Biochemical Pharmacology, Vol. 38, No. 7, pp. 1091–1095, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

DIFFERENTIAL FORSKOLIN ACTIVATION OF RAT HEART AND LUNG ADENYLATE CYCLASE

DEPENDENCE ON MEMBRANE–PROTEIN INTERACTIONS

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(*Received* 27 *May* 1988; *accepted* 18 *October* 1988)

Abstract—We have investigated whether the greater ability of forskolin to activate adenylate cyclase (EC 4.6.1.1) from rat heart compared with rat lung is due to interactions between G-proteins and catalytic units, isoforms of catalytic units or membrane–protein interactions. Interactions between Gs and catalytic units were found to be similar in both tissues with 10 μM Gpp(NH)p increasing activity up to 5-fold. While $MnCl_2$ increased the response of the lung enzyme to forskolin, it reduced the response of the cardiac enzyme and uncoupled Gs from the cardiac catalytic units indicating that Gs interactions potentiate the response to forskolin. After enzyme solubilisation with *n*-octyl-β-D-glucopyranoside, the response to forskolin was identical in heart and lung whether assayed with magnesium or manganese chloride, and not significantly different from the heart membrane enzyme. Overall, the results show that the relatively poor response of lung adenylate cyclase to forskolin is due to specific inhibitory interactions between the enzyme and lung membrane constituents.

The hormone-sensitive adenylate cyclase is primarily composed of three distinct groups of proteins: hormone receptors, G-proteins and the catalytic units, which convert ATP to cyclic AMP [1–3]. Variability in this system is seen not only with the receptors [4], but also with the G-proteins,† of which the stimulatory and inhibitory forms, Gs and Gi, are associated with adenylate cyclase [5–7]. The G-proteins are heterotrimeric and their functions are dependent on the α-subunits, of which at least two have been described for Gs [8]. The catalytic units are known to be glycoproteins of *ca.* 150,000 Da molecular weight [9–11] and calmodulin-sensitive and insensitive forms have been resolved from brain [11, 12]. The diterpene, forskolin, is a potent activator of adenylate cyclase and acts directly on the catalytic units [9, 13]. In an earlier study [14], we demonstrated that in rat heart, liver and lung the potency of forskolin was correlated with the presence and affinity of low-affinity (K_d *ca.* 1 μM) binding sites for ^{3}H-forskolin. While different isoforms of the catalytic units could account for these observations, other factors such as G-protein heterogeneity or membrane–protein interactions may have affected the activation by forskolin.

The aim of the present study was to investigate these possibilities. We examined the effects of G-protein heterogeneity by stimulation with the non-hydrolysable guanine nucleotide, Gpp(NH)p, and by promoting dissociation of the G-proteins from the catalytic units with $MnCl_2$ [4]. The effects of membrane–protein interactions were assessed by measurement of enzyme activities after solubilisation with *n*-octyl-β-D-glucopyranoside.

MATERIALS AND METHODS

Materials. [α-^{32}P]Adenosine triphosphate (10–50 Ci/mmol) and [2,8-^{3}H]adenosine 3′,5′-cyclic phosphate (30–50 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Isobutylmethylxanthine (IBMX) was purchased from Aldrich (Milwaukee, WI) and forskolin was a generous gift of Hoechst Australia Pty. Ltd. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and ethyleneglycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid (EGTA) were purchased from Calbiochem (La Jolla, CA). Guanyl-5′-yl imidodiphosphate [Gpp(NH)p] dimyristoylphosphatidylcholine (DMPC), *n*-octyl β-D-glucopyranoside and all other biochemicals were purchased from Sigma (St. Louis, MO).

Membrane preparation. Membranes from rat hearts were prepared essentially as described by Jones *et al.* [15]. Briefly, the minced hearts were homogenised (Polytron PT10, 10 sec, setting 1) in 8 vol. of 0.75 M KCl, 5 mM histidine pH 7.4 and centrifuged at 14,000 *g* for 20 min. The pelleted cell fragments were repeatedly washed by resuspension (once in 8 vol. of the original buffer and twice in 8 vol. of 10 mM $NaHCO_3$, 5 mM histidine pH 7.4) and centrifugation at 14,000 *g* for 20 min. This pellet was further homogenised (30 sec, setting 5) then centrifuged at 14,000 *g* for 20 min. Membranes were then isolated by centrifuging the supernatant at 40,000 *g* for 60 min. They were resuspended with a glass–Teflon homogeniser in 2 vol. of 50 mM HEPES, 3 mM dithiothreitol and 1 mM EGTA pH 7.5.

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† Abbreviations used: G-protein, regulatory guanine-nucleotide binding protein; Gs, stimulatory G-protein; Gi, inhibitory G-protein; Gpp(NH)p, guanyl-5′-ylimidodiphosphate; IBMX, isobutylmethylxanthine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DMPC, dimyristoylphosphatidylcholine.

Membranes of rat lung were prepared as described by Barnett *et al.* [16]. Minced lung was homogenised (Polytron PT10 setting 5, 3 × 10 sec) in 10 vol. of 50 mM Tris–HCl, 4 mM $MgCl_2$, pH 7.5, followed by four strokes of a motor-driven Teflon–glass homogeniser. The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at 500 *g* for 10 min. Membranes were isolated from the supernatant by centrifugation at 30,000 *g* for 10 min. The pelleted membranes were washed three times by resuspension with a glass homogeniser in 10 vol. of the same buffer and centrifugation at 30,000 *g* for 10 min. The final pellet was resuspended in 2 vol. 50 mM Tris–HCl pH 7.5.

Detergent solubilisation of enzymes. Enzymes were obtained from pellets prepared by centrifuging homogenates at 30,000 *g* for 30 min. Adenylate cyclase was solubilised by homogenising the pellets (Polytron PT10, setting 5, 30 sec) in 50 mM HEPES pH 7.5, 3 mM dithiothreitol, 1 mM EDTA and 25 mM *n*-octyl β-D-glucopyranoside (3 vol./g original wet wt). After centrifugation of the mixture for 30 min at 30,000 *g*, the supernatant was taken for assay of enzyme activity. In both cases, the supernatant contained 60–70% of total activity, whether unstimulated, Gpp(NH)p-stimulated or forskolin-stimulated.

Rapid gel filtration of soluble enzymes. One millilitre aliquots of the solubilised preparations were applied to 10 mm dia. columns containing 5 ml of fine Sephadex G-25 resin, previously equilibrated with 50 mM HEPES, 3 mM dithiothreitol and 1 mM EGTA pH 7.5. The sample was eluted under gravity with the same buffer and the protein peak eluting in the void volume taken for assay of enzyme activity.

Tissue preparations were kept at 0–4° at all times until assayed.

Adenylate cyclase assay. Adenylate cyclase activity was measured in 20 μl aliquots of each preparation as described previously [14]. Incubation was at 30° for 10 min in 50 mM HEPES pH 7.5, 3 mM dithiothreitol, 6.7 mM phosphoenolpyruvate, 6 U pyruvate kinase, 10 mM KCl, 1 mM IBMX and 0.25 mM adenosine triphosphate (0.45 μCi ^{32}P). $MgCl_2$ and $MnCl_2$ (final concentrations 5 mM and 4 mM respectively) were added separately from stock solutions as were Gpp(NH)p (1.5 mM in water) and forskolin (15 mM in dimethylsulfoxide). In the case of the solubilised enzymes added phospholipid (0.67 mM DMPC) was also present to improve enzyme stability [17]. Final assay volume was 150 μl and isolation and quantitation of the ^{33}P-cyclic AMP formed was as described by Salomon *et al.* [18].

Protein was assayed by the method of Lowry *et al.* [19] using bovine serum albumin as standard. All results presented are the mean ± SEM of at least three similar experiments performed in duplicate.

RESULTS

Response of membrane adenylate cyclase to Gpp(NH)p

Rat cardiac adenylate cyclase was stimulated in a concentration-dependent manner by Gpp(NH)p, reaching 4.8 ± 0.5-fold at 10 μM (Fig. 1). When $MgCl_2$ in the incubation was replaced by $MnCl_2$, the degree of stimulation fell by 87%. Gpp(NH)p-stimulated activity was not significantly different in $MgCl_2$ or $MnCl_2$. Activation of lung membrane adenylate cyclase showed a similar concentration dependency in the presence of $MgCl_2$ with maximum stimulation averaging (4.9 ± 0.2-fold). Substitution of $MnCl_2$ for $MgCl_2$ had no significant effect on the concentration-dependency or maximum activation by Gpp(NH)p of the lung enzyme (Fig. 1), as a result Gpp(NH)p-stimulated activity increased ca. 3-fold on changing from $MgCl_2$ to $MnCl_2$. Similar results were seen with homogenates of rat heart and lung (not shown).

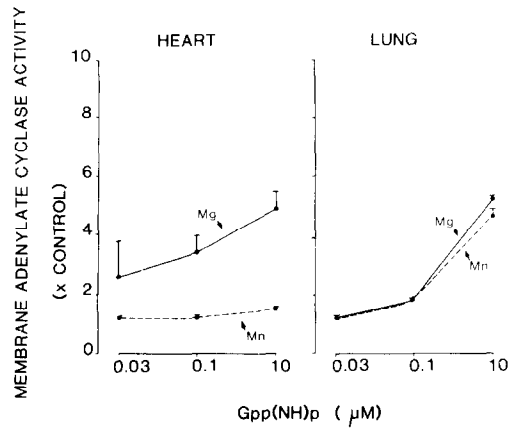

Fig. 1. Response of membrane adenylate cyclase to stimulation by guanine nucleotides. Membranes of rat heart and lung were assayed for adenylate cyclase activity in the presence of 5 mM $MgCl_2$ (solid lines) or 4 mM $MnCl_2$ (dashed lines). The increases in enzyme activity in the presence of Gpp(NH)p are expressed as multiples of the unstimulated activity (see Table 1).

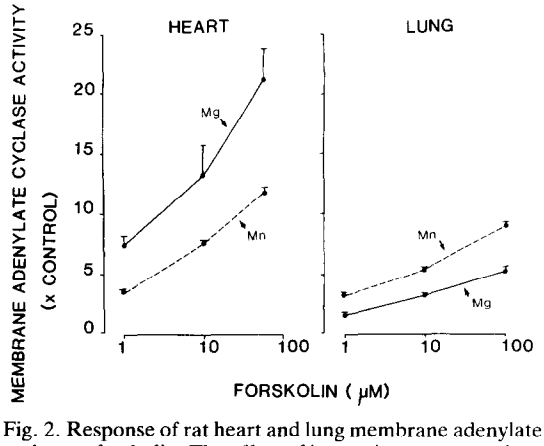

Fig. 2. Response of rat heart and lung membrane adenylate cyclase to forskolin. The effect of increasing concentrations of forskolin on rat adenylate cyclase was measured in either 5 mM $MgCl_2$ (solid lines) or 4 mM $MnCl_2$ (dashed lines). Enzyme activity is presented as a multiple of the unstimulated activity recorded in Table 1.

Response of membrane adenylate cyclase to forskolin

As previously demonstrated with homogenates [14], rat heart and lung membranes show a con-

centration-dependent increase in adenylate cyclase activity with forskolin, but heart activity is increased to a much greater extent (Fig. 2). Cardiac enzyme activity increased up to 20.7 ± 4.0-fold at 100 μM forskolin in $MgCl_2$, but lung activity increased only 4.9 ± 0.5-fold. When $MnCl_2$ was substituted for $MgCl_2$ the increase in cardiac activity fell to 11.4 ± 0.5-fold, while lung activation increased to 8.8 ± 0.3-fold. This effect is due to the action of manganese on the forskolin-stimulated activity rather than on the basal activity (Table 1). In both heart and lung membranes, the unstimulated activity was increased 3-fold by $MnCl_2$, while the difference in effect on the forskolin-stimulated activity between the tissues was independent of the forskolin concentration over the range 1–100 μM (Table 1). The response of adenylate cyclase activity to a combination of Gpp(NH)p and forskolin was found to be simply additive in both tissues (results not shown) as described in rat liver and adipocytes by Ho *et al.* [20, 21].

Response of detergent-solubilised enzymes to Gpp(NH)p

After solubilisation with the non-ionic detergent *n*-octyl-β-D-glucopyranoside, Gs interactions remained intact in both heart and lung preparations. Cardiac activity was increased 2.0 ± 0.4-fold by 10 μM Gpp(NH)p and lung activity increased 4.6 ± 0.9-fold. In the presence of $MnCl_2$, control activity increased *ca.* 7-fold while stimulation by 10 μM Gpp(NH)p was almost completely abolished, the increases in activity averaging only 14 ± 2% in heart and 81 ± 15% in lung. Thus after solubilisation, $MnCl_2$ is also capable of dissociating the lung adenylate cyclase system.

Response of detergent-solubilised enzymes to forskolin

Solubilisation of adenylate cyclase from rat heart and lung abolished all differences in their responses to activation by forskolin. Stimulation of cardiac adenylate cyclase, whether in the presence of $MgCl_2$ or $MnCl_2$, was identical to that of the membrane-bound enzyme (Fig. 3). Under these conditions stimulation by 100 μM forskolin averaged 20.1 ± 2.9-fold and 12.1 ± 1.0-fold respectively. Identical responses were seen with the solubilised lung enzyme, with stimulation by 100 μM forskolin averaging 21.0 ± 3.6 and 12.8 ± 1.1-fold in the presence of magnesium and manganese respectively.

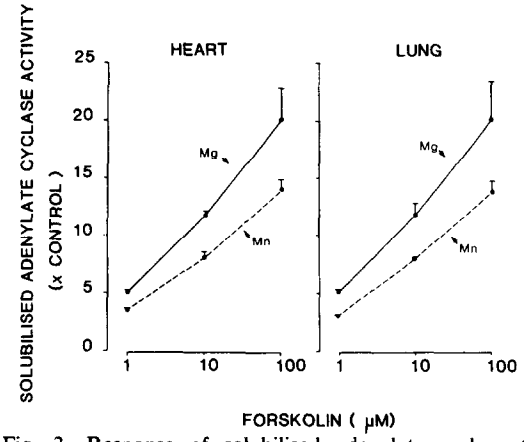

Fig. 3. Response of solubilised adenylate cyclase to forskolin. The effect of increasing concentrations of forskolin on the *n*-octyl-β-D-glucopyranoside solubilised adenylate cyclase activity of rat heart and lung was measured in the presence of either 5 mM $MgCl_2$ (solid lines) or 4 mM $MnCl_2$ (dashed lines). Enzyme activity is expressed as a multiple of the activity measured under the same conditions in the absence of forskolin.

Effect of gel-filtration on solubilised enzymes

To examine whether the presence of *n*-octyl-β-D-glucopyranoside was preventing the interaction of some inhibitory factor with the enzymes, and thus affecting the response to forskolin, the effect of removal of free detergent was examined. The high critical micelle concentration (25 mM) and small aggregate size of *n*-octyl-β-D-glucopyranoside [18] permits free detergent to be easily removed by dialysis or gel-filtration. We chose the more rapid

Table 1. Specific activities of membrane adenylate cyclase

	Forskolin concentration				Gpp(NH)p
	0 μM	1 μM	10 μM	100 μM	10 μM
Heart					
5 mM $MgCl_2$	41 ± 10	258 ± 35	434 ± 12	714 ± 27	202 ± 45
4 mM $MnCl_4$	124 ± 17	478 ± 51	913 ± 134	1423 ± 191	183 ± 21
	(3.4 ± 0.8)	(1.9 ± 0.1)	(2.1 ± 0.5)	(2.0 ± 0.3)	(1.0 ± 0.3)
Lung					
5 mM $MgCl_2$	10 ± 1.3	19 ± 2.6	32 ± 3.5	49 ± 3.9	51 ± 6
4 mM $MnCl_2$	31 ± 3.4	91 ± 7.6	162 ± 13	272 ± 27	137 ± 21
	(3.0 ± 0.3)	(5.2 ± 0.8)	(5.2 ± 0.4)	(5.5 ± 0.3)	(2.7 ± 0.5)

Specific activities of adenylate cyclase in pmol cyclic AMP/min/mg protein were determined for rat heart and lung membranes in 5 mM $MgCl_2$ or 4 mM $MnCl_2$. Control activities were measured in the absence of any stimulation of the enzyme. Figures in parentheses represent the increase in activity produced by manganese over the corresponding activity with magnesium chloride.

gel-filtration procedure on Sephadex G-25 to reduce free detergent concentration in the solubilised adenylate cyclase preparations. Removal of detergent by this procedure had no significant effect on the specific activities of the enzymes or their responses to Gpp(NH)p or forskolin (Table 2).

DISCUSSION

In this study we have confirmed in membrane preparations, the greater ability (*ca.* 5 times) of forskolin to activate rat heart adenylate cyclase than lung adenylate cyclase. The major factor influencing this difference appears to be specific inhibitory interactions between the adenylate cyclase system and components of lung membranes. Disruption of lung membranes with *n*-octyl-β-D-glucopyranoside increases the ability of forskolin to activate adenylate cyclase to levels identical with those seen with the cardiac enzyme, and facilitates uncoupling of the enzyme components by manganese ions. Association of Gs with catalytic units has been reported to potentiate the response to forskolin [23–26], and this is borne out in this study since loss of such interactions in the detergent-treated preparations and heart membranes, due to $MnCl_2$, reduces the response to forskolin. This effect is presumably due to relatively low numbers of high affinity binding sites for forskolin (K_d 10–100 nM) which have been described in rat brain and adipocytes [23, 27], since the activation by manganese relative to magnesium is independent of forskolin concentration (Table 1). These sites are associated with G-proteins and would be saturated at the forskolin concentrations (1–100 μM) used in this study.

The influence of Gi is harder to assess, since at no time was inhibition by Gpp(NH)p seen, even at the lower concentrations. However, such inhibition is usually small and best seen at low magnesium concentrations [28, 29] while major effects seem to be confined to inhibition of hormonal activation via Gs [30]. Investigations of the various resolved subunits of Gi and their action on partially purified rat brain catalytic units found no specific action on stimulation by forskolin [31]. Antisera against the βγ subunits of transducin have been found to inhibit the actions of Gs and Gi in rat brain, but to have little effect on activation by forskolin [32]. ADP-ribosylation of Gi by pertussis toxin has shown small (<50%) increases in cyclic AMP levels in mouse pituitary tumour cells [33] but we have found no effect of pertussis toxin pretreatment on the response to forskolin in cardiac and lung membranes (not shown) suggesting that Gi is not significantly involved.

The differences in response to forskolin are also not accounted for by the presence of various levels of calmodulin-sensitive catalytic units. The responses were the same whether membrane preparations or assays were performed in the presence or absence of EGTA, and neither tissue preparation was activated by μmolar concentrations of calcium. This is consistent with previous results for the cardiac enzyme [34]. The method of membrane preparation also seems unimportant, since similar results were seen in tissue homogenates, which would also rule out the effects of any soluble tissue components.

Alterations in membrane fluidity or cytoskeletal interactions have been shown to markedly influence the effect of forskolin [35, 36], but we have observed no effect on forskolin stimulation of pretreatment with linoleic acid up to 40 μg/mg protein, or the microtubule-disrupting agent, colchicine, up to 50 μM. Therefore, we must conclude that while interactions between adenylate cyclase and membrane constituents play a major role in influencing the activation by forskolin in rat lung, the nature of these interactions remains unclear. Identification of such membrane constituents would improve our understanding of mechanisms by which interactions between G-proteins and catalytic units are

Table 2. Specific activities of detergent-solubilised adenylate cyclase

	Unstimulated	Gpp(NH)p 10 μM	Forskolin 100 μM
Heart			
Soluble	1.7 ± 0.2	4.4 ± 0.6	34 ± 5.0
		(2.5 ± 0.3)	(22 ± 3.0)
Soluble post G-25	1.8 ± 0.2	4.5 ± 0.5	30 ± 3.5
		(2.4 ± 0.1)	(16 ± 1.3)
Lung			
Soluble	1.1 ± 0.2	3.8 ± 0.5	21 ± 2.3
		(3.2 ± 0.4)	(19 ± 4.9)
Soluble post G-25	1.2 ± 0.4	3.6 ± 0.4	19 ± 2.5
		(3.1 ± 0.1)	(17 ± 3.4)

Specific activities in pmol cyclic AMP/minute/mg protein of detergent-solubilised rat heart and lung adenylate cyclase were measured in the presence of 5 mM $MgCl_2$ and either alone or in the presence of the indicated concentrations of Gpp(NH)p or forskolin. Figures in parentheses represent the increase in activity as a multiple of the unstimulated activity. Activity post G-25 refers to solubilised enzymes in which free detergent concentration has been reduced by gel-filtration on Sephadex G-25. Results are the mean of three similar experiments in duplicate.

controlled, and the precise mode of action of forskolin.

Acknowledgement—This work was supported in part by an Alfred Hospital Research Scholarship to A.B.

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