Short Communications

Forskolin inhibits tension development in detergent-treated cardiac muscle fibers

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Summary. In detergent-treated cardiac muscle fibers, forskolin, a potent activator of adenylate cyclases, inhibits tension development elicited with submaximal [Ca^{2+}] and increases incorporation of ^{32}P into troponin-I. A similar reduced tension development has been observed after treatment with cAMP or the catalytic subunit of the cAMP-dependent protein kinase. It is concluded that these fibers still contain much of the enzyme cascade involved in evoking a contractile response to β-adrenergic stimulation.

Chemically skinned muscle fibers from various sources, which are devoid of a functional sarcoplasmic reticulum as a result of the skinning procedure, are widely used in evaluating processes involved in the regulation of contraction at the level of contractile proteins[2-5]. In this study we show that these fibers can also be a useful tool in studying receptor contraction coupling, i.e. events involved in the transmission of a signal from a receptor to the contractile machinery.

Forskolin, a novel cardioactive compound[6], has been described as a potent activator of all mammalian adenylate cyclases in intact and also in broken cells[7]. In intact cardiac tissue forskolin has a positive inotropic effect[5] which is associated with a marked increase in intracellular cyclic AMP levels[8] due to stimulation of the adenylate cyclase[8] thus mimicking β-adrenergic stimulation. Hormone-responsive adenylate cyclase consists of at least 3 subunits, the catalytic subunit, the guanine nucleotide regulatory subunit and a specific hormone receptor[9], and it is very likely that forskolin exerts its activity by direct action on the catalytic subunit[7] and not by stimulation of the receptor.

In intact cardiac muscle, β-adrenergic-induced increase in contractility is associated with cAMP-dependent phosphorylation of myofibrillar, sarcoplasmic and sarcolemmal proteins[10]. Phosphorylation of sarcolemmal proteins appears to open Ca^{++}-channels thus increasing the amount of Ca^{++} that enters the cell during depolarization[11]. The positive inotropic effect of forskolin may therefore be ascribed to a rise in intracellular free Ca^{++}. This effect can be eliminated by the skinning procedure. Now a possible direct effect of forskolin on the contractile apparatus can be studied without interference from membrane events. Previous studies on detergent-skinned cardiac muscle fibers showed that phosphorylation of the inhibitory subunit of troponin (TN-I) by cAMP-dependent protein kinase results in a decreased steady state affinity of troponin for Ca^{2+}, a decrease in the Ca^{2+}-sensitivity of tension development in skinned fibers[4,13] and of myofibrillar ATPase activity[14].

If these fiber preparations still contain adenylate cyclase the response to cAMP should be mimicked by forskolin. We show that, in presence of a phosphodiesterase inhibitor, forskolin decreases the Ca^{2+}-sensitivity and increases phosphorylation of TN-I. This suggests that chemically-skinned cardiac muscle fibers still contain much of the enzyme cascade involved in evoking a response of the contractile machinery to activation of the adenylate cyclase.

Materials and methods. Chemically-skinned cardiac muscle fibers were prepared from the subendocardial layer of the right ventricle of porcine hearts using Lubrol WX as detergent, and were stored as described by Herzig et al.[4]. Fiber bundles of approximately 0.1 mm diameter and 5 mm length were mounted between an adjustable glass rod mounted on a micrometer drive and a short rod extending from an AME 801 force transducer. Fibers were bathed in a 'relaxing solution' containing 10 mM ATP, 12.5 mM $MgCl_2$, 5 mM EGTA, 20 mM imidazole, 5 mM NaN_3, 10 mM phosphocreatine, 380 U/ml creatinephosphokinase (Boehringer), pH was adjusted to 6.7 at 24 °C. The 'contracting solution' had the same composition as that described above, with the exception that instead of 5 mM EGTA it contained 5 mM Ca-EGTA. The free [Ca^{++}] was increased by appropriately mixing the relaxing and contracting solutions. The free [Ca^{++}] in the solution was calculated using the apparent binding constant for EGTA given by Portzehl et al.[15]. In some experiments, ATP was replaced by [γ-^{32}P]ATP in the absence of an ATP-regenerating system. In these experiments reactions were stopped by addition of 50% trichloroacetic acid to fibers still attached to the force transducer. Denatured proteins were separated on SDS-gradient gels (6-15%) and radioactivity associated with individual bands was detected by autoradiography as described by Laemmli[16].

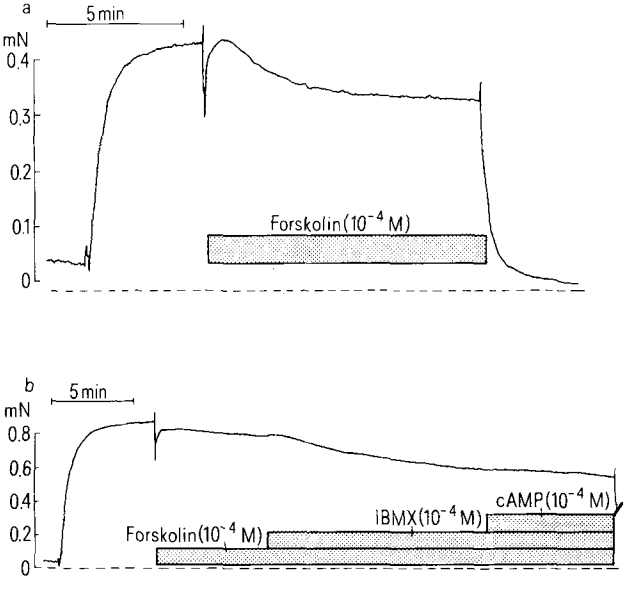

Figure 1. *a* Isometric tension of a skinned cardiac fiber bundle is recorded after raising [Ca^{++}] from pCa > 8 to pCa 6.09 in the presence of IBMX (10^{-4} M), Mg·ATP (10 mM) and an ATP regenerating system. Addition of forskolin causes partial relaxation. Complete relaxation is achieved after lowering the [Ca^{++}] to pCa > 8. *b* Submaximal contraction of a skinned preparation at pCa 6.09. Addition of forskolin in absence of IBMX has only little effect on tension development. Addition of IBMX potentiates the relaxing effect, cAMP does not further enhance relaxation.

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Results and discussion. Skinned cardiac fibers contract maximally when the $[Ca^{++}]$ in the ATP-salt solution is raised to pCa 4.67. Half-maximal activation occurs at a pCa of 6.18 ± 0.05[4]. This Ca^{++} activation behavior is readily reversible and reproducible over several hours without significant alterations[5]. The effects of forskolin on tension development are tested at pCa 6.09 and 4.67.

While maximal tension development at pCa 4.67 is not affected by forskolin in concentrations up to 10^{-4} M and in the presence of the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) (10^{-4} M), submaximal tension development at pCa 6.09 is inhibited (fig. 1a) indicating a decrease in the Ca^{++}-sensitivity of skinned cardiac fibers. The inhibition is much smaller if IBMX is omitted from the incubation medium (fig. 1b). Only after addition of IBMX to the bath is a marked decline in the isometric tension observed (fig. 1b). The potentiation of the forskolin-induced inhibition of tension development by IBMX is therefore taken to be due to blocked hydrolysis of cAMP. IBMX itself does not affect tension development indicating that the basal production of cAMP is very low. The inhibition induced by 10^{-4} M forskolin in presence of IBMX cannot be further enhanced by the addition of cAMP (10^{-4} M) (fig. 1b). The lack of additivity suggests that forskolin and cAMP inhibit tension by the same mechanism.

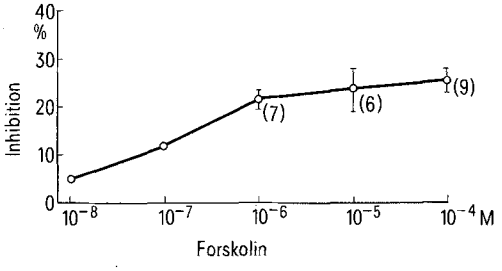

Figure 2. Dose-dependent inhibition of isometric contractile responses elicited at pCa 6.09 by forskolin. Experimental conditions as in figure 1a. Values are $\bar{x} \pm$ SEM, numbers in brackets indicate number of preparations used.

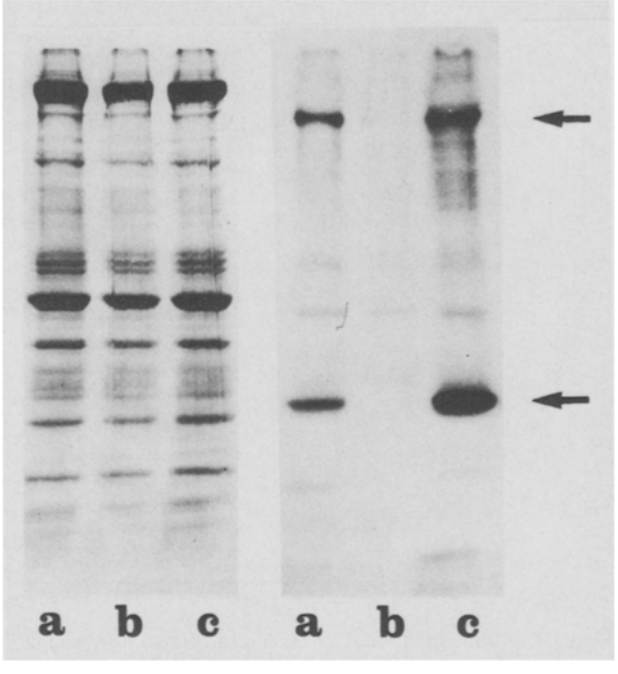

Figure 3. SDS-polyacrylamide gels (6-15% acrylamide) of individual skinned cardiac fiber bundles stained with Coomassie blue (left) and the corresponding autoradiographs (right). Fibers were incubated in relaxing solution in presence of [γ-^{32}P] ATP · Mg, with *a* 10^{-4} M forskolin, *b* 10^{-4} M IBMX, *c* 10^{-4} M forskolin and 10^{-4} M IBMX. Radioactivity is associated mainly with a M_r 28,000 and 145,000 band (←).

Figure 2 gives the inhibition of tension at pCa 6.09 induced by various concentrations of forskolin in the presence of IBMX (10^{-4} M). The inhibitory effect appears to be maximal at 10^{-6}-10^{-5} M forskolin. For comparison: The EC_{50} for forskolin activation of cAMP-generating systems of rat heart membranes is 10^{-5} M[6]. A similar small but significant ($p < 0.05$) inhibitory response is observed after incubation of skinned cardiac fibers with the catalytic subunit of the cAMP dependent protein kinase (1 µM): $22.4 \pm 3.54\%$ ($\bar{X} \pm$ SEM, $n = 6$) or with cAMP[4]. The observed decrease in tension corresponds well with biochemical experiments which showed that phosphorylation of cardiac TN-I increased $[Ca^{++}]$ required for inducing a 50% change in the fluorescence signal of 2-(4′-iodoacetamidoanilino)naphthalene-6-sulfonic acid modified TN-C[12].

Taken together, these results strongly suggest that in detergent-treated cardiac muscle fibers the catalytic subunit of adenylate cyclase is still present and can be stimulated by forskolin. The subsequent rise in cAMP then causes a shift of the calcium sensitivity via activation of the cAMP-kinase.

It is known that β-adrenergic stimulation of cardiac tissue or incubation of skinned cardiac fibers with cAMP results in phosphorylation of TN-I[4,13,18], and therefore a test was carried out to show whether the forskolin-induced inhibition of tension is associated with phosphorylation of TN-I. Figure 3 shows that there is indeed an increased incorporation of radioactivity into a M_r 28,000 band corresponding to that of troponin-I and into a band of M_r 145,000. It is not clear whether the latter band represents cardiac 'C-protein' which is also phosphorylated after β-adrenergic stimulation[19]. Note that the forskolin-induced increase in incorporation of radioactivity (fig. 3a) is greatly enhanced in the presence of IBMX (fig. 3c), while IBMX alone has no effect on phosphorylation (fig. 3b).

As in other preparations studied[6,17], the response to forskolin cannot be blocked by propranolol in concentrations up to 10^{-5} M, suggesting that in skinned fibers, too, the compound does not exert its action through the β-receptor. The question however arises whether the β-receptor itself is structurally intact in these fibers. The fact that chemically-skinned fibers no longer respond to norepinephrine (10^{-6}-10^{-4} M) or isoproterenol ($\leq 10^{-4}$ M) at submaximal $[Ca^{++}]$, strongly supports the notion that the skinning procedure alters the β-receptor.

In conclusion: Evidence is presented that detergent-treated skinned cardiac muscle preparations contain the catalytic subunit of the adenylate cyclase which can be stimulated by forskolin in the micromolar range leading to an increase in cAMP. In presence of a PDE inhibitor, this is sufficient to activate the cAMP-kinase and cause a decrease in Ca-sensitivity of the contractile apparatus comparable to the one observed after directly adding cAMP to the fibers[4]. The decrease in the Ca-sensitivity is associated with phosphorylation of troponin-I and an as yet unidentified protein component of 145,000 mol. wt. In intact cardiac muscle this effect is overridden by the increased Ca^{++} influx. Nevertheless, the decrease in Ca^{++} sensitivity of the contractile apparatus may be of physiological importance, as it may be one cause of the increased relaxation rate observed in beating hearts during β-adrenergic stimulation[12].

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Characterization and antithrombotic action of tissue plasminogen activator[1]

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Summary. An extractive fibrinolytic enzyme has been characterized and found to belong to the class of vascular plasminogen activators. The agent has been found to have an antithrombotic action in the rabbit.

We wish to report the characterization and the antithrombotic effect in the animal of a fibrinolytic agent, D44, which has recently been made available. This agent, extracted from hog ovaries, acts by converting an inert plasma protein, plasminogen, into a serine protease, plasmin, which breaks down fibrin into soluble degradation products (FDP). The body contains 2 types of plasminogen activators[3]: one, urokinaselike, secreted by non-keratinized epithelial cells and whose main action is probably to assure the passage of fluids through narrow ducts; the other, tissue activator (TA) which is produced by the vascular endothelium and has an antithrombotic action. These 2 types of activators have different properties and can be easily differentiated.

We found that D44 clearly belongs to the TA-class of plasminogen activators. The mol.wt of D44 has been determined on polyacrylamide gel electrophoresis[4]. The distance D44 had migrated (determined by testing 1 mm slices of the gel for fibrinolytic activity on fibrin plates) was recorded on a standard curve obtained by plotting the migration of reference substances against their mol.wt. The mol.wt of D44 was found to be about 70,000, (i.e. in the range of mol.wts found for TA[3]).

Experiments were made to visualize the action of D44 on plasminogen. Human plasminogen was incubated for various lengths of time together with D44, urokinase (UK) and TA (a purified tissue activator obtained from hog ovaries with another extractive method and kindly provided by P. Kok, Umeå). After incubation and reduction with β-mercapto-ethanol the mixtures were run on SDS polyacrylamide gel disc electrophoresis or on polyacrylamide gradient gel slabs (Pharmacia, Fine Chemicals, Uppsala). While UK caused the well-known pattern of limited proteolysis of plasminogen with the fading out of the plasminogen-band and the appearance of the heavy and light chains of plasmin, neiter D44 nor TA produced any significant effect on plasminogen (fig. 1). This was due to the absence of fibrin in the system. It is known that, fibrin is necessary for the activation of plasminogen by TA, but not by UK[3].

The role of fibrin in plasminogen activation by D44 was confirmed in a series of experiments with cross-immunoelectrophoresis and autoradiography. D44, UK, TA were labeled with I^{125}. Plasma incubated with D44 and run against anti-α_2-AP (α_2-antiplasmin, the main plasmin inhibitor of plasma) did not show any complex, nor did we find any mobility change of plasminogen. These results

Table 1. Mean fibrinolytic activity and range (mm^2 lysis) of resuspended euglobulin precipitate

	Before injection	After injection
Controls (10)	30 (0-50)	32 (0-60)
D44 50,000 U (10)	61 (35-150)	41 (33-48)
D44 100,000 U (5)	42 (25-64)	96 (30-169)

Table 2

	Frequency of thrombosis (%)	Frequency of occl. thrombi (%)
Controls (10)	72	50
D44 50,000 U (10)	73	28
D44 100,000 U (5)	45*	33

*$p < 0.05$ for 100,000 U compared with both 50,000 U and controls.

Table 3

	Weight (mg) of: Femoral thrombi	Jugular thrombi	Total mean
Controls (10)	1.0 (0.1-2.2)	34.4 (0.6-122.7)	24.9
D44 50,000 U (10)	1.2 (0.1-5.2)	27.6 (0.1-82.8)	18.5
D44 100,000 U (5)	1.6 (0.6-4.3)	10.6* (0.9-23)	6.6

*$p < 0.05$ for 100,000 U compared with both 50,000 U and controls.