Involvement of reduced sensitivity to Ca^{2+} in β-adrenergic action on airway smooth muscle

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Running title: Loss of Ca^{2+} sensitization in β-adrenergic action.

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Abstract

To determine the involvement of Ca^{2+} sensitization in β-adrenergic action, we examined the relationship between isometric tension and intracellular Ca^{2+} concentration (F_{340}/F_{380}) in the inhibitory action of isoproterenol (ISO) against methacholine (MCh)- induced contraction, using fura-2 loaded tracheal smooth muscle.

ISO reduces contraction more than Ca^{2+} concentration. This phenomenon was mimicked by forskolin and db-cAMP. In contrast, an inhibition in tension by SKF-96365, a non-selective Ca^{2+} channel inhibitor, was associated with that in F_{340}/F_{380}, different from ISO. In the presence of Rp-cAMP, a membrane-permeable inhibitor of protein kinase A (PKA), ISO caused an equivalent relaxation with a less reduction in F_{340}/F_{380}. The effects of ISO were not affected in the presence of Y-27632, an inhibitor of Rho-kinase, and bisindolylmaleimide, an inhibitor of protein kinase C. Even removal of Ca^{2+} from the extracellular surface, the effects of ISO was not affected. ISO also inhibits high K^+-induced contraction without lowering F_{340}/F_{380}. However, response to ISO was completely diminished in the presence of calyculin A, an inhibitor of myosin phosphatase. In conclusion, β-adrenergic action antagonizes Ca^{2+} sensitization mediated by impairment of myosin phosphatase activity. Moreover, cAMP independent process, G protein direct action, is more potent in inhibiting this Ca^{2+} sensitization than PKA.

Key words: β-adrenergic receptor agonists, Ca^{2+} sensitization, myosin phosphatase, G_s, Rho

Introduction

β-adrenergic receptor agonists (β-agonists) are well known to suppress contraction in airway smooth muscle induced by various contractile agents such as acetylcholine, histamine, and eicosanoids, and widely used clinically to relief acute asthma attacks. These contractile agents transiently bring about an augmentation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), followed by a reduction in $[Ca^{2+}]_i$ level that is higher than the control level (1, 2). This transient increase in $[Ca^{2+}]_i$ in the initial phase is due to Ca^{2+} release from the sarcoplasmic reticulum, whereas the subsequent higher $[Ca^{2+}]_i$ level than the control is due to Ca^{2+} influx from the extracellular side. Although $[Ca^{2+}]_i$ level reduces in the presence of these agonists, the tonic contraction is still sustained in airway smooth muscle (2, 3, 4). Hence, the tonic contraction in airway smooth muscle is mediated by not only Ca^{2+} influx but also Ca^{2+} sensitization. It has

been recently revealed that Rho-kinase, which is a target enzyme of Rho (a small monomeric G protein), inhibits the regulatory subunit of myosin phosphatase (5, 6, 7, 8), and that the Rho/Rho-kinase processes may result in an augmentation in sensitivity to intracellular Ca^{2+} by which agonist-induced contraction is regulated in airway smooth muscle (9, 10, 11).

Effects of β-agonists on relationship between isometric contractions and $[Ca^{2+}]_i$ have been mainly studied in mobilization of $[Ca^{2+}]_i$. β-agonists and other cAMP-related agents cause membrane hyperpolarization via a reduction in Ca^{2+} influx (12, 13, 14) and an augmentation in Ca^{2+}-activated K^+ channel activity (15, 16, 17, 18), leading to a reduction in $[Ca^{2+}]_i$. Ca^{2+} mobilization by these processes affects tone of airway smooth muscle mediated by Ca^{2+}/calmodulin- dependent myosin light chain kinase (MLCK) (19, 20). On the other hand, it is currently proposed that theophylline and cAMP may reduce Ca^{2+} sensitization to antagonize contraction by contractile agents, because cAMP-dependent protein kinase (PKA) causes an augmentation in myosin phosphate via Rho/Rho-kinase and other mechanisms (21, 22). Hence, both Ca^{2+} mobilization and Ca^{2+} sensitization may play an important functional role in the effect of cAMP-related agents on smooth muscle. However, little is still known about a reduction in Ca^{2+} sensitization elicited by β-agonists in detail, because cAMP-independent pathways are involved in the β-adrenergic action (23, 24).

This study was designed to determine involvement of Ca^{2+} sensitization in the functional antagonism between β-agonists and contractile agents. We examined causal relationship between Ca^{2+} mobilization and Ca^{2+} sensitization in the inhibitory effects of β-agonists on contraction induced by a muscarinic receptor agonist using intact tracheal smooth muscle. Moreover, we examined mechanisms underlying suppression of Ca^{2+} sensitization in β-adrenergic action.

Materials and Methods

Tissue Preparation and Solution

Male guinea pigs (300-350 g) were killed by injection of overdose of anesthetics (150 mg/kg pentobarbital, i.p.) and tracheas were excised. The tracheal rings were opened by cutting longitudinally at the cartilaginous region, and the epithelium was dissected out. The normal bathing solution was composed of (in mM):

NaCl 137, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2, and glucose 11.8, bubbled with a mixture of 99% O_2 and 1% CO_2 (pH 7.4). For the high K^+ solution (40 mM), NaCl was replaced with an equi-molar concentration of KCl. For the nominally free Ca^{2+} solution, $CaCl_2$ was replaced with an equi-molar concentration of NaCl. The bathing solution was filled in the organ bath at a constant flow of 3 ml/min. The temperature of the organ bath was maintained at 37 °C.

Isometric Tension Recording and Measurement of Fura-2 Fluorescence

The methods are essentially similar to those described previously (11, 25). Muscle strips containing four cartilaginous rings, one for isometric tension recording and three for $[Ca^{2+}]_i$ measurements, were prepared. Muscle strips were treated with 10 μM acetoxymethyl ester of fura-2 for 4 h at room temperature (22-24 °C). The non-cytotoxic detergent, pluronic F-127 (0.01% wt/vol), was added to increase the solubility of fura-2. After the loading, the chamber was filled with the normal solution at 37 °C for 50 min to wash out the extracellular fura-2 before the measurements. Isometric tension and the fura-2 fluorescence of muscle strips were measured simultaneously, using a displacement transducer and a spectrofluorometer (CAF-110; Japan Spectroscopic, Tokyo, Japan). The intensities of fluorescence due to excitation at 340 (F_{340}) and 380 (F_{380}) nm were measured after background subtraction. The absolute amount of $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} in smooth muscle cytoplasm is known to be different from that obtained *in vitro* (26). Therefore, the ratio of F_{340} to F_{380} (F_{340}/F_{380}) was used as a relative indicator of $[Ca^{2+}]_i$. MCh 1 μM was applied for 5 minutes at every 15 minutes to establish stable response to MCh, and the experiment started. Muscle tension and F_{340}/F_{380} in the resting state were taken as 0%, and the values of percent contraction and F_{340}/F_{380} were expressed by taking 1 μM MCh-induced contraction and 40 mM K^+ induced contraction at each experimental condition as 100%. The resting tone was abolished by addition to 2 μM indomethacin throughout the experiments.

Experimental Protocol

To examine relationship between relaxation and $[Ca^{2+}]_i$ in β-adrenergic action, isoproterenol (ISO) was cumulatively applied to the fura-2 loaded tissues pre contracted by 1 μM MCh. To examine relationship between relaxation and $[Ca^{2+}]_i$ in the post receptor signal transduction processes, forskolin, a direct activator of adenylate cyclase,

and dibtilyl cyclic AMP (db-cAMP), non hydrolysable cAMP, were cumulatively applied in the same way. To examine the inhibitory effects of Ca^{2+} mobilization on MCh-induced contraction, SKF-96365, an inhibiter of non-selective Ca^{2+} channels, and verapamil, voltage-dependent Ca^{2+} channel blockers, were cumulatively applied in the same way. To examine the inhibitory effects of Ca^{2+} sensitization on MCh-induced contraction, Y-27632, an inhibiter of Rho kinase, was cumulatively applied in the same way. To determine the mechanisms underlying Ca^{2+} sensitization by β-agonists, the inhibitory action of β-agonists was examined in the presence of Rp-cAMP, a membrane-permeable inhibitor of PKA, bisindolylmaleimide (BIM), an inhibitor of protein kinase C, and calyculin A, an inhibitor of myosin phosphatase. To determine the involvement of Ca^{2+} mobilization, the inhibitory action of β-agonists was examined under the condition of nominally Ca^{2+} free and 40 mM K^{+} solution in the extracellular surface.

Materials

Isoproterenol, MCh, indomethacin, forskolin, db-cAMP, SKF-96365, BIM, calyculin A, and pluronic F-127 were obtained from Sigma Chemical (St. Louis, MO). Y-27632 was a gift from Welfide Co. Ltd. (Osaka, Japan). Rp-adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMP) was obtained from BIOLOG Life Science Institute (Bremen, Germany). Fura-2 was from Dojin Laboratories (Kumamoto, Japan). Fura-2 was dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration did not exceed 0.5%. Neither drug affected the fura-2 fluorescence ratio at the concentrations used.

Statistical Analysis

All data were expressed as means ± standard deviation. The statistical significance in all data was assessed with repeated measures ANOVA with the Bonferroni *post hoc* test using all concentration. Probability below 0.05 ($P < 0.05$) was considered to be a significant difference.

Results

The inhibitory effects of β-agonists on tension and $[Ca^{2+}]_i$ induced by MCh

When ISO (0.003-1 μM) was cumulatively applied to the fura-2-loaded tissues, ISO inhibited both contraction and $[Ca^{2+}]_i$ induced by 1 μM MCh in a concentration-dependent manner. These inhibitory actions are more potent in contraction than in $[Ca^{2+}]_i$ (Fig. 1A). The values of percent contraction for 1 μM MCh with ISO inhibition were 0 ± 0%, whereas the values of percent F_{340}/F_{380} under this condition were 27.9 ± 6.6% (n = 8). The concentration-inhibition curve for ISO in tension is markedly dissociated from the curve in $[Ca^{2+}]_i$ ($P < 0.01$). The values of percent contraction for 1 μM MCh with 0.003, 0.01, 0.03, 0.1, and 0.3 μM ISO were 95.3 ± 4.8, 69.7 ± 12.3, 22.1 ± 10.7, 6.0 ± 4.7, and 0.0 ± 0.0%, respectively (n = 8; Fig. 1B). In contrast, those values of the percent F_{340}/F_{380} for 1 μM MCh with equi-molar ISO were 87.7 ± 2.8, 77.2 ± 8.6, 54.1 ± 4.5, 42.7 ± 11.6, and 33.1 ± 9.8%, respectively (n = 8; Fig. 1B). At each concentration of ISO, a reduction in tension was greater than that in $[Ca^{2+}]_i$, and this discrepancy became more remarkable as concentration of ISO was increased.

The inhibitory effects of adenylate cyclase stimulator on tension and $[Ca^{2+}]_i$ induced by MCh

Application of forskolin (0.1-10 μM) inhibited both tension and $[Ca^{2+}]_i$ induced by 1 μM MCh (Fig. 2A). However, although 10 μM forskolin caused complete inhibition, $[Ca^{2+}]_i$ was much higher than that in the basal level, similar to ISO. As shown in Fig. 2B, there was also a marked dissociation in the concentration- inhibition curves for forskolin in between tension and $[Ca^{2+}]_i$ ($P < 0.05$). The values of percent contraction for 1 μM MCh with 0.1, 0.3, 1, 3, and 10 μM forskolin inhibition were 93.9 ± 3.5, 81.4 ± 6.8, 48.7 ± 12.7, 11.7 ± 8.5, and 0.0 ± 0.0%, respectively (n = 6), whereas the values of the percent F_{340}/F_{380} for 1 μM MCh with equi-molar forskolin inhibition were 89.1 ± 1.3, 73.6 ± 3.5, 57.8 ± 3.1, 33.4 ± 9.4, and 16.4 ± 5.6%, respectively (n = 6; Fig. 2B). At each concentration of forskolin, a reduction in tension was higher than that in $[Ca^{2+}]_i$, and this discrepancy became more remarkable as concentration of forskolin was increased, similar to ISO.

The inhibitory effects of cAMP on tension and $[Ca^{2+}]_i$ induced by MCh

When 0.1-3 mM db-cAMP was cumulatively applied to the fura-2 loaded

tissues, db-cAMP caused a concentration-dependent inhibition in contraction and $[Ca^{2+}]_i$ by 1 μM MCh (Fig. 2C). 3 mM db-cAMP caused roughly complete inhibition in contraction, whereas $[Ca^{2+}]_i$ by an equi-molar of this agent also did not return to the basal level. The concentration-inhibition curve for db-cAMP in contraction was markedly dissociated from that curve in $[Ca^{2+}]_i$, similar to ISO and forskolin (Fig. 2D; $P < 0.01$). The values of percent contraction for 1 μM MCh with 0.1-3 mM db-cAMP inhibition were 100.0 ± 0.0, 86.3 ± 5.7, 35.4 ± 8.5, and 10.9 ± 7.1%, respectively (n = 6). In contrast, the values of the percent F_{340}/F_{380} for 1 μM MCh with equi-molar of this agent were 91.4 ± 5.4, 77.9 ± 5.5, 48.2 ± 8.4, and 33.1 ± 6.3%, respectively (n = 6, Fig. 2D). At each concentration of db-cAMP, a reduction in tension was higher than that in $[Ca^{2+}]_i$, and this came more remarkable as concentration of db-cAMP was increased, similar to ISO and forskolin.

The inhibitory effects of non-selective Ca^{2+} channels blocker on tension and $[Ca^{2+}]_i$ induced by MCh

A cumulative application of SKF-96365 (1-100 μM) to the fura-2 loaded tissues pre-contracted by 1 μM MCh resulted in an inhibition in tension and $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3A). When 30 μM SKF-96365 caused roughly complete inhibition, $[Ca^{2+}]_i$ by equi-molar of this agent was close to that in the basal level, different from ISO, forskolin, and db-cAMP. The concentration-inhibition curve for SKF-96365 in contraction was not dissociated from that curve in $[Ca^{2+}]_i$ (Fig. 3B). The value of percent contraction for 1 μM MCh with 1, 3, 10, 30, and 100 μM SKF-96365 inhibition were 100.0 ± 0.0, 98.5 ± 2.2, 75.9 ± 10.4, 26.3 ± 22.8, and 0.0 ± 0.0%, respectively (n = 6). Those values of the percent F_{340}/F_{380} for 1 μM MCh with equi-molar of this agent were 96.6 ± 4.9, 89.9 ± 4.7, 60.3 ± 6.1, 7.1 ± 10.0, and 0.0 ± 0.0%, respectively (n = 6; Fig. 3B). There was not a significant difference between curves of this agent.

The inhibitory effects of Rho on tension and $[Ca^{2+}]_i$ induced by MCh

When Y-27632 was cumulatively applied to the fura-2 loaded tissues pre-contracted by 1 μM MCh, Y-27632 caused an inhibition in tension by MCh in concentration-dependent manner. However, this agent modestly inhibited $[Ca^{2+}]_i$ induced by MCh (Fig. 3C). The concentration-inhibition curve for Y-27632 in contraction was markedly dissociated from that in $[Ca^{2+}]_i$ (Fig. 3D). The values of

percent contraction for MCh 1μM with 3, 10, 30, and 100 μM Y-27632 inhibition were 93.1 ± 2.7, 83.0 ± 9.4, 70.6 ± 13.9, and 51.9 ± 18.9%, respectively (n = 6). In contrast, the values of the percent F_{340}/F_{380} for 1 μM MCh with equi-molars of this agent were 100.0 ± 0.0, ±, 99.5 ± 1.2, 93.5 ± 5.0, and 85.8 ± 7.4%, respectively (n = 6, Fig. 3D). At each concentration of Y-27632, a reduction in tension was much greater than that in $[Ca^{2+}]_i$, and this discrepancy became more remarkable as concentrations of this agent were increased.

Relationships between tension and $[Ca^{2+}]_i$ in the inhibitory effects of cAMP-related agents on MCh-induced contraction

Fig. 4 shows the relationship between the Ca^{2+} mobilization and the Ca^{2+} sensitization by tension-$[Ca^{2+}]_i$ curves. In Fig. 4 the line (a) means that a reduction in tension is consistent with that in $[Ca^{2+}]_i$ concerning relaxant effects of an agent. As shown in Fig. 4A, tension-$[Ca^{2+}]_i$ curve for MCh with SKF-96365 (1-100 μM) was very close to the line (a), but this curve was upper side than the line (a). The curve for MCh with verapamil (0.1-3 μM) was upper side than SKF-96365. On the other hand, the curve for MCh with ISO (0.003-1 μM) was lower side than the line (a). The curve for MCh with Y-27632 (3-100 μM) was lower side than ISO. As shown in Fig. 4B, the curves for MCh with forskolin (0.01-10 μM), and db-cAMP (1-300 mM) were also lower side than the line (a), and these curves were significantly different from those for MCh with SKF-96365 and Y-27632. The curve for MCh with ISO was lower side than those for MCh with forskolin, and db-cAMP.

The inhibitory effects of β-agonists independent of Ca^{2+} influx

When 1 μM ISO was applied to the tissues pre contracted with 1 μM MCh in the presence of the normal bathing solution (2.4 mM Ca^{2+}), ISO inhibited MCh-induced contraction with a reduction in $[Ca^{2+}]_i$ (Fig. 5A). The values of percent contraction and percent F_{340}/F_{380} were 2.2 ± 3.1 and 18.5 ± 14.2% (n = 6), respectively (Fig. 5D).

The inhibitory effects of ISO on MCh-induced contraction were examined under the condition of a reduction in Ca^{2+} in the extracellular surface. After the tissues were contracted by 1 μM MCh in the normal bathing solution, the extracellular side was changed to the nominally Ca^{2+} free solution. Under this experimental condition, an equi-molar MCh generated force with an elevation of F_{340}/F_{380} (Fig. 5B). The values of percent contraction and percent F_{340}/F_{380} for MCh were 51.9 ± 16.0, and 49.3 ± 11.2% of

control condition (n = 6), respectively. When 1 μM ISO was applied to the tissues pre contracted by 1 μM MCh in the presence of the nominally Ca^{2+} free solution, ISO also antagonized MCh-induced contraction with a reduction in F_{340}/F_{380} (Fig. 5B). Those values percent contraction and percent F_{340}/F_{380} for MCh with ISO were 3.7 ± 2.5, and 36.4 ± 17.1% (n = 6), respectively (Fig. 5D). Inhibiting contraction by equi- molar ISO was identical, however reducing F_{340}/F_{380} by equi-molar ISO was significantly lowered.

Next, 1 μM ISO was applied to the tissues pre contracted by 40 mM K^+. As shown in Fig 5C, ISO caused an inhibition in 40 mM K^+-induced contraction with a modest reduction in $[Ca^{2+}]_i$, similar to the inhibitory effects of Y-27632 against MCh (ref. Fig 3). The values of percent contraction and percent F_{340}/F_{380} for 40 mM K^+ with ISO were 32.3, ± 8.3 and 95.2, ± 2.0% (n = 6; Fig. 5D).

Involvement of PKA, PKC, and Rho-kinase in Ca^{2+} mobilization and Ca^{2+} sensitization induced by β-agonists

Rp-cAMP 100 μM caused a modest inhibition in tension and $[Ca^{2+}]_i$ induced by MCh (Fig. 6A). When 1 μM ISO was applied to the tissues precontracted by 1 μM MCh and 100 μM Rp-cAMP, ISO antagonized MCh-induced contraction similar to the control condition. Even though ISO similarly inhibited MCh-induced contraction, a reduction in F_{340}/F_{380} by ISO was significantly lowered (Fig. 6A; ref. Fig. 5A). In the presence of 100 μM Rp-cAMP the value of percent contraction and percent F_{340}/F_{380} were 5.0 ± 3.7, and 56.2 ± 12.5% (n = 6), respectively ($P < 0.05$; Fig. 6A and 6D).

BIM 10 μM caused a modest inhibition of contraction and $[Ca^{2+}]_i$ induced by 1 μM MCh (Fig. 6B). When 1 μM ISO was applied to the tissues in the presence of MCh and BIM, ISO suppressed both contraction and F_{340}/F_{380} induced by MCh, similar to the control condition (Fig. 6B; ref. Fig. 5A). Under the experimental condition, the values of percent contraction and percent F_{340}/F_{380} for MCh with ISO were 4.0 ± 2.8, and 20.5 ± 9.2%, respectively (n = 6; Fig. 6B and 6D).

In the same way, when 1 μM ISO was applied to the tissues in the presence of 1 μM MCh and 10 μM Y-27632, ISO also suppressed both contraction and F_{340}/F_{380} induced by MCh similar to the control condition (Fig. 6C; ref. Fig. 5A). The values of percent contraction and percent F_{340}/F_{380} for MCh with Y-27632 were 70.2 ± 16.3 and 93.5 ± 5.6%, respectively. In the presence of Y-27632, the values of percent contraction and percent F_{340}/F_{380} for MCh with ISO were 2.9 ± 1.5 and 16.8 ± 8.4%, respectively (n

= 6; Fig. 6C and 6D).

The inhibitory effects of β-agonists on myosin phosphatase activity

Application of 300 nM calyculin A caused contraction without any changing in F_{340}/F_{380} (Fig. 7A). Calyculin A-induced contraction was slowly generated without phasic component, and the maximal response was observed 15 ~ 20 min after exposure. The value of percent contraction for this agent was 59.0 ± 18.2% of 1 μM MCh-induced contraction (n = 6; Fig. 7B). When 1 μM ISO was applied to tissues pre constricted by calyculin A, ISO did not inhibit it (Fig. 7A). The value of percent contraction for MCh with ISO was 57.9 ± 21.2% (n = 6; Fig. 7B).

Discussion

Our results have demonstrated for the first time that β-agonists cause an inhibition of MCh-induced contraction via not only Ca^{2+} mobilization but also Ca^{2+} sensitization, using intact tracheal smooth muscle. Force generation in smooth muscle is mediated by an increase in both concentration of intracellular Ca^{2+} and sensitivity to intracellular Ca^{2+}. These two processes are affected when β-agonists and other cAMP-related agents inhibit MCh-induced contraction. However, regulation of Ca^{2+} sensitization plays a more important role in β-adrenergic action than other cAMP-related agents in airway smooth muscle.

As shown in Figs. 1 and 2, in the inhibitory action of ISO, forskolin, and db-cAMP against MCh-induced contraction, a reduction in tension was not associated with that in $[Ca^{2+}]_i$. The concentration-percent contraction curves for MCh with β-agonists and other cAMP-related agents were significantly lower than the concentration-percent F_{340}/F_{380} curves. This phenomenon was mimicked using other contractile agents such as histamine and leukotriene D_4 (Data not shown). These results demonstrate that a reduction in not only Ca^{2+} influx but also Ca^{2+} sensitization is involved in the functional antagonism of β-agonists and other cAMP-related agents. The inhibitory action of ISO, forskolin, and db-cAMP on tension and $[Ca^{2+}]_i$ was different from that of SKF 96365 (Fig. 3), indicating that β-agonists and other cAMP-related agents do not behave as a non-selective Ca^{2+} channel blocker. This observation also supports the idea that a reduction in Ca^{2+} sensitization is involved in cAMP-dependent relaxation in airway smooth muscle.

As shown in Fig. 4A, the tension-F_{340}/F_{380} curve for SKF-96365 and verapamil existed between line (a) and (c). This area contains that a more reduction in $[Ca^{2+}]_i$ needs to obtain an equivalent relaxation by an agent. Hence, these results indicate that a reduction in $[Ca^{2+}]_i$ is insufficient to antagonize toward contraction induced by contractile agents, and that other mechanisms need to enhance their functional antagonism. In contrast, the tension-F_{340}/F_{380} curve for Y-27632 was close to the line (b) (Figs. 3 and 4A). The curve for wortmannin, an inhibitor of MLCK, was also close to the line (b) (Data not shown). This result indicates that the relationship between tension and $[Ca^{2+}]_i$ is considered to be drawn along the line (b) when agonist-induced contraction is antagonized by sensitivity to intracellular Ca^{2+}. The tension-F_{340}/F_{380} curves for ISO, forskolin, and db-cAMP existed between the line (a) and (b) (Fig. 4). This area contains that a more inhibition in contraction causes at an equivalent level of $[Ca^{2+}]_i$. This phenomenon was also mimicked by other β-agonists such as procaterol and salmeterol (Data not shown). These results also support the idea that cAMP-related agents containing β-agonists cause relaxation of airway smooth muscle mediated by not only Ca^{2+} mobilization but also Ca^{2+} sensitization, and that these agents do not behave as non-selective and voltage-dependent Ca^{2+} channel blockers.

When the nominally free Ca^{2+} solution was applied to the extracellular side, driving force in Ca^{2+} influx was lowered. However, the inhibitory effect of ISO against tension was not affected even though Ca^{2+} influx by this agonist was decreased (Fig. 5B and 5D). This observation suggests that β-agonists antagonize contraction induced by the contractile agents, such as MCh, histamine and leukotriene, in airway smooth muscle, independent of a reduction in Ca^{2+} influx. Application of ISO also resulted in an inhibition in 40 mM K^+-induced contraction with a modest reduction in $[Ca^{2+}]_i$ (Fig. 5C and 5D). Although the mechanisms underlying high K^+-induced contraction are different from mechanisms underlying contractions by these contractile agents, this result explains again that reduction of sensitivity to Ca^{2+} by β-agonists is involved in the functional antagonism induced by β-agonists.

The tension-F_{340}/F_{380} curves for MCh with ISO inhibition was closer to the line (b) than that for MCh with forskolin, and db-cAMP (Fig. 4B). These results demonstrate that an inhibition in sensitivity to Ca^{2+} is more potent in β-agonists than cAMP-related agents bypassing β-adrenergic receptors. β-adrenergic receptors are

coupled to the dual pathway of intracellular signal transduction, i.e. 1) cAMP-dependent PKA phosphorylation (15, 27, 28), 2) cAMP-independent, direct activation by G protein (G_s) (29, 30). G_s is more potent in activation of K_{Ca} than cAMP/PKA (23), and K_{Ca} channel regulation by G_s may be related to the functional antagonism between β-adrenergic receptors and muscarinic receptors (31). As shown in Fig. 6A, a reduction in F_{340}/F_{380} by ISO was markedly lowered to obtain an equivalent relaxation under the condition that PKA was inactivated by Rp-cAMP. Activation of G_s is more potent in inhibiting Ca^{2+} sensitization than that of cAMP/PKA. Reduced responsiveness to β-agonists is mediated by an augmentation in Ca^{2+} sensitization and Ca^{2+} mobilization via G_s dysfunction (14, 25, 32, 33). In tracheal smooth muscle β-adrenergic action is not affected after excessive exposure to forskolin, db-cAMP, and theophylline that elevate concentration on intracellular cAMP independent of receptor/G_s processes (14, 32, 33). Since full agonist action is closely related to G_s-mediated processes, response to full agonists is not so affected even though β-adrenergic receptor function is impaired (34, 35, 36). On the other hand, since partial agonist action is closely related to PKA-mediated processes, response to partial agonists are markedly attenuated under the condition of β-adrenergic desensitization (34, 35, 36). Hence, Ca^{2+} sensitization regulated by G_s plays a functional key role in the β-adrenergic action on airway smooth muscle.

Recently, it has been reported that theophylline and cAMP inhibit contraction of tracheal smooth muscle mediated by reduction of Rho-induced Ca^{2+} sensitization (21). On the other hand, cAMP suppresses PKC-mediated Ca^{2+} sensitization in airway smooth muscle (37). In cAMP/PKA processes mechanisms of antagonism to Ca^{2+} sensitization is still controversial. Since Ca^{2+} sensitization is generally considered to be mediated by Rho-kinase and PKC, we examined whether these pathways are involved in the Ca^{2+} sensitization antagonized by β-agonists in this study. As shown in Figs. 6B, 6C, and 6D, in the presence of Y-27632 and BIM, ISO inhibited MCh-induced contraction in the same way. Even through Rho-kinase and PKC are inactivated, the inhibitory action of β-agonists are not affected. Although these two kinases result in an augmentation in sensitivity to Ca^{2+}, other processes are involved in this phenomenon in airway smooth muscle. Both Ca^{2+} influx passing through non-selective Ca^{2+} channel and Ca^{2+} sensitization mediated by MLCK processes play an important role in this force

generation (38, 39). As shown in this study, relaxant agents may affect both Ca^{2+} mobilization and Ca^{2+} sensitization induced by contractile agents. However, when myosin phosphatase was inactivated in the presence of calyculin A, ISO completely diminished the inhibitory action against MCh-induced contraction (Fig 7). Hence, myosin phosphatase is considered to be fundamentally affected protein in the functional antagonist between β-agonists and contractile agents via Ca^{2+} sensitization. Recently, it has been revealed that ISO increases myosin phosphatase activity in a time- and concentration-dependent manner (40). This report supports the idea that β-agonists bring about a loss of sensitivity to Ca^{2+} via activation of myosin phosphatase described above. However, ISO did not cause an increase in active form of Rho (GTP-Rho), and did not phosphorylate myosin phosphatase targeting subunit (data not shown), suggesting that reduced Ca^{2+} sensitization by β-agonists is not mediated by Rho/Rho-kinase processes. Further studies are needed to clarify the mechanism underlying myosin phosphatase-induced Ca^{2+} sensitization.

In conclusions, Ca^{2+} sensitization regulated by myosin phosphatase plays an important functional role in β-adrenergic action on airway smooth muscle. cAMP-independent processes (direct action by G_s) is more potent in this phenomenon than cAMP-dependent processes (phosphorylation by PKA). Our results may provide evidence that this G_s/myosin phosphatase stimulatory linkage is a molecular target for acute asthma management.

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Acknowledgement

This work is supported by a Grant-in-Aid from the Ministry of Education Science, Sports, and Culture of Japan (15590805).

Legends

Figure 1. A reduction in sensitivity to intracellular Ca^{2+} mediated by a β-agonist. (A) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of ISO (0.003 – 1 μM) on 1 μM MCh. (B) Concentration-inhibition curves for ISO on tension (open circles) and F_{340}/F_{380} (closed circles) induced by 1 μM MCh. Tension and F_{340}/F_{380} in the resting state were taken as 0%, and those in 1 μM MCh-stimulated state were taken as 100%. The abscissa expresses molar concentration on a log scale.

Figure 2. A reduction in sensitivity to intracellular Ca^{2+} mediated by forskolin and db-cAMP. (A) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of forskolin (0.1 – 10 μM) on 1 μM MCh. (B) Concentration-inhibition curves for forskolin on tension (open circles) and F_{340}/F_{380} (closed circles) induced by MCh. (C) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of db-AMP (0.1 – 3 μM) on 1 μM MCh. (D) Concentration-inhibition curves for db-cAMP on tension (open circles) and F_{340}/F_{380} (closed circles) induced by MCh. The values of percent contraction and F_{340}/F_{380} for MCh with forskolin and db-cAMP were expressed as same way as that shown in Figure 1B. The abscissa expresses molar concentration on a log scale.

Figure 3. A reduction in sensitivity to intracellular Ca^{2+} mediated by SKF-96365 and Y-27632. (A) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of SKF-96365 (10 – 100 μM) on 1 μM MCh. (B) Concentration-inhibition curves for SKF-96365 on tension (open circles) and F_{340}/F_{380} (closed circles) induced by MCh. (C) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of Y-27632 (3 – 100 μM) on 1 μM MCh. (D) Concentration-inhibition curves for Y-27632 on tension (open circles) and F_{340}/F_{380} (closed circles) induced by MCh. The values of percent contraction and F_{340}/F_{380} for MCh with SKF-96365 and Y-27632 were expressed as same way as that shown in Figure 1B. The abscissa expresses molar concentration on a log

scale.

Figure 4. Role of Ca^{2+} mobilization and Ca^{2+} sensitization in the functional antagonism between MCh and various relaxant agents. (A) Tension- F_{340}/F_{380} curves for MCh with SKF-96365, verapamil, Y-27632, and ISO. (B) Those curves for MCh with ISO, forskolin, and db-cAMP (cAMP related agents). The values of percent contraction and F_{340}/F_{380} for MCh with these relaxant agents were expressed as same way as that shown in Figure 1B. See the contents concerning the line a, b, and c.

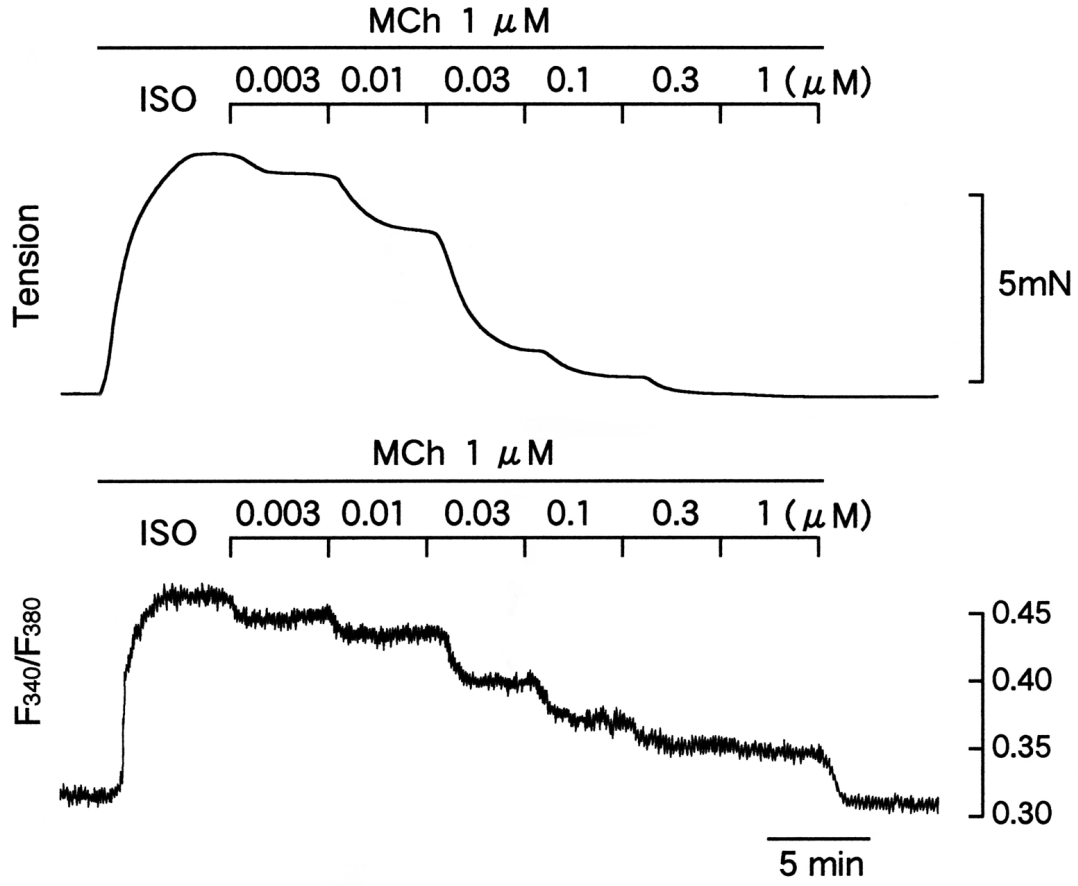
Figure 5. β-adrenergic relaxation without Ca^{2+} influx. (A) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effect of 1 μM ISO on an equi-molar MCh-induced contraction. (B) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effect of an equi-molar ISO on an equi-molar MCh-induced contraction in the presence of 0 Ca^{2+} (nominally free Ca^{2+}) at the extracellular side. (C) A representative of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effect of equi-molar ISO on high K^+ (40 mM K^+)-induced contraction. (D) The values of percent contraction (open column) and percent F_{340}/F_{380} (closed column) for MCh and 40 mM K^+ with ISO under the experimental condition shown in Figures 7A-C. Tension and F_{340}/F_{380} in the resting state were taken as 0%, and those in 1 μM MCh- and 40 mM K^+-stimulated state at each experimental condition were taken as 100%.

Figure 6. Mechanisms underlying reduced Ca^{2+} sensitization by β-agonists. (A) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of 1 μM ISO on 1 μM MCh-induced contraction in the presence of 100 μM Rp-cAMP. (B) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of an equi-molar ISO on an equi-molar MCh-induced contraction in the presence of 1 μM BIM. C) A representative of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effect of an equi-molar ISO on an equi-molar MCh-induced contraction in the presence of 10 μM Y-27632. (D) The values of percent contraction (open column) and percent F_{340}/F_{380} (closed column) for MCh with ISO under the experimental

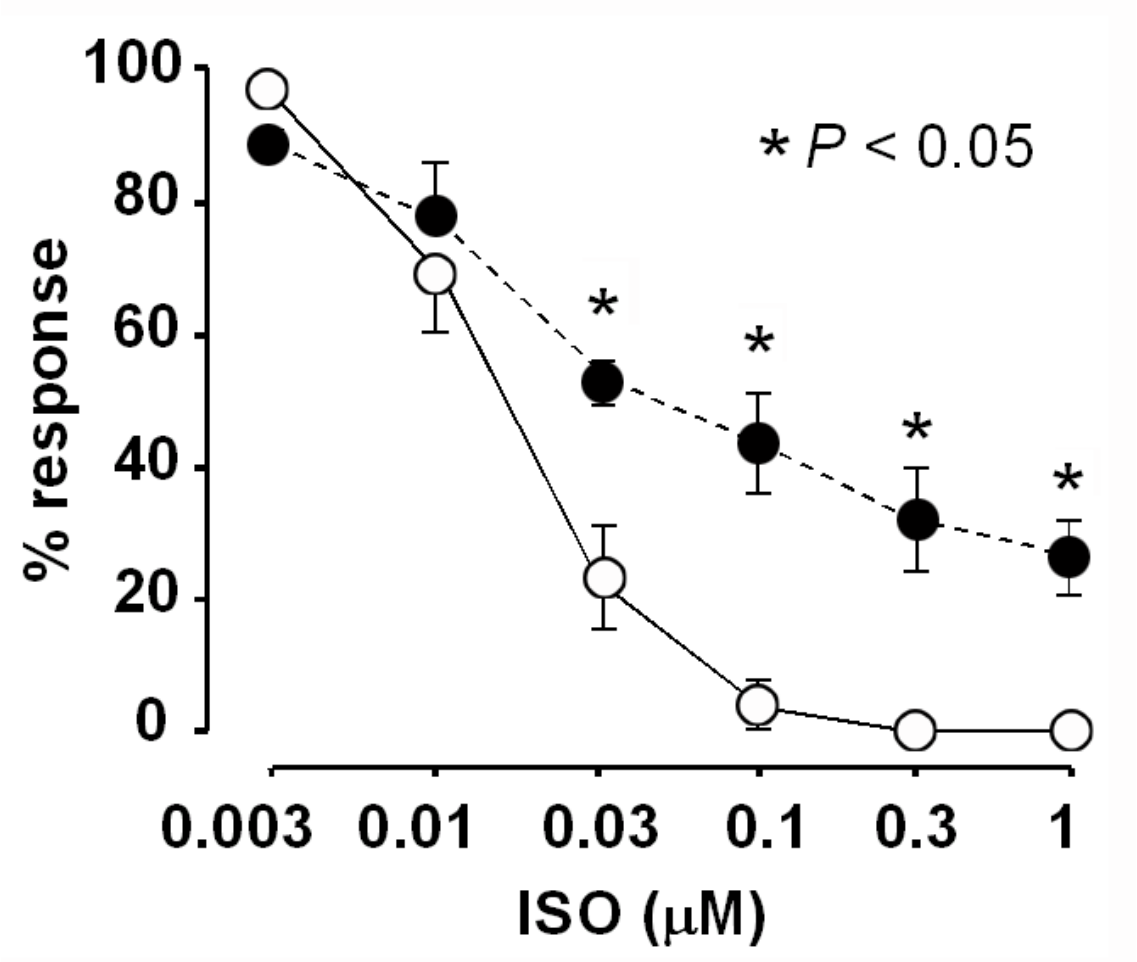
condition shown in Figures 6A-C. Those values were expressed in the same way as Figure 5D. Those values in control are identical to those values shown in the inhibitory effects of 1 μM ISO on 1 μM MCh-induced contraction in the presence of the normal bathing solution (ref. Figures 5A, and 5D).

Figure 7. Involvement of myosin phosphatase inhibition in the β-adrenergic action. (A) A typical example of simultaneous record of tension (upper trace) and F_{340}/F_{380} (lower trace) in the inhibitory effects of 1 μM ISO on 300 nM calyculin A-induced contraction. (B) The values of percent contraction for 1 μM MCh and 300 nM calyculin A with 1 μM ISO inhibition. The value of percent contraction for MCh with ISO is identical to that shown in Fig. 5D. Those values were expressed taking response to 1 μM MCh as 100%.

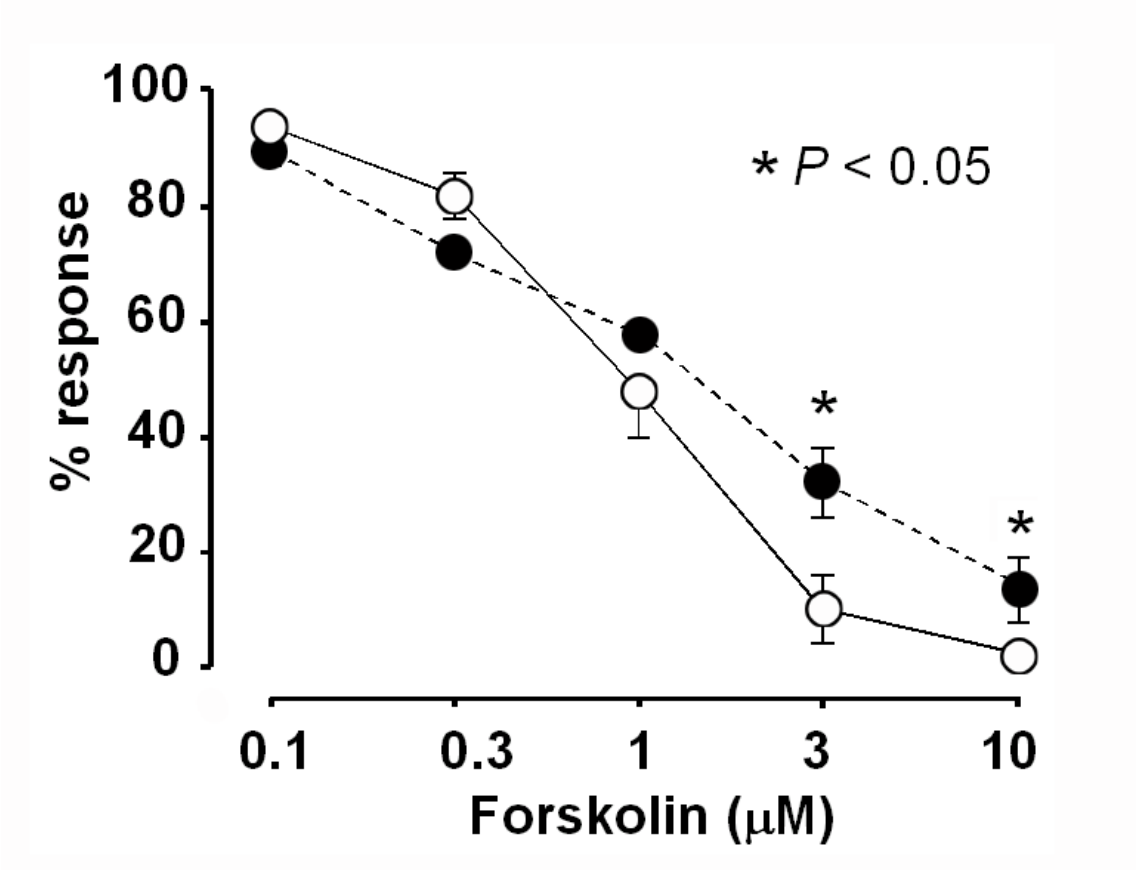
(Figure1A)


MCh 1 μM
ISO
0.003 0.01 0.03 0.1 0.3 1 (μM)
Tension
5mN
MCh 1 μM
ISO
0.003 0.01 0.03 0.1 0.3 1 (μM)
F340/F380
0.45
0.40
0.35
0.30
5 min

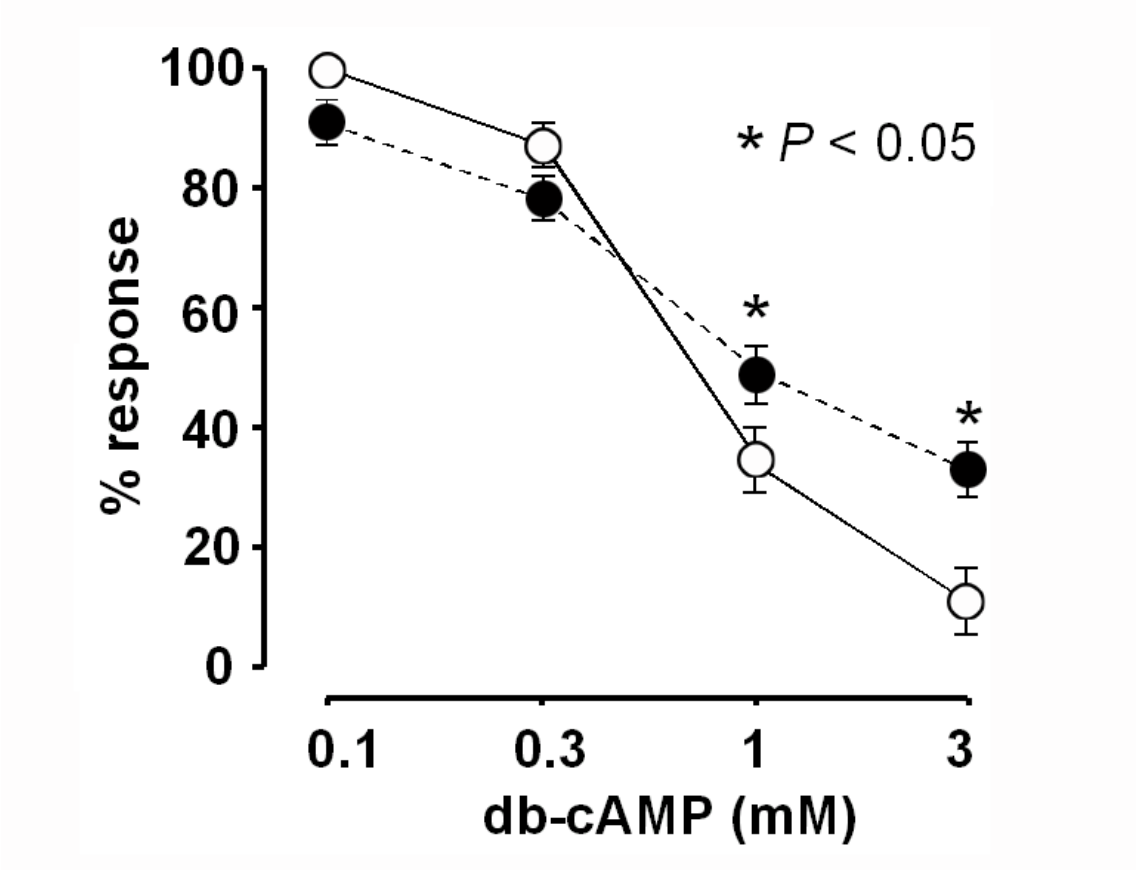
(Figure1B)



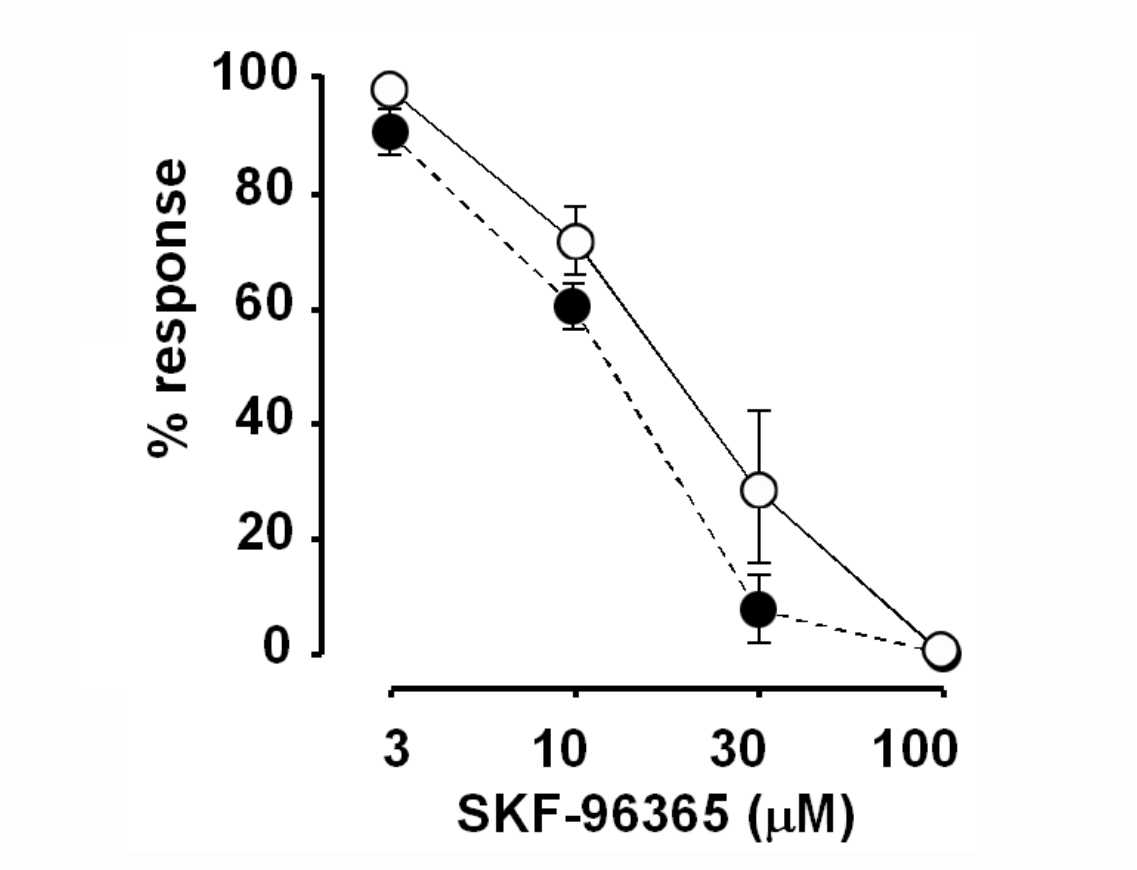
(Figure2A)



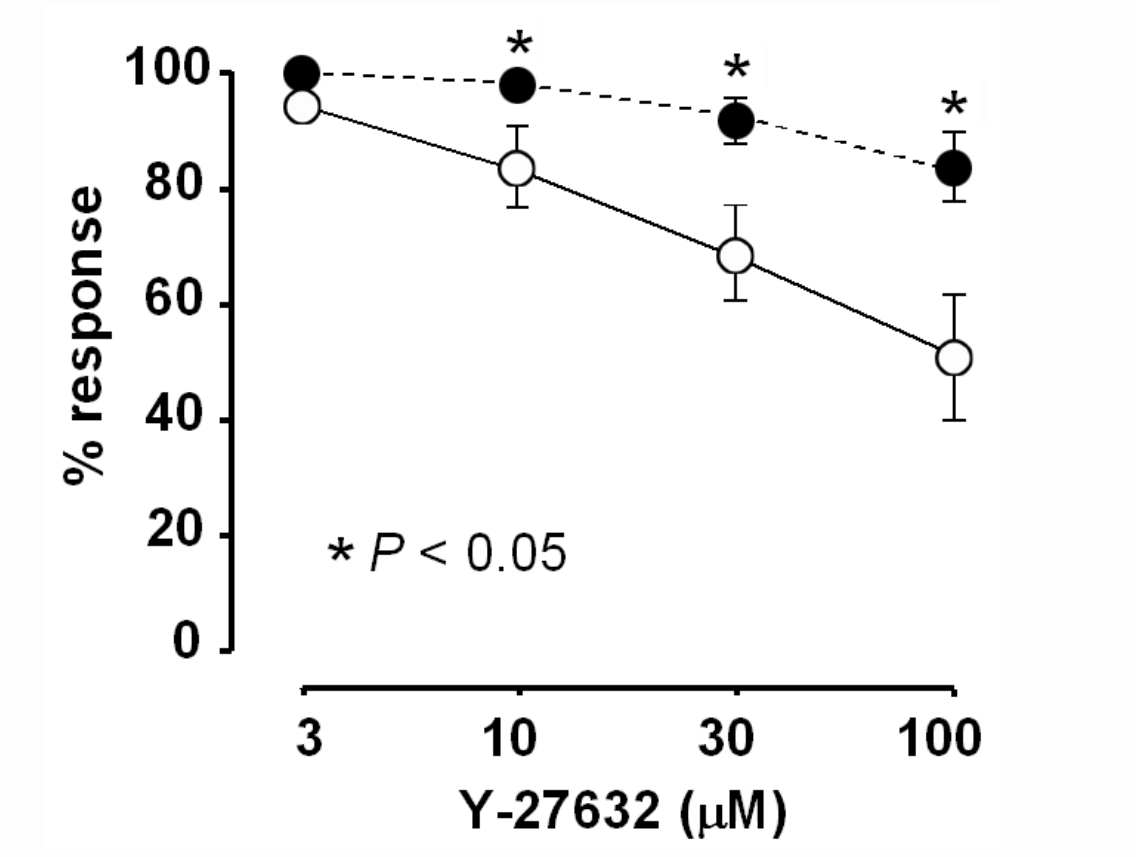
(Figure2B)



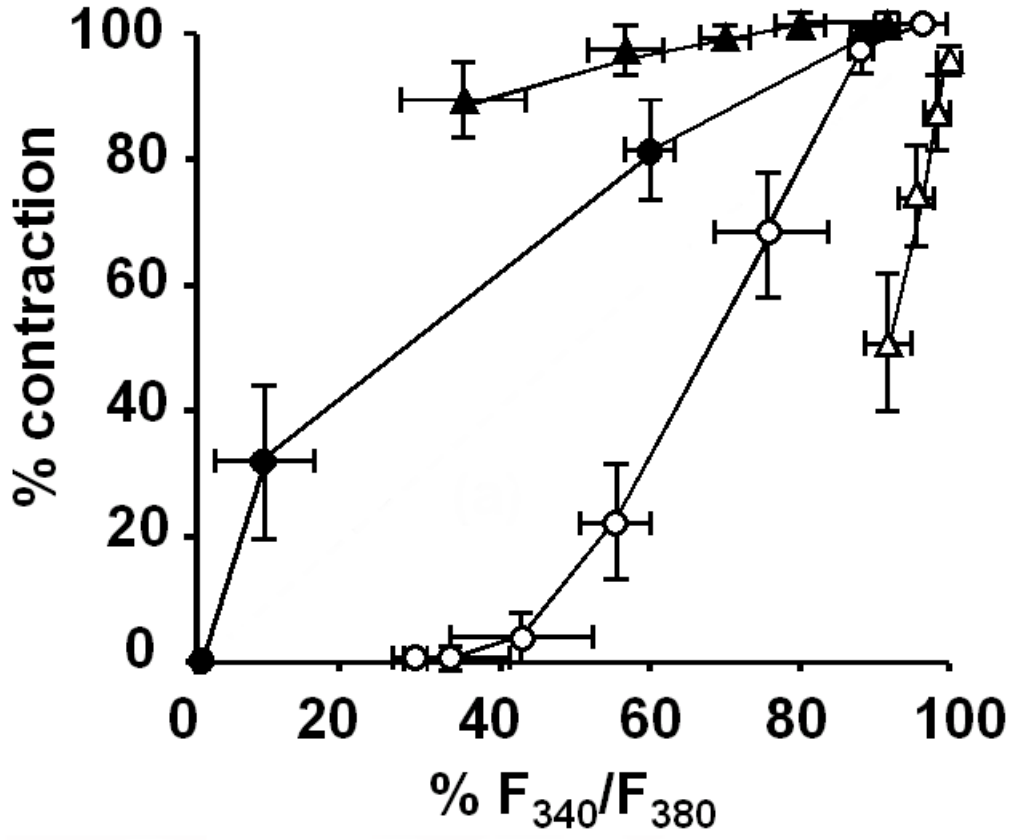
(Figure3A)



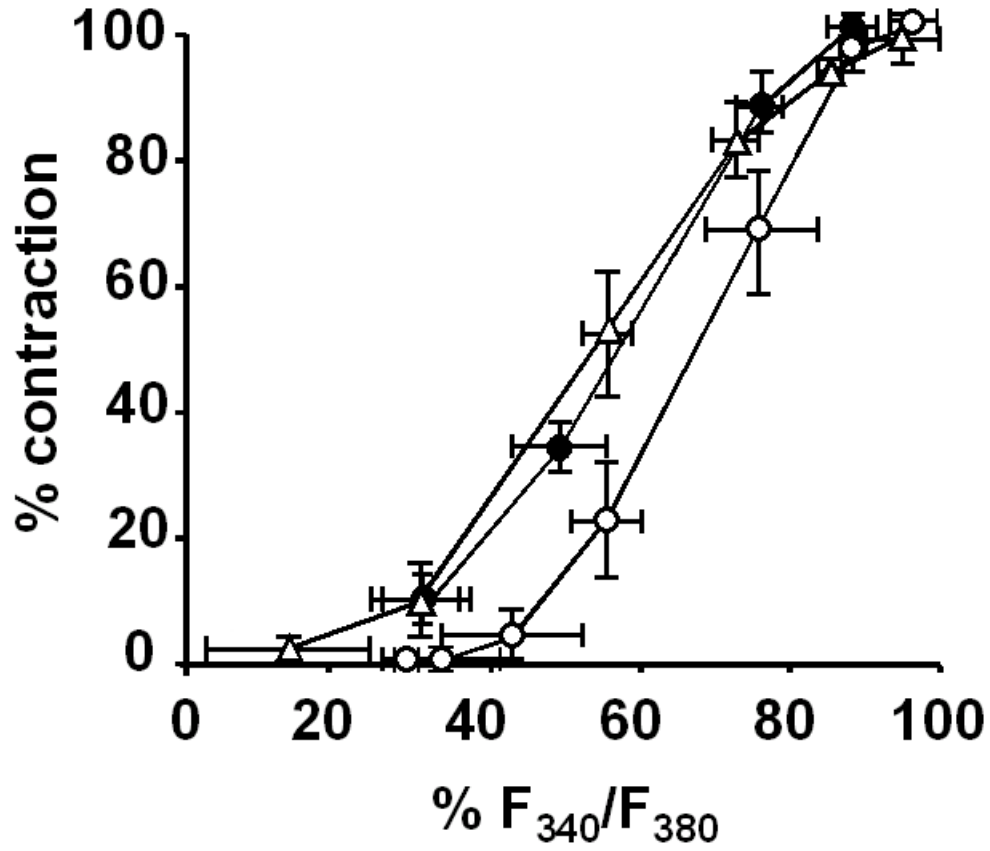
(Figure3B)



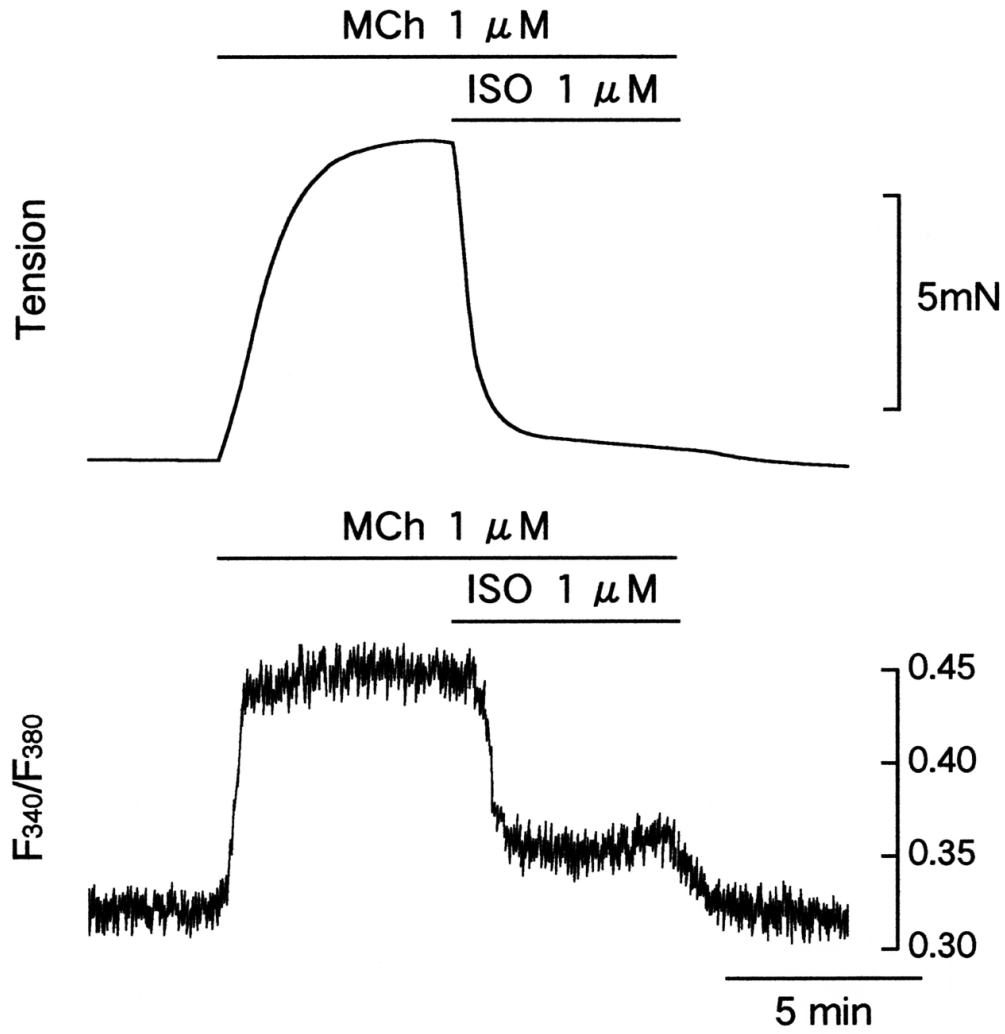
(Figure4A)



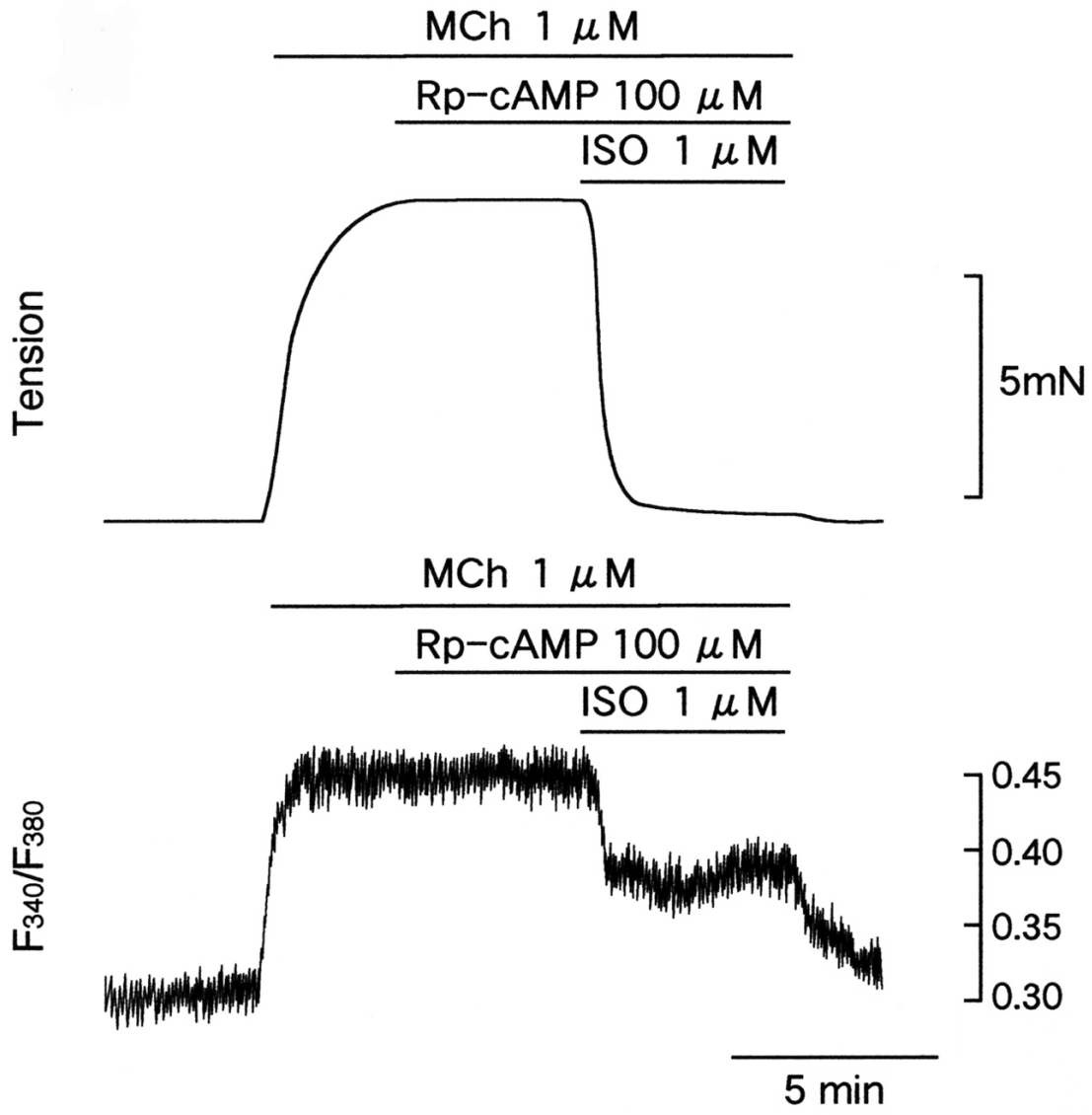
(Figure4B)



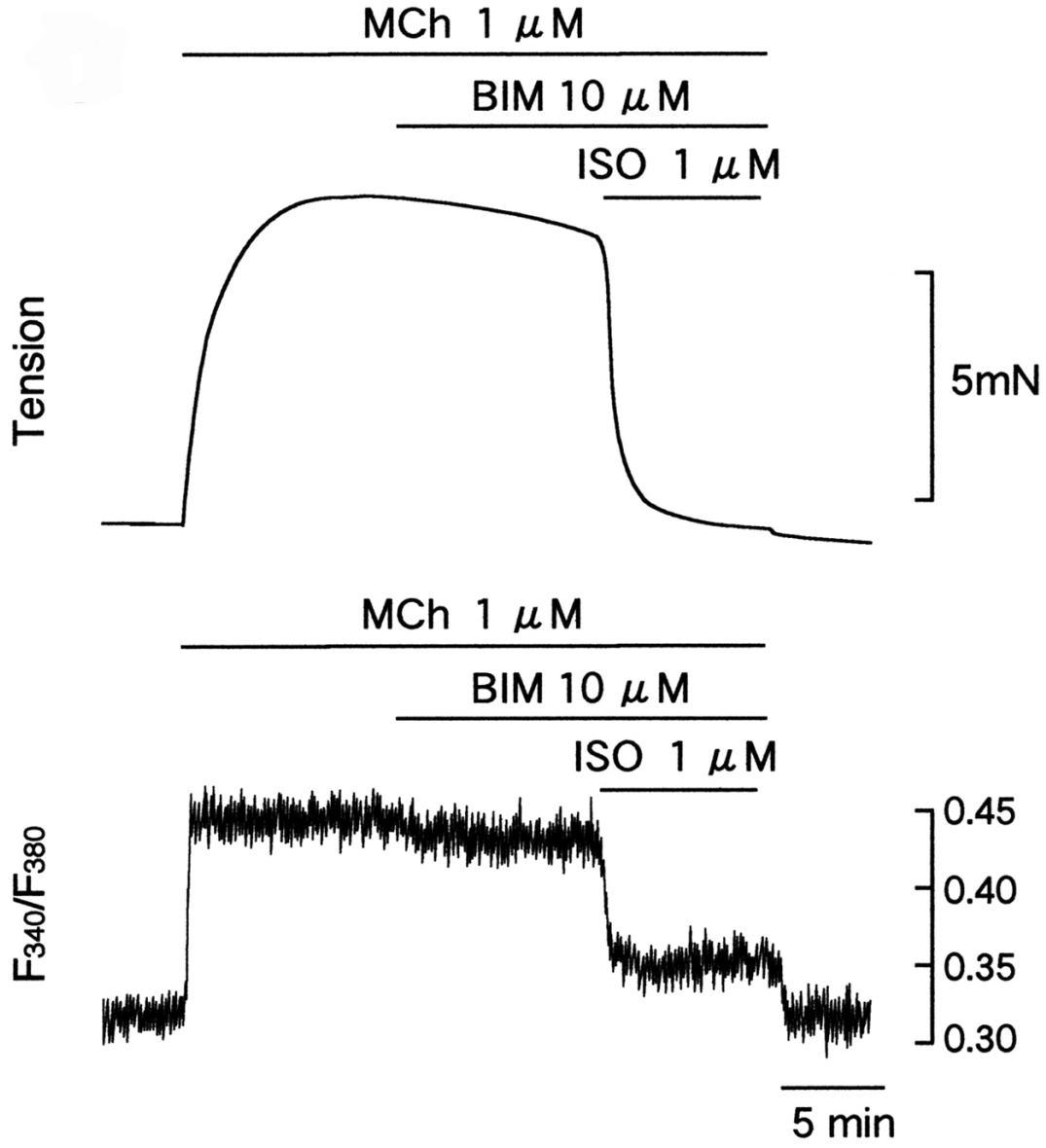
(Figure5A)


MCh 1 μM
ISO 1 μM
Tension
5mN
MCh 1 μM
ISO 1 μM
F340/F380
0.45
0.40
0.35
0.30
5 min

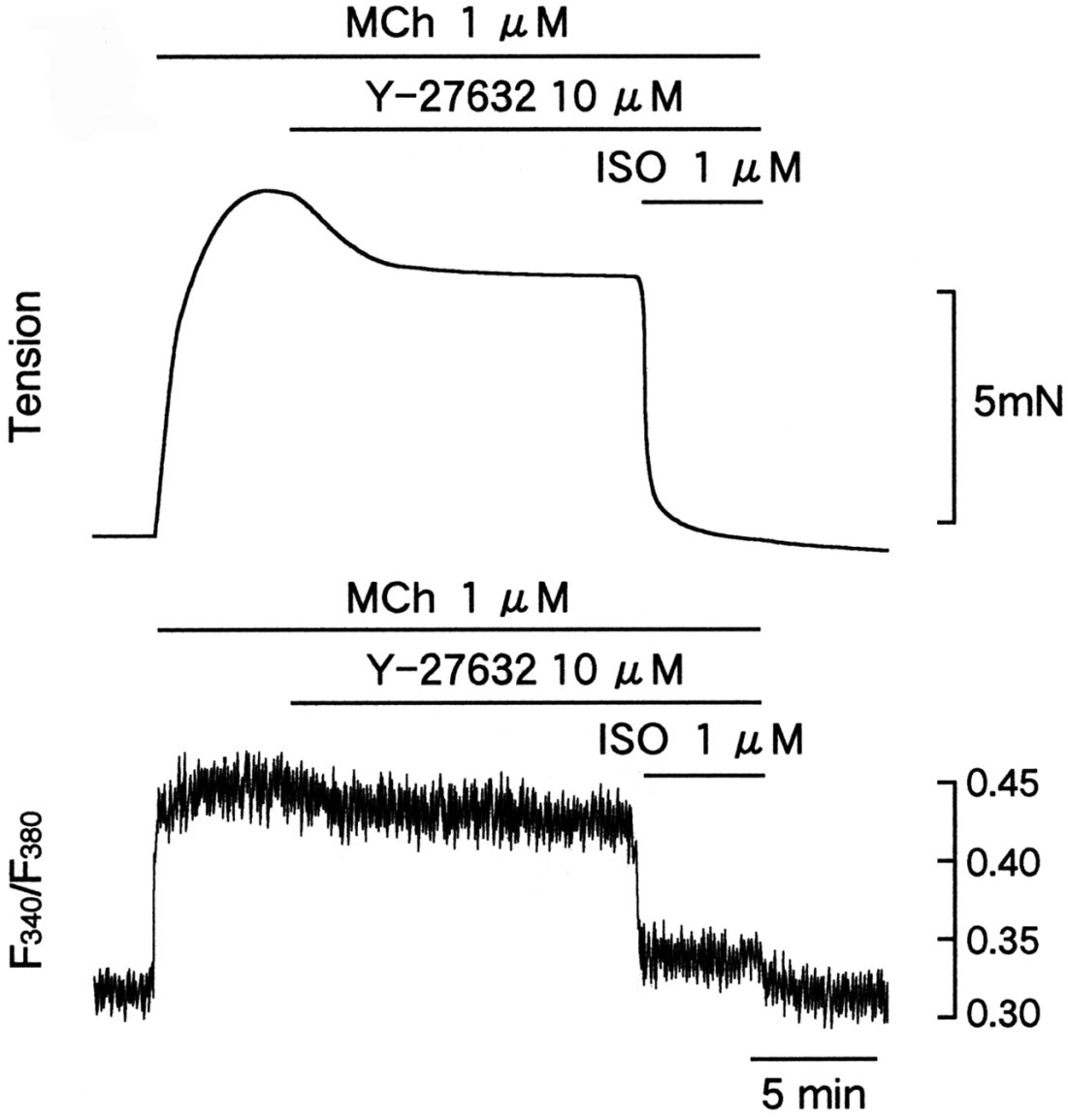
(Figure5B)


MCh 1 μM
Rp-cAMP 100 μM
ISO 1 μM
Tension
5mN
MCh 1 μM
Rp-cAMP 100 μM
ISO 1 μM
F340/F380
0.45
0.40
0.35
0.30
5 min

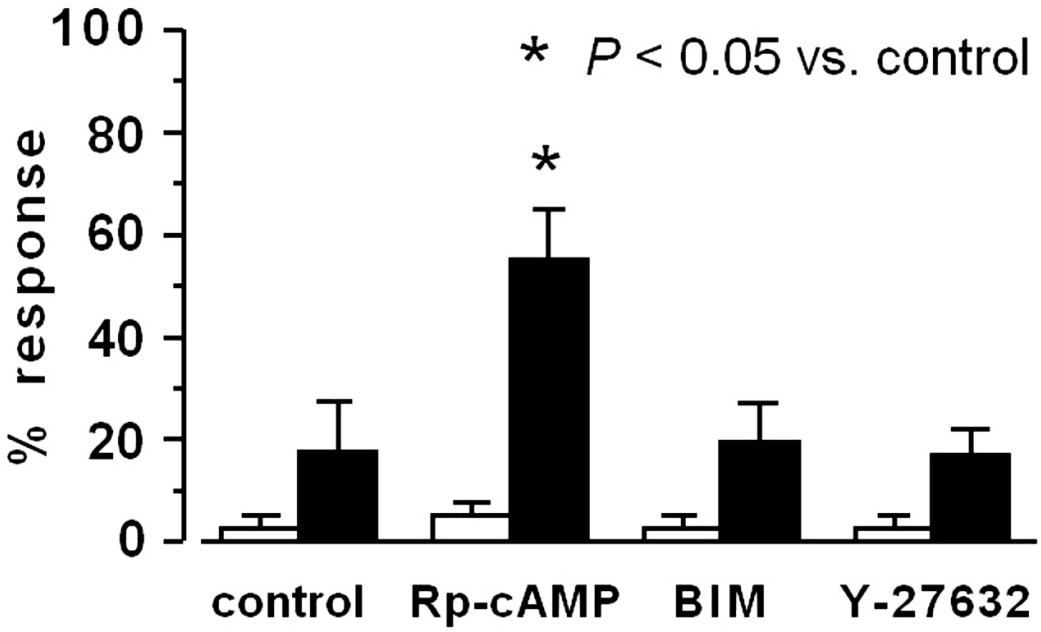
(Figure5C)


MCh 1 μM
BIM 10 μM
ISO 1 μM
Tension
5mN
MCh 1 μM
BIM 10 μM
ISO 1 μM
F340/F380
0.45
0.40
0.35
0.30
5 min

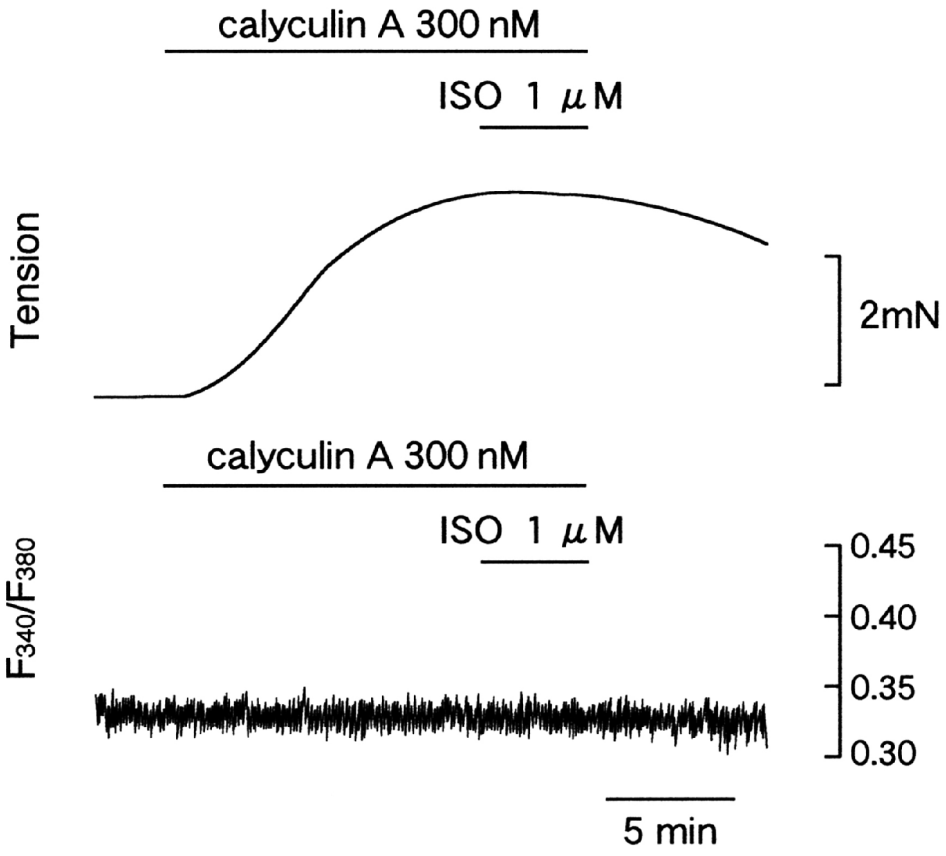
(Figure5D)


MCh 1 μM
Y-27632 10 μM
ISO 1 μM
Tension
5mN
MCh 1 μM
Y-27632 10 μM
ISO 1 μM
F340/F380
0.45
0.40
0.35
0.30
5 min

(Figure5E)


100
80
60
40
20
0
% response
* P < 0.05 vs. control
*
control
Rp-cAMP
BIM
Y-27632

(Figure6A)


calyculin A 300 nM
ISO 1 μM
Tension
2mN
calyculin A 300 nM
ISO 1 μM
F340/F380
0.45
0.40
0.35
0.30
5 min

(Figure6B)