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A cAMP INDEPENDENT INHIBITORY ACTION OF HIGH DOSES OF FORSKOLIN IN RAT LEYDIG CELLS

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Summary—In addition to well known direct stimulatory and potentiatory actions of forskolin, we have previously reported that low doses of this diterpene (10^{-9}, 10^{-12} M) markedly inhibit the production of cAMP and testosterone in rat Leydig cells through a pertussis toxin sensitive G-protein (A. Khanum and M. L. Dufau, *J. Biol. Chem.* **261**, 1986). A different type of inhibitory effect of forskolin is described in this study. Forskolin (10^{-5} M) markedly stimulates basal adenylate cyclase activity (about 200%) in rat Leydig cell membranes and potentiates the stimulatory effect of gonadotropin (10^{-9}, 10^{-7} M) on adenylate cyclase in presence or in absence of GTP (10^{-5} M). Similarly a time-dependent stimulation of forskolin (10^{-5} M) alone is noted on all cAMP pools and testosterone production. Using a supramaximal steroidogenic dose of hCG (0.26 nM) or choleragen (0.1 μM), forskolin potentiates the gonadotrophin and toxin-induced responses of all cAMP pools significantly while inhibiting testosterone production. Moreover, forskolin also inhibits 8-Bromo-cAMP stimulated steroidogenesis. In contrast, pregnenolone synthesis was not altered by the diterpene. We have demonstrated in this study that the inhibitory effect of high doses of forskolin on steroidogenesis is distal to cAMP generation, and resulted from a steroidogenic block residing beyond pregnenolone synthesis.

INTRODUCTION

Forskolin, a plant diterpene, is widely known as potent activator of adenylate cyclase in many systems including endocrine tissues [1–8]. Unlike cholera toxin or fluoride, forskolin appears to act directly and reversibly on catalytic subunit of adenylate cyclase [1, 9]. In addition, it has been shown to facilitate modulation of enzyme activity by variety of both stimulatory and inhibitory hormones, exerting its effect on receptor-mediated events via guanine nucleotide regulatory proteins: G_s and G_i [10–15]. Therefore, submaximal doses of forskolin enhance the potency and/or efficacy of the hormonal activation of adenylate cyclase and consequently increase cAMP production in number of tissues [2, 10,11]. On the other hand, forskolin induced increases in adenylate cyclase and cAMP can be attenuated by a number of inhibitory hormones [11–14]. Previous studies from our laboratory have demonstrated a novel, high affinity inhibitory action of low doses of forskolin upon adenylate cyclase and cAMP generation with involvement of G_i unit in the mediation of inhibitory action of forskolin on the basis of pertussis toxin studies [2]. Our present studies have provided evidence of yet another effect of forskolin in Leydig cells by the demonstration of an inhibitory action of high doses of this diterpene on hCG stimulated steroidogenesis while potentiating hCG-stimulated adenylate cyclase activity and cAMP production. Further this inhibitory effect was cAMP independent and found to be located beyond pregnenolone synthesis.

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MATERIALS AND METHODS

Adult male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) were killed and testes were removed and placed in ice-cold PBS, pH 7.4. Interstitial cells were obtained by collagenase digestion of decapsulated testes, as previously described [16]. Crude cell suspension was washed and then pelleted at 200 *g* for 10 min. The cell pellet was resuspended with elutriation buffer consisting of regular medium 199 with Hank's salts and L-glutamine containing 1.4 g/l $NaHCO_3$, 0.5% BSA, 1 mM EDTA, 50 U/ml heparin, 12.5 μg/ml DNAse and 50 μg/ml gentamicin,

pH 7.4. The purified cells were obtained by centrifugal elutriation [17]. Cells were centrifuged and resuspended in Medium 199 (Whittaker M. A. Bioproduct, Walkerville, Md) containing 0.1% bovine serum albumin and 0.125 mM 3-methylisobutyl xanthine (Aldrich Chemical Co., Milwaukee, Wisc.). The cells (1×10^6 cells/ml) incubated in 12×75 mm plastic tubes at 34°C with shaking at 100 cycle/min under atmosphere of $O_2:CO_2$ (95:5, v/v) in the presence and in the absence of various concentrations of hCG (hCG CR-121, preparation kindly provided by the Center for Population Research, National Institute of Child Health and Human Development, Bethesda, Md), choleragen (Schwarz-Mann, Orangeburg, N.J.) with or without forskolin and/or 8-Bromo-cAMP. The incubations were terminated by transferring the incubation tubes to an ice bath and all further steps were carried out at 0°C. The cells were sedimented at 250 *g* for 12 min, and the supernatants were saved for assay of extracellar cAMP, testosterone and pregnenolone. The cell pellets were washed with ice cold medium and processed for the analysis of intracellular and receptor-bound cAMP; and measurements of cAMP, testosterone and pregnenolone were performed by radioimmunoassays as previously described [18, 19].

Purified Leydig cell plasma membranes were prepared by the method described previously [20], and stored in liquid nitrogen until used. Adenylate cyclase assays were carried out with modifications previously described for the Leydig cell system [20]. The assay was performed in the presence or in the absence of 10^{-5} M GTP with gonadotropin (ovine LH was obtained through the Hormone Distribution Program, National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program, University of Maryland, School of Medicine, Md) and/or forskolin in a final volume of 100 μl. Reactions were initiated by the addition of 20 μg of membrane protein previously incubated at 30°C for 10 min in assay buffer without additions [20].

RESULTS

Studies were carried out to examine the time-dependent effect of 10 μM forskolin on cAMP pools and corresponding testosterone production in Leydig cells under basal conditions and during hCG-stimulation. Forskolin significantly increased intracellular and receptor-bound cAMP (Fig. 1). The action of forskolin was rapid with a marked increase at 5 min ($P < 0.005$ and $P < 0.001$ respectively) and reached to maximum level within 30 min ($P < 0.001$). Forskolin potentiated hCG-stimulated cAMP in all the compartments when cells were incubated in presence of hCG. Comparable findings were observed when adenylate cyclase activity was measured in purified Leydig cell membranes (Fig. 2). Forskolin stimulated basal adenylate cyclase activity about 200% and potentiated the stimulatory effect of gonadotropin in the presence and in the absence of GTP (10^{-5} M). In contrast to its stimulatory effects on basal and hCG-stimulated adenylate cyclase activity and cAMP production,

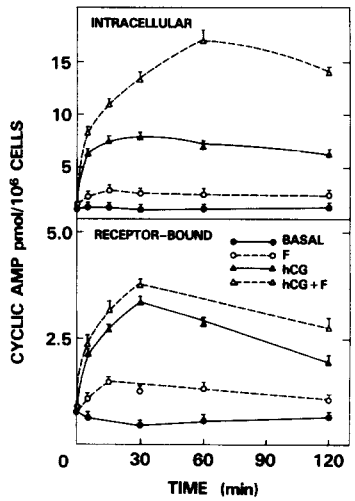

Fig. 1. Effect of forskolin on hCG-stimulated cAMP production. Leydig cells (10^6 cells/ml) were incubated with hCG (0.26 nM) in the presence and in the absence of forskolin [F] (10 μM) for 0–120 min. Each point represents the mean ± SE of triplicate incubations in this and figures below.

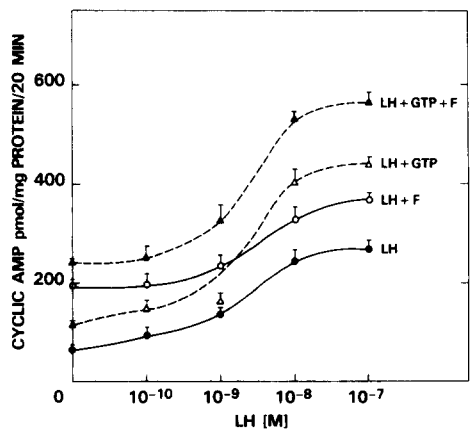

Fig. 2. Effect of forskolin on hCG-stimulated adenylate cyclase activity. The Leydig cells membranes were incubated with GTP (10^{-5} M) and LH (10^{-10}–10^{-7} M) in the presence and in the absence of forskolin (10 μM) for 20 min.

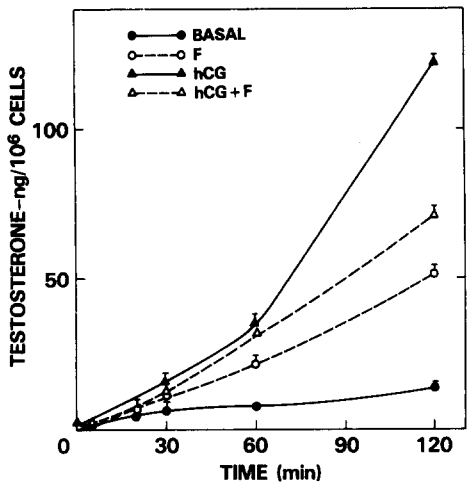

Fig. 3. Effect of forskolin on hCG-stimulated testosterone production. Leydig cells (10^6 cell/ml) were incubated with hCG (0.26 nM) in the presence and in the absence of forskolin (10 μM) for 0–120 min.

forskolin showed an inhibitory effect on hCG-stimulated testosterone production (Fig. 3). Significant inhibition ($P < 0.025$) was observed as early as 30 min of incubation and was more pronounced at 120 min ($P < 0.001$). Figure 4 shows that cells incubated with hCG alone caused maximal testosterone production as a result of increase in receptor-bound cAMP. On the other hand, incubation with forskolin alone

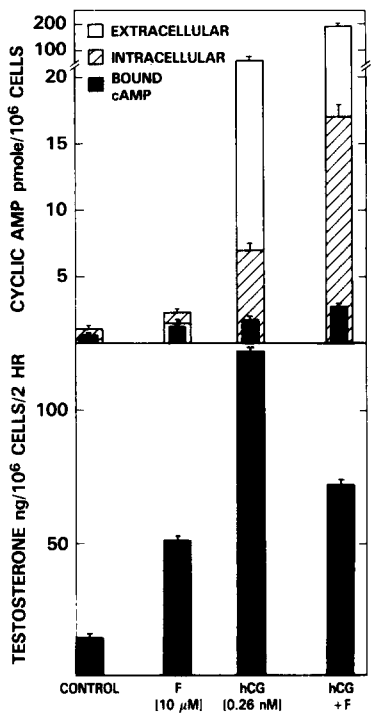

Fig. 4. Effect of forskolin on hCG-stimulated cAMP and testosterone production. Leydig cells (10^6 cells/ml) were incubated for 60 min in case of cAMP and for 120 min in case of testosterone with hCG (0.26 nM) in the presence and in the absence of forskolin (10 μM).

(10 μM) elicited lower levels of testosterone (42% of hCG alone), results which were consistent with the levels of receptor-bound cAMP (56% of hCG alone). When cells were incubated in the presence of both stimuli, hCG and forskolin, the expected stimulatory effect on testosterone production was not observed, though there was an additive increase in receptor-bound cAMP. Instead, a significant decrease in androgen formation by 41% was noted. Additional studies were carried out to determine the effect of forskolin on hCG-stimulated testosterone production at two dose levels 1 and 10 μM (Fig. 5). Forskolin 1 μM also has a significant inhibitory effect ($P < 0.01$) on testosterone production stimulated with a supramaximal dose of hCG (0.26 nM). This inhibitory effect was more pronounced when hCG stimulated cells were incubated with 10 μM forskolin ($P < 0.001$). In further studies, we investigated the effect of forskolin on choleragen-stimulated cAMP and testosterone production. As noticed in previous studies [21] there was a marked increase in receptor-bound cAMP ($P < 0.001$) by a low dose of choleragen (20 pM), while minor but significant increase in testosterone production was observed (Fig. 6). Addition of forskolin after 60 min preincubation itself showed 18-fold increase in testosterone production with further significant increase in receptor-bound cAMP ($P < 0.001$, forskolin vs choleragen). Thus, incubation of cells prior to addition of 10 μM forskolin enhanced significantly cAMP and testosterone responses to the diterpene. Cells incubated in presence of both stimuli (choleragen and forskolin), showed stimulatory effect on receptor-bound cAMP ($P < 0.025$, forskolin vs forskolin plus choleragen) without any significant effect on testosterone production. In other

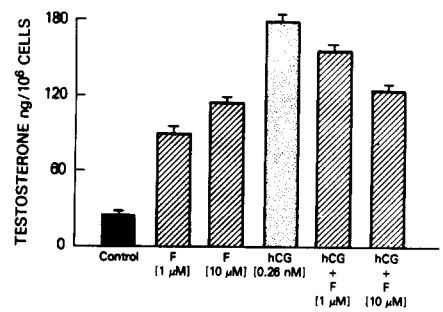

Fig. 5. Effect of forskolin (1 and 10 μM) on hCG-stimulated testosterone production. Leydig cells (10^6 cells/ml) were incubated for 120 min with the supramaximal hCG concentration (0.26 nM) in the presence and in the absence of 1 and 10 μM of forskolin.

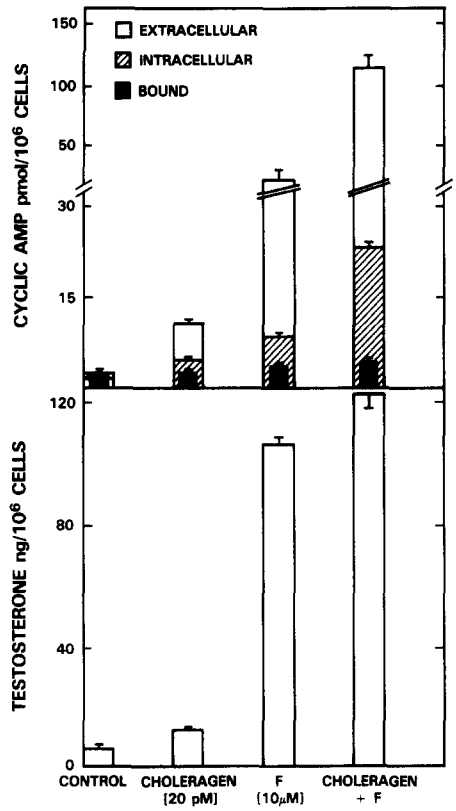

Fig. 6. Effect of forskolin on choleragen-stimulated cAMP and testosterone production. Leydig cells (10^6 cells/ml) were incubated for 60 min in presence or in absence of choleragen (20 pM) followed by 120 min in the presence or in the absence of forskolin (10 μM).

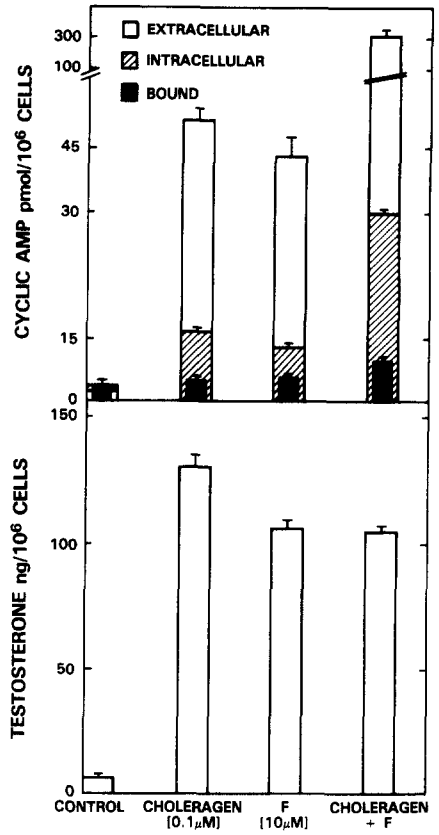

Fig. 7. Effect of forskolin on choleragen-stimulated cAMP and testosterone production. Leydig cells (10^6 cells/ml) were incubated for 60 min in presence or in absence of choleragen (0.1 μM) followed by 120 min in the presence or in the absence of forskolin (10 μM).

studies, using a supramaximal steroidogenic dose of choleragen (0.1 μM) it was observed that despite of significant stimulation of receptor-bound cAMP by incubation of Leydig cells with forskolin and toxin ($P < 0.005$, forskolin vs forskolin plus choleragen), there was significant decrease in testosterone production ($P < 0.005$, choleragen vs choleragen plus forskolin) (Fig. 1). The effects of forskolin on choleragen stimulated steroidogenesis resembled those noted for hCG (Fig. 4). In both instances, increase in receptor-bound cAMP were not capable to sustain testosterone production. Subsequently, when the cells were exposed to 8-Bromo-cAMP in presence of forskolin and hCG, the inhibitory effect of forskolin was not reversed (Fig. 8). The inhibitory action of high doses of forskolin on hCG and choleragen stimulated testosterone production is distal to cAMP generation and could not be restored to normal by addition of exogenous cAMP. In subsequent studies, we observed that forskolin did not affect the early biosynthetic pathway since comparable stimulation by hCG on pregnenolone production by Leydig cells in presence or absence of the diterpene was observed (Fig. 9). These studies, therefore, led us to conclude that the inhibitory effect of forskolin on testosterone production resides beyond pregnenolone synthesis and is not due to inavailability of cAMP formation.

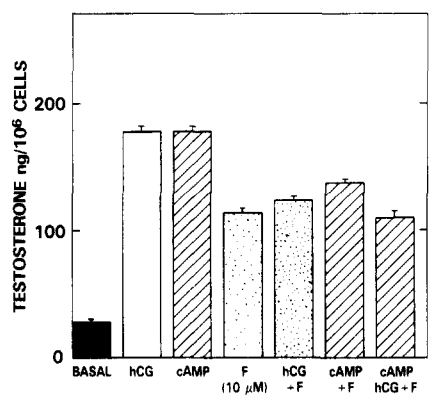

Fig. 8. Effect of 8-Bromo-cAMP on forskolin-induced inhibition on testosterone production. Leydig cells (10^6 cells/ml) were incubated for 120 min with 8-Bromo-cAMP (1 mM) in the presence and in the absence of hCG and/or forskolin.

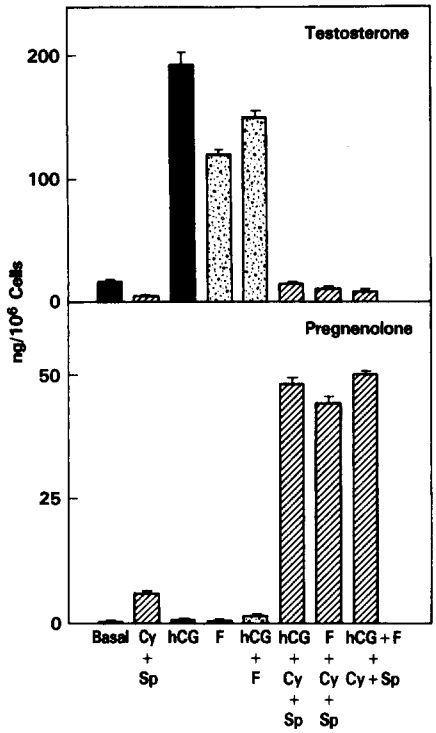

Fig. 9 Effect of forskolin on hCG-stimulated pregnenolone production. Leydig cells (10^6cells/ml) were preincubated for 15 min in presence and absence of cyanoketone [Cy] (10^{-6} M) and spironolactone [Sp] (10^{-5} M). Incubation was further carried out for 120 min with 0.26 nM hCG in the presence and in the absence of forskolin (10 μM). The two inhibitors used in this experiment effectively inhibit 3β-hydroxysteroid dehydrogenase Δ 5 isomerase and 17α-hydrolase activity respectively and cause pregnenolone accumulation in control incubations and during gonadotropin stimulation [9]. Pregnenolone in the incubation media was measured by radioimmunoassay.

DISCUSSION

Our present studies have further confirmed that forskolin stimulates cAMP production in all cellular compartments and also potentiates the hCG-stimulated cAMP production in intact Leydig cells. These results are consistent with our earlier studies on the effects of forskolin in purified Leydig cells [2] and with studies by others in various tissues [4, 5, 12–24].

In this paper, we have demonstrated that high concentration of forskolin caused inhibition on hCG-stimulated testosterone production. Forskolin (10 μM) stimulated testosterone production about 4–5-fold over the basal level, whereas, with a supramaximal dose of hCG, forskolin showed significant inhibition on steroidogenesis while still potentiating its effect at the level of adenylate cyclase and cAMP pools. Similarly forskolin inhibited choleragen-stimulated testosterone production while causing additive stimulation on cAMP pools. This inhibitory action of forskolin was not reversed by the addition of 8-Bromo-cAMP, indicating a block induced by the diterpene on steroidogenesis distal to cAMP generation. Furthermore, the level of inhibition was found to be beyond pregnenolone synthesis. This was demonstrated conclusively, measuring the accumulation of pregnenolone in presence of cyanoketone and spironolactone which effectively inhibit pregnenolone metabolism [19].

Inhibitory actions of high doses of forskolin have also been noticed in some other systems including ACTH-stimulated corticosterone production in rat adrenals [5], parathyroid hormone-stimulated Ca-release in bone [14], LH-stimulated progesterone production in rat luteal cells [22] glucagon-stimulated ketogenic effect in hepatocytes from euthyroid and hypothyroid rats [23], insulin-stimulated glucose metabolism and glucose transport in adipocytes [24, 25] and IgE-mediated release of histamine and leukotriene in human basophils and mast cells [26]. The finding that LH-stimulated progesterone production was inhibited by forskolin in luteal cells coupled to our observations of intactness of the early steroidogenic pathway in the Leydig cells, indicates that forskolin could influence either the 3β-hydroxysteroid dehydrogenase step of pregnenolone metabolism, or more distal steps involved in androgen biosynthesis. Although the precise mechanism of hormonal inhibition in the presence of high doses of forskolin is unknown, it may also be possible that production of high concentration of cAMP by forskolin plus hCG/choleragen or exogenous added cAMP might interfere with intracellular compartmentalization of cAMP [21, 24]. Direct effects of high levels of cAMP, of a competitive and non-competitive nature has been shown to inhibit 3β-hydroxysteroid dehydrogenase in rat ovarian mitochondria and microsomes and in rat adrenal microsomes [27, 28]. Forskolin has been demonstrated to cause rapid reduction of surface expression of pig intestinal amino-peptidase N by postranslational degradation of the enzyme [29]. The very rapid effects of the diterpene in inhibiting steroidogenesis points to a direct or indirect effect (through cAMP) of the diterpene on the steroidogenic enzymes activity or at the post/or translational level, however, we cannot exclude effects at the transcriptional level.

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