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Forskolin Reverses Tachyphylaxis to the Bronchodilator Effects of Salbutamol: An In-vitro Study on Isolated Guinea-pig Trachea

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Abstract

The objective of this study was to assess the relaxant responses of salbutamol, a β_2 agonist, and forskolin, an activator of adenylate cyclase, and the possible role of forskolin in reversing tachyphylaxis to salbutamol.

The in-vitro bronchodilator effects of salbutamol and forskolin (10^{-9}–10^{-5} M) were tested on isolated guinea-pig tracheal rings precontracted with carbachol (10^{-7} M). Both salbutamol and forskolin elicited concentration-dependent relaxation. Potency (EC50; the dose resulting in 50% relaxation) was determined from cumulative concentration–response curves. Salbutamol was more potent than forskolin in relaxing the tracheal preparations (−log molar EC50 $7{\cdot}68 \pm 0{\cdot}14$ and $6{\cdot}3 \pm 0{\cdot}17$, respectively). Reproducible relaxant responses to salbutamol could be elicited after 24 h incubation in Krebs solution. Tachyphylaxis to the relaxant effects of salbutamol was experimentally induced by incubation (24 h) of the preparations in Krebs solution containing salbutamol (10^{-6}, 3×10^{-6} or 10^{-5} M). This pretreatment of the tissues resulted in a significant reduction in the potency of salbutamol. The potency of salbutamol was reduced to $6{\cdot}85 \pm 0{\cdot}2$, $6{\cdot}8 \pm 0{\cdot}1$ and $5{\cdot}9 \pm 0{\cdot}27$ after 24 h incubation with salbutamol 10^{-6}, 3×10^{-6} or 10^{-5}M, respectively. The potency of salbutamol was increased from $7{\cdot}35 \pm 0{\cdot}2$ to $7{\cdot}76 \pm 0{\cdot}28$ by addition of forskolin (3×10^{-7} M) under control conditions. Moreover, forskolin (3×10^{-7} M) reversed the development of tachyphylaxis to salbutamol-induced relaxation in tissues pretreated with salbutamol. The potency of salbutamol was increased to $7{\cdot}29 \pm 0{\cdot}41$, $7{\cdot}37 \pm 0{\cdot}17$ and $7{\cdot}23 \pm 0{\cdot}35$ after the addition of forskolin (3×10^{-7} M) to preparations pre-incubated (24 h) with salbutamol 10^{-6}, 3×10^{-6} or 10^{-5} M respectively.

These results show that in guinea-pig tracheal ring preparations, forskolin shares with salbutamol the ability to relax airway smooth muscle and produces an apparent reversal of tachyphylaxis to the bronchodilator effects of salbutamol, particularly in the low concentration range. This effect could provide an alternative therapy for long term use, particularly with high doses of β_2 agonists in bronchial asthma.

Forskolin, a diterpene isolated from the roots of *Coleus forskohlii*, activates adenylate cyclase by direct stimulation, by-passing adrenergic receptors on the cell surface (Seamon et al 1981). Forskolin does not interact with β-adrenergic receptors, neither does it inhibit the breakdown of cyclic AMP (Christenson et al 1991). Forskolin is believed to activate adenylate cyclase by direct stimulation of the catalytic component because forskolin stimulates cyclase activity in mutant cells which lack a functional guanine nucleotide regulatory protein (Darfler et al 1982; Brooker et al 1983) and in membrane cyclase preparations devoid of hormone receptors or a regulatory subunit (Metzger & Lindner 1981).

Tachyphylaxis to the bronchodilator effects of β_2 agonists has been reported in normal and asthmatic patients (Harvey & Tattersfield 1982; Larsson et al 1992). It has been suggested that the development of tachyphylaxis or tolerance to the bronchodilator effects of β_2 agonists after long-term use might predispose patients to use excessive amounts of rescue medication in the event of an acute attack (Sears & Taylor 1994). The development of tachyphylaxis is independent of the density of the innervation, endothelial cell function and synthesis

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of and response to a particular second messenger. Rather, it is intimately dependent on type (or subtype) of receptors (Miao & Lee 1992).

Activation of adenylate cyclase seems to be an integral link in the mediation of the physiological response to β-adrenergic agonists (Lefkowitz et al 1982; Abramson & Molinoff 1984). The aim of this study was to investigate how forskolin interacts with salbutamol in isolated guinea-pig preparations under tachyphylaxis conditions. The effectiveness of forskolin in reversing tachyphylaxis to salbutamol was also examined.

Materials and Methods

The study was performed on mature male guinea-pigs, 5 months, 800 g (approx.). The animals were killed by decapitation under light ether anaesthesia. The tracheas were isolated, excess connective tissue was removed, and the tracheas were cut into rings 4 mm in length. The preparations were mounted in organ-baths containing 25 mL Krebs-Henseleit solution at pH 7·4. A single tracheal ring was suspended in each organ-bath. The tissue bath solution was maintained at 37°C and was oxygenated with 95% O_2–5% CO_2. Isometric tension was recorded on a Lectromed UFI-dynamometer and recorder system. The preparations were left for 45 min with the Krebs solution being changed at 15-min intervals, thereafter a pre-tension of 1·5 g was applied. The preparations were left for a stabilization period of 45 min until stable baseline tone was obtained.

Drugs and chemicals

The composition of the Krebs-Henseleit solution was (mM): NaCl (118·3), KCl (4·7), $CaCl_2$ (2·5), $MgSO_4$ (1·2), $NaHCO_3$ (25), KH_2PO_4 (1·2), glucose (11·2). Salbutamol hydrochloride and carbachol were from Sigma (St Louis, MO). Forskolin was a gift from Hoechst, Germany. Forskolin was dissolved in dimethylsulphoxide; salbutamol and carbachol were dissolved in distilled water. Dilutions of forskolin were prepared in distilled water.

In all experiments the final concentration of the solvent in the organ-baths did not exceed 0·1%, which did not have any effect on tissue responses (tested in preliminary experiments).

Effects of salbutamol and forskolin

The guinea-pig tracheal preparations were precontracted with a submaximum concentration of carbachol (10^{-7} M), before cumulative concentration–response curves for salbutamol or forskolin (10^{-9}–3×10^{-5} M) were established. The contact time of carbachol was 15 min, enough to produce a steady-state level of contraction.

The possible synergistic effect of forskolin on salbutamol-induced relaxant effects was also examined. Potentiation of salbutamol-induced relaxation was tested by pretreatment of the tissues with forskolin as described by Satake & Shibata (1997). In our study, the preparations were precontracted with carbachol (10^{-7} M) followed by forskolin (3×10^{-7} M) added to the tissue bath for 20 min before a cumulative dose–response curve for salbutamol (10^{-9}–10^{-6} M) was established.

Induction of tachyphylaxis

Tachyphylaxis to salbutamol was induced in the isolated tracheal preparations according to the method described by Ljusegren et al (1988), with modifications in the period of incubation and the concentration of drug in the incubation medium. Segments of the isolated and cleaned trachea were incubated in oxygenated Krebs solution containing different concentrations of salbutamol (10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6} or 10^{-5} M) for 24 h, control specimens incubated with drug-free oxygenated Krebs solution were always run in parallel. All the tissues were kept at 4°C in oxygenated incubation medium. After termination of the incubation period, the preparations were mounted in the organ-baths, as described previously, and were washed with Krebs solution every 10 min for 1 h. Thereafter, the tracheal segments were precontracted with carbachol (10^{-7} M) and cumulative concentration–response curves for salbutamol (10^{-9}–3×10^{-6} M) were established. The effect of forskolin (3×10^{-7} M) on the bronchodilator effect of salbutamol was tested as described in the previous section.

Statistical analysis

Dose–response curves were analysed using GraphPad Prism software. Non-linear regression analysis was performed on the data obtained in our experiments. Results are presented as means ± standard error of the mean ($n = 4$–10). Standard statistical evaluation of the data was performed with Student's *t*-test; a value of $P < 0.05$, was considered to be indicative of significance.

Results

Salbutamol and forskolin (10^{-9}–3×10^{-5} M) induced concentration-dependent relaxation of

guinea-pig tracheal preparations precontracted with carbachol (10^{-7} M) (Figure 1). The EC50 values (doses resulting in 50% relaxation) for the two relaxant agents are shown in Table 1. Salbutamol was found to be more potent than forskolin. To study any synergistic effect of forskolin on salbutamol-induced relaxation, several concentrations of forskolin were tested. Forskolin (3×10^{-7} M) was chosen because at higher concentrations a strong relaxant effect of forskolin abolished the pre-set tone. Therefore, it would not be possible to test the relaxant effects to salbutamol. Pretreatment of the guinea-pig tracheal preparations with forskolin (3×10^{-7} M) did not potentiate the relaxation induced by salbutamol (10^{-9}–3×10^{-6} M) (Figure 2). The potency and maximum relaxant response to salbutamol were not significantly enhanced ($P > 0.05$) (Table 1).

Reproducibility of the bronchodilator effect

Salbutamol (10^{-9}–3×10^{-6} M) elicited concentration-dependent relaxation of guinea-pig tracheal preparations. The relaxant effect of salbutamol was maintained after the incubation period (24 h) in oxygenated Krebs solution (Figure 3). This preparation was therefore used as control for comparison with the effects of salbutamol on preparations incubated in salbutamol-containing solution.

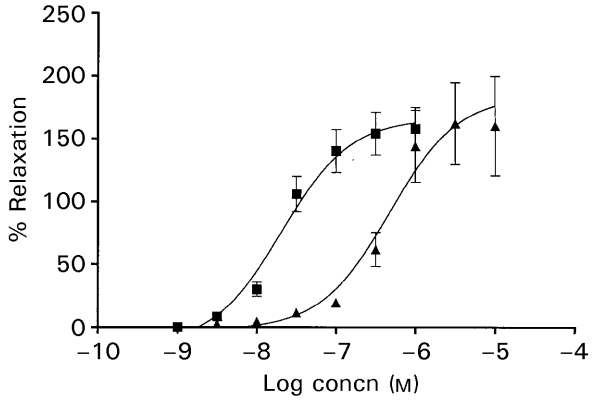

Figure 1. Cumulative dose–response curves for the action of salbutamol (■) and forskolin (▲) (10^{-9}–3×10^{-6} M) on isolated guinea-pig tracheal rings precontracted by carbachol (10^{-7} M) (n = 9–10).

Table 1. Potency and relaxation induced by forskolin and salbutamol determined from cumulative relaxation concentration–response curves obtained from fresh guinea-pig tracheal preparations.

	Potency (EC50, −log M)	Relaxation (%)
Forskolin	6·30 ± 0·17	159·4 ± 29·3
Salbutamol	7·68 ± 0·14***	158·2 ± 17·2
Salbutamol + forskolin (3×10^{-7} M)	7·76 ± 0·28	125·0 ± 15

EC50 = Dose resulting in 50% relaxation. ***$P < 0.001$ compared with result for forskolin alone (n = 9–10).

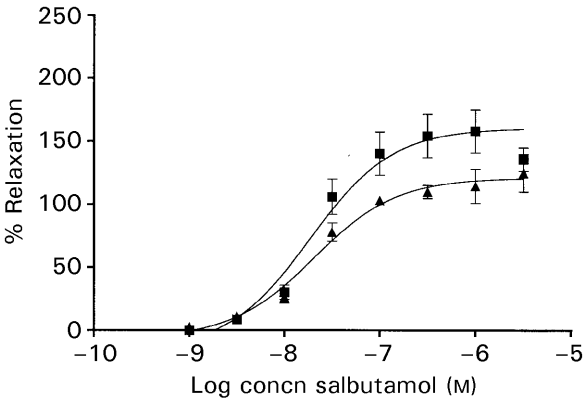

Figure 2. The effect of forskolin 10^{-7} M (▲) on the relaxation induced by salbutamol (10^{-9}–3×10^{-6} M) (■) in guinea-pig tracheal preparations precontracted by carbachol (10^{-7} M) (n = 9).

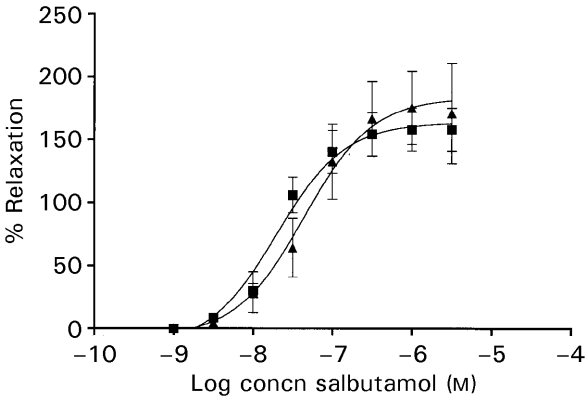

Figure 3. Cumulative dose–response curves for the action of salbutamol (10^{-9}–3×10^{-6} M) on fresh (■) and overnight-incubated preparations (▲) of guinea-pig isolated tracheal rings precontracted by carbachol (10^{-7} M) (n = 8–9).

Development of tachyphylaxis

Salbutamol (10^{-9}–3×10^{-6} M) elicited concentration-dependent relaxation of tissues incubated overnight in salbutamol-containing Krebs solution (3×10^{-8}, 10^{-3}, 3×10^{-7}, 10^{-6}, 3×10^{-6} or 10^{-5} M), or tissues incubated in drug-free medium. Salbutamol at concentrations lower than 10^{-6}M failed to elicit any reduction of salbutamol-induced relaxant response, but there was a significant reduction in preparations incubated with salbutamol 10^{-6}, 3×10^{-6} or 10^{-5} M (Figure 4). The amount of tachyphylaxis as expressed in reduced

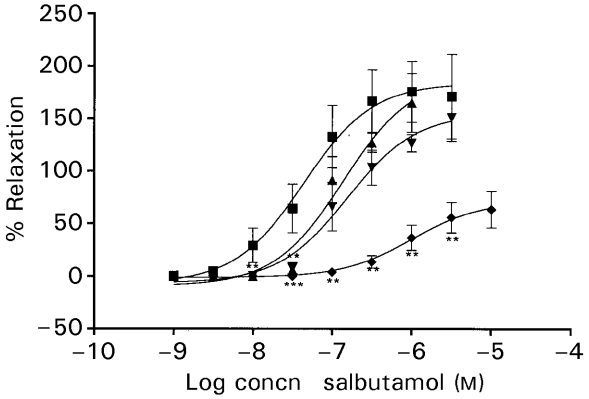

Figure 4. Development of tachyphylaxis to the salbutamol (10^{-9}–10^{-6} M) relaxant response (■) induced by pretreatment with salbutamol (▲, 10^{-6} M; ▼, 3×10^{-6} M; ◆, 10^{-5} M) in guinea-pig tracheal preparations precontracted by carbachol (10^{-7} M). ***$P < 0.001$, **$P < 0.01$, significantly different from the control result (n = 4–8).

Table 2. Potency of salbutamol determined from cumulative relaxation concentration–response curves of control and salbutamol-treated guinea-pig tracheal preparations before and after addition of forskolin (3×10^{-7} M).

	Potency (EC50, −log M)	
	Before addition of forskolin	After addition of forskolin
Control	7·35 ± 0·20	7·76 ± 0·28
+10^{-6} M salbutamol	6·85 ± 0·20	7·29 ± 0·41
+3×10^{-6} M salbutamol	6·80 ± 0·10	7·37 ± 0·17
+10^{-5} M salbutamol	5·90 ± 0·27**	7·23 ± 0·35*

Preparations were used after 24 h incubation. EC50 = dose resulting in 50% relaxation. *$P < 0.05$, **$P < 0.001$ compared with control result (n = 4–10).

potency values for salbutamol is shown in Table 2. The potency of salbutamol was significantly reduced after incubation with 10^{-5} M salbutamol ($P < 0.001$). In addition, the maximum relaxation to salbutamol was reduced in parallel with the increase in the concentration of salbutamol in the incubation medium (Figure 4).

Forskolin reverses tachyphylaxis to salbutamol

Addition of forskolin (3×10^{-7} M) to the preparations after induction of tachyphylaxis, elicited a significant increase in the relaxant response to salbutamol. The potentiation of salbutamol-induced relaxation was significant at the three concentrations of salbutamol tested (10^{-6}, 3×10^{-6} or 10^{-5} M) (Figure 5). The potency of salbutamol was notably increased, particularly at the lower concentration range of salbutamol. The highest

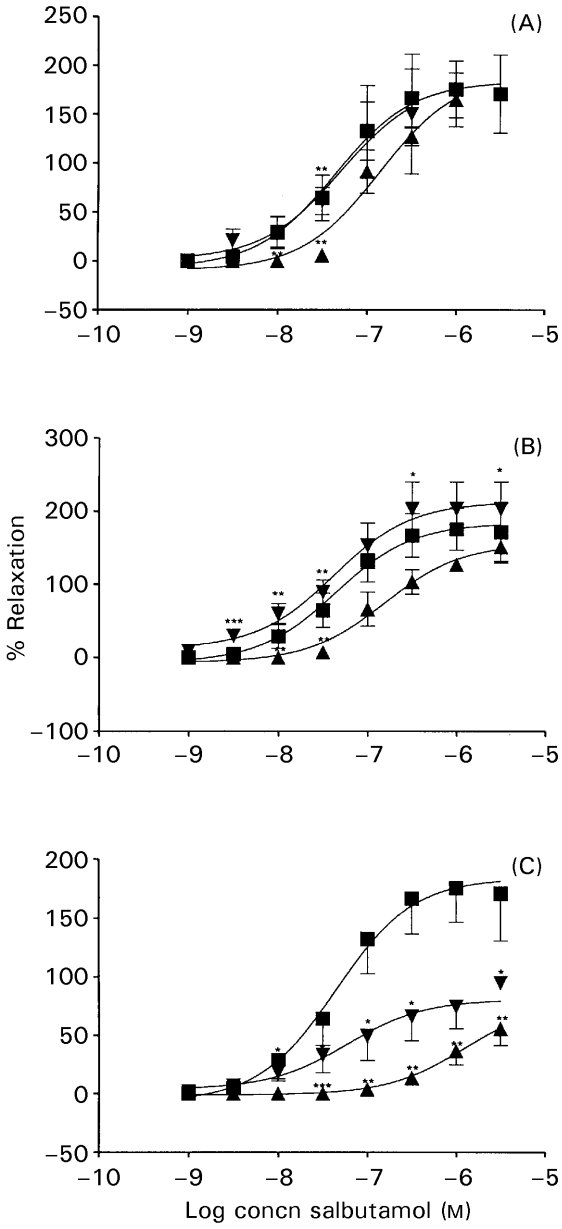

Figure 5. Effect of forskolin (3×10^{-7} M) (▼) on development of tachyphylaxis to salbutamol relaxant response (10^{-9}–10^{-6} M) (■) induced by pretreatment with salbutamol (A, 10^{-6} M; B, 3×10^{-6} M; C, 10^{-5} M; ▲) in guinea-pig tracheal preparations precontracted by carbachol (10^{-7} M). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control result (n = 4–8).

increase in potency was achieved after incubation with salbutamol (10^{-5} M) ($P < 0.05$) (Table 2).

Forskolin was tested under similar conditions, after pre-incubation with salbutamol. Development of tachyphylaxis to salbutamol had no significant effect on the forskolin-induced bronchodilator response. The potency and maximum relaxation of forskolin was not significantly altered.

Discussion

Forskolin, a diterpene isolated from the roots of *Coleus forskohlii*, activates adenylate cyclase in membrane preparations and intact cells from a variety of tissues (Adnot et al 1982; Litosch et al 1982). Forskolin also augments receptor-mediated increases in cyclic AMP (Moriwaki et al 1982; Siegl et al 1982) and has been shown to activate adenylate cyclase in many systems, thereby eliciting cellular responses which are dependent on cyclic AMP activation (Metzger & Lindner 1981; Seamon & Daly 1981). Forskolin has been an attractive agent because of its ability to stimulate adenylate cyclase directly (Seamon & Daly 1986). It by-passes cell membrane receptors to activate adenylate cyclase and raises tissue levels of cyclic AMP (Kreutner et al 1985).

In this study both forskolin and salbutamol elicited concentration-dependent relaxant responses in isolated guinea-pig tracheal preparations precontracted with carbachol. Salbutamol was significantly more potent than forskolin; however, results indicated that forskolin is a potent bronchodilator in-vitro compared with salbutamol. Addition of forskolin to the physiological solution did not significantly potentiate the bronchodilator effects of salbutamol in-vitro. This is in accordance with results from in-vivo studies reported by Kreutner et al (1985).

The development of tachyphylaxis or desensitization in a tissue upon repeated application of an agonist is a well established phenomenon (Miao & Lee 1992). Whereas β_2 agonists remain essential for relief of breakthrough symptoms, long-term use, particularly with high doses of potent agents, seems to be detrimental (Sears & Taylor 1994).

The procedure of tachyphylaxis induction in-vitro was established in this study to determine the effectiveness of forskolin in reversing the development of tachyphylaxis to salbutamol. Different concentrations of salbutamol in the incubation medium and different periods of incubation were tested in preliminary experiments. Salbutamol at concentrations less than 10^{-6} M in the incubation medium failed to reduce salbutamol-induced relaxant response significantly. Tachyphylaxis to salbutamol-induced relaxant response was established after 24 h incubation in oxygenated Krebs solution containing 10^{-6}, 3×10^{-6} or 10^{-5} M salbutamol. Control experiments using tracheal preparations incubated in drug-free Krebs solution were always run in parallel. A significant reduction in salbutamol-induced relaxant response was recorded from preparations incubated in salbutamol-containing incubation medium. The potency of salbutamol was significantly reduced. However, forskolin-induced relaxant response was not affected in tracheal preparations incubated under similar experimental conditions. Addition of forskolin to the preparations after inducing tachyphylaxis caused a significant reversal of the reduced potency of salbutamol. The reversal of tachyphylaxis was either complete, at the lower concentrations of salbutamol (10^{-6}, 3×10^{-6} M) in the incubation medium, or partial, as shown at the highest concentrations of salbutamol used to induce tachyphylaxis (10^{-5} M). The potency of salbutamol was significantly increased in the presence of forskolin upon induction of tachyphylaxis.

These results suggest that treatment of asthmatics with a combination of forskolin and β_2 adrenergic agonists might provide an alternative therapy to increasing agonist dose upon development of tachyphylaxis. Such a combination might have the advantage of eliminating the serious side effects which result from high doses of anti-asthma β_2 agonists.

References

Abramson, S. N., Molinoff, P. B. (1984) in vitro interactions of agonists and antagonists with β-adrenergic receptors. Biochem. Pharmacol. 33: 869–875

Adnot, S., Desmier, M., Ferry, N., Hanoune, J., Sevenet, T. (1982) Forskolin, a powerful inhibitor of human platelet aggregation. Biochem. Pharmacol. 31: 4071–4074

Brooker, G., Pedone, C., Barovsky, K. (1983) Selective reduction of forskolin-stimulated cyclic AMP accumulation by inhibitors of protein synthesis. Science 220: 1169–1170

Christenson, J. T., Thulesius, O., Owunwanne, A., Nazzal, M. (1991) Forskolin impregnation of small calibre PTFE grafts lowers early platelet graft sequestration and improves potency in a sheep model. Eur. J. Vas. Surg. 5: 271–275

Darfler, F., Mahan, L., Koachman, A., Insel, P. (1982) Stimulation by forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. J. Biol. Chem. 257: 11901–11907

Harvey, J. E., Tattersfield, A. E. (1982) Airway response to salbutamol: effect of regular salbutamol inhalations in normal, atopic and asthmatic subjects. Thorax 37: 280–287

Kreutner, W., Chapman, R., Gulbenkian, A., Tozzi, S. (1985) Bronchodilator and antiallergy activity of forskolin. Eur. J. Pharmacol. 111: 1–8

Larsson, K., Martinsson, A., Hjemdahl, P. (1992) Influence of β-adrenergic receptor function during terbutaline treatment on allergen sensitivity and bronchodilator response to terbutaline in asthmatic subjects. Chest 101: 953–960

Lefkowitz, R. J., Caron, M., Michel, T., Stadel, J. (1982) Mechanisms of hormone receptor-effector coupling: the β-adrenergic receptor and adenylate cyclase. Fed. Proc. 41: 2664

Litosch, I., Hudson, T., Mills, I., Li, S., Fain, J. (1982) Forskolin as an act of cyclic AMP accumulation and lipolysis in rat adipocytes. Mol. Pharmacol. 22: 109–115

Ljusegren, M. E., Ahlner, J., Axelsson, K. (1988) Studies on vascular smooth muscle tolerance to different cGMP-mediated vasodilators and cross-tolerance to glyceryl trinitrate. Pharmacol. Toxicol. 62: 302–307

Metzger, H., Lindner, E. (1981) The positive inotropic-acting forskolin, a potent adenylate cyclase activator. Arzneim. Forsch. 31: 1248–1250

Miao, F. J., Lee, T. J. (1992) Regional variation in acute vascular homologous tachyphylaxis. J. Pharmacol. Exp. Ther. 260: 644–651

Moriwaki, K., Itoh, Y., Iida, S., Ichihara, K. (1982) Forskolin potentiates adrenocorticotropin-induced cyclic AMP production and steroidogenesis in isolated rat adrenal cells. Life Sci. 30: 2235–2240

Satake, N., Shibata, S. (1997) Potentiation of the relaxing action of isoproterenol by forskolin in aortic rings: the involvement of β_2-adrenoceptors. Gen. Pharmacol. 28: 753–756

Seamon, K., Daly, J. (1981) Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. 7: 201–224

Seamon, K., Daly, J. (1986) Forskolin: its biological and chemical properties. Adv. Cyclic Nucleotides Protein Phosphorylation Res. 20: 1–150

Seamon, K., Padgett, W., Daly, J. (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl Acad. Sci. USA 78: 3363–3367

Sears, M. R., Taylor, D. (1994) The β_2 agonist controversy. Observations, explanations and relationship to asthma epidemiology. Drug Safety 11: 259–283

Siegl, A., Daly, J., Smith, J. (1982) Inhibition of aggregation and stimulation of cyclic AMP generation in intact human platelets by the diterpene forskolin. Mol. Pharmacol. 21: 680–687