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# FORSKOLIN AS A NOVEL LIPOLYTIC AGENT*

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**ABSTRACT:** Forskolin is a novel lipolytic agent which elevates cAMP and FFA release in rat adipocytes in a manner different from existing lipolytic factors. This effect of Forskolin is potentiated by all lipolytic hormones tested, i.e. epinephrine, ACTH, and glucagon and is also reversible. The same batch of adipocytes can be repeatedly stimulated after washing. The effective concentration of Forskolin is in the micromolar range. Its action is due to an activation of cAMP synthesis by adenylate cyclase. There is no effect on cAMP hydrolysis. In contrast to stimulation by lipolytic hormones, Forskolin-activated membrane adenylate cyclase was not further stimulated by GPP(NH)P. These results suggest that Forskolin may be a useful analytical agent in the study of adenylate cyclase mediated function in intact adipocytes.

## INTRODUCTION

Forskolin, a diterpene derivative, has been known for its action on the cardiovascular system (1). Our interest in this compound was derived from a report (Fed. Proc. 40, abs. 2267, 1981) by Daly et al., in which Forskolin was stated to elevate cellular cAMP and stimulate the membrane adenylate cyclase of rat cerebral cortex. Whether cAMP mediates the lipolytic or antilipolytic action of a number of hormones on adipocytes is still not agreed on (2-5). We think this new adenylate cyclase activator may help solve such questions. This paper reports the findings regarding the effect of Forskolin on intact adipocytes and isolated adipocyte plasma membranes. Its action is compared with NaF, GPP(NH)P, and epinephrine. Forskolin appears to be a potent, novel lipolytic agent and an excellent tool for the study of adenylate cyclase activation in a cell-free system and for the study of cAMP mediated function in the intact cells.**

## METHODS AND MATERIALS

Preparation of adipocyte and adipocyte plasma membrane. Adipocytes from rat epididymal adipose tissue were prepared according to the method of Rodbell (6)

Abbreviations used are: cAMP, adenosine cyclic 3',5'-monophosphate; GPP(NH)P, 5'-guanylimidodiphosphate; FFA, free fatty acids; cAMP-PDE, cAMP-phosphodiesterase.

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with minor modification. Hormone-sensitive adipocyte plasma membranes were isolated according to the method of Laudat et al. (7), also with minor modification. The hormone-sensitive adenylate cyclase was stable for at least 6 months when stored in liquid nitrogen.

Incubation of isolated adipocytes with hormone and Forskolin. The incubation medium was Krebs-Ringer bicarbonate buffer, pH 7.4, containing 2% defatted bovine serum albumin (fraction V) equilibrated with a gas mixture ($O_2$, 95%; and $CO_2$, 5%). The incubation volume was 2.6 ml. Forskolin and/or hormone was added to the vial and the reaction was started by the addition of adipocytes. The incubation method with multiple stimulation of the adipocyte was similar to that already described (8). The reaction was stopped at given time intervals and portions of the incubation medium were taken for determination of cAMP and FFA. Total lipids of cell suspension were extracted as by Dole and Meinertz (9) and determined gravimetrically. Lipolysis is expressed as the number of μmoles of FFA released per g. of total lipids during a specified time interval. Cyclic AMP levels in adipocytes are expressed as nmoles/g lipids.

Determination of long-chain free fatty acids. FFA release from adipocyte during incubation was determined by a slightly modified method of Ho and Meng (10), using [$^{57}$Co]-labelled cobaltous nitrate as tracer.

Assay for adenylate cyclase activity. Adenylate cyclase activity was assayed essentially according to Rall and Sutherland (11) with modification. 4 mM ATP and 8 mM $Mg^{2+}$ were used as substrates.

Determination of cAMP. Determination of the amount of cAMP in adenylate cyclase assays followed the method of Gilman (12) with minor modification. The binding protein was prepared according to Miyamoto et al. (13).

Determination of cAMP-phosphodiesterase activity. Whether cAMP phosphodiesterase (PDE) in adipocyte homogenate was influenced by added Forskolin was determined by the tracer method of Thompson et al. (14). The adenosine product was separated from cAMP on a short column of QAE-Sephadex (15).

MATERIALS

[2, 8-$^3$H] Adenosine cyclic 3',5'-monophosphate, 34 ci/mmole, and [8-$^3$H] adenosine 3',5'-monophosphate were purchased from New England Nuclear Corp., MA. Dowex AG50 Wx8, 100-200 mesh was purchased from Bio-Rad Laboratories, Richmond, CA. Forskolin (7b-aceoxy-8, 13-epoxy-1a,6b,9a-trihydroxy-labd-14-en-11-one) was obtained from Calbiochem-Boehringer Corp., San Diego, CA. 5'-Guanylylimidodiphosphate, or GPP(NH)P was obtained from Sigma Chemical Company, Saint Louis, MO. 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Aldrich Chemical Co., Inc. and recrystallized from ethanol before use. [$^{57}$Co]-labelled cobaltous chloride was purchased from New England Nuclear Corp., MA. It was converted into the nitrate form in our laboratory.

## RESULTS

Effect of Forskolin on cAMP levels in rat adipocytes incubated with or without epinephrine, ACTH, and glucagon. It was observed that micromolar Forskolin reproducibly stimulated cAMP levels in intact adipocytes by as much as 2-fold. However, much more dramatic effects were seen when Forskolin was supplemented by epinephrine (1 μM), ACTH (25 mU/ml), or glucagon (5 ug/ml) (Fig. 1A). At 10 μM of Forskolin and 1 μM epinephrine, cAMP was 25 fold greater than that due to epinephrine alone, 50 fold greater than that due to 10 μM Forskolin, and 60 fold greater than that of the basal levels (Fig. 1a). Forskolin has a similar ability to potentiate cAMP levels with ACTH (Fig. 1B) and with glucagon (Fig. 1C) to elevate cAMP Levels above the basal by 94 and 49 fold respectively.

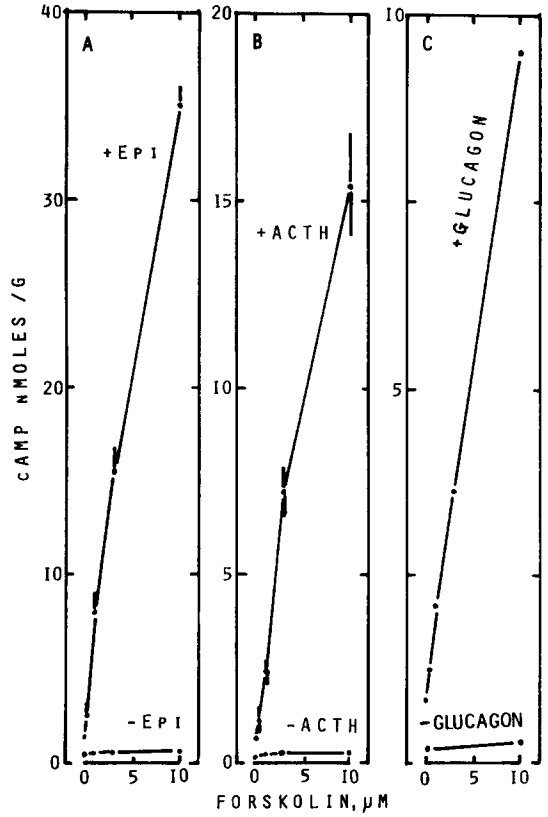


Fig. 1. Potentiation of cAMP elevation effect of epinephrine, ACTH, or glucagon by Forskolin.

Rat adipocytes were incubated with varied concentrations of Forskolin ranging from 0 to 10 μM with or without 1 μM epinephrine (Fig. 1A), 10 mu/ml ACTH (Fig. 1B) or 5 ug/ml glucagon (Fig. 1C). Reaction was started with the addition of Forskolin (or solvent for Forskolin). Incubation was carried out at 37 C for 4 minutes. Results of cAMP is expressed as nmoles/g lipid. Each point is the mean of 4-8 incubation experiments.

Reversible and repeated stimulation of cAMP levels in rat adipocyte by ACTH, epinephrine, or its combination with Forskolin. Fig. 2 shows experiments which demonstrate that the high cAMP levels induced by the combination of 10 μM Forskolin and 10 mu/ml ACTH or 1 μM epinephrine are rapidly reduced to baseline levels by a simple washing procedure; moreover, following the wash procedure, the cells can be restimulated (Fig. 2A and 2B). The second stimulation of Forskolin + ACTH led to a response similar to that of the first stimulation (Fig. 2A). In the case of Forskolin and epinephrine, the second stimulation gave a 2-fold higher level of cAMP (Fig. 2B).

Stimulation of lipolysis by Forskolin. As shown in Fig. 3A, Forskolin stimulated FFA release from intact adipocytes occurs in a concentration dependent manner. Fig. 3B shows that Forskolin potentiates epinephrine stimulated FFA release. The potentiation represents a dramatic lipolytic

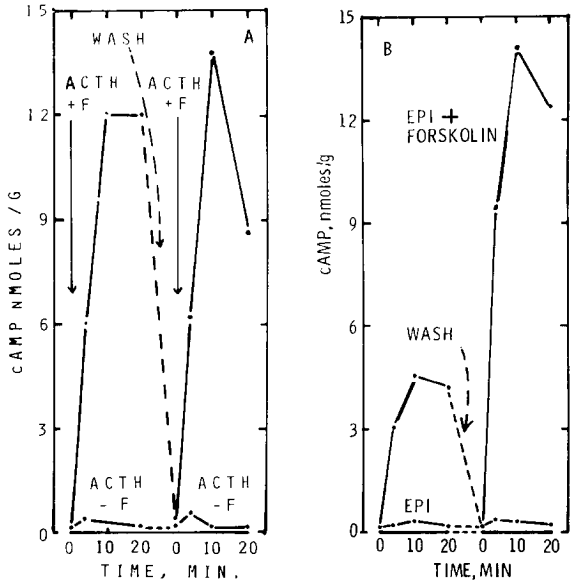


Fig. 2. Reversible and repetitive stimulation of cAMP elevation in adipocytes by ACTH, epinephrine, Forskolin and NaF.

The experiments were designed to test the reversibility of Forskolin-enhanced hormone action in terms of cAMP elevation in rat adipocytes. There are two-20 min time course studies, with the adipocytes subjected to washes in between. Adipocytes were stimulated with ACTH 10 mu/ml, ACTH and Forskolin 10 μM (Fig. 2A); epinephrine 1 μM, epinephrine and Forskolin 10 μM (Fig. 2B). Samples for cAMP determination were taken at 0, 4, 10 and 20 minutes of incubation. Then the adipocytes were wash-incubated for three times with a medium free from hormone and Forskolin. Results are the mean of 2-6 experiments and are expressed as nmoles/g fat cell lipid.

effect which resembles the effect on cAMP. The effect is reduced by washing, and the cells can be restimulated.

Activation of adenylate cyclase of rat adipocyte plasma membrane by Forskolin and its potentiation by epinephrine. Forskolin, in a concentration-dependent manner, stimulated adenylate cyclase activity of rat adipocyte plasma membranes. At 100 μM Forskolin, the enzyme activity was stimulated 10 fold above the basal activity (Fig. 4, lower curve). Epinephrine potentiated the effect of Forskolin. Such potentiation is also dependent on the concentration of Forskolin (Fig. 4, upper curve). Epinephrine, in the absence of Forskolin, stimulates the enzyme by 2-3 fold. At 100 μM Forskolin and 230 μM epinephrine, the adenylate cyclase activity was 22 fold higher than the basal and 7 fold higher than epinephrine alone; epinephrine enhanced the $V_{max}$ of such Forskolin effect without changing the apparent $K_m$ for Forskolin.

Fig. 5 shows the effect of increasing the concentration of epinephrine on the activation of rat adipocyte plasma membrane adenylate cyclase incubated in presence or absence of Forskolin (100 μM). Epinephrine at 230 μM stimulated adenylate cyclase activity by 3 fold above the basal rate (lower curve). The apparent $K_m$ for epinephrine is approximately 20-30 μM. With

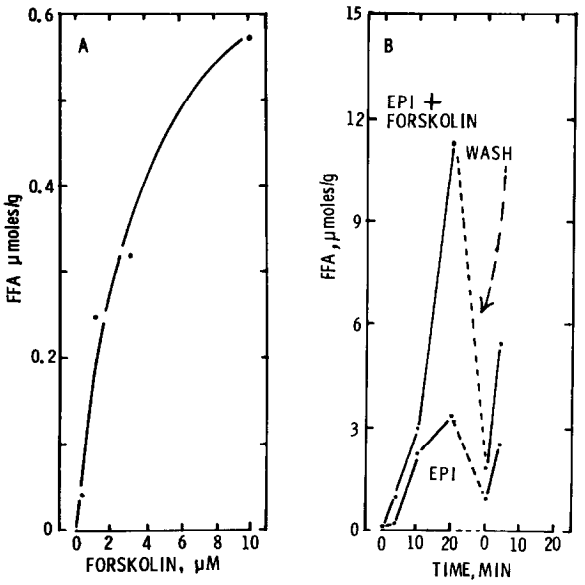


Fig. 3. Effect of Forskolin epinephrine and NaF on FFA release from rat adipocytes.

Fig. 3A: rat adipocytes were incubated as that described in Fig. 1. At the end of 4 minutes incubation, FFA in the incubation system was extracted and determined. Results are expressed in µmole/g lipid.

Fig. 3B: adipocyte were incubated in the manner described in Fig. 2. Results presented are FFA released from adipocyte, which is the difference of FFA level above the zero time level. And second time course after washing only lasted for 4 minutes of incubation.

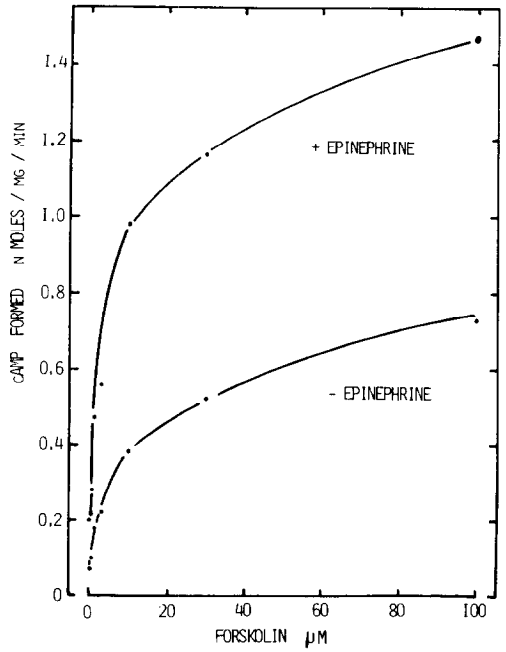


Fig. 4. Effects of varying concentrations of Forskolin on adenylate cyclase activity of rat adipocyte plasma membrane in the presence or absence of epinephrine.

Plasma membrane was incubated with varying concentrations of Forskolin ranging from 0-100 µM with or without epinephrine 230 µM. Results are the mean of 4 experiments.

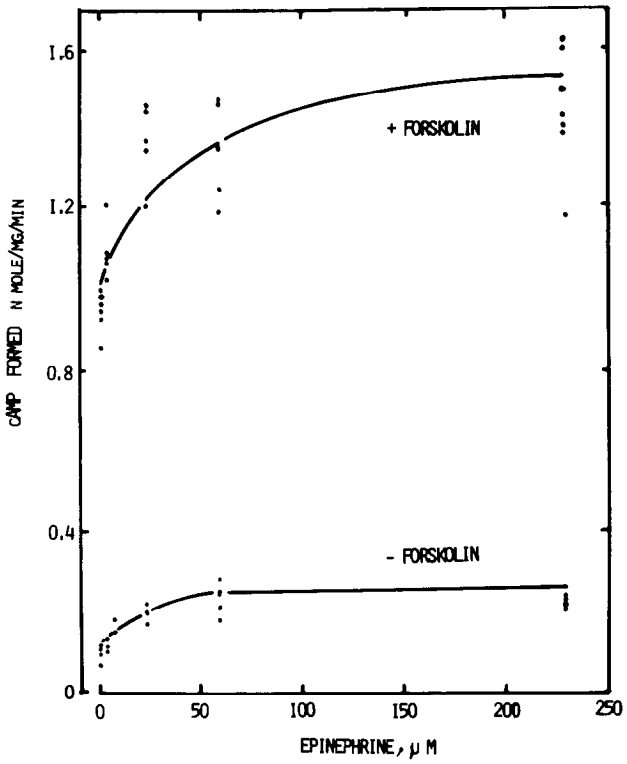


Fig. 5. Effect of varying concentrations of epinephrine on adenylate cyclase of rat adipocyte plasma membrane in the presence or absence of Forskolin.
Plasma membrane was incubated with varied concentration of epinephrine ranging from 0-230 μM with or without Forskolin 100 μM. Results are mean of 4 experiments.

epinephrine in combination with Forskolin (100 μM), a further activation adenylate cyclase was observed (upper curve). This increase in adenylate cyclase in the presence of Forskolin was dependent on the concentration of epinephrine. Forskolin at 100 μM has no effect on cAMP phosphodiesterase (results not shown).

The action of GPP(NH)P on adenylate cyclase of rat adipocyte plasma membrane: lack of potentiation of Forskolin stimulatory effect, potentiation of epinephrine effect and inhibition of NaF stimulatory effect. Forskolin, 100 μM, epinephrine, 230 μM, NaF, 100 mM, and GPP(NH)P 10 μM significantly stimulated the enzyme activity by 1276, 195, 288 and 24% respectively (Table 1). The combined effects of Forskolin and epinephrine was significantly greater than either Forskolin or epinephrine alone and was 65% more than their additive effect. The combined effects of Forskolin and GPP(NH)P was the same as of Forskolin alone. GPP(NH)P inhibited the effect of NaF. The combined effect of NaF and GPP(NH)P was 46% of that of NaF alone.

TABLE I

ADENYLATE CYCLASE ACTIVITY OF RAT ADIPOCYTE PLASMA MEMBRANE IN THE PRESENCE OF DIFFERENT ACTIVATORS

| F $\mu$M | Epi $\mu$M | G $\mu$M | NaF mM | Adenylate cyclase cAMP, nmole/mg/min | P < | Effect (%) |
|---|---|---|---|---|---|---|
| - | - | - | - | 0.042+0.005(32) | - | 0 |
| 100 | - | - | - | 0.578+0.075(19) | 0.01 | 1276 |
| - | 230 | - | - | 0.124+0.011(20) | 0.01 | 195 |
| - | - | 10 | - | 0.052+0.005(24) | 0.01 | 24 |
| - | - | - | 10 | 0.163+0.006(20) | 0.01 | 288 |
| 100 | 230 | - | - | 1.060+0.103(20) | 0.01 | 2424 |
| 100 | - | 10 | - | 0.597+0.046(8) | 0.01 | 1321 |
| - | 230 | 10 | - | 0.266+0.008(16) | 0.01 | 533 |
| - | - | 10 | 10 | 0.098+0.009(8) | 0.01 | 133 |

Adenylate cyclase activity were tested in a 500 l assay system, incubated at 30°C for 10 min. F, Forskolin; Epi, epinephrine, G, GPP(NH)P; NaF, sodium floride.

## DISCUSSION

The role of cAMP in the mediation of hormone stimulated lipolysis involves cAMP-dependent phosphorylation of the hormone-sensitive lipase (16) via the activation of a protein kinase (17. This is how all known lipolytic agents act, such as lipolytic hormone, cholera toxin, and phosphodiesterase inhibitor. We have demonstrated that Forskolin is a potent and novel lipolytic agent. Its action is apparently mediated by its ability to increase cAMP levels in the intact adipocyte. Studies of isolated membranes show that Forskolin increases cAMP levels by activation of adenylate cyclase. In other experiments no inhibition of cAMP phosphodiesterase by Forskolin could be demonstrated. Similar to lipolytic hormone (8), the effect of Forskolin is rapid and reversible, and the same batch of adipocytes can be repeatedly stimulated. This properties of Forskolin in intact adipocytes is different from cholera toxin which is not readily reversible.

There are three major components of the adenylate cyclase complex (18) - the hormone receptor, the G/F regulatory proteins, and the adenylate cyclase itself. Forskolin does not require a specific receptor at the membrane surface; it activates adenylate cyclase which has been separated from the receptors (Ho, Shi and Wang, Fed. Proc. 41, no. 6666, 1982). The receptor mediated activators-lipolytic hormones-are potentiated by GPP(NH)P (18, 19), but the action of Forskolin is not effected by this nucleotide. Furthermore, Seamon and Daly (20) showed that Forskolin activated adenylate cyclase in cyc-

49 cell membrane which is known to lack the G/F protein. Forskolin has also been shown to activate membrane adenylate cyclase from several mammalian tissues including rat cerebral cortex (21, 22). All these evidence clearly show that Forskolin acts on adenylate cyclase with the involvement of neither specific receptors at the membrane surface nor the well known coupler, G/F protein. It appears to act on adenylate cyclase directly. Therefore, Forskolin is the first lipolytic agent which acts on adenylate cyclase directly. In view of its mode of action, this novel lipolytic agent may be very useful as a tool in the study of cAMP-mediated action in adipocyte.

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