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The effect of forskolin on the isometric contraction of the isolated hemidiaphragm of the rat

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1 The effects of forskolin on the parameters of the isometric contraction alone and in combination with aminophylline and isoprenaline were studied on the isolated hemidiaphragm of the rat during direct electrical stimulation.

2 Forskolin (2.6–18.2 μmol l^{-1}) produced a concentration-dependent increase in tension developed (*Td*) and, to a lesser extent, in the maximum rate of rise in tension (*dT/dt max*).

3 The dose-response curve for the action of forskolin (2.6–18.2 μmol l^{-1}) on *Td* was shifted to the left in the presence of a standard concentration of aminophylline (0.32 mmol l^{-1}).

4 Forskolin (5.2 μmol l^{-1}) produced a further and significant increase in both *Td* and *dT/dt max* in the presence of isoprenaline (0.24 μmol l^{-1}) in the bath.

5 In a calcium-free medium, the effects of forskolin (7.80 and 18.2 μmol l^{-1}) on *Td* and *dT/dt max* were significantly weaker than in a medium containing calcium.

6 These data indicate that forskolin increases the isometric contraction of the isolated hemidiaphragm probably by activating the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generating system. These effects are possible only in the presence of calcium.

Introduction

Many sympathomimetic substances and aminophylline are known to modulate cyclic AMP metabolism, and at the same time, to influence the basal characteristics of the skeletal muscle contraction (Varagić & Kentera, 1977; 1978; Varagić *et al.*, 1979; 1980). It has been found that stimulation of adrenoceptors in both fast and slow-contracting skeletal muscles produces a rise in the cellular content of cyclic AMP (Al-Jeboory & Marshall, 1978; Fellenius *et al.*, 1980).

Bowman & Nott (1974) reported that the effects of some β_2-agonists on contractions of the skeletal muscle were potentiated by a range of cyclic nucleotide phophodiesterase inhibitors, and that the effects on contractility were mediated via cyclic AMP (Rodger & Bowman, 1983).

Forskolin has already been found to stimulate adenylate cyclase selectively in a variety of different cell types, leading to large increases in intracellular cyclic AMP concentration (Seamon & Daly, 1981; Rodger & Shahid, 1984).

Therefore, it was of interest to study the effects of forskolin, alone and in a combination with isoprenaline and aminophylline, on tension developed (*Td*) and the maximum rate of rise of tension (*dT/dt max*) of the isolated hemidiaphragm of the rat during direct electrical stimulation.

Methods

The isolated hemidiaphragm of male or female rats (200–250 g) was suspended in an isolated organ bath of 15 ml capacity. Tyrode solution was used with a double amount of glucose and bubbled with a mixture of 97% O_2 and 3% CO_2. The composition of Tyrode solution was as follows (mmol l^{-1}): NaCl 136, KCl 2.81, $MgCl_2$ 0.105, $CaCl_2$ 1.8, NaH_2PO_4 0.417, $NaHCO_3$ 11.9 and dextrose 11.1. The temperature of this solution was about 36°C. The preparation was stimulated directly by supramaximal single pulses of 0.8–1 ms duration. The frequency of stimulation was 0.15 Hz. The isometric contractions were recorded with a microdisplacement myograph transducer (F 50, Narco-Bio-Systems, Inc.) and displayed on paper (Physiograph IV polygraph). Both tension (*Td*) and the maximum rate of rise of tension (*dT/dt max*) were recorded simultaneously. The differential of tension with respect to time was recorded by a differentiator Coupler Type 7301 (Narco-Bio-Systems, Inc.), which

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provides the true mathematical derivative of analogue signal voltages. Two paladore wires (paladium 30% and silver 70%) were used for delivering the pulses for direct electrical stimulation. The diaphragm was secured to one of these wires at several points. The other electrode was placed around the upper part of the diaphragm, but was not in contact with the muscle.

A separate series of experiments was performed in a Ca-free medium, which was made by adding 0.025 mM disodium edetate (di-Na-EDTA) to a calcium-free solution. The observations in Ca^{2+}-free solution were bracketed by observations in normal Ca^{2+}. Thus, the effects of increasing concentrations of forskolin (2.60–18.2 μmol l^{-1}) were observed in a medium containing calcium chloride. The effects of the same concentration of forskolin were then recorded in a Ca^{2+}-free medium, and once again in normal Ca^{2+}.

Drugs

The following drugs were used: aminophylline, dimethyl sulphoxide (DMSO) (Fluka), di-sodium-EDTA (Merck), isoprenaline hydrochloride and forskolin (Pharma Biochemie, Hoechst A.G., generously provided by Dr H. Metzger). This substance was dissolved in DMSO to provide a stock solution (4.1 mg ml^{-1}), which was thereafter diluted in phosphate buffer (pH 7).

Statistics

Results are expressed as the mean ± s.e.mean of n determinations, and the difference between means was assessed for significance by Student's t test (unpaired). Values of $P < 0.05$ were taken as statistically significant.

Results

The effect of forskolin on tension (Td) *and the maximum rate of rise of tension* (dT/dt max).

The increasing concentrations of forskolin added to the organ bath (2.60–18.2 μmol l^{-1}) produced a concentration-dependent increase in both *Td* and *dT/dt max* (Figure 1). The increase in *dT/dt max* was less pronounced than the increase in *Td*. The content of the buffer-diluted DMSO in the final volume of organ bath ranged from 0.026 to 0.11%. The effect of forskolin occurred slowly after 1 to 3 min, reaching the maximum after 5 min. This effect was completely reversible after washing out the drug from the organ bath.

Higher concentrations of forskolin (7.80 and 18.2 μmol l^{-1}) produced a significant increase in *Td* of the isolated hemidiaphragm of the rat in comparison

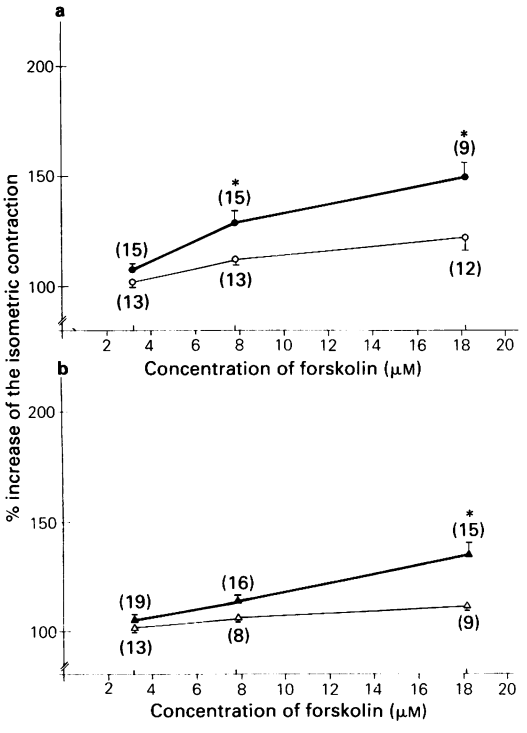

Figure 1 (a) The effect of increasing concentrations of forskolin on the tension (*Td*) of the isolated hemidiaphragm of the rat. Abscissa scale: concentration of forskolin. Ordinate scale: % increase of the isometric contraction of the isolated hemidiaphragm of the rat in comparison with the control, prior to addition of the drug, taken as 100%. The response of forskolin itself is represented by (●), and the effect of solvent alone by (○). Each point is the mean of 9–15 experiments. The number of experiments is given in parentheses. Vertical bars show the s.e.mean. *Significantly different from the effect of solvent alone (Student's t test; $P < 0.05$). (b) The effect of forskolin on the maximum rate of rise of tension (*dT/dt max*) of the isolated hemidiaphragm of the rat. Abscissa scale: concentration of forskolin (in μmol l^{-1}). Ordinate scale: % increase of the isometric contraction, in comparison with the control, prior to addition of the drug, taken as 100%. The response to forskolin is represented by (▲), and the effect of solvent by (△). Each point is the mean of 8–19 experiments. The number of experiments is given in parentheses. Vertical bars show the s.e.mean. *Significantly different from the effect of solvent alone (Student's t test; $P < 0.05$).

with the effect of the solvent alone ($P < 0.05$). The low concentration of forskolin used in our experiments (2.6 μmol l^{-1}) also produced an increase in *Td* compared with the effect of the solvent alone, but this difference was not significant. On the other hand, the

highest concentration of forskolin (18.2 μmol l^{-1}) produced a significant increase in *dT/dt max*, compared with the effect of the solvent alone. In these experiments, the solvent was used in the same volume as the corresponding concentration of forskolin. This means that the solvent alone was diluted by phosphate buffer to produce the same concentration as that which was present when forksolin was applied.

Time-paired controls have shown that at a particular time of year (summer), the effect of forskolin was fairly weak. Thus, the concentration of 7.8 μmol l^{-1} produced an increase of less than 10% in *Td* in 6 out of 15 experiments. In another series performed during winter and spring, the same concentration of forskolin produced an increase in *Td* of 35% in 9 out of 15 experiments. Therefore, the mean increase in *Td* after this concentration of forskolin for the whole group of 15 experiments was 28.8 ± 5.9%.

Forskolin and aminophylline (AMPh)

Aminophylline (0.32 mmol l^{-1}) added to the organ bath, and then left for 5 to 7 min, produced an increase in both *Td* and *dT/dt max* of the isolated hemidiaphragm of the rat during direct electrical stimulation. The addition of forskolin (2.6 μmol l^{-1}) after the addition of aminophylline, produced a further increase in both parameters of the isometric contraction (Figure 2). The effects of a combination of AMPh and forskolin on *Td* and *dT/dt max* during direct electrical stimulation were significantly different from the effects of AMPh alone ($P < 0.05$ and $P < 0.05$, respectively). The effects of a combination of AMPh and forskolin on *Td* and *dT/dt max* were also significantly different from the effects of a combination of AMPh and the solvent ($P < 0.05$ and $P < 0.05$, respectively).

It should be pointed out that the second addition of AMPh at the same concentration of 0.32 mmol l^{-1}, but in the presence of forskolin, produced a significant increase in both *Td* ($P < 0.01$) and *dT/dt max* ($P < 0.01$) in comparison with the effect of AMPh alone. The effect of the second addition of AMPh, in the presence of forskolin, produced a significantly larger effect both on *Td* ($P < 0.05$) and *dT/dt max* ($P < 0.01$), in comparison with the control experiments with the corresponding volume of solvent (DMSO + phosphate buffer), as shown in the right hand columns of Figure 2.

The effect of forskolin in the presence of a standard concentration of AMPh (0.32 mmol l^{-1}) was studied in a separate series of experiments. AMPh was left in the organ bath for 5 min, and then, forskolin in increasing concentrations (2.6 to 18.2 μmol l^{-1}) was added to the bath. The results for *Td* of these experiments are shown in Figure 3. It can be seen that the responses at each concentration of forskolin (*Td*) were distinctly increased in amplitude. The maximum

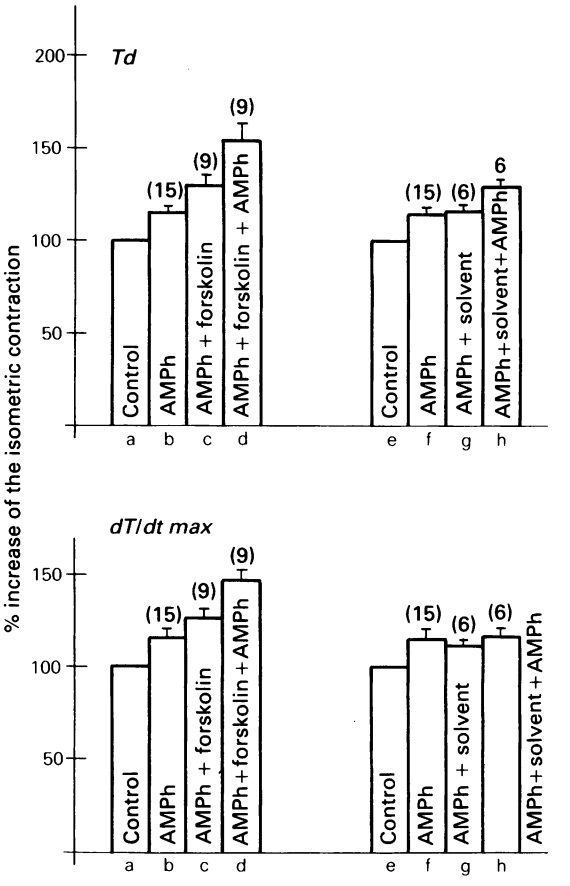

Figure 2 The effect of forskolin and aminophylline on *Td* and *dT/dt max* of the isolated hemidiaphragm of the rat. The ordinate scale represents the increase in the isometric contraction of the isolated hemidiaphragm of the rat in comparison with the control, prior to addition of the drug, taken as 100%. The upper panel represents changes in *Td*; the lower panel represents changes in *dT/dt max*. Aminophylline (AMPh, 0.32 mmol l^{-1}) was added to the organ bath, and left for 5–7 min. It produced an increase in both *Td* and *dT/dt max*. The addition of forskolin (2.6 μmol l^{-1}) to the bath produced a further increase in both *Td* and *dT/dt max*. Five minutes after the addition of forskolin to the bath, aminophylline (0.32 mmol l^{-1}) again was added and its effect was recorded for another 7 min. The second addition of the same concentration of AMPh to the bath produced a further increase in both *Td* and *dT/dt max*. The number of experiments is given at the top of the vertical bars, which represent s.e.mean. The control experiments with the solvent are represented on the right side of both panels. The significance of the results was as follows: Upper panel, $P(b:c) < 0.05$; $P(b:d) < 0.01$; $P(c:g) < 0.05$; $P(d:h) < 0.05$. Lower panel, $P(b:c) < 0.05$; $P(b:d) < 0.01$; $P(c:g) < 0.05$; $P(d:h) < 0.01$.

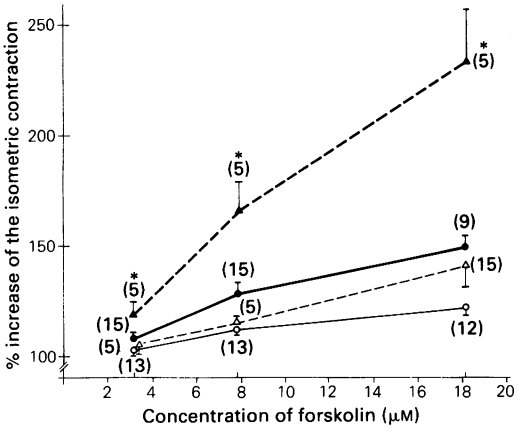

Figure 3 The effect of forskolin on *Td* of the isolated hemidiaphragm of the rat, in the presence of a standard concentration of aminophylline. Abscissa scale: concentration of forskolin (μmol l^{-1}). Ordinate scale: % increase of the isometric contraction of the isolated hemidiaphragm of the rat in comparison with the control, prior to addition of the drug, taken as 100%. The response to forskolin is represented by (●), and the response to forskolin in the presence of the standard concentration of aminophylline (0.32 mmol l^{-1}) by (▲). The corresponding control experiments are represented by (○) (solvent alone) and (△) (aminophylline + solvent). Each point is the mean ± s.e.mean (vertical bars). The number of experiments is given in parentheses. *Effects of forskolin in the presence of a standard concentration of aminophylline were significantly different from the effects of forskolin alone ($P < 0.05$). The effects of forskolin (7.2 and 18.2 μmol l^{-1}) were significantly different from the effects of the solvent alone. This is indicated in Figure 1a but not in this Figure. The effects of forskolin in the presence of a standard concentration of aminophylline were significantly different from the effects of the solvent in the presence of aminophylline (not shown by * on the Figure).

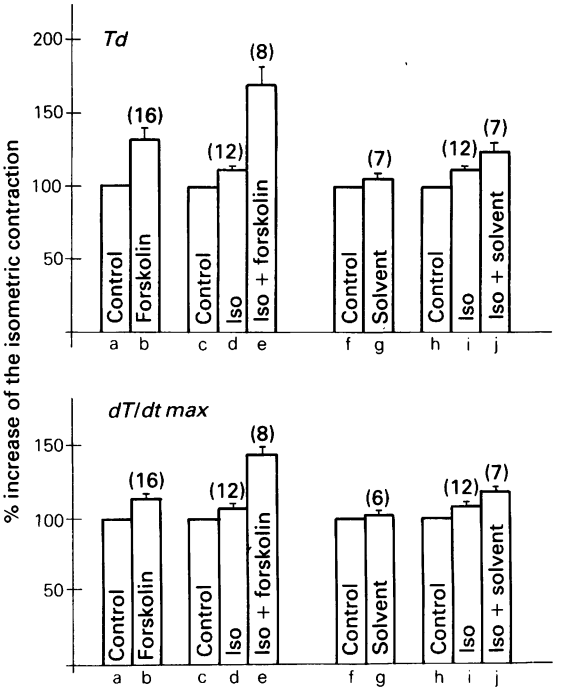

Figure 4 The influence of isoprenaline on the effects of forskolin on the isolated hemidiaphragm of the rat. Ordinate scale: percentage increase of the isometric contraction of the isolated hemidiaphragm of the rat in comparison with the control, prior to addition of the drug, taken as 100%. The upper panel represents changes in *Td*; the lower panel represents changes in *dT/dt max*. The concentration of forskolin used in these experiments was 5.2 μmol l^{-1}, and of isoprenaline 0.24 μmol l^{-1}. Each column represents the mean with s.e.mean (vertical bars). The number of experiments is given at the top of the columns. The control experiments with the solvent are represented on the right side of both panels. The significance of the results was as follows: Upper panel, P(b:e) < 0.05; P(b:g) < 0.05; P(d:e) < 0.05; P(e:j) < 0.05. Lower panel, P(b:e) < 0.001; P(b:g) < 0.05; P(d:e) < 0.05; P(e:j) < 0.05.

rate of rise of tension (*dT/dt max*) was also increased by the joint action of AMPh and forskolin.

Forskolin and isoprenaline

In a separate series of experiments, the effect of forskolin (5.2 μmol l^{-1}) on the parameters of the isometric contraction of the isolated hemidiaphragm of the rat, was studied in the presence of a standard concentration of isoprenaline in the bath (0.24 μmol l^{-1}). The addition of isoprenaline to the bath, produced an insignificant increase in both *Td* and *dT/dt max* (111 ± 1.3 and 108 ± 2.0%, respectively), the increase in *dT/dt max* being less pronounced than the increase in *Td*. Isoprenaline was left in the bath for 3 min after which, forskolin (5.2 μmol l^{-1}) was added to the bath and left for 7 to 10 min. The results of these experiments are shown in Figure 4.

The addition of forskolin to the bath in the presence of isoprenaline, produced a further increase of the parameters of the isometric contraction of the isolated hemidiaphragm of the rat. The effects of forskolin (5.2 μmol l^{-1}) in the presence of 0.24 μmol l^{-1} isoprenaline were significantly different from the effects of forskolin alone ($P < 0.05$ for *Td* and $P < 0.001$ for *dT/dt max*).

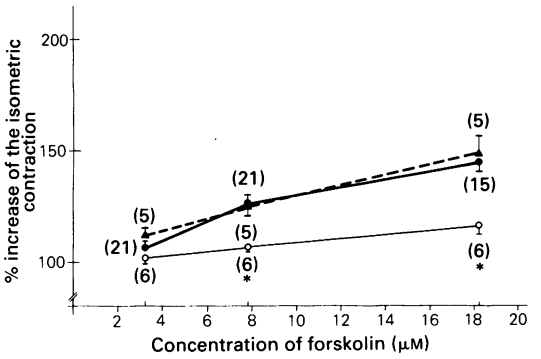

Figure 5 The effect of forskolin (2.60–18.2 μmol l^{-1}) on *Td* of the isolated hemidiaphragm of the rat in a Ca^{2+}-free medium. The observations in a Ca^{2+}-free solution (○) were bracketed by observations in normal Ca^{2+} ((●) before and (▲) after). Each point represents the mean with s.e.mean (vertical bars). The number of experiments is given in parentheses. Abscissa scale: concentration of forskolin in μM. Ordinate scale: % increase of *Td* in comparison with the control prior to addition of drugs, taken as 100%.

The effects of forskolin in a calcium-free medium

The effects of forskolin in a calcium-free medium were studied in a separate series of experiments. First, the cumulative dose-response curve for forskolin (2.60–18.2 μmol l^{-1}) was obtained in a medium containing calcium chloride 1.8 mmol l^{-1}. Second, the dose-response curve for the same concentrations of forskolin (2.60–18.2 μmol l^{-1}) was obtained in a calcium-free medium, made as described in the Methods. Third, the effect of increasing concentrations of forskolin was once again observed in the medium containing calcium chloride. One of these experiments (for *Td*) is shown in Figure 5. Similar results were obtained for *dT/dt max* (not shown in the figure). The effects of forskolin (7.80 and 18.2 μmol l^{-1}) on *Td* were significantly weaker in a calcium-free solution ($P<0.05$ and $P<0.05$, respectively), than in the medium containing Ca^{2+}.

It should be pointed out that the effect of forskolin in the Ca^{2+}-free solution was qualitatively different from its effect in the medium with Ca^{2+}, i.e. the effect of forskolin occurred rapidly, almost immediately after its addition to the organ bath, reaching its peak in about 30 s to 1 min, and after that, depression of the isometric contractions slowly occurred.

The effects of forskolin (2.60–18.2 μmol l^{-1}) on *Td* and *dT/dt max* of the same muscle after restoring the Ca concentration in the medium were completely restored (Figure 5).

Discussion

Forskolin, at concentrations ranging from 2.6 to 18.2 μmol l^{-1}, produced an increase in the isometric contraction of the isolated hemidiaphragm of the rat during direct electrical stimulation. This increase was particularly significant in tension developed (*Td*), whereas the maximum rate of rise of tension (*dT/dt max*) was also increased, but to a lesser extent. The solvent itself, consisting of a mixture of DMSO and phosphate buffer, also produced a small but insignificant increase in both parameters of the isometric contraction of the isolated hemidiaphragm. The amount of solvent added to the bath was always between 0.026 and 0.11% of the volume in the organ bath.

It has already been found that forskolin stimulates adenylate cyclase in a variety of different cell types, leading to large increases in intracellular cyclic AMP concentration (Seamon & Daly, 1981; Vegesna & Diamond, 1983; Rodger & Shahid, 1984; Marshall & Fain, 1985). Many β-adrenoceptor agonists have been found to increase the isometric contraction of the isolated hemidiaphragm (Kentera & Varagić, 1975; Varagić & Kentera, 1977; 1978), and at the same time to produce an increase in the concentration of cyclic AMP in the muscle (Al Jeboory & Marshall, 1978; Varagić, 1980). It is therefore possible that the potentiating action of forskolin on the isometric contraction of the isolated hemidiaphragm may be due to its action on cyclic AMP metabolism in this type of muscle.

The effect of forskolin on *Td* is even more pronounced if combined either with isoprenaline or with aminophylline. In the presence of a standard concentration of aminophylline (0.32 mmol l^{-1}), the increasing concentration of forskolin (2.6–18.2 μmol l^{-1}) produced a stronger effect, shifting the dose-response line to the left. In our experiments, isoprenaline (0.24 μmol l^{-1}) added to the organ bath and left for 3 min, produced a slight increase in *Td* and *dT/dt max* of the isolated hemidiaphragm of the rat during direct electrical stimulation, amounting to 11% for *Td* and 8% for *dT/dt max*. Other authors (Bowman & Raper, 1967; Bowman & Zaimis, 1958) showed that the rate of rise of tension is unaffected (rabbit tibialis anterior and cat tibialis anterior), or is initially slightly slowed down by adrenaline (Goffart & Ritchie, 1952; rat diaphragm). In another series of experiments, the response to a standard concentration of forskolin (5.2 μmol l^{-1}), which by itself produced a significant increase in *Td* ($P<0.05$) and *dT/dt max* ($P<0.05$) compared with the corresponding volume of the solvent alone, was significantly increased in the presence of isoprenaline (0.24 μmol l^{-1}) in the organ bath, compared with the effect of forskolin alone ($P<0.05$ for *Td* and $P<0.001$ for *dT/dt max*). These results might have been expected if all these substances

(forskolin, isoprenaline and aminophylline) were presumed to act via a cyclic AMP dependent mechanism(s).

Bowman *et al.* (1985) found, on the isolated soleus muscle (a typical representative of slow-contracting muscles) that forskolin failed to elicit a sympathomimetic response, despite the fact that it significantly increased the intracellular concentration of cyclic AMP. At the same time, forskolin failed to potentiate the effects induced by isoprenaline. These authors have suggested that, in the skeletal muscle, adenylate cyclase may be compartmentalized in much the same way as has been proposed for cardiac muscle (Zahler, 1983). If this is so, it may be possible that in spite of a huge rise of cellular cyclic AMP content produced by forskolin, the particular adenylate cyclase system governing the calcium sequestration might be unaffected.

If this idea of compartmentalization of adenylate cyclase in skeletal muscle cells is correct, then in the isolated hemidiaphragm of the rat, which is a representative of fast-contracting muscles, forskolin acts on the very compartment which is reponsible for Ca sequestration, thus producing an increase in the isometric contraction, as measured by an increase in *Td*. Further experiments are necessary in order to establish or deny the participation of different adenylate cyclase systems in the hemidiaphragm of the rat and the soleus of the guinea-pig.

It was also shown in the present experiments that the potentiating action of forskolin on the isometric contraction of the hemidiaphragm during direct electrical stimulation was significantly depressed in a Ca^{2+}-free medium. Seamon & Daly (1984), while studying the mechanism of activation of adenylate cyclase by forskolin, suggested that this substance either exerted its activity by a direct action on the catalytic subunit of the enzyme, or by an indirect action mediated via an as yet unidentified protein. Whatever the mechanism(s) may be, our experiments indicate a possible role of Ca^{2+} in the action of forskolin on the isolated hemidiaphragm of the rat.

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