Role of Chloride and Inhibitory Action of Inorganic Nitrate on Gonadotropin-Stimulated Steroidogenesis in Mouse Leydig Tumor Cells

N.S. Panesar

The involvement of adenylate cyclase–cyclic adenosine monophosphate (AC-cAMP) in gonadotropin-stimulated testicular steroidogenesis is well known. Little is known about the role of guanylate cyclase–cyclic guanosine monophosphate (GC-cGMP) or early chloride conductance stimulated by gonadotropins in steroidogenesis. Human chorionic gonadotropin (hCG) 1 IU/L caused significant androgen secretion without a discernible effect on cAMP production. Despite negligible intracellular cAMP, the protein kinase A inhibitor H89 blocked basal and hCG-stimulated steroidogenesis. The GC inhibitors methylene blue (MB) and LY83583 decreased androgen secretion, but hCG did not stimulate cGMP production and there was not a steroidogenic response to exogenous cGMP. A chloride-channel inhibitor, diphenylamine-2-carboxylate (DPC), at concentrations up to 0.6 mmol/L stimulated basal steroid secretion and hCG 10 IU/L stimulated cAMP production, but higher concentrations had an inhibitory effect. Substitution of chloride by gluconate enhanced basal steroid secretion, but nitrate completely abolished the effect of 1 IU/L hCG on androgen secretion, which could be partially overcome by increasing the gonadotropin concentration. In conclusion, chloride, perhaps by activating AC-cAMP, mediates the steroidogenic action of gonadotropins in mouse Leydig tumor cells (MLTC-1). Inorganic nitrate probably inhibited steroidogenesis via conversion to nitric oxide (NO) without involving the GC-cGMP pathway. Nevertheless, the results obtained with GC inhibitors suggest a role for the GC-cGMP pathway in Leydig cell steroidogenesis.

THE MAIN MEDIATOR of steroidogenesis in Leydig cells in response to either luteinizing hormone (LH) or human chorionic gonadotropin (hCG), which share the same receptor, is believed to be cyclic adenosine monophosphate (cAMP). cAMP signal transduction involves dissociation of the α-subunit of Gs protein and activation of adenylate cyclase (AC).[1] However, the gonadotropin action on steroidogenesis also involves other signaling pathways. Calcium is essential for testosterone secretion[2] and is mobilized during the gonadotropin action,[3] but a recent study based on single-cell recordings opposes the involvement of calcium in the mediation of gonadotropin message in Leydig cells.[4] Duchatelle and Joffre[5] described three types of chloride conductance in rat Leydig cells, gonadotropin- and cAMP-independent conductance (ie, hyperpolarization- and calcium-activated) and a dependent conductance that they speculated may have a role in steroid hormone release. More recently, Noulin and Joffre[6] proposed that the hyperpolarization-activated chloride conductance could be modulated by cAMP. Choi and Cooke[7] proposed that chloride depletion stimulates steroidogenesis at a low concentration of gonadotropins independently of cAMP, and that cAMP is only involved in steroidogenesis at higher concentrations of gonadotropins. It is uncertain if the chloride conductances activated by gonadotropins precede the activation of AC and cAMP production, but chloride is necessary for dissociation of the α-subunit of Gs protein.[8] Therefore, it is plausible that the early chloride conductance may be involved in the activation of AC and cAMP production and steroidogenesis.

Nitric oxide (NO) has also been reported to inhibit steroidogenesis in Leydig cells[9,10] and granulosa-luteal cells.[11] We serendipitously found that the substitution of chloride with inorganic nitrate inhibited steroidogenesis in response to a low concentration of hCG (reported herein). A probable mechanism for the inhibition of steroidogenesis by nitrates may be modeled on their ability to cause vasodilatation. The sequential process involves the conversion of nitrate to NO, activation of guanylate cyclase (GC), cGMP production, sequestration of cytoplasmic calcium, smooth muscle relaxation, and resultant vasodilatation.[12,13] The GC-cGMP pathway is involved in corticotropin-stimulated adrenal steroidogenesis[14,15] and in atrial natriuretic peptide–stimulated testosterone synthesis in Leydig cells,[16] the latter involving the calcium/calmodulin messenger pathway.[17] cGMP is also involved in ion channels.[18] However, it is not known if gonadotropin action in Leydig cell steroidogenesis also involves GC-cGMP.

In this study, we have evaluated the role of chloride, gluconate, sulfate, and nitrate in basal and hCG-stimulated steroidogenesis in relation to other signaling events in mouse Leydig tumor cells (MLTC-1). In our attempts to elucidate the mechanism by which inorganic nitrate inhibits steroidogenesis, we have also studied the involvement of GC-cGMP in Leydig cell steroidogenesis. Although MLTC-1 are reported to produce mainly progesterone and little testosterone,[19] we also examined testosterone production by these cells.

MATERIALS AND METHODS

MLTC-1 cells were obtained from the American Type Tissue Collection (Rockville, MD). Hanks balanced salt solution ([HBSS] with and without calcium and magnesium), tissue cultureware, hCG (3,000 IU/mg), and sodium salts of cAMP and cGMP were obtained from Sigma Chemical (St Louis, MO). Growth media, penicillin-streptomycin, trypsin-EDTA, and glutamine for cell culture were obtained from GIBCO BRL (Gaithersburg, MD). Diphenylamine-2-carboxylate (DPC), H89, and LY83583 were gifts from various colleagues at the Chinese University of Hong Kong ([CUHK] see Acknowledgment). The testosterone antiserum was provided by the Division of Steroid Endocrinology, The University of Leeds (Leeds, UK). [1,2,6,7-^{3}H]testosterone (specific activity, 94 Ci/mmol), [8-^{3}H]cAMP, ammonium salt (specific activity, 29 Ci/mmol), and the Amerlax M cGMP [^{125}I]radioimmunoas-

From the Department of Chemical Pathology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong.
Submitted March 3, 1998; accepted January 20, 1999.
Supported by a grant from the Croucher Foundation.
Address reprint requests to N.S. Panesar, PhD, Department of Chemical Pathology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong.

0026-0495/99/4806-0005$10.00/0

say (RIA) kit were obtained from Amersham International (Amersham, Buckinghamshire, UK). Histochemical-grade methylene blue (MB) was obtained from the Department of Anatomical and Cellular Pathology, CUHK. Analytical-grade salts and steroids were obtained from the British Drug Houses (Poole, Dorset, UK), Sigma, or Merck (Darmstadt, Germany). Analytical-grade diethyl ether for extracting steroids was obtained from Merck.

Culture of MLTC-1 Cells

MLTC-1 cells were cultured in RPMI 1640 containing 25 mmol/L HEPES and supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, penicillin G (100 U/mL), and streptomycin (100 μg/mL), pH 7.3, at 37°C in a 5% CO_2 incubator. At 40% to 50% confluency, the cells were disaggregated with trypsin-EDTA (0.05% and 0.53 mmol/L, respectively) dissolved in calcium and magnesium–free HBSS. After washing once with the culture medium, approximately 66,000 viable cells (viability, 90% to 95%) in 1 mL growth medium were seeded per well in a 24-well plate and grown for 2 days before the experiments. For studying the production of cGMP in response to hCG, 2×10^5 cells in 2 mL growth medium were plated in 12-well plates and cultured for 2 days before hCG stimulation.

Experimental Conditions and Assays for Testosterone and Cyclic Nucleotides

MLTC-1 cells were incubated with HEPES–phosphate-buffered Ringer solution containing 135 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 0.83 mmol/L Na_2HPO_4, 3.33 mmol/L NaH_2PO_4, 5 mmol/L HEPES, 10 mmol/L glucose, and 0.5% bovine serum albumin (pH 7.4) in the absence or presence of various concentrations of hCG for 1 hour to stimulate steroidogenesis. The effects of various anions on steroidogenesis were tested by preincubating the cells in medium without or with chloride substituted by equimolar concentrations of gluconate, nitrate, or sulfate while maintaining the cation concentration the same. The experiment was performed under similar anion conditions without or with hCG. The effects of DPC, H89, MB, or LY83583 were tested by preincubating the cells for 1 hour in a medium containing the agent alone and then adding the medium containing the agent without or with hCG. All experiments were performed on two to five separate occasions in triplicate or quadruplicate.

After incubation, the medium from each well was removed and stored at −20°C until analysis for androgens. One milliliter of 1.7% HCl in ethanol (HCl/EtOH) was added per well to lyse the cells, and the plates were stored at −20°C overnight. The acid-alcohol extract was dried using a speed-vacuum and reconstituted in Tris-EDTA buffer (50 mmol/L Tris hydrochloride and 4 mmol/L EDTA, pH 8.0) for determination of intracellular cAMP by the protein kinase binding assay.[1] cGMP was assayed according to the manufacturer's instructions using a one-fourth volume of HCl/EtOH cell extract. Androgen levels were measured by an in-house testosterone RIA essentially according to Ooi et al.[20] The intraassay and interassay coefficient of variation for these assays was 8%. The percentage cross-reactivity of testosterone antiserum for the following steroids was as follows: dihydrotestosterone, 40%; androstenedione, 0.27%; progesterone, 0.002%; and cortisol, 17β-estradiol, and 17α-hydroxyprogesterone, all less than 0.002%.

To standardize androgen and cAMP production to a constant figure, a 24-well plate with MLTC-1 cells was subjected to the same procedural maneuvers as the cells in the experimental plates. The cells in all wells were trypsinized for 10 minutes, harvested, pooled together, washed once with culture medium, and counted using a hemacytometer. From the cell count obtained, the average cell number per well was calculated and used for standardization of the cell number.

MLTC-1 and Testosterone Production

To determine if MLTC-1 cells produce testosterone in response to hCG, cells were grown in a 24-well plate and stimulated with 1 to 10^4 IU/L hCG in quadruplicate as before. A pool of 500 μL was made by taking 125 μL of the incubation medium from each of the replicate assays. From this pool, two 25-μL aliquots were saved for direct measurement of androgen levels by the testosterone RIA. To the remaining 450 μL, 5,000 dpm purified tritiated testosterone was added, and two 25-μL aliquots were taken for determination of radioactivity. The remainder of the pool was extracted with 4 mL diethyl ether for 3 minutes at maximum speed on a multitube vortexer (Baxter Diagnostics, Deerfield, IL). The extract was concentrated by drying 1-mL aliquots under a stream of nitrogen. The residue was suspended in 50 μL diethyl ether and applied to a precoated aluminum-backed silica gel thin-layer chromatography plate with fluorescent dye (Merck) along with steroid markers. The plate was chromatographed in a cyclohexane: ethyl acetate (60:40) solvent system. After developing the plate, the steroids were located under UV light. The spots corresponding to testosterone marker were cut and eluted with 1 mL ethanol. Radioactivity was determined in two 50-μL aliquots of eluates, and two 50-μL aliquots were used for testosterone RIA. After correcting for recovery, the specific testosterone was expressed as a percentage of the total value recorded by testosterone RIA.

Statistical Analysis

All data are expressed as the mean ± SD normalized to 10^5 cells per incubation. Testosterone was transformed to a logarithmic scale while the square root of the cAMP level was obtained to stabilize their variances among different concentrations of hCG before analysis. The amount of testosterone secreted and the intracellular cAMP level at different concentrations of hCG were compared using one-way ANOVA models separately for each concentration of other agents such as H89, MB, LY83583, and DPC. Differences in anion content were also analyzed using one-way ANOVA models at each hCG level. If there were significant differences ($P < .05$), a post hoc test was used. P values were subjected to Bonferroni adjustment. The concentrations of agents causing 50% inhibition (IC_{50}) of testosterone were calculated using the sigmoidal dose-response (variable slope) model.

RESULTS

MLTC-1 and Testosterone Production

Thin-layer chromatography showed two prominent UV-absorbing steroids, namely progesterone (retardation factor [Rf] 0.66) and androstenedione (Rf 0.54), in the supernates of cells stimulated with hCG. Despite different concentrations of hCG, the steroid recovered from the spots corresponding to the testosterone marker (Rf 0.43) accounted for 37% to 41% (five observations) of the steroid measured directly by testosterone RIA. Because of its 135-fold higher cross-reactivity relative to progesterone (0.27% *v* 0.002%), androstenedione would have contributed substantially to the steroid pool measured by the testosterone RIA. In view of the constancy of testosterone production by MLTC-1 and contribution by androstenedione, the results hereafter are reported as androgen production.

Effects of Varying Concentrations of hCG on Androgen Secretion and Intracellular Cyclic Nucleotide Levels in MLTC-1 Cells in the Normal Medium

Figure 1 shows the effects of varying concentrations of hCG on androgen secretion and intracellular cAMP levels in MLTC-1 cells. Androgen secretion appeared to show a concentration-

dependent response to hCG, but the secretion was not significantly different above 10 IU/L hCG. At greater than 10,000 IU/L, there was inhibition of androgen and cAMP production. Relative to the basal incubations without hCG, 1 IU/L hCG caused a significant ($P < .0001$) increase in androgen production, amounting to nearly a half-maximal response, without a discernible effect on intracellular cAMP levels. hCG 10 IU/L yielded only one fourth of the maximal cAMP response obtained with 10,000 IU/L hCG, but this in turn was not significantly different from 100 IU/L hCG. Although 10 IU/L was submaximal, this concentration was subsequently used in all experiments for the sake of economy.

The concentration of cGMP in MLTC-1 cells was almost 1,000-fold lower than cAMP, and there was no significant change in cGMP production in response to hCG (data not shown).

Effects of Protein Kinase A Inhibitor H89 on hCG-Stimulated Androgen Secretion and Intracellular cAMP Levels in MLTC-1 Cells

Figure 2a shows the dose-dependent decrease of androgen secretion by H89. Androgen secretion was significantly ($P < .0005$) different in basal and 1- and 10-IU/L hCG–stimulated cells at all concentrations of H89 except 100 μmol/L. Post hoc testing showed an insignificant difference between 1- and 10-IU/L hCG–stimulated androgen secretion, although there was more androgen produced with the higher concentration of hCG. IC_{50} values for H89 for basal and 1-IU/L and 10-IU/L hCG–stimulated cells were 16.5, 15.9, and 18.5 μmol/L, respectively.

Despite the inhibition of steroidogenesis by H89, intracellular cAMP increased with the increasing dose of protein kinase A inhibitor (Fig 2b). At all concentrations of H89, intracellular cAMP was significantly ($P < .0005$) higher in cells stimulated by 10 IU/L hCG versus basal and 1-IU/L–stimulated cells.

Effects of GC Inhibitors MB and LY83583 on hCG–Stimulated Androgen Secretion by MLTC-1 Cells and Direct Effect of cGMP on Steroidogenesis

Figure 3 shows the dose-dependent inhibition of androgen secretion by GC inhibitors. The difference in androgen secretion from basal and 1-IU/L–stimulated cells was significant ($P < .0005$) at all concentrations of MB. At a concentration of 1 μmol/L LY83583 or less, there was significantly ($P < .0005$) higher androgen secretion from cells stimulated by 1 IU/L hCG. At 10 μmol/L LY83583, there was still significantly ($P < .016$) higher androgen secretion from hCG-stimulated cells, but at higher concentrations, the difference became insignificant. The IC_{50} for MB and LY83583 at 1 IU/L hCG was 14.5 and 3.6 μmol/L, respectively.

Native cAMP at a concentration of 1.4 mmol/L significantly increased androgen secretion by MLTC-1, but a similar concentration of native cGMP had no effect. The basal and cyclic nucleotide–stimulated androgen secretion (n = 4) was as follows: cAMP, 53.7 ± 9.1 versus 305.9 ± 29.6 pg/well ($P < .0005$); and cGMP, 38.2 ± 4.6 versus 48.6 ± 7.3 pg/well ($P = .08$).

Effects of Chloride-Channel Inhibitor DPC on hCG-Stimulated Androgen Secretion and Intracellular cAMP Levels in MLTC-1 Cells

Figure 4a shows the dose-dependent effects of DPC on basal and hCG-stimulated androgen secretion from MLTC-1 cells. Basal androgen secretion showed a biphasic response to DPC, increasing at lower concentrations of the chloride-channel inhibitor and decreasing at higher concentrations. ANOVA showed significant differences in androgen secretion between different concentrations of DPC for the basal cells ($P < .0005$). The findings of post hoc tests are shown in Table 1. DPC caused a dose-dependent decrease in hCG-stimulated androgen secretion. The difference in androgen secretion between basal and hCG-stimulated cells was highly significant ($P < .0005$) up to a concentration of 0.6 mmol/L DPC, but above this concentration, the difference became insignificant. The IC_{50} for DPC at 1 and 10 IU/L hCG was 0.84 and 0.75 mmol/L DPC, respectively.

Figure 4b shows intracellular cAMP levels in the three groups of cells. There was no significant difference in cAMP production between basal and 1-IU/L hCG–stimulated cells. The 10-IU/L–stimulated cells showed a biphasic response in cAMP production, increasing at lower concentrations of DPC and decreasing at higher concentrations. At all concentrations of the chloride-channel inhibitor, the difference was significant compared with basal and 1-IU/L hCG–stimulated cells. Table 2 shows the pairwise difference in cAMP production in 10-IU/L–stimulated cells at different concentrations of DPC.

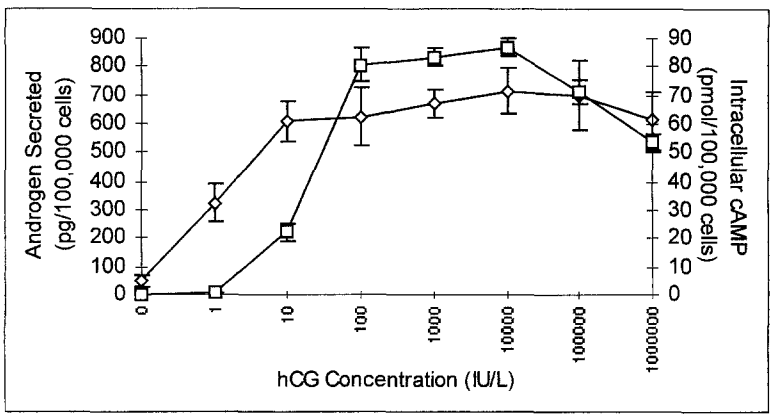

Fig 1. Androgen secretion (◇) and intracellular cAMP level (□) in MLTC-1 cells in response to various concentrations of hCG plotted logarithmically (mean ± SD, n = 4).

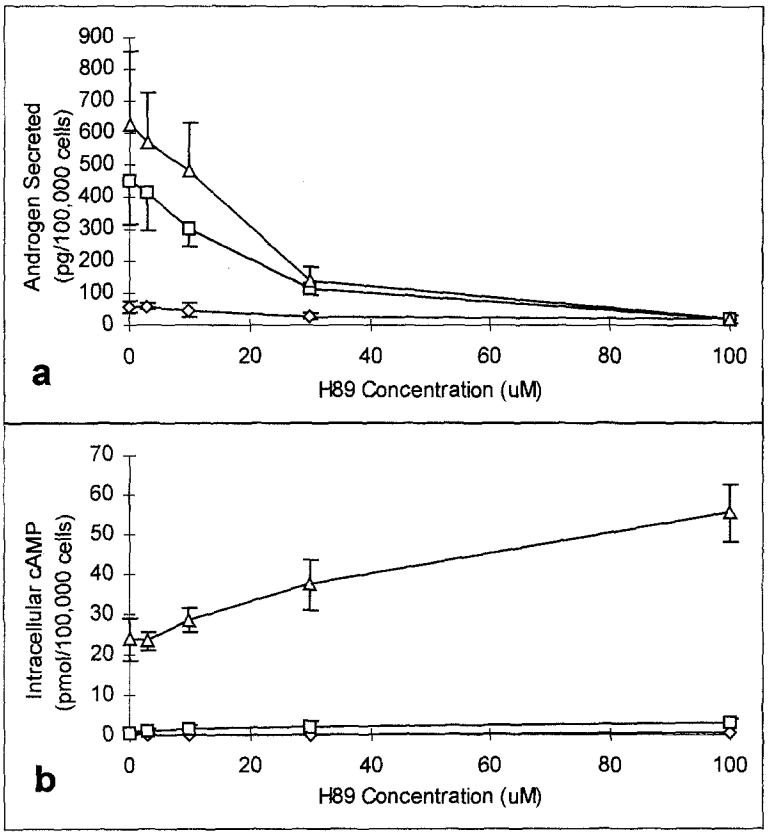

Fig 2. Androgen secretion (a) and intracellular cAMP level (b) in basal (◇) and 1-IU/L hCG (□)- and 10-IU/L hCG (△)-stimulated MLTC-1 cells in the absence or presence of various concentrations of H89, a protein kinase A inhibitor (mean ± SD, n = 9).

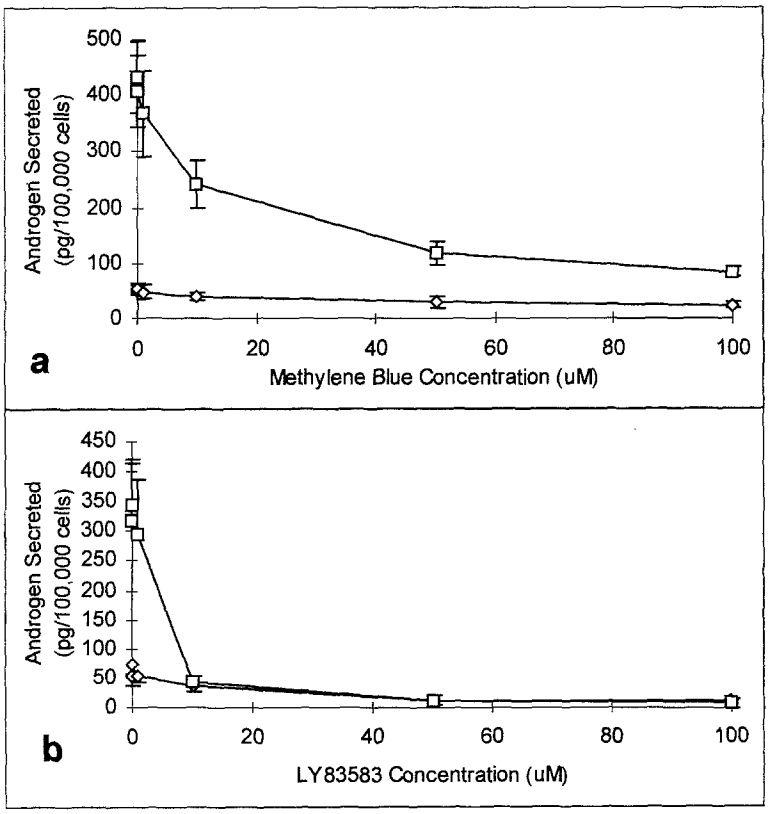

Fig 3. Androgen secretion from basal (◇) and 1-IU/L hCG (□)-stimulated MLTC-1 cells in the absence or presence of various concentrations of MB (a) and LY83583 (b), guanylate cyclase inhibitors (mean ± SD, n = 9).

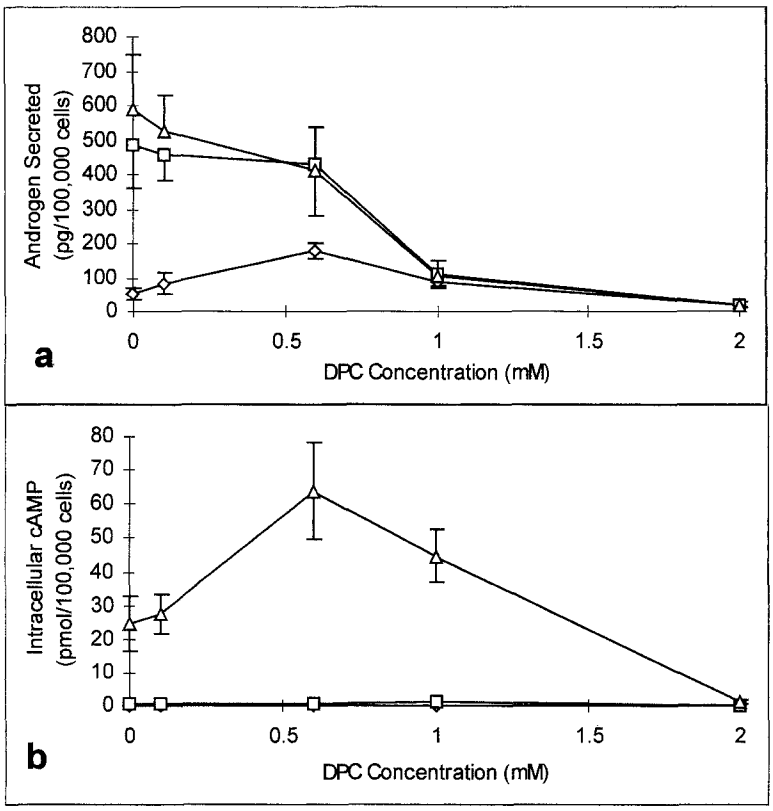

Fig 4. Androgen secretion (a) and intracellular cAMP level (b) in basal (◇) and 1-IU/L hCG (□)- and 10-IU/L hCG (△)-stimulated MLTC-1 cells in the absence or presence of various concentrations of DPC, a chloride-channel inhibitor (mean ± SD, n = 12).

Effects of Substituting Chloride With Nitrate, Sulfate, or Gluconate on hCG-Stimulated Androgen Secretion and Intracellular cAMP Levels in MLTC-1 Cells

Figure 5a shows the effects of different anions on basal and hCG-stimulated androgen secretion by MLTC-1 cells. Under basal conditions, cells incubated in the presence of gluconate secreted significantly ($P < .007$ or better) more androgen compared with chloride and sulfate incubation, but the difference with nitrate was insignificant. When stimulated with 1 IU/L hCG, androgen secretion in the presence of sulfate was significantly ($P < .009$ or better) lower than with chloride and gluconate. However, the most significant ($P < .0005$) inhibition of androgen secretion occurred in the presence of nitrate. With 10 IU/L hCG as the stimulant, androgen secretion was still significantly ($P < .015$ or better) lower in the presence of nitrate.

Figure 5b shows the effects of anions on basal and hCG-stimulated intracellular cAMP levels in MLTC-1 cells. There was little cAMP produced under basal and 1-IU/L hCG–stimulated conditions, with gluconate causing a significantly higher yield of the second messenger in the presence of the gonadotropin. With 10 IU/L hCG, cAMP production increased dramatically, but cells incubated in the presence of nitrate had significantly ($P < .0005$) less intracellular cAMP.

Table 1. Post Hoc Pairwise Comparisons of Basal Androgen Secretion Between Different Concentrations of DPC

	DPC (mmol/L)			
DPC (mmol/L)	0	0.1	0.6	1
0.1	$P = .006$	—		
0.6	$P < .0005$	$P < .0005$	—	
1	$P = .001$	$P = .99$	$P < .0005$	—
2	$P < .0005$	$P < .0005$	$P < .0005$	$P < .0005$

DISCUSSION

MLTC-1, a mouse Leydig cell line, is reported to secrete progesterone and little testosterone in response to gonadotropins.[19] However, 40% of the steroid measured by testosterone RIA was indeed testosterone. Thin-layer chromatography also showed a substantial production of androstenedione. Considering its 0.027% cross-reaction with the testosterone antiserum, androstenedione must have contributed to the measured androgen result. Thus, the MLTC-1 cells used in these studies secreted C-19 androgens in response to hCG.

The minimum hCG concentration that stimulated substantial androgen synthesis had no effect on intracellular cAMP production by MLTC-1, which has previously been reported.[21] The hCG concentration causing the peak androgen response only yielded one fourth of the maximal cAMP response. Above certain hCG concentrations, submaximal androgen and cAMP responses occurred, probably as a result of desensitization.[22] Choi and Cooke[7] reported similar findings in rat Leydig cells with LH, and in the same study, 4-acetoamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), a chloride-channel blocker, inhibited testosterone production at low LH concentration but not at high concentration. However, SITS did not inhibit dibutryl-cAMP and forskolin-stimulated testosterone production. The investigators concluded that there were two pathways regulating steroidogenesis in rat Leydig cells: a pathway mediated through chloride channels at a low physiological concentration of LH (≤1 ng/mL) and a cAMP-dependent

Table 2. Post Hoc Pairwise Comparisons of 10-IU/L hCG–Stimulated cAMP Production Between Different Concentrations of DPC

	DPC (mmol/L)			
DPC (mmol/L)	0	0.1	0.6	1
0.1	$P = .99$	—		
0.6	$P < .0005$	$P < .0005$	—	
1	$P < .0005$	$P = .001$	$P = .004$	—
2	$P < .0005$	$P < .0005$	$P < .0005$	$P < .0005$

pathway stimulated by higher concentrations of gonadotropin. However, we found that the inhibition of cAMP-dependent protein kinase A by H89 blocked both the basal and 1-IU/L hCG–stimulated androgen production by MLTC-1 cells, when there was no discernible cAMP production. Our findings thus suggest that cAMP is involved in steroidogenesis even at low concentrations of gonadotropins, and at concentrations for which cAMP production becomes measurable, steroidogenesis is well under way. This also explains why a threefold increase of cAMP in the presence of isobutylmethyl xanthine had no additional stimulatory effect on androgen synthesis (data not shown). Similarly, Choi and Cooke[7] reported higher cAMP production with a higher LH concentration, but no increase in testosterone production.

Recently, various chloride conductances in response to LH in Leydig cells have been reported.[5,6] In a separate study, we have demonstrated the efflux of chloride in response to hCG from various cells with functional lutropin receptors, including MLTC-1 (C.W. Poon, N.S. Panesar, A. Hidaka, L.D. Kohn, unpublished data, 1997). Since chloride is necessary for dissociation of the α-subunit of the Gs protein complex,[8] the conductance/efflux of the ion in response to a gonadotropin may thus be involved in the activation of AC and cAMP production. A possible scenario is a momentary increase of chloride around Gs protein, leading to an increase in cAMP. DPC, another chloride-channel inhibitor, at concentrations up to 0.6 mmol/L, increased basal androgen production and 10-IU/L hCG–stimulated cAMP production. These results suggest that a partial block of chloride conductance resulting in higher intracellular chloride may facilitate increased α-subunit dissociation and consequently increased AC activity and cAMP production. Paradoxically, DPC also inhibited hCG-stimulated steroidogenesis. This suggests that besides potentiating the cAMP response, chloride conductance may also regulate steroidogenesis through a cAMP-independent pathway, which is not involved in basal steroidogenesis. The decrease in the extracellular androgen concentration with DPC was also noted for the intracellular androgen (data not shown). This further suggests that chloride conductance is involved in steroidogenesis.

The enhancement of gonadotropin response following depletion of extracellular chloride by gluconate[7] also occurred with basal and 1-IU/L hCG–stimulated androgen production in this study. Since gluconate is a membrane-impermeable anion,[23] the enhancement may have been due to increased cAMP production in response to chloride efflux. The substitution of monovalent chloride by divalent sulfate and particularly nitrate inhibited

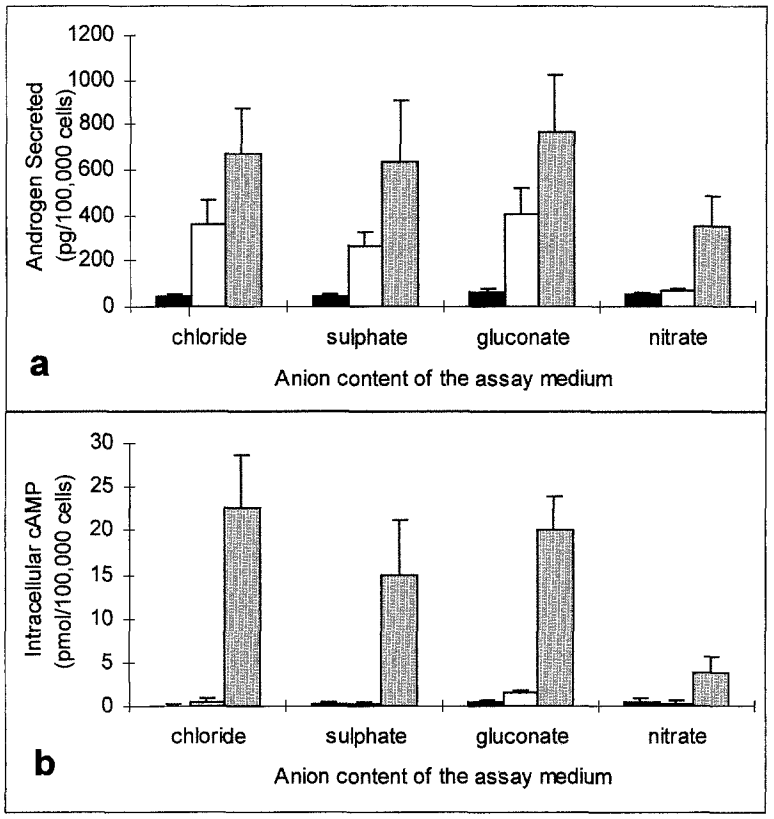

Fig 5. Androgen secretion (a) and intracellular cAMP level (b) in basal (■) and 1-IU/L hCG (□)- and 10-IU/L hCG (▨)-stimulated MLTC-1 cells in the presence of different anions (mean ± SD, n = 15).

androgen and cAMP production. Unlike gluconate, nitrate is membrane-permeable, which argues against the valency factor as the cause of inhibition. Some chloride channels are permeable to nitrate,[24,25] and therefore, nitrate may have abolished the effect of chloride on cAMP production.

Alternatively, the inorganic nitrate may have acted through its conversion to NO, with activation of the GC-cGMP pathway resulting in decreased intracellular calcium[12,13,18] and the latter inhibiting steroidogenesis.[3] Welch et al[9] found decreased cGMP production with increased testosterone yield in response to NO synthase inhibitors in a rat Leydig cell preparation; however, dibutryl-cGMP did not stimulate testosterone synthesis. In contrast, Punta et al[10] could not demonstrate increased cGMP production in response to NO donors. Our studies to elucidate the involvement of GC-cGMP in steroidogenesis also produced conflicting results. HCG did not stimulate cGMP production and there was not a steroidogenic response to exogenous cGMP, but both GC inhibitors, MB and LY83583, inhibited androgen synthesis. Therefore, the inhibitory action of inorganic nitrate may not have involved the NO-activated GC-cGMP pathway. The more likely mechanism is the conversion of the ion to the gaseous inhibitor. NO blocks specific cytochrome P-450 enzymes,[10] and NO formed during the metabolism of 18-nitrooxyandrostenedione by a cytochrome P-450 inhibited aldosterone biosynthesis.[26] It remains to be shown if inorganic nitrate is indeed converted to NO in Leydig cells. Moreover, since cGMP activates ion channels,[18] the chloride conductance associated with gonadotropins may involve the GC-cGMP pathway. This possibility also needs further study.

Nitrate was used to deplete intracellular chloride based on a buffer recipe of a research collaborator.[27] However, there may be other studies in which nitrates may have been used without realizing the probable inhibitory effect(s) of the anion on cellular mechanisms: for instance, whether patients receiving nitrates for angina pectoris show changes in steroid hormones before and after treatment. This is also relevant for sildenafil (Viagra; Pfizer), which also involves an interplay between NO and cGMP for its mode of action.[28] Returning to chloride, a clinical implication of chloride conductance in steroidogenesis may concern cystic fibrosis (CF). Besides the pulmonary and pancreatic complications in CF, the disease is also characterized by a short stature and delayed puberty, supposedly the result of a malnutrition-induced delay in maturation of the hypothalamic-pituitary-gonadal axis.[29,30] However, there is a delayed increase in testosterone secretion[31] and a subnormal response to gonadotropin-releasing hormone,[32] implying that the gonadal steroidogenesis is compromised. The electrolyte disturbances in CF occur because of decreased chloride permeability across the cell membrane.[33] The defect lies in the CF transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel[34] that is inhibited by DPC.[35,36] We cannot say if the chloride channel inhibited by DPC in MLTC-1 cells is CFTR, but the present findings lend support to the contention that the short stature and delayed puberty in CF may ensue from impaired steroidogenesis due to defective chloride conductance.[37] Similarly, impaired iodide transport into thyrocytes may be a cause of subclinical hypothyroidism in CF.[38]

In conclusion, we propose that chloride conductance, AC-cAMP, and GC-cGMP are all involved in gonadotropin-stimulated steroidogenesis in Leydig cells. A probable mechanism is as follows: the interaction of LH/hCG with lutropin receptor stimulates chloride efflux (perhaps via the GC-cGMP pathway), a momentary increase of chloride around Gs protein, dissociation of the α-subunit, activation of AC, cAMP production, and finally steroidogenesis.

ACKNOWLEDGMENT

I am indebted to Dr C.W. Poon, K.W. Chan, and Winnie Wong for technical assistance, and to Peggo Lam (Center for Clinical Trials and Epidemiological Research) for help with the statistics. I am also grateful to Professor Patrick Wong (Department of Physiology) for DPC, Dr Helen Wise (Department of Pharmacology) for H89, and Dr Vincent Yeung (Department of Medicine) for LY83583.

REFERENCES

1. Ferndale RW, Allan LM, Martin BR: Adenylate cyclase and cAMP, in Milligan G (ed): Signal Transduction: A Practical Approach. New York, NY, IRL, 1992, pp 75-102

2. Janszen FHA, Cooke BA, Van Driel MJA, et al: The effects of calcium ions on testosterone production in Leydig cells from rat testis. Biochem J 160:433-437, 1976

3. Sullivan MHF, Cooke BA: The role Ca^{2+} in steroidogenesis in Leydig cells. Stimulation of intracellular free Ca^{2+} by lutropin (LH), luliberin (LHRH) agonist and cyclic AMP. Biochem J 236:45-51, 1986

4. Tomic M, Dufau ML, Catt KJ, et al: Calcium signaling in single rat Leydig cells. Endocrinology 136:3422-3429, 1995

5. Duchatelle P, Joffre M: Potassium and chloride conductances in rat Leydig cells: Effects of gonadotropins and cyclic adenosine monophosphate. J Physiol (Lond) 428:15-37, 1990

6. Noulin JF, Joffre M: Characterization and cyclic AMP–dependence of a hyperpolarization-activated chloride conductance in Leydig cells from mature rat testis. J Membr Biol 133:1-15, 1993

7. Choi MS, Cooke BA: Evidence for two independent pathways in the stimulation of steroidogenesis by luteinizing hormone involving chloride channels and cyclic AMP. FEBS Lett 261:402-404, 1990

8. Toyoshige M, Basi NS, Rebois RV: Chloride effects on Gs subunit dissociation. Fluoroaluminate binding to Gs does not cause subunit dissociation in the absence of chloride ion. J Biol Chem 271:8791-8795, 1996

9. Welch C, Watson ME, Poth M, et al: Evidence to suggest nitric oxide is an interstitial regulator of Leydig cell steroidogenesis. Metabolism 44:234-238, 1995

10. Punta KD, Charreau ED, Pignataro OP: Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137:5337-5343, 1996

11. Van Voorhis BJ, Dunn MS, Snyder GD, et al: Nitric oxide: An autocrine regulator of human granulosa-luteal cell steroidogenesis. Endocrinology 135:1799-1806, 1994

12. Parker JO: Nitrates and angina pectoris. Am J Cardiol 72:3C-8C, 1993

13. Kukovetz WR, Holzmann S, Romanin C: Mechanism of vasodilation by nitrates: Role of cyclic GMP. Cardiology 1:12-19, 1987

14. Perchellet JP, Sharma RK: Mediatory role of calcium and guanosine 3′,5′-monophosphate in adrenocorticotropin-induced steroidogenesis by adrenal cells. Science 203:1259-1261, 1979

15. Perchellet JP, Sharma RK: Metabolic regulation of steroidogenesis in isolated adrenocortical carcinoma cells. ACTH regulation of

guanosine cyclic 3′:5′-monophosphate levels. Biochem Biophys Res Commun 78:676-683, 1977

16. Leitman DC, Murad F: Atrial natriuretic factor receptor heterogeneity and stimulation of particulate guanylate cyclase and cyclic GMP accumulation. Endocrinol Metab Clin North Am 16:79-105, 1987

17. Mukhopadhyay AK, Helbing J, Leidenberger FA: The role of Ca^{2+} and calmodulin in the regulation of atrial natriuretic peptide–stimulated guanosine 3′,5′-cyclic monophosphate accumulation by isolated mouse Leydig cells. Endocrinology 125:686-692, 1989

18. Lincoln TM, Cornwell TL: Intracellular cyclic GMP receptor proteins. FASEB J 7:328-338, 1993

19. Rebois RV: Establishment of gonadotropin-responsive murine Leydig tumor cell line. J Cell Biol 94:70-76, 1982

20. Ooi LSM, Panesar NS, Masarei JRL: Urinary excretion of testosterone and estradiol in Chinese men and relationships with serum lipoprotein concentrations. Metabolism 45:279-284, 1996

21. Hsueh AJ, Dufau ML, Catt KJ: Gonadotropin-induced regulation of luteinizing hormone receptors and desensitization of testicular 3′:5′-cyclic AMP and testosterone responses. Proc Natl Acad Sci USA 74:592-595, 1977

22. Cooke BA, Choi MC, Dirami G, et al: Control of steroidogenesis in Leydig cells. J Steroid Biochem Mol Biol 43:445-449, 1992

23. Ramnath HI, Peterson S, Michael AE, et al: Modulation of steroidogenesis by chloride ions in MA-10 mouse tumor Leydig cells: Roles of calcium, protein synthesis, and the steroidogenic acute regulatory protein. Endocrinology 138:2308-2314, 1997

24. Dinudom A, Komwatana P, Young JA, et al: A forskolin-activated Cl^- current in mouse mandibular duct cells. Am J Physiol 268:G806-G812, 1995

25. Guinamard A, Chraibi A, Teulon J: A small conductance Cl^- channel in the mouse ascending limb that is activated by ATP and protein kinase A. J Physiol (Lond) 485:97-112, 1995

26. Delaforge M, Piffeteau A, Boucher JL, et al: Nitric oxide formation during the cytochrome P-450–dependent reductive metabolism of 18-nitro-oxyandrostenedione. J Pharmacol Exp Ther 274:634-640, 1995

27. Huang SJ, Chan HC, Wong PY: Adrenaline-regulated Cl^- transport in cultured single rat epididymal cells measured by an entrapped $Cl^-(-)$ sensitive fluorophore. J Physiol (Lond) 474:183-191, 1994

28. Boolell M, Allen MJ, Ballard SA, et al: Sildenafil: An orally active type 5 cyclic GMP–specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. Int J Impot Res 8:47-52, 1996

29. Landon C, Rosenfeld RG: Short stature and pubertal delay in male adolescents with cystic fibrosis. Androgen treatment. Am J Dis Child 138:388-391, 1984

30. Landon C, Rosenfeld RG: Short stature and pubertal delay in cystic fibrosis. Pediatrician 14:253-260, 1987

31. Boas SR, Cleary DA, Lee PA, et al: Salivary testosterone levels in male adolescents with cystic fibrosis. Pediatrics 97:361-363, 1996

32. Reiter EO, Stern RC, Root AW: The reproductive endocrine system in cystic fibrosis: II. Changes in gonadotrophins and sex steroids following LHRH. Clin Endocrinol (Oxf) 16:127-137, 1982

33. Quinton PM, Bijman J: Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with cystic fibrosis. N Engl J Med 308:1185-1189, 1983

34. Riordan JR: The cystic fibrosis transmembrane conductance regulator. Annu Rev Physiol 55:609-630, 1993

35. Schwiebert EM, Flotte T, Cutting GR, et al: Both CFTR and outwardly rectifying chloride channels contribute to cAMP-stimulated whole cell chloride currents. Am J Physiol 266:C1464-C1477, 1994

36. Vandorpe D, Kizer N, Ciampollilo F, et al: CFTR mediates electrogenic chloride secretion in mouse inner medullary collecting duct (mIMCD-K2) cells. Am J Physiol 269:C683-C689, 1995

37. Panesar NS: Could growth retardation in cystic fibrosis be partly due to deficient steroid and thyroid hormonogenesis? Med Hypotheses (in press)

38. Devuyst O, Goldtein PE, Sanches MV, et al: Expression of CFTR in human and bovine thyroid epithelium. Am J Physiol 272:C1299-C1308, 1997