Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused rat heart

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Continuous perfusion of rat hearts with concentrations of forskolin between 0.1 and 12 μM resulted in transient increases in tension after 45 s, followed by a return to the control value after 5 min. In contrast, the content of cyclic AMP increased linearly with time over this period, reaching values up to 35 times control after 5 min. Increases in contractile force, intracellular cyclic AMP concentration and the proportion of phosphorylase in the *a* form were dependent on the concentration of forskolin when measured 45 s and 120 s after initiation of perfusion. In hearts perfused for 45 s with various concentrations of forskolin, the measured cyclic AMP-dependent protein kinase activity ratio and phosphorylase *a* content for a given measured intracellular cyclic AMP concentration were both much less than the corresponding values in hearts perfused for 30 s with various concentrations of isoprenaline. The phosphorylation of the contractile proteins troponin-I and C-protein also showed a concentration-dependent increase in hearts perfused with forskolin. There was a strong correlation between the cyclic AMP-dependent protein kinase activity ratios and the phosphorylation of the contractile proteins under all perfusion conditions. These results suggest that cyclic AMP is compartmented in perfused rat heart, and that much of the cyclic AMP produced in response to forskolin is unavailable to activate cyclic AMP-dependent protein kinase.

INTRODUCTION

The major physiological mechanism for enhancing myocardial contractility is the stimulation of adenylate cyclase by catecholamine through the β -adrenoceptor. This results in an increase in cytosolic cyclic AMP and an activation of cyclic AMP-dependent protein kinase (cAMP-PrK), giving an increased phosphorylation of both membrane and contractile proteins. Acute hormonal control of cardiac contraction occurs predominantly by changes in the concentration of Ca^{2+} in the cytoplasm (ter Keurs, 1983). This is mediated by phosphorylation of proteins in both the sarcolemma and the sarcoplasmic reticulum (Huggins & England, 1985). Phosphorylation of protein(s) in the sarcolemma results in an increased opening of the voltage-dependent Ca^{2+} channel, and an increased influx of Ca^{2+} during depolarization (Osterreider *et al.*, 1982). Proteins that have been identified as possible targets for cAMP-PrK include phospholamban (Huggins & England, 1983), a protein of M_r 15000 (Presti *et al.*, 1985), and the Ca^{2+} channel itself (Curtis & Catterell, 1985). In the sarcoplasmic reticulum, phosphorylation of phospholamban (Tada *et al.*, 1974; Katz *et al.*, 1975) results in an increased uptake of Ca^{2+} during diastole, contributing both to an increased rate of relaxation and to an increased release of Ca^{2+} during subsequent systole.

In addition, two contractile proteins are phosphorylated in response to catecholamine stimulation. Troponin-I, a component of the troponin complex on the thin filament (Ebashi, 1966), and C-protein, a thick-filament protein (Starr & Offer, 1971), are phosphorylated over the same time course as for the increase in contraction (England, 1975, 1976; Jeacocke & England,

1980a). In addition, both proteins are excellent substrates for cAMP-PrK (Cole & Perry, 1975; England *et al.*, 1984). Phosphorylation of troponin-I decreases the affinity of the troponin complex for Ca^{2+} (Ray & England, 1976), and is probably a major determinant in the increase in the rate of relaxation of the heart induced by catecholamines (Robertson *et al.*, 1982; see England, 1983). The function of C-protein is not known, although the protein is a major substrate for cAMP-PrK in heart (Jeacocke & England, 1980a).

Forskolin is a diterpene that is a specific and potent activator of adenylate cyclase in a variety of tissues (Seamon & Daly, 1981), including rabbit heart (Metzger & Lindner, 1981) and human cardiac muscle (Bristow *et al.*, 1984). It has been reported to produce powerful positive inotropic responses in isolated guinea-pig hearts (Lindner *et al.*, 1978) and rabbit papillary muscle (Rodger & Shahid, 1984). The latter authors also reported that the positive inotropic effects of forskolin were associated with large increases in the intracellular concentration of cyclic AMP. The effects of forskolin on cardiac contraction are similar to those produced by catecholamines, in that it decreases the time to peak contraction, increases the rate of tension development, and enhances the rate of relaxation (Giembycz *et al.*, 1985). In addition, the electrophysiological effects of catecholamines are also mimicked by forskolin (Spah, 1984).

In spite of the many similarities in the physiological actions of forskolin and catecholamines, the changes in cyclic AMP concentrations in muscle in response to these agents differ widely (Vegesna & Diamond, 1983; Rodger & Shahid, 1984). The present study was undertaken to attempt to explain these differences, and particularly whether there was evidence for compartmentation of

Abbreviation used: cAMP-PrK, cyclic AMP-dependent protein kinase.

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cyclic AMP in heart muscle. In addition, a study of the mode of action of forskolin on protein phosphorylation was undertaken, to investigate whether the inotropic actions of forskolin could be explained by the same mechanisms as those suggested for catecholamines. A preliminary account of part of this work has previously appeared in abstract form (England & Shahid, 1985).

METHODS AND MATERIALS

Perfusion of hearts

Hearts from Wistar rats (220–260 g) were perfused by the Langendorff technique at a pressure of 6 kPa with modified Krebs–Henseleit bicarbonate-buffered medium containing 0.234 mM- P_i and 11 mM-glucose, and gassed with $O_2:CO_2$ (19:1) (England, 1975). After a 5 min pre-perfusion, the hearts were perfused with medium containing 0.5 MBq of [^{32}P]P $_i$ for 15 min in a recycling system. The hearts were then perfused for 1 min with non-radioactive medium, during which time contractile force was measured by a force-displacement transducer (Ormed Engineering, Welwyn Garden City, Herts., U.K.) attached to the apex of the heart. Perfusion was continued with control medium, or medium containing forskolin or isoprenaline at concentrations indicated in the Results section. At appropriate times hearts were freeze-clamped and stored at $-70^\circ C$. For measurement of cyclic AMP concentrations and cAMP-PrK activity ratios, hearts were either perfused by the same protocol in the absence of [^{32}P]P $_i$, or the ^{32}P was allowed to decay for 4–6 months at $-70^\circ C$ before assay.

Analysis of protein phosphorylation

Samples of frozen heart were prepared for polyacrylamide-gel electrophoresis in the presence of SDS as described by Jeacocke & England (1980b). Electrophoresis was performed as described by Laemmli (1970), in gels containing 12.5% polyacrylamide (for troponin-I and myosin P-light chain) or 5% polyacrylamide (for C-protein). Gels were stained with Coomassie Brilliant Blue, dried and autoradiographed at $-70^\circ C$ for 4–7 days. Protein-bound ^{32}P was quantified by densitometry with a Joyce–Loebl Chromoscan 3 linked to a Hewlett–Packard 9845 minicomputer. Corrections were applied for variations in the specific radioactivity of the γ -phosphate group of ATP of individual hearts, measured as described by England & Walsh (1976). The autoradiographs were within the range of proportionality for absorbance and radioactivity. The positions of C-protein, troponin-I and myosin P-light chain on the gels were determined by co-migration of purified samples of these proteins. The ^{32}P content of the proteins has been expressed relative to that present in myosin P-light chain in control perfusions, since previous work has shown that the phosphorylation of this protein remains constant over a wide range of perfusion conditions (Holroyde *et al.*, 1979; Jeacocke & England, 1980b; Herring & England, 1986).

Enzyme assays and metabolite determinations

The proportion of phosphorylase in the *a* form was determined as described by England (1976). cAMP-PrK activity ratios in frozen hearts were measured by the method of Corbin (1983).

ATP and phosphocreatine were measured in deproteinized tissue extracts as described by Lamprecht &

Trautschold (1965) and Lamprecht & Stein (1965) respectively. Cyclic AMP in deproteinized extracts was measured either by the method of Brown *et al.* (1971) or by using the protein-binding kit from Amersham International (Amersham, Bucks., U.K.). Assays of the effluent perfusate showed that less than 0.05% of the cyclic AMP measured in frozen heart extracts was present in the extracellular space.

Protein was determined by the biuret method, or, for quantification of protein loadings for electrophoresis, by the method of Zamen & Verwilghen (1979).

Values are quoted as means $\pm$ S.E.M. (*n*) throughout. The significance of differences were calculated by Student's *t* test for unpaired data.

Materials

[^{32}P]P $_i$ and cyclic [3H]AMP were obtained from Amersham International. Forskolin was from Calbiochem (Cambridge Bioscience, Cambridge, U.K.). Histone type IIA and (–)-isoprenaline were from Sigma Chemical Co., Poole, Dorset, U.K. The cyclic AMP binding protein was kindly provided by Dr. Sarah Pay, Department of Pharmacology, University of Bristol. All other reagents for assays of enzymes and metabolites were from Boehringer, Lewes, Sussex, U.K. (±)-Isoprenaline was from Kodak, Kirkby, Lancs., U.K.

RESULTS

Time course and dose–response relationships of the effects of forskolin on tension development, cyclic AMP concentrations and phosphorylase activity

Initial studies examined the time course of the positive inotropic action of forskolin. This was done to aid the selection of time points most suitable for later experiments. As shown in Fig. 1, there was a delay in the onset of action of forskolin (12 μM) of approx. 10–15 s. Maximum tension (170% of control) was achieved 45 s after the start of perfusion with forskolin. This was followed by a slow decline over the subsequent 4 min period back to the initial tension value. This response was not significantly modified by propranolol (0.1 μM), indicating the non-involvement of β -adrenoceptors in the action of forskolin (results not shown). For comparison, perfusion with (±)-isoprenaline (0.1 μM) caused an increase in tension to $174 \pm 4\%$ (6) of control after 25 s. Forskolin therefore takes considerably longer to produce a positive inotropic response than with isoprenaline, probably because forskolin has to penetrate the cell membrane in order to activate the catalytic entity of adenylate cyclase (Wong & Martin, 1983). Both isoprenaline and forskolin elicited positive chronotropic responses, producing a 20–30% elevation in heart rate. With forskolin, the increased chronotropy was maintained throughout the perfusion period, in contrast with the progressive fall in tension between 1 and 5 min.

This fall in tension was not caused by a decrease in the concentrations of ATP or phosphocreatine (Fig. 2). The ATP concentration remained constant at 30–32 $\mu mol/g$ of protein throughout the perfusion period. The phosphocreatine concentration showed a transient fall of 40% during the first 2 min of forskolin perfusion, but then recovered to 90% of control by 5 min. The fall in phosphocreatine concentration in fact occurred when the increase in tension development was maximal, suggesting that changes in the concentration of phosphocreatine

Fig. 1. Time course of changes in tension, phosphorylase α and cyclic AMP in hearts perfused with $12 \mu\text{M}$ -forskolin

Hearts were perfused as described in the Methods and materials section before perfusion with $12 \mu\text{M}$ -forskolin continuously for the times shown. At the end of the perfusion period the hearts were freeze-clamped, and assayed for cyclic AMP and phosphorylase α as described in the Methods and materials section. Tension was measured by a force displacement transducer attached to the apex of the heart. ●, Tension; □, cyclic AMP; ▲, phosphorylase α . Error bars show S.E.M. values for $n = 4$.

itself had no effect on tension development. A similar but more dramatic fall was also seen during perfusions with isoprenaline (Fig. 2), where the phosphocreatine decreased to 25% of control at the peak of the inotropic response. However, the ATP concentration again remained constant. The reason for the transient fall in phosphocreatine is probably that the increase in ATP production by the mitochondria, required to balance the increased contractile activity, occurs more slowly than the increase in myofibrillar ATPase activity (McCormack & England, 1983). This will lead to a transient imbalance in the rates of ATP production and utilization, with phosphocreatine being used to maintain the ATP concentration. During longer periods of perfusion, the production of ATP by mitochondria is increased sufficiently for the phosphocreatine concentration to return to the control value.

The transient nature of the increase in the force of contraction observed with forskolin (Fig. 1) was not so marked with β -adrenoceptor agonists. Although perfusion with maximal concentrations of isoprenaline caused a transient increase in tension to approx. 170% of control at 30 s, subsequently the tension was maintained at 130–150% for several minutes (results not shown). We have no obvious explanation for the difference in response to the two agents, particularly as forskolin causes a prolonged elevation of cyclic AMP concentrations (see below). However, as noted above, there was an increase in the chronotropic response throughout the 5 min of forskolin perfusion, indicating that the decrease in tension was not due to any inactivation or loss of forskolin. Furthermore, it is conceivable that the sustained increase in frequency of contraction, which can produce a negative inotropic response in the rat heart

Fig. 2. Time course of changes in ATP and phosphocreatine in hearts perfused with $12 \mu\text{M}$ -forskolin or $0.1 \mu\text{M}$ -($\pm$)-isoprenaline

Hearts were perfused and freeze-clamped as described in the legend to Fig. 1, before being assayed for (●, ○) ATP or (▲, △) phosphocreatine. ●, ▲, Perfusions in the presence of $12 \mu\text{M}$ -forskolin; ○, □, perfusions in the presence of $0.1 \mu\text{M}$ -($\pm$)-isoprenaline. Error bars show S.E.M. values for $n = 4$.

(Benforado, 1958), may have partially obscured the action of forskolin on tension development.

Fig. 1 also shows the time course of the changes of cyclic AMP concentration and the proportion of phos-

Table 1. Cyclic AMP-dependent protein kinase activity ratio and the concentration of cyclic AMP in hearts perfused with forskolin

Hearts were perfused with forskolin or isoprenaline at the concentration and for the time indicated, and freeze-clamped as described in the Methods and materials section. * $P < 0.05$, ** $P < 0.01$ for differences from the control-perfused hearts ($n > 3$ for all groups).

Inotrope	Concn. (μM)	Time of perfusion (s)	cAMP-PrK activity ratio	[Cyclic AMP] (nmol/g of protein)
Control	—	—	0.13 ± 0.01	2.3 ± 0.1
($\pm$)-Isoprenaline	(0.1)	30	$0.74 \pm 0.03^{**}$	$13.2 \pm 1.1^{**}$
Forskolin	(0.1)	45	0.14 ± 0.02	$5.4 \pm 0.3^*$
	(0.6)	45	0.19 ± 0.01	5.7 ± 1.8
	(3.0)	45	0.27 ± 0.02	$11.9 \pm 2.9^*$
	(12.0)	45	$0.34 \pm 0.02^*$	$14.3 \pm 2.1^*$
Forskolin	(0.1)	120	0.16 ± 0.01	$9.9 \pm 2.3^*$
	(0.6)	120	$0.30 \pm 0.04^*$	$21.3 \pm 5.0^*$
	(3.0)	120	$0.56 \pm 0.11^*$	$41.8 \pm 5.4^{**}$
	(12.0)	120	$0.74 \pm 0.05^{**}$	$53.7 \pm 8.2^{**}$

phorylase in the *a* form during perfusion with $12 \mu\text{M}$ -forskolin. After 45 s the cyclic AMP concentration had increased 6.2-fold over the control value. This is very comparable with the 5.7-fold increase measured after 30 s perfusion with $0.1 \mu\text{M}$ -($\pm$)-isoprenaline (see Table 1). However, with forskolin the cyclic AMP concentration continued to rise, so that after 5 min there was a 35-fold increase over control. Phosphorylase *a* also increased rapidly during the initial 45 s of perfusion with forskolin, reaching the value of 53% phosphorylase in the *a* form. Subsequently however, there was only a small further increase to give a maximum of 61% phosphorylase *a*. This was slightly less than the amount of phosphorylase in the *a* form [$71 \pm 9\%$ (6)] after 30 s of perfusion with $0.1 \mu\text{M}$ -($\pm$)-isoprenaline (results not shown).

In order to study the concentration-response relationship for forskolin on cyclic AMP and phosphorylase, two times of perfusion were chosen. Fig. 3(a) shows the results obtained after 45 s of perfusion with forskolin (the peak of the tension response) and Fig. 3(b) after 120 s (at which time there was a large increase in cyclic AMP and a maximal change in phosphorylase *a*). At both times there was a concentration-dependent increase in each of the three parameters.

After 45 s of perfusion with forskolin, the increases in cyclic AMP concentration were relatively small when compared with those observed at longer times of perfusion (see below). However, at $12 \mu\text{M}$ -forskolin the increase in cyclic AMP was comparable with that measured after 30 s perfusion with $0.1 \mu\text{M}$ -($\pm$)-isoprenaline (Table 1). The increases in tension and phosphorylase *a* were also dose-dependent, the maximal observed increase in tension at $12 \mu\text{M}$ -forskolin relative to control being slightly less than that observed on perfusion for 30 s with $0.1 \mu\text{M}$ -($\pm$)-isoprenaline ($177 \pm 4\%$, $n = 4$).

After 120 s of perfusion with forskolin, there were much larger, although still dose-dependent, increases in cyclic AMP concentration when compared with those after 45 s of perfusion (Fig. 3b). This shows that the progressive increase in cyclic AMP concentrations seen on perfusion with $12 \mu\text{M}$ -forskolin (Fig. 1) could be obtained at all forskolin concentrations. The increases in tension, although showing a dose-dependent relation-

Fig. 3. Concentration-response relationships of tension, cyclic AMP and phosphorylase *a* in hearts perfused with forskolin

Hearts were perfused as described in the legend to Fig. 1 with the concentration of forskolin shown for (a) 45 s or (b) 120 s before being freeze-clamped. ●, Increase in tension (%); □, cyclic AMP; ▲, phosphorylase *a*. Error bars show S.E.M. values for $n = 4$.

ship, were much less than those at 45 s, confirming the transient response observed in Fig. 1. The increases in phosphorylase *a* were somewhat greater than those after 45 s of perfusion with forskolin.

Fig. 4. Relationship between the concentration of cyclic AMP and cyclic AMP-dependent protein kinase activity ratios or the proportion of phosphorylase in the *a* form in hearts perfused with forskolin or isoprenaline

Hearts were perfused as described in the legend to Fig. 1 and in the text with various concentrations of either forskolin for 45 s ($\square$) or with (—)-isoprenaline for 30 s ($\bullet$) before being freeze-clamped. (a) Relationship between cyclic AMP content and cAMP-PrK activity ratios. (b) Relationship between cyclic AMP content and the proportion of phosphorylase in the *a* form. Error bars are S.E.M. values for $n > 3$.

Relationships between cyclic AMP concentration, cyclic AMP-dependent protein kinase activity ratio, and the proportion of phosphorylase in the *a* form

Examination of the data of Fig. 3 suggested that on perfusion with forskolin, the increase in tension and phosphorylase *a* obtained for a given increase in cyclic AMP concentration was less than that observed for a similar increase in cyclic AMP in hearts perfused with isoprenaline (England, 1976). A more detailed investigation of this phenomenon was therefore carried out. Hearts were perfused with various concentrations of either forskolin (0.1–12 μ M) for 45 s, or (—)-isoprenaline (1–30 nM) for 30 s before being freeze-clamped and subsequently assayed for cyclic AMP and phosphorylase *a* content, and measurement of cAMP-PrK activity ratios. The times were chosen as being at the peak of the tension responses to the two inotropes, and therefore directly comparable in terms of functional response. This also avoided the very high concentrations of cyclic AMP that occurred on prolonged perfusion with forskolin.

The cAMP-PrK activity ratio as measured in these studies gives an indication of the amount of the enzyme in an active state at the time of freeze-clamping (Corbin, 1983), even though only the soluble activity is assayed. Since the only mechanism whereby cyclic AMP can affect

the functioning of a cell is by activation of cAMP-PrK (Krebs & Beavo, 1979), changes in the activity ratio can provide a sensitive measure of the response of the heart to changes in cyclic AMP concentration. In addition, any sequestration of cyclic AMP into a compartment not accessible for binding to cAMP-PrK can be assessed from a comparison of the activity ratio with the concentration of cyclic AMP in the same heart.

Fig. 4(a) shows the relationship between cyclic AMP content and cAMP-PrK activity ratio after perfusion with forskolin or isoprenaline. The values of the activity ratio in control hearts and on perfusion with isoprenaline were similar to those reported by Keely *et al.* (1975), although the maximum value obtained in the present study (0.74) was somewhat higher than that previously reported. There was an approximately linear relationship between the cyclic AMP content and the cAMP-PrK activity ratio. In the forskolin-perfused hearts this relationship was again linear. However, a much lower cAMP-PrK activity ratio was observed for a given cyclic AMP content than in the isoprenaline perfusions. Thus, whereas with isoprenaline an activity ratio of 0.38 was observed with a doubling of the control value of cyclic AMP content (from 2.3 to 5.2 nmol/g of protein), a similar activity ratio in perfusions with forskolin was only seen with a cyclic AMP content of 14.3 nmol/g. A doubling of the cyclic AMP content in the forskolin perfusions, in contrast, gave no statistically significant increase in the activity ratio. These results suggest that in forskolin perfusions much of the cyclic AMP that is produced is unavailable to activate cAMP-PrK, at least that proportion of the enzyme present in the soluble fraction after homogenization.

This apparent lack of activation of cAMP-PrK by increases in cyclic AMP induced by forskolin was also reflected in the proportion of phosphorylase *a* in the tissue (Fig. 4b). The conversion of phosphorylase *b* into *a* is dependent on both Ca^{2+} and cyclic AMP (Chan & Graves, 1984). However, in rat heart the increase in phosphorylase *a* induced by an increase in cytoplasmic Ca^{2+} alone (McCormack & England, 1983) is considerably less than that induced by isoprenaline, which increases both cytoplasmic Ca^{2+} and cyclic AMP. Fig. 4(b) shows that forskolin was much less effective at stimulating the conversion of phosphorylase *b* into *a* for a given tissue content of cyclic AMP than was isoprenaline, suggesting that much of the cyclic AMP produced in response to forskolin is apparently unavailable to activate cAMP-PrK. This difference between isoprenaline and forskolin cannot be explained by forskolin not causing an increase in cytoplasmic Ca^{2+} , since even the lowest concentrations of forskolin used caused an increase in tension (Fig. 3a), and therefore by inference an increase in cytoplasmic Ca^{2+} .

Table 1 shows the data for cyclic AMP content and cAMP-PrK activity ratios after perfusion with forskolin for 120 s. With the very large increases in cyclic AMP content seen at this time, the cAMP-PrK activity ratios approached those seen with isoprenaline. It would thus appear that when the cyclic AMP content is elevated to very high values by forskolin, sufficient cyclic AMP becomes available to activate cAMP-PrK to the same extent as isoprenaline. However, in forskolin perfusions the discrepancy between the actual cyclic AMP content and the expected activity ratios is still apparent at this longer perfusion time. It is noteworthy that, even when

Fig. 5. Densitometer traces of autoradiographs of polyacrylamide gels of whole heart proteins after perfusion with $[^{32}\text{P}]\text{P}_i$

Hearts were perfused by recycling with $[^{32}\text{P}]\text{P}_i$ as described in the Methods and materials section, followed by perfusion with: CON, control medium; FOR, $12\text{ }\mu\text{M}$ -forskolin for 45 s; ISO, $0.1\text{ }\mu\text{M}$ -($\pm$)-isoprenaline for 30 s. Details of the preparation of proteins for electrophoresis and the subsequent autoradiography are given in the Methods and materials section. Gels: (a) 5% polyacrylamide; (b) 12.5% polyacrylamide. The positions of known phosphorylated cardiac proteins are indicated: Tn-I, troponin-I; P-LC, myosin P-light chain. O, gel origin. Note that the traces for isoprenaline- and forskolin-perfused hearts have been moved approx. 0.4 and 0.8 absorbance unit upwards respectively to improve clarity. The mobilities of standard proteins are indicated by the numbered arrows. Proteins used (with M_r values) were: 1, myosin heavy chain (180 000); 2, phosphorylase *b* (94 000); 3, actin (45 000); 4, cardiac myosin light chain 1 (27 000); 5, cardiac myosin light chain 2 (19 000); 6, cytochrome *c* (12 400).

the cyclic AMP content is elevated 23-fold above the control perfusion, there is still not 100% activation of the kinase.

Effects of forskolin perfusion on contractile protein phosphorylation

In view of the apparent lack of activation of cAMP-PrK in forskolin-perfused hearts, two contractile proteins whose phosphorylation is dependent on cAMP-PrK, namely troponin-I and C-protein (England, 1983), were examined. Fig. 5 shows typical densitometric scans

of autoradiographs of SDS/polyacrylamide gels of whole heart proteins used to quantify the ^{32}P content of the contractile proteins. Table 2 shows the effects of forskolin on the phosphorylation of troponin-I, C-protein and myosin P-light chain after 45 s and 120 s of perfusion. Forskolin caused no change in the phosphorylation of myosin P-light chain, in agreement with other studies showing a constant extent of phosphorylation of this protein during inotropic interventions (Holroyde *et al.*, 1979; Perry *et al.*, 1979; Jeacocke & England, 1980b; High & Stull, 1980).

Forskolin caused both time- and concentration-dependent increases in the phosphorylation of troponin-I and C-protein at the higher concentrations used. At lower concentrations there was no increase in the phosphorylation of these proteins, in agreement with the lack of activation of cAMP-PrK shown in Fig. 4. Overall, there were very close correlations between the cAMP-PrK activity ratios from Fig. 4(a) and Table 1, and the extents of phosphorylation of troponin-I ($r = 0.94$) and C-protein ($r = 0.97$) from Table 2. These results, in conjunction with the data on measurements of phosphorylase *a*, strongly suggest that the cAMP-PrK activity ratio as measured in this study is a good indicator of the proportion of cAMP-PrK active in the tissue.

DISCUSSION

The experiments described in this paper show that the positive inotropic effects of forskolin were generally associated with large increases in the concentration of cyclic AMP in perfused rat heart, in agreement with previous cardiac studies (Rodger & Shahid, 1984). Increases in cyclic AMP induced by catecholamines have been shown to be associated with increased phosphorylation of sarcolemmal (Huggins & England, 1983; Presti *et al.*, 1985), sarcoplasmic reticulum (Kranias & Solaro, 1982) and contractile proteins (England *et al.*, 1984). These are believed to be responsible for both increased Ca^{2+} availability and a decrease in the Ca^{2+} -sensitivity of the myofibrils (Katz, 1979; Haiech & Demaille, 1983; England, 1983), leading to increased contractility. The data in the present paper show that the inotropic effects of forskolin are accompanied by increases in contractile-protein phosphorylation, in agreement with this hypothesis. Although no direct measurements of membrane-protein phosphorylation were made in this study, there are indications in Fig. 5 that proteins migrating with apparent M_r approx. 11 000–15 000 are phosphorylated in response to forskolin. These proteins are probably phospholamban and/or a 15 000- M_r sarcolemmal protein (Huggins & England, 1983; Presti *et al.*, 1985). Moreover, Fliegel & Drummond (1985) have reported the phosphorylation of a 25 000- M_r membrane protein in guinea-pig hearts perfused with forskolin, this protein presumably being phospholamban migrating in its pentameric form under the electrophoresis conditions used.

Although the overall response to forskolin was consistent with this postulated role of cyclic AMP and protein phosphorylation in the control of contraction, there were clearly a number of major differences in the responses to forskolin and isoprenaline. One noticeable difference was that forskolin produced much larger increases in cyclic AMP than did isoprenaline, par-

Table 2. Phosphorylation of troponin-I, C-protein and myosin P-light chain in hearts perfused with forskolin

Hearts were perfused with [32 P]P_i as described in the Methods and materials section, and then with the inotrope at the concentration and for the time indicated. Analysis of 32 P content by electrophoresis and autoradiography is described in the Methods and materials section. All values are expressed relative to the amount of 32 P in myosin P-light chain in the control perfusions. * $P < 0.05$, ** $P < 0.01$ versus control values ($n > 3$ for all groups).

Inotrope	Concn. (μ M)	Time of perfusion (s)	32 P content relative to P-light chain control		
			Troponin-I	C-protein	Myosin P-light chain
Control		—	0.10 $\pm$ 0.02	0.13 $\pm$ 0.02	1.00 $\pm$ 0.12
($\pm$)-Isoprenaline	(0.1)	30	0.75 $\pm$ 0.02	0.81 $\pm$ 0.99**	1.01 $\pm$ 0.15
Forskolin	(0.1)	45	0.06 $\pm$ 0.01	0.09 $\pm$ 0.01	0.92 $\pm$ 0.08
	(0.6)	45	0.09 $\pm$ 0.01	0.15 $\pm$ 0.04	0.88 $\pm$ 0.12
	(3.0)	45	0.33 $\pm$ 0.04**	0.34 $\pm$ 0.01**	1.02 $\pm$ 0.12
	(12.0)	45	0.62 $\pm$ 0.02**	0.54 $\pm$ 0.04**	0.94 $\pm$ 0.12
Forskolin	(0.1)	120	0.09 $\pm$ 0.02	0.15 $\pm$ 0.01	0.81 $\pm$ 0.12
	(0.6)	120	0.22 $\pm$ 0.04	0.28 $\pm$ 0.01*	0.79 $\pm$ 0.12
	(3.0)	120	0.65 $\pm$ 0.02**	0.62 $\pm$ 0.01**	0.92 $\pm$ 0.08
	(12.0)	120	1.97 $\pm$ 0.07**	0.92 $\pm$ 0.08**	1.06 $\pm$ 0.12

ticularly when present for long periods. Thus, in the example shown in Fig. 1, after 5 min of perfusion with 12 μ M-forskolin there was a 26-fold increase in the cyclic AMP concentration. Such large increases in cyclic AMP have been reported previously (Vegesna & Diamond 1983; Rodger & Shahid, 1984), and indicate that stimulation of adenylate cyclase by forskolin does not result in desensitization, in contrast to the situation with catecholamines (see Sibley & Lefkowitz, 1985). A consequence of this continuing increase in cyclic AMP is that any correlation made between a functional or metabolic parameter and the concentration of cyclic AMP will vary, depending on the time at which the hearts were frozen. In particular, correlations made after long periods of perfusion with the drug are likely to be meaningless, owing to the very large increases in cyclic AMP which occur. Therefore, in the studies reported in this paper attention was focused on short perfusion times, particularly at 45 s, when the tension increase was just maximal, and 120 s, where only at the highest forskolin concentrations had the cyclic AMP concentrations increased beyond that obtained with maximum concentrations of β -adrenergic agonists. By using these short perfusion times, therefore, it is probable that more appropriate comparisons between the actions of forskolin and catecholamines can be made.

The most interesting observation in this study is that forskolin can cause a significant elevation in the concentration of cyclic AMP with little or no activation of cAMP-PrK. This conclusion is supported both by measurements of cAMP-PrK activity ratios in heart extracts and by measurements of protein phosphorylation in the intact hearts. Perfusion with forskolin for 45 s caused either no activation of the kinase, or much less than would be expected for the measured concentration of cyclic AMP. This was also true, although less noticeable, after perfusion for 120 s with forskolin. A dissociation between tension development and cyclic AMP concentrations has been previously noted in rabbit papillary muscle (Rodger & Shahid, 1984). Similar results

have also been obtained in other tissues. Thus a dissociation between activation of cAMP-PrK and increased cyclic AMP has been reported in smooth muscle when incubated with forskolin (Vegesna & Diamond, 1983; Do Khac *et al.*, 1986), and in skeletal muscle forskolin caused a 20-fold increase in cyclic AMP, with no effect on sub-tetanic contractions (Bowman *et al.*, 1985).

It has been suggested (Palmer *et al.*, 1980) that the cAMP-PrK activity ratio as measured by the method of Corbin (1983) and used in the present study is not a good estimate of the actual activity of cAMP-PrK in the tissue at the time of freezing. This is because the extraction conditions used possibly may not maintain the activity ratio present in the tissue, and that association or dissociation of the kinase may occur during the assay. In the present study, if this were the case then there would be expected to be a close correlation between the content of cyclic AMP in the tissue and the cAMP-PrK activity ratio measured. However, it is clear from the results of Fig. 4(a) and Table 1 that there is no such correlation. In addition, the extent of phosphorylation of phosphorylase α , troponin-I and C-protein are all much more closely related to the degree of activation of cAMP-PrK, as measured by the kinase activity ratio, than to the intracellular concentration of cyclic AMP. These results strongly suggest that the cAMP-PrK activity ratio as measured in this study is a good estimate of the activity actually present in the tissue, and is not an artefact of the assay conditions.

The observations reported in this paper can be interpreted as forskolin inducing the formation of cyclic AMP in a functional compartment separate from that containing soluble cAMP-PrK. Compartmentation of cyclic AMP and cAMP-PrK in heart has been previously postulated by Hayes *et al.* (1979, 1980) from experiments in which hearts were perfused with prostaglandin E₁. Thus many of the results described above can be explained if it is postulated that forskolin predominantly activates a subfraction of adenylate cyclase not activated

by β -adrenoceptor agonists, particularly at low concentrations. The cyclic AMP thus produced would be in a different functional compartment from cAMP-PrK, at least that fraction of the kinase responsible for the phosphorylation of contractile proteins and phosphorylase (Brunton *et al.*, 1979). At higher concentrations, forskolin would also activate the subfraction of adenylate cyclase activated by isoprenaline, resulting in the phosphorylation of the substrate proteins. Overall, the stimulation of these two separate subfractions of adenylate cyclase, each one linked to separate intracellular functional compartments of cyclic AMP, would explain the large increases in cyclic AMP observed with only relatively small effects on cardiac function. This idea is further supported by the observation that low concentrations of forskolin ($< 1 \mu\text{M}$), despite increasing cyclic AMP, failed to potentiate the effects of isoprenaline in various muscle tissues (Vagesna & Diamond, 1983; Bowman *et al.*, 1985; Waldeck & Widmark, 1985). Indeed, the evidence for the selective activation of adenylate cyclase in regions of certain cells was reviewed by Earp & Steiner (1978). Whether the subfraction of adenylate cyclase stimulated by low concentrations of forskolin is the same as that stimulated by prostaglandin E_1 is not known at present.

Compartmentation of cyclic AMP and cAMP-PrK could explain the observations in Fig. 3 that low concentrations of forskolin caused some increase in the force of contraction, with no apparent increase in cAMP-PrK activation or contractile-protein phosphorylation. Thus, if it is proposed that forskolin caused selective activation of a small, sarcolemmal-bound, fraction of cAMP-PrK, phosphorylation of the membrane proteins controlling the voltage-operated Ca^{2+} channel would occur (Huggins & England, 1983; Presti *et al.*, 1985; Curtis & Catterell, 1985). However, most of the cell cAMP-PrK would not be activated, and hence no contractile-protein phosphorylation would be observed.

Evidence for the functional compartmentation of cyclic AMP in heart has also been provided by the use of selective cyclic nucleotide phosphodiesterase inhibitors. Rolipram (Schering ZK 62711) has been shown to be a potent and selective inhibitor of a newly described cyclic AMP-specific phosphodiesterase present in heart (Reeves *et al.*, 1987). Perfusion of guinea-pig and cat hearts with Rolipram (Gristwood & Owen, 1986; Gristwood *et al.*, 1986) caused an approx. 2-fold increase in cyclic AMP concentration, with no increase in the force of contraction. In contrast, perfusion of hearts with agents (e.g. SK&F 94120) which inhibit the so-called 'low- K_m ' or 'cyclic GMP-inhibited' phosphodiesterase (Harrison *et al.*, 1986) caused large inotropic effects, with only a 50% increase in the concentration of cyclic AMP (Gristwood & Owen, 1986).

At present it is not known whether the postulated compartments of cyclic AMP or cAMP-PrK in the heart represent spatially localized regions, or a more functional compartmentation resulting from close proximity of the relevant enzymes. It is unlikely that the compartmentation results from different cell types, since, if most of the cyclic AMP produced in response to forskolin were not in the myocytes, concentrations greater than 0.5 mM would have to be present in these other cells.

There is now a considerable body of evidence that cyclic AMP is compartmented in several different cell types, and the use of a particular agent to increase

cyclic AMP may have quite different effects, depending on the compartment affected. Forskolin clearly does not always exactly mimic the actions of hormones which interact with β -adrenoceptors, and its use as an agent to circumvent the hormone-receptor/G-protein system of adenylate cyclase should take due regard of these problems.

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