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PRELIMINARY REPORT

Low Temperature Blocks the Stimulatory Effect of Human Chorionic Gonadotropin on Steroidogenic Acute Regulatory Protein mRNA and Testosterone Production But Not Cyclic Adenosine Monophosphate in Mouse Leydig Tumor Cells

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Low temperatures slow down metabolism, partly because the kinetic energy of molecules is reduced and enzymes may be structurally impaired. We now report that relative to its maximal activity at 37°C, adenylate cyclase (AC) still retained 25% functionality (determined as cyclic adenosine monophosphate [cAMP] production) at 4°C in mouse Leydig tumor cells (MLTC-1) in response to 50 IU/L human chorionic gonadotropin (hCG), whereas steroidogenic acute regulatory (StAR) protein mRNA and testosterone production were completely impaired. The incubation of MLTC-1 with the phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine; IBMX) resulted in significantly increased intracellular cAMP concentration at all 3 temperatures, but this had no impact on testosterone production. AC, cAMP, and phosphodiesterase form an important intracellular second-messenger mechanism in many organisms, some that inhabit very low temperature niches. The cold-resistance of AC and phosphodiesterase may thus have evolved to cope with adverse conditions. Although hibernation may lead to decreased steroid hormone production, it is also likely that cold-mediated decreased steroid hormone production induces hibernation.

ENZYMES, natural catalysts, allow biochemical reactions to occur with less expenditure of energy by lowering the energy of activation required by substrate molecules to enter transition states.[1] The latter can also be achieved by raising system temperature, which increases the kinetic energy of molecules, leading to greater collision between them. For an enzyme and its substrate, a 10°C rise in temperature roughly doubles an enzyme's rate of reaction ($Q_{10} \simeq 2$).[1] Enzymes function more efficiently at higher temperatures, but being proteins undergo denaturation at temperatures above 56°C.[2] However, enzymes in thermophilic bacteria can tolerate higher temperatures.[3]

On the other hand, low temperatures reduce the kinetic energy of molecules, making it more difficult for them to enter transition states. Low temperatures also may change the shapes of enzymes, and for the cold-sensitive, cause loss of activity. However, enzymes that resist cold may be important in the survival of organisms, particularly those exposed to periodic cold temperatures, the hibernators.[4] The recrudescence from a winter slumber in hibernating animals may depend on these enzymes to "kick-start" other cellular mechanisms.[5]

We have studied the in vitro effects of different temperature on cyclic adenosine monophosphate (cAMP; without or with 3-isobutyl-1-methylxanthine [IBMX]), steroid hormones, and steroidogenic acute regulatory (StAR) protein mRNA production by a cultured mouse Leydig tumor cells (MLTC-1) and freshly isolated Syrian hamster Leydig cells, from a nonhibernator and a hibernator rodent, respectively, in response to human chorionic gonadotropin (hCG). We report that adenylate cyclase (AC) activity determined as cAMP production in MLTC-1 persists partially at 4°C, whereas testosterone and StAR mRNA productions were completely blocked.

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MATERIALS AND METHODS

The materials and methods used for growing MLTC-1 and studying testosterone and cAMP production, along with reverse-transcriptase polymerase chain reaction (RT-PCR) for StAR and L19 (housekeeping gene) mRNA have all been described.[6] In the present study, 24-well plates containing MLTC-1 cells either were directly incubated at 4°C, 22°C, and 37°C with 0 to 50 IU/L hCG for 1 hour in 200 μL of buffer or were preincubated at the aforementioned temperatures for 1 hour, followed by stimulation with hCG at 37°C for 1 hour. The latter experiment was done to rule out detrimental effects of cold temperature on cells. The effect of the phosphodiesterase inhibitor on cAMP and testosterone was determined by preincubating cells without or with 0.5 mmol/L IBMX for 1 hour at 37°C, followed by hCG (0 to 50 IU/L) stimulation in the absence or presence of the inhibitor at 4°C, 22°C, and 37°C for 1 hour. Similarly the effect of exogenous cAMP (0 to 1mg/mL) on testosterone production was also studied. An attempt was also made to study the in vitro effect of cold temperature on Leydig cells of Syrian hamsters (*Microcricetus auratus*), bred in our Animal Laboratory facility, on a 12-hour light:12-hour dark cycle. The Leydig cells were isolated similar to a procedure used for Balb/c mice.[6]

Data were analyzed by means of analysis of variance (ANOVA) and Student's *t* test using the Statistica software (Statsoft, Tulsa, OK). *P* values less than .05 were regarded as significant.

RESULTS

First, the incubation of MLTC-1 cells at lower temperatures had no detrimental effects on cells. The cells preincubated at 4°C, 22°C, and 37°C for 1 hour and then stimulated with hCG at 37°C had similar testosterone and cAMP yields (data not shown). Figure 1A and B show the production of testosterone and cAMP in the absence and presence of 0.5 mmol/L IBMX. At 37°C, whereas testosterone production was already in the maximal range in response to the lowest concentration of hCG, ie, 12.5 IU/L, cAMP increased gradually, but had still not reached a plateau at 50 IU/L hCG. Relative to the maximal production stimulated with 50 IU/L hCG at 37°C, testosterone and cAMP yields decreased to 26% and 4%, and 58% and 25% at 22°C and 4°C, respectively. Inclusion of IBMX caused a significant ($P < .000000$, ANOVA) increase in intracellular cAMP concentration in response to hCG at all 3 temperatures (Fig 1B), but this had no significant ($P > .05$, ANOVA) effect on testosterone production (Fig 1A). At 37°C, the highest concentration of exogenous cAMP stimulated testosterone production, achieving levels similar to the maximal production stimulated by hCG. However, the testosterone yield was only 17% and 0% at 22°C and 4°C, respectively, relative to the maximal response achieved in response to the highest concentration of exogenous cAMP at 37°C (Fig 1C). Relative to L19 mRNA, StAR mRNA production increased significantly in response to 1-hour stimulation with 100 IU/L hCG compared to basal production at 37°C, but at 4°C and 22°C, its production either remained unchanged or was decreased (Fig 2). There was no change in testosterone, progesterone or cAMP production in Syrian hamster Leydig cells in response to 0 to 100 IU/L hCG (data not shown).

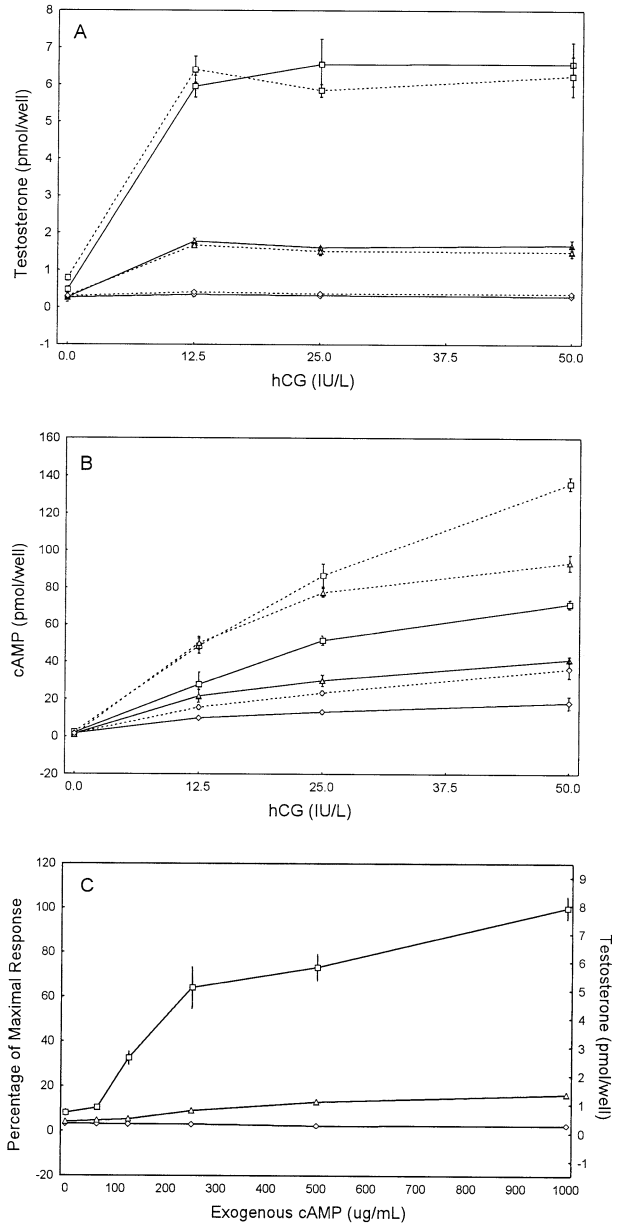

Fig 1. (A) Testosterone and (B) cAMP production by MLTC-1 following preincubation without (····) or with (—) 0.5 mmol/L IBMX for 1 hour at 37°C, followed by stimulation with 0 to 50 IU/L hCG for 1 hour at 4°C (◇), 22°C (△), and 37°C (□). (C) Testosterone production by MLTC-1 in response to 0 to 1,000 μg/mL exogenous cAMP for 1 hour at 4°C (◇), 22°C (△), and 37°C (□) plotted as a percentage of maximal response (ie, stimulation with 1,000 μg/mL cAMP at 37°C). Right Y-axes represent the absolute quantity of testosterone produced per well. Results are means ± 1 SD (n = 4).

DISCUSSION

Steroidogenesis is a complex process and steps leading to it may be represented as follows: the binding of a pituitary tropic hormone to its specific receptor coupled to a G stimulatory heterotrimeric protein complex (Gs); the activation of Gs in presence of halide ions,[7] whereby guanosine triphosphate (GTP) displaces guanosine diphosphate (GDP) on the α-subunit of the heterotrimer, with a resultant dissociation of the activated α-subunit from $\beta\gamma$-subunits; the activation of AC by α-subunit and the conversion of adenosine triphosphate (ATP) to cAMP (the α-subunit action is terminated by the conversion

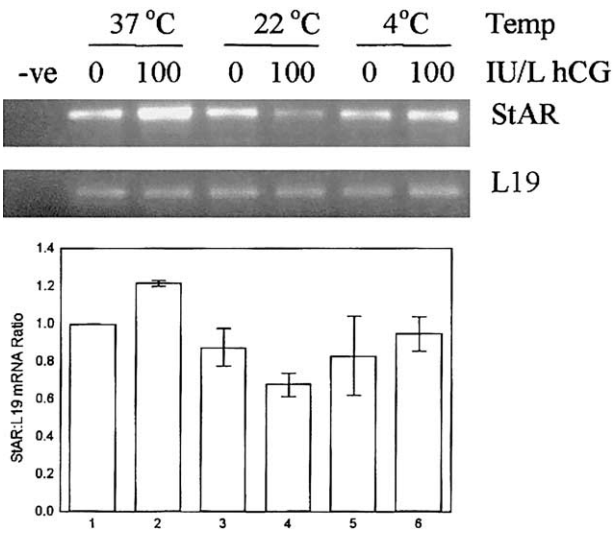

Fig 2. The StAR (980 bp) and L19 (395 bp) mRNA RT-PCR products viewed under UV-transillumination following the incubation of MLTC-1 in the absence or presence of 100 IU/L hCG at 37°C, 22°C, and 4°C for 1 hour. Bars (1-6) below the autoradiograms represent the StAR:L19 mRNA ratio for each corresponding experimental condition. StAR mRNA production was significantly ($P < .02$ or lower by Student's *t* test, n = 2) different between the following pairs: 1 *v* 2; 1 *v* 4; 2 *v* 3; and 2 *v* 4.

of GTP to GDP by GTPase, and the reformation of the heterotrimer); the activation of protein kinases by cAMP leading to the de novo synthesis of StAR protein, as well as the hydrolysis of cholesterol esters to free cholesterol (FC); and finally the StAR-mediated transfer of FC to the inner mitochondrial membrane where cytochrome P450 side chain cleaving (450scc) enzyme performs the first reaction and starts steroidogenesis.[8]

From a thermodynamic standpoint, all processes should have been impaired by the low temperature, but this study has clearly shown that processes leading up to the activation of AC and cAMP production are more cold resistant. Even at 22°C, when testosterone production decreased by 75%, cAMP yield was down by only 42%. These findings are interesting considering that cAMP production normally lags behind testosterone in response to gonadotropins,[9] but even at very low concentrations of the ligand when cAMP cannot be detected, the second messenger is indispensable for steroidogenesis.[10] Although cAMP-dependent protein kinase could be activated by DL-isoproterenol in mouse thymocytes at 4°C,[11] the processes downstream of protein kinase activation were not studied. Low temperature also changes the Hill coefficient for cAMP dissociation of the ground squirrel protein kinase A holoenzyme, making the release of catalytic subunits at low temperature more responsive to small changes in cAMP levels.[4] However, neither the persistent cAMP production in response to hCG at 4°C, which was further increased by the inclusion of IBMX in the incubation mixture, nor the addition of exogenous cAMP, stimulated processes downstream of protein kinase action, ie, StAR mRNA and subsequently testosterone production at low temperature in the present study. The fact that cells incubated under steady-state conditions without IBMX had significantly lower intracellular cAMP compared to those with the inhibitor suggests that phosphodiesterase activity is also viable at 4°C. Since phosphodiesterase is part of the second-messenger signaling mechanism, terminating the message by inactivating cAMP, its persistence at low temperature is thus not surprising. AC, cAMP, and phosphodiesterase form an important intracellular second-messenger signaling mechanism in many prokaryotic and eukaryotic organisms,[12] some that inhabit niches where temperatures are very low and water pressure very high.[13] Their ability to resist cold must have evolved because of these adverse conditions.

Animals hibernate to escape harsh conditions imposed by the cold weather, when the availability and foraging for food becomes difficult. Although photoperiodism[14] coupled to melatonin release from the pineal gland[15] is implicated in the induction of hibernation, the process can be inhibited in Turkish hamsters by the administration of physiological amounts of testosterone.[16] The latter suggests that cold induced decreased steroidogenesis may be a factor in hibernation. Exposure of toads (*Bufo melanostictus*) to cold for 2 and 3 weeks reduced testicular 17β-hydroxydehydrogenase activity and circulating testosterone, respectively.[17] Our attempts to study the in vitro effect of hCG on cAMP and steroid hormone production by Syrian hamster Leydig cells at different temperatures produced no response. Hamster Leydig cells are apparently less sensitive to human and ovine gonadotropins.[18,19] However, we have previously shown that the pattern of cAMP and testosterone production in MLTC-1 cells and freshly isolated Balb/c mouse Leydig cells were similar.[6] Since the mechanism of cAMP and steroid production is similar in different organisms, we believe the results of this study can be applied to other mammals that also hibernate, eg, *Jaculus orientalis*, *Marmota flaviventris*, *Eptesicus fuscus*, and *Spermophilus lateralis*. The decreased testosterone production under cold environment may thus be a factor in the induction of hibernation.

We conclude that AC and phosphodiesterase in mouse Leydig cells are cold-resistant enzymes. Since AC-cAMP, along with phosphodiesterase, constitutes an important intracellular signaling mechanism in many organisms, some that inhabit very low temperature niches, the ability to resist cold may have evolved to allow an organism's survival in the inhospitable environment.

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