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3′,5′-Cyclic Adenosine Monophosphate as an Intracellular Second Messenger of Luteinizing Hormone: Application of the Forskolin Criteria

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The role of adenosine 3′,5′-cyclic monophosphate (cAMP) as an intracellular second messenger of luteinizing hormone (LH) was reinvestigated in vitro with diterpene forskolin, a highly specific activator of adenylate cyclase. Treatment of cultured testicular cells from adult hypophysectomized rats with increasing concentrations (10^{-7}–10^{-4} M) of forskolin produced dose-dependent increments in cAMP and testosterone accumulation. Concomitant blockade of cAMP-phosphodiesterase activity with 3-isobutyl-1-methyl-xanthine (10^{-4} M) resulted in significant ($P < 0.05$) enhancement of the forskolin effect for all but the 10^{-4} M forskolin dose. Potency evaluation as judged by half-maximal stimulation of testosterone accumulation revealed median effective doses (mean ± SE) of $1.25 \pm 0.2 \times 10^{-5}$, $1.7 \pm 0.5 \times 10^{-5}$, and $2.5 \pm 0.4 \times 10^{-10}$ M for forskolin, N^6, $O^{2'}$-dibutyryl cAMP (Bt_2cAMP), and human chorionic gonadotropin (hCG), respectively. Examination of the time requirements of forskolin disclosed time-dependent increments in the accumulation of extracellular cAMP and testosterone, the earliest significant ($P < 0.05$) increases being noted by 6 hr of treatment. In comparison, a minimal time requirement of ≤12hr was noted for hCG- and choleragen-stimulated androgen biosynthesis, whereas the apparent onset of action of Bt_2cAMP was delayed to the 24-hr time point. Although 10^{-7} M of forskolin by itself did not alter the accumulation of testosterone, its addition resulted in substantial amplification of the hCG effect, producing a 4.6-fold reduction in the median effective dose (ED_{50}) of hCG. Moreover, concurrent treatment with this functionally inert dose of forskolin rendered steroidogenically inert doses of hCG (eg, 10^{-11} or 3×10^{-11} M) steroidogenically potent. However, combined treatment with maximally stimulatory doses of Bt_2cAMP (10^{-4} M) and one of several testicular cell agonists [forskolin (10^{-4} M), choleragen (10^{-9} M) or hCG (10^{-9} M)] did not prove additive. Taken together, our findings indicate that forskolin, like LH, is capable of stimulating testicular cAMP generation as well as androgen biosynthesis and that a functionally inert low dose of forskolin can significantly amplify LH hormonal action. Inasmuch as forskolin-stimulated and forskolin-

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amplified hormonal action are acceptable as novel criteria of cAMP dependence, our observations provide new evidence in keeping with the notion that cAMP may be in intracellular second messenger of LH.

Key words: forskolin-stimulated hormonal action, forskolin-amplified hormonal action

Although the primary role of luteinizing hormone (LH) in the regulation of testicular biosynthesis is well accepted, the role of adenosine 3′,5′-cyclic monophosphate (cAMP) as its intracellular second messenger remains under investigation. Indeed, it has been repeatedly observed that relatively low concentrations of LH are capable of producing a maximal steroidogenic response in the face of a relatively limited increase in intracellular cAMP accumulation [1,2]. However, this apparent dissociation between cAMP and androgen biosynthesis has been resolved by the demonstration that Leydig cell steroidogenesis is preceded by increments in the binding of cAMP to the cytosolic protein kinase [3]. Other lines of evidence supporting an intermediary role for cAMP in LH action include the ability of LH to stimulate interstitial cell adenylate cyclase activity [4,5] as well as intracellular cAMP accumulation [1–3,6]. In addition, testicular androgen biosynthesis may be reproduced by increasing intracellular cAMP levels via nondegradable cAMP analogs or cAMP-phosphodiesterase (PDE) inhibitors [6–8]. Thus, it is generally held that the steroidogenic response of interstitial cells to LH is mediated via a highly compartmentalized activation pathway, wherein limited increments in intracellular cAMP result in dose-dependent occupancy and activation of protein kinase, leading in turn to androgen biosynthesis.

In an effort to revaluate the intermediary role of cAMP in LH action, we have used to advantage the diterpene forskolin, a rapid, potent, and reversible activator of adenylate cyclase of tissues of diverse origin [9,10], including rat testis [10]. Most importantly, forskolin constitutes a highly specific activator of adenylate cyclase, having been shown to be without effect on a wide range of other enzymes including guanylate cyclase, cAMP-PDE, or cAMP-dependent protein kinase [9]. This unique specificity of forskolin, has prompted the suggestion that forskolin-stimulated hormonal action and forskolin-amplified hormonal action (a distinct but as yet poorly understood forskolin-hormone synergism) be adopted as new criteria of cAMP dependence [11]. As such, these so-called "forskolin" criteria are intended to complement rather than replace the traditional criteria proposed by Sutherland et al [12]. Our observations that forskolin, like LH, stimulates testicular cAMP generation and androgen biosynthesis by itself and that it is capable of amplifying LH action lend additional credence to the possibility that cAMP may be an intracellular second messenger of LH.

MATERIALS AND METHODS

Animals

Adult (50–70-day-old) hypophysectomized male rats of the Sprague-Dawley strain were purchased from Johnson Laboratories Inc. (Bridgeview, IL) and delivered on the fifth postoperative day. The animals were housed in air-conditioned quarters and given physiological saline (0.9% NaCl) solution and pellets of rodent laboratory chow 5001 from Ralston Purina Co. (St. Louis, MO) ad libitum. A 14-hr light, 10-hr

dark cycle was maintained, with the light cycle starting at 0600 hr. The animals were sacrificed by decapitation 10–15 days after hypophysectomy.

Reagents and Hormones

McCoy's 5a medium (modified, without serum), DNase (bovine pancreas; 2,100 units/mg), penicillin-streptomycin solution, L-glutamine (29.2 mg/ml), bovine serum albumin (BSA), and trypan blue stain (0.4%; wt/vol) were obtained fron Grand Island Biological Company (Grand Island, NY). Collagenase was from Worthington Biochemical Corporation (Freehold, NY). Cholera holotoxin (choleragen), N^6,$O^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphate (Bt_2cAMP), and 3-isobutyl-1-methyl-xanthine (MIX), were obtained from Sigma Chemical Co. (St. Louis, MO). Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one) was from Calbiochem-Behring (La Jolla, CA). Highly purified human chorionic gonadotropin (hCG; CR-121; 13,450 IU/mg) was generously provided by Dr. R.E. Canfield through The Center for Population Research, National Institute of Child Health and Human Development.

Testicular Cell Preparation

Testicular cell suspensions from adult (50–70-day-old) hypophysectomized male rats were prepared as previously described [13]. Testes were dissected free of fat, decapsulated, and dispersed in an enzyme solution containing 0.4% (wt/vol) collagenase, 10 μg/ml DNase, and 0.1% (wt/vol) BSA. The testes were incubated at 37°C for 1.5 hr, during which time they were dissociated into a cell suspension by repeated pipetting every 30 min with a graded series of micropipettes (inside diameters: Ca. 0.5–1.0 mm). At the end of the incubation, the cells were collected by centrifugation at 250g for 5 min, washed three times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (137 mM NaCl, 5mM KCl, 0.7 mM Na_2HPO_4, 25mM HEPES, 10 mM glucose, 360 μM $CaCl_2$, pH 7.2) containing 0.1 % (wt/vol) BSA, and then resuspended into a known volume of McCoy's 5a medium. Aliquots of the cell suspension were diluted with equal volumes of trypan blue stain and samples were taken for counting in a hemacytometer. Cell viability was consistently >80%.

Tissue Culture Procedures

Testicular cells (1.5–3.0 $\times$ 10^6 viable cells/culture) from adult hypophysectomized male rats were plated onto tissue culture dishes (35 $\times$ 10 mm; Falcon Plastics, Los Angeles, CA) containing 1 ml McCoy's 5a medium (modified, without serum) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). Cell cultures were maintained at 37°C under a water-saturated atmosphere of 5% CO_2 and 95% air.

The cells were initially cultured without treatment for 8 days, during which time the media were collected and replaced every 2 days. This treatment-free period is necessary for the recovery and stabilization of the responsiveness of Leydig cells of hCG in this culture system as previously described [13]. At the end of this period, the cells were reincubated for an additional 72 hr (unless indicated otherwise), during which time the various treatments were applied. Hormones were diluted in sterile culture medium and added in 50 μl aliquots. Forskolin was initially dissolved in 99.5% (vol/vol) ethanol, followed by subsequent dilution with sterile culture medium. The final ethanol concentration in the dish did not exceed 0.5% (vol/vol). At the

conclusion of the incubation period, the media were collected and stored frozen at −20°C until assayed for their testosterone content by RIA. Media intended for the measurement of the extracellular content of cAMP were collected, boiled for 10 min in a water bath, and stored frozen at −20°C until assayed for their cAMP content by RIA.

Radioimmunoassay

Medium testosterone content was determined by the use of an antiserum raised against testosterone-3-(O-carboxymethyl)-oxime-bovine serum albumin, generously provided by Dr. A. Avinoam Kowarski (University of Maryland School of Medicine, Baltimore, MD). The cross reactivities of this antiserum and assays performance characteristics were as previously described [14]. The extracellular content of cAMP in the medium was determined with a cAMP RIA kit (NEX-132) obtained from New England Nuclear (Boston, MA), as previously described [15].

Data Analysis

All experimental data are presented as the mean ± SE of duplicated measurements of triplicate cultures. RIA data were analyzed with a computer program by Davis et al [16]. Dose-response curve fitting and determination of median effective dose (ED_{50}) were carried out by fitting the results through a sigmoidal calibration curve with a computer program based on the 4-parameter logistic equation [17], generously provided by Dr. David Rodbard (Biophysical Endocrinology Section, Endocrinology and Reproductive Research Branch, NICHHD, NIH, Bethesda, MD). Statistical significance was determined by Student's paired 2-tailed t-test and analysis of variance (ANOVA) as indicated [18].

RESULTS

Forskolin-Stimulated Accumulation of Extracellular cAMP and Testosterone: Dose-Dependence

To evaluate the dose requirements of forskolin, testicular cells were cultured in the absence or presence of increasing concentrations (10^{-7}–10^{-4} M) of forskolin with or without MIX at the 10^{-4} M dose level (Fig. 1A,B).Whereas the basal accumulation of extracellular cAMP or testosterone was negligible, treatment with increasing concentrations of forskolin produced dose-dependent increments in cAMP and testosterone accumulation. The extracellular accumulation of cAMP and testosterone in response to treatment with the highest dose (10^{-4} M) of forskolin tested reached 11.4 ± 1.0 pmol/culture and 16.1 ± 5 ng/culture, respectively. Concomitant blockade of cAMP-PDE activity with MIX resulted in significant ($P < 0.05$) enhancement of the forskolin effect for all dose studied other than the 10^{-4} M forskolin dose. These findings indicate that forskolin is capable of effecting dose-dependent increments in the accumulation of extracellular cAMP and testosterone, an effect further augmented through cAMP-PDE blockade.

Forskolin, hCG-, and Bt_2cAMP-Stimulated Testosterone Accumulation: Potency Evaluation

To evaluate the potency and efficacy of forskolin vis a vis hCG and Bt_2cAMP, testicular cells were cultured in the absence or presence of increasing concentrations

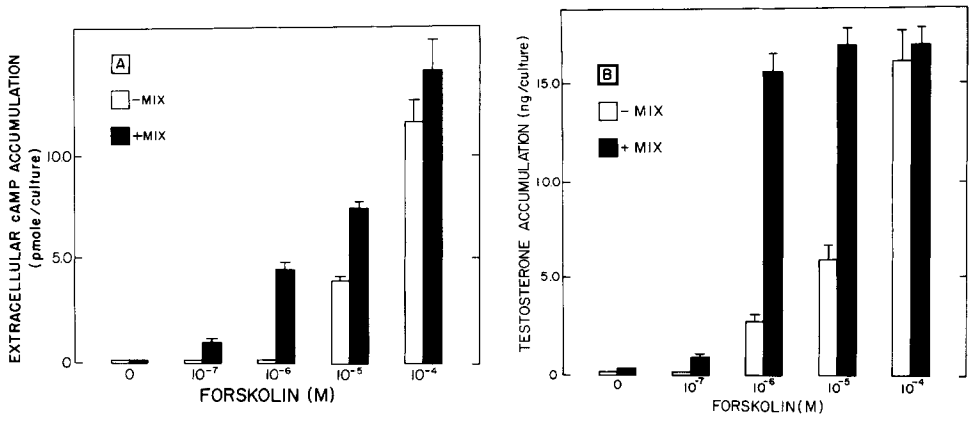

Fig. 1. Forskolin stimulated accumulation of extracellular cAMP and testosterone: dose dependence. Testicular cells (2×10^6 viable cells/culture) were obtained from adult (50–70 days old) hypophysectomized male rats. The cells were initially cultured for 8 days without treatment, during which time the media were collected and replaced every 2 days. At the end of this period, the cells were reincubated for an additional 72 hr in the absence or presence of increasing concentrations (10^{-7}–10^{-4} M) of forskolin, with or without MIX (10^{-4} M). Collected media were assayed for their cAMP (A) and testosterone (B) content by RIA. The results represent the mean ± SE of three separate determinations.

of forskolin (10^{-6}–10^{-4} M; Fig. 2A), hCG (10^{-11}–10^{-9} M; Fig. 2B), or Bt_2cAMP (10^{-6}–10^{-4}; Fig. 2C). Treatment with increasing concentrations of all experimental agents resulted in dose-dependent increments in testosterone accumulation, with apparent ED_{50s} of $1.25 \pm 0.2 \times 10^{-5}$, $2.5 \pm 0.4 \times 10^{-10}$, and $1.7 \pm 0.5 \times 10^{-5}$ M for forskolin, hCG, and Bt_2cAMP, respectively. The highest dosage of forskolin (10^{-4} M), hCG (10^{-9} m), and Bt_2cAMP (10^{-4} M) proved maximally stimulatory in that treatment with higher doses of these agents did not yield additional increments in testosterone accumulation (not shown). Efficacy evaluation (as assessed by maximal testosterone accumulation) revealed a rank order of Bt_2cAMP > forskolin > hCG (24.6:4.7:1.0).

Forskolin-Stimulated Accumulation of Extracellular cAMP and Testosterone: Time Dependence

To study the time requirements of forskolin, MIX (10^{-4} M)-treated testicular cells were cultured for the duration indicated (1–72 hr) in the presence of forskolin at the 10^{-4} M dose level (Fig. 3A,B). Extracellular cAMP accumulation during the first 12 hr (Fig. 3A) and testosterone accumulation during the first 7 hr (Fig. 3B) are shown in the corresponding insets. Treatment with forskolin produced time-dependent increments in the extracellular accumulation of cAMP, the earliest significant ($P < 0.05$) increase being observed by 6 hr of treatment, at which time forskolin-treated cells accumulated 5.5-fold more cAMP than untreated controls. A similar time course was observed for the accumulation of testosterone, significant ($P < 0.05$) increments being noted by 6 hr of treatment, at which point forskolin-treated cells accumulated 14.5-fold more testosterone than untreated controls. These finidngs suggest that forskolin is capable of effecting time-dependent increments of extracellular cAMP and testosterone with a minimal time requirement of $\leqslant$ 6 hr.

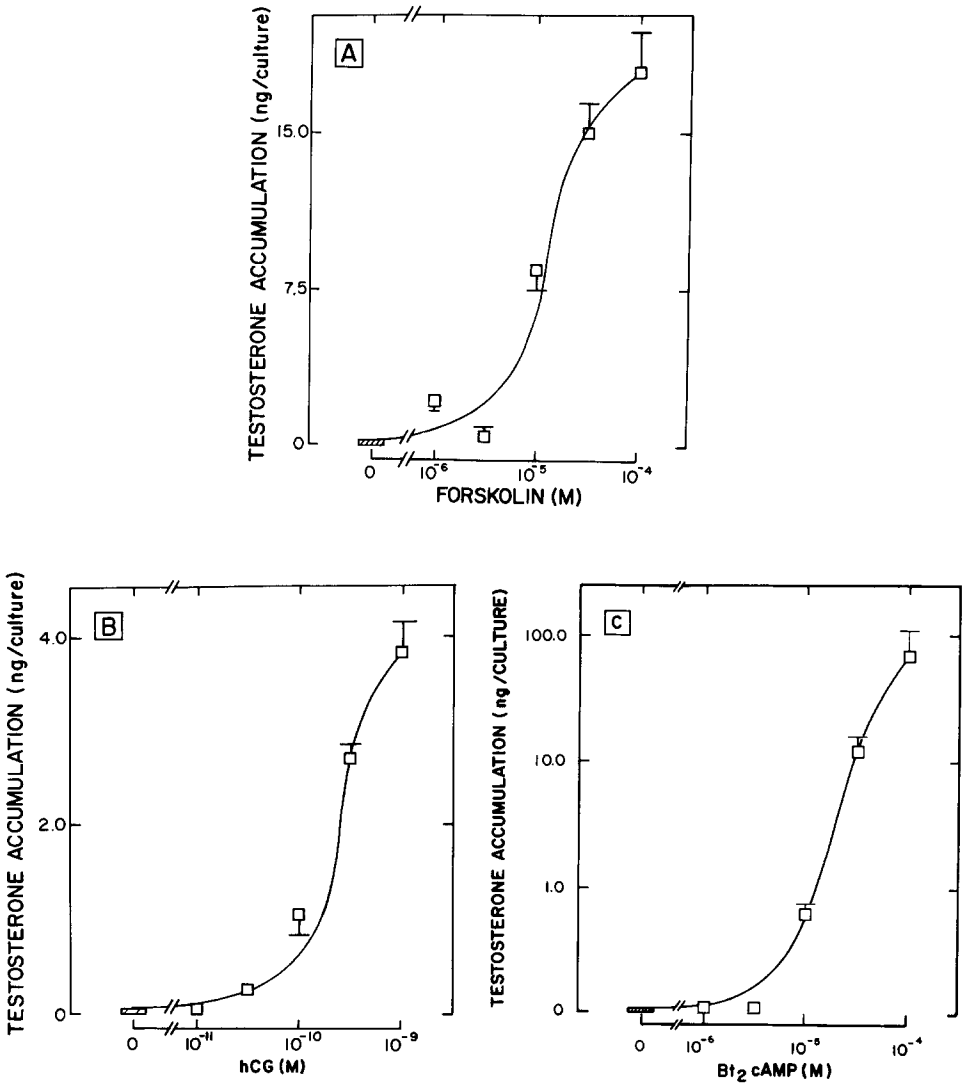

Fig. 2. Forskolin-, hCG-, and Bt_2cAMP-stimulated testosterone accumulation: relative potency and efficacy comparison. Testicular cells were obtained as described in Figure 1 and initially cultured without treatment for 8 days, during which time the media were collected and replaced every 2 days. At the end of this period, the cells were reincubated for additional 72 hr in the absence or presence of increasing concentrations of forskolin (A) (10^{-6}–10^{-4} M), hCG (B) (10^{-11}–10^{-9} M), or Bt_2cAMP (C) (10^{-6}–10^{-4} M; note logarithmic scale). Collected media were assayed for their testosterone content by RIA. The results represent the mean ± SE of three separate determinations.

Forskolin-, hCG-, Choleragen-, and Bt_2cAMP-Stimulated Testosterone Accumulation: Time Course Evaluation

To evaluate the time requirements of forskolin, hCG, choleragen, and Bt_2cAMP, MIX (10^{-4} M)-treated testicular cells were cultured for the duration indicated (2–48 hr) in the presence of forskolin (10^{-4} M), hCG (10^{-9} M), choleragen (10^{-9} M), or

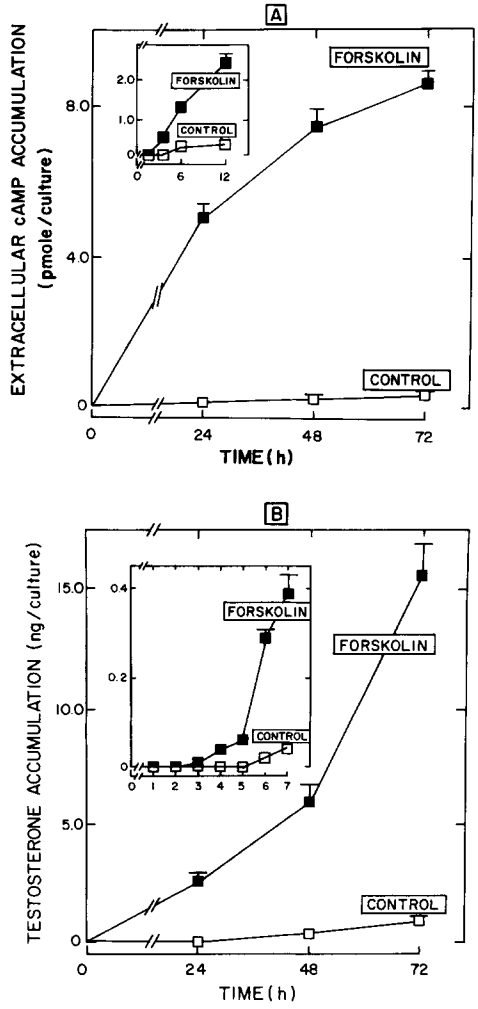

Fig. 3. Forskolin-stimulated accumulation of extracellular cAMP and testosterone: time dependence. Testicular cells were obtained as described in Figure 1 and initially cultured without treatment for 8 days, during which time the media were collected and replaced every 2 days. At the end of this period, the cells were cultured for the duration indicated (1-72 hr) in the presence of MIX (10^{-4} M) and forskolin (10^{-4} M). Extracellular cAMP accumulation during the first 12 hr (A) and testosterone accumulation during the first 7 hr (B) are shown in the corresponding insets. Collected media were assayed for their cAMP (A) and testosterone (B) content by RIA. The results represent the mean ± SE of three separate determinations.

Bt_2cAMP at the 10^{-4} M dose level (Fig. 4). Although treatment with all agents resulted in time-dependent increments in testosterone accumulation, distinct differences were observed with respect to their apparent onset of action. As shown earlier (Fig. 3B), the earliest significant ($P < 0.05$) increase in forskolin-stimulated testosterone accumulation was observed by 6 hr of treatment. In contrast, the earliest detectable testosterone increments in response to treatment with either hCG or cholera gen were observed by 12 hr of treatment. Onset of action was most delayed for

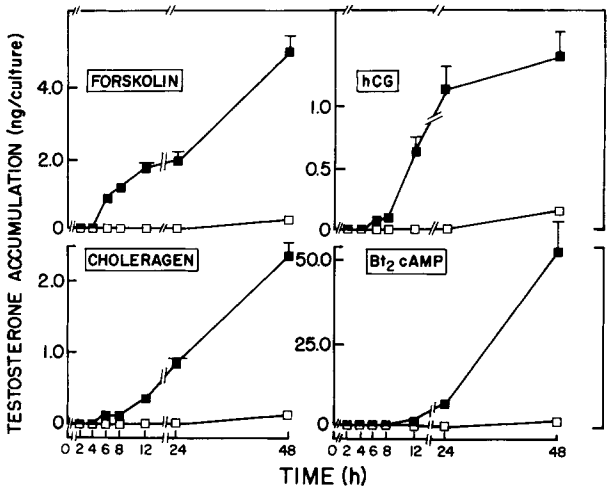

Fig. 4. Forskolin, hCG-, choleragen-, and Bt_2cAMP-stimulated testosterone accumulation: time course comparison. Testicular cells were obtained as described in Figure 1 and initially cultured without treatment for 8 days, during which time the media were collected and replaced every 2 days. At the end of this period, MIX (10^{-4} M)-treated testicular cells were cultured for the duration indicated (2–48 hr) in the presence of forskolin (10^{-4} M), hCG (10^{-9} M), choleragen (10^{-9} M), or Bt_2cAMP (10^{-4} M). Collected media were assayed for their testosterone content by RIA. The results represent the mean ± SE of three separate determinations.

Bt_2cAMP for which no significant increments in testosterone accumulation could be observed prior to 24 hr of treatment. These findings suggest a faster onset of action for forskolin relative to that of hCG and choleragen, with Bt_2cAMP displaying a minimal time requirement of $\leqslant$24 hr.

Forskolin-Stimulated Testosterone Accumulation: Reversibility Studies

To study the reversibility of the forskolin effect, testicular cells were cultured for 72 hr in the absence or presence of forskolin (10^{-4} M). At the conclusion of this pretreatment period, the media were collected, the cells were washed twice with 2-ml portions of medium, and they were reincubated for an additional 72 hr in the absence or presence of forskolin (10^{-4} M). Forskolin-stimulated testosterone accumulation during the pretreatment period was 14.5 ± 1.3 ng/culture. The basal accumulation of testosterone was negligible. Repeat stimulation of forskolin-pretreated cells produced testosterone levels of 13.2 ± 2.6 ng/culture as compared with negligible accumulation by unstimulated testosterone accumulation upon initial and repeat stimulation and its lack of effect following withdrawal are in keeping with the reversible nature of the forskolin effect.

hCG- and Choleragen-Stimulated Testosterone Accumulation: Effect of a Functionally Inert Low Dose of Forskolin

To study the capacity of forskolin to amplify hCG- and choleragen-stimulated testosterone accumulation, testicular cells were cultured in the absence or presence of increasing concentrations (10^{-11}–10^{-9} M) of hCG or choleragen, with or without forskolin at the 10^{-7} M dose level (Fig. 5A,B). Treatment of testicular cells with hCG produced dose-dependent increments in the acccumulation of testosterone, with

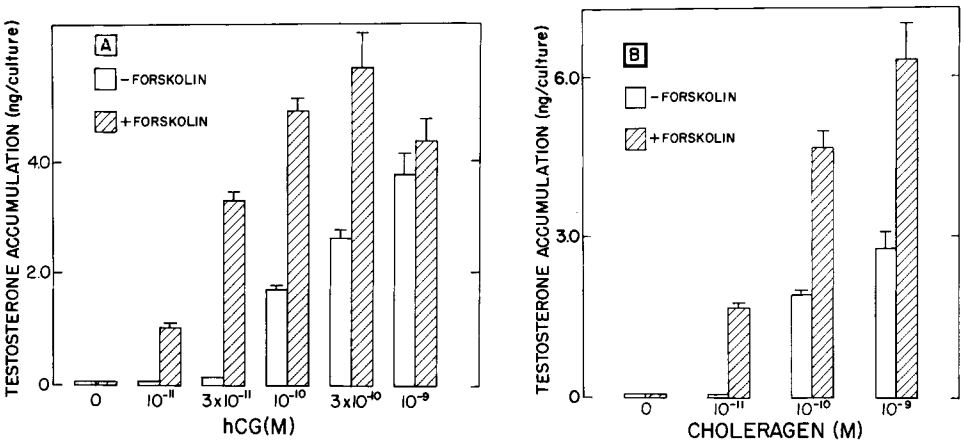

Fig. 5. hCG-and choleragen-stimulated testosterone accumulation: effect of a functionally inert low dose of forskolin. Testicular cells were obtained as described in Figure 1 and initially cultured without treatment for 8 days, during which time the media were collected and replaced every 2 days. At the end of this period, testicular cells were cultured in the absence or presence of increasing concentrations (10^{-11}–10^{-9} M) of hCG (A) or choleragen (B), with or without forskolin (10^{-7} M). Collected media were assayed for their testosterone content by RIA. The results represent the mean ± SE of three separate determinations.

an ED_{50} of 1.2 ± 0.2 × 10^{-10} M. The 10^{-9} M dose of hCG proved maximally satisfactory, higher doses producing no further increments in testosterone accumulation (not shown). Whereas 10^{-7} M forskolin by itself did not alter the accumulation of testosterone (or cAMP; Fig. 1A), its addition resulted in amplification of the hCG effect for all but the 10^{-9} M hCG dose, producing a 4.6-fold reduction in the ED_{50} of hCG to 2.5 × 10^{-11} M. Moreover, steroidogenically inert doses of hCG (eg, 10^{-11} M or 3 × 10^{-11} M), were rendered steroidogenically potent upon the addition of forskolin. Similarly, concurrent treatment with this functionally inert dose of (10^{-7} M) forskolin produced significant ($P < 0.05$) amplification of the choleragen effect for all doses studied, converting a steroidogenically inert dose of choleragen (10^{-11} M) into a steroidogenically potent one.

Bt_2cAMP-Stimulated Testosterone Accumulation: Additivity Studies With cAMP-Generating Agonists

To evaluate further the possible role of cAMP in LH action, testicular cells were cultured in the presence of a maximal stimulatory dose of Bt_2cAMP (10^{-4} M) in combination with maximal stimulatory doses of either forskolin (10^{-4} M), choleragen (10^{-9} M), or hCG (10^{-9} M). However, as shown in (Table I), combined treatment did not prove additive for any of the cAMP-generating agonists employed. These findings suggest that Bt_2cAMP, forskolin, choleragen, and hCG enhance testicular cell testosterone biosynthesis via a common, saturable mechanism of action. These observations provide additional support to the notion that cAMP may play an intermediary role in the mechanism of LH action.

DISCUSSION

The intermediary role of cAMP in the mechanism of LH action was reinvestigated in vitro by the use of a primary culture of rat testicular cells. Our findings

TABLE I. Bt_2cAMP-Stimulated Testosterone Accumulation: Additivity Studies With cAMP-Generating Agonists*

Treatment	Testosterone accumulation (ng/culture)
None	Nondetectable
Bt_2cAMP (10^{-4} M)	97.2 ± 5.6
Forskolin (10^{-4} M)	13.8 ± 2.1
Choleragen (10^{-9} M)	5.2 ± 1.6
hCG (10^{-9} M)	4.1 ± 1.1
Bt_2cAMP (10^{-4} M) + forskolin (10^{-4} M)	91.2 ± 6.1
Bt_2cAMP (10^{-4} M) ± choleragen (10^{-9} M)	89.8 ± 7.2
Bt_2cAMP (10^{-4} M) + hCG (10^{-9} M)	101.3 ± 6.4

*Testicular cells were obtained as described in Figure 1 and initially cultured without treatment for 8 days, during which time the media were collected and replaced every 2 days. At the end of this period, the cells were reincubated for additional 72 hr in the absence or presence of Bt_2cAMP (10^{-4} M), forskolin (10^{-4} M), choleragen (10^{-9} M), hCG (10^{-9} M), and combinations thereof. Collected media were assayed for their testosterone content by RIA. The results represent the mean ± SE of three separate determinations.

indicate that the adenylate cyclase-specific probe forskolin, like LH, is capable of stimulating testicular cAMP generation as well as androgen biosynthesis in a dose- and time-dependent fashion. As such, forskolin proved to be a rapid, potent, and reversible activator of testicular androgen production. Morover, a functionally inert low dose of forskolin proved capable of significant amplification of LH hormonal action.

As demonstrated in other tissues, forskolin proved a potent, rapid, and reversible activator of testicular androgen biosynthesis. Indeed, forskolin stimulated testicular androgen biosynthesis with an ED_{50} of $1.25 \pm 0.2 \times 10^{-5}$ M, a value well within the range of 10–40 μM reported for a wide variety of eukaryotic cells [11], including steroidogenically active cells [15,19–24]. Our findings also suggest a faster onset of action for forskolin relative to that of hCG and choleragen, further underscoring forskolin's rapidity of action. In addition, the ability of forskolin to stimulate comparable increments in testosterone accumulation upon initial and repeat stimulation suggests that the effect of forskolin in testicular cells, as with other tissues, is reversible in nature [11]. Thus, the interaction of forskolin with testicular cells appears to conform in its general characteristics to the pattern observed for other eukaryotic tissues thus far studied.

Our findings indicate that forskolin and is approximately five orders of magnitude less potent than hCG. These findings are in keeping with the relative binding affinity of these agonists to their respective recognition sites. Indeed, forskolin has been reported to display binding affinity in the micromolar range [25–27], whereas the binding affinity of hCG to its testicular receptor is generally thought to be in the range of 2×10^{-11} M [6]. In contrast, our findings suggest that in terms of efficacy, Bt_2cAMP is approximately 25-fold more efficient than hCG and about fivefold more efficient than forskolin. Similar findings were reported by Malaska and Payne wherein cAMP proved more effective than LH in stimulating microsomal P-450 enzymes of cultured Leydig cells [28]. Although the reason(s) underlying this rank order of efficacy remain uncertain, it is tempting to speculate that it may be related to diminished LH/hCG binding consequent to hypophysectomy and culture conditions. Alternatively, the extent of cAMP binding to the regulatory subunit of protein kinase

may be at play given that the delivery of high concentrations of cAMP by means of a lipophilic nondegradable derivative (ie, butyrate) may result in increased occupancy and hence activation of protein kinase. In contrast, forskolin and hCG heavily rely on the generation of endogenous cAMP requiring efficient compartmentalization and delivery for maximal action. The faster onset of action of forskolin relative to that of hCG and choleragen may be related to its ability to interact at a level beyond the cell surface receptors. By comparison, the relatively slow onset of action of Bt_2cAMP is in all likelihood related to the dynamic partition equilibrium within the lipid bilayer wherein this lipophilic cAMP derivative must achieve a critical intramembranous concentration before it is able to traverse into the cytosol.

The culture system used in this investigation constitutes a whole testicular dispersate [13]. The relative representation of the Leydig cells is of the order of 20% as estimated by 3β-hydroxysteroid dehydrogenase/isomerase staining [13]. The relative representation of Sertoli cells and the identity and representation of the remaining cell types are not known. Consequently, one cannot conclude that the testicular effects of forskolin are exerted solely at the Leydig cell level. Although testosterone accumulation, a Leydig cell function, in all likelihood accurately reflects the interaction forskolin with the Leydig cell, one cannot exclude the possibility that forskolin activates Sertoli cell adenylate cyclase, which may therefore contribute to the extracellular accumulation of cAMP. However, preliminary studies with highly purified FSH, an established Sertoli cell agonist, proved to be without effect in this culture system as assessed by increased cAMP or androgen binding protein accumulation (not shown). Thus, although Sertoli cells may be present in the present culture system, the likely major target of forskolin is the Leydig cell.

The role of cAMP as an intracellular second messenger of LH has been the subject of rigorous investigation. For the most part, the assignment of an intermediary role for cAMP in the mechanism of LH action required that the criteria proposed by Sutherland et al [35] be satisfied. Although these criteria have been met for the case of LH-stimulated androgen biosynthesis, it is now possible to assess the role of cAMP through the use of forskolin, taking advantage of its unique and apparently specific interaction with adenylate cyclase. Accordingly, forskolin-stimulated and forskolin-potentiated hormonal action have been proposed as additional criteria of cAMP dependence [31]. Inasmuch as these criteria are acceptable as a measure of cAMP dependence, our observations provide new evidence in keeping with the notion that cAMP may be an intracellular second messenger of LH.

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