Forskolin refractoriness

Exposure to the diterpene alters guanine nucleotide-dependent adenylate cyclase and calcium-uptake activity of cells cultured from the rat aorta

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Cells with the morphological properties of endothelial cells were cultured from the rat aorta. The cultured cells accumulated 46Ca2+ from the medium in a manner which was stimulated by forskolin and by 8-bromo-cyclic AMP. Pretreating the cultures for 20 h with forskolin diminished forskolin-dependent Ca2+-uptake activity. Adenylate cyclase activity of cultured cell homogenates was stimulated by guanosine 5’-[β,γ-imido]triphosphate (p[NH]ppG) and forskolin, and by isoprenaline in the presence, but not in the absence, of guanine nucleotide. p[NH]ppG increased forskolin sensitivity and caused a leftward shift in the forskolin dose–response curve. Pretreating the cultured cells with forskolin for 20 h, conditions that decreased forskolin-dependent Ca2+ uptake, increased basal and guanine nucleotide-dependent adenylate cyclase activity, but not forskolin-dependent activity determined in the absence of p[NH]ppG. Forskolin pretreatment diminished p[NH]ppG’s capacity to increase forskolin sensitivity, but did not have a significant effect on either the sensitivity of adenylate cyclase to p[NH]ppG or its responsiveness to isoprenaline. These results suggest that the Ca2+ uptake mechanism is cyclic AMP-dependent and that guanine nucleotides mediated forskolin-dependent cyclic AMP production by the intact cells. In addition, there may be different guanine nucleotide requirements for hormone–receptor coupling and forskolin activation.

INTRODUCTION

Sensitivity to forskolin is a ubiquitous property of hormone- and neurotransmitter-sensitive adenylate cyclase in mammals, and it was proposed originally that forskolin activated the adenylate cyclase catalytic subunit directly (Seamon et al., 1981; Daly, 1984). Since then, however, evidence has accumulated from a variety of sources that suggests that activation by forskolin and activation by receptors for hormones or neurotransmitters share components of adenylate cyclase besides the catalytic subunit. Both the stimulatory and inhibitory forms of the N regulatory protein that couple receptor and catalytic subunits, as well as more poorly characterized components of the adenylate cyclase system, have been suggested as mediators of forskolin-dependent cyclic AMP production (Green & Clark, 1982; Downs & Aurbach, 1982; Seamon & Daly, 1982; Brooker et al., 1983; Wong & Martin, 1983; Barber & Goka, 1985).

Forskolin increases the hormone-sensitivity of a variety of cell types and has the capacity to reverse agonist-dependent desensitization in some types of cells (Harper, 1983–84; Darler et al., 1982; Daly, 1984). Although protracted exposure to the diterpene decreases the hormone-sensitivity of cyclic AMP production in some cells, forskolin treatment does not always desensitize adenylate cyclase, although it may diminish the steady-state rate of cyclic AMP production by increasing phosphodiesterase activity (Darler et al., 1982; Clark et al., 1982; Dix et al., 1984; Rich et al., 1984). Since desensitization in a variety of cell types has been shown to have a cyclic AMP-dependent component, this latter property of forskolin seems incongruous with respect to its ability to cause a protracted increase in cyclic AMP (Su et al., 1976; Moylan & Brooker, 1981; Moylan et al., 1982; Nambi et al., 1984). We therefore examined the effects of prolonged forskolin exposure on vascular cells which retain several hormone-sensitive properties in tissue culture, including receptor-mediated cyclic AMP synthesis (D’Amore & Shepro, 1977; Brotherton et al., 1982; Hong & Deykin, 1982).

MATERIALS AND METHODS

Aortic-cell culture

Thoracic aortae were removed from female Sprague–Dawley rats (200–250 g), placed in freshly gassed (O2/CO2, 19:1) Hanks buffered salt solution (pH 7.4) and inverted with forceps. Both ends of the inverted vessel were tied with 3-0 silk thread, and these sealed tubes were transferred to the cell-dispersion medium, which was Medium 199 (Earle’s modified salts) containing 1.0 mg of type II collagenase (Sigma, St. Louis, MO, U.S.A.)/ml and 0.5 mg of elastase (Calbiochem–Behring, San Diego, CA, U.S.A.)/ml.

The incubation mixture was shaken for 2 h at 37 °C with continuous gassing (O2/CO2, 19:1). The resultant cell suspension was filtered through a single layer of cloth mesh and the cells were collected by centrifugation at 2000 g for 15 min. The cell pellet was washed twice by suspension in Medium 199 and collected each time by centrifugation. The washed cells were finally suspended in fresh medium containing 20% (w/v) bovine calf serum and plated at a density of 25000–50000 cells/ml.

The cultured cells reached confluence within 7–10...

days, when they assumed the cobblestone shape that characterizes vascular endothelial cells in tissue culture (Gospodarowicz et al., 1980).

**Determination of Ca\(^{2+}\)-uptake activity**

Suspensions of cells were prepared from confluent cultures by replacing the growth medium with fresh Medium 199 without serum and which contained 0.05% trypsin and 0.02% EDTA. After 15 min the cells were transferred with the aid of a scraper and collected by centrifugation at 1000 g for 15 min. The cell pellet was centrifugally washed twice by resuspension in fresh medium without trypsin or EDTA, and twice by resuspension in Ca\(^{2+}\)-PSS, which was: 5 mM-Hepes/NaOH, pH 7.2, 0.14 mM-NaCl, 5 mM-KCl, 1 mM-MgCl\(_2\), 0.01 mM-dextrose and 0.765 mM-CaCl\(_2\). The washed cells were suspended in Ca\(^{2+}\)-PSS at a density of 1 \(\times\) 10\(^6\) cells/ml for use in the uptake assay.

\(^{45}\)Ca\(^{2+}\) (New England Nuclear, Boston, MA, U.S.A.) was added to 0.25 ml portions of the cell suspension so that the final specific radioactivity was 10 \(\mu\)Ci/ml. The assay was started by adding 0.025 ml of either forskolin dissolved in ethanol or ethanol alone to the cell suspension at 37 °C. The reaction was stopped after 2 min by the addition of 1.5 ml of La\(^{3+}\)-PSS, which was the same as Ca\(^{2+}\)-PSS but with 0.01 mM-LaCl\(_3\) and no CaCl\(_2\). The diluted cell suspensions were rapidly filtered under vacuum on to nitrocellulose filters (0.045 \(\mu\)m pore size; HAWP; Millipore Corp., Boston, MA, U.S.A.) which were washed with 3 \(\times\) 2 ml of La\(^{3+}\)-PSS. The filters were dissolved and counted for radioactivity in Bray's scintillation cocktail.

**Determination of adenylyl cyclase activity**

Cells suspended and washed in Ca\(^{2+}\)-PSS as described above were concentrated by centrifugation and resuspended and homogenized at 2 °C with a glass/Teflon homogenizer in adenylyl cyclase homogenization buffer, which was: 0.05 mM-Hepes/NaOH, pH 7.6, 1 mM-EGTA and 10% (v/v) dimethyl sulphoxide. The homogenates were stored at −90 °C for up to 3 weeks. Enzyme activity of 0.04 ml of the frozen and thawed homogenates was determined as the conversion of \([\alpha-\text{\textsuperscript{32}P}]\text{ATP}\) into cyclic \([\text{\textsuperscript{32}P}]\text{AMP}\) in 5 min at 37 °C, followed by purification of the radioactive product by double column chromatography by the method of Salomon et al. (1974) as described by Fortier et al. (1983).

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

**Expression of the results**

All values are those obtained with three or more separate preparations. The results of some experiments are expressed as \(v/V\), the enzyme activity at a fraction of the maximum concentration of the activator divided by the activity of the maximum concentration of the same activator.

**RESULTS**

Several important functions of the vascular endothelial cell are Ca\(^{2+}\)-dependent (Hong & Deykin, 1982; Brotherton & Hoak, 1982) and, like endothelial cells, the cells cultured from the rat aorta were able to take up \(^{45}\)Ca\(^{2+}\) in measurable quantities from the incubation medium (Fig. 1). The \(^{45}\)Ca\(^{2+}\)-uptake activity was variable in different cultures, but was increased significantly (\(P < 0.025\)) by the labdane diterpene forskolin (Fig. 1). This suggested that Ca\(^{2+}\) uptake by these cells was a cyclic AMP-mediated process, and addition of low concentration of 8-bromo-cyclic AMP to the cells significantly increased \(^{45}\)Ca\(^{2+}\) uptake (Table 1). Pretreating the cultures by exposing them to forskolin for 20 h decreased the sensitivity of the \(^{45}\)Ca\(^{2+}\)-uptake activity to subsequent stimulation by forskolin (Fig. 1). This further suggested that forskolin-dependent Ca\(^{2+}\) uptake was cyclic-AMP-mediated, although variability in the activity of the cultured cells precluded determining whether 8-bromo-cyclic AMP significantly increased the Ca\(^{2+}\)-uptake activity of the cells pretreated with forskolin (results not shown). The loss of forskolin

![Fig. 1. Ca\(^{2+}\)-uptake activity of the cultured aortic cells](image-url)

Ca\(^{2+}\)-uptake activity was quantified as described in the Materials and methods section by using suspensions of cells incubated for 20 h under control conditions (○) or in the presence of 25 \(\mu\)M-forskolin (●). The results are means \(\pm\) S.E.M. for four experiments. **Significantly less than the control values (\(P < 0.01\)) compared by unpaired \(t\) test; *significantly greater than the control values (\(P < 0.025\)) compared by unpaired \(t\) test.

**Table 1. Stimulation of Ca\(^{2+}\)-uptake activity by exogenous addition of cyclic AMP**

<table>
<thead>
<tr>
<th>Additions to the cultured cells</th>
<th>Ca(^{2+})-uptake activity (nmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.57 (\pm) 0.38</td>
</tr>
<tr>
<td>10 (\mu)M-8-Bromo-cyclic AMP</td>
<td>2.72 (\pm) 0.74*</td>
</tr>
<tr>
<td>10 (\mu)M-8-Bromo-cyclic GMP</td>
<td>1.09 (\pm) 0.23</td>
</tr>
</tbody>
</table>
Table 2. Properties of aortic-cell adenylate cyclase

Results are means ± S.E.M. for four determinations.

<table>
<thead>
<tr>
<th>Additions to assay</th>
<th>Activity (nmol of cyclic AMP formed/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal)</td>
<td>0.214 ± 0.009</td>
</tr>
<tr>
<td>p[NH]ppG alone</td>
<td>1.800 ± 0.045</td>
</tr>
<tr>
<td>Forskolin alone</td>
<td>1.661 ± 0.022</td>
</tr>
<tr>
<td>p[NH]ppG + forskolin alone*</td>
<td>3.247 ± 0.050</td>
</tr>
<tr>
<td>Forskolin + p[NH]ppG</td>
<td>3.364 ± 0.177</td>
</tr>
</tbody>
</table>

* The sum of the activity with each of the activators alone.

sensitivity was further investigated, therefore, at the level of forskolin-sensitive adenylate cyclase.

Adenylate cyclase activity of cell homogenates was stimulated by guanine nucleotide and by forskolin (Table 2). When tested in saturating concentrations (300 and 100 μM respectively), there was not a significant difference (P > 0.05) between p[NH]ppG and forskolin in the degree to which they stimulated adenylate cyclase. Adenylate cyclase activity was further enhanced by the addition of p[NH]ppG and forskolin together, but the stimulation was additive (Table 2).

As shown in Fig. 2(a), p[NH]ppG markedly increased the sensitivity of adenylate cyclase to stimulation by forskolin in homogenates of cells which had not been exposed to forskolin previously (control incubation). In four separate experiments, 300 μM-p[NH]ppG decreased from 10 to 0.2 μM the concentration of forskolin that was required to give half-maximal stimulation of adenylate cyclase. Consequently, in the aortic cells, as in the other cell types, the guanine nucleotide coupling apparatus contributes to maximum forskolin sensitivity, even though the diterpene by itself is capable of stimulating catalysis.

A relationship between activation of adenylate cyclase by forskolin and by guanine nucleotides was also suggested by the changes that were induced in adenylate cyclase by culturing the cells in the presence of forskolin. Exposure to forskolin for 20 h, the conditions that diminished forskolin-dependent Ca++-uptake activity, also caused a significant (P < 0.01) increase in basal adenylate cyclase activity when this was determined in the presence of Mg++ (Table 3). Forskolin exposure also increased guanine nucleotide-dependent adenylate cyclase activity when the activity in the presence of p[NH]ppG and Mg++ was corrected by subtraction of basal activity (activity in the presence of Mg++ alone). In contrast with the effects that forskolin exposure had on basal and p[NH]ppG-dependent adenylate cyclase acti-

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Table 3. Effect of forskolin pretreatment on aortic-cell adenylate cyclase activity

Results are means ± S.E.M. for four to seven separate experiments. Cells were incubated for 24 h in the presence of 25 μM-forskolin dissolved in ethanol or with the vehicle alone. * ** significantly greater (P < 0.05; **P < 0.001) than the control value when compared by unpaired t test.

<table>
<thead>
<tr>
<th>Cell culture conditions</th>
<th>Activity (nmol of cyclic AMP formed/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>+p[NH]ppG†</td>
</tr>
<tr>
<td>+forskolin‡</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.168 ± 0.130</td>
</tr>
<tr>
<td>0.436 ± 0.045*</td>
<td>3.581 ± 0.143**</td>
</tr>
<tr>
<td>2.802 ± 0.615†</td>
<td></td>
</tr>
</tbody>
</table>

* Assayed in the presence of 300 μM-p[NH]ppG and corrected by subtraction for activity in its absence.
† Assayed in the presence of 100 μM-forskolin and corrected by subtraction for activity in its absence.

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Fig. 2. Effect of exposure to forskolin on forskolin-dependent adenylate cyclase activity

Cell cultures were pretreated for 20 h in the absence (○, □) or presence (●, ■) of 25 μM-forskolin before harvesting and homogenizing as described in the Materials and methods section. (a) The dose-dependent effect of forskolin on adenylate cyclase activity of the homogenates was determined in the absence (○, ●) or presence (□, ■) of 300 μM-p[NH]ppG, as indicated. (b) Forskolin-dependent adenylate cyclase activity determined in the absence (○, ●) or presence (□, ■) of 300 μM-p[NH]ppG and calculated by the subtraction, respectively, of the basal activity or the activity in the presence of p[NH]ppG alone. The results are means ± S.E.M. for four experiments. **Significantly different (P < 0.025) from the activity in cells not pretreated by exposure for 20 h to forskolin (control incubation) when compared by unpaired t test.
Adenylyl cyclase activity of cells first incubated in the absence (△) or presence (▲) of 25 μM-forskolin was determined at the indicated concentrations of [p(NH)ppG as described in the Materials and methods section. The results (means ± S.E.M. for four experiments) have been corrected for the activity in the presence of Mg²⁺ alone (basal activity).

Adenylyl cyclase activity, culturing the cells in the presence of forskolin had no significant effect on forskolin-dependent activity determined in the absence of p[NH]ppG (Table 3).

Exposing the cells to forskolin for 20 h had a measurable effect on forskolin-dependent adenylyl cyclase activity, however, when this was determined in the presence of p[NH]ppG (Fig. 2). Exposure to forskolin marked diminished the ability of p[NH]ppG to increase the sensitivity of adenylyl cyclase to forskolin, as determined by comparison of the effect of the guanine nucleotide on the forskolin dose–response curves of cells incubated in the absence or presence of forskolin (Fig. 2b). Compared with these effects on p[NH]ppG-dependent forskolin sensitivity, the effects of forskolin exposure on adenylyl cyclase sensitivity to stimulation by p[NH]ppG were small and not significant (P > 0.05), as reflected by the similarity between the dose–response curves of the two treatment groups (Fig. 3).

Besides guanine nucleotides and forskolin, cultured cell adenylyl cyclase was also stimulated by the β-adrenergic catecholamine agonist isoprenaline in the presence, but not in the absence, of an exogenously added guanine nucleotide such as p[NH]ppG or GTP (results not shown). We therefore determined whether the effect of forskolin exposure on catalytic subunit activation encompassed its regulation by the β-adrenergic receptor. As shown in Table 4, isoprenaline in the presence of GTP stimulated adenylyl cyclase about 2-fold above the effect of GTP alone whether the cells had been exposed to forskolin or only to the vehicle. Moreover, isoprenaline-dependent activity, determined by subtracting the activity in the presence of GTP from that in the presence of isoprenaline and GTP, was not significantly different (P > 0.05) in forskolin-treated compared with control cells.

**DISCUSSION**

Prolonged exposure of aortic cells that morphologically resembled vascular endothelial cells to forskolin in cell culture increased guanine nucleotide-dependent adenylyl cyclase activity, but decreased the capacity of guanine nucleotide to modulate the forskolin sensitivity of catalysis. Prolonged exposure to the diterpene also decreased forskolin-dependent Ca²⁺ uptake by the cells. Since the Ca²⁺-uptake activity was also stimulated by 8-bromo-cyclic AMP, the results suggest that all of the changes induced by exposure to forskolin reflect changes in a common component of the adenylyl cyclase system. Alterations in one or more of the components of the N regulatory protein that accounts for the guanine nucleotide sensitivity of mammalian adenylyl cyclase may account for all of the changes induced by exposure to forskolin (Gilman, 1984). Several observations suggest that some subunits of N protein were affected by forskolin exposure, although neither p[NH]ppG sensitivity nor β-adrenergic-receptor coupling seem to have been much altered.

First, the pretreated cells had significantly higher guanine nucleotide-dependent activity, but the same forskolin-dependent activity, compared with cells that were not first pretreated with forskolin. If forskolin sensitivity measured in the absence of guanine nucleotides reflects the relative abundance of the adenylyl cyclase catalytic subunits, as implied originally by Seamon et al. (1981), then the changes caused by forskolin exposure which we have characterized did not involve the catalytic subunits and probably occurred proximally to them. Next, changes at the N-regulatory-protein level might account for the loss of forskolin sensitivity of adenylyl cyclase, which could only be demonstrated in the presence of p[NH]ppG. Finally, the effect of forskolin exposure on the Ca²⁺-uptake process (which was also sensitive to 8-bromo-cyclic AMP) in the intact cells

![Graph](image-url)
suggests that some components of N protein functioned both in forskolin action in the intact cells and in regulating forskolin sensitivity by p[NH]ppG in the broken-cell adenylate cyclase assay. Forskolin-induced changes in these enzyme components probably therefore accounted for the decrease in forskolin-dependent Ca\(^{2+}\) uptake as well as changes in the guanine nucleotide-sensitive adenylate cyclase system.

Glucagon desensitization induces forskolin refractoriness in the rat hepatocyte, and forskolin exposure is associated with a loss of MDCK-cell adenylate cyclase sensitivity to the same peptide hormone as well as to series E prostaglandins (Rich et al., 1984; Noda et al., 1984). Forskolin treatment also induces refractoriness to lutropin in Leydig-tumour cells (Dix et al., 1984). These characterizations of adenylate cyclase refractoriness to regulation by receptors coupled by the N regulatory protein, and the present report, may all reflect different aspects of the importance of the N complex in the forskolin response in intact cells, as initially suggested by Downs & Aurbach (1982). The effect of forskolin exposure at the level of the N regulatory protein in the cultured aortic cells did not seem to extend, however, to the coupling of the \(\beta\)-adrenergic receptor with the catalytic subunit. Subunits of the N protein different than those that couple receptors with the catalytic subunit may therefore function in forskolin activation. Other hormone- and neurotransmitter-sensitive cells may share this property.

For instance, partially purified N protein restored NaF sensitivity, but failed to restore sensitivity to glucagon or to series E prostaglandins to MDCK-cell membranes desensitized with glucagon (Rich et al., 1984). The failure to recouple desensitized receptors occurs despite the fact that detergent extracts of membranes enriched in the N regulatory protein augment the activation of catalysis by forskolin in several cell types (Green & Clark, 1982; Bender & Neer, 1983; Levine et al., 1984). In addition, one of us argued previously from kinetic evidence that, since forskolin increased both the \(K_m\) and the \(V_{\text{max}}\) of adenylate cyclase activation by p[NH]ppG, the deter-
pene probably bound at a (second) site that was different from the guanine nucleotide binding site, which resides on the \(\alpha\)-subunit of the N-regulatory-protein complex (Krall, 1984).

The large effect of p[NH]ppG on the apparent activation constant of aortic-cell adenylate cyclase by forskolin, and the absence of a detectable effect of forskolin exposure on forskolin-dependent adenylate cyclase, except in the presence of p[NH]ppG, further suggests that two separate sites account for forskolin–guanine-nucleotide interaction. Although both sites may reside in the same heterogeneous N-regulatory-protein complex, it is difficult to account for all of the observed properties by a common (single) site of guanine nucleotide and diterpene action such as that proposed by Barber & Goka (1985). Although purely kinetic analysis may distinguish between sites but not subunits, other studies found forskolin responsiveness more sensitive than receptor coupling to protein-synthesis inhibition, and therefore suggested the existence of a putative forskolin coupling factor within hormone-sensitive enzyme systems (Brooker et al., 1983).

The observation that forskolin-dependent adenylate cyclase activity in the cell homogenates only reflected the loss of Ca\(^{2+}\)-uptake sensitivity by the intact cells if enzyme activity was determined in the presence of guanine nucleotide deserves emphasis. It supports the contention of Darfler et al. (1982) that cell disruption leads to subtle changes in the adenylate cyclase system that decrease the guanine nucleotide requirements of its forskolin sensitivity. Moreover, it suggests that the interpretation of forskolin sensitivity of adenylate cyclase may be unreliable unless the contribution of the \(N\) subunits can be taken into account in some fashion.

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