphoproteins of baculoviral origin are considered to be sufficiently phosphorylated (28, 29). However, no difference in enzyme activity was noted between these two preparations. To further examine the effects of phosphorylation on 20αHSD activity, the dephosphorylated rec enzyme was phosphorylated with serine/threonine- or tyrosine-specific kinases. 20αHSD was a good substrate for both PKA and PKC. In contrast, Src- and insulin receptor-associated tyrosine kinases failed to act on 20αHSD, and no phosphorylation on tyrosine residues was detected. Interestingly, neither PKA nor PKC induced phosphorylation of rec 20αHSD had any significant effect on its activity. These findings together with the results we previously reported (18, 20, 34) suggest that hormonal regulation of 20αHSD activity is not through a phosphorylation/dephosphorylation mechanism, but, rather, through regulation of 20αHSD gene expression. PRL inhibits the activity of 20αHSD and markedly downregulate 20αHSD gene expression and the level of its protein, whereas LH stimulates 20αHSD enzyme activity, which is accompanied by increased levels of 20αHSD messenger RNA.

In the current studies, we failed to detect any difference in activity between bacterially expressed (nonglycosylated) and insect cell-expressed (presumably glycosylated) forms of 20αHSD. These indirect studies led us to conclude that glycosylation of 20αHSD may not be required for the catalytic function of the enzyme. The follow-up studies are in progress to more directly examine the glycosylation of 20αHSD and further assess the role of glycosylation in enzyme catalysis.

In summary, this report describes the high level expression, purification, and characterization of 20αHSD using *E. coli* and baculovirus expression systems. The rec enzyme preparations are essentially indistinguishable from that isolated from rat ovary, except that bacterially expressed enzyme is nonglycosylated and possibly poorly phosphorylated. The kinetic parameters of both bacterially expressed and insect cell expressed rec enzymes have been determined and suggest that phosphorylation and glycosylation of 20αHSD may not be required for enzyme catalysis. Finally, the fact that the rec 20αHSD can be expressed and readily purified, indicates that the bacterial and viral systems are excellent choices for exploring the detailed mechanism of action of this multifunctional enzyme protein.

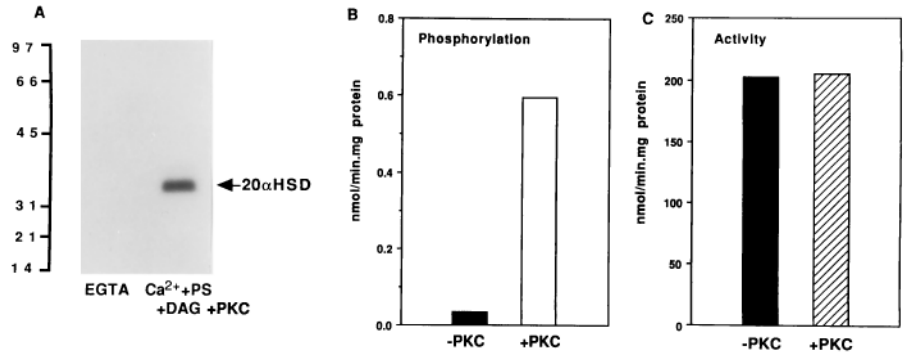

FIG. 7. Phosphorylation and catalytic function of 20αHSD. A, PKC-mediated ^{32}P phosphorylation of rec, dephosphorylated, bacterially expressed 20αHSD was performed as described in *Materials and Methods* and analyzed by SDS-PAGE and autoradiography. *Left lane*, Basal (EGTA); *right lane*, Ca^{2+}, DAG, phosphatidylserine, and PKC. B, Aliquots of rec dephosphorylated 20αHSD (0.9 μg) were phosphorylated with [γ-^{32}P]ATP-Mg^{2+} with or without PKC, DAG, and Ca^{2+} *in vitro* as described in *Materials and Methods*. The incorporation of ^{32}P into 20αHSD was quantified by liquid scintillation spectrometry. C, Aliquots of rec dephosphorylated 20αHSD were phosphorylated as described in B, except [γ-^{32}P]ATP was replaced with a similar concentration of unlabeled ATP. The catalytic activities of both phosphorylated and dephosphorylated forms were quantified by following the conversion of [^3H]20α-hydroxyprogesterone to [^3H]progesterone. Other details are given in *Materials and Methods*.

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