Journal of Cellular Biochemistry 83:147–154 (2001)

Stimulatory Effect of Lactate on Testosterone Production by Rat Leydig Cells

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Abstract Previously we found that the increased plasma testosterone levels in male rats during exercise partially resulted from a direct and luteinizing hormone (LH)-independent stimulatory effect of lactate on the secretion of testosterone. In the present study, the acute and direct effects of lactate on testosterone production by rat Leydig cells were investigated. Leydig cells from rats were purified by Percoll density gradient centrifugation subsequent to enzymatic isolation of testicular interstitial cells. Purified rat Leydig cells (1×10^5 cells/ml) were in vitro incubated with human chorionic gonadotropin (hCG, 0.05 IU/ml), forskolin (an adenylyl cyclase activator, 10^{-5} M), or 8-bromo-adenosine-3′:5′-cyclic monophosphate (8-Br-cAMP, 10^{-4} M), SQ22536 (an adenylyl cyclase inhibitor, 10^{-6}–10^{-5} M), steroidogenic precursors (25-hydroxy-cholesterol, pregnenolone, progesterone, and androstenedione, 10^{-5} M each), nifedipine (a L-type Ca^{2+} channel blocker, 10^{-5}–10^{-4} M), or nimodipine (a potent L-type Ca^{2+} channel antagonist, 10^{-5}–10^{-4} M) in the presence or absence of lactate at 34°C for 1 h. The concentration of medium testosterone was measured by radioimmunoassay. Administration of lactate at 5–20 mM dose-dependently increased the basal testosterone production by 63–187% but did not alter forskolin- and 8-Br-cAMP-stimulated testosterone release in rat Leydig cells. Lactate at 10 mM enhanced the stimulation of testosterone production induced by 25-hydroxy-cholesterol in rat Leydig cells but not other steroidogenic precursors. Lactate (10 mM) affected neither 30- nor 60-min expressions of cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory (StAR) protein. The lactate-stimulated testosterone production was decreased by administration of nifedipine or nimodipine. These results suggested that the physiological level of lactate stimulated testosterone production in rat Leydig cells through a mechanism involving the increased activities of adenylyl cyclase, cytochrome P450scc, and L-type Ca^{2+} channel. J Cell. Biochem. 83: 147–154, 2001.

Key words: lactate; testosterone; rat; Leydig cells

It has been well known that brief, intense physical exertion increases the circulating testosterone level [Guezennec et al., 1986; Cumming et al., 1987; Maresh et al., 1988]. Although the elevation in serum testosterone is generally followed by gonadotropin stimulation, plasma level of luteinizing hormone (LH) is unchanged after exercise [Sutton et al., 1973; Dessypris et al., 1976; Gawel et al., 1976; Morville et al., 1979]. Hemoconcentration and decreased metabolic clearance have been suggested as mechanisms to explain the exercise-associated testosterone increase [Sutton et al., 1978; Keizer et al., 1980]. Such non-specific mechanisms may apply to other steroid hormones including testosterone. However, an increase of whole blood lactate is accompanied by an increase of serum testosterone in human after heavy resistance exercise [Kraemer et al., 1991].

In rats an increase of lactate production following exercise has been reported [Lin et al., 1994; Kelly et al., 1995; Lu et al., 1996, 1997]. Recently, we have demonstrated that the

Grant sponsor: National Science Council (NSC), Republic of China; Grant number: 85-2331-B-010-053; Grant sponsor: Medical Research and Advancement Foundation in memory of Dr. Chi-Shuen Tsou (ROC), to P.S.W.

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Received 14 March 2001; Accepted 4 May 2001

DOI 10.1002/jcb.1213

increased plasma testosterone levels in male rats during exercise partially resulted from a direct and LH-independent stimulatory effect of lactate on the release of testosterone by the testicular tissues via increasing the production of adenosine 3′:5′-cyclic monophosphate (cAMP) [Lu et al., 1997]. However, the detail mechanisms of lactate action on testosterone production by Leydig cells are not clear yet.

Leydig cells from the testis are the source of testosterone, which is responsible for maintaining spermatogenesis and secondary sexual characteristics in the male. Testosterone biosynthesis is dependent on both acute and chronic stimulation of Leydig cells by the trophic hormone, LH. Binding of LH to its receptors on the Leydig cell surface results in activation of adenylyl cyclase, followed by increased production of intracellular cAMP [Dufau, 1988]. Both acute and chronic effects of LH are mediated by increases in cAMP levels. The acute response is rapid and involves the cAMP-dependent steroidogenic acute regulatory protein (StAR) [Stocco and Clark, 1997], which provides the tonic production of steroid hormone by regulating cholesterol translocation from the outer to the inner mitochondrial membrane, the site containing the P450 side-chain cleavage enzyme (P450scc) that converts cholesterol to pregnenolone. Chronic stimulation of Leydig cells by LH or cAMP is required for optimal expression of enzymes required for steroidogenesis, including the enzyme involved in the first step, P450scc. Both StAR protein and P450scc are involved in the rate-limiting step of the testosterone biosynthesis pathway [Brownie et al., 1972; Crivello and Jefcoate, 1980; Privalle et al., 1983], stimulation of which should have a rapid and marked effect on testosterone production.

In the present study, we explored the effects of lactate on testosterone production in rat Leydig cells. We found that the physiological dosages of lactate (e.g. 10^{-2} M) increased the basal and cholesterol-induced production of testosterone in rat Leydig cells and the stimulation of lactate might be related to the activities of Ca^{2+} channel and P450scc.

MATERIALS AND METHODS

Animals

Male rats of the Sprague–Dawley strain weighing 300–350 gm were housed in a temperature controlled room (22±1°C) with 14 h of artificial illumination daily (06 h 00 min–20 h 00 min) and given food and water ad libitum.

Preparation of Leydig Cells

The method for preparation of rat Leydig cells has been described elsewhere [Huang et al., 2001]. Rat testicular interstitial cells, dispersed using collagenase as described by Lin et al. [1998], were gently applied to the upper layer of a continuous Percoll gradient (20 ml/tube; composed of 9 parts of Percoll and 11 parts of 1.8-fold Medium 199/0.1% bovine serum albumin (BSA) generated by centrifugation at 20,000*g* for 60 min at 4°C, [Browning et al., 1981] and centrifuged at 800*g* for 20 min at 4°C. The Leydig cell layer, found in 10% height of the centrifuge tube from the bottom, was removed, diluted to 5 ml, then centrifuged at 100*g* for 10 min at 4°C. After two further washes, the cell pellet was resuspended in a final volume of 10 ml in incubation medium consisting of medium 199 containing 1% BSA, 25 mM N- 2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), 2.2 gm/l of sodium bicarbonate, 100 IU/l of penicillin-G, 100 μg/l of streptomycin sulfate, and 2,550 USP U/l of heparin, pH 7.3, aerated with 95% O_2 and 5% CO_2. The cell concentration (1×10^5 cells/ml) and viability (over 98%) were determined using a hemocytometer and the Trypan blue method. The 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method [Dirami et al., 1991; Krummen et al., 1994]. was used to measure the proportion of Leydig cells in the preparation. When the cells (1×10^5 cells/ml) were incubated for 90 min at 34°C with 0.05 M PBS, pH 7.4, containing 0.2 mg/ml of nitro blue tetrozolium, 0.12 mg/ml of 5-androstane-3β-ol-one, and 1 mg/ml of NAD^+, a blue formazan deposit was seen in Leydig cells. The proportion of Leydig cells in the preparation, determined using a hemocytometer, was approximately 87±3% (n = 3).

Effect of Lactate on cAMP-Related Testosterone Production

Cell suspensions (1×10^5 cells/ml) were preincubated for 1 h and then incubated for 1 h with lactate (0, 2.5, 5, 10, and 20 mM) in the presence or absence of forskolin (an adenylyl cyclase activator, 10^{-5} M), 8-Br-cAMP (a membrane-permeable analog of cAMP, 10^{-4} M), or SQ22536 (an adenylyl cyclase inhibitor, 10^{-6}–10^{-5} M). Administration of Leydig cells with

hCG (0.05 IU/ml) was used as a positive control. At the end of incubation, 0.5 ml ice-cold gelatin-phosphate buffer saline (GPBS) were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Effect of Lactate on Testosterone Steroidogenesis

Cell suspensions were preincubated for 1 h and then incubated for 1 h with or without lactate at 10 mM in the presence or absence of four steroidogenic precursors (10^{-5} M). These precursors included 25-hydroxy-cholesterol (membrane-permeable cholesterol, 25-OH-C), pregnenolone (Δ_5P), progesterone (P), and androstenedione (Δ_4). At the end of the incubation, 0.5 ml ice-cold GPBS buffer were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Western Blotting

After 30 or 60 min incubation with lactate (10 mM), Leydig cells were washed three times with fresh normal saline, then extracted with homogenization buffer consisting of 1.5% Na-lauroylsarcosine, 2.5×10^{-3} mol/L Tris-base, 1×10^{-3} mol/l EDTA, 0.68% phenylmethylsulfonyl fluoride (PMSF), and 2% proteinase inhibitors, pH 8.0, and disrupted by sonication (Heat System, Farmingdale, NY) in an ice-bath. The cell extracts were centrifuged for 12 min at 10,000*g*, then the supernatants were collected and the protein concentrations determined using the Bradford method [Zor and Selinger, 1996]. The extracted protein was denatured by boiling for 10 min in SDS buffer (0.125 mol/l Tris-base, 4% SDS, 0.001% bromphenol blue, 12% sucrose, 0.15 mol/l dithiothreitol). Western blotting and gel electrophoresis were performed as described by Kau et al. [1999]. The proteins in the samples were separated by electrophoresis on a 12% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE) at 50 V for 20 min, then at 100 V for 60 min, and electrophoretically transferred for 50 min at 60 mA (8 × 10 mm membrane) to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Inc., Boston, MA) using a Trans-Blot SD semi-dry transfer cell (170-3940, Bio-Rad, Hercules, CA) and transfer solution (0.025 mol/l Tris-base, 0.2 mol/l glycine, and 10% methanol, pH 8.3).

The membranes were immersed in TBS-T buffer (0.8% NaCl, 0.02 mol/l Tris-base, and 0.3% Tween-20, pH 7.6) for 5 min, then blocked for 60 min in blocking buffer (TBS-T buffer containing 5% nonfat dry milk) at room temperature. They were then incubated overnight at 4°C with antibodies against P450scc (1:1,000, rabbit), StAR (1:2,000, rabbit), or β-actin (1:8,000, mouse) diluted in blocking buffer. After three 10 min washes with TBS-T buffer, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:6,000 for anti-P450scc and anti-StAR antibodies) or anti-mouse IgG (1:8,000 for anti-β-actin antibodies) in blocking buffer, washed three times with TBS-T buffer, and the three proteins visualized by chemiluminescence (ECL Western blotting detection reagents, Amersham International Plc., UK) by exposure for 5–10 sec to BioMax films (Eastman Kodak Company, Rochester, NY). Band intensity was quantified using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

Effect of Ca^{2+} Channel Blocker on Lactate-Increased Testosterone Production

Cell suspensions were preincubated and then incubated for 1 h with or without lactate at 10 mM in the presence or absence of nifedipine (a L-type Ca^{2+} channel blocker, 10^{-5}–10^{-4} M), or nimodipine (a potent L-type Ca^{2+} channels antagonist, 10^{-5}–10^{-4} M). At the end of the incubation, 0.5 ml ice-cold GPBS buffer were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

RIA of Testosterone

The concentrations of testosterone in medium samples were determined by RIA as described elsewhere [Wang et al., 1994; Lin et al., 1998]. With anti-testosterone serum No. W8, the sensitivity of testosterone RIA was 2 pg/assay tube. The intra- and interassay coefficients of variation (CV) were 10.6 (n = 5) and 6.2% (n = 7), respectively.

Materials

Bovine serum albumin (BSA), N-2-hydroxyethylpiperazine-N′-2-ethane-sulphonic acid

(HEPES), Hank's balanced sodium salt (HBSS), Medium 199, sodium bicarbonate, penicillin-G, streptomycin, heparine, collagenase, human chorionic gonadotropin (hCG), forskolin, 8-Br-cAMP, SQ22536, 25-hydroxy-cholesterol, pregnenolone, progesterone, androstenedione, nifedipine, nimodipine, and lactic acid were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-testosterone was obtained from Amersham International Plc. (Bucks, UK). The doses of drugs were expressed in their final molar concentrations in the flask.

Statistical Analysis

All values are given as the mean±standard error of the mean (SEM). In some cases, the means of treatment were tested for homogeneity by a two-way analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan's multiple-range test [Steel and Torrie, 1960]. In other cases, Student's t-test was employed. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Effect of Lactate on cAMP-Related Testosterone Production

Lactate at 5–20 mM resulted in a dose-dependent increase of testosterone production ($P < 0.01$) (Fig. 1) by rat Leydig cells. Administrations of hCG (0.05 IU/ml), forskolin (10^{-5} M), and 8-Br-cAMP (10^{-4} M) significantly increased testosterone production ($P < 0.01$, 0.01, and 0.05, respectively) by rat Leydig cells (Figs. 1 and 2). However, lactate did not alter the forskolin- and 8-Br-cAMP-stimulated production of testosterone by rat Leydig cells (Fig. 2). SQ22536 at 10^{-5} M decreased the stimulation of testosterone production induced by lactate (10 mM) ($P < 0.05$) (Fig. 3).

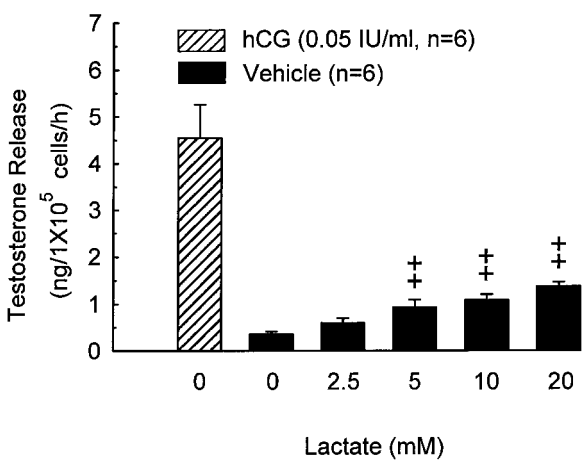

Fig. 1. Effects of lactate on the production of testosterone by rat Leydig cells. Each value represents the mean±SEM. ++, $P < 0.01$ compared with vehicle group treated with 0 M lactate.

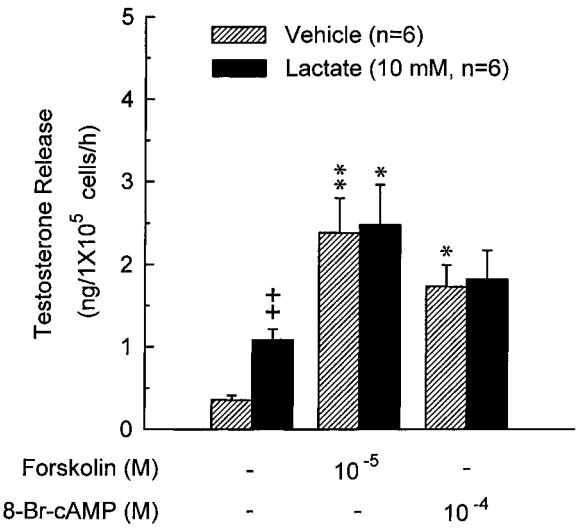

Fig. 2. Effects of lactate on the testosterone production in response to forskolin and 8-Br-cAMP by rat Leydig cells. Each value represents the mean±SEM. *, **$P < 0.05$ and $P < 0.01$ compared with corresponding basal groups, respectively. ++, $P < 0.01$ compared with vehicle group.

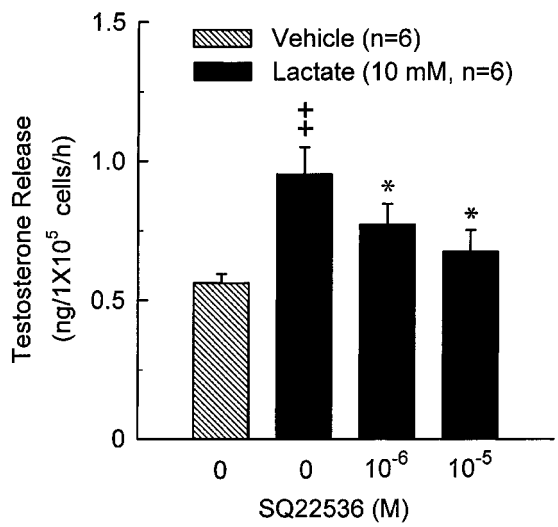

Fig. 3. Effects of SQ22536 on lactate-stimulated production of testosterone by rat Leydig cells. Each value represents the mean±SEM. *$P < 0.05$ compared with lactate-treated control group. ++, $P < 0.01$ compared with vehicle group.

Effects of Lactate on Testosterone Steroidogenesis

In the concentration of 10^{-5} M, the four testosterone precursors tested each increased testosterone production by rat Leydig cells ($P < 0.05$ or 0.01) (Fig. 4). Lactate at 10 mM enhanced the stimulation of testosterone production by 25-OH-cholesterol in Leydig cells ($P < 0.01$). However, lactate did not alter testosterone production induced by any other three precursors tested.

Effects of Lactate on the Expressions of StAR Protein and P450scc

To determine whether the stimulatory effects of lactate on Leydig cells were correlated to the altered StAR or P450scc protein expressions, the levels of these proteins in Leydig cells were assessed by Western blotting and densitometry after administration of lactate (10 mM) for 30 or 60 min (Fig. 5), each experiment being repeated three times. No matter whether it was 30 or 60 min incubation, lactate (10 mM) did not alter the expressions of P450scc and StAR protein.

Effect of Calcium Channel Blocker on Lactate-Increased Testosterone Production

The lactate (10 mM)-stimulated testosterone production by rat Leydig cells was reduced by the administration of nifedipine (a L-type Ca^{2+} channel blocker, 10^{-4} M, $P < 0.01$), or nimodipine (a potent L-type Ca^{2+} channels antagonist, 10^{-5}–10^{-4} M, $P < 0.01$) (Figs. 6 and 7).

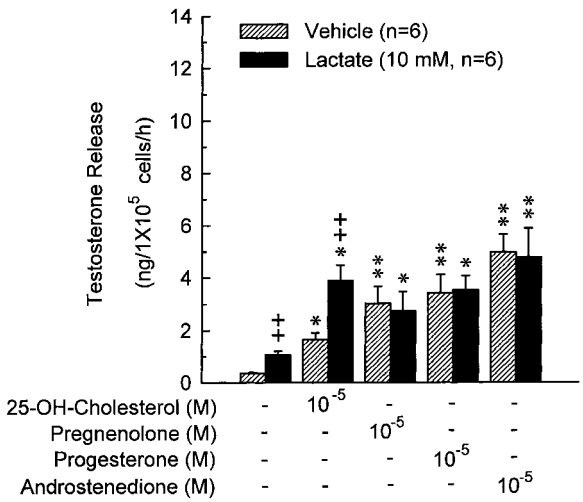

Fig. 4. Effects of lactate on the testosterone production in response to steroidogenic precursors by rat Leydig cells. Each value represents the mean±SEM. *, **$P < 0.05$ and $P < 0.01$ compared with corresponding basal groups (i.e., without steroidogenic precursor), respectively. ++, $P < 0.01$ compared with vehicle group.

DISCUSSION

It has been well known that the level of plasma lactate increases when exercise intensity exceeds 75–120% of maximum oxygen uptake in humans [Karlsson, 1971; Hermansen and Vaage, 1977]. In rats an increase in lactate production has been demonstrated following exercise [Lin et al., 1994; Kelly et al., 1995; Lu et al., 1996, 1997]. Meanwhile, an increase in plasma testosterone levels in humans has been reported after exercise [Wilkerson et al., 1980; Guezennec et al., 1986; Cumming et al., 1987; Maresh et al., 1988]. Our previous studies have demonstrated that the increased plasma testosterone levels in male rats during exercise is at least a result of a direct and LH-independent stimulatory effect of lactate on the secretion of testosterone by the testicular tissues [Lu et al., 1997]. In the present study, we found that lactate in vitro dose-dependently increased the spontaneous production of testosterone by acting directly on rat Leydig cells. Since the physiological levels of lactate in rat plasma are within a range of 2–10 mM [Lu et al., 1996, 1997], the effective doses (2.5–20 mM) of lactate utilized in vitro in the present study are physiological doses. Actually, we found that 100 mM of lactate, a dose greater than physiological dose decreased rather than increased the production of testosterone (data not shown).

Numerous reports indicate that hCG is an effective stimulant for testosterone secretion both in vivo [Padron et al., 1980; Wang et al., 1994] and in vitro [Sutton et al., 1973; Simpson et al., 1987], as well as for cAMP generation in testes [Avallet et al., 1987; Petersson et al., 1988; Wang et al., 1994]. An increase of testosterone release induced by forskolin (an adenylyl cyclase activator) and 8-Br-cAMP (a membrane permeable analog of cyclic AMP) has been demonstrated in rat testicular interstitial cells [Lin et al., 1998]. In the present study, the administration of lactate at physiological doses did not alter the stimulatory effects caused by forskolin, or 8-Br-cAMP, on testosterone production, however, SQ22536, an adenylyl cyclase inhibitor, effectively inhibited the stimulatory effects of lactate. It is apparent that the stimulatory effects of lactate might relate to

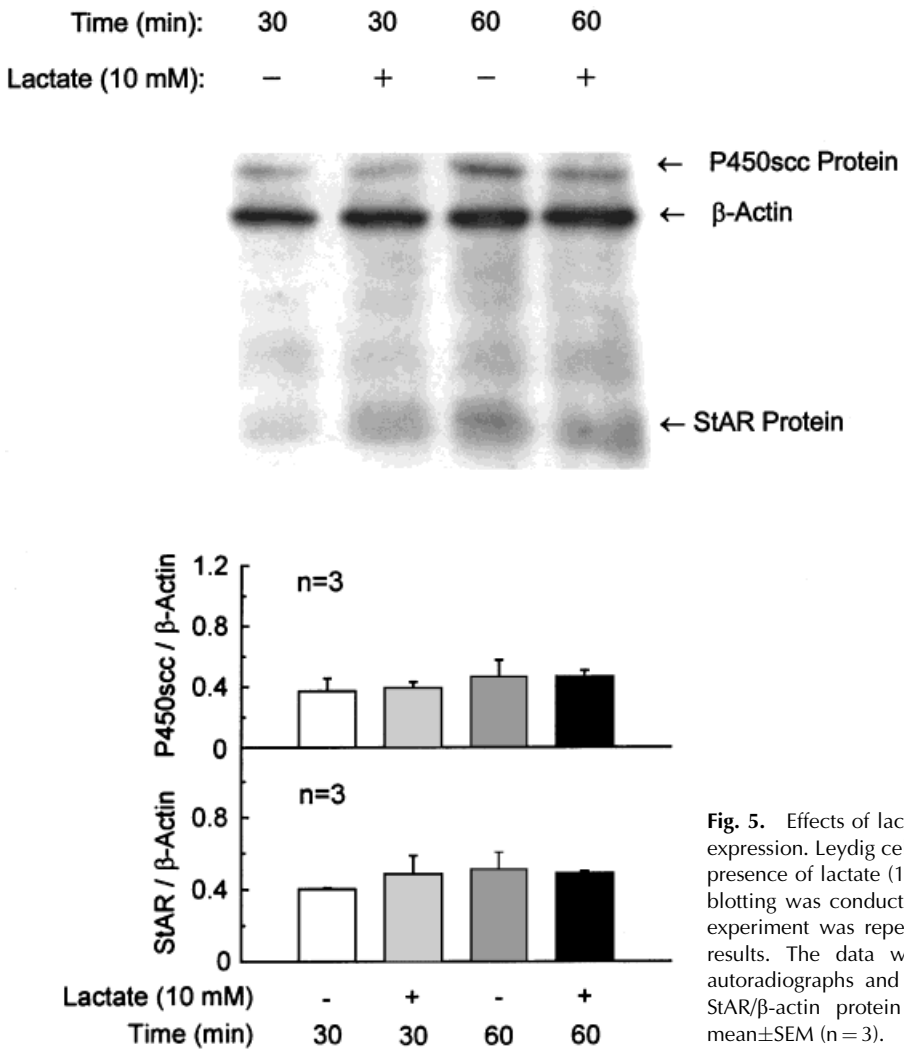

Fig. 5. Effects of lactate on StAR and P450scc protein expression. Leydig cells were cultured in the absence or presence of lactate (10 mM) for 30 or 60 min. Western blotting was conducted as described in Methods. This experiment was repeated three times with the similar results. The data was obtained by scanning three autoradiographs and expressed as P450scc/β-actin or StAR/β-actin protein ratio. Each column represents mean±SEM (n = 3).

the activation of adenylyl cyclase on testosterone production in rat Leydig cells.

Since lactate further enhanced testosterone production increased by cholesterol but did not influence that increased by the other steroidogenic precursors tested in rat Leydig cells, our data suggested that P450scc might be a key target for lactate. In response to steroidogenic stimuli, testosterone biosynthesis is initiated by cleavage of the side chain of cholesterol to form pregnenolone. This reaction is catalyzed by P450scc, located on the matrix side of the inner mitochondrial membrane [Yago and Ichii, 1969]. Delivery of cholesterol to P450scc in the inner mitochondrial membrane is considered to be the rate-limiting step in steroidogenesis [Crivello and Jefcoate, 1980; Privalle et al., 1983]. Clark et al. [1994] identified a 30 kDa protein that was suggested to regulate cholesterol translocation from the outer to the inner mitochondrial membrane and named it the steroidogenic acute regulatory (StAR) protein. When LH binds to its receptor, it activates Gs protein and stimulates adenylyl cyclase, increasing the concentration of intracellular cAMP. StAR protein and steroidogenic enzymes, including P450scc, are cAMP-dependent, and are regulated by upstream trophic signals, such as LH. Our results showed that lactate did not alter the expressions of P450scc and StAR protein but might enhance the activity of P450scc to stimulate testosterone production in rat Leydig cells.

The increase of intracellular Ca^{2+} is involved in the enhancement of testosterone production in rat Leydig cells [Hall et al., 1981; Themmen et al., 1986; Foresta and Mioni, 1993; Foresta et al., 1995]. The present results indicated that

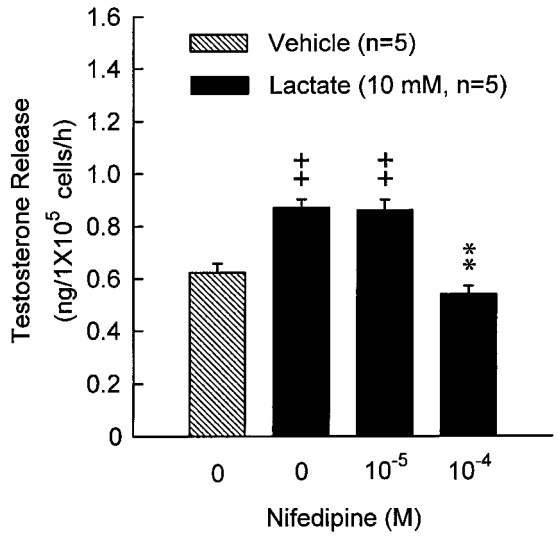

Fig. 6. Effects of nifedipine on lactate-stimulated production of testosterone by rat Leydig cells. Each value represents the mean±SEM. $^{**}P < 0.01$ compared with lactate-treated control group. ++, $P < 0.01$ compared with vehicle group.

the increase of testosterone production in rat Leydig cells by 10 mM lactate was significantly reduced by administration of nifedipine and nimodipine, both are L-type Ca^{2+} channel blockers. These data suggested that the activation of L-type Ca^{2+} channel in rat Leydig cells involved in the increase of testosterone by lactate.

In summary, the results demonstrated that lactate at physiological doses acts directly on rat Leydig cells to increase testosterone production. Its actions were effected at least in part by increasing the activities of adenylyl cyclase, P450scc, and L-type Ca^{2+} channel in rat Leydig cells.

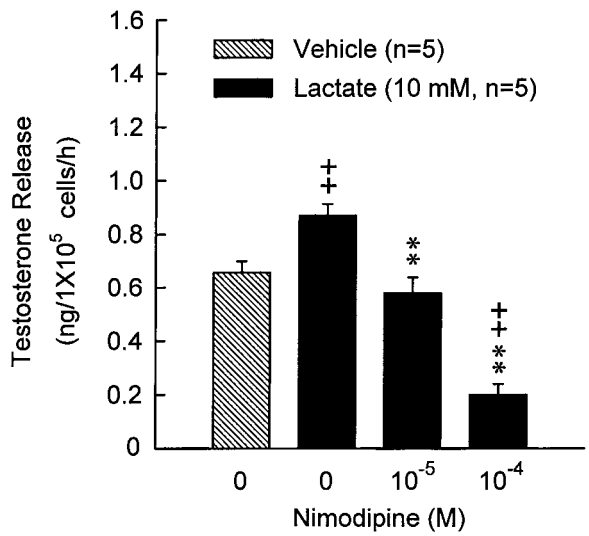

Fig. 7. Effects of nimodipine on lactate-stimulated production of testosterone by rat Leydig cells. Each value represents the mean±SEM. $^{**}P < 0.01$ compared with lactic acid-treated control group. ++, $P < 0.01$ compared with vehicle group.

ACKNOWLEDGMENTS

The authors wish to thank Dr. D.M. Stocco, Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas and Dr. Bon-Chu Chung, Institute of Molecular Biology, Academia Sinica, ROC for kindly providing anti-StAR protein and anti-cytochrome P450scc antibody, respectively.

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