MOLECULAR PHARMACOLOGY, 28:502–507

MOL PHARM

Forskolin Potentiation of Cholera Toxin-Stimulated Cyclic AMP Accumulation in Intact C6-2B Cells

Evidence for Enhanced G_s-C Coupling

KENNETH BAROVSKY AND GARY BROOKER

Department of Biochemistry, Georgetown University Medical Center, Washington, D. C. 20007

Received May 7, 1985; Accepted September 25, 1985

SUMMARY

Forskolin directly stimulates adenylate cyclase activity and acts synergistically with receptor-mediated agonists which stimulate cyclic AMP production. We have previously observed that a 3-hr incubation of C6-2B rat astrocytoma cells with 6 nM cholera toxin in the presence of 1 μM forskolin results in cyclic AMP accumulation 9-fold greater than in the absence of forskolin. Since the action of cholera toxin is mediated by the stimulatory guanine nucleotide-binding regulatory component (G_s) of the adenylate cyclase complex, we proposed that the mechanism by which forskolin augments hormone responses involves an enhanced coupling of G_s with the adenylate cyclase catalytic component (C). In the present communication, we report the detailed characterization of the synergistic interaction between forskolin and cholera toxin as effectors of cyclic AMP accumulation in intact C6-2B cells. After a 3-hr incubation, maximal cholera toxin-stimulated cyclic AMP accumulation was 990 ± 34 pmol/mg of protein. In the presence of 1 μM forskolin, the response to cholera toxin increased to 13,137 ± 1,595 pmol of cyclic AMP/mg of protein. The half-maximally effective cholera toxin concentrations estimated by nonlinear least squares regression analysis determined in the absence or presence of 0.1 mM forskolin were 56.6 and 57.5 pM, respectively. The highly reproducible lag in forskolin-stimulated cyclic AMP accumulation in C6-2B cells was abolished by cholera toxin pretreatment, indicating a possible role for G_s-associated GTPase in the mechanism of forskolin action. Cholera toxin treatment markedly augmented forskolin-stimulated cyclic AMP accumulation and shifted the forskolin concentration-response curve to the left approximately 1.5 log units. When C6-2B cells were treated for 1 min with 10 nM cholera toxin, the response to forskolin was significantly potentiated by 10 min. No significant increase in cellular cyclic AMP content in the absence of a forskolin challenge was apparent for up to 45 min. It appears that prior promotion of G_s-C coupling by cholera toxin treatment enhances the ability of forskolin to stimulate cyclic AMP accumulation. Whether or not forskolin interacts (i.e., binds) exclusively to C remains to be proven. However, the actions of forskolin to stimulate cyclic AMP formation and potentiate agonist-stimulated cyclic AMP formation are modulated by the activity state of G_s, and at least part of the response to forskolin is mediated by G_s.

INTRODUCTION

Hormonal stimulation of adenylate cyclase-catalyzed cyclic AMP formation in eukaryotic organisms involves the interaction of at least three functionally and physically separable components (1): the stimulatory hormone receptor, to which hormones initially bind and which acts to discriminate between agonists and antagonists; the stimulatory coupling component, G_s,[1] a multi-subunit complex (2) which binds and hydrolyzes GTP, thereby regulating both the hormone-receptor interaction and the activity state of adenylate cyclase; and the catalytic component, C, by which ATP is enzymatically converted

This work was supported by a grant from the Nation's Capital Affiliate of the American Heart Association (K. B.) and by National Institutes of Health Grant HL28940 (G. B.).

[1] The abbreviations used are: G_s, G_i, the guanine nucleotide-binding regulatory complexes of adenylate cyclase which mediate hormonal stimulation or inhibition, respectively; C, the catalytic component of adenylate cyclase; EC_{50}, half-maximally effective drug concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine.

0026-895X/85/060502-06$02.00/0
Copyright © 1985 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

to cyclic AMP. The free α-subunit of G_s can activate C (3, 4). Stimulation of adenylate cyclase activity can be effected by agents which interact directly with G_s, e.g., cholera toxin, an enterotoxin from *Vibrio cholerae* (2), or with C, e.g., forskolin, a diterpene derived from an extract from the roots of *Coleus forskohlii* (5).

The mechanism by which cholera toxin stimulates adenylate cyclase activity has been extensively investigated (see Refs. 6 and 7 for review). Cholera toxin is made up of two major components. The B-component, consisting of five similar subunits, functions to bind to ganglioside Gm_1, the cell surface receptor for choleragen. The subsequent activation of adenylate cyclase by the A-subunit follows a lag which may be due to the time required for the A-subunit to traverse the cell membrane. Cholera toxin catalyzes the NAD-dependent ADP-ribosylation of the α-subunit of G_s (2). The consequent stimulation of GTP release from G_s (8) and inhibition of G_s-associated GTP hydrolysis, which normally functions as the cyclase inactivation process, results in a long-lived activation of adenylate cyclase (9, 10). In addition, cholera toxin-catalyzed ADP-ribosylation of the α-subunit of G_s promotes its dissociation from the β-subunit (11), which may directly account for toxin stimulation of adenylate cyclase activity.

The mechanism by which forskolin stimulates adenylate cyclase activity is less clear. It appears that forskolin can interact directly with C to stimulate cyclic AMP synthesis (12–18), although the presence of G_s may be required for the full expression of forskolin action (18–22). In addition, at concentrations below those which directly stimulate cyclic AMP formation, forskolin acts synergistically with hormones as effectors of cyclic AMP production (5). The mechanism of the synergistic activation of adenylate cyclase by hormones and forskolin remains enigmatic but has been proposed to be the result of a forskolin-altered state of C (17).

In intact C6-2B rat glioma cells, forskolin stimulates cyclic AMP accumulation with an EC_{50} estimated to be greater than 50 μM (23). Forskolin also acts synergistically with the β-adrenergic agonist, isoproterenol, to produce an increase in both efficacy and potency of isoproterenol-stimulated cyclic AMP accumulation. The EC_{50} for forskolin-increased potency of the response to isoproterenol is approximately 20 nM (23). In consideration of this greater than three orders of magnitude difference in forskolin concentrations required to half-maximally stimulate cyclic AMP accumulation and increase the sensitivity to isoproterenol, we proposed a two-site model for forskolin action in C6-2B cells (23, 24). It appears that forskolin acts to stimulate cyclic AMP production via a low affinity site residing on a rapidly turning-over protein which may be closely associated with C. Forskolin acts synergistically with other agents which stimulate cyclic AMP formation via a high affinity site which appears to be separate from the low affinity site but has yet to be localized. However, the preliminary observation that forskolin acts synergistically with cholera toxin (23) suggests that an enhanced G_s-C interaction may be the mechanism by which forskolin enhances hormone-stimulated adenylate cyclase activity.

In the present communication, the interaction of forskolin and cholera toxin as effectors of cyclic AMP accumulation in intact C6-2B cells is examined in greater detail. It has been proposed that the synergistic action of forskolin is due to a forskolin-induced alteration in the state of C (17). Our present results suggest that, whether or not forskolin interacts (i.e., binds) exclusively to C, the actions of forskolin are modulated by the activity state of G_s and that at least part of the response to forskolin is mediated by G_s.

EXPERIMENTAL PROCEDURES

Materials. (−)-Isoproterenol and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO). (−)-Isoproterenol was dissolved in 5 mM HCl containing 0.1% sodium metabisulfite such that 10 μl added to 1 ml of medium (0.001% final sodium metabisulfite) gave the desired (−)-isoproterenol concentration. Cholera toxin was purchased from Schwarz/Mann (Spring Valley, NY) or List Biological Laboratories (Campbell, CA). Forskolin (7β-acetoxy-8,13-epoxy-1α-6β-9α-trihydroxy-labd-14-en-11-one) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Forskolin (10 mM) was dissolved in 95% ethanol and stored at −20°. Forskolin dilutions were made in 95% ethanol such that 10 μl added to 1 ml of medium (0.95% ethanol final concentration) gave the desired final forskolin concentration. Other chemicals were ACS reagent grade or better. Solutions were prepared with deionized, glass-distilled water.

Cell culture. C6-2B rat glioma cells were grown as monolayers on 16-mm 24-well cluster plates (Nunc; Vangard International, Inc., Neptune, NJ) in Ham's F-10 nutrient medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% donor calf serum (M. A. Bioproducts; Walkersville, MD) in a humidified atmosphere of 95% air, 5% CO_2 at 37° (25). All assays were performed on confluent culture wells containing 0.9–1.3 × 10^6 cells/well. Cells were used between passages 16 and 36. Cells were washed free of serum using Ham's F-10 medium containing 20 mM HEPES buffer (pH 7.4) immediately before all experimental manipulations, which were done in a humidified 37° warm-room. The phosphodiesterase inhibitor IBMX (0.1 mM) was added to the serum-free Ham's F-10/HEPES culture medium.

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 M HCl containing 0.1 mM $CaCl_2$. The culture plates were then agitated on an orbital shaker for 15 min at room temperature. The HCl extract of the cells was removed from the plates and stored at −20° until assayed for cyclic AMP concentration using the Gammaflow automated radioimmunoassay system (26) after acetylation by the method of Harper and Brooker (27). 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.* (28) with bovine serum albumin as the protein standard. Cellular cyclic AMP content is expressed on a per mg of protein basis.

The data are presented as the mean ± standard error of triplicate determinations. The absence of error bars indicates that the magnitude of the error is less than the size of the symbol in the graph. Statistical comparisons were performed using an unpaired *t* test. Differences with *p* values less than 0.05 were considered significant. The half-maximally effective concentrations of cholera toxin were determined using nonlinear least squares regression analysis of primary, untransformed data for parameter and confidence interval estimation (29). The data were fit to the hyperbolic function $R = (R_{max} * X)/(K_x + X)$, where R_{max} is the maximal response, X is the cholera toxin concentration, R is the response at a given cholera toxin concentration, and K_x is the half-maximally effective concentration of cholera toxin. The data are plotted as $\log_{10}$[cholera toxin] versus cellular cyclic AMP content. The calculated half-maximally effective cholera toxin concentrations are re-

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

ported along with 65% confidence intervals, representing approximately 1 SD.

RESULTS

Effect of forskolin on cholera toxin-stimulated cyclic AMP accumulation. Our previous studies have shown that co-incubation of intact C6-2B rat astrocytoma cells for 3 hr with 6 nM cholera toxin and 1 μM forskolin results in a 9-fold greater increase in cyclic AMP accumulation than with cholera toxin alone (23). To explore further the mechanism of the synergistic interaction between cholera toxin and forskolin as effectors of cyclic AMP accumulation, we determined the effect of forskolin on the time course and concentration-response relationship for cholera toxin-stimulated cyclic AMP accumulation. In intact C6-2B cells, cholera toxin-stimulated cyclic AMP accumulation proceeds following a lag of 60–90 min (30). In the presence of the phosphodiesterase inhibitor, IBMX (0.1 mM), the time course of the response to a submaximal concentration of cholera toxin (0.1 nM) was qualitatively similar whether determined in the absence or the presence of 1 μM forskolin (Fig. 1A). In the absence of forskolin, cholera toxin-stimulated cyclic AMP accumulation increased after a 60-min delay to a maximum level of 565 ± 55 pmol of cyclic AMP/mg of protein by 3 hr. In the presence of forskolin, a significant elevation of cyclic AMP levels was also not observed until 60 min of incubation with cholera toxin. A maximum level of cyclic AMP accumulation (4649 ± 622 pmol of cyclic AMP/mg of protein) was attained after 3 hr with cholera toxin plus forskolin. A marked synergy between cholera toxin and forskolin as effectors of cyclic AMP formation was observed at all time points at which cholera toxin-stimulated cyclic AMP levels were elevated above basal.

The effect of forskolin on the concentration-response relationship of cholera toxin-stimulated cyclic AMP accumulation was determined in the presence of IBMX using a 3-hr incubation duration (Fig. 1B). The maximum responses to cholera toxin in the absence or presence of 0.1 mM forskolin were 990 ± 34 and 13,137 ± 1,595 pmol of cyclic AMP/mg of protein, respectively. The EC_{50} values (and 65% confidence intervals) determined by nonlinear least squares regression analysis as described under "Experimental Procedures" for cholera toxin-stimulated cyclic AMP accumulation in the absence or presence of forskolin were 56.6 (41.3, 77.1) or 57.5 (45.8, 72.1) pM, respectively. Thus, forskolin produced a more than 13-fold increase in cholera toxin efficacy but no alteration in cholera toxin potency.

Effect of cholera toxin pretreatment on forskolin-stimulated cyclic AMP accumulation. As we have reported previously (23), forskolin stimulates cyclic AMP accumulation in intact C6-2B cells following a reproducible lag of 1–2 min. The lag in the response to forskolin was completely abolished after a 3-hr treatment with 10 nM cholera toxin (Fig. 2).

Forskolin alone stimulates cyclic AMP accumulation in C6-2B cells at concentrations greater than 1 μM (23). The forskolin concentration-dependent increase in cyclic AMP levels does not reach a maximum at the highest concentration tested (0.1 mM). The EC_{50} for forskolin-stimulated cyclic AMP accumulation in C6-2B cells could only be estimated as at least 25 μM in the experiment depicted in Fig. 3, assuming that the response to 0.1 mM forskolin represents a maximal response. The forskolin response was markedly augmented after a 3-hr treatment

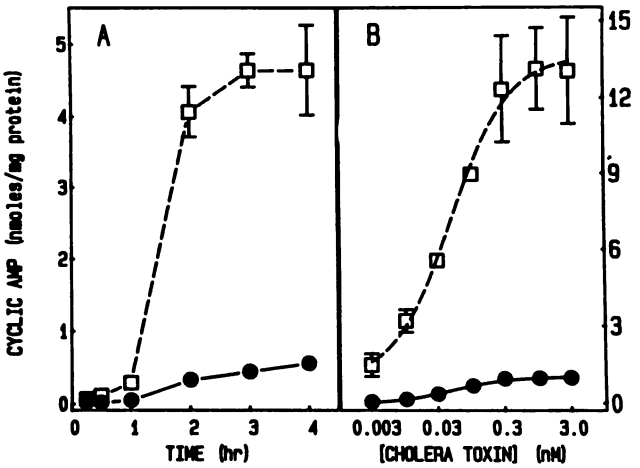

FIG. 1. *Effect of forskolin on the time course and dose-response relationship of cholera toxin-stimulated cyclic AMP accumulation*

A, C6-2B cells were incubated with 0.1 nM cholera toxin in the presence (□) or absence (●) of 1 μM forskolin for the indicated times. B, C6-2B cells were incubated for 3 hr with the indicated concentrations of cholera toxin in the absence (●) or presence (□) of 0.1 mM forskolin. The data are presented as the means ± standard errors of triplicate determinations and are representative of five (A) or four (B) similar experiments. The curves in B were calculated from nonlinear least squares regression analysis of primary experimental data as described under "Experimental Procedures."

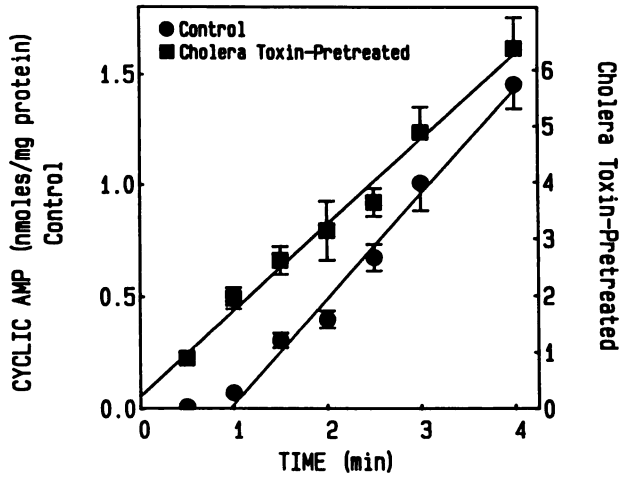

FIG. 2. *Effect of cholera toxin pretreatment on the time course of forskolin-stimulated cyclic AMP accumulation*

C6-2B cells were incubated for 3 hr without (●) or with (■) 10 nM cholera toxin. After washing five times with 1 ml of fresh medium per well to remove free toxin, cells were incubated with 0.1 mM forskolin for the indicated times. The lines depict the *maximal* rates of cyclic AMP accumulation as determined by linear regression analysis. The time delay in the response to forskolin was determined as the *x*-intercept of the calculated line. The data are presented as the means ± standard errors of triplicate determinations and are representative of nine similar experiments.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

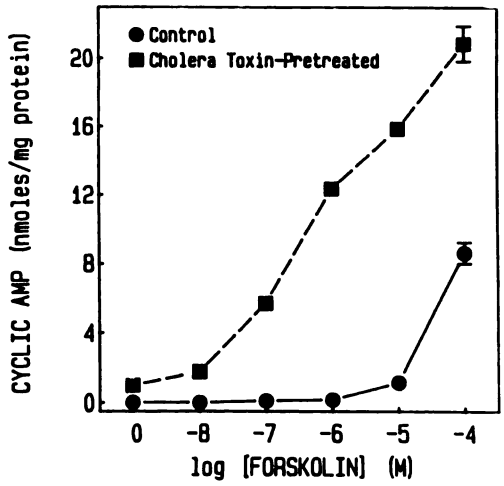

FIG. 3. *Effect of cholera toxin pretreatment on the concentration-response relationship of forskolin-stimulated cyclic AMP accumulation*

C6-2B cells were incubated for 3 hr with 0.3 nM cholera toxin. After washing as described in the legend to Fig. 2 to remove free toxin, cells were incubated for 30 min with the indicated concentrations of forskolin. The data are presented as the means ± standard errors of triplicate determinations and are representative of six similar experiments.

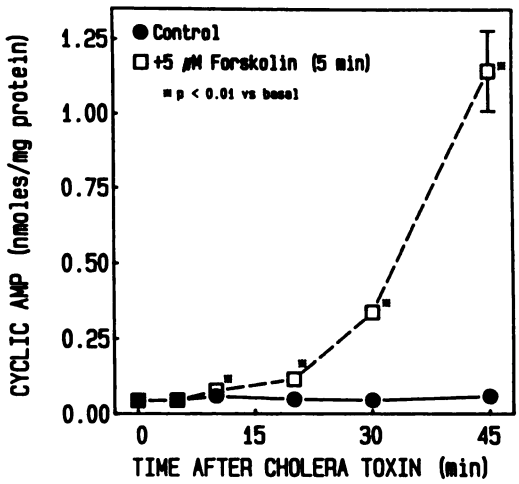

FIG. 4. *Time course of development of the synergistic interaction between cholera toxin and forskolin as effectors of cyclic AMP accumulation*

C6-2B cells were incubated with 10 nM cholera toxin for 1 min and then washed as described in the legend to Fig. 2 to remove free toxin. At the indicated times, the culture medium was rapidly changed to medium without (●) or with (□) 5 μM forskolin, and the incubation was continued for 5 min. The data are presented as the means ± standard errors of triplicate determinations and are representative of three similar experiments.

of C6-2B cells with 0.3 nM cholera toxin. A significant response to 10 nM forskolin was observed after toxin treatment, and the forskolin concentration-response curve was shifted at least 1.5 log units to the left. The EC_{50} for forskolin-stimulated cyclic AMP accumulation after cholera toxin treatment was estimated to be at least 0.6 μM, again assuming that the response to 0.1 mM forskolin represents a maximal response.

It was not necessary to preincubate C6-2B cells for 3 hr with cholera toxin to see the synergistic interaction between cholera toxin and forskolin as effectors of cyclic AMP accumulation. In the experiment depicted in Fig. 4, C6-2B cells were incubated with 10 nM cholera toxin for 1 min and washed to remove free toxin. After the indicated incubation times, the culture medium was rapidly changed to one without or with 5 μM forskolin and the incubation was continued for an additional 5 min. No increase in cyclic AMP accumulation was seen in cells treated with cholera toxin that were not subsequently challenged with forskolin. In cells which had been pretreated with cholera toxin, the response to the forskolin challenge was significantly elevated above basal level after 10 min (45 ± 2 versus 79 ± 3 pmol of cyclic AMP/mg of protein). It appears that, despite the lack of an observable direct effect of cholera toxin on cyclic AMP levels for up to 45 min, the potentiation of the response to forskolin by cholera toxin treatment was apparent by 10 min.

DISCUSSION

Forskolin appears to stimulate cyclic AMP synthesis via a direct interaction with C, the catalytic component of the adenylate cyclase complex (12–18). It has become apparent that G_s, the guanine nucleotide-binding component of adenylate cyclase which mediates the effects of stimulatory hormone receptor-agonists, also plays a significant role in the mechanism of forskolin action. Results from numerous investigations indicate that G_s is required for the full expression of forskolin-stimulated adenylate cyclase activity (18–22).

In addition to directly stimulating cyclic AMP production, forskolin augments hormone-stimulated cyclic AMP synthesis in many systems. The biochemical mechanism of this potentiative action of forskolin is poorly understood. It appears that G_s also plays a significant role in the ability of forskolin to act synergistically with stimulatory hormone receptor-agonists. Darfler *et al.* (20) observed that forskolin can restore the response to isoproterenol in the S49 murine lymphoma cell variant, H21a, in which the G_s-C interaction is defective, but not in the S49 cell variant, UNC, in which the receptor-G_s interaction is defective, or in the G_s-deficient S49 cell variant, *cyc*$^-$. These findings indicate that forskolin-augmented hormone responses may be the result of an enhanced interaction between G_s and C. Seamon and Daly (31) observed that forskolin and the metabolically stable GTP analog, Gpp(NH)p, acted synergistically as activators of adenylate cyclase in rat striatum membranes after the inhibitory hormone receptor-G_i-C pathway was antagonized by manganese. Our results, using intact C6-2B rat astrocytoma cells, confirm the hypothesis that the potentiative effect of forskolin is due to enhanced G_s-C coupling. Indeed, the ability of forskolin to increase hormone receptor-agonist potency is enhanced after the G_s-C interaction is promoted by cholera toxin treatment.[2]

The hypothesis that forskolin enhances G_s-C coupling is supported by the observation that forskolin increases the efficacy of cholera toxin-stimulated cyclic AMP accumulation. Cholera toxin stimulation of cyclic AMP

[2] K. Barovsky and G. Brooker, unpublished observation.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

production is a result of the specific, toxin-catalyzed ADP-ribosylation of the α-subunit of G_s (2). The consequent stimulation of GTP release from G_s (8), inhibition of the G_s-associated GTPase, which functions as the enzyme inactivation process (9, 10), and stimulation of the dissociation of the α-subunit of G_s from the β-subunit (11) produces a long-lived activation of adenylate cyclase. The activation of adenylate cyclase by cholera toxin in intact cells is therefore similar to the persistent activation by metabolically stable GTP analogs in broken cells (32) or reconstituted systems (33). Co-incubation of intact C6-2B cells with cholera toxin and a concentration of forskolin which has little direct stimulatory action produced responses markedly greater than additive at all time points at which cyclic AMP levels were above basal levels. Since these experiments were done in the presence of the phosphodiesterase inhibitor, IBMX, it appears that the effect of forskolin cannot be attributed to an inhibition of phosphodiesterase. Forskolin has not been found to have a direct effect on phosphodiesterase activity (34, 35).

Forskolin did not affect the lag typically seen in the response to cholera toxin. Thus, it does not appear that forskolin has an effect on the binding of cholera toxin to its cellular receptor, ganglioside Gm_1, or the subsequent translocation of the catalytically active A-subunit across the cell membrane. The synergistic interaction between forskolin and cholera toxin is expressed at times as short as 10 min after a 1-min incubation with cholera toxin, when no measurable effect of cholera toxin alone can be observed. Thus, forskolin may be enhancing the rate at which cholera toxin catalyzes the ADP-ribosylation reaction. This does not appear to be a likely mechanism for the potentiative action of forskolin since full expression of the synergistic interation between forskolin and cholera toxin can be observed after the maximum response to cholera toxin is attained. Berthillier *et al.* (36) reported that, in rat brain synaptosomes, forskolin has no direct effect on cholera toxin-catalyzed ADP-ribosylation of membrane proteins. It therefore appears that forskolin effects the enhancement of cholera toxin-stimulated cyclic AMP production, and also the enhancement of the response to hormone receptor-agonists, by a mechanism which would lead to enhanced signal transduction between G_s and C.

Forskolin-stimulated cyclic AMP accumulation in C6-2B cells proceeds following a reproducible 1–2-min lag. A similar lag in forskolin-stimulated cyclic AMP accumulation has been observed in wild-type S49 cells (18). In addition, adenylate cyclase activation by forskolin in wild-type S49 cell membranes proceeds following a lag (19). The lag is not observed in the variant S49 cell, *cyc*$^-$ (18), which is deficient in G_s (37), or in membranes from S49 *cyc*$^-$ cells (19). Reconstitution of *cyc*$^-$ membranes with G_s derived from wild-type S49 cell membranes restores the lag (19). Martin *et al.* (22) reported a lag in forskolin-stimulated adenylate cyclase activity in canine basolateral renal cortical membranes. The lag is decreased by Mg^{2+} concentrations above those required for adenylate cyclase catalytic activity. Since, at these higher concentrations, Mg^{2+} apparently acts as an allosteric effector of adenylate cyclase activity mediated via G_s (3, 38), these authors proposed that the site of action of forskolin may be at or closely related to G_s. Co-incubation of C6-2B cells with forskolin and isoproterenol (23) or wild-type S49 cells with forskolin and epinephrine (18) also eliminates the time delay in the response to forskolin. In the present report, we show that pretreatment of C6-2B cells with cholera toxin abolished the 1–2-min lag seen in forskolin-stimulated cyclic AMP accumulation. Catecholamines (9) and cholera toxin (8) have been shown to induce the release of bound guanine nucleotide from G_s, thus facilitating the exchange of bound and exogenous nucleotide. This would account for the action of catecholamines and cholera toxin to reduce the lag typically seen in the activation of adenylate cyclase by Gpp(NH)p in broken cell preparations (39). In addition, cholera toxin-catalyzed ADP-ribosylation of G_s leads to the dissociation of the α-subunit from the β-subunit (11). The promotion of the dissociation of the subunits of G_s has also been proposed as a mechanism for hormonal stimulation of adenylate cyclase (40). The reduction by cholera toxin or isoproterenol of the lag in the response to forskolin in C6-2B cells indicates that the mechanisms of forskolin action may involve the guanine nucleotide binding-hydrolysis-release cycle on G_s and/or the dissociation of the subunits of G_s.

Our data are consistent with the findings of Bender and Neer (17) that forskolin potentiates the activation of resolved bovine caudate nucleus C by purified, Gpp(NH)p-activated G_s (G_s*). In this system, forskolin *must* be added to C before activation by G_s* in order to see the synergistic effect. Bender and Neer (17) proposed, therefore, that the transduction of signals between G_s and C is bidirectional, i.e., that the activity state of the adenylate cyclase system depends not only on the activity state of G_s, but also on the state of activity of the catalytic unit. We assume that cholera toxin catalyzes the stoichiometric ADP-ribosylation of G_s to form a long-lived activated state of G_s since cholera toxin-stimulated cyclic AMP accumulation reaches a maximum at 0.3 nM and does not increase with 10-fold higher toxin concentrations. However, the maximal response to cholera toxin is considerably less than that to forskolin. Thus, maximal stimulation by cholera toxin would not be expected to result in 100% conversion of C to the activated G_s-C complex. In addition, if activated G_s acted catalytically to form the transient, active species G_s-C, each G_s would then activate more than one C. In either case, forskolin could interact with free C, thereby altering the activity state of C before its activation by G_s. The lag in the response to forskolin in C6-2B cells, S49 cells (18) and membranes (19), and canine basolateral renal cortical membranes (22) may be due to the time required for the signal transmission from C to G_s leading to the promotion of an active G_s-C complex following the interaction of forskolin with free C. "Priming" the system with catecholamines, cholera toxin or Mg^{2+} would decrease the lag by promoting the interaction of G_s with C.

We have previously proposed a two-site model for forskolin action in C6-2B cells (23, 24): a low affinity site residing on a rapidly turning-over protein distinct

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

from but closely associated with the catalytic polypeptide by which forskolin stimulates cyclic AMP synthesis and a high affinity site by which forskolin acts to augment hormone responses. Although the high affinity site appears to be physically separate from the low affinity site responsible for direct stimulation of cyclic AMP synthesis by forskolin, we have no evidence to suggest whether the high affinity site is associated with G_s, C, or another component of the adenylate cyclase system. However, based on our previous findings that treatment of intact C6-2B cells with cycloheximide decreases the direct stimulatory effect of forskolin but not the ability of forskolin and cholera toxin to act synergistically as effectors of cyclic AMP accumulation (23, 24), we can conclude that the enhanced G_s-C coupling observed in this report only involves the high affinity forskolin site.

REFERENCES

1. Ross, E. M., and A. G. Gilman. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49**:533–564 (1980).
2. Northup, J. K., P. C. Sternweis, M. C. Smigel, L. S. Schleifer, E. M. Ross, and A. G. Gilman. Purification of the regulatory component of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **77**:6516–6520 (1980).
3. Northup, J. K., M. D. Smigel, P. C. Sternweis, and A. G. Gilman. The subunits of the stimulatory regulatory component of adenylate cyclase: resolution of the activated 45,000-dalton (α) subuint. *J. Biol. Chem.* **258**:11369–11376 (1983).
4. Northup, J. K., P. C. Sternweis, and A. G. Gilman. The subunits of the stimulatory regulatory component of adenylate cyclase: resolution, activity and properties of the 35,000-dalton (β) subunit. *J. Biol. Chem.* **258**:11361–11368 (1983).
5. Seamon, K. B., and J. W. Daly. Forskolin, a unique diterpene activator of cyclic AMP generating systems. *J. Cyclic Nucleotide Res.* **7**:201–224 (1981).
6. Gill, D. M. Mechanism of action of cholera toxin. *Adv. Cyclic Nucleotide Res* **8**:85–118 (1977).
7. Moss, J., and M. Vaughan. Activation of adenylate cyclase by choleragen. *Annu. Rev. Biochem.* **48**:581–600 (1979).
8. Burns, D. L., J. Moss, and M. Vaughan. Choleragen-stimulated release of guanyl nucleotides from turkey erythrocyte membranes. *J. Biol. Chem.* **257**:32–34 (1984).
9. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation through the β-adrenergic receptor: catecholamine-induced displacement of bound GDP by GTP. *Proc. Natl. Acad. Sci. USA* **75**:4155–4159 (1978).
10. Selinger, Z., and D. Cassel. Role of guanine nucleotides in hormonal action of adenylate cyclase. *Adv. Cyclic Nucleotide Res.* **14**:15–22 (1981).
11. Kahn, R. A., and A. G. Gilman. ADP-ribosylation of G_s promotes the dissociation of its α and β subunits. *J. Biol. Chem.* **259**:6235–6240 (1984).
12. Seamon, K. B., W. Padgett, and J. W. Daly. Forskolin: unique diterpene activator of adenylate cyclase in membranes and intact cells. *Proc. Natl. Acad. Sci. USA* **78**:3363–3367 (1981).
13. Seamon, K. B., and J. W. Daly. Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *J. Biol. Chem.* **256**:9799–9801 (1981).
14. Pfeuffer, T., and H. Metzger. 7-*O*-Hemisuccinyl-deacetyl forskolin-Sepharose: a novel affinity support for purification of adenylate cyclase. *FEBS Lett.* **146**:369–379 (1982).
15. Pfeuffer, T., R. M. Dreher, H. Metzger, and T. Pfeuffer. Catalytic unit of adenylate cyclase: purification and identification by affinity crosslinking. *Proc. Natl. Acad. Sci. USA* **82**:3086–3090 (1985).
16. Ross, E. M. Phosphatidylcholine-promoted interaction of the catalytic and regulatory proteins of adenylate cyclase. *J. Biol. Chem.* **257**:10751–10758 (1982).
17. Bender, J. L., and E. J. Neer. Properties of the adenylate cyclase catalytic unit from caudate nucleus. *J. Biol. Chem.* **258**:2432–2439 (1983).
18. Clark, R. B., T. J. Goku, D. A. Green, R. Barber, and R. W. Butcher. Differences in the forskolin activation of adenylate cyclases in wild-type and variant lymphoma cells. *Mol. Pharmacol.* **22**:609–613 (1982).
19. Green, D. A., and R. B. Clark. Direct evidence for the role of the coupling proteins in forskolin activation of adenylate cyclase. *J. Cyclic Nucelotide Res.* **8**:337–346 (1982).
20. Darfler, F. J., L. C. Mahan, A. M. Koachman, and P. A. Insel. Stimulation by forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. *J. Biol. Chem.* **257**:11901–11907 (1982).
21. Morris, S. A., and J. P. Bilezikian. Evidence that forskolin activates turkey erythrocyte adenylate cyclase through a noncatalytic site. *Arch. Biochem. Biophys.* **220**:628–636 (1983).
22. Martin, K. J., T. J. Stokes, Jr., and C. L. McConkey, Jr. Influence of forskolin on the parathyroid hormone dependent adenylate cyclase system of canine kidney: evidence for noncatalytic effects of forskolin. *Endocrinology* **115**:1678–1682 (1984).
23. Barovsky, K., C. Pedone, and G. Brooker. Distinct mechanisms of forskolin-stimulated cyclic AMP accumulation and forskolin-potentiated hormone responses in C6-2B cells. *Mol. Pharmacol.* **25**:256–260 (1984).
24. Brooker, G., C. Pedone, and K. Barovsky. Selective reduction of forskolin-stimulated cyclic AMP accumulation by inhibitors of protein synthesis. *Science (Wash. D. C.)* **220**:1169–1170 (1983).
25. Terasaki, W. L., and G. Brooker. [^{125}I] Iodohydroxybenzylpindolol binding sites on intact rat glioma cells: evidence for β-adrenergic receptors of high coupling efficiency. *J. Biol. Chem.* **253**:5418–5425 (1978).
26. Brooker, G., W. L. Terasaki, and M. G. Price. Gammaflow: a completely automated radioimmunoassay system. *Science (Wash. D. C.)* **194**:270–276 (1976).
27. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res* **1**:207–218 (1976).
28. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
29. Johnson, M. L., and S. G. Frasier. Nonlinear least squares analysis. *Methods Enzymol.*, in press.
30. Nickols, G. A., and G. Brooker. Induction of refractoriness to isoproterenol by prior treatment of C6-2B rat astrocytoma cells with cholera toxin. *J. Cyclic Nucleotide Res.* **5**:435–447 (1979).
31. Seamon, K. B., and J. W. Daly. Guanosine 5'-(β,γ-imido)triphosphate inhibition of forskolin-activated adenylate cyclase is mediated by the putative inhibitory guanine nucleotide regulatory protein. *J. Biol. Chem.* **257**:11591–11596 (1982).
32. Schramm, M. The catecholamine-responsive adenylate cyclase system and its modification by 5'-guanylylimidodiphosphate. *Adv. Cyclic Nucleotide Res.* **5**:105–115 (1975).
33. Strittmatter, S., and E. J. Neer. Properties of the separated catalytic and regulatory units of brain adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **77**:6344–6348 (1980).
34. Ho, R. J. and Q. H. Shi. Forskolin as a novel lypolytic agent. *Biochem. Biophys. Res. Commun.* **107**:157–164 (1982).
35. Litosch, I., T. H. Hudson, I. Mills, S.-Y. Li, and J. N. Fain. Forskolin as an activator of cyclic AMP accumulation and lipolysis in rat adipocytes. *Mol. Pharmacol.* **22**:109–115 (1982).
36. Berthillier, G., J. d'Alayer, and A. Monneron. ADP-ribosylation of brain synaptosomal proteins correlates with adenylate cyclase activation. *Biochem. Biophys. Res. Commun.* **109**:297–304 (1982).
37. Johnson, G. L., H. R. Kaslow, and H. R. Bourne. Genetic evidence that cholera toxin substrates are regulatory components of adenylate cyclase. *J. Biol. Chem.* **253**:7120–7123 (1978).
38. Iyengar, R., and L. Birnbaumer. Hormone receptor modulates the regulatory component of adenylyl cyclase reducing its requirement for Mg^{2+} and enhancing its extent of activation by guanine nucleotides. *Proc. Natl. Acad. Sci. USA* **79**:5179–5183 (1982).
39. Lad, P. M., T. B. Nielsen, M. S. Preston, and M. Rodbell. The role of guanine nucleotide exchange reaction in the regulation of the β-adrenergic receptor and in the actions of catecholamines and cholera toxin on adenylate cyclase in turkey erythrocyte membranes. *J. Biol. Chem.* **255**:988–995 (1980).
40. Smigel, M., T. Katada, J. K. Northup, G. M. Bokoch, M. Ui, and A. G. Gilman. Mechanisms of guanine nucleotide-mediated regulation of adenylate cyclase activity. *Adv. Cyclic Nucleotide Protein Phosphor. Res.* **17**:1–18 (1984).

Send reprint requests to: Dr. Kenneth Barovsky, Department of Biochemistry, Georgetown University Medical Center, 3900 Reservoir Road, NW, Washington, D.C. 20007.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012