INHIBITORY EFFECT OF AN ANTI-LIPOOXYGENASE AGENT ON IMMUNOGLOBULIN G-MEDIATED ANAPHYLACTIC CONTRACTION OF SINGLE SMOOTH MUSCLE CELLS

Kayo Nemoto and Tadao Okamura

Department of Pharmacology, Nippon Medical School

Intracellular signal transduction during anaphylactic contractions of smooth muscle and the site of signal generation, or antigen-antibody reaction, participating in the appearance of these contractions were studied. The taenia coli was removed from a guinea-pig which had previously been sensitized with anti-egg albumin (EA) rabbit serum or anti-EA rabbit immunoglobulin G (IgG) fraction. Dispersed smooth muscle cells, obtained after enzymatic digestion, were used as the specimen.

The dispersed single smooth muscle cells sensitized with anti-EA rabbit IgG fraction showed anaphylactic contractions in response to antigen administration. The method using enzyme-linked antibody revealed binding of IgG by these single muscle cells. Anaphylactic contractions of the single cells were not inhibited by antihistamines or anti-5-hydroxytryptamine (5HT) agents, but they were suppressed by Y-19,432 [1-butyl-5-hydroxy-2-methyl-N-[1-(2-phenylethyl)-4-piperidyl] indole-3-carboxamide hydrochloride hydrate], an inhibitor of 5-lipoxygenase.

The anaphylactic contractions of the single smooth muscle cells of the guinea-pig taenia coli appear to be initiated in response to IgG binding with the antigen bound to the smooth muscle plasma membrane. For contraction development, the lipoxygenase pathway was shown to participate as a part of the intracellular signal transduction.

Key words: anaphylactic contraction — guinea-pig taenia coli — IgG — lipoxygenase — single smooth muscle cell

INTRODUCTION

Experiments observing anaphylactic contractions of isolated smooth muscle cells are useful to elucidate the mechanism of smooth muscle contraction by Schultz-Dale reactions at the cellular level. The fact that single smooth muscle cells obtained from immunized animals contract upon exposure to antigen in vitro, strongly suggests that antigen-antibody reactions occur on the surface of the muscle cells, which in turn trigger the contraction. However, it is not known how antigen-cell antibody binding generates the biological signal to transduct information intracellularly, leading to the development of the anaphylactic contraction.

The rise of Ca^2+ concentration within the smooth muscle cell appears to cause the muscle contraction through a series of biochemical reactions involving activation of Ca^2+-phospholipid dependent protein kinase (C-kinase) and phosphorylation of Ca^2+-calmodulin dependent myosin light chain kinase (MLCK) and increase of actomyosin ATPase activity. Serhan et al. and Volpi et al. reported that the mechanism of the increase in intracellular Ca^2+ concentration may depend on the action of leukotriens B4 (LTB4), a metabolic product by 5-lipoxygenase, as an endogenous Ca^2+ ionophore. Facilitation of Ca^2+ uptake during anaphylactic contractions of the guinea-pig taenia coli has also been reported.

Based on these findings, we studied the site of antigen-antibody reaction and type of antibodies related to signal generation using single smooth
muscle cells obtained from the guinea-pig taenia coli. Using Y-19,432 [1-butyl-5-hydroxy-2-methyl-N-[1-(2-phenylethyl)-4-piperidyl] indole-3-carboxamide hydrochloride hydrate] a 5-lipoxygenase inhibitor, the role of the lipoxygenase pathway as an intracellular signal transduction system was also studied.

MATERIALS AND METHODS

1. Preparation of dispersed cells:
Dispersed cells were prepared by the technique reported previously by Shonai et al. Briefly, taenia coli removed from a female Hartley guinea-pig weighing 300 to 400 g was minced and digested in modified Krebs' buffered solution containing 0.1% collagenase, 0.1% soybean trypsin inhibitor, 1% bovine serum albumin (BSA), and 1% Eagle MEM amino acids and vitamins at 31°C for 20 min. The buffered solution was adjusted to pH 7.4 and 297 m osmol. After digestion, the muscle segments were washed three times in this buffer containing 4% BSA. The cells were dispersed at 31°C for 20 min under a stream of 100% oxygen to obtain a cell suspension. The cell density was adjusted to $2 \times 10^6$/ml.

2. Measurement of dispersed cell contraction:
A 500 μl of the cell suspension was incubated at 31°C for 20 min with 0.01% neutral red to judge the viability of the dispersed cells. Thereafter, histamine, 5HT or antigen was added to the suspension to induce contraction. After a certain period, 2.5% glutaraldehyde was added to the suspension, which fixes cells at in situ length. In the experiments of antihistamines or anti-5HT agents, the cell suspension was incubated with one of these antagonists 3 min prior to the application of the agonists or antigen. In the case of 5-lipoxygenase inhibitor, the cell suspension was incubated with the inhibitor for a certain period before addition of the antigen. All the experiments were performed within one hour after cell dispersion.

The length of each of the 150–200 fixed cells was measured on a hemocytometer plate and the cells were grouped by indicated length to elucidate contractile response. The cells were divided approximately into 25 μm increments. The number of cells within each length increment was counted to prepare a cumulative frequency distribution curve (CFD curve). The extent of contractile response was assessed as a decrease in median cell length in the CFD curve. The decrease was expressed as a percentage change from the control cell length.

3. Preparation of antisera and procedure of passive immunization:
A mixture of equal amounts of complete Freund's adjuvant (CFA) and 1% egg albumin (EA) was subcutaneously injected into a rabbit. The anti-EA rabbit serum was far obtained was incubated with BSA for 2 hr at 37°C to adsorb antibodies cross reacting with BSA. The antisera was then centrifuged at 2,000 ×g for 10 min to remove the unwanted antibodies. The supernatant was intravenously administered to a guinea-pig at a dose of 4 ml/kg body weight as a passive sensitization. The taenia coli was removed 20 hr after the injection and dispersed as described above.

A mixture of 0.001% EA and 0.5% aluminium hydroxide gel was injected intraperitoneally at a dose of 4 ml/kg body weight into a guinea-pig. Using the prepared anti-EA guinea-pig serum or the anti-EA rabbit IgG fraction (20 mg protein/kg body weight), passive sensitization was performed. From the taenia coli, a muscle cell suspension was prepared according to the method described above. Administration of antigen to the single muscle cell preparation was performed at a final concentration of 1% EA.

4. Identification of antibodies bound to the surface of single smooth muscle cells:
A smear specimen of non-sensitized or sensitized single muscle cells was prepared on a glass slide for the identification of antibodies using the method for enzyme-linked antibody. To the non-sensitized single muscle cells, the smear was added either anti-EA rabbit IgG fraction (0.5 mg protein/ml) or anti-EA rabbit serum as the primary antibody. The mixture was incubated at 37°C for 30 min in a moist chamber, followed by the addition of biotinylated anti-rabbit Ig antibody as the link antibody. To the sensitized single
muscle cell smear specimen, obtained from guinea-pigs passively sensitized over 3 hr with either anti-EA rabbit IgG fraction or anti-EA rabbit serum, was added the link antibody. Streptavidin labelled with high affinity biotin-bound peroxidase was then added to the smear specimen incubated with the link antibody. Utilizing the peroxidase activity, the primary antibody binding sites were stained for observation by light microscopy.

5. Chemicals and statistics:

The chemicals used in the present study were as follows: BSA (fraction V), EA, soybean trypsin inhibitor (type I-S) and mepyramine (pyrilamine maleate, Sigma); collagenase (184 units/mg solid, Worthington); Eagle MEM amino acids and vitamins (Nissui); histamine dihydrochloride, neutral red and glutaraldehyde (Wako Pure Chemicals); 5HT (serotonin-creatinine sulfate, Daiichi Kagaku); diphenhydramine hydrochloride (Kowa); chlorpheniramine maleate (Sankyo); methysergide hydrogenmaleate (Sandoz); cyproheptadine hydrochloride (Nippon Merck Banyu); Y-19,432 (1-butyl-5-hydroxy-2-methyl-N-[1-(2-phenylethyl)-4-piperidyl] indole-3-carboxamide hydrochloride hydrate, Yoshitomi); anti-EA rabbit IgG fraction (Cappel); StrAviGen B-SA rabbit universal kit (BioGenex Lab.).

The data is expressed as the mean ± SE. Statistical analysis of the results was made by Student's t-test.

RESULTS

1. Time course of anaphylactic contractions in dispersed cells:

As previously reported11, addition of the antigen (1% EA) to the dispersed smooth muscle cells obtained from a passively sensitized guinea-pig resulted in a contraction comparable to that caused by $9 \times 10^{-7}$ to $9 \times 10^{-6}$ M histamine. This anaphylactic contraction began immediately after antigen administration and reached a maximum level 60 sec later (Fig. 1).

2. Effects of antihistamines, anti-5HT agents and

![Graph](image)

**Fig. 1.** Time course of contractile response induced by antigen. Single smooth muscle cells subjected to passive sensitization were incubated with antigen (1% EA) or with no additions (control) at 31°C for times specified. Cells were then fixed with 2.5% glutaraldehyde, and for each data point in experiment mean cell length of 150-200 cells was determined by micrometry. Contraction is expressed as percentage decrease in mean cell length from control. Each point represents the mean ± SE of 4 experiments.
a lipoxygenase inhibitor on anaphylactic contractions in dispersed cells:

Antihistamines and 5HT antagonists inhibited the development of contractions induced by histamine and 5HT, respectively, while they did not affect anaphylactic contractions (Fig. 2). On the other hand, when the cells were treated with $5 \times 10^{-5}$ M of Y-19,432 for 5 min, the development of contractions in response to antigen was inhibited by 50.7% of the control values.

As shown in Fig. 3, the effect of Y-19,432 was dependent on pretreatment-time with the cells. Significant inhibition of anaphylactic contractions was obtained when the cells were pretreated for more than 5 min. Pretreatment for 30 min resulted in suppression of as low as 5.18% of control values. The pD$_2$ values, as non-competitive antagonistic activity, after 5 and 10 min of pretreatment were 4.29 ± 0.14 and 4.48 ± 0.13, respectively.

The CFD curve of dispersed cells in response to the antigen shifted parallel to the right depending on the concentration of Y-19,432 for 10 min of pretreatment (Fig. 4).

3. Anaphylactic contraction of dispersed cells from guinea-pigs passively sensitized with IgG or IgE:

The addition of antigen (1% EA) to dispersed cells from a guinea-pig passively sensitized with anti-EA rabbit IgG fraction caused contractions resulting in anaphylactic contraction.

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**Fig. 2.** Inhibitory action of Y-19,432 on anaphylactic contractions of single smooth muscle cells. The ordinate indicates percentage of contractions induced by antigen, histamine or 5HT. A: effects of diphenhydramine ($3.9 \times 10^{-7}$ M), chlorpheniramine ($3.6 \times 10^{-8}$ M), mepyramine ($1 \times 10^{-8}$ M) and Y-19,432 ($5 \times 10^{-5}$ M) on antigen- or histamine-induced contractions. B: effects of methyserglide ($2.8 \times 10^{-6}$ M), cyproheptadine ($3.5 \times 10^{-7}$ M) and Y-19,432 ($5 \times 10^{-5}$ M) on antigen- or 5HT-induced contractions. Each point represents the mean ± SE of 3-5 experiments. (*) $p<0.05$ and (**) $p<0.01$, compared with the contractile response induced by histamine ($9 \times 10^{-7}$ M), 5HT ($5.7 \times 10^{-7}$ M) or antigen (1% EA).
Fig. 3. Anaphylactic contraction after incubation for various time-periods with Y-19,432. Dispersed cells were incubated with Y-19,432 (5 × 10^{-5} M) as an inhibitor of 5-lipoxygenase at 31°C for times specified, and then antigen (1% EA) was added to the suspension of single muscle cells. The ordinate indicates the anaphylactic contraction expressed as percentage in cell length after exposure to antigen for 60 sec. Each point represents the mean ± SE of 4 experiments. (*) $p<0.05$ and (**) $p<0.01$, compared with antigen-induced contraction.

Fig. 4. Cumulative frequency distribution curves of lengths of dispersed muscle cells incubated in the presence or absence of Y-19,432 during anaphylaxis. Cells from taenia coli of sensitized guinea-pigs were incubated with various concentrations of Y-19,432 for 10 min at 31°C, and then antigen (1% EA) added to the suspension of single muscle cells. Shift of the curves to the left indicates increase in numbers of the contracted cells. The abscissa is expressed as cell lengths divided into 25 μm increment. The ordinate indicates percentage of cells whose length was less than the given lengths on the abscissa. Each point represents the mean ± SE of 4 experiments.
similar to those cells sensitized with anti-EA rabbit serum (Fig. 5). After passive sensitization with anti-EA guinea-pig serum which probably consists of IgE (see Methods), administration of the antigen failed to cause a contraction in the dispersed cells (Fig. 5).

4. Identification of antibodies bound to the surface of isolated smooth muscle cells:

As the primary antibody, either anti-EA rabbit IgG fraction (Fig. 6A) or anti-EA rabbit serum (Fig. 6B) was added to the smear specimens of isolated smooth muscle cells from nonsensitized guinea-pig taenia coli. This was followed by the addition of the enzyme-linked antibody. The single smooth muscle cells were stained red in response to IgG binding with the antigen bound to the cells (Fig. 6A, B). Red staining was observed in smear specimens of single cells from guinea-pigs treated with either anti-EA rabbit IgG fraction (Fig. 6C) or anti-EA rabbit serum (Fig. 6D) for 3 hr.

**DISCUSSION**

Much still remains unknown about the mechanism of the development of the Schultz-Dale reaction using intestinal tissue specimens. In the present study, we have examined the development of contractions of single smooth muscle cells to elucidate the mechanisms of intracellular signal transduction leading to anaphylactic contractions. The fact that an anaphylactic contraction can be induced in single smooth muscle cells raises the question related to an exogenous factor or an intrinsic factor in the smooth muscle itself.10

At present, little is known about the mechanism in anaphylactic contractions by which the signal, generated by antigen-antibody reactions on the smooth muscle cell membrane, reaches the contractile element of the smooth muscle cell. Since intracellular Ca²⁺ rises during anaphylactic contractions of the taenia coli specimens,10 the main signal transduction system of this contractile reaction is assumed to be mediated by intracellular Ca²⁺ mobilization.

The present study confirmed the results pr-
Fig. 6. Photomicrographs of single smooth muscle cells stained with the enzyme-linked antibody. The dispersed cells from taenia coli were sensitized with anti-EA rabbit IgG fraction in vitro (A), in vivo (C) or with anti-EA rabbit serum in vitro (B), in vivo (D). The cell smear specimens were added by the biotinylated anti-rabbit immunoglobulins as the link antibody. In the four experimental conditions as to the combination of immunizations and antibodies above mentioned, each resultant photograph showed that anti-EA rabbit IgG binding sites on the cells were stained red in response to the link antibody.

Previously reported that antihistamines and anti-5HT agents blocked histamine- and 5HT-induced contractions, respectively, indicating the presence of H₁ and 5HT₂ receptors on single smooth muscle cell membranes. However, it was found that the blockers did not affect anaphylactic contractions. If it is assumed that anaphylactic contractions develop by a mechanism of contraction which differs from that of histamine- and 5HT-induced contraction, the difference in the contraction mechanism must lie in the antigen-antibody reaction site or the intracellular signal transduction system of the smooth muscle cell. At the present time, the signal transduction pathway for smooth muscle contraction is thought to involve phospholipase C—inositol triphosphate (IP₃)γ−¹−²—diacylglycerol (DG)—C kinase²−¹−² and C kinase—MLCK⁶−⁷ pathway. Each activation of these metabolic processes is related to an increase in intracellular Ca²⁺. Serhan et al¹¹ and Volpi et al¹², suggest the possibility that the Ca²⁺ ionophore-like action of LTB₄, a metabolic product of arachidonic acid, may play an important role in intracellular Ca²⁺ influx. Like LTB₄, lipoxin A₄, a metabolic product of arachidonic acid, produced by the activation of 5-lipoxygenase, was reported by Hansson et al¹² to activate C-kinase. Therefore, it is possible that the lipoxygenase pathway contributes to the development of anaphylactic contractions in the smooth muscle as an intracellular signal transduction system.

Besides having a strong antihistaminic activ-
Lipoxygenase and Anaphylactic Contraction

Y-19,432 was reported to inhibit fifty percent level of 5-lipoxygenase activity by means of the pretreatment with $10^{-5}$ M of the agent, not of 12-lipoxygenase or cyclooxygenase. Furthermore, the present experiment showed that $5 \times 10^{-5}$ M of Y-19,432 inhibited the development of contraction induced by histamine, 5HT and antigen respectively. In Fig. 2, anaphylactic contraction was strongly suppressed by Y-19,432. Thus, it is likely that this inhibitory action is not due to the antihistaminic or anti-5HT effect of Y-19,432. Some reports indicate the presence of the lipoxygenase pathway in vascular smooth muscle cells. Considering these reports, our experiment suggests that the 5-lipoxygenase pathway plays a role in the suppression of anaphylactic contractions by Y-19,432. This further suggests the possibility that the lipoxygenase pathway participates in the intracellular signal transduction system in guinea-pig taenia coli.

Though the concentration of Y-19,432 used in the present study was the same as that reported to inhibit more than 90% of 5-lipoxygenase activity in polymorphonuclear leukocytes from guinea-pig ascites, the suppression of anaphylactic contractions was incomplete (see Fig. 4). It is unlikely that the signal generated by antigen-antibody binding caused a MLCK phosphorylation reaction via the lipoxygenase pathway alone.

Anti-EA rabbit IgG was bound to the cell membranes of single smooth muscle cell (Fig. 6) and a contraction occurred in response to antigen exposure (Fig. 5). These results directly indicate the participation of IgG in Schultz-Dale reaction of Type I allergy. The site of antigen-antibody binding in Schultz-Dale reaction or the site of occurrence of signal in anaphylactic contractions was also shown to be on cell membranes of smooth muscle cells. Further studies are required to determine whether the mechanism of IgG binding to smooth muscle cells resembles the mechanism of binding between IgE and mast cells and basophils and lymphocytes.

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REFERENCES


IgG を介した単離平滑筋細胞のアナフィラキシー性収縮に
及ぼすリポキシゲナーゼ阻害薬の抑制作用

日本医科大学薬理
根本香代　岡村忠夫

平滑筋のアナフィラキシー性収縮（A収縮）発現に関与するシグナル発生部位と細胞内情報伝達について検討した。anti-egg albumin（EA）rabbit serum か anti-EA rabbit IgG fraction で被動的に感作したモルモットから盲腸縦を摘出し、酵素消化で得た平滑筋分散細胞を使用した。
anti-EA-rabbit IgG fraction 感作細胞は抗原で A収縮を示し、酵素抗体法により分散細胞への IgG の結合が確認できた。
分散平滑筋細胞の A収縮は抗ヒスタミン薬や抗5-HT薬で抑制されず、5-lipoxygenase 阻害薬 Y-19,432で抑制された。
以上から、モルモット盲腸縦平滑筋細胞の A収縮は平滑筋細胞膜に結合した IgG と抗原との結合によって収縮発現のシグナルを発生させると思われる。収縮発現の細胞内情報伝達には、lipoxygenase pathway が含まれる可能性のあることが示唆された。