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SQ22536 and W-7 inhibit forskolin-induced cAMP elevation but not relaxation in newborn ovine pulmonary veins

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Abstract

The role of cAMP in forskolin-induced relaxation was studied in isolated pulmonary veins of newborn lambs (7–12 days). In vessels preconstricted with endothelin-1, forskolin at concentrations $\leq 10^{-7}$ M had no effect on cAMP content and adenylyl cyclase activity but caused up to 50% relaxation. At higher concentrations, forskolin markedly elevated cAMP content and adenylyl cyclase activity and caused a further relaxation. SQ22536 [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; an adenylyl cyclase inhibitor] and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide; a calmodulin-dependent adenylyl cyclase inhibitor] had no significant effect on forskolin-induced relaxation but markedly inhibited the elevation of cAMP content and adenylyl cyclase activity caused by forskolin. Rp-8-CPT-cAMPS [8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate; an inhibitor of cAMP-dependent protein kinases] and Rp-8-Br-PET-cGMPS (β-phenyl-1, N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate; an inhibitor of cGMP-dependent protein kinases) attenuated the relaxation caused by a cAMP analog but not that caused by forskolin. These results suggest that cAMP may not play a major role in forskolin-induced relaxation of pulmonary veins of newborn lambs. © 2001 Published by Elsevier Science B.V.

Keywords: cAMP; Adenylyl cyclase; Smooth muscle, Vascular; Pulmonary vein

1. Introduction

Forskolin is a diterpene which directly activates the catalytic subunit of adenylyl cyclases (Seamon et al., 1981). It causes marked increases in cAMP content and potent relaxation of various smooth muscle types (Murray, 1990). It is thought that the elevation in cAMP content is the major factor leading to smooth muscle relaxation. Such a causal relationship, however, has not been well established.

If forskolin-induced relaxation is mediated mainly by cAMP, an inhibition of adenylyl cyclase activity would reduce the cAMP content and attenuate the relaxation. This has so far not been determined and therefore was investigated in the present study. SQ22536 [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; an adenylyl cyclase inhibitor] (Haslam et al., 1978) and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide; a calmodulin-dependent adenylyl cyclase inhibitor] (Ahlijanian and Cooper, 1987) were employed to inhibit the activity of adenylyl cyclases. The response induced by forskolin was examined using isolated pulmonary veins of newborn lambs. Our previous studies show that forskolin is a potent dilator of this vessel type (Gao et al., 1998).

2. Materials and methods

2.1. Pulmonary vein preparations

Twenty-one newborn lambs (7–12 days old, either sex) from Nebeker Ranch (Lancaster, CA, USA) were used. They were anesthetized with ketamine hydrochloride (30 mg/kg, i.m.) and then sacrificed with an overdose of pentobarbital (100 mg/kg, i.v.). The method used to sacrifice the new born lambs and study protocols were reviewed and approved by the Harbor-UCLA Animal Care and Use Review Committee.

The lungs were immediately removed and fourth generation pulmonary veins (outside diameter: 1.5–2.5 mm) were dissected free of parenchyma and cut into rings (length: 5 mm). To eliminate the involvement of endoge-

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nous nitric oxide, the endothelium was removed by gently rubbing the luminal surface with the tips of a watchmaker's forceps. Removal of endothelium was confirmed by lack of relaxation to 3×10^{-5} M acetylcholine (Gao et al., 1995).

2.2. Vessel tension study

Vessel rings were suspended in organ chambers filled with 10 ml of modified Krebs–Ringer bicarbonate solution [composition (in mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.1] maintained at 37 ± 0.5°C and aerated with 95% O_2–5% CO_2 (pH = 7.4). Each ring was suspended by two stirrups passed through the lumen. One stirrup was anchored to the bottom of the organ chamber; the other one connected to a strain gauge (model FT03C, Grass Instrument, Braintree, MA, USA) for the measurement of isometric force (Gao et al., 1995).

At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension. This was achieved by step-wise stretching in 0.1-g increment until active contraction of the vessel ring to 100 mM KCl reached a plateau. The optimal resting tension of pulmonary veins was 0.28 ± 0.03 g ($n = 19$).

After the vessels were brought to their optimal resting tension, 1 h of equilibration was allowed. Indomethacin [10^{-5} M; an inhibitor of cyclooxygenase (Vane, 1978)] was then administered to exclude the possible involvement of endogenous prostanoids (Gao et al., 1996). Indomethacin caused an increase in resting tension by 0.51 $\pm$ 0.11 g ($n = 6$) in these veins.

Effect of forskolin was determined in vessels preconstricted with endothelin-1 (3×10^{-9} M to 2×10^{-8} M). Experiments were performed under control conditions and in the presence of SQ22536 [3×10^{-4} M; an inhibitor of adenylyl cyclases) (Haslam et al., 1978)] or W-7 [3×10^{-4} M; an inhibitor of Ca^{2+}-calmodulin-dependent adenylyl cyclases (Ahlijanian and Cooper, 1987)]. All experiments were performed in a parallel manner. For each vessel ring, only one concentration–response curve to forskolin was tested.

2.3. Cyclic AMP assay

Vessel rings of pulmonary veins without endothelium were incubated in modified Krebs–Ringer bicarbonate solution (37°C, 95% O_2 and 5% CO_2, pH 7.4) in the presence of indomethacin (10^{-5} M) to prevent the interference of endogenous prostanoids (Gao et al., 1996).

After 1 h of equilibration, SQ22536 (3×10^{-4} M), W-7 (3×10^{-4} M), or control solvent was added. Thirty minutes later, endothelin-1 (2×10^{-8} M for W-7 group and 3×10^{-9} M for others) was given. Endothelin-1 was added to make the conditions for cAMP assay similar to those of the vessel tension studies where endothelin was used to preconstrict the vessels. Concentrations of endothelin-1 used were similar to those in vessel tension study. Thirty minutes after endothelin-1 was given, forskolin (10^{-9} M to 10^{-5} M) was added. Vessel rings were rapidly frozen in liquid nitrogen at various time points after the administration of different concentrations of forskolin. Frozen vessels were thawed in trichloroacetic acid (6%), homogenized in glass with a motor-driven teflon pestle, sonicated for 5 s, and centrifuged at $13{,}000 \times g$ for 15 min. The supernatant was extracted with four volumes of water-saturated diethyl ether and lyophilized. The lyophilized samples were resuspended in 0.5 ml of sodium acetate buffer (0.05 M, pH 6.2) and the content of cAMP was determined using a cAMP radioimmunoassay kit (Biomedical Technologies, Stoughton, MA, USA). Cyclic AMP content is expressed as pmol/mg protein of the tissues. Protein content was determined by Bradford method using bovine serum albumin as the standard (Bradford, 1973).

2.4. Adenylyl cyclase assay

Adenylyl cyclase activity was determined as previously described (Gao et al., 1998). Briefly, pulmonary veins were homogenized in 5 volumes of ice-cold Tris/HCl buffer (50 mM, pH 7.6) containing dithiothreitol (2 mM), EDTA (5 mM), phenymethylsulfonyl fluoride (1 mM), antipain (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin (10 μg/ml). The homogenate was then centrifuged at $500 \times g$ for 10 min to remove the unbroken cells and cell nuclei. The protein concentration of the homogenate was determined by Bradford method using bovine serum albumin as the standard (Bradford, 1973).

Pulmonary vessel homogenate (30 μg protein) was incubated at 30°C for 10 min in 50 mM Tris HCl (pH 7.4), dithiothreitol (0.1 mM), ATP (1 mM), $MgCl_2$ (10 mM), creatine phosphate (20 mM), creatine phosphokinase (150 U/ml), ascorbic acid (0.5 mM), and isobutylmethylxanthine (1 mM) in the presence or absence of forskolin (10^{-7} to 10^{-6} M). In some vials, SQ22536 (3×10^{-4} M) or W-7 (3×10^{-4} M) was included. The total incubation volume was 150 μl. The reaction was terminated by placing the assay tubes in a boiling water bath for 5 min. The samples were then centrifuged at $13{,}000 \times g$ for 15 min. The supernatant was taken for cAMP measurement using a radioimmunoassay kit (Biomedical Technologies, Stoughton, MA, USA). Adenylyl cyclase activity is expressed as pmol cAMP/min/mg protein. Preliminary experiments confirmed the linearity of adenylyl cyclase activity at the protein concentrations and incubation times mentioned above.

2.5. Drugs

The following drugs were used (unless otherwise specified, all were obtained from Sigma, St. Louis, MO, USA):

L-ascorbic acid, 8-Br-cAMP (8-bromoadenosine-3′,5′-cyclic monophosphate, Rp-8-CPT-cAMPS [8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; Biolog Life Science Institute, La Jolla, CA, USA), Rp-8-Br-PET-cGMPS (β-phenyl-1, N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; Biolog Life Science Institute), endothelin-1 (American Peptide, Sunnyvale, CA, USA), indomethacin, forskolin bitartrate salt, SQ22536 [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine], and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide HCl; Biomol Research Laboratories, Plymouth Meeting, PA, USA].

W-7 was dissolved in 50% ethanol/water (final concentration of ethanol, 0.9%). SQ22536 was dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO, 0.18%). Preliminary experiments indicated that the solvents at these concentrations did not significantly affect contraction of the tissue to endothelin-1 nor the relaxation to forskolin (data not shown). Indomethacin (10^{-5} M) was prepared in equal molar Na_2CO_3. This concentration of Na_2CO_3 did not significantly affect the pH of the solution in the organ chamber. Vessels were exposed to inhibitors and antagonists for at least 30 min prior to testing their effects.

2.6. Data analyses

Data are shown as means ± S.E.M. When mean values of two groups were compared, Student's *t* test for unpaired observations was used. When the mean values of the same group before and after stimulation were compared, Student's *t* test for paired observations was used. Comparison of mean values of more than two groups from same vessel type was performed with one-way ANOVA test with Student–Newman–Keuls test for post hoc testing of multiple comparison. All these analyses were performed using a commercially available statistics package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). Statistical significance was accepted when the *P* value (two-tailed) was less than 0.05. In all experiments, *n* represents the number of animals.

3. Results

3.1. Effects of forskolin on vessel tension and cAMP

In pulmonary veins preconstricted with endothelin-1 [3×10^{-9} M; tension, 1.97 ± 0.28 g ($n = 6$)], forskolin induced a concentration-dependent relaxation. At concentrations $\leq 3 \times 10^{-7}$ M, forskolin reduced vessel tension by up to $75.3 \pm 7.6\%$ ($n = 6$) without an effect on the intracellular content of cAMP. The basal level of cAMP of pulmonary veins was 16.6 ± 3.1 pmol/mg protein, which was not affected by endothelin-1 (3×10^{-9} M; data not shown, $n = 6$, $P > 0.05$). In the presence of endothelin-1, forskolin at $\geq 10^{-6}$ M caused a marked increase in cAMP and a further moderate reduction in tension (Fig. 1).

The time-course relationship for relaxation and cAMP accumulation evoked by forskolin was also examined. One minute after the administration of forskolin (10^{-6} M), vessel tension was reduced by $27.8 \pm 4.3\%$ ($n = 5$) without a significant change in cAMP content. At 2 min, vessel tension was reduced by $64.9 \pm 5.1\%$ ($n = 5$) accompanied by a moderate increase in cAMP (4.4 ± 2.1 pmol/mg protein; $n = 5$). Maximal changes in tension and cAMP evoked by forskolin were obtained at about 5 min (Fig. 2).

3.2. Effects of SQ22536 and W-7 on forskolin-induced relaxation

In vessels treated with SQ22536 (3×10^{-4} M) and W-7 (3×10^{-4} M) relaxation induced by forskolin was not significantly different from control (Fig. 3). SQ22536 (3×10^{-4} M) had no significant effect on basal tension nor contraction of the veins to endothelin-1 (3×10^{-9} M) (data not shown, $n = 6$ for each group, $P > 0.05$). W-7 (3×10^{-4} M) reduced basal tension of the veins by 0.55 ± 0.07 g ($n = 6$, $P < 0.05$). In the presence of W-7, a higher concentration of endothelin-1 (2×10^{-8} M) was used so as to raise the vessel tension to a level comparable to that in control vessels (1.90 ± 0.23 g vs. 1.94 ± 0.28; $n = 6$ for each group, $P > 0.05$).

3.3. Effects of SQ22536 and W-7 on forskolin-induced cAMP elevation

In pulmonary veins treated with SQ22536 (3×10^{-4} M) and W-7 (3×10^{-4} M), forskolin (10^{-6} M) caused no significant change in cAMP level (Fig. 4). Basal cAMP content was not affected by SQ22536 (3×10^{-4} M) but

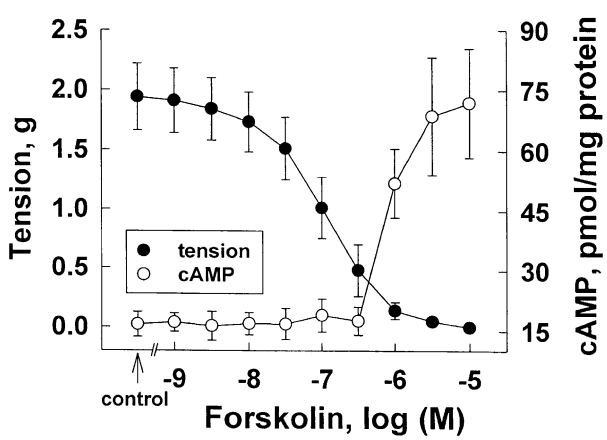

Fig. 1. Concentration-dependent effects of forskolin on tension and the intracellular content of cAMP of isolated ovine newborn pulmonary veins preconstricted with endothelin-1 (3×10^{-9} M). Vessel tension and the intracellular cAMP content were determined 5 min after the administration of forskolin. Data are shown as means ± S.E.M.; $n = 6$ for each group.

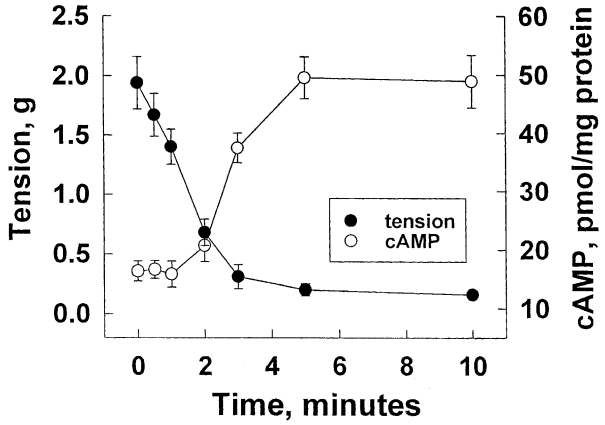

Fig. 2. The time-course relationships for relaxation and cAMP accumulation caused by forskolin (10^{-6} M). Vessels were preconstricted with endothelin-1 (3×10^{-9} M). Data are shown as means ± S.E.M.; $n = 5$ for each group.

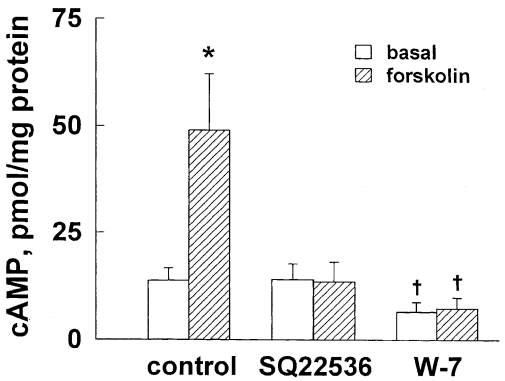

Fig. 4. Effect of forskolin (10^{-6} M, 5 min) on the intracellular content of cAMP of isolated ovine newborn pulmonary veins under control conditions and in the presence of SQ22536 (3×10^{-4} M) and W-7 (3×10^{-4} M). Assay was performed in the presence of endothelin-1 (2×10^{-8} M for W-7 group and 3×10^{-9} M for others). Data are shown as means ± S.E.M.; $n = 6$ for each group. * Significantly different from basal; †Significantly different from control ($P < 0.05$).

was significantly reduced by W-7 (3×10^{-4} M)(Fig. 4). The effect of these inhibitors was determined in the presence of endothelin-1 (2×10^{-8} M for W-7 group and 3×10^{-9} M for the others). Endothelin-1 had no significant effect on the basal cAMP content (data not shown; $n = 6$ for each group, $P > 0.05$).

3.4. Effects of SQ22536 and W-7 on forskolin-induced increase in adenylyl cyclase activity

In the crude membrane preparations of pulmonary veins, the basal activity of adenylyl cyclases was 12.5 ± 1.7 pmol/min/mg protein ($n = 5$). In the presence of SQ22536 (3×10^{-4} M) and W-7 (3×10^{-4} M), the adenylyl cyclase activity was reduced to 6.5 ± 0.9 pmol/min/mg protein ($n = 5$) and 6.8 ± 1.2 pmol/min/mg protein ($n = 5$), respectively ($P < 0.05$).

Forskolin at concentrations $\geq 3 \times 10^{-7}$ M caused a significant increase in adenylyl cyclase activity. Such an effect was largely eliminated by treatment with SQ22536 (3×10^{-4} M) or W-7 (3×10^{-4} M) (Fig. 5).

3.5. Effects of Rp-8-CPT-cAMPS and Rp-8-Br-PET-cGMPS on forskolin-induced relaxation

Cyclic AMP may cause vasodilation via cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) (Jiang et al., 1992; Dhanakoti et al., 2000). In vessel tension study, relaxation of pulmonary veins induced by 8-Br-cAMP [3×10^{-4} M; a cell membrane permeable analog of cAMP (Meyer and Miller, 1974)] was attenuated by Rp-8-CPT-cAMPS (3×10^{-5} M) and Rp-8-Br-PET-cGMPS (3×10^{-5} M), selective inhibitors of PKA and PKG, respectively (Butt et al., 1995; Gjertsen et al.,

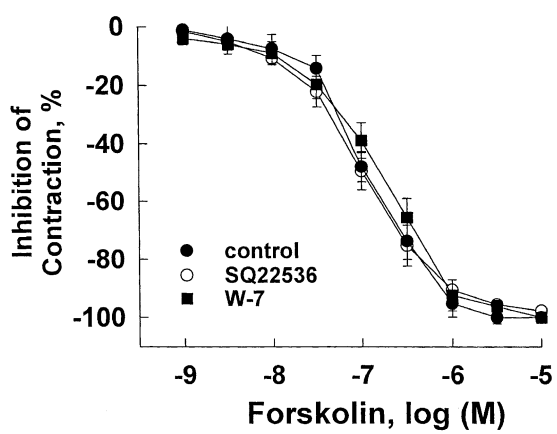

Fig. 3. Effects of SQ22536 (3×10^{-4} M) and W-7 (3×10^{-4} M) on relaxation of isolated ovine newborn pulmonary veins induced by forskolin. Vessels were preconstricted with endothelin-1 (2×10^{-8} M for W-7 group and 3×10^{-9} M for the others) to a similar tension level. Data are shown as means ± S.E.M.; $n = 6$ for each group.

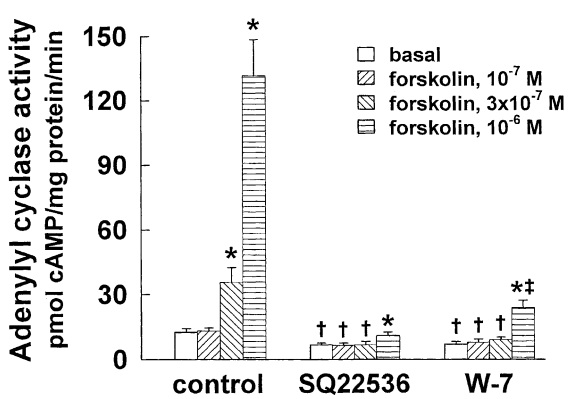

Fig. 5. Effect of forskolin on adenylyl cyclase activity of crude membrane preparations of isolated ovine newborn pulmonary veins under control conditions and in the presence of SQ22536 (3×10^{-4} M) or W-7 (3×10^{-4} M). Data are shown as means ± S.E.M.; $n = 5$ for each group. * Significantly different from basal; †Significantly different from control; ‡Significantly different from those treated with SQ22536 ($P < 0.05$).

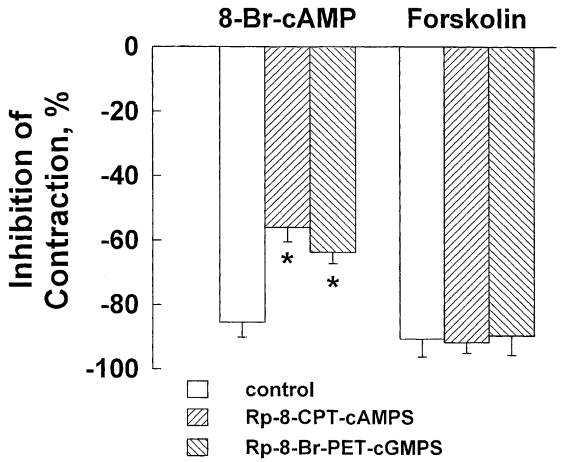

Fig. 6. Effect of Rp-8-CPT-cAMP (3×10^{-5} M) and Rp-8-Br-PET-cGMPS (3×10^{-5} M) on relaxation of isolated ovine newborn pulmonary veins induced by 8-Br-cAMP (3×10^{-4} M) and forskolin (10^{-6} M). Vessels were preconstricted with endothelin-1 (3×10^{-9} M) to a similar tension level. Data are shown as means ± S.E.M.; $n=6$ for each group. * Significantly different from control ($P<0.05$).

1995). However, these inhibitors had no significant effect on relaxation caused by forskolin (10^{-6} M) (Fig. 6). These experiments were performed in vessels preconstricted with endothelin-1 (3×10^{-9} M) to a comparable tension level (1.76 ± 0.19 g to 1.87 ± 0.21; $n=6$ for each group, $P>0.05$). Rp-8-CPT-cAMPS (3×10^{-5} M) and Rp-8-Br-PET-cGMPS (3×10^{-5} M) had no significant effects on the basal tension and endothelin-induced contraction of the vessels (data not shown, $n=6$ for each group, $P>0.05$).

4. Discussion

In the present study, forskolin at lower concentrations ($\leq10^{-7}$ M) reduced tension of pulmonary veins of newborn lambs by up to about 50% without affecting cAMP level or adenylyl cyclase activity. These observations are in agreement with some other studies, e.g., forskolin at 10^{-7} M causes rabbit aorta to relax with no significant elevation of cAMP (Vegesna and Diamond, 1986). In bovine coronary arteries, forskolin at a concentration which elevates cAMP levels by 5.5-fold has no effect on the vessel tension (Vegesna and Diamond, 1983). Thus, it seems that, at least in certain vessel types, cAMP may not play a large role in relaxation induced by low concentrations of forskolin.

At higher concentrations, forskolin-induced relaxation of pulmonary veins were accompanied by an elevation in cAMP content. However, the relaxation occurred earlier than cAMP elevation. Furthermore, forskolin-induced relaxation was not affected by SQ22536 or W-7 which abolished the elevation in cAMP and largely abolished the increase in adenylyl cyclase activity caused by forskolin. Therefore, in ovine pulmonary veins, a cAMP-dependent mechanism may not play a larger role in forskolin-induced relaxation. In our study, forskolin at 3×10^{-7} M had no significant effect on the cAMP content but caused an increase in adenylyl cyclase activity. In contrast to adenylyl cyclase assay which reveals the direct effect of forskolin on the activity of the enzymes, the cAMP content we measured reflects the net effect of cAMP production by adenylyl cyclases and cAMP degradation by phosphodiesterases (Murray, 1990). We did not use an inhibitor of phosphodiesterases to artificially augment intracellular cAMP content as it would mask the true effect of forskolin on cAMP levels under physiological conditions.

Two agents were used to inhibit adenylyl cyclases. Among them SQ22536 is a specific inhibitor of the enzymes (Haslam et al., 1978) while W-7 is a calmodulin antagonist which inhibits Ca^{2+}-calmodulin-dependent isoforms of adenylyl cyclases (Ahlijanian and Cooper, 1987). In mammalian cells, adenylyl cyclases consist of at least 10 isoforms (Sunahara et al., 1996). Some isoforms are stimulated by Ca^{2+}-calmodulin (Mons et al., 1998) and inhibited by calmodulin antagonists such as W-7 (Ahlijanian and Cooper, 1987). Since the increase in cAMP content and in adenylyl cyclase activity caused by forskolin was largely inhibited not only by SQ22536 but also by W-7, it appears that the isoforms of adenylyl cyclases in pulmonary veins of the newborn lamb are predominantly Ca^{2+}-calmodulin-dependent (Sunahara et al., 1996).

Cyclic AMP may cause smooth muscle relaxation via cAMP- and cGMP-dependent protein kinases (Jiang et al., 1992; Dhanakoti et al., 2000). In the present study, 8-Br-cAMP—but not forskolin-induced relaxation—was attenuated by inhibitors of PKA and PKG [Rp-8-CPT-cAMPS and Rp-8-Br-PET-cGMPS, respectively (Butt et al., 1995; Gjertsen et al., 1995)]. These data provide further evidence against a major role for cAMP in forskolin-induced relaxation of pulmonary veins of newborn lambs. Such a mechanism seems contradictory to the fact that this diterpene causes a marked increase in cAMP content. However, in our study, a large portion of the relaxation caused by forskolin occurred at concentrations that did not elevate cAMP levels. Furthermore, the components of cAMP pathway exist in distinct subcellular compartments such that cAMP generated by certain stimuli may not have access to enzymes which are involved in tension modulation (Zhou et al., 1992). This would explain why SQ22536 and W-7 abolished cAMP elevation but not the relaxation caused by forskolin. It has been recently shown that, in guinea-pig aortas, SQ22536 eliminated the elevation of cAMP induced by an iloprost (a prostaglandin I_2 analog) but had no effect on relaxation induced by this agent (Turcato and Clapp, 1999).

There are few studies on cAMP-independent relaxation of smooth muscle induced by forskolin. In rat pulmonary arteries, forskolin-induced relaxation was inhibited by 4-aminopyridine and by decreasing extracellular Cl^- concentration, suggesting that forskolin may cause vasodilation

by acting on voltage-dependent K^+ channels and Cl^- channels (Zhao et al., 1998). This study did not explore whether or not the effects of forskolin are cAMP-independent (Zhao et al., 1998). However, in many cell types forskolin can act on Ca^{2+} and K^+ channels in a cAMP-independent manner (Watanabe and Gola, 1987; Hoshi et al., 1988; Harris-Warrick, 1989; Park and Kim, 1996; Zerr et al., 1996; Gandia et al., 1997).

In conclusion, the present study demonstrates that cAMP does not play a major role in forskolin-induced relaxation of ovine newborn pulmonary veins. Forskolin has been used extensively to elicit cAMP-dependent physiological responses (Daly, 1984; Seamon and Daly, 1986; Murray, 1990). However, depending on the concentrations of forskolin, the subcellular locations of cAMP accumulation, and the heterogeneity of the cell types being studied, cAMP may not be an obligatory mediator in forskolin-induced relaxation.

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