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Essential Role of Adenosine Triphosphate in Activation of 17β-Hydroxysteroid Dehydrogenase in the Rat Leydig Cell

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ABSTRACT

The forskolin-induced steroidogenic block of testosterone production residing beyond pregnenolone synthesis in rat Leydig cells was localized to the level of the 17β-hydroxysteroid dehydrogenase (17βHSD) reaction in this study. The use of forskolin analogs that discriminate between the diterpene's inhibitory effect on the glucose transporter(s) (1,9-dideoxyforskolin) and its activation of adenylate cyclase (6-aminoethyl carbamyl forskolin) revealed that the block is related to inhibition of glucose transporter(s). 1,9-Dideoxyforskolin, but not 6-aminoethyl carbamyl forskolin, caused a significant inhibition of basal and hCG-stimulated testosterone production with accumulation of androstenedione. Glucose-deficient media produced the same metabolic block in the absence of forskolin, with a significant reduction in 17βHSD activity and increases in the apparent K_m for androstenedione. In contrast, metabolic steps before testosterone formation were not affected. Glucose-induced 17βHSD activation was mimicked by the addition of ATP or GTP in glucose-deficient media, but not by nonhydrolyzable triphosphate analogs or NADPH. A decrease in 17βHSD activity caused by KT-5720, a specific inhibitor of protein kinase A and the calmodulin antagonist W-7, indicates that the ATP requirement may be related to the participation of protein kinases in the activation of 17βHSD. ATP levels derived from alternative (nonglycolytic) pathways are adequate to support basal and hormone-stimulated enzymatic activities in the metabolism of cholesterol to androstenedione. However, the integrity of the glucose transport system with subsequent ATP generation is required for activation of 17βHSD in the final step of androgen biosynthesis. In conclusion, the conversion of androstenedione to testosterone requires the contribution of the glycolytic pathway to meet ATP requirements for 17βHSD activity. (*Endocrinology* **138:** 1612–1620, 1997)

ANDROGEN production by the Leydig cell is dependent on androstenedione, the immediate precursor of testosterone synthesis, and the activity of the microsomal enzyme, 17β-hydroxysteroid dehydrogenase (17βHSD), that converts androstenedione to testosterone. The early steps in steroidogenesis leading to androgen production are stimulated by the ligand-activated LH receptor that is coupled primarily through G_s to adenylyl cyclase, leading to stimulation of cAMP production.

Previous studies have demonstrated inhibition of testosterone production in Leydig cells treated with high concentrations of forskolin (1). Such cells showed increased basal and potentiated hCG-stimulated adenylate cyclase activity and cAMP production, indicating that the inhibitory action of forskolin on steroidogenesis was not related to changes in cyclase activation. Furthermore, the metabolic site of this forskolin-induced inhibition was beyond pregnenolone synthesis (1). Since this effect of forskolin was not related to cAMP generation, it was important to determine its location and biochemical mechanism. Forskolin has been shown to interact with membrane proteins other than the adenylate cyclase system, including channels and transporters (2–8). Several studies have shown that forskolin inhibits the glucose transporter in a number of different cells, including rat and human adipocytes (2, 3), human erythrocytes (4), platelets (5), and Leydig tumor cells (6). It has been previously shown that glucose is required for optimal hCG-stimulated testosterone production in the testis and in Leydig cell cultures (9, 10). Such findings raise the possibility that forskolin inhibition is related to its effect on glucose transporter(s) in the Leydig cell. On the basis of the structural similarity between α-D-glucose and forskolin, it has been postulated that forskolin binds to the glucose transporter through its β-face, whereas the interaction with adenylate cyclase occurs through its α-face (2). Analogs of forskolin with selective specificity for the glucose transporter or adenylate cyclase, namely the naturally occurring analog 1,9-dideoxyforskolin (1,9-DDF) and the synthetic analog 6-aminoethyl carbamyl forskolin (6-AEC-F), respectively (2, 11), were used in this study to elucidate the mechanism of forskolin's effect on steroidogenesis. The inhibitory action of forskolin on steroidogenesis was found to be related to the diterpene's inhibition of the glucose transporter and the metabolic block was localized to the 17βHSD reaction. These studies demonstrate an ATP requirement for stimulation of 17βHSD activity.

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Materials and Methods

Cell preparation, incubation, and assays

Leydig cells were prepared from adult male Sprague-Dawley rats by collagenase dispersion and purified by centrifugal elutriation (12). The

cells were centrifuged and resuspended in medium 199 (Microbiological Associates, Bethesda, MD) containing 0.1% BSA and 0.125 mM 3-methylisobutylxanthine (Aldrich Chemical Co., Milwaukee, WI). The cells (1×10^6 cells/ml) were incubated in 12×75-mm plastic tubes at 34 C for the designated time periods (see figure legends) with shaking at 100 cycles/min under 95% O_2-5% CO_2 in the presence or absence of hCG (CR121, preparation kindly provided by the Center for Population Research, NICHHD, Bethesda, MD) with or without forskolin, 1,9-DDF (Sigma Chemical Co., St. Louis, MO) or 6-AEC-forskolin (kindly provided by Dr. K. B. Seamon, FDA, NIH, Bethesda, MD). The incubations were terminated by transferring the tubes to an ice bath, and all further steps were performed at 0–2 C. The cells were then sedimented at $250 \times g$ for 12 min, and the supernatant was saved for the assay of extracellular cAMP, pregnenolone, progesterone, 17α-hydroxyprogesterone, androstenedione, and testosterone. The cell pellets were washed with ice-cold medium and processed for the analysis of intracellular cAMP. The measurements of cAMP and steroids were performed by RIA as previously described (13).

The effect of glucose on the steroidogenic pathway was determined by preincubating the cells in HEPES buffer, pH 7.4, containing 5.8 mM glucose for 120 min. The cells were washed three times with glucose-free HEPES buffer and then resuspended in the same buffer containing either 5.8 mM glucose or no glucose. The cells were further incubated in the presence or absence of hCG (260 pM) for 120 min at 34 C. After completion of the incubation period, the cells were processed as previously described for the measurement of various steroids.

For glucose uptake studies, Leydig cells were preincubated for 120 min in HEPES buffer (5 mM; pH 7.4) containing 4 mM KCl, 140 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.8 mM glucose, and 0.1% BSA. Before performing the uptake assay, cells were washed three times with glucose-free HEPES buffer. Leydig cells (10^6/ml) were then resuspended in glucose-free HEPES buffer, and glucose uptake was measured by adding 0.1 mM [1,2-^{3}H]2-deoxy-D[]-GLUCOSE OR 0.1 mM L-[1-^{3}H]glucose and various additives. The uptake reactions were terminated by adding 2 ml ice-cold glucose-free HEPES buffer followed by two washes with 1 ml ice-cold buffer. The cells were then solubilized in 400 μl 0.2 N NaOH and counted for radioactivity after adding Exscint II (National Diagnostics, Atlanta, GA). Cell-associated radioactivity was determined by adding ice-cold glucose-free HEPES buffer before the addition of labeled sugar at zero time.

All experiments were performed at least three times in triplicate; results are the mean ± SD unless otherwise specified.

Cell permeabilization

The Leydig cells were permeabilized to assess the effect of ATP, GTP, their nonhydrolyzable analogs (App[NH]p, Gpp[NH]p, and guanosine 5′-O-(-3-thiotriphosphate) GTPγS), various other nucleotides (including CTP, UTP, and TTP), and NADPH before incubation. The permeabilization was performed with an electroporation system (Cell-Porater, Life Technologies, Bethesda, MD). Leydig cells (10^6 cells/ml) were resuspended in HEPES-buffered saline (21 mM HEPES, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; and 6 mM glucose) and permeabilization was carried out at a 200–300 V and a capacitance of 10 μF with a 0.2-msec pulse length at 0–2 C. Incubation of permeabilized cells was performed in the presence or absence of various additives, and steroid measurements were performed as described above.

ATP measurement

For the measurement of total cellular ATP, the cells were preincubated in HEPES buffer, pH 7.4, containing 5.8 mM glucose for 60 min. The cells were washed three times with glucose-free buffer containing either 5.8 mM glucose or no glucose with or without hCG for 120 min. After completion of the incubation period, ATP was extracted as described previously (13) and measured with the ATP bioluminescent assay (Sigma).

Kinetic analysis of 17βHSD

The effect of glucose on 17βHSD was measured by preincubating Leydig cells (10^6 cells/ml) in HEPES buffer, pH 7.4, containing 5.8 mM glucose for 120 min, then washing the cells three times and further incubating with various concentrations of androstenedione (0–100 μM) along with a fixed concentration of [^{14}C]androstenedione (0.2 μM) in HEPES buffer (pH 7.4) in the presence or absence of glucose, with or without hCG and with or without ATP, App[NH]p, or GTP (1 mM). The incubation was carried out for 120 min at 34 C. Steroids in medium were extracted with ethyl acetate and separated on a silica-coated TLC plates with chloroform and ethyl acetate (3:1, vol/vol) as previously described (14). Quantitation of radioactive product was performed by PhosphorImage:SF (Molecular Dynamics, Sunnyvale, CA). Kinetic analysis was performed with ENZFITTER (Elsevier Biosoft, Amsterdam, The Netherlands).

To understand the mechanism of activation of 17βHSD and its regulation, we used a model in which the steroidogenic pathway was blocked before and after the 17βHSD reaction with aminoglutethimide (AG; Ciba-Geigy, Ardsley, NY), which has been reported to interfere with the conversion of cholesterol to pregnenolone through inhibition of the cholesterol side-chain cleavage reaction, and aromatization of androstenedione by inhibition of aromatase (15). After preincubating the cells in HEPES buffer (pH 7.4) containing 5.8 mM glucose for 120 min and washing three times, the cells were incubated in the presence or absence of glucose for 20 min with 100 μg/ml AG. Further incubation was carried out with various concentrations of androstenedione in the presence or absence of hCG with or without ATP for 120 min. In some experiments, after AG incubation, cells were further incubated for 60 min in the presence or absence of various inhibitors of protein kinase A (PKA), protein kinase C (PKC), and Ca^{2+}-calmodulin kinase (CaMK) before the addition of androstenedione and ATP. The PKC inhibitors (bisindolylmaleimide and staurosporine), the PKA inhibitors [H-89, Rp-cAMP (the R isomer of a sulfur-modified cAMP) antagonist, and KT-5720], and the CaMK inhibitors (KN-62, KN-93, and W-7) were purchased from Calbiochem (La Jolla, CA). To determine the effect of calcium, in some experiments the cells were preincubated for 120 min, then washed three times with glucose- and calcium-free HEPES buffer containing 2.5 mM EGTA. After completion of incubation, the production of testosterone was measured as described above.

Statistical significance was evaluated by ANOVA followed by Duncan's multiple range test.

Results

Initial studies were performed to locate the metabolic block in the steroidogenic pathway beyond pregnenolone synthesis that is caused by high doses of forskolin in hCG- or 8-bromo-cAMP (cAMP)-stimulated rat Leydig cells (1). Measurement of steroids in the biosynthetic pathway between pregnenolone and testosterone revealed that the forskolin-induced metabolic block occurs at the 17βHSD reaction, with accumulation of the substrate androstenedione and marked reductions in testosterone levels (Fig. 1, F, hCG+F). The significant stimulation of testosterone in the presence of hCG or cAMP (Fig. 1, hCG, cAMP) was not evident with forskolin *per se* (Fig. 1, F). More importantly, forskolin greatly reduced hCG-stimulated testosterone production by 50–70% (Fig. 1, hCG+F) and to a lesser extent reduced cAMP-stimulated testosterone production by 30–50% (Fig. 1, cAMP+F). Forskolin increased the steroid precursors progesterone and 17α-hydroxyprogesterone to hCG-stimulated levels (Fig. 1, F), and enhanced these levels upon simultaneous addition with hCG (Fig. 1, hCG+F). However, conversion of androstenedione to testosterone was severely impaired in the presence of forskolin. Thus, the forskolin block was localized to the 17βHSD reaction.

Two analogs of forskolin with selective specificities for adenylate cyclase (6-AEC-F) or for the glucose transporter (1,9-DDF) were employed to investigate whether the observed effects of forskolin on testosterone production were derived from its interaction with glucose transporter pro-

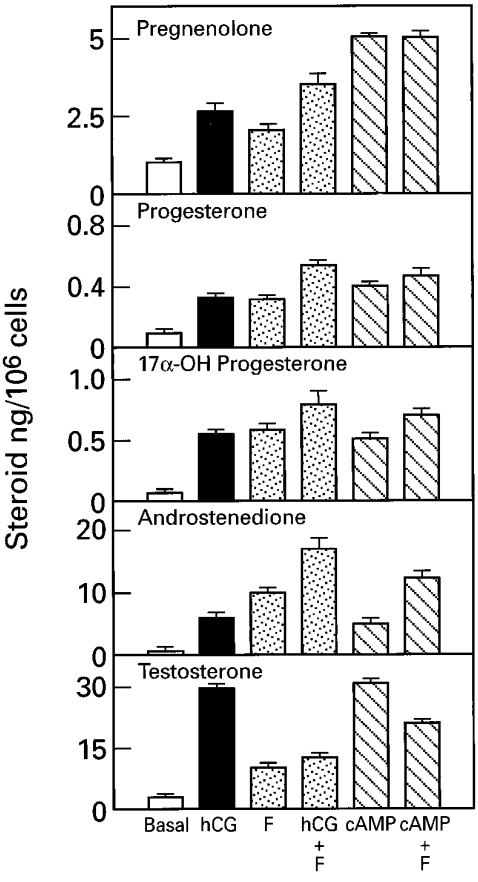

FIG. 1. Effect of forskolin (F) on metabolic products of the steroidogenic pathway in basal, hCG-stimulated, or cAMP-stimulated Leydig cells. Cells (10^6 cells/ml) were incubated for 120 min in the presence or absence of hCG (260 pM) or 8-bromo-cAMP (1 mM) with or without forskolin (10 μM). Each *point* represents the mean ± SE of incubations performed in triplicate in this and experiments in the following figures.

teins in the Leydig cell. The forskolin analog 6-AEC-F caused a small, but significant, stimulation of extracellular cAMP [basal, 0.06 ± 0.0004 pmol; 1 μM 6-AEC-F, 0.1 ± 0.002 pmol ($P < 0.02$); 10 μM 6-AEC-F, 0.16 ± 0.003 pmol ($P < 0.00005$)] and intracellular cAMP levels [basal, 0.12 ± 0.009 pmol; 1 μM 6-AEC-F, 0.21 ± 0.006 pmol ($P < 0.005$); 10 μM 6-AEC-F, 0.39 ± 0.03 pmol ($P < 0.00007$)] and markedly potentiated hCG-stimulated cAMP generation in a dose-dependent manner (Fig. 2, *left*). In all cases, forskolin was a more potent stimulant of cAMP than 6-AEC-F. Forskolin alone stimulated testosterone production in a dose-dependent manner to levels below those observed with the supramaximal concentration of hCG, whereas 6-AEC-F (10 μM) caused a minor, but significant, stimulation of androgen production ($P < 0.00007$), that was consistent with the degree of stimulation of cAMP by this analog. 1,9-DDF, which interacts exclusively with glucose transporter(s) (2), did not show any effect on cAMP pools at the doses employed (Fig. 2, *right*). Under basal conditions, 1,9-DDF caused significant, inhibition of testosterone production (basal, 1.1 ± 0.04 ng; 1,9-DDF, 0.62 ± 0.015 ng; $P < 0.00008$). It also exerted a profound inhibitory effect on hCG-stimulated testosterone production, which was comparable to that caused by forskolin.

The forskolin analog 1,9-DDF, which mimicked the inhibitory effect of forskolin on testosterone production (Fig. 2, *right*), did not increase basal or hCG-stimulated levels of pregnenolone, progesterone, or 17α-hydroxyprogesterone (Fig. 3). Forskolin, which possesses both cyclase-activating and glucose transporter inhibitory functions (2), increased the production of progesterone and 17α-hydroxyprogesterone (Fig. 3, basal *vs.* F *vs.* hCG), and enhanced hCG-stimulated production of these metabolites (Fig. 3, hCG *vs.* hCG+F). Both forskolin and 1,9-DDF markedly blocked the production of testosterone, with consequent accumulation of its precursor androstenedione in hCG-stimulated Leydig cells (Fig. 3, hCG *vs.* hCG+1,9-DDF; hCG *vs.* hCG+F). Basal testosterone levels were also significantly inhibited by 1,9-DDF (Fig. 3; basal, 3.1 ± 0.17 ng; 1,9-DDF, 1.6 ± 0.07 ng; $P < 0.001$).

To determine whether forskolin and 1,9-DDF inhibit testosterone production through inhibition of glucose transporter, glucose uptake was measured and was found to be significantly enhanced in hCG-stimulated cells from 2.2 ± 0.06 to 3.0 ± 0.02 nmol/h·10^6 cells ($P < 0.0002$); this increase was markedly reduced by 1,9-DDF to basal levels (2.29 ± 0.03 nmol/h·10^6 cells; $P < 0.006$). To monitor the direct effect of glucose deficiency on hCG-stimulated steroidogenesis, the steady state levels of the metabolites in the steroidogenic pathway were measured in the presence or absence of exogenous glucose. The results clearly demonstrate that the specific inhibition of testosterone production by forskolin and 1,9-DDF at the level of the 17βHSD reaction can be mimicked by incubating the Leydig cells in the absence of glucose (Fig. 4). Glucose deficiency, however, did not affect pregnenolone, progesterone, or 17α-hydroxyprogesterone levels, whereas it markedly inhibited testosterone levels, with accumulation of its precursor androstenedione (Fig. 4). Furthermore, the conversion ratio of testosterone and androstenedione was not significantly different in cells incubated in the absence of glucose with and without 1,9-DDF (0.13 ± 0.007 *vs.* 0.11 ± 0.011). These studies suggest that forskolin or 1,9-DDF inhibits testosterone production through the reduction of intracellular levels of glucose. No significant difference in the level of intracellular cAMP was observed in either the presence or absence of glucose (data not shown). Thus, within the steroidogenic pathway, the absence of glucose from the incubation media markedly affected 17βHSD activity in basal (2.2 ± 0.06 *vs.* 0.7 ± 0.06 ng testosterone; $P < 0.0001$) and hCG-stimulated cells, whereas it did not influence the production of precursors of the androgen pathway (Fig. 4).

The glucose requirement for 17βHSD activity was further investigated to determine whether byproducts of glucose metabolism, such as ATP (16, 17) or NADPH (18, 19), were responsible for activation of 17βHSD. Permeabilized Leydig cells were incubated in the presence or absence of glucose, with or without hCG, and ATP or NADPH. The addition of ATP to control and hCG- or 8-bromo-cAMP-stimulated cells, incubated in the absence of glucose, effectively stimulated the conversion of androstenedione to testosterone to the levels observed in the presence of glucose (Fig. 5, *left* and *right*). Thus, the addition of ATP increased 17βHSD activity in the absence of glucose, whereas glucose-deficient cells without

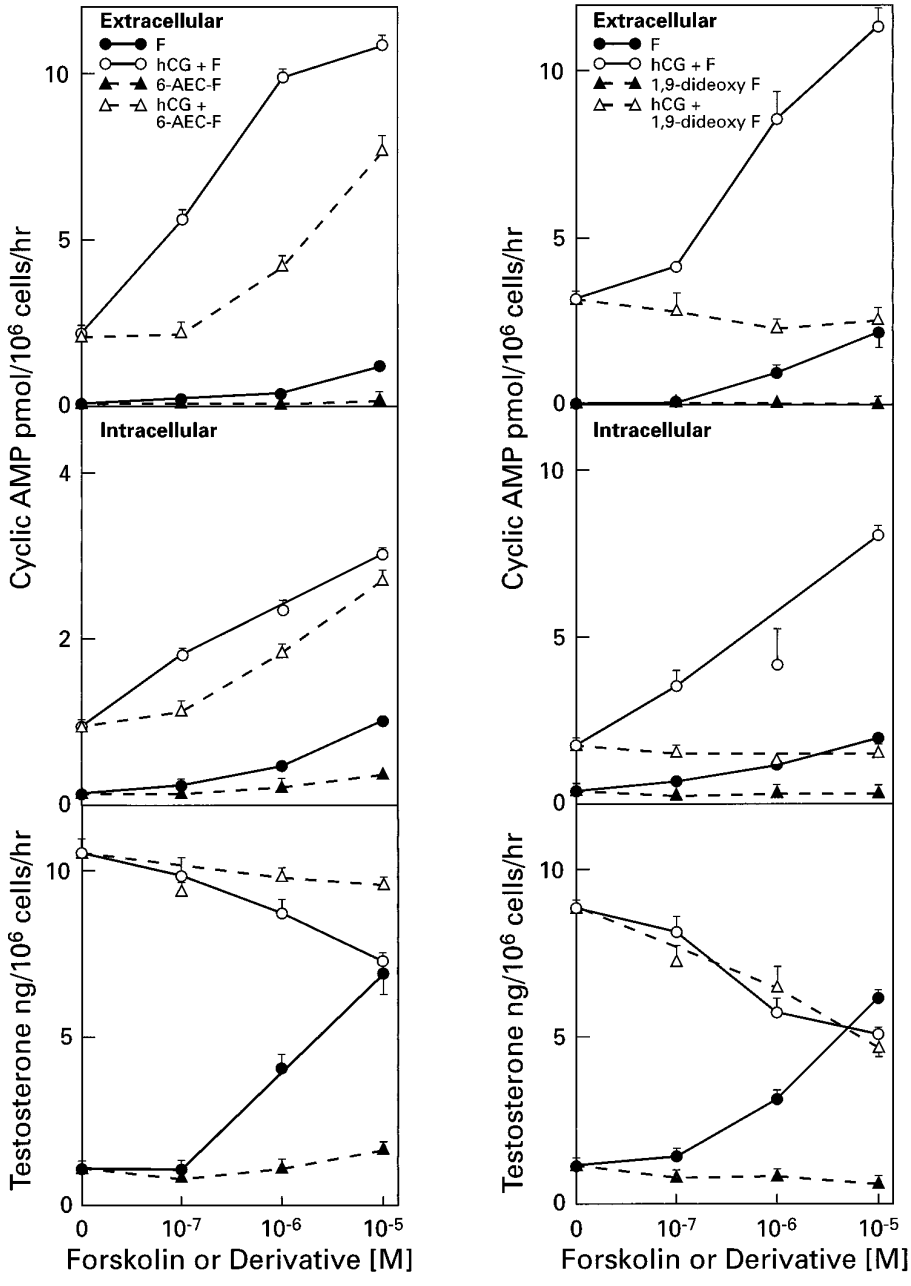

FIG. 2. Effect of forskolin and its analogs (6-AEC-F and 1,9-DDF) on cAMP pools and testosterone production. Results shown in *left* and *right panels* are from experiments performed at different times. Leydig cells (10^6 cells/ml) were incubated in the presence or absence of hCG (260 pM) with or without various doses of forskolin or its analogs (0.1–10 μM) for 60 min. *Left*, Effect of forskolin and 6-AEC-forskolin. *Right*, Effect of forskolin and 1,9-DDF.

the addition of exogenous ATP exhibited a marked inhibition of testosterone production. This experiment demonstrates that ATP is essential for the conversion of androstenedione to testosterone. In contrast, no recovery of 17βHSD activity was observed with the addition of NADPH to the medium (data not shown). Furthermore, Fig. 5 substantiates the observation in Fig. 1 that cAMP, but not hCG, is capable of inducing partial (50%) reversal of forskolin-blocked 17βHSD activity (Fig. 5, ATP, *lower panel*). This is consistent with the demonstration that ATP availability is a limiting factor in forskolin-treated cells, as hCG induction requires ATP for cAMP production.

The implication of these results is that glucose deficiency leads to a functional ATP deficiency that only affects the final reaction in steroidogenesis. Figures 1 and 4 indicate that endogenous ATP levels are sufficient for all reactions through the P450 17α-hydroxylase, but consistently are not sufficient for the final 17βHSD activity. Intracellular ATP levels in rat Leydig cells after 2-h incubation with or without glucose-rich medium was measured to establish whether an ATP deficiency exists in the absence of glucose. A statistically significant, but modest, reduction in ATP levels from 2.6 ± 0.45 to 1.3 ± 0.15 × 10^{-15} moles/cell ($P < 0.004$) was determined in cells incubated in the presence or absence of glucose-rich media, respectively. Although this change was minor and presumably compartmentalized, it may account for the activation of the 17βHSD reaction. Furthermore, endogenous ATP levels in the absence of glucose are sufficient for the metabolic steps of steroidogenesis before testosterone

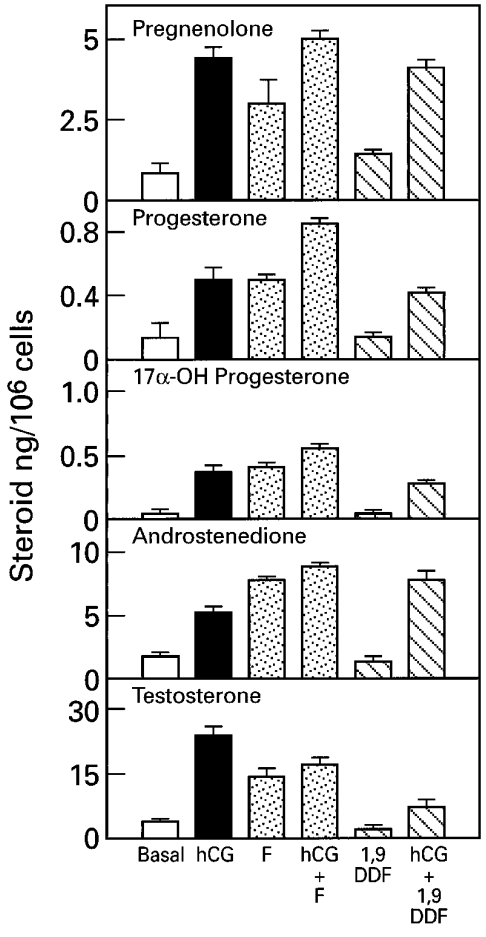

FIG. 3. Effects of forskolin and 1,9-DDF on metabolic products of the steroidogenic pathway in basal and hCG-stimulated Leydig cells. Cells (10^6 cells/ml) were incubated for 120 min in the presence or absence of hCG (260 pM) with or without forskolin (10 μM) or 1,9-DDF (10 μM).

formation, including hCG activation of the early reactions through an increase in cyclase activity.

We then evaluated the effects of glucose and ATP on the kinetic parameters of 17βHSD activity. Analysis of testosterone production showed that 17βHSD inhibition caused by the absence of glucose [or an inhibition of the glucose transporter(s)] is reflected as an increase in the apparent K_m (300%) of the enzyme, and a decrease in the apparent specificity constant (kcat/K_m), whereas no significant difference in kcat was observed (Table 1). However, with the addition of ATP or GTP, the apparent K_m and kcat/K_m of the enzyme reverted to control levels in the presence of glucose. The apparent K_m for 17βHSD in controls in the presence of glucose was similar to that reported for the rat testicular microsomal 17βHSD (20). Addition of nonhydrolyzable analogs of ATP did not reduce the apparent K_m of the enzyme. Similarly, 1,9-DDF induced increases in the apparent K_m (200%) in cells incubated in the presence of glucose, supporting the involvement of a defective glucose transporter(s) in the observed increase in apparent K_m of the 17βHSD. These kinetic parameters suggest the presence of high and low affinity 17βHSDs that are interconvertible through the addition or depletion of glucose or ATP.

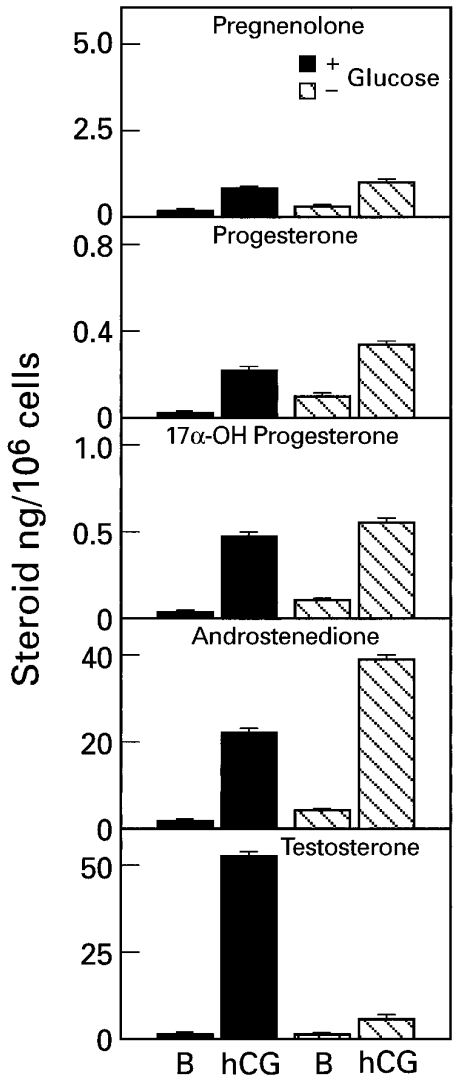

FIG. 4. Effect of glucose on metabolic products of the steroidogenic pathway in basal and hCG-stimulated Leydig cells. Cells (10^6 cells/ml) were preincubated for 120 min. After washing three times, the cells were incubated for an additional 120 min in the presence or absence of glucose (5.8 mM) with or without hCG (260 pM). B, Basal condition.

To determine whether the ATP requirement for 17βHSD activity is specific for a hydrolyzable form and requires the breakdown of ATP to a high energy phosphate, we tested several nucleotides, including nonhydrolyzable analogs of ATP and GTP and the cofactor, NADPH, in cells that were incubated in glucose-deficient medium. It is evident from our results that only ATP or GTP supported testosterone production (Table 2). The nonhydrolyzable analogs of ATP and GTP (App[NH]p, Gpp[NH]p, and GTPγS, respectively), other nucleotides (CTP, UTP, and TTP), and NADPH did not stimulate the conversion of androstenedione to testosterone (Table 2). Furthermore, in the presence or absence of exogenous ATP, hCG did not have a significant influence on the conversion ratio (testosterone/androstenedione) of the 17βHSD reaction in either the presence or absence of glucose. Additional experiments using cells incubated in the presence

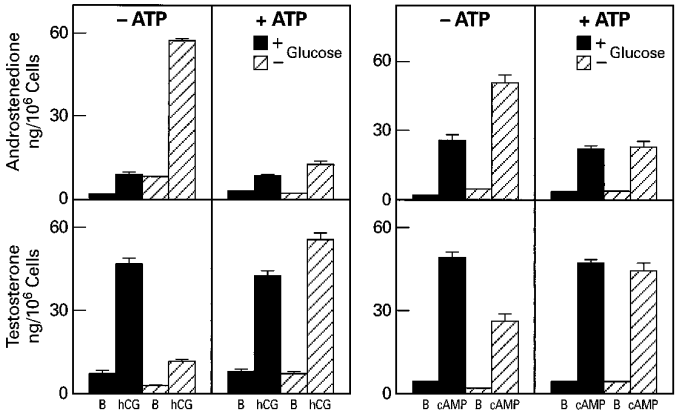

FIG. 5. Effect of ATP on hCG- and 8-bromo-cAMP-stimulated testosterone synthesis in the presence or absence of glucose in Leydig cells. Cells (10^6 cells/ml) were permeabilized as described in *Materials and Methods*. The conditions of preincubation and incubation in this and the following experiments were same as those described in Fig. 4 with or without ATP (1 mM) along with other additives. B, Basal condition. *Left*, Effect of hCG. *Right*, Effect of 8-bromo-cAMP.

TABLE 1. Kinetic parameters (apparent) of the 17βHSD reaction

Treatments	K_m (μM)	kcat (h^{-1})	kcat/K_m
+G	34 ± 3.7	4.7 ± 0.6	0.14
−G	110 ± 3.5	5.8 ± 0.5	0.05
−G + ATP	37 ± 5.0	6.4 ± 1.1	0.17
−G + App[NH]p	104 ± 12.7	4.0 ± 0.4	0.04
−G + GTP	31 ± 4.8	4.8 ± 0.3	0.15
+G + DDF	73 ± 4.6	7.14 ± 0.3	0.09
+G + hCG	51 ± 5.8	6.2 ± 0.7	0.12
−G + hCG	176 ± 16.7	7.97 ± 1.8	0.05
−G + hCG + ATP	80 ± 0.04	7.2 ± 0.001	0.09
+G + hCG + DDF	86 ± 4.8	8.13 ± 1.3	0.09

Leydig cells (10^6/ml) were preincubated for 120 min in the presence of glucose. After washing three times, the cells were incubated for 120 min in the presence or absence of glucose with or without various additives as described in *Materials and Methods*. Each *point* represents the mean ± SE of four experiments, each performed in triplicate.

of AG further confirm the studies presented in Table 2 (Table 3).

It was apparent that further studies required isolation of the 17βHSD reaction from the metabolic pathway to permit controlled addition of the substrate androstenedione. This was accomplished by the use of aminoglutethimide (AG), an enzyme inhibitor that blocks cholesterol metabolism, depletes the steroid substrates from the cells, and prevents aromatization of androstenedione to estrone. In the presence of AG, androstenedione is metabolized exclusively to testosterone. Increasing concentrations of androstenedione were added in the presence or absence of glucose, hCG, and ATP to cells that contained unndetectable precursors in the steroidogenic pathway. The production of testosterone was used as a marker for 17βHSD activation. Addition of androstenedione itself caused a dose-dependent increase in the basal activity of 17βHSD, whereas no further significant increase was noted in hCG-stimulated cells (Table 4). Addition of ATP markedly enhanced the basal activity of enzyme at 1 and 10 μM androstenedione. Again, no further increase over basal activity in hCG-stimulated cells was observed after the addition of ATP, similar to the results obtained in the noninhibited system (minus AG) with measurement of the conversion ratio (Table 2). These results were also obtained in cells incubated in the presence of glucose, where the extent of stimulation after the addition of ATP was more pronounced in cells incubated in the absence of glucose (Table 4). This observation further emphasizes the requirement of ATP for optimal androgen biosynthesis and corroborates the studies performed in the absence of the metabolic inhibitor AG.

The suggestion of a high energy phosphorylated intermediate was explored with kinase inhibitors that would block the proposed phosphorylation of 17βHSD. These studies were carried out using cells with an AG blockade of the steroidogenic pathway. Inhibitors specific for PKA, PKC, and CaMK were added to the cells in an effort to evaluate the role of kinases in enhancing the basal activity of 17βHSD in the presence of ATP. Various potential phosphorylation sites for PKC, casein kinase (CK), and PKA have been detected in the recently cloned 17βHSD isoenzyme type I [human placenta (21) and rat ovary (22)], type II (human prostate) (23), and type III (human testis) (24). For these studies, the PKC inhibitors, bisindolylmaleimide (25) and staurosporine (26), the PKA inhibitors H-89 (27) and KT-5720 (28), the cAMP inhibitor, Rp-cAMP (29) and CaMK inhibitors KN-62 (30), KN-93 (31), and W-7, the calmodulin antagonist (32) were added to the incubation medium. Initial studies using concentrations of the inhibitor that were close to the reported K_i of the selective kinase reaction (25–32) demonstrated that PKA and CaMK, but not PKC inhibitors, elicited effective inhibition of the 17βHSD reaction (data not shown). Both KT-5720 and W-7 markedly reduced ATP-induced testosterone production from androstenedione by 30–50% ($P < 0.00006$; Fig. 6). The PKA inhibitor, H-89, however, did not show inhibition of testosterone production at its reported K_i (48 nM) for purified enzyme, although when the concentration was increased to 1 μM, significant inhibitions of 30% (basal; $P < 0.0009$) and 18% (plus ATP; $P < 0.0003$) were observed. Similarly, the cAMP antagonist Rp did not show inhibition until concentrations reached 1 mM (K_i = 11 μM). Under these conditions, testosterone levels were reduced by 28% (basal; $P < 0.0002$) and 10% (plus ATP; $P < 0.02$). Although H-89 and KT-5720 are both ATP antagonists, the mode of action and molecular structures of the inhibitors are quite different and have diverse effects in various experimental cell types (28, 33). Furthermore, Rp is a weak inhibitor of cAMP in Leydig cells.

As the KT-5720 and W-7 inhibitors did have a profound effect on testosterone production, we investigated whether the W-7 calmodulin antagonist simply depleted the divalent cation requirement for ATP binding or acted in an independent mode. The inhibitory effects of KT-5720 and W-7 were additive, causing reductions in testosterone to near-basal levels (Fig. 6). This complete inhibition of 17βHSD activity suggests that multiple kinases may play a role in the activation of 17βHSD (Fig. 6, *left*). In the studies performed in the absence of Ca^{2+}, in which the CaMK activity presumably is inoperative, the addition of KT-5720 caused a complete inhibition of 17βHSD activity. The contribution of KT-5720 to enzyme activation increased from 50% in the presence of calcium to 100% in the absence of calcium, suggesting com-

TABLE 2. Effects of nucleotides on the testosterone/androstenedione (T/A) conversion ratio in basal and hCG-stimulated Leydig cells

Treatments	Basal (T/A ± SE)	+hCG (T/A ± SE)
+G	2.1 ± 0.23	2.9 ± 0.31
+G + 1 mM ATP	3.60 ± 0.35	3.60 ± 0.25
−G	0.50 ± 0.05	0.33 ± 0.03
−G + 1.0 mM ATP	3.10 ± 0.5	3.10 ± 0.42
−G + 0.1 mM ATP	1.54 ± 0.21	1.70 ± 0.16
−G + 0.01 mM ATP	1.95 ± 0.05	1.41 ± 0.05
−G + 1.0 mM App[NH]p	0.97 ± 0.13	0.72 ± 0.13
−G + 0.1 mM App[NH]p	1.05 ± 0.15	0.85 ± 0.1
−G + 1.0 mM GTP	3.20 ± 0.06	3.20 ± 0.007
−G + 0.1 mM GTP	2.25 ± 0.39	2.14 ± 0.27
−G + 0.1 mM Gpp[NH]p	0.97 ± 0.14	0.78 ± 0.08
−G + 0.01 mM Gpp[NH]p	0.65 ± 0.05	0.40 ± 0.04
−G + 0.01 mM GTPγS	0.84 ± 0.15	0.61 ± 0.09
−G + 1.0 mM CTP	0.68 ± 0.02	0.59 ± 0.11
−G + 1.0 mM UTP	0.42 ± 0.17	0.26 ± 0.03
−G + 1.0 mM TTP	0.58 ± 0.05	0.36 ± 0.07
−G + 1.0 mM NADPH	1.10 ± 0.15	0.87 ± 0.05

Cells (10^6 cells/ml) were incubated with or without the designated nucleotides in the presence or absence of hCG in medium devoid of glucose. See *Materials and Methods* for details.

pensation and/or the activation of a common 17βHSD site through different pathways. The compensation between kinases is also suggested by the observation that basal and ATP-stimulated testosterone levels were similar in the presence and absence of calcium (Fig. 6).

Discussion

These studies provide evidence for a steroidogenic block at the level of conversion of androstenedione to testosterone by 17βHSD in rat Leydig cells that is induced by forskolin or its analog 1,9-DDF. The forskolin block at the 17βHSD reaction was attributed to impairment of the glucose transport system, as this block was also present in cells incubated in glucose-deficient medium. The glucose effect was related to ATP, a byproduct of glucose metabolism, as the block at the 17βHSD level was effectively removed with the addition of ATP/GTP, but not with nonhydrolyzable ATP/GTP analogs. Furthermore, the loss of ATP activation by inhibitors of PKA (28) and CaMK (32) suggests the participation of kinases in activation of the 17βHSD reaction.

These studies demonstrate that in the absence of glucose, the hormone-stimulated steroidogenic pathway proceeds only to the accumulation of androstenedione, and an increase in the concentration of this steroid precursor is not sufficient to activate the 17βHSD reaction. Our studies infer that 17βHSD activation is highly compartmentalized and separate from the rest of the steroidogenic pathway, because glucose deficiency impacts only the 17βHSD reaction in steroidogenesis, and total intracellular ATP levels are sufficient for all metabolic steps from cholesterol to androstenedione, except the 17βHSD reaction. The ATP generated from nonglycolytic alternative pathways in cells incubated in the absence of glucose appears to be adequate for maintaining all metabolic steps before androstenedione formation and for the reduced, but detectable, levels of 17βHSD activity observed in the absence of glucose. These cells are capable of using alternative routes to generate ATP in the absence of glucose, although not at optimal levels, perhaps through the

TABLE 3. Effects of nucleotides on testosterone production (nanograms per 10^6) in basal and hCG-stimulated Leydig cells treated with AG

Treatments	Basal	hCG
−G + A	79 ± 2.9	90 ± 7.0
−G + A + ATP	209 ± 20	234 ± 8.4
−G + A + GTP	236 ± 6.4	233 ± 4.4
−G + A + CTP	83 ± 4.0	84 ± 6.5
−G + A + UTP	69 ± 4.3	54 ± 4.1
−G + A + TTP	91 ± 4.4	63 ± 5.9
−G + A + NADPH	104 ± 2.0	97 ± 4.8

The incubation methods were described in *Materials and Methods* for isolation of the 17βHSD reaction by treatment of cells with AG. The concentration of androstenedione (A) was 1 μM for all nucleotides, and that of NADPH was 1 mM. Values are the mean ± SE of triplicate incubations (n = 2).

phosphoenolpyruvate kinase, lactate dehydrogenase, or transamination of gluconeogenic amino acid pathways. Furthermore, addition of GTP by itself brings about maximal steroidogenesis, most likely acting as a direct phosphate donor and not through NDP kinase, which catalyzes the transfer of terminal phosphate from nucleoside triphosphate to nucleoside diphosphate (34, 35). The final conversion of androstenedione to testosterone by the 17βHSD reaction requires activation of the glycolytic pathway for optimal provision of functional ATP under the conditions used in these studies.

Total intracellular ATP levels in the Leydig cells in the presence or absence of glucose shows a statistically significant, but not large, decrease with glucose-deficient medium. The ATP values in Leydig cells (2×10^{-15} mol/cell) are in the range observed for hepatocytes (6×10^{-15} mol/cell) (36) and are consistent with an apparent K_m for testosterone production of 2 μM (10^6 cells/ml). The apparent K_a for activation of phosphorylase phosphatase activity that involves conversion of inactive forms of the enzyme is 2 μM (37). However, the intracellular ATP concentration may not be a reliable index of ATP availability for the 17βHSD reaction. ATP is required at multiple levels for both the early and late reactions of steroidogenesis, and it is apparent in our studies that the ATP levels, in the absence of glycolysis, are sufficient through the P450 17α-hydroxylase reaction and androstenedione production, but not for the 17βHSD step. The measurement of steady state levels of ATP within the cell does not provide specific information about utilization of ATP in the early *vs.* the late reactions or localized reduction of ATP in the microsomal compartment.

Although hCG stimulation of androstenedione levels in Fig. 1 is evident and is presumably due to the induction of cyclase activity, the elevated level of cAMP causing activation of androstenedione does not reverse forskolin-induced inhibition of testosterone formation. Similarly, forskolin itself induces cyclase activity for the activation of a step(s) before testosterone formation in addition to inhibiting the glucose transporter, and this cAMP should support 17βHSD activation if the observed effect is through PKA. This cyclase-induced activation of androstenedione levels may account for the differences between F or hCG+F and basal testosterone levels in Fig. 1, and the addition of greater amounts of cAMP stimulates the reaction further (cAMP+F). How-

TABLE 4. Testosterone production in Leydig cells treated with aminoglutethimide

Treatments	+G		−G	
	Basal	hCG	Basal	hCG
No addition	ND	ND	ND	ND
Andr (0.1 μM)	12 ± 0.4	14 ± 0.6	4 ± 0.3	5 ± 0.5
Andr (0.1 μM) + ATP (1 mM)	19 ± 0.6	18 ± 0.75	11 ± 0.4	10 ± 0.08
Andr (1 μM)	94 ± 2.9	89 ± 1.0	39 ± 0.9	39 ± 2.8
Andr (1 μM) + ATP (1 mM)	137 ± 4.5	134 ± 1.5	92 ± 4.5	84 ± 7.0
Andr (10 μM)	463 ± 7.6	433 ± 0.5	205 ± 26	213 ± 2.0
Andr (10 μM) + ATP (1 mM)	632 ± 26	663 ± 37	519 ± 25	485 ± 49

The incubation methods were described in *Materials and Methods* for isolation of the 17βHSD reaction by treatment of cells with AG. Andr, Androstenedione. Each *point* represents the mean ± SE of triplicate incubations.

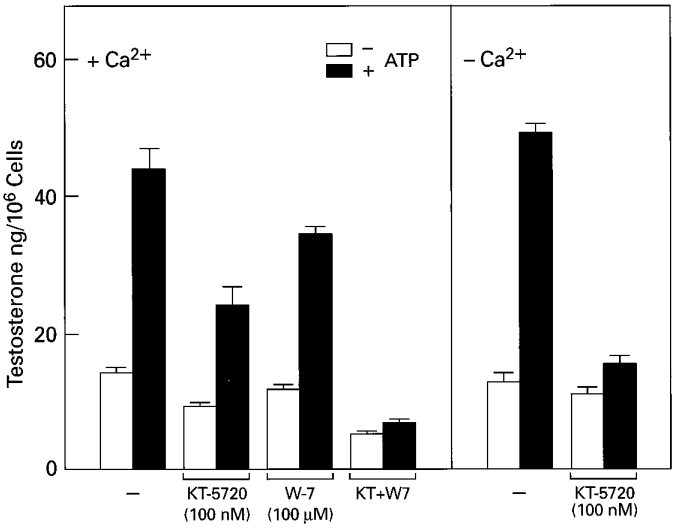

FIG. 6. *Left*, Effects of kinase inhibitors added individually or together on testosterone production in AG-treated cells after the addition of androstenedione. *Right*, Effect of PKA inhibitor on testosterone production in the absence of Ca^{2+} in AG-treated cells after the addition of androstenedione. The incubations were performed as described in *Materials and Methods*, except that 0.1 mM ATP was added before the final 120-min incubation along with 1 μM androstenedione (A).

ever, the inability of hCG to increase the utilization of endogenous or exogenous ATP to stimulate testosterone production at the 17βHSD step in the presence of the metabolic inhibitor AG indicates that ATP action may involve nucleotide sites on the enzyme itself and perhaps a regulatory mechanism consisting of nucleotide competition similar to that of glycogen phosphorylase and phosphatase proteins that also exists in different activation states (37).

The studies with the ATP antagonist KT-5720 suggest but by no means prove a PKA contribution to 17βHSD activity, as inhibition was observed at levels close to the K_i for PKA. The studies in Figs. 1 and 5 suggest that the reversal of forskolin inhibition of testosterone synthesis by ATP has some connection with cAMP and presumably PKA, as cAMP ameliorated forskolin-induced testosterone inhibition by 50%, and this correlates well with the 50% inhibition observed by KT-5720 in the presence of calcium.

This effect was not duplicated with hCG, perhaps because the use of hCG necessitates the utilization of at least 1 extra mol of ATP compared with addition of cAMP in the cyclase reaction. When ATP is added to glucose-deficient cells, this discrepancy between testosterone production in the presence of added 8-bromo-cAMP or hCG is not apparent. In addition, 17βHSD activity was inhibited by the calmodulin antagonist W-7, which inhibits calmodulin kinases (38). The additive inhibition of the 17βHSD by an ATP and calmodulin antagonist (KT-5720 and W-7) supports the hypothesis that the inhibitors are affecting 17βHSD activity through inhibition of their selective kinases, perhaps in the same domain. In this regard, it is of interest to note that both PKA and CaMK were reported to phosphorylate the same serine (133) in the cAMP response element-binding protein that is required for transcriptional activation (39). As the differences observed in the 17βHSD enzyme activity correlated closely with the apparent K_m values of the enzyme for its substrate androstenedione, it is conceivable that the activated state could reflect differences in the state of phosphorylation. However, further definition of the activating mechanism must await isolation of the rat Leydig cell 17βHSD complementary DNA.

In a number of studies, phosphorylation has been shown to be involved in regulating enzyme activities, including the NAD- and NADP-dependent dehydrogenases (40–42). The human placental (type I), prostatic (type II), testicular (type III), and rat ovarian (type I) 17βHSD have been cloned and sequenced (21–24). A potential PKA phosphorylation site (Arg-Arg-X-Ser) has been identified in the deduced amino acid sequence of the human placental and rat ovarian type I 17βHSD, suggesting the possibility of PKA-dependent phosphorylation for enzyme activation in this isoform (21, 22). The type I forms of the 17βHSD have a higher affinity for estrone than androstenedione and catalyze the conversion of estrone to estradiol. In a recent *in vitro* study, efficient phosphorylation of recombinant 17βHSD (placental type I isozyme) by PKA was shown in addition to an *in vivo* decrease in enzyme activity after cellular treatment with bacterial alkaline phosphatase (43). At this time, the rat testicular 17βHSD has not been cloned, although it is unlikely that it will be a type I reductase (21, 22) because of its substrate specificity. More likely, it would display a closer similarity to the type III human testis form (24).

These studies demonstrate that the 17βHSD activity is under ATP control, and that glucose plays an important role in the regulation of Leydig cell 17βHSD activity. Forskolin has proven to be a valuable tool for evaluating the role of glucose/ATP-mediated events in 17βHSD activity.

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