Stimulatory and Inhibitory Effects of Forskolin on Adenylate Cyclase in Rat Normal Hepatocytes and Hepatoma Cells

Kenichi MIYAMOTO, Fujiko SANAE, Ryozo KOSHIURA,* Takayuki MATSUNAGA,** Kenzo TAKAGI, Tatsuo SATAKE,*** and Takaaki HASEGAWA*4

Third Division of the Research Laboratory for Development of Medicine, School of Pharmacy, Hokuriku University,* Ho-3 Kanagawa-machi, Kanazawa, 920-11, Japan, Toyama Prefectural Institute for Pharmaceutical Research,** 17-1 Nakataikoyama, Kosugi, Toyama, 939-03, Japan and Second Department of Internal Medicine*** and Department of Hospital Pharmacy,*4 School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya, 466, Japan

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Forskolin synergistically potentiated adenosine 3',5'-cyclic monophosphate formation by prostaglandin E\textsubscript{1} (PGE\textsubscript{1}) in rat normal hepatocytes freshly prepared by collagenase digestion and rat ascites hepatoma AH66 cells, but dose-dependently inhibited the accumulation by PGE\textsubscript{1} in AH66F cells. Forskolin activated adenylate cyclase in a dose-dependent manner in homogenates of all cell lines. In normal hepatocytes and AH66 cells, simultaneous addition of forskolin and other adenylate cyclase activators [isoproterenol (IPN), PGE\textsubscript{1}, guanosine 5'-triphosphate sodium salt (GTP), 5'-guanylimidodiphosphate sodium salt (Gpp (NH)p), NaF, cholera toxin, islet activating protein and MnCl\textsubscript{2}] gave greater than additive responses. On the other hand, in AH66F cells, the effect of forskolin on adenylate cyclase was hardly influenced by GTP, but forskolin diminished the activities induced by high concentrations of GTP to that by the diterpene alone. Forskolin also significantly inhibited the PGE\textsubscript{1}-stimulated and the guanine nucleotide binding regulatory protein-stimulated activities. Because AH66F cells were insensitive to IPN, the combination with forskolin and IPN gave similar activity to that obtained with the diterpene alone. The effect of forskolin on the activation by manganese ion was neither synergistic nor inhibitory but was additive in AH66F cells. These results suggest that forskolin promotes the interaction between the stimulatory guanine nucleotide binding regulatory protein and the catalytic unit in normal hepatocytes and AH66 cells, but in AH66F cells forskolin interferes with the coupling of the two components of adenylate cyclase.

Keywords — forskolin; adenylate cyclase; stimulation; inhibition; rat normal hepatocyte; rat ascites hepatoma; AH66 cell; AH66F cell

Introduction

Forskolin, a plant diterpene, is an important research tool in the investigation of the adenosine 3',5'-cyclic monophosphate (cyclic AMP) production system because it is a potent activator of adenylate cyclase.\textsuperscript{1,2} In 1981, Seamon and Daly\textsuperscript{3} suggested that forskolin perhaps acts directly on the catalytic unit of adenylate cyclase. Since then, many investigators have presented evidence that this diterpene modifies the interaction between the guanine nucleotide binding regulatory protein (N) and the catalytic unit of adenylate cyclase.\textsuperscript{4-7} On the other hand, there are reports that forskolin inhibited adenylate cyclase activity stimulated by guanine nucleotides in rat basophilic leukemia\textsuperscript{8} and human platelets.\textsuperscript{9} Hudson and Fain\textsuperscript{10} have shown that the forskolin-activated cyclase was inhibited by guanine nucleotides in rat adipocytes.

We have previously indicated that in rat ascites hepatoma AH66 cells forskolin markedly increased cyclic AMP level and augmented the elevation of the nucleotide by cholera toxin (CT), islet activating protein (IAP) and prostaglandin E\textsubscript{1} (PGE\textsubscript{1}), but in AH66F cells the diterpene produced little cyclic AMP and suppressed the elevation by these agents.\textsuperscript{11} We have now investigated the effect of forskolin on adenylate cyclase activated by several activators in AH66 cells and AH66F cells, compared to that in rat normal hepatocytes, and report here that forskolin inhibits the N-mediated activation of adenylate cyclase in AH66F cells, while the combination of the diterpene and other adenylate cyclase activators synergistically activates adenylate cy-
clase in normal hepatocytes and AH66 cells.

Materials and Methods

Chemicals — Guanosine 5'-triphosphate sodium salt (GTP), 5'-guanylimidodiphosphate sodium salt [Gpp(NH)p], 1-isoproterenol hydrochloride (IPN), CT (Sigma Chemical Co., St. Louis, U.S.A.), sodium fluoride (NaF), manganese chloride (MnCl₂, Wako Pure Chemicals, Osaka) and IAP (Kaken Seiyaku, Tokyo) were purchased from the indicated sources. PGE₁ and forskolin were kindly provided by Fuji Yakuhin Co., Ltd., Takaoka and Nihon Kayaku Co., Ltd., Tokyo, respectively. Materials except forskolin were dissolved in Tris-HCl buffer (pH 7.4) just before use. Forskolin was dissolved in ethanol, diluted with Tris-HCl buffer and used so as to give a final ethanol concentration of below 1.0%.

Cells — Hepatocytes were isolated from female Donryu rats (5 to 6 weeks old, Shizuoka Laboratory Animal Center) by collagenase digestion in situ as described elsewhere. AH66 cells and AH66F cells were maintained serially by intraperitoneal passage at weekly intervals in female Donryu rats. The AH66F cell line had been derived from the AH66 cell line in 1959.

Treatment with CT and IAP — Cells suspended in Eagle’s minimum essential medium containing 10% fetal bovine serum were treated with CT (1 μg/ml) or IPA (1 μg/ml) for 3 h at 37 °C in a CO₂ incubator.

Determination of Cyclic AMP — Cells (3 × 10⁶/ml) were treated with forskolin alone or with the combination of forskolin and PGE₁ in the presence of 10⁻³ M theophylline at 37 °C for 15 min. After the treatment, the cells were chilled, collected by centrifugation and homogenized in 6% trichloroacetic acid. The extracted cyclic AMP was assayed with a cyclic AMP assay kit (Yamasa Shoyu, Choshi) following the procedure developed by Honma et al.

Adenylate Cyclase Assay — Cells were homogenized in 30 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl₂ using a Teflon-glass homogenizer. The enzyme activity in the Cell homogenate was assayed in the absence or presence of activators as described in the previous paper.

Protein Assay — Protein contents were estimated by the method of Lowry et al. with bovine serum albumin as the standard. All experiments were performed in triplicate, and results were expressed as the mean ± S.E. Statistical significance of difference was evaluated by using Student’s t-test.

Results

Effect on Intracellular Cyclic AMP Level

Figure 1 shows the changes of cyclic AMP level in normal hepatocytes, AH66 cells and AH66F cells simultaneously treated with graded concentrations of forskolin alone and in combination with 10⁻⁵ M PGE₁. Forskolin dose-dependently increased the cyclic AMP level maximally about 4-fold in both hepatocytes and AH66 cells. In AH66F cells, while the cyclic AMP content in the non-treated condition was higher than that in the other cells, forskolin induced only a little elevation even at 10⁻⁴ M.

![Fig. 1. Effect of Simultaneous Treatment with Forskolin and PGE₁ on Intracellular Cyclic AMP Level](image-url)
**Effect of Forskolin on Adenylate Cyclase**

**Table I. Effect of Forskolin on the Activation of Adenylate Cyclase by Other Effectors in Rat Normal Hepatocytes, AH66 Cells and AH66F Cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Hepatocytes Without Forskolin</th>
<th>Hepatocytes With Forskolin</th>
<th>AH66 cells Without Forskolin</th>
<th>AH66 cells With Forskolin</th>
<th>AH66F cells Without Forskolin</th>
<th>AH66F cells With Forskolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>3.4±0.4</td>
<td>33.8±1.9</td>
<td>3.7±1.1</td>
<td>84.4±3.2</td>
<td>20.5±1.4</td>
<td>45.6±2.4</td>
</tr>
<tr>
<td>GTP</td>
<td>10⁻⁵ M</td>
<td>4.7±0.5</td>
<td>68.9±10.6</td>
<td>20.0±3.4</td>
<td>185±12</td>
<td>60.8±8.1</td>
<td>50.2±4.6</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>10⁻⁵ M</td>
<td>23.5±1.7</td>
<td>104±8.1</td>
<td>57.6±1.9</td>
<td>431±13</td>
<td>265±17</td>
<td>61.1±4.6</td>
</tr>
<tr>
<td>NaF</td>
<td>10⁻² M</td>
<td>16.5±2.3</td>
<td>60.9±3.8</td>
<td>157±10</td>
<td>513±54</td>
<td>197±28</td>
<td>99.9±11.2</td>
</tr>
<tr>
<td>CT</td>
<td>1 μg/ml</td>
<td>29.9±1.8</td>
<td>206±14</td>
<td>56.4±3.4</td>
<td>405±21</td>
<td>137±2</td>
<td>52.6±1.6</td>
</tr>
<tr>
<td>IAP</td>
<td>1 μg/ml</td>
<td>7.7±2.4</td>
<td>92.2±5.2</td>
<td>81.0±6.7</td>
<td>421±19</td>
<td>85.6±9.0</td>
<td>52.7±3.1</td>
</tr>
<tr>
<td>PGE₁</td>
<td>10⁻⁵ M</td>
<td>75.5±2.8</td>
<td>203±16</td>
<td>52.6±7.3</td>
<td>393±63</td>
<td>181±39</td>
<td>68.2±13.6</td>
</tr>
<tr>
<td>IPN</td>
<td>10⁻⁴ M</td>
<td>15.3±1.2</td>
<td>97.5±21.1</td>
<td>37.5±5.1</td>
<td>353±37</td>
<td>62.0±3.2</td>
<td>51.5±3.3</td>
</tr>
</tbody>
</table>

a) Adenylate cyclase activity was determined in the presence of GTP (10⁻⁵ M) with or without forskolin (10⁻⁵ M). b) Cells were treated with each agent for 3 h, washed and homogenized, and then the adenylate cyclase activity was determined in the presence of GTP (10⁻⁵ M). Each value represents the mean ± S.E. (cyclic AMP pmol/min/mg protein) of three experiments; values for c) are significantly different from values in the absence of forskolin, p < 0.01.

The cell lines tested in this study were all sensitive to PGE₁ and the maximum increase of cyclic AMP in each cell line was obtained at 10⁻⁵ M PGE₁; about 3-fold increase in hepatocytes and AH66 cells and about 10-fold in AH66F cells. The combined treatment with forskolin and PGE₁ elevated the intracellular cyclic AMP levels additively or more in hepatocytes and AH66 cells, but in AH66F cells forskolin dose-dependently inhibited the accumulation by PGE₁.

**Effect on Adenylate Cyclase Activation**

Table I shows the adenylate cyclase activity after the simultaneous treatment with forskolin and several effectors. Forskolin (10⁻⁵ M) activated adenylate cyclase in the homogenate of AH66F cells, but the extent of increase was less than those in hepatocytes and A66 cells. Other agents which act on the N, such as GTP, Gpp(NH)p, NaF, CT and IAP, all activated the enzyme in each cell line. Adenylate cyclase of hepatocytes and AH66 cells was sensitive to the receptor stimulation by PGE₁ and IPN, but that of AH66F cells was less sensitive to IPN-stimulation. These stimulated activities in hepatocytes and AH66 cells were synergistically aug-

**Table II. Effect of Forskolin on the Activation of Adenylate Cyclase by Manganese Ion in Rat Normal Hepatocytes, AH66 Cells and AH66F Cells**

<table>
<thead>
<tr>
<th>Concentration of MnCl₂ (mM)</th>
<th>Hepatocytes Without Forskolin</th>
<th>Hepatocytes With Forskolin</th>
<th>AH66 cells Without Forskolin</th>
<th>AH66 cells With Forskolin</th>
<th>AH66F cells Without Forskolin</th>
<th>AH66F cells With Forskolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.2±0.5</td>
<td>30.6±2.5</td>
<td>5.1±2.2</td>
<td>78.3±5.1</td>
<td>18.6±1.1</td>
<td>38.4±2.2</td>
</tr>
<tr>
<td>0.2</td>
<td>8.6±0.7</td>
<td>51.5±5.2</td>
<td>6.3±1.2</td>
<td>122±10</td>
<td>22.5±2.6</td>
<td>39.8±2.3</td>
</tr>
<tr>
<td>0.5</td>
<td>11.9±0.7</td>
<td>82.4±9.5</td>
<td>8.5±1.0</td>
<td>168±15</td>
<td>28.4±1.8</td>
<td>40.6±3.3</td>
</tr>
<tr>
<td>1.0</td>
<td>14.5±0.5</td>
<td>102±14</td>
<td>10.4±0.8</td>
<td>287±18</td>
<td>33.4±2.0</td>
<td>42.6±2.9</td>
</tr>
<tr>
<td>2.0</td>
<td>17.2±1.1</td>
<td>167±27</td>
<td>19.9±1.2</td>
<td>325±23</td>
<td>46.4±4.4</td>
<td>60.6±3.1</td>
</tr>
<tr>
<td>5.0</td>
<td>20.2±1.2</td>
<td>183±17</td>
<td>52.0±3.6</td>
<td>400±21</td>
<td>167±18</td>
<td>207±36</td>
</tr>
</tbody>
</table>

Adenylate cyclase activity was determined in the absence or presence of forskolin (10⁻⁵ M) without GTP. Each value represents the mean ± S.E. (cyclic AMP pmol/min/mg protein) of three experiments; values for a) and b) are significantly higher than the values in the absence of forskolin, p < 0.01 and p < 0.05, respectively.
Fig. 2. Effect of Simultaneous Treatment with Forskolin and GTP on Adenylate Cyclase Activity of Rat Normal Hepatocytes

The cell homogenate was incubated with various concentrations of forskolin in the absence (○) or presence of 10^{-5} M GTP (●) (left panel) and various concentrations of GTP in the absence (○) or presence of 10^{-5} M forskolin (●) (right panel) for 10 min at 37 °C. Values are the mean ± S.E. (bar) of three experiments.

Fig. 3. Effect of Simultaneous Treatment with Forskolin and GTP on Adenylate Cyclase Activity of AH66 Cells

The cell homogenate was incubated with various concentrations of forskolin in the absence (○) or presence of 10^{-5} M GTP (●) (left panel) and various concentrations of GTP in the absence (○) or presence of 10^{-5} M forskolin (●) (right panel) for 10 min at 37 °C. Values are the mean ± S.E. (bar) of three experiments.
Effect of Forskolin on Adenylate Cyclase

![Graphs showing the effect of forskolin and GTP on adenylate cyclase activity.]

Fig. 4. Effect of Simultaneous Treatment with Forskolin and GTP on Adenylate Cyclase Activity of AH66F Cells

The cell homogenate was incubated with various concentrations of forskolin in the absence (○ — ○) or presence of $10^{-5}$ M (● — ●) or $10^{-4}$ M (● — ●) GTP (left panel) and various concentrations of GTP in the absence (○ — ○) or presence of $10^{-5}$ M forskolin (● — ●) (right panel) for 10 min at 37°C. Values are the mean ± S.E. (bar) of three experiments.

mented by additional stimulation with forskolin. However, in AH66F cells forskolin-stimulated activity was not affected by other effectors and forskolin apparently diminished the activities induced by effectors except IPN. The activity after treatment with the combination with IPN and forskolin was the same as the activity with the diterpene alone.

Table II shows the effect of forskolin on manganese ion-stimulated activity. Adenylate cyclase of each cell line was activated by manganese ion. The combination of forskolin and manganese ion in hepatocytes and AH66 cells led to synergistic stimulation, but in AH66F cells the effect was additive.

**Effect on GTP-Stimulated Activity**

Each of forskolin and GTP dose-dependently activated adenylate cyclase of all cell lines. In hepatocytes and AH66 cells, each of these agents potentiated the activity stimulated by the other (Figs. 2 and 3). In AH66F cells, forskolin lowered the activities stimulated by $10^{-5}$ and $10^{-4}$ M GTP to the levels given by $3 \times 10^{-6}$ and $10^{-5}$ M forskolin alone, respectively, and the above concentrations of forskolin in the presence of GTP provided the same activity as obtained with forskolin alone. The forskolin-stimulated activity was hardly affected by GTP (Fig. 4).

**Discussion**

This study has shown that forskolin can accumulate cyclic AMP and activate adenylate cyclase in rat normal hepatocytes, AH66 cells and AH66F cells. Two types of action of forskolin on the cyclic AMP formation and the stimulated adenylate cyclase activities were found, namely potentiation and inhibition. In hepatocytes and AH66 cells forskolin significantly potentiated the activation of adenylate cyclase through the hormone receptors by PGE$_1$ and IPN, the N by NaF, GTP and its unhydrolyzable analogue, Gpp(NH)p, and the catalytic unit by manganese.
ion. The diterpene also further activated the cyclase in these cells treated with CT and IAP which activate the enzyme by stimulation of the stimulatory N (Ns) and inhibition of the inhibitory N (Ni), respectively. It has been proposed that forskolin modifies the interaction between the Ns and the catalytic unit of adenylate cyclase. Bouhelal et al. have concluded that forskolin may stabilize the complex between the GTP-binding protein and the catalytic unit in a reversible manner. The potentiation by forskolin of the stimulated cyclase activity in rat hepatocytes and AH66 cells may result from the closer interaction between the Ns and the catalytic unit.

On the other hand, the inhibitory effect of forskolin was observed in AH66F cells. Seamon and Wetzel have suggested in a review that adenylate cyclase activity induced by forskolin is dually affected by guanine nucleotides; one mode of action is inhibitory regulation via the Ni by low concentrations of the nucleotides and the other is stimulatory regulation via the Ns by high concentrations of the nucleotides. Jakobs and Watanabe described the inhibition of adenylate cyclase by forskolin in rat basophilic leukemia cells and human platelets, and suggested that this inhibition is not mediated by the Ni, but may be due to an action of the diterpene at the catalytic unit. In this study using AH66F cells, forskolin dose-dependently activated the adenylate cyclase but suppressed the intracellular cyclic AMP accumulation and the adenylate cyclase activity stimulated by PGE1 and other activators except IPN and manganese ion. AH66F cell line was insensitive to IPN while the other two cell lines showed high sensitivity. The action of forskolin on adenylate cyclase in AH66F cells exhibits the following characteristics: a) the forskolin-stimulated activity was not affected by other effectors including guanine nucleotides; b) the diterpene inhibited the activation through the receptor and the N, namely it abolished the Ni-mediated activation; c) it did not inhibit but additively increased the manganese-stimulated activity. The possibility of the Ni-mediated inhibition of forskolin in AH66F cells may be ruled out from the findings of no inhibition by itself, no effect on the activities induced by low concentrations of GTP and inhibition of the increased activity by inactivation of the Ni by IAP. These data suggest that forskolin directly activates the catalytic unit but interferes with the stimulatory interaction between the Ns and the catalytic unit in AH66F cells. Although the AH66F cell line is a kind of subclone of the AH66 cell line, AH66F cells appear to have quite different properties from the parent cells and normal hepatocytes. Some functional or structural changes on the Ns and the catalytic unit in AH66F cells may cause the impaired interaction.

We conclude from the present study that forskolin acts on the catalytic unit and promotes the interaction between the Ns and the catalytic unit in rat normal hepatocytes and AH66 cells, whereas the diterpene inhibits the interaction in AH66F cells.

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References

Effect of Forskolin on Adenylate Cyclase

(1985).


