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Protein Kinase C Increases 11β-Hydroxysteroid Dehydrogenase Oxidation and Inhibits Reduction in Rat Leydig Cells

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ABSTRACT: Glucocorticoid hormone controls Leydig cell steroidogenic function through a receptor-mediated mechanism. The enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) plays an important role in Leydig cells by metabolizing glucocorticoids, and catalyzing the interconversion of corticosterone (the active form in rodents) and 11-dehydrocorticosterone (the biologically inert form). The net direction of this interconversion determines the amount of biologically active ligand, corticosterone, available for glucocorticoid receptor binding. We hypothesize that 11βHSD oxidative and reductive activities are controlled separately in Leydig cells, and that shifts in the favored direction of 11βHSD catalysis provide a mechanism for the control of intracellular corticosterone levels. Therefore, in the present study, we tested the dependency of 11βHSD oxidative and reductive activities on protein kinase C (PKC) and calcium-dependent signaling pathways. 11βHSD oxidative and reductive activities were measured in freshly isolated intact rat Leydig cells using 25 nM radiolabeled substrates after treatment with protein kinase modulators. We found that PKC and calcium-dependent signaling had opposing effects on 11βHSD oxidative and reductive activities. Stimulation of PKC using the PKC activator, 6-[*N*-decylamino]-4-hydroxymethylinole (DHI), increased 11βHSD oxidative activity from a conversion rate of 5.08% to 48.23% with an EC_{50} of 1.70 ± 0.44 μM (mean ± SEM), and inhibited reductive activity from 26.90% to 3.66% conversion with an IC_{50} of 0.22 ± 0.05 μM. This indicated that PKC activation in Leydig cells favors 11βHSD oxidation and lower levels of corticosterone. The action of DHI was abolished by the PKC inhibitor bisindolylmaleimide I. In contrast, addition of calcium to Leydig cells increased 11βHSD reductive activity while decreasing oxidative activity, thereby favoring reduction and conversion of inert 11-dehydrocorticosterone into active corticosterone. The opposite effect was seen after elimination of calcium-dependent signaling, including removal of calcium by EGTA or addition of the calmodulin (calcium binding protein) inhibitor SKF7171A, or the calcium/calmodulin-dependent protein kinase I (CaMK II) inhibitor, KN62. We conclude that 11βHSD oxidative and reductive activities are separately regulated and that, in contrast to calcium-dependent signaling, PKC stimulates 11βHSD oxidation while inhibiting 11βHSD reduction. Maintenance of a predominantly oxidative 11βHSD could serve to eliminate adverse glucocorticoid-induced action in Leydig cells.

Key words: Calcium, calmodulin, glucocorticoid action, oxidoreductases, steroid metabolism, stress.

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Glucocorticoid hormone is known to affect cell proliferation, metabolism, and differentiation (Schmid et al, 1995). In the testis, glucocorticoids directly inhibit testosterone biosynthesis in Leydig cells via a receptor-mediated mechanism (Bambino and Hsueh, 1981; Hales and Payne, 1989; Monder et al, 1994a). Access of glucocorticoids to their receptors within target cells is controlled by the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) (Monder and White, 1993; Monder et al, 1994a). When testicular 11βHSD is inhibited, excessive glucocorticoid action suppresses testosterone production in Leydig cells (Monder et al, 1994a). In particular, men who ingest the 11βHSD inhibitor glycyrrhetinic acid in licorice, have reduced serum testosterone levels (Armanini et al, 1999). To date, two distinct forms of 11βHSD have been identified: type I 11βHSD (11βHSD-I), which was first purified from rat liver and later cloned, has both oxidative and reductive activities (Lakshmi and Monder, 1988; Agarwal et al, 1989); and Type II 11βHSD (11βHSD-II), first identified in kidney and later cloned, is exclusively oxidative with a high affinity for glucocorticoids (Rusvai and Naray-Fejes-Toth, 1993; Albiston et al, 1994; Zhou et al, 1995). Leydig cells have been shown to express only 11βHSD-I and not 11βHSD-II (Phillips et al, 1989; Li et al, 1996; Gao et al, 1997; Ge et al, 1997a). In Leydig cells, 11βHSD-I catalyzes the interconversion of active glucocorticoid, corticosterone, and its biologically inert metabolite, 11-dehydrocorticosterone.

The prevailing direction of 11βHSD interconversion controls the amount of active ligand, corticosterone, available for glucocorticoid receptor binding. If the net direction of 11βHSD-I in Leydig cells was to favor oxidation, this enzyme would inactivate corticosterone, thereby lowering its action. On the other hand, if the net direction favored reduction, the enzyme would generate more ac-

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tive corticosterone from circulating 11-dehydrocorticosterone. We hypothesize that 11βHSD oxidative and reductive activities are controlled separately in Leydig cells, and that shifts in the predominant direction of 11βHSD catalysis are a mechanism by which corticosterone levels are controlled.

Luteinizing hormone (LH) and glucocorticoid each induce opposing changes in the oxidative versus reductive activities of 11βHSD in Leydig cells (Gao et al, 1997). Because these hormones regulate Leydig cell function through more than one intracellular signal transduction pathway, including the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA; Cooke, 1996), calcium (Janzen et al, 1976; Gudermann et al, 1992a,b), protein kinase C (PKC; Nikula et al, 1987; Majercik and Puett, 1991), and tyrosine protein kinase (Wuerther et al, 1995), 11βHSD oxidation and reduction may be separately controlled. In the present investigation, after testing a series of modulators of PKA, calcium, PKC, and tyrosine protein kinase, we demonstrated that 11βHSD oxidation and reduction in rat Leydig cells are controlled in opposing fashion by PKC and calcium-dependent signaling.

Materials and Methods

Chemicals

Sodium bicarbonate, HEPES, trypsin inhibitor, ethylenediaminetetraacetic acid (EDTA), bovine serum album (BSA, fraction V), Dulbecco modified Eagle medium:F12 Ham (DMEM/F12, 1:1 mixture without phenol red), dibutyryl cyclic adenosine monophosphate (cAMP), albumin, Percoll, etiocholan-3β-ol-17-one, nicotinamide adenine dinucleotide (NAD), nitro blue tetrazolium, and gentamycin and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Sigma Chemical Company (St Louis, Mo). [1,2,6,7-*N*-^{3}H]corticosterone (specific activity 88 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, Mass). 11-dehydro[1,2,6,7-*N*-3H]corticosterone was prepared from [^{3}H]corticosterone as described earlier (Lakshmi and Monder, 1985). Corticosterone and 11-dehydrocorticosterone were purchased from Steraloids (Wilton, NH). Fluphenazine-*N*-2-chloroethane (SKF 7171A), 1-[*N*,*O*-bis-(isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 6-[*N*-decylamino]-4-hydroxymethylinole (DHI), and bisindolmaleimide I were from Calbiochem (La Jolla, Calif). Thin-layer chromatography plates (Polygram Silica Gel/UV254) were obtained from Baker-Flex (Phillipsburg, NJ).

Animals

Sprague-Dawley adult rats weighing 250 to 300 g from Charles River Laboratories (Wilmington, Mass) were used for isolation of adult Leydig cells. The animals were killed by asphyxiation with CO_2. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Rockefeller University (protocol 91200R2).

Cell Isolation and Incubation

Leydig cells were isolated from adult rats by collagenase dispersion and purified by centrifugal elutriation as described previously (Klinefelter et al, 1993). Purities of Leydig cell fractions were evaluated by histochemical staining for 3β-hydroxysteroid dehydrogenase activity, with 0.4 mM etiocholanolone as the steroid substrate (Payne et al, 1980). More than 95% of adult Leydig cells isolated were intensely stained. Cells were resuspended in phenol red-free DMEM/F12 (1:1) medium buffered with HEPES containing 0.5% (g/dL) BSA.

Analysis of 11βHSD Oxidative and Reductive Activities

11βHSD oxidative and reductive activities were measured in intact Leydig cells as previously described (Ge et al, 1997c). For the assay of 11βHSD oxidative activity, isolated intact Leydig cells (0.1×10^6) were incubated with 25 nM [^{3}H]corticosterone in 0.5 mL phenol-red free medium (DMEM/F12 1:1) at 34°C for 60 minutes. In assays of 11βHSD reductive activity, 25 nM [^{3}H]11-dehydrocorticosterone was used in place of [^{3}H]corticosterone. No cofactors, NADP or NADPH, were added to the reaction. The reaction was stopped by adding 2 mL of ice-cold ethyl acetate to the incubation mixture. The steroids were extracted, and the organic layer was dried under nitrogen. The steroids were separated chromatographically on thin layer plates in chloroform: methanol (90:10), and the radioactivity was measured with a scanning radiometer (System 200/AC3000; Bioscan Inc, Washington, DC). The percentage of conversion of corticosterone to 11-dehydrocorticosterone and 11-dehydrocorticosterone to corticosterone was calculated by dividing the radioactive counts identified as 11-dehydrocorticosterone (or corticosterone, respectively) by the total counts associated with these 2 steroids.

Effects of Protein Kinase Modulators on 11βHSD Oxidative and Reductive Activities

To establish the dose dependency of protein kinase modulators on 11βHSD, intact rat Leydig cells were preincubated with different concentrations of modulators of protein kinases for 2 hours. The 11βHSD activities were measured by incubation with substrates limited to 60 minutes, because in the absence of additives, 11βHSD reduction exceeds oxidation after 60-minute incubations with substrate (Ge and Hardy, 2000). To establish the onset of the effects of the PKC activator DHI and calmodulin inhibitor SKF 7171A and calcium/calmodulin-dependent protein kinase II inhibitor KN62, Leydig cells were preincubated with these compounds for timed intervals ranging from 5 minutes to 2 hours. The concentrations used have been shown not to affect cell viability (Grove and Mastro, 1991; Kozlowski and Ashford, 1992; Nikodijevic and Guroff, 1992). The cells were then incubated with corticosterone or 11-dehydrocorticosterone for an additional 40 minutes to measure 11βHSD activities. To determine whether PKC and calcium/calmodulin modulators affected Leydig cell viability, testosterone production was measured after incubations in vitro. Aliquots of Leydig cells were incubated in triplicate at a concentration of 0.1×10^6 cells/mL in DMEM/

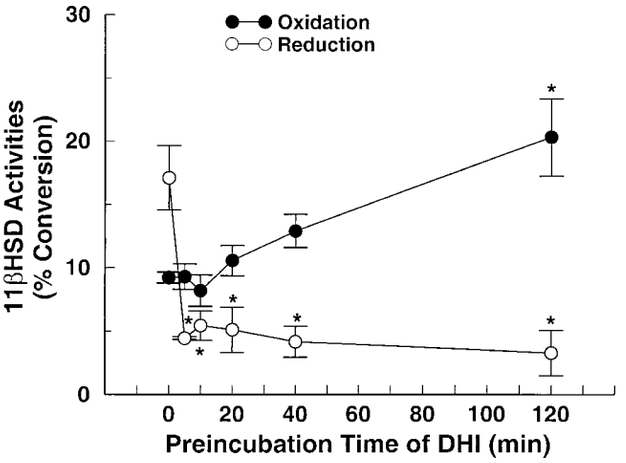

Figure 1. The 11βHSD oxidative and reductive activities in intact rat Leydig cells after addition of the PKC activator DHI. Aliquots of 0.1×10^6 intact rat Leydig cells were preincubated with 10 μM DHI at timed intervals from 5 minutes to 120 minutes, and 11βHSD oxidative and reductive activities were then assayed after incubation with 25 nM [^{3}H]corticosterone (oxidation, ●——●) or [^{3}H]11-dehydrocorticosterone (reduction, ○——○) in DMEM/F12 for 60 minutes. Values are means ± SEM (n = 6). The asterisks designate a significant difference compared with control (zero time) at $P < .05$.

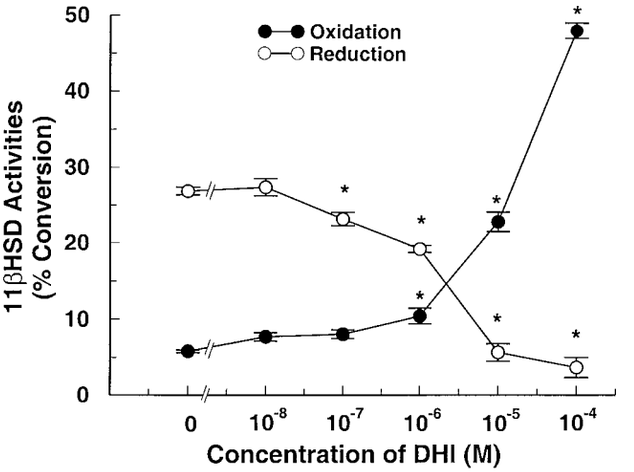

Figure 2. Dose-response for the PKC activator DHI on 11βHSD oxidative and reductive activities in intact Leydig cells. Aliquots of 0.1×10^6 intact rat Leydig cells were preincubated with DHI at concentrations ranging from 10 nM to 100 μM for 2 hours, and 11βHSD oxidative and reductive activities were then assayed after incubation with 25 nM [^{3}H]substrate in DMEM/F12 for 60 minutes. Values are means ± SEM (n = 6–27). The asterisks designate a significant difference compared with control (no DHI) at $P < .05$.

F12 under basal and LH-stimulated (100 ng/mL) conditions. Several concentrations of DHI and SKF7171A were evaluated to test for dose-dependent effects on testosterone production. At the end of 2 hours, the samples were centrifuged at $500 \times g$ and the supernatants were stored at −20°C until radioimmunoassay.

Data Analysis

In each experiment, data were obtained from triplicate assays and the results expressed as the mean ± standard errors of the mean (SEM). Statistical analysis of the changes in 11βHSD oxidative and reductive activities was performed by Kruskal-Wallis analysis of variance followed by multiple comparisons testing to identify significant differences between groups (Sokal and Rohlf, 1995).

Results

Effects of PKC Signaling Pathways On 11βHSD

Previously, 11βHSD activity in intact Leydig cells was shown to switch from predominant oxidation to reduction after 40 minutes of incubation with substrates in DMEM/F12 culture medium (Ferguson et al, 1999; Ge and Hardy, 2000). To examine whether PKC signaling pathways can restore predominance of 11βHSD oxidation in Leydig cells after prolonged incubations, a specific PKC activator, DHI, which binds to PKC at the same sites as phorbol-12,13-dibutyrate (Grove and Mastro, 1991; Kozlowski and Ashford, 1992), was used to stimulate kinase activity. Leydig cells were preincubated with 10 μM DHI for time intervals ranging from 5 minutes to 2 hours, and the 11βHSD activities were then measured after 60-minute incubations with substrates. The PKC activator DHI stimulated 11βHSD oxidation and inhibited reduction. After 5 minutes of incubation with DHI, 11βHSD in Leydig cells switched from predominance of reduction to oxidation (Figure 1). This suggested that PKC is required for maintenance of 11βHSD oxidative predominance in Leydig cells.

Stimulation of 11βHSD oxidation occurred over a period of 2 hours but 11βHSD reduction fell by 50% after only 5 minutes, suggesting that, in comparison to the oxidase, 11βHSD reductive activity is more sensitive to modulation by DHI. To further determine the sensitivity of 11βHSD oxidation and reduction to DHI, dose-dependent effects on the 11βHSD oxidase and reductase were examined after incubation of Leydig cells with various concentrations of DHI, ranging from 10 nM to 100 μM, for 2 hours. 11βHSD oxidative activity underwent a concentration-dependent increase, with an EC_{50} of 1.70 ± 0.44 μM. Simultaneously, there was an inhibition of the 11βHSD reductive activity, with an IC_{50} of 0.22 ± 0.05 μM (Figure 2). This corroborated the hypothesis that the 11βHSD reductase is more sensitive to DHI than the oxidase.

The influence of PKC signaling pathways on 11βHSD activities in Leydig cells was also examined with a selective PKC inhibitor, bisindolylmaleimide I, which acts as a competitive inhibitor at the adenosine triphosphate-binding site (Muid et al, 1991; Gekeler et al, 1996). Treatment with bisindolylmaleimide I did not affect 11βHSD oxidase, but increased reductase activity in a dose-depen-

Table 1. *Effects of bisindolmaleimide I on 11β HSD oxidative and reductive activities in intact rat Leydig cells**

Treatments	11β HSD (% control)	
	Oxidation	Reduction
Control	100.00 ± 1.65	100.00 ± 5.74
Bisindolmaleimide I		
0.1 μM	88.90 ± 4.92	113.68 ± 3.78
1 μM	82.93 ± 4.93	121.44 ± 1.46†
10 μM	72.92 ± 2.95	133.92 ± 6.05†

* Leydig cells (0.1×10^6) were incubated with bisindolmaleimide I for 2 hours. 11β HSD oxidation and reduction were measured by 60-minute incubation with 25 nM substrates. Values indicate means ± SE (n = 6).
† Indicates significant difference compared with control in each column at $P < .05$.

dent manner (Table 1). When Leydig cells were incubated with bisindolylmaleimide I and DHI together, the DHI-induced effects on 11βHSD activities were partially alleviated (Figure 3). These data suggest that PKC is involved in the maintenance of 11βHSD oxidative activity and suppression of the 11βHSD reductase.

Effects of Calcium Signaling Pathways on 11βHSD

Calcium is believed to be a second messenger controlling steroidogenesis in Leydig cells (Janzen et al, 1976; Gudermann et al, 1992b). Two basic mechanisms contribute to the elevation of cytosolic calcium levels: 1) movement of calcium from the extracellular space into the cell through the cell membrane, and 2) release of calcium from intracellular storage sites. In order to examine whether calcium-signaling pathways are involved in the regulation of 11βHSD activities, 11βHSD activities were measured in both calcium-free phosphate buffer and DMEM/F12 medium. After addition of 1 mM calcium into calcium-free phosphate buffer, there was a decrease in oxidative activity and reductive activity increased (Figure 4A). Treatment with the chelating agent EGTA was employed to deplete calcium from the medium, blocking movement of calcium ions into the Leydig cell. Depletion of calcium by addition of 5 mM EGTA increased 11βHSD oxidation by 76% and decreased reduction by 42% (Figure 4B).

Because calcium-mediated signaling typically works in concert with the binding protein calmodulin (LaPorte et al, 1980; Kuznicki et al, 1981; Sarmiento et al, 1987), the involvement of calmodulin in the regulation of 11βHSD oxidation and reduction was examined by treating Leydig cells with a calmodulin-specific inhibitor, SKF7171A (Nikodijevic and Guroff, 1992). The time of onset of SKF7171A action was estimated by preincubation of cells with SKF7171A over a range of times, from 5 minutes to 2 hours. SKF7171A stimulated 11βHSD oxidation and inhibited reduction (Figure 5). Stimulation of 11βHSD oxidation required 2 hours but 11βHSD reduction fell by

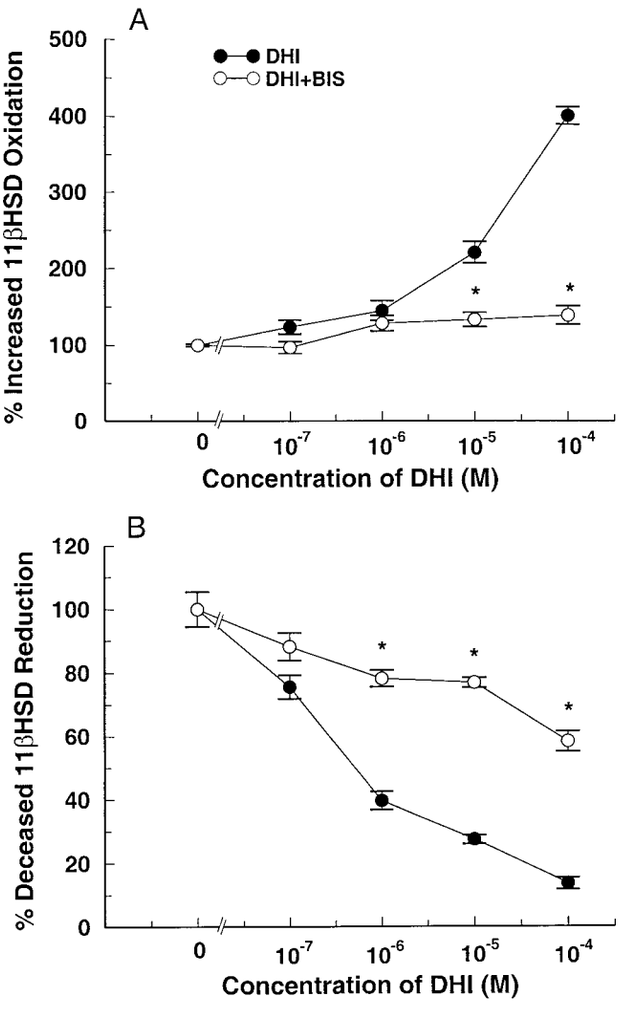

Figure 3. Antagonistic effect of bisindolmaleimide I on DHI-induced changes in 11βHSD oxidative and reductive activities. Aliquots of 0.1×10^6 intact rat Leydig cells were preincubated with DHI at concentrations ranging from 0.1 μM to 100 μM in the presence of 1 μM bisindolmaleimide I for 2 hours, and 11βHSD oxidative **(A)** and reductive **(B)** activities were then assayed after incubation with 25 nM [^{3}H]substrate in DMEM/F12 for 60 minutes. Values are means ± SEM (n = 6). The asterisks designate a significant difference compared with control (no DHI) at $P < .05$.

50% after only 20 minutes. This supported the hypothesis that 11βHSD oxidative and reductive activities are separately regulated, with the 11βHSD reductase being more sensitive to the influence of SKF7171A. When Leydig cells were incubated with 10 μM SKF7171A for 2 hours, there was a concentration-dependent increase in 11βHSD oxidase, with an EC_{50} of 20.33 ± 3.47 μM. Simultaneously, an inhibition of the 11βHSD reductase occurred, with an IC_{50} of 1.77 ± 0.33 μM (Table 2). Because the intracellular effects of calcium are typically mediated by calcium/calmodulin-dependent protein kinase II (CMK II), the involvement of CMK II in the regulation of 11βHSD activities in Leydig cells was evaluated using a CMK II-

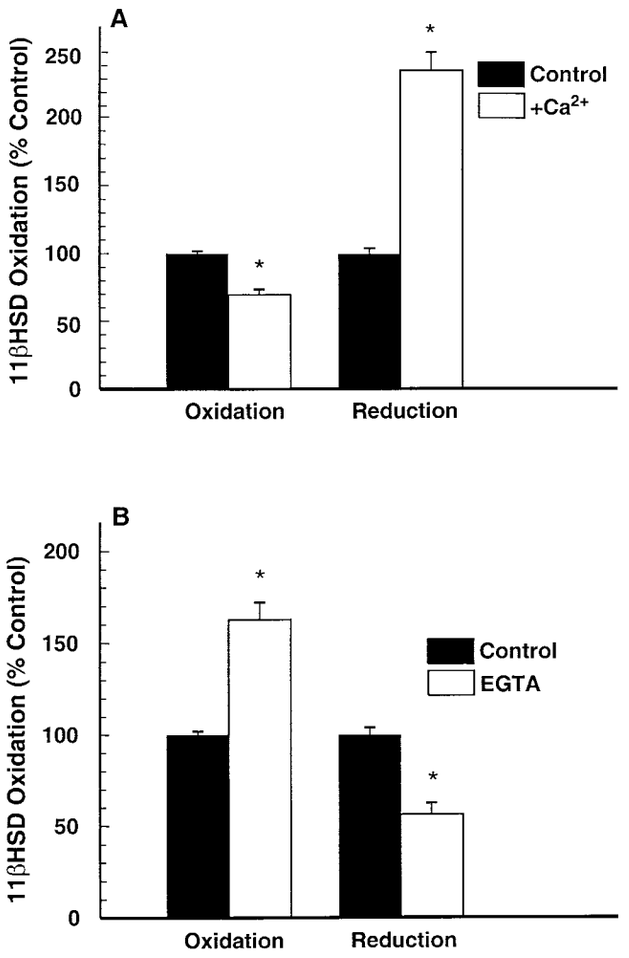

Figure 4. Regulation of 11βHSD oxidative and reductive activities in intact Leydig cells by calcium. Aliquots of 0.1×10^6 intact rat Leydig cells were incubated with phosphate buffer (control) and phosphate buffer plus 1 mM Ca^{2+} **(A)** and DMEM/F12 medium (control) and DMEM/F12 medium plus 5 mM ETGA **(B)**. The 11βHSD oxidative and reductive activities were assayed after incubation with 25 nM [3H]substrate in DMEM/F12 for 60 minutes. Values are means ± SEM (n = 6). The asterisks designate significant differences compared with control at $P < .05$.

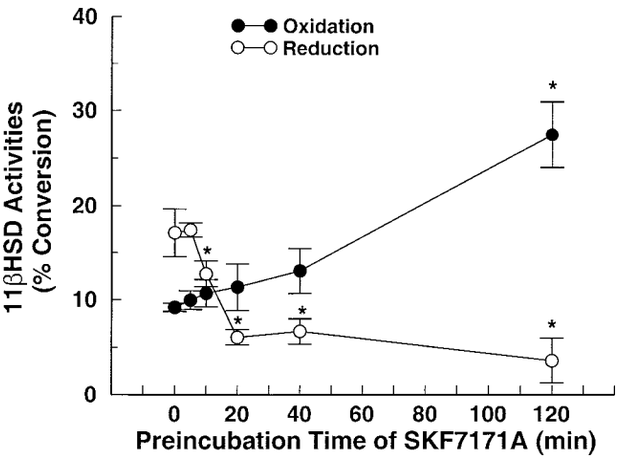

Figure 5. The 11βHSD oxidative and reductive activities in intact Leydig cells after addition of calmodulin inhibitor SKF7171A. Aliquots of 0.1×10^6 intact rat Leydig cells were preincubated with 10 μM SKF7171A for 5 minutes to 120 minutes, and 11βHSD oxidative and reductive activities were then assayed after incubation with 25 nM [3H]corticosterone (oxidation, ●———●) or [3H]11-dehydrocorticosterone (reduction, ○———○) in DMEM/F12 for 60 minutes. Values are means ± SEM (n = 6). The asterisks designate a significant difference compared with control at $P < .05$.

specific inhibitor, KN62 (Tokumitsu et al, 1990; Ito et al, 1991). Treatment with KN62 significantly increased 11βHSD oxidase activity while inhibiting reductase activity in a concentration-dependent manner (Table 3). These results indicated that calcium signaling pathways stimulate 11βHSD reductase activity while concurrently inhibiting the oxidase, in direct contrast to the results for the PKC-mediated pathway.

To establish whether the protein kinase modulators DHI and SKF7171A affected testosterone production in Leydig cells, their effects were tested under basal and LH-stimulated conditions. The PKC activator DHI had no effect on LH-stimulated testosterone production, but inhibited basal testosterone production in a dose-dependent manner. In contrast, SKF7171A had no effect on basal testosterone production, but inhibited LH-stimulated testosterone production in a dose-dependent manner (Table 4). The 11β-HSD activities were affected at submicromolar doses of the modulators, whereas effects on testosterone production were seen only at doses of 1 μM and higher. In additions, both DHI and SKF7171A had similar effects on 11β-HSD oxidative and reductive activities, whereas their effects on basal and LH-stimulated testosterone production were different. This suggests that the effects of the modulators on 11β-HSD activity are not related to their effects on testosterone production in Leydig cells.

Discussion

The present study demonstrated that 11βHSD oxidative and reductive activities in rat Leydig cells are regulated independently by 2 separate intracellular signaling pathways, PKC and calcium. This may explain why the direction of catalysis for this enzyme is sensitive to culture conditions and differs according to cell type (Kotelevtsev et al, 1997; Leckie et al, 1998; Ferguson et al, 1999; Ge and Hardy, 2000). PKC signaling stimulated the 11βHSD oxidase while inhibiting the reductase, maintaining a predominance of 11βHSD oxidation over reduction. In contrast, calcium-dependent signaling had the opposite effect, stimulating 11βHSD reductive activity while inhibiting the oxidase, thereby enforcing a predominance of 11βHSD reduction over oxidation. In this way, these 2 signaling pathways determine the direction of 11βHSD

Table 2. *Effects of SKF7171A on 11β-HSD oxidative and reductive activities in intact rat Leydig cells**

Treatments	11β HSD (% conversion)		Ox/Rd
	Oxidation	Reduction	
Control	5.80 ± 0.19	26.90 ± 0.52	0.22
0.01 μM SKF7171A	6.92 ± 0.82	22.19 ± 1.09	0.31
0.1 μM SKF7171A	11.16 ± 0.83†	11.94 ± 0.99†	0.93
1 μM SKF7171A	15.23 ± 0.94†	8.04 ± 0.73†	1.89
10 μM SKF7171A	29.96 ± 1.28†	8.10 ± 1.17†	3.70
100 μM SKF7171A	33.07 ± 1.17†	2.94 ± 0.71†	11.25

* Leydig cells (0.1×10^6) were incubated with SKF7171A for 2 hours. 11β HSD oxidation and reduction were measured by 60-minute incubation with 25 nM substrates. Values indicate means ± SE (n = 3).

† Indicates significant difference compared with control in each column at $P < .05$.

catalysis, thus controlling the availability of active glucocorticoid to its receptors in Leydig cells. The physiological significance of regulatory changes in 11βHSD lies in the fact that Leydig cells contain glucocorticoid receptors and are the primary target of glucocorticoid action in the testis (Hales and Payne, 1989; Schultz, 1989; Stalker et al, 1989; Ge et al, 1997b). Because glucocorticoid-induced suppression of testosterone production is mediated by glucocorticoid receptors (Hales and Payne, 1989, Monder et al, 1994b), the intracellular concentration of glucocorticoid within the Leydig cell should determine the magnitude of its effect. The present data indicate that regulation of 11βHSD through PKC and calcium-signaling system may profoundly affect testosterone production in Leydig cells.

PKC and calcium/calmodulin-dependent protein kinases belong to a class of modulators that regulate cell function via protein phosphorylation. PKC (Dufau and Catt, 1974; Cooke et al, 1977; Themmen et al, 1986) and calcium/calmodulin-dependent signaling (Hall et al, 1981; Majercik and Puett, 1991; Wuerther et al, 1995) have already been shown to be involved in the regulation of steroidogenesis. The physiological relevance of PKC and calcium signaling pathways in the regulation of 11βHSD activities lies in their potential for setting the levels of glucocorticoid metabolism in Leydig cells. Activation of the PKC pathway results in predominant 11βHSD oxidation, opposing the glucocorticoid-induced inhibition of testosterone production (Monder et al, 1994b). In contrast, activation of the calcium signaling pathway will lead to predominance of 11βHSD reduction, potentiating glucocorticoid action, which may be important for Leydig cell development, as has been demonstrated for lung (Schmid et al, 1995).

PKC and calcium signaling systems are postulated to be intracellular mediators of hormones that control 11βHSD activities in Leydig cells. PKC, a key signaling system in many cell types, is known to be present in Leydig cells where it regulates testosterone biosynthesis (Nikula et al, 1987). PKC signaling is activated by arachidonic acid and the latter induces steroidogenic acute regulatory protein, leading to increased steroidogenesis (Janzen et al, 1976; Gudermann et al, 1992b). Calcium fluxes, triggered by LH binding, have also been shown to activate Leydig cell steroidogenesis (Janzen et al, 1976; Sullivan and Cooke, 1985; Gudermann et al, 1992b; Wang et al, 2000). In the present study, we observed that cal-

Table 3. *Effects of KN62 on 11β HSD oxidative and reductive activities in intact rat Leydig cells**

Treatments	11β HSD (% conversion)	
	Oxidation	Reduction
Control	100.00 ± 1.65	100.00 ± 5.47
0.01 μM KN62	109.05 ± 5.74	78.40 ± 3.18†
0.1 μM KN62	117.06 ± 11.11	60.54 ± 4.44†
1 μM KN62	149.65 ± 7.56	52.39 ± 1.92†
10 μM KN62	486.86 ± 54.47†	16.42 ± 0.90†

* Leydig cells (0.1×10^6) were incubated with KN62 for 2 hours. 11β HSD oxidation and reduction were measured by 60-minute incubation with 25 nM substrates. Values indicate means ± SE (n = 6).

† Indicates significant difference compared with control in each column at $P < .05$.

Table 4. *Effects of protein kinase modulators on testosterone production in intact rat Leydig cells**

Treatments	Testosterone production (ng/10^6 cells/2h)	
	Basal	LH-stimulated
Control	18.65 ± 3.27	163.80 ± 27.51
0.1 μM SKF7171A	19.87 ± 3.60	166.00 ± 24.83
1 μM SKF7171A	17.80 ± 2.24	101.60 ± 12.39†
10 μM SKF7171A	17.09 ± 4.42	89.89 ± 27.07†
100 μM SKF7171A	20.87 ± 3.50	30.20 ± 10.71†
0.1 μM DHI	16.23 ± 3.62	202.6 ± 39.68
1 μM DHI	10.39 ± 2.70†	163.90 ± 30.08
10 μM DHI	5.56 ± 1.95†	162.30 ± 14.48
100 μM DHI	5.07 ± 1.68†	163.60 ± 17.48

* Leydig cells (0.1×10^6) were incubated with or without SKF7171A or DHI for 2 hours. Testosterone production was measured by radioimmunoassay. Values indicate means ± SE (n = 4).

† Indicates significant difference compared with control in each column at $P < .05$.

cium-dependent signal transduction was required for stimulation of 11βHSD reductive activity. Depletion of extracellular calcium by EGTA, blocking movement of calcium from the extracellular space into Leydig cells, decreased 11βHSD reductive activity while increasing oxidative activity. Calcium binds to calmodulin within cells, causing this protein to undergo a conformational change, exposing a hydrophobic region that typically binds to phosphorylase kinase (Kuznicki et al, 1981). In the present study, treatment with the potent calmodulin inhibitor SKF7171A resulted in a dramatic inhibition of the 11βHSD reductase and increase in 11βHSD oxidation. KN62, a calmodulin kinase II inhibitor, stimulated 11βHSD oxidative activity while inhibiting the 11βHSD reductase. The data support the hypothesis that the effects of calcium on 11βHSD involve the activation of calcium/calmodulin-dependent protein kinase II.

LH, the primary hormone required for maintenance of Leydig cell steroidogenesis, has been shown to suppress 11βHSD oxidative activity and increase reductive activity (Gao et al, 1997). Given that LH increases calcium flux within Leydig cells, we infer that its regulation of 11βHSD activities is calcium-dependent. Consistent with this hypothesis, neither cAMP nor inhibitors of PKA affected 11βHSD activities in Leydig cells (data not shown), although cAMP acts in the major signaling pathways for LH-stimulated Leydig cell steroidogenesis.

The present data establish that 11βHSD oxidative and reductive activities in Leydig cells are regulated independently by PKC and calcium-dependent kinase. The effects of the PKC and calcium-dependent kinase pathways on 11βHSD oxidative and reductive activities could be exerted directly or indirectly. An indirect mechanism is seen, for example, in the case of testosterone itself, which has been shown to decrease 11βHSD oxidative activity and increase reductive activity (Gao et al, 1997). Both PKC and calcium signaling have been shown to regulate testosterone production (Janzen et al, 1976; Nikula et al, 1987; Gudermann et al, 1992b). Both PKC and calcium/calmodulin modulators could affect 11βHSD via the action of testosterone. Our data show that the PKC activator (DHI) and calcium/calmodulin inhibitor (SKF7171A) had differential effects on testosterone production, although they both increased 11βHSD oxidative activity and reduced reductive activity (Table 4). The effects of both modulators on testosterone production were not correlated with their influence on 11βHSD. Both DHI and SKF7171A affected 11βHSD activities even at concentrations below 1 μM, which had no detectable effects on testosterone production. It is unlikely, therefore, that two modulators influence 11βHSD activities by changing Leydig cell viability.

The use of inhibitors, as in the present study, may create interpretational issues due to nonspecific effects on energy metabolism (ATP) or intracellular redox potential ($NADP^+$/NADPH). The modulators may have changed the levels of ATP and/or NADPH present in Leydig cells, either of which could alter 11βHSD activities. However, we have been unable to observe effects of ATP on 11βHSD activity (data not shown). Moreover, it has been reported that wide variations in the intracellular NADPH concentrations do not affect 11βHSD activity (Jamieson et al, 1995). It seems likely, therefore, that PKC and calcium-dependent kinase regulate 11βHSD in Leydig cells via mechanisms that are independent of energy metabolism and redox potential. One possibility is that the two pathways separately phosphorylate the 11βHSD serine/threonine residues near the basic residues, arginine and lysine, resulting in opposite changes of 11βHSD activities. Type I 11βHSD contains a potential PKC motif (Threonine-X-Arginine, residues 61–62) in the cofactor binding domain (Valine 37 to Valine 71) and another (residues 192–194) within the stretch of amino acids (Serine 166-Arginine 194) that is believed to be a dimerization site (Tsigelny and Baker, 1995, 1996). Phosphorylation of these amino acid residues could regulate cofactor binding and protein dimerization. In the case of cofactor binding, if phosphorylation of amino acid residues promotes binding of the $NADP^+$ cofactor, the oxidative activity of the enzyme would predominate over the reductase. In contrast, if phosphorylation promoted binding of the NADPH cofactor, then type I 11βHSD would be predominately reductive.

In summary, 11βHSD oxidative and reductive activities were inversely regulated by PKC and calcium. These two pathways of signal transduction comprise opposing controls of 11βHSD oxidative and reductive activity in Leydig cells. PKC stimulated 11βHSD oxidative activity and inhibited the reductase, whereas the calcium-dependent system in Leydig cells stimulated 11βHSD reductive activity, and inhibited oxidation. Accordingly, the intracellular regulation of 11βHSD-I in Leydig cells was seen to be flexible with the net direction of catalysis adapted to the changing needs for normal cell function. 11βHSD reduction could be stimulated to increase glucocorticoid action during Leydig cell development and, alternatively, 11βHSD oxidation could be stimulated to ameliorate the deleterious effects of glucocorticoids during stress.

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