Korean J Physiol Pharmacol
Vol 13: 189−194, June, 2009

Forskolin Changes the Relationship between Cytosolic Ca^{2+} and Contraction in Guinea Pig Ileum

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This study was designed to clarify the mechanism of the inhibitory effect of forskolin on contraction, cytosolic Ca^{2+} level ($[Ca^{2+}]_i$), and Ca^{2+} sensitivity in guinea pig ileum. Forskolin (0.1 nM~10 μM) inhibited high K^+ (25 mM and 40 mM)- or histamine (3 μM)-evoked contractions in a concentration-dependent manner. Histamine-evoked contractions were more sensitive to forskolin than high K^+-evoked contractions. Spontaneous changes in $[Ca^{2+}]_i$ and contractions were inhibited by forskolin (1 μM) without changing the resting $[Ca^{2+}]_i$. Forskoln (10 μM) inhibited muscle tension more strongly than $[Ca^{2+}]_i$ stimulated by high K^+, and thus shifted the $[Ca^{2+}]_i$-tension relationship to the lower-right. In histamine-stimulated contractions, forskolin (1 μM) inhibited both $[Ca^{2+}]_i$ and muscle tension without changing the $[Ca^{2+}]_i$-tension relationship. In α-toxin-permeabilized tissues, forskolin (10 μM) inhibited the 0.3 μM Ca^{2+}-evoked contractions in the presence of 0.1 mM GTP, but showed no effect on the Ca^{2+}-tension relationship. We conclude that forskolin inhibits smooth muscle contractions by the following two mechanisms: a decrease in Ca^{2+} sensitivity of contractile elements in high K^+-stimulated muscle and a decrease in $[Ca^{2+}]_i$ in histamine-stimulated muscle.

Key Words: Fosrkolin, Cytosolic Ca^{2+}, Ca^{2+} sensitivity, Guinea pig ileum

INTRODUCTION

It is generally accepted that adenosine 3',5'-cyclic monophosphate (cAMP) is the intracellular messenger by which many smooth muscle relaxants inhibit the generation or maintenance of muscle tension (Murray, 1990; Lincoln and Cornwell, 1991; Ozaki et al., 1992; Murthy, 2006; Porter et al., 2006). Smooth muscle relaxation induced by cAMP occurs after activation of cAMP-dependent protein kinase (Eckly-Michel et al., 1997; Porter et al., 2006). In smooth muscle, cAMP decreases resting tone and inhibits contraction induced by various agonists, although the mechanism of the inhibitory effect is not fully understood. One of the possibilities is that cAMP decreases $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx or by activating Ca^{2+} sequestration (Abe and Karaki, 1988; Cooper, 2003). Another possibility is that cAMP-dependent protein kinase phosphorylates myosin light chain kinase and inhibits the activity of this kinase, thus inhibiting contractile elements (Adelstein et al., 1986; Woodrum et al., 1999). Forskolin, a diterpene isolated from the roots of *Coleus forskohli*, is known to stimulate adenylate cyclase directly, resulting in an increase of intracellular cAMP levels, and to relax vascular smooth muscles (Seamon and Daly, 1986; Rembold and Chen, 1998). This provides a good pharmacologic tool to investigate the role of cAMP.

Although the rise in $[Ca^{2+}]_i$ initiates contractions in smooth muscle, there is not a simple relationship between $[Ca^{2+}]_i$ and tension. Several agonists have been shown to alter the sensitivity of the contractile apparatus to Ca^{2+}. For example, high K^+ induces less contractile tension for a given increase in $[Ca^{2+}]_i$ than agonists, such as norepinephrine in ferret portal veins, norepinephrine, prostaglsndins, and endothelin-1 in rat aortas, thromboxane analogues and phenylephrine in rabbit pulmonary arteries, carbachol in canine tracheas, and pilocarpine in ileum (Karaki, 1989). Furthermore, several reports have suggested that receptor-agonists induce greater myosin light chain phosphorylation than high K^+ at a given $[Ca^{2+}]_i$ (Ratz, 1999; Porter et al., 2006). A decrease in Ca^{2+} sensitivity due to cAMP has also been reported (Rembold and Chen, 1998; Rembold et al., 2001). Therefore, it is possible that modulation of smooth muscle contraction by cAMP is due to the decrease in $[Ca^{2+}]_i$ and Ca^{2+} sensitivity.

Based on these findings, we now assume that cAMP induces relaxation by suppressing cytosolic Ca^{2+} concentration and by decreasing Ca^{2+} sensitivity in intestinal smooth muscle. In order to clarify the precise mechanism of the relaxant effect of forskolin on intestinal smooth muscle, we examined its effect on $[Ca^{2+}]_i$ and muscle tension, as well as an α-toxin-permeabilized preparation.

Received April 28, 2009, Revised May 15, 2009,
Accepted June 2, 2009

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ABBREVIATIONS: $[Ca^{2+}]_i$, cytosolic Ca^{2+} level; PSS, physiologic salt solution; cAMP, 3'5'-cyclic monophosphate; fura-2/AM, fura-2 acethoxymethyl ester; FCCP, carbonylcyanide p-(trifluoro-methoxy)phenylhydrazone.

METHODS

Tissue preparation

Male guinea pigs (250~300 g) were killed by a sharp blow to the neck and exsanguination. A section of the ileum was isolated and placed in a physiologic salt solution (PSS). The lumen of the ileum was cleaned gently with PSS. A glass pipette, 7 mm in diameter, was inserted in the lumen of the ileum and the longitudinal muscle layer was separated from the underlying circular muscle layer. Segments of the longitudinal muscle layer (approximately 10 mm), were used for experiments.

Measurements of muscle tension

A muscle strip was attached to a holder under a resting tension of 0.5 g. After equilibration for 60~90 min in PSS, each strip was repeatedly exposed to 40 mM KCl solution until the responses became stable. The high K^+ solution was prepared by replacing NaCl with equimolar KCl. Muscle contraction was recorded isometrically with a force-displacement transducer and recorded with a pen recorder. The PSS contained the following compositions (in mM): 136.9 NaCl, 5.4 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 23.8 $NaHCO_3$, 0.01 ethylenediamine tetraacetic acid (EDTA), and 5.5 glucose. These solutions were saturated with a 95% O_2 and 5% CO_2 mixture at 37°C to maintain the pH at 7.4.

Fura-2 loading and simultaneous measurements of tension and $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured according to the method described by Kwon et al. (2000b) using the fluorescent Ca^{2+} indicator, fura-2. Muscle strips were exposed to the acethoxymethyl ester of fura-2 (fura-2/AM [5 μM]) in the presence of 0.02% cremophor EL for 3~4 hr at room temperature. After loading, the muscle strip was washed with PSS at 37°C for 20 min to remove uncleaved fura-2/AM and was held horizontally in a temperature-controlled, 7 ml organ bath. One end of the muscle strip was connected to a force-displacement transducer to monitor the muscle contraction. The muscle strips were illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm). The intensity of the 500 nm fluorescence (F340 and F380) was measured using a fluorimeter (CAF-100; Jasco, Tokyo). The ratio of F340 to F380 (F340/F380) was calculated as an indicator of $[Ca^{2+}]_i$. The absolute Ca^{2+} concentration was not calculated in this experiment because the dissociation constant of the fluorescence indicator for Ca^{2+} in the cytosol may be different from that obtained *in vitro* (Karaki, 1989). Therefore, the ratio obtained in resting and 40 mM KCl-stimulated muscle was taken as 0% and 100%, respectively.

Permeabilized preparation

Permeabilized smooth muscle was prepared, as described by Kwon et al. (2000a). A thin bundle of ileum (120~150 μm in diameter and 2~3 mm in length) was dissected parallel to the longitudinal muscle fiber under a binocular microscope. The muscle strip was soaked for 5~10 min at room temperature (22~24°C) in a relaxing solution containing *Staphylococcus aureus* α-toxin (10 μM protein/ml). One end of the muscle strip was fixed to the chamber, and the other end was attached to a force transducer under a resting tension of 50 mg and equilibrated for 60~90 min. During this period, 10 μM Ca^{2+} was repeatedly applied until the peak force became reproducible. The contractile force of the permeabilized muscle was recorded isometrically. The Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA was considered to be 1 μM at pH 6.8 and 20°C. The relaxing solution used for the permeabilized tissue contained the following compositions (in mM): 130 potassium proprionate, 4 $MgCl_2$, 5 Na_2-ATP, 2 creatine phosphate, 10 creatine phosphokinase, 20 Tris-maleate (pH 6.8), and 2 EGTA. Added to the relaxing solution were the mitochondrial inhibitor, 1 μM carbonyl cyanide p-(trifluoro-methoxy)phenylhydrazone (FCCP), and the protease inhibitors, 1 μM E-64 and 1 μM leupeptin.

Statistics

The results of the experiments were expressed as the mean±SE mean. An unpaired Student's t-test was used for statistical analysis of the results and the number of preparations taken from separate animals was indicated by n. p values$<$0.05 were considered to be significantly different.

RESULTS

The effect of forskolin on high K^+- and histamine-evoked contractions

Fig. 1 shows the concentration-response relationship of the inhibitory effects of forskolin on high K^+ (25 and 40 mM)- and histamine (3 μM)-evoked sustained contractions. Forskolin inhibited histamine-evoked contractions more strongly than high K^+-evoked contractions from 25 mM to 40 mM. The inhibitory effect of forskolin was reduced by increasing the K^+ concentration.

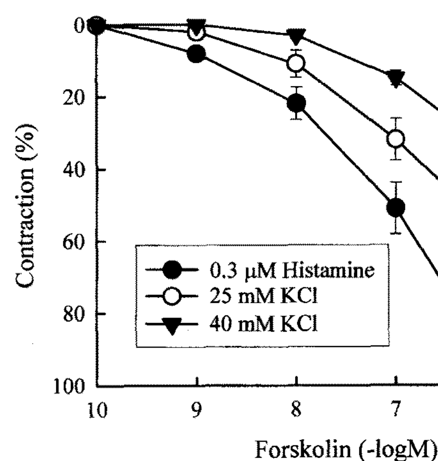

Fig. 1. Concentration-response relationship for the inhibitory effect of forskolin on contractions induced by 25 mM KCl, 40 mM KCl, and 3 μM histamine. Forskolin (0.1 nM~10 μM) was cumulatively added after the contractions reached a steady state.

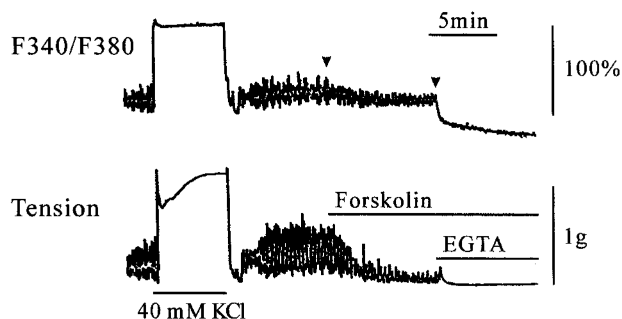

Fig. 2. The effect of forskolin (1 μM) on $[Ca^{2+}]_i$ (upper trace) and muscle tension (lower trace) in spontaneously active ileum. 100% represents the steady state $[Ca^{2+}]_i$ in the presence of 40 mM KCl. Forskolin (1 μM) inhibited rhythmic increases in $[Ca^{2+}]_i$ and tension without changing the basal $[Ca^{2+}]_i$. EGTA (4 mM) decreased basal $[Ca^{2+}]_i$ below the resting level with no further decrease in muscle tension.

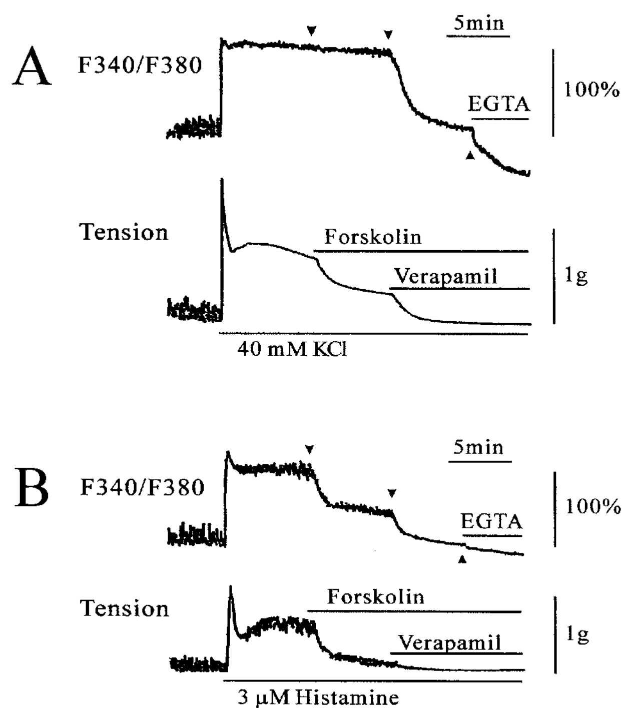

Fig. 3. Effects of forskolin on 40 mM KCl (A)- and 3 μM histamine (B)-stimulated $[Ca^{2+}]_i$ (upper trace) and muscle tension (lower trace). 100% represents the 40 mM KCl- or histamine-induced increases in $[Ca^{2+}]_i$ before addition of forskolin. In panel A, after 40 mM KCl-stimulated $[Ca^{2+}]_i$ and muscle tension reached a steady state level, 10 μM forskolin and verapamil were sequentially added. Forskolin partially inhibited contraction without changing $[Ca^{2+}]_i$. Verapamil (10 μM), on the other hand, inhibited $[Ca^{2+}]_i$ and tension to the resting level. In panel B, when the $[Ca^{2+}]_i$ and muscle tension induced by histamine (3 μM) reached a steady state level, 1 μM forskolin and 10 μM verapamil were sequentially added. Forskolin (1 μM) significantly inhibited $[Ca^{2+}]_i$ and contractions. Verapamil (10 μM) inhibited $[Ca^{2+}]_i$ and tension to the resting level.

The effect of forskolin on spontaneous activities

Fig. 2 shows the inhibitory effect of 1 μM forskolin on the spontaneous rhythmic changes in $[Ca^{2+}]_i$ and muscle tension. Forskolin inhibited the rhythmic changes in $[Ca^{2+}]_i$ and contractions without changing the basal $[Ca^{2+}]_i$. The addition of EGTA decreased the basal $[Ca^{2+}]_i$ below the resting level and inhibited spontaneous rhythmic contractions.

The effect of forskolin on high K^+- and histamine-evoked changes in $[Ca^{2+}]_i$ and contractions

Fig. 3A shows the effect of forskolin on high K^+ (40 mM)-evoked responses in the ileum. The addition of 10 μM forskolin inhibited muscle tension by 44.1±9.7% (n=6), with a small decrease in $[Ca^{2+}]_i$ (3.8±0.8; n=6). Addition of verapamil (10 μM) decreased the remaining $[Ca^{2+}]_i$ and contractions to the resting level.

Fig. 3B shows the inhibitory effect of forskolin on the histamine-evoked changes in $[Ca^{2+}]_i$ and contractions. Forskolin (1 μM) inhibited the histamine-induced contractions by 68.4±7.7%, with a decrease in $[Ca^{2+}]_i$ by 32.3±7.7% (n=6).

The effect of forskolin on the $[Ca^{2+}]_i$-tension relationship

Fig. 4A shows that K^+ (10, 15, 20, 30, and 40 mM) evoked concentration-dependent increases in $[Ca^{2+}]_i$ and muscle

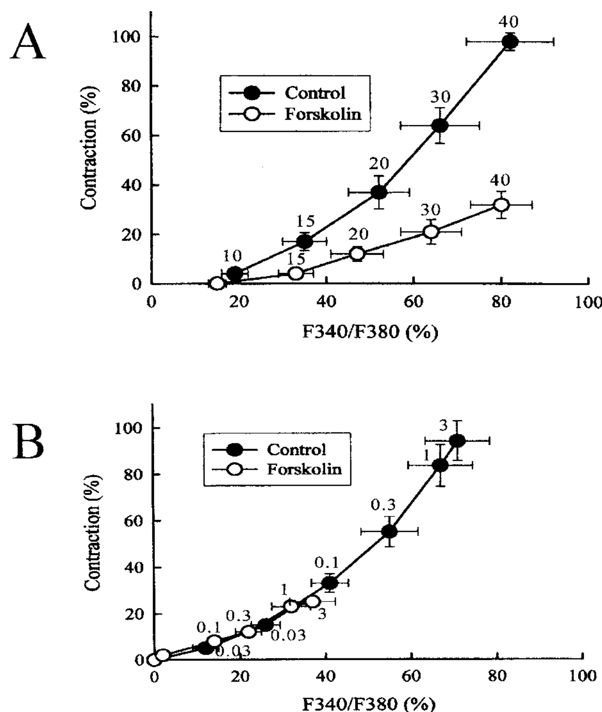

Fig. 4. Effect of forskolin (10 μM, open circles) on the relationship between $[Ca^{2+}]_i$ (abscissa) and muscle tension (ordinate) in the presence various concentrations of KCl (10, 15, 20, 30, and 40 mM) or histamine (0.03, 0.1, 0.3, 1, and 3 μM). 100% represents 40 mM KCl-induced increases in $[Ca^{2+}]_i$ and muscle tension measured before cumulative addition of the stimuli. Each point represents the mean of 7~10 experiments, and the SR mean is shown by vertical and horizontal bars.

tension. The relationship between $[Ca^{2+}]_i$ and muscle tension suggests that there is a threshold $[Ca^{2+}]_i$ for contractions (approximately 20% $[Ca^{2+}]_i$). When the $[Ca^{2+}]_i$ increased above this level, there was a positive correlation between $[Ca^{2+}]_i$ and muscle tension. Forskolin inhibited the high K^+ (30 and 40 mM)-induced contractions without changing $[Ca^{2+}]_i$, whereas $[Ca^{2+}]_i$ stimulated by 10, 15, and 20 mM of K^+ was decreased. Thus, forskolin shifted the $[Ca^{2+}]_i$-tension curve to the lower-right and increased the threshold for contractions, suggesting that forskolin decreased the Ca^{2+} sensitivity of contractile elements.

Fig. 4B shows the effect of 1 μM forskolin on histamine-evoked changes in $[Ca^{2+}]_i$ and muscle tension. There is a positive correlation between $[Ca^{2+}]_i$ and muscle tension in the presence of histamine (0.03, 0.1, 0.3, 1, and 3 μM) in a concentration-dependent manner. In the presence of forskolin, the effects of histamine to increase $[Ca^{2+}]_i$ and muscle tension were inhibited. Since forskolin inhibited both $[Ca^{2+}]_i$ and muscle tension proportionally, the slope of the $[Ca^{2+}]_i$-tension relationship did not change. These results suggest that forskolin inhibits histamine-induced contraction primarily by decreasing $[Ca^{2+}]_i$.

The effect of forskolin on Ca^{2+}-evoked contractions in permeabilized muscle

Fig. 5A shows the pCa^{2+}-tension relationship in the presence and absence of 10 μM of forskolin. In permeabilized ileum treated with α-toxin, the cumulative addition of 0.01 ~10 μM Ca^{2+} induced graded contractions in a concentration-dependent manner. The contractile responses were minimally affected by pretreatment of muscle fibers with 10 μM forskolin (Fig. 5B).

As shown in Fig. 5C, treatment of muscle with 0.1 mM GTP potentiated the contractions induced by 0.3 μM Ca^{2+}. Forskolin (10 μM) inhibited the 0.3 μM Ca^{2+}-evoked contractions in the presence of 0.1 mM GTP.

DISCUSSION

In the present study, we compared the inhibitory effects of forskolin on the contractions induced by high K^+ and histamine in guinea-pig ileum. The results indicate that forskolin inhibited the increase in $[Ca^{2+}]_i$ and muscle tension induced spontaneously or by stimulation with histamine. Forskolin inhibited high K^+-evoked contractions without or with only a small inhibition in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$, which was not inhibited by forskolin, was reduced by verapamil to the basal level, suggesting that this $[Ca^{2+}]_i$ is due to Ca^{2+} entry from voltage-dependent Ca^{2+} channels. Furthermore, the inhibitory effect of forskolin was attenuated when the K^+ concentration was increased. Histamine-induced contractions were more sensitive to forskolin than that induced

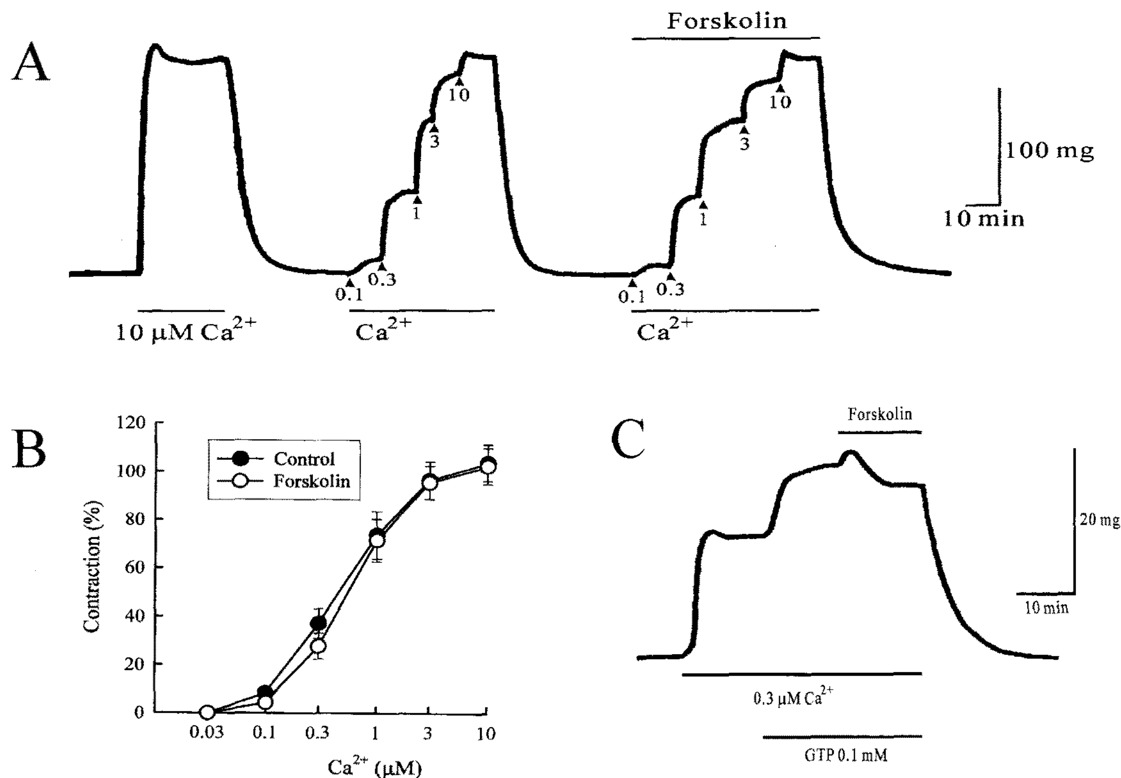

Fig. 5. (A) Effect of 10 μM forskolin on the contraction evoked by Ca^{2+} in α-toxin-permeabilized ileum. Ca^{2+} was cumulatively applied. The experiments were repeated after application of 10 μM forskolin. As a control, 10 μM Ca^{2+} was applied. (B) The pCa^{2+}-tension relationship observed before (●) and after application of 10 μM forskolin (○). The amplitude of 10 μM Ca^{2+} was taken as 100%. Each point represents the mean of 6 experiments and the SE mean is shown by a vertical bar. (C) Effects of 10 μM forskolin treated with 0.1 mM GTP. After permebilizing the tissues, 0.3 μM Ca^{2+} was applied. After the contraction induced by 0.3 μM Ca^{2+} had reached a steady level, 0.1 mM GTP and forskolin were sequentially applied.

by high K^+-induced contractions. The effects of forskolin on the $[Ca^{2+}]_i$-tension relationship in the presence of histamine showed that the inhibitory effect is mainly due to the decrease in $[Ca^{2+}]_i$, although this was not the case with the high K^+-evoked contractions.

The decrease in $[Ca^{2+}]_i$ may be due to the following: 1) inhibition of Ca^{2+} influx, 2) inhibition of Ca^{2+} release from sarcoplasmic reticulum, and 3) activation of Ca^{2+} sequestration into intracellular stores. In smooth muscles, forskolin may decrease $[Ca^{2+}]_i$ by the following two mechanisms: 1) direct inhibition of voltage-dependent Ca^{2+} channels and 2) indirect inhibition of Ca^{2+} channels following the activation of voltage- or Ca^{2+}-dependent K^+ channels. The first possibility may be less likely since forskolin did not reduce high K^+-induced increases in $[Ca^{2+}]_i$, which is readily inhibited by L-type Ca^{2+} channel blockers (Karaki and Weiss, 1984). A probable site of action is K^+ channels; indeed, an increase in K^+ current hyperpolarizes the membrane. Higher concentrations of K^+ may attenuate the membrane hyperpolarization due to opening K^+ channels, and also by decreasing the transmembrane K^+ gradient. This may be the reason why the inhibitory effects of forskolin on $[Ca^{2+}]_i$ were less in the presence of high K^+. Forskolin inhibited the increases in $[Ca^{2+}]_i$ and muscle tension induced spontaneously or by stimulation with histamine and this may be due to the inhibition of pacemaker activity by membrane hyperpolarization and/or the inhibition of Ca^{2+} influx by the indirect inhibition of voltage-dependent Ca^{2+} channels. Rembold and Chen (1998) reported that membrane hyperpolarization is involved in the inhibitory effect of forskolin. Forskolin significantly increased the cyclic AMP content, suggesting that the inhibitory effect of forskolin is mainly mediated by cyclic AMP (Lincoln and Cornwell, 1991; Kwon et al., 1993). It has also been reported that Ca^{2+}-activated K^+ channels may be modulated by cAMP in various tissues, including vascular and visceral smooth muscle (Wellman et al., 2001; Ise et al., 2003). It is therefore possible that the increase in cAMP production in response to forskolin might activate K^+ channels via protein kinase A-mediated protein phosphorylation.

The second possibility for the forskolin-induced decrease in histamine-stimulated $[Ca^{2+}]_i$ is the cAMP-mediated inhibition of Ca^{2+} release from intracellular stores. It is known that cAMP can regulate $[Ca^{2+}]_i$ mobilization from sarcoplasmic reticulum in a number of ways, including phosphorylation of IP_3 and ryanodine receptors, and modulating the accumulation of Ca^{2+} in the sarcoplasmic reticulum. The inhibition by forskolin and dibutyryl cyclic AMP of phosphatidyl-inositol hydrolysis has been demonstrated in tracheal and vascular smooth muscles (Hall et al., 1989; Ahn et al., 1992). Previous reports have also suggested that elevation of cAMP leads to an increase in sarcoplasmic reticulum Ca^{2+} content and/or the probability of open ryanodine receptor channels (Wellman et al., 2001; Morales et al., 2004).

It is also possible that the reduction in $[Ca^{2+}]_i$ caused by forskolin could be related to an enhancement in Ca^{2+} uptake into sarcoplasmic reticulum (Bulbring and Tomita, 1987). cAMP has been reported to increase Ca^{2+} uptake or binding by microsomal fractions from intestinal smooth muscle (Andersson and Nilson, 1977), blood vessels (Bhalla et al., 1978), and uterine tissue (Nishikori and Maeno, 1979). However, this mechanism may not play a major role because forskolin showed little effect on muscles depolarized with high K^+. In support of this hypothesis, forskolin does not change the membrane Ca^{2+} pump activity in cultured vascular smooth muscle (Fukukawa et al., 1988).

Smooth muscle tone is regulated not only by levels of $[Ca^{2+}]_i$, but also by the Ca^{2+} sensitivity of the contractile elements (Somlyo et al., 1999). Therefore, the inhibitory effects of forskolin do not appear to be limited to a decrease in $[Ca^{2+}]_i$. Forskolin inhibited high K^+-induced contractions with little decrease in $[Ca^{2+}]_i$, suggesting that forskolin decreased the Ca^{2+} sensitivity of contractile elements to Ca^{2+}. In premeabilized ileum, forskolin did not affect the pCa^{2+}-tension relationship in the absence of GTP, although it decreased Ca^{2+} sensitivity in the presence of GTP, indicating that GTP is required for this action, likely so as to serve as the substrate of adenylate cyclase. In vascular and tracheal smooth muscles, elevation of cAMP inhibits Ca^{2+}-induced contraction by the decrease in Ca^{2+} sensitivity of contractile elements (Pfitzer et al., 1984; Nishimura and van Breemen, 1989; Rembold and Chen, 1989). On the other hand, the $[Ca^{2+}]_i$-tension relationship in the presence of histamine showed that the inhibitory effect of forskolin was mainly due to the decrease in $[Ca^{2+}]_i$. Ozaki et al. (2002) reported that inhibitors of voltage-dependent Ca^{2+} channels, such as verapamil and nifedipine, completely inhibit agonist-induced contraction in the guinea-pig ileum, suggesting that histamine-induced contraction is mediated by Ca^{2+} influx through voltage-dependent Ca^{2+} channels. This result suggests that inhibition of L-type Ca^{2+} channels by forskolin in histamine-induced contractions could be a mechanism to induce relaxation.

In summary, we demonstrated that forskolin, through cyclic AMP-dependent signaling pathways, inhibits smooth muscle contraction by decreasing $[Ca^{2+}]_i$, resulting from, inhibition of Ca^{2+} influx and Ca^{2+} sensitivity in guinea pig ileum.

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