Biochemical Pharmacology Vol 35 No 13, pp 2247–2251 1986
Printed in Great Britain

0006-2952/86 $3.00 + 0.00

FORSKOLIN-MEDIATED ACTIVATION OF CARDIAC, LIVER AND LUNG ADENYLATE CYCLASE IN THE RAT

RELATION TO [^{3}H]FORSKOLIN BINDING SITES

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(*Received* 14 *November* 1985, *accepted* 21 *January* 1986)

Abstract—We investigated whether differences in binding sites for [^{3}H]forskolin could account for the low potency of forskolin on adenylate cyclase (EC 4.6.1.1) from rat lung compared with heart or liver adenylate cyclase. Forskolin (0.1 mM) increased basal adenylate cyclase activity 41-fold in heart, 27-fold in liver, but only 3-fold in lung. The low potency in lung could not be accounted for by any lack of enzyme or stimulatory nucleotide-binding protein, since sodium fluoride (10 mM) increased basal activity 9–12-fold in all three tissues. The effectiveness of forskolin on adenylate cyclase appears to be related to the presence of specific [^{3}H]forskolin binding sites. [^{3}H]Forskolin binding in both heart and liver membranes was consistent with single binding sites with dissociation constants of 0.74 ± 0.25 μM and 1.43 ± 0.21 μM respectively. No such binding sites were detected in lung membranes. The binding was of low affinity (>100 μM) and showed no tendency to saturate. These results are not consistent with the hypothesis that the nucleotide-binding protein influences stimulation of adenylate cyclase by forskolin, rather [^{3}H]forskolin binding sites appear to be an important determinant of the effect of forskolin in different tissues.

Forskolin, a diterpene isolated from *Coleus forskohlii*, is a potent cardiac inotrope, vasodilator and activator of adenylate cyclase (EC 4.6.1.1) [1–4]. It is able to activate this enzyme, to various degrees, both in intact cells as well as in cell-free systems [3, 5–7] and is also capable of potentiating the effects of various hormones on adenylate cyclase [5, 8, 9]. Little is known about how it exerts these effects on adenylate cyclase. Experiments with mutant S-49 cells [8–11] and partially purified enzyme preparations [12–15] suggest that forskolin interacts with either the catalytic subunit of adenylate cyclase or some other as yet unrecognised protein subunit. It has also been suggested that guanine nucleotide stimulatory proteins (N_s) might also be important in influencing the magnitude by which forskolin stimulates adenylate cyclase in various tissues. Forskolin is a weak activator of bovine sperm adenylate cyclase which lacks N_s protein. Some sensitivity to forskolin is gained upon complementation of bovine sperm membranes with human erythrocyte membranes containing the N_s protein [16, 17]. However, the lack of N_s protein cannot explain the poor responsiveness of rat lung adenylate cyclase to forskolin, compared with adenylate cyclase in either heart, brain or liver [18]. The aim of the present investigation was to examine whether other factors such as an apparent lack of forskolin binding sites on rat lung tissue might account for its poor responsiveness compared with heart and liver.

MATERIALS AND METHODS

Materials. (α-^{32}P)Adenosine triphosphate (10–50 Ci/mmol) was purchased from Amersham International Ltd. (Amersham, U.K.). [2,8-^{3}H]-Adenosine-3′,5′-cyclic phosphate (cyclic AMP) (30–50 Ci/mmol) and [12-^{3}H]forskolin (31.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Isobutylmethylxanthine (IBMX) was obtained from Aldrich (Milwaukee, WI) and forskolin was a generous gift of Hoechst Australia Ltd. 1-Acetylforskolin and 7-desacetylforskolin were synthesised from forskolin by the methods of Bhat *et al.* [19, 20]. Stock solutions (15 mM) of forskolin and its derivatives were prepared in dimethylsulfoxide (DMSO) and stored at −20°. These were diluted as required. *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) were obtained from Calbiochem (La Jolla, CA) and guanosine-5′-triphosphate (GTP) and all other biochemicals from Sigma (St. Louis, MO).

Preparation of homogenates and crude membranes. Homogenates of heart, liver and lung for estimating adenylate cyclase activity were prepared from male Wistar rats (150–250 g) by homogenising (Polytron, PT10, setting 2.5, 30 sec) the tissues in 10 vol. of ice-cold 50 mM HEPES pH 7.5 buffer containing 3 mM dithiothreitol and 1 mM EGTA [21]. The homogenate was passed through two layers of gauze prior to assay for adenylate cyclase activity. Protein concentration of heart homogenates averaged 22 mg/ml, while those of lung and liver averaged 15 mg/ml and 26 mg/ml respectively.

Crude membrane fractions for use in the [^{3}H]forskolin binding studies were obtained by homogenising (Polytron PT10, setting 2.5, 30 sec) the three tissues in 4–10 vol. of TEB buffer (10 mM Tris HCl, pH 8.0, 1 mM EGTA, 1 mM thioglycollic acid and 2 mM $MgCl_2$) containing 10% sucrose. The homogenates were initially centrifuged at 500 *g* (15 min) and the resulting pellets carefully discarded

The supernatant was centrifuged at 30,000 *g* (15 min) and the pellet collected and resuspended in either 1 vol (heart and lung) or 3 vol (liver) of TEB per original unit weight of tissue Protein concentrations in crude cardiac membrane suspensions averaged approximately 7 mg/ml, while those for lung and liver averaged 3 5 mg/ml and 17 mg/ml respectively

Estimates of protein in tissue homogenates and membrane preparations were performed by the method of Lowry *et al* [22] after overnight hydrolysis in 1 M NaOH (37°) Bovine serum albumin was used as the standard. Both tissue homogenates and membrane suspensions were used immediately after preparation

Adenylate cyclase Adenylate cyclase activities in homogenates were measured with [α-^{32}P]ATP as the substrate according to Little *et al* [21]. The incubation mixtures contained 50 mM HEPES pH 7 5, 6 7 mM phosphoenolpyruvate, 6U pyruvate kinase, 100 μM GTP, 5 mM $MgCl_2$, 10 mM KCl, 1 mM isobutylmethylxanthine, 0 25 mM ^{32}P-ATP (0 45 μCi) and 15 μl tissue homogenates in a total volume of 150 μl Forskolin and its derivatives were added to the incubation mixtures in 5% DMSO in 50 mM HEPES buffer pH 7 5 The final concentration of DMSO in the incubation was 0 67% This and higher concentrations of DMSO have been found to have minimal effects on adenylate cyclase activities [23] Incubations were carried out at 30° for 10 min in a shaking water bath The incubation was terminated by transfer to a 0° bath and addition of 100 μl of a solution containing 40 mM ATP, 1 4 mM cyclic AMP and 2% sodium dodecylsulfate in 50 mM HEPES pH 7 5 Cyclic AMP (^{3}H, 20,000 dpm) was also added to each sample to monitor recovery of cyclic AMP which was isolated as described by Salomon *et al* [24]

[^{3}H]*forskolin binding* The binding of [^{3}H]forskolin to the membrane preparations was carried out by a rapid filtration assay similar to that described for [^{3}H]dihydroforskolin [23] In a typical assay 100 μl of membranes was added to glass tubes containing 400 μl 50 mM Tris HCl pH 7 5 buffer containing 1 mM $MgCl_2$, 0 67% DMSO and [^{3}H]forskolin Concentrations of forskolin greater than 0 1 μM were obtained by adding unlabelled forskolin The incubations were carried out at 30° for 10 min and terminated by rapidly filtering the samples (~2 sec) under vacuum through Whatman GF/C filters The filters were rapidly washed with 3 × 3 3 ml of ice-cold 50 mM Tris HCl, pH 7 5 containing 1 mM $MgCl_2$ The filters were dried by suction and counted at similar efficiencies (*ca* 35%) in a liquid scintillation counter

"Non-specific" binding of [^{3}H]forskolin was determined in the presence of 0 1 mM forskolin and was predominantly due to binding of [^{3}H]forskolin to the filters In a typical experiment with liver membranes and 10 nM [^{3}H]forskolin, total membrane binding averaged 3200 cpm with 124 cpm bound to filters (3.4 and 0 125% of cpm added, respectively) Filter blanks were determined in each experiment by carrying out parallel experiments in tubes containing no membranes

Saturation specific binding isotherms were analysed directly by computer as described by Parker and Waud [26] as well as by using linearized transformations of the binding isotherms (Eadie–Hofstee plot) [27] [^{3}H]forskolin displacement curves were analysed as described by Parker and Waud [26] The value for maximum binding capacity (B_{max}) derived from the Eadie–Hofstee plot was used to estimate the Hill coefficient (^nH) from the Hill plot [31] All results are the means ± S.E

RESULTS

Adenylate cyclase activation by forskolin and derivatives

The ability of forskolin to stimulate adenylate cyclase in homogenates prepared from rat heart, liver and lung is illustrated in Fig 1 Basal activity of cardiac adenylate cyclase is increased 9-fold by 0 1 μM forskolin whilst 0.1 mM forskolin increased activity 41-fold It was not possible to determine whether this represented maximum levels of stimulation since 0 1 mM forskolin is close to its limit of solubility in the incubation medium [25] All con-

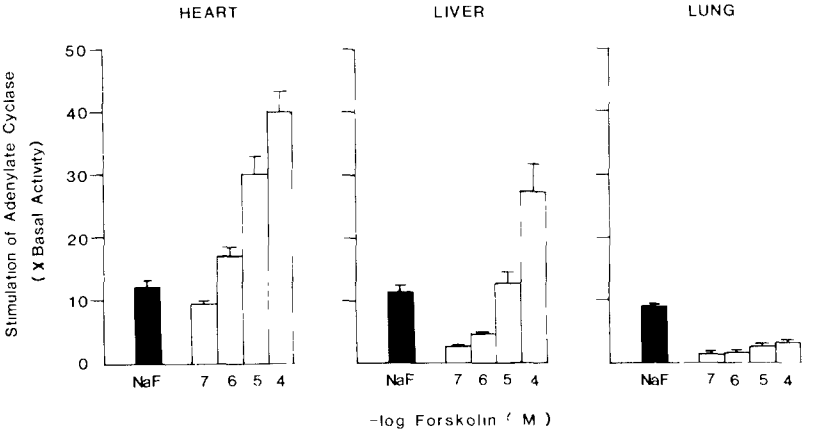

Fig 1 Stimulation of adenylate cyclase by increasing concentrations of forskolin and 10 mM fluoride in homogenates of rat heart, liver and lung Basal adenylate cyclase activities in these homogenates averaged 8 1 ± 1 6 pmol/min/mg for heart, 15 4 ± 3 1 pmole/min/mg for lung and 3 0 ± 0 54 pmole/min/mg for liver Results are the mean ± S E of three similar experiments

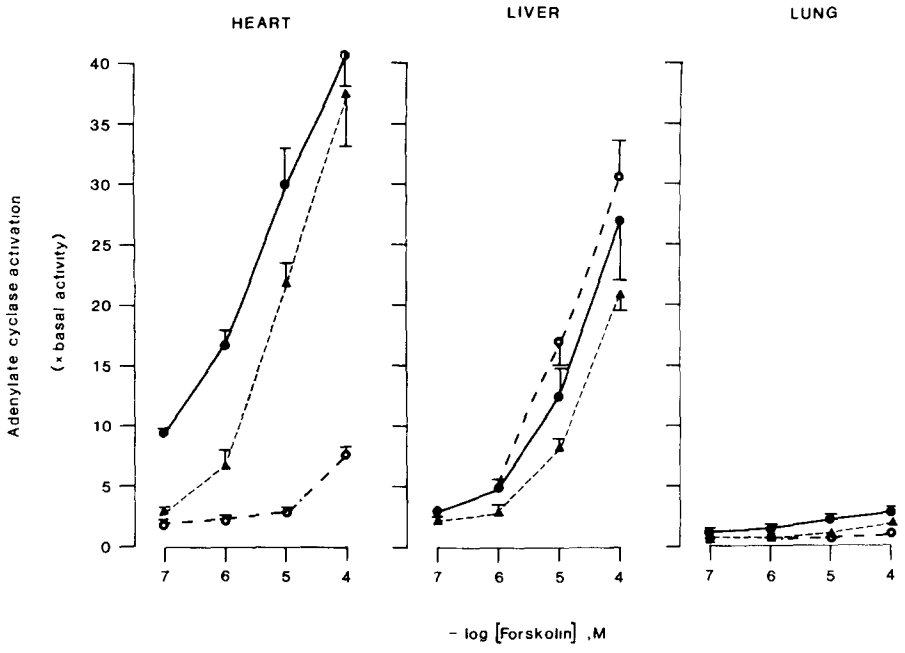

Fig 2 Comparison of the relative abilities of forskolin (●), 1-acetylforskolin (○) and 7-desacetylforskolin (▲) to increase adenylate cyclase activities of rat heart, liver and lung homogenates Results are the means ± S E of three experiments.

centrations of forskolin exerted a greater effect on cardiac adenylate cyclase activity than on liver and lung adenylate cyclase (Fig 1). Although forskolin always stimulated adenylate cyclase activity in homogenates of rat lung, reaching 3-fold with 0.1 mM forskolin, this represented only about 7% of the stimulation achieved with cardiac adenylate cyclase. In contrast to the poor ability of forskolin to stimulate rat lung adenylate cyclase compared with either heart or liver, sodium fluoride was a potent activator of lung adenylate cyclase. Sodium fluoride elevated lung adenylate cyclase approximately 9-fold (Fig. 1).

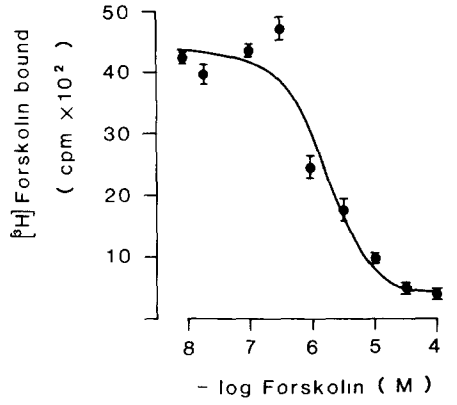

Fig 3 Displacement of [^{3}H]forskolin from liver membranes by unlabelled forskolin [^{3}H]Forskolin was incubated with liver membranes in the presence of increasing concentrations of unlabelled forskolin and the amount of [^{3}H]forskolin bound to membranes determined as described in the methods Results (mean ± S E) are typical of three similar experiments performed in triplicate

Cardiac and liver adenylate cyclase were elevated between 11- and 12-fold by sodium fluoride

The effects of 7-desacetylforskolin and 1-acetylforskolin on adenylate cyclase activity were also examined in the three tissues. Although 7-desacetylforskolin was somewhat less potent than forskolin (Fig. 2), it exerted a similar potency profile in the three tissues. The order of potency for stimulating adenylate cyclase was heart > liver ≫ lung. In contrast, 1-acetylforskolin appeared most potent in stimulating adenylate cyclase in liver homogenates This appeared due to the rapid hydrolysis by liver homogenates, of 1-acetylforskolin to forskolin. This was confirmed by TLC analysis of the products of the membrane mixture at the end of the incubation

[^{3}H]*forskolin binding*

The ability of forskolin to displace [^{3}H]forskolin from cell surface membranes was examined in rat liver (Fig 3) The displacement isotherm is consistent with [^{3}H]forskolin binding to a single, saturable site on liver membranes. The Hill coefficients (^{n}H) of the binding isotherms (Fig. 4) averaged 0.90 ± 0 05. The dissociation constant for [^{3}H]forskolin was 1.43 ± 0.21 μM with maximum binding on liver membranes being 5.2 ± 0 4 pmoles [^{3}H]forskolin/mg protein (Fig. 4)

Results of comparisons of [^{3}H]forskolin binding to rat heart, liver and lung are shown in Fig 5 [^{3}H]forskolin also bound to heart in a manner consistent with the presence of specific [^{3}H]forskolin binding sites on crude membranes Specific [^{3}H]forskolin binding tended to be higher in crude liver than heart membranes (Fig 5) However, as with liver, [^{3}H]forskolin displacement curves were consistent with a single binding site, ^{n}H =

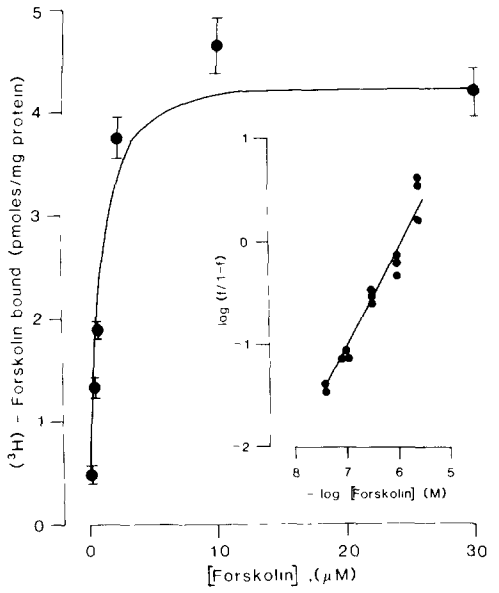

Fig 4 Specific binding of [^{3}H]forskolin to rat liver membranes Binding was determined by incubating rat liver membranes with [^{3}H]forskolin as described in the methods The result shown is representative of three similar experiments performed in triplicate Data is consistent with a single binding site for [^{3}H]forskolin (K_D = 0 94 μM and B_{max} = 4 43 pmole/mg protein) Inset depicts a Hill plot of the same data with slope (n_H) = 0 95

0.78 ± 0 21, with a mean dissociation constant of 0 74 ± 0 25 μM Although [^{3}H]forskolin bound to rat lung membranes, binding was of very low affinity (dissociation constant > 100 μM). The binding sites showed no tendency to saturate even with forskolin concentrations approaching 100 μM (Fig. 5) It was not possible to detect significant concentrations of higher (~1 μM) affinity binding sites on rat lung membranes

DISCUSSION

We have confirmed previous observations on the differing degrees to which adenylate cyclase from rat heart, liver and lung are stimulated by forskolin [18] Forskolin was most potent in stimulating cardiac adenylate cyclase followed by liver adenylate cyclase It was least effective in stimulating rat lung adenylate cyclase In contrast to the markedly different responses to forskolin, all three adenylate cyclase preparations were activated to a similar degree by sodium fluoride This suggests that the low potency of forskolin on adenylate cyclase from rat lung is not due to any lack of guanine nucleotide stimulatory protein (N_s) or catalytic units The ability of forskolin to stimulate adenylate cyclase seems to be more related to the presence of saturable forskolin binding sites.

The precise mechanism by which forskolin activates adenylate cyclase is, as yet, unknown It has been suggested that N_s proteins may be required for forskolin to exert its stimulatory effects on the enzyme. Evidence in support of this hypothesis has been based on complementation studies of human erythrocytes containing N_s protein with bovine sperm membranes, which do not contain N_s protein and whose adenylate cyclase is not activated by forskolin [16] Additional evidence for the involvement of N_s proteins has been obtained from comparisons of adenylate cyclase activities and their stimulation by forskolin in S-49 "wild type" WT strain and the cyc$^-$ variant of S-49 cells which lack an effective N_s protein [8, 9]. Both basal and forskolin activated adenylate cyclase activity are lower in the cyc$^-$ variant of S-49 cells than in the "wild type" WT strain However, when stimulation is expressed as a multiple of the

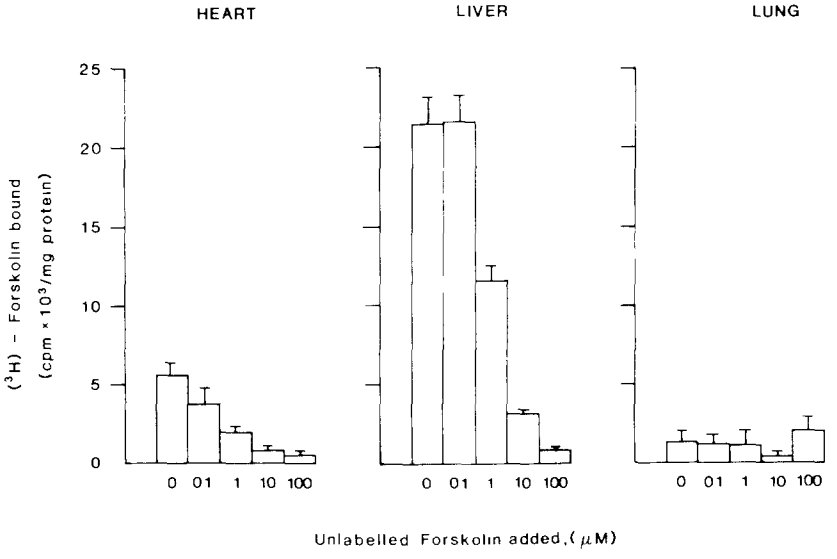

Fig 5 Comparison of the binding characteristics of [^{3}H]forskolin to crude cardiac liver and lung membranes [^{3}H]Forskolin (0 1 μM) was incubated with tissue membranes in the presence of increasing concentrations of unlabelled forskolin [^{3}H]Forskolin bound to membranes was determined as described in the methods Results are the means ± S E of three separate experiments performed in triplicate

corresponding basal activity, the degree by which forskolin stimulated adenylate cyclase in the two cell strains is similar These results suggest that N_s may influence basal adenylate cyclase activity independently from forskolin stimulation. More recently, it has been reported that forskolin is capable of stimulating solubilised adenylate cyclase catalytic units free of N_s protein [12–15]. Our results are consistent with the latter experiments suggesting that N_s protein is not essential for adenylate cyclase activation by forskolin. The limited response of lung adenylate cyclase to high concentrations of forskolin compared with cardiac adenylate cyclase, was not limited by the responsiveness of the N_s protein.

The responsiveness of adenylate cyclase to forskolin appeared to be qualitatively related to the number of [^{3}H]forskolin binding sites present on the membrane preparations In crude cardiac and liver membranes [^{3}H]forskolin binding sites with mean affinity constants of 0.74 μM and 1 43 μM respectively could easily be detected. This order of affinities is in good agreement with the relative potency of forskolin on adenylate cyclase in the two tissues (Fig. 1). However, binding of [^{3}H]forskolin to rat lung membranes appears to be of very low affinity with no indication of saturability Forskolin causes small elevations in adenylate cyclase activity in this tissue. It was not possible in the present study to completely exclude the presence of low levels of higher (~1–2 μM) affinity binding sites for [^{3}H]forskolin in rat lung membranes. However, the limited responsiveness of adenylate cyclase to forskolin is consistent with there being a very small number of binding sites on this tissue

Recently two binding sites for [^{3}H]forskolin have been identified on rat brain membranes [28, 29] It has been suggested that the lower affinity site ($K_D \sim 0$ 1 μM) may be associated with the direct activation of adenylate cyclase whilst the higher affinity site ($K_D \sim 15$ nM) might be associated with its ability to potentiate hormonal activation [28]. In rat liver membranes we could detect only a single low affinity binding site for [^{3}H]forskolin. Despite the presence of N_s protein in liver membranes and its ability to interact with the catalytic unit of adenylate cyclase, no high affinity sites could be detected. Our results of a single binding site for [^{3}H]forskolin in liver are consistent with recent reports of the presence of a single site for forskolin binding, identified by [^{3}H]14,15-dihydroforskolin on rat adipocytes and liver membranes [23, 30]. We did not examine whether a high affinity [^{3}H]forskolin binding site was present on cardiac or lung membranes. However, our results obtained with liver do not support the hypothesis that the high affinity site for [^{3}H]forskolin found on rat brain membranes represents an interaction between the forskolin binding site and the N_s protein [28]

In the present study, we have confirmed that forskolin selectively activates adenylate cyclase in certain tissues It is a potent activator of adenylate cyclase in rat heart and liver whilst in rat lung it only causes relatively modest elevations in enzyme activity. The ability of forskolin to activate adenylate cyclase in these tissues appeared dependent upon the presence of [^{3}H]forskolin binding sites Both the location and nature of these binding sites within the adenylate cyclase system will have to be determined before an understanding of the mechanism by which forskolin activates adenylate cyclase is achieved

Acknowledgements—We are grateful for financial support to A B from an Alfred Hospital Scholarship

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