Stimulation of steroidogenesis by forskolin in rat adrenal zona glomerulosa cell preparations

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ABSTRACT

The actions of forskolin have been investigated to determine to what extent its effects on steroidogenesis in rat adrenal preparations are dependent on activation of adenylate cyclase. In zona glomerulosa preparations, stimulation of both aldosterone and corticosterone production was obtained at concentrations of forskolin between 1 and 10 μmol/l. The effects of 10 μmol forskolin/l were additive with those of low doses (1 pmol/l) of corticotrophin (ACTH), but not with those of high doses (1 nmol/l) of ACTH. In contrast, in zona fasciculata/reticularis cells, doses of forskolin up to 10 μmol/l produced no significant stimulation of corticosterone production either alone or in the presence of ACTH (1 pmol/l and 1 nmol/l). The response to 1 nmol ACTH/l was attenuated in the presence of forskolin (10 μmol/l) in both zona glomerulosa and zona fasciculata/reticularis cell preparations. Cyclic AMP production increased progressively with dose up to 100 μmol forskolin/l in zona glomerulosa cells, whereas corticosterone production was maximal between 10 and 30 μmol forskolin/l and decreased at 100 μmol forskolin/l. In zona fasciculata/reticularis cells, cyclic AMP production was also increased by forskolin (1 and 10 μmol/l).

The stimulation of zona glomerulosa steroidogenesis by forskolin (1–10 μmol/l) and ACTH (1–100 pmol/l) were both reduced by the adenylate cyclase inhibitor, N6-phenylisopropyladenosine (100 μmol/l). The calcium channel inhibitor, nifedipine, only reduced the steroidogenic response to forskolin (3 μmol/l) at doses of 300 μmol/l whereas the response to 8·4 mmol K⁺/l was inhibited at 10 μmol nifedipine/l. Although there is some dissociation between the effects of forskolin on cyclic AMP and steroidogenesis, the results are generally consistent with the view that the effects of forskolin in rat zona glomerulosa cells are mainly dependent on activation of adenylate cyclase. This contrasts with the effects of forskolin in bovine fasciculata cells which are reported to be mediated by activation of voltage-regulated calcium channels.


INTRODUCTION

The diterpene compound, forskolin, activates adenylate cyclase in mammalian cells, and has found wide use as a tool with which to probe the intracellular mechanisms coupled to cyclic AMP generation (Seamon & Daly, 1981, 1983, 1986; Laurenza, McHugh Sutkowski & Seamon, 1989). It has been shown that the actions of forskolin are not mediated through interaction with any of the major classes of receptors, nor through the guanine nucleotide regulatory unit of hormone-sensitive adenylate cyclase. In most, but not all situations the actions of forskolin appear to be consistent with a direct action on the catalytic subunit (Seamon & Daly, 1983). Recently, however, a number of alternative mechanisms have been proposed, in which forskolin may produce cyclic AMP-independent effects by interactions with channel and transport proteins in the cell membrane (Laurenza et al. 1989).

Forskolin has been reported to stimulate cyclic AMP and steroid hormone production in ovarian, testicular and adrenal preparations on many occasions (Moriwaki, Itoh, Iida & Ichihara, 1982; Hedin &
Rosenberg, 1983; Schimner & Tsao, 1984; Sullivan & Cooke, 1984; Mikami, Nishikawa & Strott, 1985; Kenny & Robinson, 1986; de Foresta, Rogard & Gallay, 1987; Langlois, Saez & Begeot, 1987; Jammes, Llosa-Hermier, Martinet & Hermier, 1988; Yanagibashi, Papadopoulos, Masaki et al. 1989). The interaction of forskolin with adrenocorticotrophin (ACTH)- and cholera toxin-stimulation of corticosterone production by rat adrenocortical cells has been described. The potentiation of the responses obtained in the presence of forskolin was taken to indicate that all three agents acted by increasing adenylate cyclase activity (Moriwaki et al. 1982). However, this view has been challenged by evidence which suggests that the stimulation of steroidogenesis produced by forskolin in bovine adrenocortical cells is instead mediated by activation of voltage-dependent calcium channels (Yanagibashi et al. 1989). The authors also reported that forskolin did not stimulate corticosterone production in rat zona fasciculata/reticularis preparations and suggested that the absence of calcium channels accounted for this phenomenon (Yanagibashi, 1979; Yanagibashi, Kawamura & Hall, 1990). In contrast with the unresponsiveness of rat fasciculata/reticularis cells, studies in this laboratory have shown forskolin to be an effective stimulant of steroid production by rat zona glomerulosa cells (Purdy & Whitehouse, 1987). We have, therefore, investigated the mechanisms by which forskolin stimulates steroid production in rat glomerulosa cells, to determine whether its effects on steroidogenesis in zona glomerulosa preparations are dependent on increased cyclic AMP production. The effects of an inhibitor of adenylate cyclase and of a calcium channel blocker on forskolin-stimulated effects have been investigated, and the correlation between cyclic AMP production and steroidogenesis has been examined.

MATERIALS AND METHODS

Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one) was obtained from Sigma Chemical Co. Ltd, Poole, Dorset, U.K. It was stored at -10°C in ethanol at a concentration of 10 mmol/l and diluted as required in Krebs-Ringer bicarbonate buffer (3.6 mmol K+/l; 11.1 mmol glucose/l) containing 1% (w/v) bovine serum albumin (KRBA medium) for addition to incubations. Synacthen (ACTH(1-24)) was obtained from Ciba Geigy, Horsham, Sussex, U.K.; Worthington type I collagenase from Cambridge Bio-Science, Cambridge, U.K.; bovine serum albumin, fraction V (BSA), nifedipine, N⁶-phenylisopropyladenosine (PIA) and isobutyl-methylxanthine (IBMX) from Sigma. 1,9-Dideoxy-

forskolin was obtained from Calbiochem Corporation, San Diego, CA, U.S.A.

Adrenals were obtained from both male and female Sprague-Dawley rats (body weights 250–350 g) bred and maintained in the Kensington Campus animal house. The animals were killed by stunning and cervical dislocation and the glands rapidly removed. After removal of excess fat, the adrenals were separated into capsule (mainly zona glomerulosa) and inner zone (mainly zona fasciculata/reticularis) fractions by squashing between glass plates. The dispersed cell preparations were obtained by collagenase treatment following the method of Haning, Tait & Tait (1970). In brief, capsules and inner zone fractions of 16–20 adrenals were incubated separately in 5 ml KRBGA medium with 2 mg collagenase/ml for 1 h at 37°C under 95% O₂ and 5% CO₂. Tissue was dispersed by repeated pipetting and filtered through nylon gauze (60 μm mesh). After centrifugation at 100 g, the pelletted cells were washed with KRBGA and centrifuged again. The cells were resuspended and incubated in 1 ml KRBGA in glass vials (approximately 2 × 10⁴ zona glomerulosa cells per ml and 5 × 10⁴ fasciculata/reticularis cells per ml) for 2 h in the presence or absence of forskolin (1 nmol/l–10 μmol/l), ACTH (10 fmol/l–10 nmol/l), PIA (100 μmol/l) or nifedipine (10–300 μmol/l). After incubation, the media and cells were extracted with ethyl acetate and the aldosterone and corticosterone contents assayed by radioimmunoassay as previously described (Vinson, Laird, Whitehouse & Hinson, 1989). For corticosterone the lower detection limit of the assay was 2 nmol/l, and the intra- and interassay coefficients of variation were 3.5 ± 0.45% and 3.1% at 20 nmol corticosterone/l (n = 3). The lower detection limit of the aldosterone assay was 0.5 nmol/l, and the intra- and interassay coefficients of variation were 7.9 ± 2.5% and 2.9% at 5 nmol aldosterone/l (n = 3).

Separate 30-min incubations were carried out in parallel for the measurement of cyclic AMP production; these were preceded by a 60-min preincubation period in 10 ml KRBGA. The cells were recovered by centrifugation and the series of experimental tubes set up in duplicate as described above. The cyclic AMP incubations were terminated by heating to 95°C for 15 min after the addition of 1 ml theophylline (2 mmol/l) as described by Fujita, Aguilera & Catt (1979). The cyclic AMP content of the medium was measured by radioimmunoassay using the method of Steiner, Parker & Kipnis (1972) including the acetylation procedure detailed by Harper & Brooker (1975). The lower detection limit of the assay was 250 pmol cyclic AMP/l, and the intra- and interassay coefficients of variation were 7.3 ± 2.1% and 8.5% at 2.5 nmol cyclic AMP/l (n = 3).

All incubations were stored at -10°C before assay. Results are expressed as means ± s.e.m. The
RESULTS

Effects of forskolin on steroid and cyclic AMP production by rat adrenal cells

Figure 1 illustrates the effect of doses of forskolin ranging from 1 nmol/l to 10 µmol on steroid
production by rat adrenal capsular and inner zone preparations in the presence and absence of ACTH at two dose levels (1 pmol/l and 1 nmol/l). It can be seen that forskolin significantly stimulated both aldosterone and corticosterone production at concentrations above 1 μmol/l and that its effects were additive with those of a low dose of ACTH (1 pmol/l; Fig. 1a,b). In four similar experiments with 3 μmol forskolin/l the mean stimulation ratio (stimulated/basal) for aldosterone was 1.83 ± 0.04 and for corticosterone 1.94 ± 0.13. Forskolin had no significant effect on corticosterone production by zona fasciculata/reticularis cells at any dose either under control conditions or in the presence of 1 pmol/l ACTH (Fig. 1c). There was a significant attenuation of the response to 1 nmol ACTH/l at doses of 10 μmol forskolin/l in both capsular and inner zone preparations (Fig. 1a,b,c).

The effects of 3 μmol forskolin/l on the cyclic AMP and corticosterone responses to ACTH (1 pmol/l and 1 nmol/l) in zona glomerulosa cells are shown in Fig. 2a and b. Cyclic AMP production was increased in the presence of forskolin to a small but significant extent at both levels of ACTH, whereas corticosterone production was further increased by forskolin at 1 pmol ACTH/l but not at 1 nmol ACTH/l.

Table 1 compares the corticosterone and cyclic AMP responses to forskolin concentrations up to 100 μmol/l, and shows that cyclic AMP production increased progressively with dose, whereas corticosterone production reached a maximum between 10 and 30 μmol forskolin/l and declined at 100 μmol/l. 1,9-Dideoxyforskolin did not affect corticosteroid production by rat adrenal zona glomerulosa cells at doses between 1 and 30 μmol/l nor did 30 μmol and 100 μmol forskolin/l affect corticosterone production by zona fasciculata/reticularis cells (data not shown). The effects of the phosphodiesterase inhibitor, IBMX (5 mmol/l), on the cyclic AMP and corticosterone responses to forskolin were also tested in the zona fasciculata/reticularis preparations. Cyclic AMP production was significantly increased by exposure to 1 and 10 μmol forskolin/l (control: 7.92 ± 0.14 pmol/tube; 1 μmol forskolin/l: 11.51 ± 0.86 pmol/tube, 10 μmol forskolin/l: 22.49 ± 0.27 pmol/tube), and this response was potentiated in the presence of 5 mmol IBMX/l (control: 9.63 ± 0.66 pmol/tube; 1 μmol forskolin/l: 20.11 ± 0.83 pmol/tube, 10 μmol forskolin/l: 42.67 ± 2.25 pmol/tube); however, neither agent affected corticosterone production.

Effect of PIA and nifedipine on rat zona glomerulosa cell responses

Figure 3a shows that the adenylate cyclase inhibitor, PIA (100 μmol/l), significantly attenuated the response of zona glomerulosa cells to ACTH (1, 10 and 100 pmol/l), without having any effect on basal steroid production. PIA also significantly reduced the response to forskolin at 1, 3 and 10 μmol/l (Fig. 3b). The mean reduction of ACTH stimulation (1 nmol/l) of corticosterone production in three experiments was 51 ± 6% and for forskolin stimulation (3 μmol/l) 48 ± 5%.

The results of an experiment in which the ability of the calcium channel antagonist, nifedipine (10–300 μmol/l), to inhibit the responses of zona glomerulosa cells was investigated, are shown in Fig. 4a and b. A clear dose-related reduction in the corticosterone and aldosterone response to stimulation by 8.4 mmol K⁺/l was seen with no changes in basal steroid production. However, only at the highest dose of nifedipine (300 μmol/l) was there any significant reduction in the stimulation of corticosterone production produced by forskolin (3 μmol/l) and ACTH (10 pmol/l). In three experiments the mean reduction of forskolin and ACTH stimulation of corticosterone production produced by 30 μmol nifedipine/l was 15 ± 4% and 22 ± 5%, compared with a reduction of K⁺ stimulation of 56 ± 4%.

DISCUSSION

The results obtained in this study show that forskolin is an effective stimulant of rat zona glomerulosa cells, and confirm that it is without effect on steroidogenesis in rat zona fasciculata/reticularis cells. An inhibitory action of low levels (picomolar to nanomolar) of forskolin on cyclic AMP production and steroidogenesis was revealed in a study of the effects of the
Figure 3. Effect of N^{6}-phenylisopropyladenosine (PIA; 100 µmol/l) and (a) ACTH or (b) forskolin on corticosterone production by rat adrenal zona glomerulosa cells. ACTH or forskolin alone (open bars); ACTH or forskolin plus PIA (100 µmol/l; hatched bars). Values are means ± S.E.M., n = 5. *P < 0·01 compared with no PIA (ANOVA and Dunnett’s t-test).

drug on Leydig cells (Khanum & Dufau, 1986). There was no suggestion of an equivalent effect in either adrenocortical cell type in the present series of experiments. Rat zona fasciculata/reticularis preparations were also unresponsive to forskolin in the presence of low doses (1 pmol/l) of ACTH, whereas the effects of the two stimulants were approximately additive in zona glomerulosa preparations, although the maximal stimulation did not exceed that produced by a high level of ACTH (1 nmol/l; Fig. 1a,b,c). Stimulation of steroidogenesis in zona glomerulosa cells by forskolin reached a plateau value at 10 µmol/l with a decline at 100 µmol/l (Table 1). In both zona glomerulosa and zona fasciculata/reticularis preparations a reduction of the response to 1 nmol ACTH/l is seen in the presence of 10 µmol forskolin/l; similar results were obtained by Moriwaki et al. (1982). A related phenomenon has been observed with forskolin and luteinizing hormone (LH) stimulation of steroid production in testicular Leydig cells and ovarian luteal cells (Sullivan & Cooke, 1984; Kenny & Robinson, 1986). The fact that the diterpene also inhibits glucose transport (upon which steroidogenesis is dependent) in the cells has been put forward as an explanation for these inhibitory actions of high doses of forskolin.

Figure 4. Effect of nifedipine and forskolin (3 µmol/l) or K+ (8·4 mmol/l) or ACTH (10 pmol/l) on (a) corticosterone and (b) aldosterone production by rat adrenal zona glomerulosa cells. Values are means ± S.E.M., n = 5. *P < 0·05 compared with no nifedipine (NFD) (ANOVA and Dunnett’s t-test).
increases (Amrolia, Sullivan, Garside et al. 1988). It seems unlikely that the inhibition of corticosterone production is associated with depletion of ATP as suggested by Moriwaki et al. (1982) since cyclic AMP production increases linearly with dose of forskolin up to 100 μmol/l (Table 1).

The conventional view which holds that the actions of forskolin in adrenal cells are mediated by activation of adenylate cyclase has been challenged by the recent report by Yanagibashi et al. (1989) in which it was proposed that the stimulation of corticosteroid production by forskolin in bovine fasciculata cells was instead mediated by activation of voltage-dependent calcium channels. The aim of the present study was to determine to what extent this interpretation was applicable to rat zona glomerulosa cells and to this end the effects of PIA, an inhibitor of adenylate cyclase (Shima, 1986), on the responses to ACTH and forskolin were compared. In contrast to bovine cells, the results obtained with rat zona glomerulosa cells show a clear reduction of the response to forskolin in the presence of PIA (100 μmol/l; Fig. 3). In addition, the 1,9-dideoxy derivative of forskolin which does not activate adenylate cyclase and which can serve as a ‘negative control’ (Seamon & Daly, 1986; Laurenza et al. 1989) did not stimulate steroidogenesis in zona glomerulosa preparations. Evidence suggesting that forskolin stimulates steroidogenesis in bovine zona fasciculata cells by activating voltage-dependent calcium channels was provided by the observation that the actions of forskolin were inhibited by calcium channel antagonists, such as nifedipine (Yanagibashi et al. 1989). Again, contrasting results were obtained with rat zona glomerulosa cells, where inhibition of forskolin stimulation by nifedipine was not obvious: only at very high concentrations of nifedipine (300 μmol/l) was the steroidogenic response to both forskolin and ACTH reduced. This may be compared with the 30-fold lower dose of nifedipine required to reduce the response to 8.4 mmol K⁺/l, an action known to be mediated by activation of voltage-regulated calcium channels (Aguilera & Catt, 1986; Quinn, Cornwall & Williams, 1987; Barrett, Bollag, Isales et al. 1989). On balance, therefore, the results obtained with rat zona glomerulosa cells are consistent with the view that the activation of adenylate cyclase by forskolin is of prime importance in mediating its effects.

Some inconsistencies still remain, however, particularly in the lack of correlation between the stimulation of cyclic AMP production and stimulation of steroidogenesis, which is manifest even in zona glomerulosa cells (Fig. 2, Table 1). It is at its most extreme in the inner zone preparations where, as has previously been noted by Yanagibashi et al. (1989), the failure of forskolin to stimulate steroid production in zona fasciculata/reticularis cells is not accompanied by a failure to stimulate cyclic AMP production. Moreover, inclusion of the phosphodiesterase inhibitor, IBMX, in these experiments led to still further increases in cyclic AMP production in the presence of forskolin, without any change in corticosterone production. This is an extremely unusual phenomenon, particularly when considering that similar increases in cyclic AMP production are produced by 1 nmol ACTH/l and result in maximum stimulation of steroidogenesis (B. J. Whitehouse & D. R. E. Abayasekara, unpublished observations). Yanagibashi et al. (1989, 1990) have attributed the failure of forskolin to stimulate steroidogenesis in fasciculata preparations to the absence of voltage-regulated calcium channels from the cells. The results obtained here with rat zona glomerulosa cells where activation of such calcium channels does not seem to be an important component of forskolin action would suggest that this is not the only explanation and that other mechanisms should continue to be sought.

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