MOLECULAR PHARMACOLOGY, 22:609–613

Differences in the Forskolin Activation of Adenylate Cyclases in Wild-Type and Variant Lymphoma Cells

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Received May 25, 1982; Accepted July 23, 1982

SUMMARY

The ability of the diterpene forskolin to stimulate cyclic AMP accumulation in intact cell and membrane preparations of wild-type S49 lymphoma cells (WT) and a number of variants has been confirmed. Additionally, a number of salient new findings have emerged: (a) A time delay in forskolin stimulation of cyclic AMP accumulation and adenylate cyclase ($t_{1/2} \cong 1.5$ min) occurred in all hormone-sensitive WT and variant cell and membrane preparations tested. (b) The time delay was missing in the adenylate cyclase-deficient variant (*cyc*$^-$) of the S49 lymphoma cell, which also lacks functional adenylate cyclase-coupling proteins. (c) The simultaneous addition of epinephrine and forskolin to WT cells or to membrane preparations eliminated the time delay. (d) Forskolin stimulation of intact WT cells did not appear to desensitize adenylate cyclase. (e) The activation of WT adenylate cyclase by forskolin was biphasic with respect to concentration, with both high- and low-affinity components being apparent. In *cyc*$^-$, only the low-affinity component was detected.

INTRODUCTION

Recently, Seamon and his co-workers (1–3) demonstrated that forskolin activated adenylate cyclase in membrane preparations from a wide range of cell types, including the *cyc*$^-$ variant[1] of S49 lymphoma cells, and also in the soluble fraction of testis homogenates. Since *cyc*$^-$ is deficient in the coupling protein (G/F) (4, 5), and the testis enzyme appears to be a free catalytic unit (6), these results suggested that forskolin could stimulate the catalytic unit independently of functional G/F or receptor components. However, Seamon *et al.* (1) and Seamon and Daly (3) showed that stimulation by forskolin and hormone together was synergistic, implying that the details of forskolin action on adenylate cyclase may not be that simple. These intriguing results demonstrated the potential importance of the compound for the study of both adenylate cyclase and cyclic AMP-regulated processes.

Very little is known about the presumed interaction between G/F and the catalytic unit (7, 8). The reported synergism between hormone and forskolin activation suggested that the interaction of G/F with the catalytic unit might modify the effects of forskolin and vice versa. One might expect, therefore, that studies of forskolin activation of adenylate cyclase in intact cells and in membrane preparations would provide clues to the interactions of the components of the adenylate cyclase system. In the present communication we present such studies contrasting the forskolin responses of WT cells replete with functional G/F and the *cyc*$^-$ S49 variant.

This research was supported in part by Grant AM 26943-03 (to R. W. B.) from the National Institutes of Health.

[1] The abbreviations used are: *cyc*$^-$, adenylate cyclase-deficient S49 variant lymphoma cell; G/F, adenylate cyclase-coupling proteins; WT, wild-type S49 lymphoma cell; 24.4.6, kinase$^-$ low-phosphodiesterase variant lymphoma cell; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MIX, 1-methyl-3-isobutylxanthine.

EXPERIMENTAL PROCEDURES

Materials

(−)-Epinephrine bitartrate was purchased from K & K Biochemicals (Plainview, N. Y.), and prostaglandins E_1 and E_2 were a gift from Dr. John Pike, of The Upjohn Company (Kalamazoo, Mich.). Forskolin was purchased from Calbiochem (San Diego, Calif.). [8-^{3}H]Adenine (17 Ci/mmole), [8-^{14}C]adenine (55 mCi/mmole), [U-^{14}C]ATP (501 mCi/mmole), [8-^{14}C]cyclic AMP (52.1 mCi/mmole), and [α-^{32}P]ATP (25 mCi/mmole) were purchased from the New England Nuclear Corporation (Boston, Mass.). Other chemicals were of the highest grade commercially available.

Methods

Cell culture. The WT and *cyc*$^-$ cultures were grown in 1-liter roller bottles at 37° with Dulbecco's modified Eagle's medium (GIBCO, Grand Island, N. Y.), which had been supplemented with antibiotics (GIBCO) and either 5% horse serum (Flow Laboratories, Rockville, Md.) for WT and *cyc*$^-$ or 10% for 24.4.6. The population

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density was maintained at between 1.5 and 2×10^6 cells/ml. Fresh medium was added daily.

S49 WT and 24.4.6 were kindly provided by Dr. Henry Bourne, of the University of California at San Francisco; WT and *cyc*$^-$ were provided by Dr. Mary Bordelon, of Baylor College of Medicine (Houston, Tex.).

Experimental incubations. Cellular cyclic AMP accumulation was measured as described previously (9), with minor modifications being necessitated by the use of S49 cells. The cell incubations were carried out at 24° in serum-free Dulbecco's medium without $NaHCO_3$ but with 20 mM Hepes and 37 mM NaCl. Since the S49 cells were grown in suspension the experiments were terminated by centrifugation for approximately 10 sec in an Eppendorf Microfuge. The medium was rapidly removed by aspiration. The packed cells were loosened by agitation on a Vortex mixer. An ice-cold stopping solution (0.5 ml) containing 5% trichloroacetic acid and radioactive tracers to monitor cyclic AMP and ATP recoveries was added. After centrifugation to remove the protein residue, the ATP and cyclic AMP in the cell extracts were isolated as previously described (9). Cyclic AMP levels were expressed as percentage conversion; i.e., ([^{3}H]cyclic AMP × 100)/([^{3}H]cyclic AMP + [^{3}H]ATP).

Plasma membrane preparations. Plasma membrane fractions were prepared from WT and *cyc*$^-$ by the procedure of Ross *et al.* (10), modified as follows: the crude particulate was resuspended in 10% sucrose in HME buffer [20 mM Hepes, (pH 8.0), 2 mM $MgCl_2$, and 1 mM EDTA], and then layered over 40% sucrose in HME buffer and centrifuged at 80,000 × *g* for 80 min. The membranes floating over the 40% sucrose were collected, diluted with 5–6 volumes of HME buffer, and centrifuged at 45,000 × *g* for 20 min. Membrane pellets were resuspended in HME buffer with 1.0 mM dithiothreitol, frozen, and stored at −70°. Prior to use, the membrane suspension was thawed and centrifuged at 45,000 × *g* for 15 min, and the pellet was resuspended in HME buffer for adenylate cyclase assays.

Adenylate cyclase assay. The adenylate cyclase assay was carried out as previously described (11). The assay solution was composed of 40 mM sodium Hepes (pH 7.5), 6 mM $MgCl_2$, 8 mM creatine phosphate, creatine phosphokinase (16 units/ml), 0.5 mM ATP, 10 μM GTP, 0.1 mM MIX, and 1.5 μCi of [α-^{32}P]ATP in a final volume of 100 μl (including the adenylate cyclase preparation). The protein concentration used for the assay was 1–2 mg/ml. All incubations were carried out at 30°. Protein was determined either by the procedure of Lowry *et al.* (12) with bovine serum albumin as the standard, or with the Bio-Rad protein reagent using γ-globulin as the standard. Comparable results were obtained with either method.

RESULTS

Time delay in the effects of forskolin. Time courses of cyclic AMP levels in intact S49 WT cells exposed to several concentrations of forskolin are shown in Fig. 1A. Aside from the concentration dependence of the cyclic AMP response to forskolin, the results were notable in that there was a distinct lag in the establishment of the maximal rate of cyclic AMP accumulation. The duration of the lag was independent of forskolin concentration.

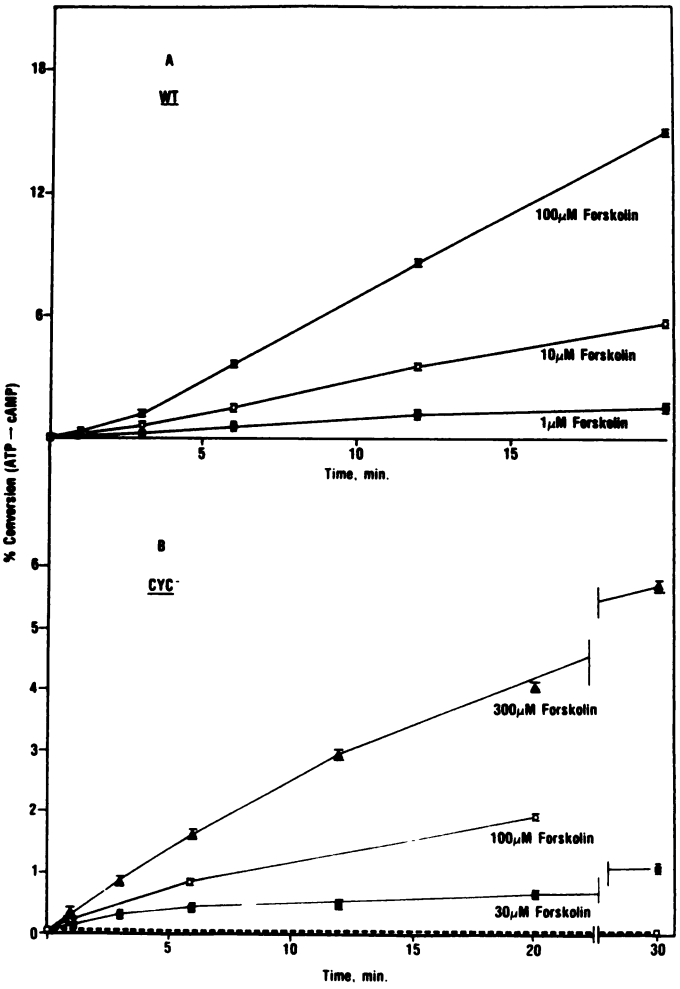

FIG. 1. *Forskolin-stimulated cyclic AMP accumulation in WT and cyc*$^-$ *cells*

Intact WT (A) or *cyc*$^-$ cells (B) were stimulated with various forskolin concentrations as shown. The incubations involving *cyc*$^-$ cells contained 0.5 mM MIX. Values shown are the means ± standard error of the mean. □- - -□, Basal cyclic AMP accumulations.

This was demonstrated by showing that the ratio of the accumulations between the various time points did not differ significantly for the three forskolin concentrations. The lag was not affected by the inclusion of MIX (0.5 mM, data not shown). Similar experiments were performed with intact *cyc*$^-$ cells (Fig. 1B) except that 0.5 mM MIX was present in all incubations. Cyclic AMP levels were increased by incubation with forskolin; however, the levels of cyclic AMP achieved were much lower than those found in WT cells. Furthermore, the lag observed in the WT cells was absent in the *cyc*$^-$ variant.

As in the intact cells, adenylate cyclase activities in membrane preparations from WT showed a lag in establishing the rate of cyclic AMP formation in response to forskolin (Fig. 2A) which was independent of the forskolin concentration. Once a stable rate of adenylate cyclase activity was achieved, it was constant for at least 30 min. The lag did not occur in membranes prepared from *cyc*$^-$ cells (Fig. 2B) when incubated with forskolin at concentrations over a 100-fold range.

Forskolin and epinephrine interactions on cyclic AMP metabolism. The combination of 0.1 μM epineph-

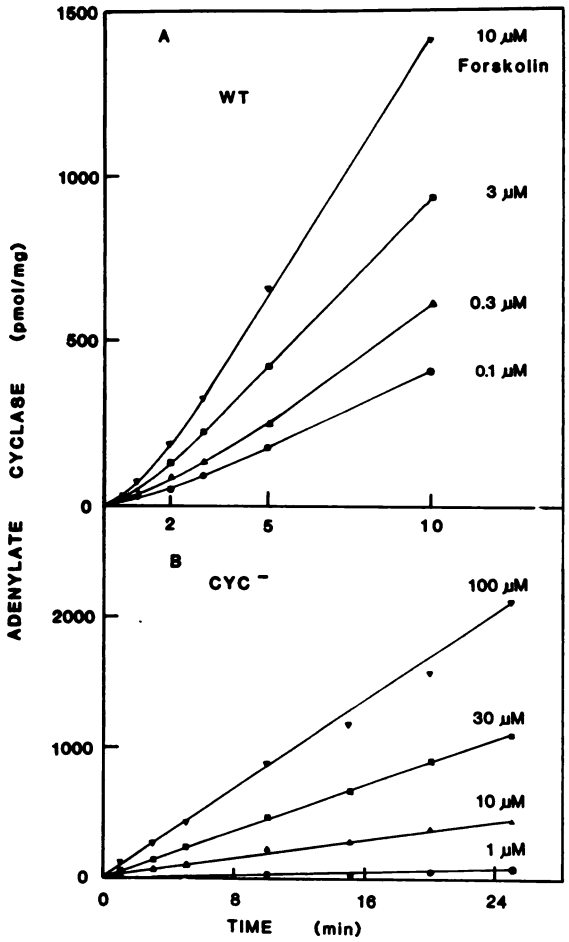

FIG. 2. *Forskolin-stimulated adenylate cyclase activity in membrane preparations from WT and cyc⁻ cells*

Membranes from WT (A) or *cyc*⁻ cells (B) were stimulated with various forskolin concentrations as indicated, and adenylate cyclase activity was measured from 0–30 min. To show more clearly the lag observed with WT membranes, only the 0–10 min data are shown. Values represent single determinations. Linear regression analysis of the 5–30 min WT data resulted in the following abscissa (x) intercepts indicating the lag time [correlation coefficients (r^2) are also given]: 10 μM forskolin, $r^2 = 0.999$, $x = 1.7$ min; 3 μM, $r^2 = 0.996$, $x = 1.6$ min; 1 μM, $r^2 = 0.998$, $x = 1.6$ min; 0.3 μM, $r^2 = 0.999$, $x = 1.2$ min. The assay mixture contained membrane protein (1 mg/ml).

rine and 100 μM forskolin caused a rate of cyclic AMP accumulation in intact WT cells which was greater than the rate with either alone (Fig. 3A). The time delay characteristic of forskolin activation was abolished by the inclusion of epinephrine. Similar results were obtained in experiments measuring the adenylate cyclase activity in membrane preparations isolated from epinephrine-responsive S49 lines (Fig. 3B). In control experiments, (data not shown) the forskolin stimulation of cyclic AMP accumulation in intact or cell-free preparations of *cyc*⁻ cells was unaffected by epinephrine.

In contrast to the effects of epinephrine (13), forskolin did not cause desensitization in WT cells. The rate of cyclic AMP accumulation in response to forskolin alone was nearly linear after the lag period (Fig. 3A). Any decrease with time in the rate of cyclic AMP accumulation in cells exposed to constant hormonal stimulation is a composite of desensitization and k_e (the destruction constant) (9). However, even assuming that the entire decrease in rate with time is due to desensitization rather than to breakdown, the maximal forskolin desensitization could not have been greater than 20%. By contrast, as shown in Fig. 3A, incubation of WT cells with epinephrine routinely produced significant desensitization. Independent measurements of turnover showed that WT cells were routinely desensitized by at least 50% after 30 min of incubation with epinephrine (data not shown). The inability of the diterpene to produce desensitization was most obvious in the low-phosphodiesterase mutant 24.4.6 (see Table 1). The lower rate of accumulation at short times was indicative of the lag in forskolin action after which there was no indication of a significant fall in the rate of accumulation.

This argument could not be used in the *cyc*⁻ system, where the nonlinearity of cyclic AMP accumulations was

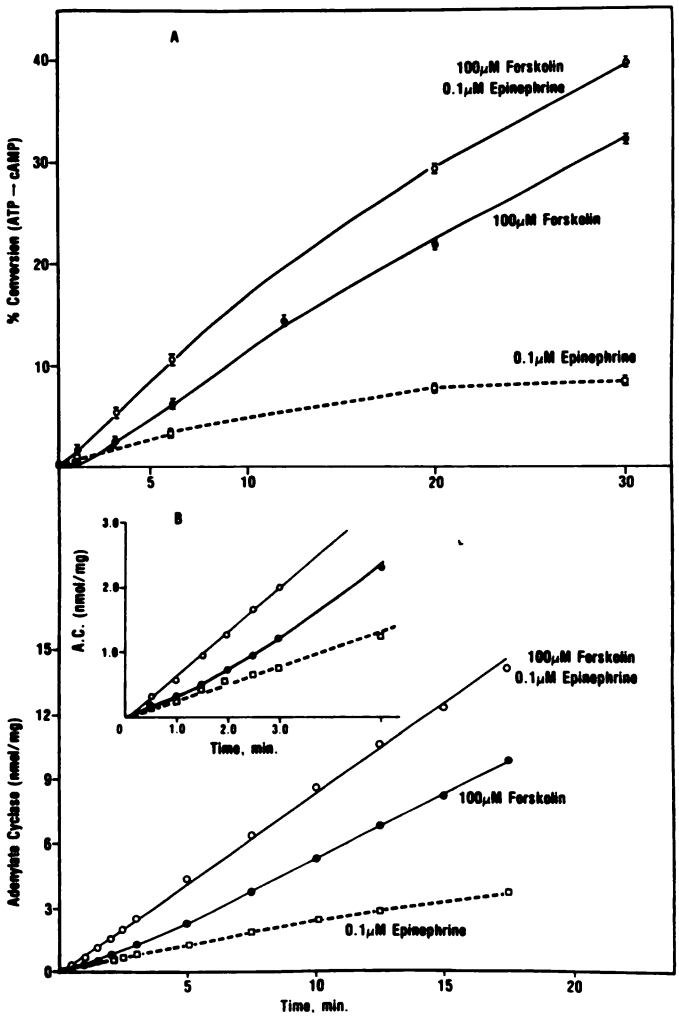

FIG. 3. *Effects of epinephrine on forskolin-stimulated cyclic AMP accumulation and adenylate cyclase activity*

Intact WT cells (A) or membrane preparations from WT cells (B) were stimulated with 0.1 μM epinephrine, 100 μM forskolin, or the combination of the two. The *inset* (B) shows the 0–5 min adenylate cyclase (*A.C.*) data on an expanded scale. Values shown for the intact cell cyclic AMP accumulations are the means ± standard error of the mean. Adenylate cyclase data were obtained from single determinations. The assay contained membrane protein (1 mg/ml).

TABLE 1

Rate of accumulation of cyclic AMP in the 24.4.6 variant of S49 cells

The ranges of time quoted are for periods subsequent to the addition of 100 μM forskolin at $t = 0$.

Time range	Mean rate of cyclic AMP accumulation
min	*% conversion ATP → cyclic AMP/min*
1–3	0.368 ± 0.045
3–6	0.417 ± 0.035
6–10	0.605 ± 0.106
10–20	0.721 ± 0.073
20–30	0.635 ± 0.102

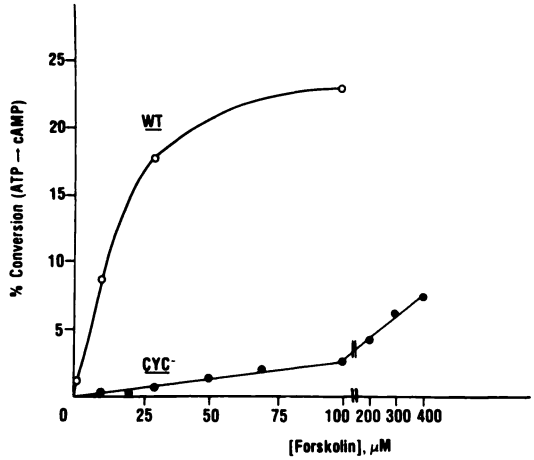

FIG. 4. *Accumulation of cyclic AMP in WT and cyc⁻ cells at various concentrations of forskolin*

The determinations were made after 30-min incubations at 24°. The forskolin was added dissolved in ethanol and the final concentration of ethanol in all incubations was 4%. The incubations involving *cyc⁻* cells contained 0.5 mM MIX.

readily apparent (Fig. 1B). However, the data do not unequivocally demonstrate desensitization in these cells. The accumulations obtained with this variant are relatively low. A preliminary communication has suggested that the turnover constant in S49 cells is greater at low cyclic AMP accumulations (14). Thus, the deviation from linear accumulation of cyclic AMP due to hydrolysis should have been greater at the lower concentrations of cyclic AMP. This view was supported by the fact that the greatest departure from linear accumulation occurred at the lowest forskolin stimulation, the opposite of what was to be expected if it were a function of desensitization.

MIX was used in the experiments with these cells because cyclic AMP accumulation was very low in its absence. The large effects of this compound on accumulation was an indication that cyclic AMP was rapidly hydrolyzed in its absence. MIX, while greatly reducing cyclic AMP hydrolysis in cellular systems, does not eliminate it (15). The contribution of escape to the total cyclic AMP turnover in the *cyc⁻* system has not been determined.

Different sensitivities of WT cells and the cyc⁻ variant to forskolin. The assessment of the potency of forskolin on different S49 cells was complicated because maximal rates of activation could not be achieved in all cases, presumably because of the limited solubility of the diterpene. As shown in Fig. 4, an apparent maximal rate of cyclic AMP accumulation occurred with 100 μM forskolin in WT cells. However, in the *cyc⁻* cells cyclic AMP accumulation was increased in parallel with increasing concentrations of forskolin. The WT cells were considerably more sensitive to forskolin than the *cyc⁻* cells. The half-maximal response in WT cells occurred at concentrations between 15 and 20 μM, whereas in *cyc⁻* cells the half-maximal concentration could not have been less than 100 μM.

The adenylate cyclase activities in membrane preparations from WT and *cyc⁻* cells were determined in the presence of various forskolin concentrations (Fig. 5). In

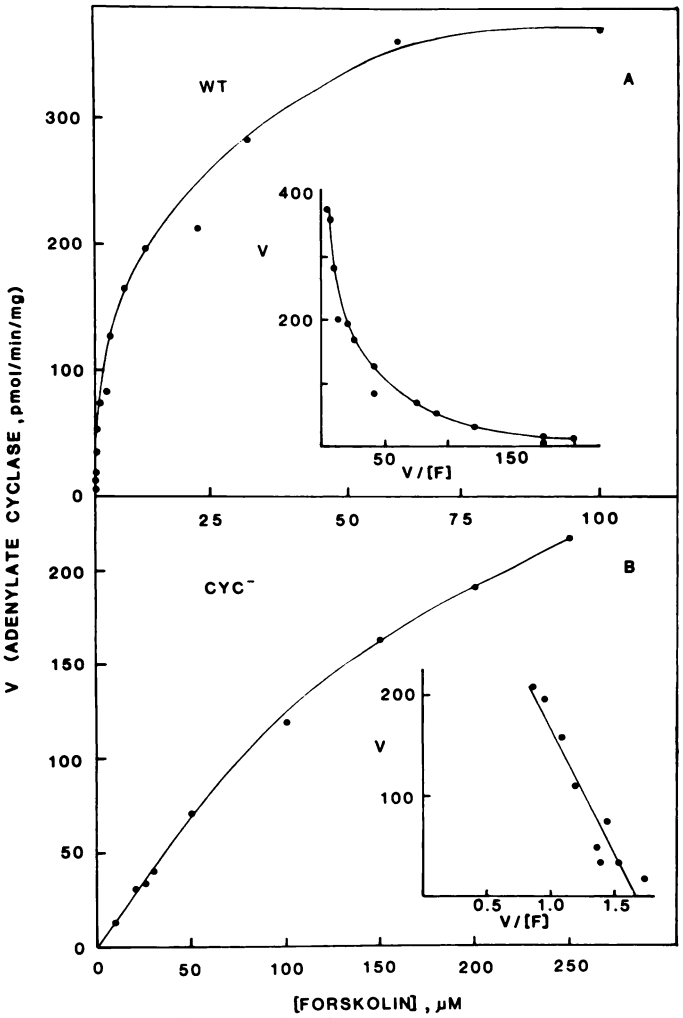

FIG. 5. *Forskolin activation of adenylate cyclase with various concentrations of forskolin*

The adenylate cyclase from WT (A) or *cyc⁻* membranes (B) at a protein concentration of 0.8 mg/ml was stimulated with various concentrations of forskolin for 20 min. The adenylate cyclase data are shown with the basal activities subtracted (29 pmoles/min/mg for WT and 0.34 pmole/min/mg for *cyc⁻*). The ethanol concentration in the *cyc⁻* assay was 2.5% throughout to maintain the solubility of the highest forskolin concentrations. Control experiments with WT membranes demonstrated that 2.5% ethanol did not affect the activity of adenylate cyclase. *Insets*: the data plotted by the Eadie-Hofstee method, where V is the adenylate cyclase activity (picomoles of cyclic AMP per minute per milligram) with basal activity subtracted, and $[F]$ is the forskolin concentration.

WT preparations, plots of V versus V/[forskolin] resulted in biphasic curves (see Fig. 5A, *inset*). This could be interpreted as suggesting two components in the forskolin response, one with an apparent high affinity for forskolin ($K_{app} \cong 0.35$ μM) and the other with a low affinity for forskolin ($K_{app} \cong 22.0$ μM). The K_{app} values were obtained by linear regression analysis of the Eadie-Hofstee plots at forskolin concentrations of 0.03–0.3 μM (high affinity) and 20–100 μM (low affinity). The biphasic kinetics was not a function of the lag, which was independent of forskolin concentration.

By contrast, forskolin activated adenylate cyclase in the *cyc*$^-$ membranes with kinetics described by a rectangular hyperbola. V versus V/[forskolin] plots of the data were linear (see Fig. 5B, *inset*) with K_{app} for activation of the enzyme system in *cyc*$^-$ membranes at around 244 μM. Values for V_{max} of the low-affinity component, estimated for both *cyc*$^-$ and WT by extrapolation on these plots, were 412 pmoles/min/mg for *cyc*$^-$ and 468 pmoles/min/mg for WT. This suggested that, although the adenylate cyclase system from WT cells was more sensitive to forskolin, the maximal achievable stimulations of preparations from both cell types were similar.

DISCUSSION

The description of the time delay in forskolin stimulation of cyclic AMP accumulation and adenylate cyclase in WT reported here is the first detailed evidence of this property of the diterpene. The duration of the lag was independent of the forskolin concentration and was eliminated by the presence of 0.1 μM epinephrine. It occurred without exception in four different clones of the S49 lymphoma WT and in a number of variants (all with functional G/F): the kinase$^-$ and low-phosphodiesterase line, 24.4.6; the kinase$^-$ line, 24.6.1; the high-phosphodiesterase line, K30a; and the cyclic AMP escape line, U200.95. In addition to the lymphoma cells, we found a lag in forskolin activation of intact human lung fibroblasts (WI-38) and crude particulate preparations of the neuroblastoma × glioma hybrid NG108-15. Seamon and Daly (3) reported a slight lag in the forskolin activation of adenylate cyclase in rat cerebral cortical membranes. Thus, the lag is certainly not an isolated property of the lymphoma cells and thus far we have found it to be absent only in the *cyc*$^-$ which lacks a functional G/F.

There were two additional differences between the responses of WT and *cyc*$^-$ cells to forskolin. First, preparations of WT cells were more sensitive to forskolin than those from *cyc*$^-$. Second, the V versus V/[forskolin] plots suggested that there were two components in WT membrane preparations with different affinities for forskolin. These characteristics of the forskolin activation of WT cells or membranes (that is, the time delay in activation, the independence of the lag with various forskolin concentrations, the elimination of the lag in the presence of epinephrine, and the presence of biphasic kinetics) are consistent with the idea that activated G/F plays a role in forskolin stimulation. One might speculate that forskolin stimulation of adenylate cyclase is associated with the stabilization of activated G/F. The lag would then be a reflection of the slow activation of G/F at basal rates. The acceleration of G/F activation by the addition of epinephrine would be expected to eliminate the time delay in adenylate cyclase activity.

Evidence that forskolin stimulated cyclic AMP accumulation without causing any appreciable desensitization in intact cell preparations of a variety of S49 cells has been presented here. Green and Clark (13) have shown that exposure of *cyc*$^-$ cells to epinephrine caused rapid desensitization of the adenylate cyclase complex without increasing cellular cyclic AMP levels. This finding, together with the data presented in this communication, localizes acute catecholamine desensitization to the *beta*-adrenergic receptor, at least in S49 cells. Furthermore, a role for cyclic AMP per se in epinephrine-specific, rapid desensitization of S49 cells is excluded.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of Mary Ellen Whitworth and Jacqueline Friedman.

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