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Distinct Mechanisms of Forskolin-Stimulated Cyclic AMP Accumulation and Forskolin-Potentiated Hormone Responses in C6-2B Cells

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SUMMARY

Forskolin activates a variety of adenylate cyclase systems and acts synergistically with receptor-mediated agonists which stimulate cyclic AMP production. The mechanism(s) and site(s) of forskolin action remain unclear. In C6-2B rat astrocytoma cells, forskolin stimulated greater than a 100-fold increase in cellular cyclic AMP content with a half-maximally effective concentration (EC_{50}) of greater than 50 μM. Incubation of C6-2B cells with forskolin plus (−)-isoproterenol resulted in an increase in (−)-isoproterenol efficacy and potency. The EC_{50} for the forskolin-induced increase in (−)-isoproterenol potency was 22 nM, greater than 3 orders of magnitude lower than the EC_{50} for direct forskolin-stimulated cyclic AMP accumulation. Forskolin had no effect on *beta*-receptor affinity for (−)-isoproterenol as measured by competition for (−)-[^{125}I]iodopindolol binding sites. Forskolin also augmented the responses to prostaglandin E_1 and cholera toxin. Inhibition of protein synthesis with cycloheximide markedly reduced forskolin-stimulated cyclic AMP accumulation with little or no effect on the responses to (−)-isoproterenol, prostaglandin E_1, or cholera toxin. The ability of forskolin to act synergistically with these agents was unaffected by cycloheximide treatment. These observations are compatible with a two-site model of forskolin action in C6-2B cells: a low-affinity site which mediates the direct action of forskolin to increase cellular cyclic AMP accumulation and a high-affinity site which mediates the potentiative action of forskolin. The low-affinity forskolin site appears to reside on a protein which is closely associated with the catalytic adenylate cyclase moiety and has a relatively shorter half-life than other components of the cyclase system. The high-affinity site resides on a more stable component of the adenylate cyclase system. The synergistic action of forskolin may involve an enhancement of the interaction between the guanine nucleotide-binding regulatory component and the catalytic component of the adenylate cyclase complex.

INTRODUCTION

The currently accepted model of the hormone-sensitive adenylate cyclase system in eukaryotic cells involves the interaction of at least three membrane-associated components: hormone receptor, guanine nucleotide-binding regulatory proteins (G)[2] and the catalytic component (C) (1). The diterpene forskolin, which stimulates cyclic AMP production in a variety of mammalian tissues and cell types, has been proposed to activate adenylate cyclase via a direct interaction with C or a closely associated protein (2). Forskolin also acts to augment hormone-stimulated cyclic AMP production in many model systems (2). This synergism is manifest as an increase in agonist potency or efficacy or as increases in both efficacy and potency.

Forskolin stimulates cyclic AMP accumulation in intact C6-2B rat astrocytoma cells (3). We recently reported that prolonged treatment of C6-2B cells with protein synthesis inhibitors causes a progressive and marked diminution of forskolin-stimulated cyclic AMP accumulation with minimal effects on the responses to (−)-isoproterenol or cholera toxin (3). In this communication, we now report the basic characteristics of the direct effects of forskolin to stimulate cyclic AMP accumulation and its ability to potentiate the responses to (−)-isoproterenol in C6-2B rat astrocytoma cells. The half-maximally effective concentration of forskolin for stimulating cyclic AMP accumulation was found to be at least 3 orders of magnitude greater than the concentra-

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[2] The abbreviations used are: G, the guanine nucleotide-binding regulatory proteins of adenylate cyclase; C, the catalytic adenylate cyclase protein; PGE_1, prostaglandin E_1; IBMX, 3-isobutyl-1-methylxanthine; [^{125}I]IPIN, (−)-[^{125}I]iodopindolol.

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tion of forskolin necessary for half-maximal potentiation of the response to (−)-isoproterenol. Prolonged treatment with cycloheximide, which produced a 90% reduction in the direct stimulatory effect of forskolin, did not reduce the ability of forskolin to potentiate (−)-isoproterenol-, PGE_1- or cholera toxin-stimulated cyclic AMP accumulation. These results indicate that the stimulatory and potentiative actions of forskolin in C6-2B cells are separable and involve different sites within the adenylate cyclase complex.

EXPERIMENTAL PROCEDURES

Materials. (−)-Isoproterenol, (±)-propranolol, PGE_1, IBMX, and cycloheximide were purchased from Sigma Chemical Company (St. Louis, Mo.). Cholera enterotoxin was purchased from Schwarz/Mann, Inc. (Spring Valley, N. Y.). Forskolin (7β-acetoxy-8,13-epoxy-1α-6β-9α-trihydroxy-labd-14-en-one) was purchased from Calbiochem-Behring Corporation (La Jolla, Calif.). Forskolin (10 mM) was dissolved in 95% ethanol and stored at −20°. Except as otherwise noted, forskolin dilutions were made in 95% ethanol such that 10 μl added to 1 ml of incubation medium (0.95% ethanol final concentration) gave the desired final concentration of forskolin. Ten microliters of 95% ethanol were added to control cells in the absence of forskolin. (−)-Pindolol was generously provided by Dr. Daniel Hauser (Sandoz, Basel, Switzerland). Carrier-free $Na^{125}I$ was purchased from Amersham Corporation (Arlington Heights, Ill.). Other chemicals were ACS reagent grade or better. Solutions were prepared with deionized, glass-distilled water.

Cell culture. Confluent monolayers of C6-2B rat astrocytoma cells were grown on 16-mm 24-well cluster plates (Flow Laboratories, McLean, Va.) in Ham's F-10 nutrient medium (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 10% donor calf serum (M. A. Bioproducts, Walkersville, Md.) in a humidified atmosphere of 95% air/5% CO_2 at 37° as previously described (4). Cells were used between passages 16 and 37. Cells were washed free of serum immediately before all experimental manipulations, which were carried out at 37° in a humidified atmosphere. Cells which were to be incubated with cycloheximide (5 μg/ml) were changed to serum-free medium 24 hr before the initiation of the experiment. This concentration of cycloheximide inhibited [^{3}H]leucine incorporation into trichloroacetic acid-precipitable protein by 93% (3). Except as otherwise noted, the data are presented as the mean of triplicate determinations. Standard errors were within 10% of the mean values shown.

Cyclic AMP assay. The cyclic AMP content of C6-2B cells was measured following drug treatment by rapidly aspirating the culture medium and treating the cells with 0.1 N HCl containing 0.1 mM $CaCl_2$. The plates were then agitated on an orbital shaker for 15 min at room temperature. The acid extracts were removed from the plates and stored at −20° until assayed. Samples were assayed for cyclic AMP content using the Gammaflo automated radioimmunoassay system (5) after acetylation as described by Harper and Brooker (6). The protein precipitated by treatment with HCl was dissolved in 1 ml of 0.2 N NaOH and measured using an automated form of the assay of Lowry *et al.* (7), using bovine serum albumin as the protein standard. Cellular cyclic AMP content is expressed on a per milligram of cell protein basis.

Binding of [^{125}I]IPIN. The radioiodination of (−)-pindolol to yield [^{125}I]IPIN and the binding of [^{125}I]IPIN to intact C6-2B cells were performed as previously described (8). (−)-Isoproterenol displacement of [^{125}I]IPIN was determined by adding increasingly greater concentrations of (−)-isoproterenol immediately after changing the cells to serum-free medium containing [^{125}I]IPIN. The concentration of (−)-isoproterenol which half-maximally inhibited the binding of [^{125}I]IPIN (IC_{50}) was determined graphically from displacement curves constructed using at least seven different concentrations of (−)-isoproterenol.

RESULTS

Forskolin-stimulated cyclic AMP accumulation in intact C6-2B rat astrocytoma cells reached a peak by 30 min and declined thereafter (Fig. 1). The maximal rate of cyclic AMP accumulation with 100 μM forskolin was attained after an approximately 2-min lag (Fig. 1, *inset*). This delay is similar to that observed in intact wild-type S49 murine lymphoma cells (9). A similar time course was observed when C6-2B cells were incubated concurrently with 1 μM forskolin and 0.01 μM (−)-isoproterenol (Fig. 1); however, there was no lag in the response to forskolin plus (−)-isoproterenol (Fig. 1, *inset*), and the response was about 10-fold greater than the response to 100 μM forskolin alone. In the presence of 0.95% ethanol, which had no effect on the basal or (−)-isoproterenol-stimulated cyclic AMP content of C6-2B cells (data not shown), a 30-min challenge with forskolin (100 μM) produced greater than a 100-fold increase in cellular cyclic AMP content (Table 1). The lowest concentration of forskolin that increased cyclic AMP accumulation was 10 μM. Under these conditions (0.95% ethanol), 100 μM forskolin was the highest concentration tested, since this approached the limit of forskolin solubility. The half-maximally effective concentration of forskolin could therefore only be estimated as greater than 50 μM. In the presence of 3% ethanol, higher forskolin concentrations could be attained, but the responses were considerably diminished as compared with 0.95% ethanol (3% ethanol, 320 μM forskolin: 1134 ± 336 pmoles of cyclic AMP per milligram of protein versus 0.95% ethanol, 100 μM for-

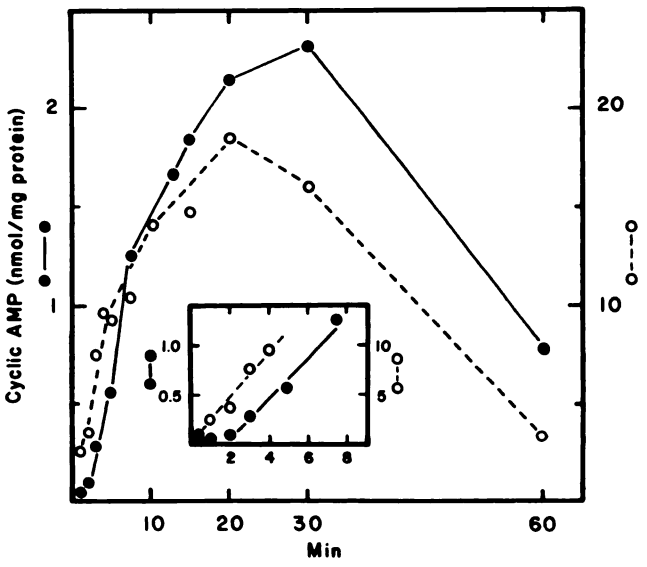

FIG. 1. *Time course of forskolin-stimulated cyclic AMP accumulation*
Intact C6-2B cells were incubated with 100 μM forskolin (●——●) or 1 μM forskolin plus 0.01 μM (−)-isoproterenol (○– – –○) for the time periods indicated. For time periods of 5 min or less, drugs were added to cells while the cluster plates were being rotated on a heating block to provide mixing and temperature maintenance. The *inset* shows 0–8 min on an expanded scale. The lines depict the maximal rates of cyclic AMP accumulation as determined by linear regression analysis. The lag in the response to forskolin was determined as the *x*-intercept of the calculated line.

TABLE 1

Effect of forskolin on cyclic AMP accumulation, (−)-isoproterenol potency, and beta-receptor affinity after 24-hr pretreatment of C6-2B cells with or without cycloheximide

For cyclic AMP accumulation, intact C6-2B cells were incubated for 24 hr with or without cycloheximide (CH) (5 μg/ml) and then for 30 min with forskolin or an equivalent concentration of ethanol (0.95%) and/or (−)-isoproterenol. The data are presented as the mean ± standard error of the mean of triplicate determinations. The half-maximally effective (−)-isoproterenol concentrations (EC_{50}) were determined graphically from the data in Fig. 2. For competition with [^{125}I]IPIN, intact C6-2B cells were incubated with (−)-isoproterenol in the presence of forskolin or an equivalent concentration of ethanol (0.95%), and the concentration of (−)-isoproterenol which half-maximally inhibited [^{125}I]IPIN binding (IC_{50}) was determined as described under Materials and Methods.

Forskolin	Cyclic AMP		Isoproterenol EC_{50}		Isoproterenol IC_{50}
	− CH	+ CH	− CH	+ CH	
μM	pmoles/mg protein		nM		μM
0	34 ± 7	47 ± 4	11.2	37.6	26
0.001	27 ± 2	44 ± 5	12.6	35.5	ND[a]
0.01	12 ± 3	40 ± 2	6.7	24.3	ND
0.1	16 ± 3	40 ± 4	4.3	15.0	25
1.0	26 ± 4	49 ± 3	1.0	2.5	26
10.0	60 ± 4	47 ± 8	0.6	1.2	ND
30.0	536 ± 97	ND	ND	ND	ND
100.0	4978 ± 330	435 ± 55	0.3	1.0	24

[a] ND, not determined.

skolin: 4949 ± 174 pmoles of cyclic AMP per milligram of protein, $n = 3$). All subsequent experiments were done in 0.95% ethanol.

Forskolin acted to increase (−)-isoproterenol-stimulated cyclic AMP accumulation when C6-2B cells were incubated with both agents simultaneously (Fig. 2A). This effect was manifest as an increase in (−)-isoproterenol efficacy, as evidenced by the greater than additive effect of forskolin with maximal (−)-isoproterenol concentrations. Forskolin also increased (−)-isoproterenol potency, which was apparent as a decrease in the half-maximally effective concentration of (−)-isoproterenol (EC_{50}) with increasing concentrations of forskolin (Table 1). When the (−)-isoproterenol EC_{50} values were plotted versus forskolin concentration, the EC_{50} for the action of forskolin to increase (−)-isoproterenol potency was determined to be 22 nM. Forskolin potentiation of the response to (−)-isoproterenol thus occurred at an EC_{50} at least 3 orders of magnitude lower than the EC_{50} for forskolin-stimulated cyclic AMP accumulation. When competition for [^{125}I]IPIN binding sites was determined, the IC_{50} for (−)-isoproterenol was 26 μM. In the presence of forskolin, the (−)-isoproterenol competition curves were identical with that determined in the absence of forskolin, as were the IC_{50} values (Table 1). Forskolin thus had no effect on *beta*-receptor affinity for (−)-isoproterenol, which, combined with the decrease in the (−)-isoproterenol EC_{50}, resulted in an apparent increase in receptor-cyclase coupling efficiency (IC_{50}/EC_{50}).

We have previously reported that prolonged inhibition of C6-2B cellular protein synthesis causes a progressive and profound decrease in forskolin-stimulated cyclic AMP accumulation with no effect on basal cellular cyclic AMP content and little or no effect on the responses to maximal concentration of forskolin was reduced by greater than 90% after a 24-hr treatment with cycloheximide (Table 1). The response to (−)-isoproterenol was not affected after treatment of cells for 24 hr with cycloheximide. A 24-hr treatment with cycloheximide had little effect on (−)-isoproterenol efficacy (Fig. 2B) but increased the EC_{50} for (−)-isoproterenol-stimulated cyclic AMP accumulation several-fold (Table 1). After cycloheximide treatment, the response to (−)-isoproterenol in the presence of higher concentrations of forskolin was decreased approximately 20% (Fig. 2). This is accounted for by the loss of the direct stimulatory effect of these concentrations of forskolin after cycloheximide treatment. The ability of forskolin to increase (−)-isoproterenol potency was unaffected by inhibition of protein synthesis (Table 1). The EC_{50} for forskolin to increase (−)-isoproterenol potency after cycloheximide treatment was 27 nM. Thus, although a 24-hr pretreatment with cycloheximide markedly decreased the direct action of forskolin to stimulate cyclic AMP accumulation in C6-2B cells, inhibition of protein synthesis had no effect on the action of forskolin to increase (−)-isoproterenol efficacy or potency.

The potentiation of PGE_1-stimulated cyclic AMP accumulation by forskolin was also unaffected by treatment of C6-2B cells with cycloheximide (Table 2). PGE_1 (10 μM) stimulated only modest increases in cellular cyclic AMP content in the absence of a phosphodiesterase inhibitor. However, in the presence of a concentration of IBMX, which alone had no effect on cyclic AMP accumulation (100 μM), PGE_1 produced a 4-fold increase in cellular cyclic AMP content. Forskolin markedly potentiated the response to PGE_1 in the absence or presence (−)-isoproterenol or cholera toxin (3). The response to a

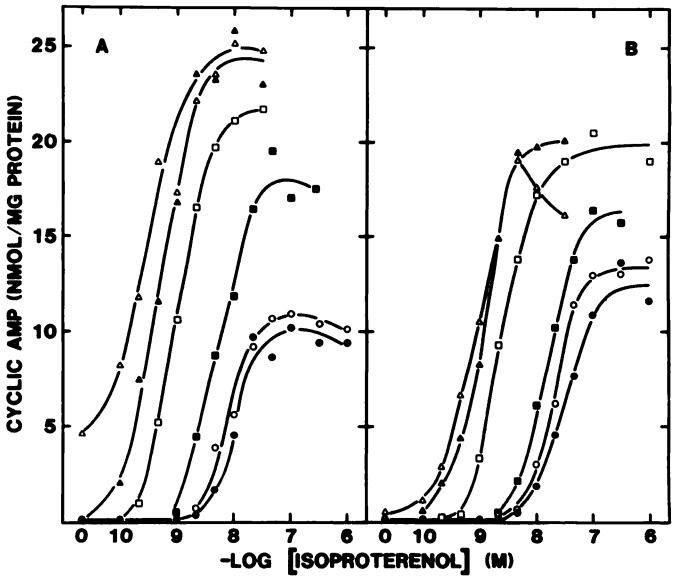

FIG. 2. *Effect of forskolin on cellular cyclic AMP content and (−)-isoproterenol-stimulated cyclic AMP accumulation*

Intact C6-2B cells were incubated for 24 hr in the absence (A) or presence (B) of cycloheximide (5 μg/ml) and then for 30 min with the indicated concentrations of (−)-isoproterenol in the presence of 0.95% ethanol (●) or 0.01 (○), 0.1 (■), 1.0 (□), 10.0 (▲) or 100.0 (△) μM forskolin.

TABLE 2

Effect of 24-hr pretreatment of C6-2B cells with cycloheximide on forskolin potentiation of PGE_1- and cholera toxin-stimulated cyclic AMP accumulation

Intact C6-2B cells were incubated for 24 hr with or without cycloheximide (5 μg/ml). Cellular cyclic AMP content was determined after a 15-min challenge with PGE_1 (10 μM) in the presence or absence of IBMX (100 μM), forskolin (1 μM), or an equivalent concentration of ethanol (0.95%). In a separate experiment, cellular cyclic AMP content was determined in control or cycloheximide-treated cells after a 3-hr challenge with cholera toxin (6 nM) in the absence or presence of forskolin or ethanol (0.95%). In order to compare with the 24-hr cycloheximide-treated cells, cycloheximide was included during the control 3-hr challenge with cholera toxin since previous studies have shown that cycloheximide increases the response to cholera toxin (10). In the absence of cycloheximide, a 3-hr challenge with cholera toxin increased cyclic AMP levels to 666 ± 91 pmoles/mg of protein. The data are presented as the mean ± standard error of the mean of triplicate determinations.

	Cyclic AMP			
	Ethanol		Forskolin	
	Control	Cycloheximide	Control	Cycloheximide
	pmoles/mg protein			
Basal	10 ± 1	11 ± 2	17 ± 2	12 ± 4
IBMX	14 ± 2	16 ± 3	43 ± 6	20 ± 4
PGE_1	15 ± 1	18 ± 1	250 ± 23	293 ± 32
PGE_1 + IBMX	41 ± 1	53 ± 8	528 ± 28	574 ± 64
Basal	30 ± 2	23 ± 2	44 ± 4	42 ± 8
Cholera toxin	1,271 ± 257	2,545 ± 328	11,370 ± 639	12,887 ± 640

of IBMX. Pretreatment of the cells with cycloheximide for 24 hr had no effect on these responses.

We have previously shown that cycloheximide has no effect on the cyclic AMP response of C6-2B cells to a 3-hr challenge with cholera toxin (3). When forskolin is added during the incubation with cholera toxin (6 nM), a marked potentiation of this response was observed (Table 2). The ability of forskolin to potentiate the response to cholera toxin was unaffected by treatment with cycloheximide.

DISCUSSION

Seamon and Daly (2) proposed that forskolin stimulates adenylate cyclase activity via a direct interaction with the catalytic moiety of the hormone receptor-guanine nucleotide regulatory proteins-catalytic cyclase complex. The observation that low concentrations of forskolin act synergistically with receptor-mediated activators of the system led Daly *et al.* (11) to conclude that forskolin may interact with more than one site within the adenylate cyclase complex to prime the system for further activation. Alternatively, a kinetic model was proposed (11) in which the binding of forskolin increases the binding and action of receptor-mediated agonists. The data in the present report support a two-site model of forskolin action in C6-2B cells.

Forskolin stimulated cyclic AMP accumulation in intact C6-2B cells with an EC_{50} of greater than 50 μM. Forskolin augmented the response to (−)-isoproterenol at much lower concentrations and induced an increase in (−)-isoproterenol potency with an EC_{50} of 22 nM. Forskolin, at concentrations as high as 100 μM, did not alter the affinity of the *beta*-receptor for (−)-isoproterenol, as measured by competition for [^{125}I]IPIN binding sites (IC_{50}). Forskolin thus increased the receptor/cyclase coupling efficiency, defined as the ratio IC_{50}/EC_{50} for (−)-isoproterenol binding affinity and stimulation of cyclic AMP accumulation (4), at concentrations of forskolin well below those which stimulated an increase in cellular cyclic AMP content. Treating C6-2B cells with protein synthesis inhibitors has only minimal effect on (−)-isoproterenol-stimulated cyclic AMP accumulation and no apparent effect on the response to cholera toxin (3) or PGE_1. It thus appears that the hormone receptor(s)-G-C interactions remain intact after treatment with cycloheximide. In contrast, the response to forskolin alone is noticeably diminished by treatment with cycloheximide for periods as short as 1 hr (3). The response to forskolin is progressively and markedly diminished with longer treatments with cycloheximide, with up to a 90% loss of response after 24 hr with cycloheximide. In a one-site model, one would expect to see not only a reduction in forskolin-stimulated cyclic AMP accumulation but also a concomitant reduction in forskolin-potentiation of hormone responses, or at least a decrease in the potency of forskolin to augment these responses. This was not the case, however, since the efficacy of submaximal forskolin concentrations to augment the responses to (−)-isoproterenol, PGE_1, or cholera toxin and to potentiate the response to (−)-isoproterenol was unaffected by prolonged inhibition of protein synthesis. It therefore appears that forskolin stimulates cyclic AMP production via a low-affinity site residing on a protein which is closely associated with the catalytic moiety of the adenylate cyclase complex and which has a relatively shorter half-life than other components of the system. The high-affinity site with which forskolin interacts to augment the responses to drugs and hormones remained intact after cycloheximide treatment and thus may reside on one of the remaining components or possibly another unrecognized component.

It has become increasingly apparent that guanine nucleotide regulatory proteins play a significant role in the mechanism of forskolin action. The lag in the response seen in C6-2B cells and wild-type S49 cells and membranes is similar to that seen with nonhydrolyzable GTP analogues and is absent in G-deficient *cyc*$^-$ S49 cells and membranes (9). The lag in the forskolin response can be

observed in *cyc*⁻ cell membranes after reconstitution with wild-type G (12). In turkey erythrocytes, forskolin decreases *beta*-receptor affinity (13), again similar to the effect of guanine nucleotides. But in S49 (14) and C6-2B cells, forskolin has no effect on *beta*-receptor affinity, although the proportion of *beta*-receptors in the "high-affinity" state is increased by forskolin in S49 cells (14). An effect of forskolin on receptor affinity does not appear to be a universal explanation for the potentiation of *beta*-receptor-mediated responses. Forskolin can restore the response to (−)-isoproterenol in the S49 cell variant, H21a, in which the G-C interaction is defective, but not in *cyc*⁻ or the S49 cell variant *unc*, in which the receptor-G interaction is defective (14). Since forskolin also augmented the response of C6-2B cells to cholera toxin, which is known to stimulate adenylate cyclase activity via an interaction with G (15, 16), the forskolin-induced potentiation of hormone responses may involve the interaction between G and C.

Pfeuffer and Metzger (17) have partially purified C from rabbit myocardium by affinity chromatography using a biologically active forskolin derivative coupled to a Sepharose support matrix. Duck and pigeon erythrocyte adenylate cyclases, which are insensitive to forskolin, do not bind to the forskolin affinity column, leading Pfeuffer and Metzger (17) to consider the existence of a forskolin-binding component which is closely associated with C. Turkey erythrocyte adenylate cyclase is also insensitive to forskolin alone (13). Forskolin does act in this system to augment the responses to isoproterenol. Recognizing that C6-2B cells, S49 cells, and avian erythrocytes are diverse cell types, the responses of these systems to forskolin, considered in concert, are consistent with the hypothesis that forskolin stimulates adenylate cyclase activity indirectly through a protein closely associated with C and augments hormone responses by enhancing the G-C interaction via a site residing on G, C, or another unrecognized component of the adenylate cyclase system. The protein component through which forskolin is proposed to stimulate adenylate cyclase activity appears to be tightly bound to C, as forskolin sensitivity is retained in C prepared from rabbit hepatic membranes by cholate solubilization at high ionic strength followed by gel filtration (18). On the other hand, adenylate cyclase preparations which are not responsive to forskolin, such as mammalian sperm (19, 20) or *Bordetella pertussis* (21), may be missing this protein. The identification of a protein component of adenylate cyclase which would confer forskolin sensitivity to these preparations remains to be accomplished.

In summary, the data in the present report supports a two-site model of forskolin action in C6-2B cells. This hypothesis is based on (a) the markedly different concentration dependence of forskolin-stimulated cyclic AMP accumulation and forskolin-augmented hormone responses and (b) the selective effect of protein synthesis inhibition to produce a marked decrease in forskolin stimulation of cellular cyclic AMP content with no effect on forskolin potentiation of hormone responses. The effect of low concentrations of forskolin to potentiate the action of (−)-isoproterenol may involve an enhanced G-C interaction since (a) forskolin had no effect on the affinity of the *beta*-receptor for (−)-isoproterenol and (b) the response to cholera toxin can be potentiated by forskolin. It thus appears that a low-affinity forskolin site resides on a protein with a relatively shorter half-life than other components of the cyclase system, as we have previously postulated (3). This protein would be required to mediate the action of forskolin to increase cellular cyclic AMP accumulation. A high-affinity forskolin site which mediates the potentiative action of forskolin appears to reside on a more stable component of the receptor-cyclase system.

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