

INTRACELLULAR SIGNALS IN IgG-MEDIATED ANAPHYLACTIC CONTRACTION
OF SINGLE SMOOTH MUSCLE CELLS

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Single smooth muscle cells from the taenia coli of guinea pigs passively sensitized with anti-egg albumin IgG fraction were used to evaluate the intracellular signal transduction system during anaphylactic contraction. ^{125}I -IgG was bound to single smooth muscle cells, and sensitized smooth muscle cells contracted in response to antigen treatment. In response to neuraminidase treatment, ^{125}I -IgG binding to the cells was decreased, along with an inhibition of anaphylactic contraction. Pretreatment with islet-activating protein (IAP), neomycin, quercetin and H-7 inhibited anaphylactic contraction, whereas pretreatment with phorbol 12, 13-dibutylate augmented the contraction.

The signal produced by the interaction between IgG bound to the smooth muscle cells and antigen appears to be transmitted to the intracellular contractile element via the signal transduction system involving G protein. Phospholipase-C and -A_2 appear to be the effectors in the signal transduction system for anaphylactic contractions. Protein kinase C, activated with reference to phospholipase-C, also may be involved in anaphylactic contraction.

Key words: anaphylactic contraction — G protein — guinea pig taenia coli — IgG — single smooth muscle cell

INTRODUCTION

Antigen-antibody reaction induced smooth muscle contractile response is thought to be caused by chemical mediators via $\text{IgE}^{(1)}$. $\text{IgG}^{(2)(3)}$ - and $\text{IgG}_1^{(4)(5)}$ -mediated anaphylactic contractions of airway smooth muscle and of single smooth muscle cells from guinea pigs passively sensitized by IgG have been reported $^{(6)(7)}$. The mode of transmission of the signal generated by the IgG-antigen interaction via the signal transduction pathway to

the contractile elements of smooth muscle remains unclear.

Recently, the role of guanine nucleotide-binding regulatory proteins (G protein) as a cell signal transducer through the actions of autacoids $^{(8)(9)}$, neurotransmitters $^{(10)(11)}$ and hormones $^{(12)(13)}$, has received attention with reference to the formation of second messengers and coupling between G protein and various effectors such as enzymes and ion channels $^{(14)}$. The present study was conducted to explore IgG participation in the signal formation which induces anaphylactic contraction of single smooth muscle cells from guinea pig taenia coli. In addition, the signal transduction system of the cell membrane involving G protein effectors and signal transmission to the intracellular contractile element was explored.

MATERIALS AND METHODS

1. *Passive sensitization of guinea pigs:*

Female Hartley strain guinea pigs weighing 300–400 g were intravenously injected with 20 mg/kg body weight of anti-egg albumin (EA) rabbit IgG. The time for sensitization was estab-

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Abbreviations: BSA bovine serum albumin; cAMP cyclic adenosine 3',5'-monophosphate; cGMP cyclic guanosine 3',5'-monophosphate; CFD curve cumulative frequency distribution curve; EA egg albumin; G protein guanine nucleotide-binding regulatory protein; H-7 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IAP islet-activating protein; Ig immunoglobulin; IP_3 inositol triphosphate; NAase neuraminidase; PCA passive cutaneous anaphylaxis; PDBu phorbol 12,13-dibutylate; PL-A_2 , -C phospholipase- A_2 , -C

lished based on the result of a heterologous PCA test conducted 3, 20, 48 and 168 hr after a subcutaneous injection of anti-EA rabbit IgG in the backs of the guinea pigs. The results of this preliminary experiment demonstrated the most intense response 3 hr after intracutaneous administration of IgG, and the time for passive sensitization was set at 3 hr. The experiments were started 3 hr after the intravenous injection using passively sensitized animals.

2. *Preparation of single smooth muscle cells:*

Passively sensitized and non-sensitized guinea pigs were sacrificed by a quick blow to the head and then exsanguinated. The method for preparing single smooth muscle cells was principally the same as previously described by the authors⁶⁾. Briefly, the taenia coli were removed and minced. The minced tissue was incubated twice at 31°C for 20 min in modified Krebs' buffered solution containing 0.1% collagenase and 0.1% soybean trypsin inhibitor in sequence. Then it was washed 3 times with the buffered solution containing 4% bovine serum albumin (BSA), followed by incubation in the buffered solution at 31°C for 20 min under 100% oxygen flow for dispersion. The concentration of the dispersed cells in suspension was adjusted to 2×10^5 cells/ml. Even though other tissue cells are mixed up with muscle cells in the preparation, they have no effect to the contractile response of smooth muscle cells as previously reported⁶⁾. The composition of modified Krebs' buffered solution was as follows (in mM): HEPES, 24.5; NaCl, 110.5; KCl, 5; CaCl₂, 1.8; MgCl₂, 1.2; NaH₂PO₄, 1; glucose, 11.5; glutamine, 2; sodium pyruvate, 5; sodium fumarate, 5; sodium glutamate, 5; 1% Eagle MEM amino acids and vitamins mixture and 0.1% BSA; pH 7.4, 297 mOsm/l.

3. *Measurement of the contractile response of single smooth muscle cells:*

To test viability, a 500 μ l of the cell suspension was incubated with 0.01% neutral red at 31°C for 20 min. Then, EA as an antigen at a final concentration of 1% was added to this suspension. Exactly 1 min later, the cells were fixed to stop the contractile response by the addition of 2.5% glutaraldehyde. The length of the fixed cells was measured on a hemocytometer, averaging 200–250

cells for one measurement.

In experiments in which some chemicals were used to dissect a mechanism underlying the anaphylactic contraction, the cell suspension was pretreated with a chemical, and the cells after addition of the antigen were fixed as described above.

The length of the fixed cells was measured, and a cumulative frequency distribution (CFD) curve for cell length was prepared by counting the number of cells in each 25 μ m length increment. The percent decrease in contractile response was calculated by indexing the median cell length in the antigen-sensitized group to that in the antigen-nonsensitized (control) group.

4. *Measurement of cyclic nucleotides:*

A 900 μ l of cell suspension was incubated with antigen or various chemicals. After 5 min of incubation at 31°C under 100% oxygen flow, 6% trichloroacetic acid was added to stop the reaction. This suspension was homogenized and centrifuged at 4°C for 20 min at 2000 $\times$ g. Diethylether was added to extract the supernatant, and after separation the upper ether layer was dried under a stream of nitrogen at 60°C. The residue was dissolved in 0.05 M acetate buffer 500 μ l and the solution was used for radioimmunoassay. Control samples were prepared using modified Krebs' buffered solution in the place of the sample. For radioimmunoassay of the cyclic nucleotides, ¹²⁵I-cAMP and ¹²⁵I-cGMP assay system were used.

5. *Detection of IgG binding to single smooth muscle cells:*

A 4.5 ml of cell suspension was incubated with 5×10^{-6} g/ml of neuraminidase (NAase) and the mixture was incubated at 31°C for 30 min under 100% oxygen flow. After centrifugation at 57 $\times$ g for 5 min, the cells were washed with modified Krebs' buffered solution to prepare a suspension of NAase-treated smooth muscle cells. A 600 μ l of this suspension was incubated with 3.7 KBq ¹²⁵I-goat anti-rabbit IgG/5.85 μ g protein at 31°C for 30 min. The cells were washed and centrifuged for 10 min at 2000 $\times$ g. This process was repeated four times. The bound ¹²⁵I-IgG was measured using a γ -ray counter (Beckman). The control cells were treated only with modified Krebs' buffered solu-

tion and measured using the same technique.

by Student's t-test.

6. Chemicals and statistics:

The chemicals used in the present study were as follows: islet-activating protein (IAP, Kaken); neuraminidase (0.6 U/mg protein, Baehringer Mannheim); H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, MBL]; neomycin sulfate, quercetin dihydrate, histamine dihydrochloride, theophylline, HEPES (Wako Pure Chemicals); 1-isoproterenol hydrochloride (Nikken Chemicals); phorbol 12, 13-dibutyrate, BSA (fraction V), EA, soybean trypsin inhibitor (type I-S, Sigma); cyclic nucleotides assay system (RPA509, RPA525, Amersham); ^{125}I -goat anti-rabbit IgG (ICN Biomedical Inc.); collagenase (184 U/mg, Worthington); anti-EA rabbit IgG fraction (Cappel); and Eagle MEM amino acids and vitamins (Nissui).

The data are expressed as the mean $\pm$ SE. Statistical analysis of the results was evaluated

RESULTS

1. Influence of islet-activating protein (IAP) on anaphylactic contractions:

The single smooth muscle cells prepared from the taenia coli of guinea pigs passively sensitized with anti-EA rabbit IgG contracted in response to antigen administration (Fig. 1). After a 120-minute pretreatment with IAP, anaphylactic contractions were significantly inhibited in a concentration-dependent manner (Fig. 1, 2). When the duration of pretreatment with 3×10^{-9} M IAP was less than 90 min, no contraction inhibition was noted (Fig. 3).

2. Intracellular cyclic nucleotides during anaphylactic contraction:

The basal levels of intracellular cAMP and

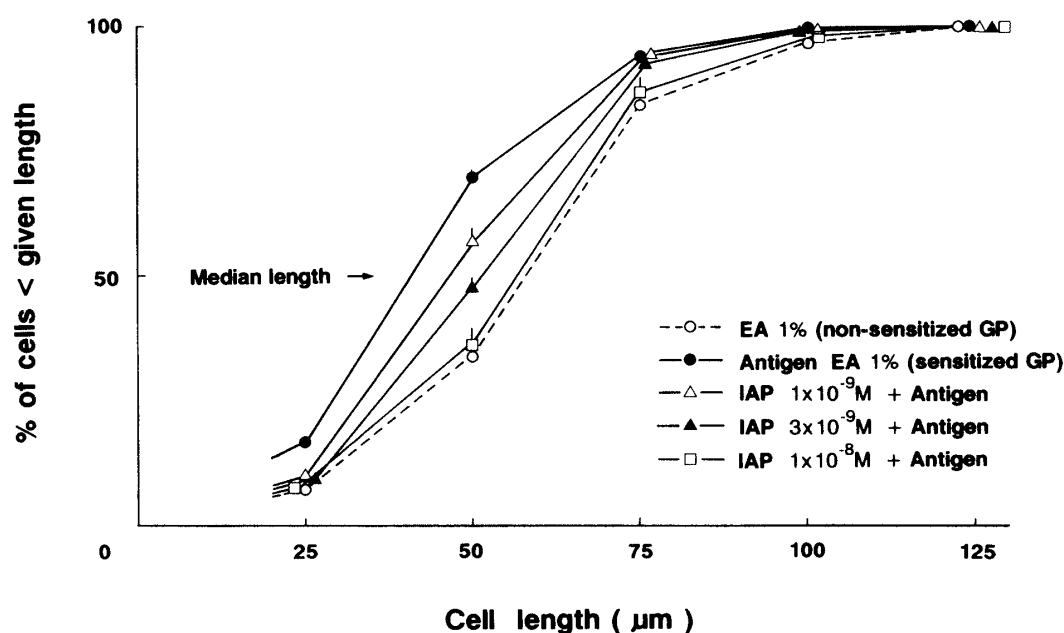


Fig. 1. Influence of islet-activating protein (IAP) on the anaphylactic contraction of single smooth muscle cells from the taenia coli of passively sensitized guinea pigs. The cumulative frequency distribution (CFD) curve of cell length after antigen administration (1% EA) is shifted to the left after a 120-minute IAP treatment. The CFD curve obtained after 1% EA administration to non-sensitized guinea pigs served as a control, because the curve had not significant difference from that obtained by the addition of modified Krebs' solution alone. The shift of curves to the left indicates the disappearance of relaxed cells and the appearance of a greater proportion of contracted cells. The decreased median cell length compared to the control shown by the arrow represents an anaphylactic contraction. Each point represents the mean $\pm$ SE of 4 experiments.

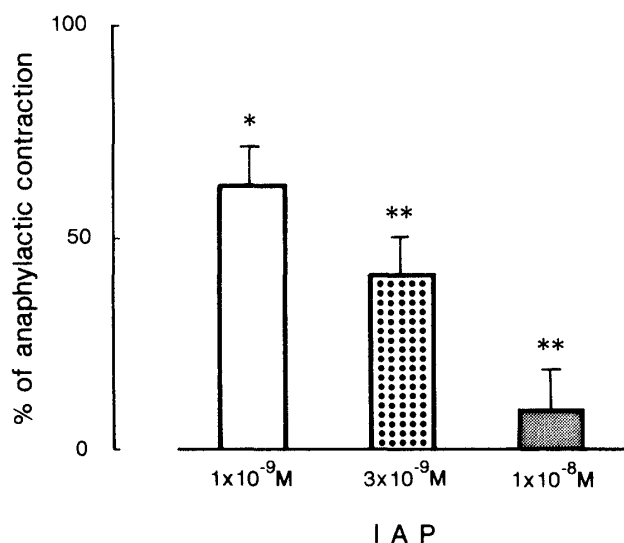


Fig. 2. Anaphylactic contraction inhibition by a 120-minute IAP pretreatment. The ordinate indicates the anaphylactic contraction expressed as a percentage in the median cell length after exposure to antigen for 1 min. Data represent the mean $\pm$ SE of 4 experiments. (*) $p < 0.05$ and (**) $p < 0.01$, compared with antigen-induced contraction.

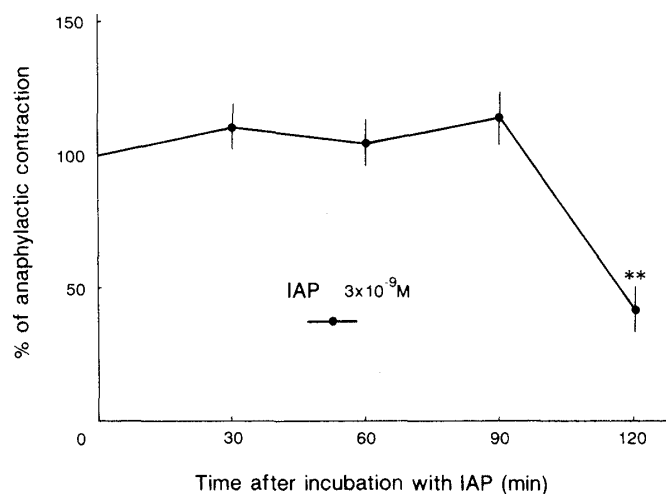


Fig. 3. Relationship of anaphylactic contraction inhibition to the duration of $3 \times 10^{-9} \text{ M}$ IAP pretreatment. The ordinate indicates the anaphylactic contraction expressed as a percentage in the median cell length after exposure to antigen for 1 min. Each point represents the mean $\pm$ SE of 4 experiments. (**) $p < 0.01$, compared with antigen-induced contraction.

cGMP in the sensitized single smooth muscle cells were 1.92 ± 0.21 and $7.99 \pm 0.37 \text{ pmol}/10^5 \text{ cells}$, respectively (Fig. 4). At 5 min after antigen administration, cAMP and cGMP contents were $2.21 \pm$

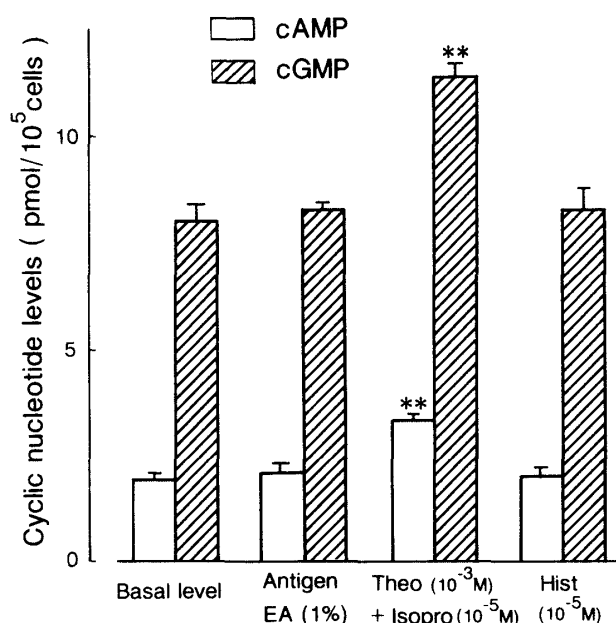


Fig. 4. Cyclic nucleotide content in single smooth muscle cells measured by radioimmunoassay after a 5-minute treatment with antigen, theophylline and histamine and a 10-minute treatment with isoproterenol. Control values (basal levels) were obtained after treatment with modified Krebs' buffered solution. Data represent the mean $\pm$ SE of 4-6 experiments. (**) $p < 0.01$, compared with the basal level.

0.15 and $8.92 \pm 0.14 \text{ pmol}/10^5 \text{ cells}$, respectively, with no significant difference from basal levels. Treatment with 10^{-5} M histamine resulted in no change in the cyclic nucleotide content. Contrarily, 10^{-5} M isoproterenol resulted in a significant increase of cyclic nucleotide content ($p < 0.01$) in the cells pretreated with 10^{-3} M theophylline.

3. Influence of various enzyme inhibitors and an activator on anaphylactic contraction:

The antigen-induced contraction of the sensitized single smooth muscle cells was significantly inhibited ($p < 0.01$) in a concentration-dependent manner by a 10-minute pretreatment with neomycin, a phospholipase-C inhibitor (Fig. 5A). Anaphylactic contraction was also inhibited by pretreatment with quercetin, a phospholipase- A_2 and lipoxygenase inhibitor (Fig. 5B); H-7, a protein kinase C inhibitor (Fig. 6A); or NAase, which dissociates sialic acid bound to the ends of cell membrane sugar chains (Fig. 7). Pretreatment for 10 min with $1 \times 10^{-7} \text{ M}$ phorbol 12, 13-dibutyrate

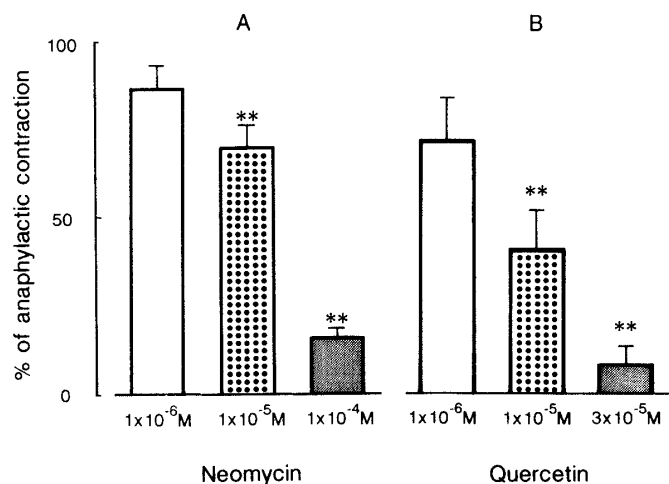


Fig. 5. Anaphylactic contraction inhibition by a 10-minute pretreatment of neomycin or quercetin. A: inhibitory action of neomycin on anaphylactic contraction of single smooth muscle cells. B: inhibitory action of quercetin on the contraction. Data represent the mean $\pm$ SE of 4 experiments. (**) $p < 0.01$, compared with antigen-induced contraction.

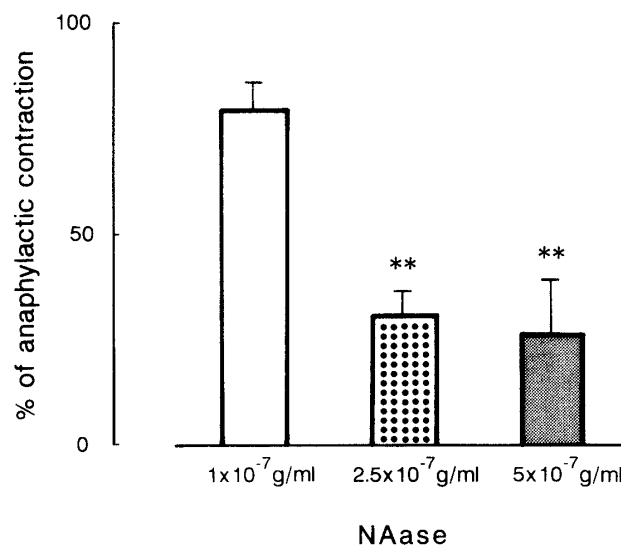


Fig. 7. Anaphylactic contraction inhibition by a 30-minute neuraminidase (NAase) pretreatment. Data represent the mean $\pm$ SE of 3-4 experiments. (**) $p < 0.01$, compared with antigen-induced contraction.

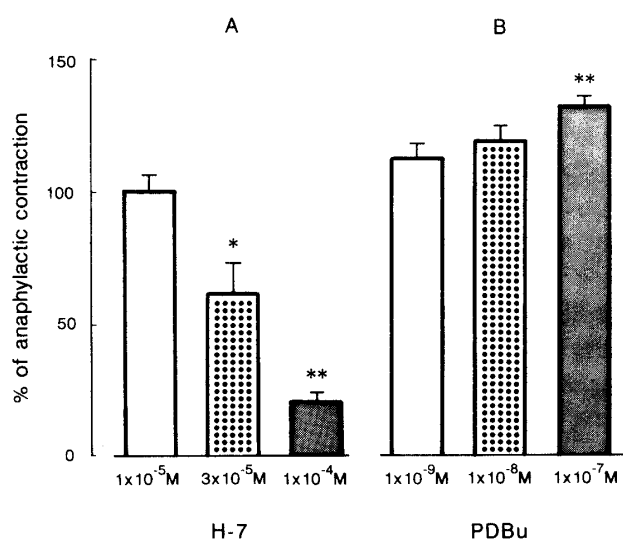


Fig. 6. Effects of H-7 and phorbol 12, 13-dibutylate (PDBu) on anaphylactic contraction of single smooth muscle cells. A: anaphylactic contraction inhibition by a 20-minute H-7 pretreatment. B: anaphylactic contraction augmentation by a 10-minute PDBu pretreatment. Data represent the mean $\pm$ SE of 4 experiments. (*) $p < 0.05$ and (**) $p < 0.01$, compared with antigen-induced contraction.

(PDBu), a protein kinase C activator significantly augmented anaphylactic contractions (Fig. 6B). Table 1 summarizes the concentration of chemicals which inhibited anaphylactic contractions to

Table 1 IC_{50} values of various chemicals on the anaphylactic contraction of single smooth muscle cells

Chemical	IC_{50}
IAP	$(1.91 \pm 0.40) \times 10^{-9}$ M
Neomycin	$(1.51 \pm 0.46) \times 10^{-5}$ M
Quercetin	$(4.07 \pm 1.14) \times 10^{-6}$ M
H-7	$(3.39 \pm 0.79) \times 10^{-5}$ M
NAase	$(2.63 \pm 0.80) \times 10^{-7}$ g/ml

IC_{50} : concentration of each chemical required to cause 50% inhibition of anaphylactic contraction of single smooth muscle cells from passively sensitized guinea pigs. Data represent the mean $\pm$ SE of 3-4 experiments.

50% (IC_{50}).

4. IgG binding to single smooth muscle cells:

By incubation of 10^4 single smooth muscle cells and 5.85 pg ^{125} I-IgG for 30 min, 1.27 pg IgG protein was bound to the cells, representing about 22% binding (Table 2). This IgG binding was significantly decreased after treatment with 5×10^{-6} g/ml NAase for 30 min. When the dispersed cells from taenia coli were sensitized with anti-EA rabbit IgG in vitro or in vivo, the addition of ^{125} I-goat anti-rabbit IgG to the cells caused radioactive binding similar to non-sensitized cells (data not

Table 2 Inhibitory effect of neuraminidase (NAase) on ^{125}I -IgG binding to single smooth muscle cells

	^{125}I -IgG binding (pg protein/ 10^4 cells)
Control	1.27 ± 0.53
NAase (5×10^{-6} g/ml)	$0.67 \pm 0.31^*$

After incubation of 1×10^4 cells with 5.85 pg ^{125}I -goat anti-rabbit IgG for 30 min, free ^{125}I -IgG was removed and the bound IgG was measured. Data represent the mean $\pm$ SE of 4 experiments. (*) $p < 0.05$, compared with control.

shown).

DISCUSSION

Anaphylactic contraction of guinea pig taenia coli is the result of activation of intracellular contractile protein in response to a signal generated by the interaction between antigen and antibody on the cell membrane⁷⁾. The transmission pathway of this signal to the contractile element in the smooth muscle remains unclear.

The first step toward understanding the cellular signal transduction of anaphylactic contraction is signal generation. The possibility that IgG participates in the formation of this signal has been previously reported by the authors^{6,7)}. In the present study, even though the value of ^{125}I -IgG bound to the cells includes the non-specific binding, 22% of ^{125}I -IgG was bound to the single smooth muscle cells. Inhibition of ^{125}I -IgG binding by NAase pretreatment suggests a role of sialic acid¹⁵⁾ occupying a terminal position on cell membrane glycoproteins. A difference, however, was noted between the NAase concentration which inhibited ^{125}I -IgG binding by 50% and the concentration which inhibited anaphylactic contraction by 50% (Fig. 7, Table 1, 2). By separating sialic acid from the cells, which contributes to the negative charge of the cell membrane, NAase decreases IgG binding below that required to cause signal formation at the cell membrane and inhibits anaphylactic contraction. Other unknown mechanisms which inhibit anaphylactic contractions may also participate.

G protein incorporated into the cell membrane

acts as a transducer for signals from hormones and neurotransmitters, activating effectors to form second messengers and participating in the cellular response⁸⁾⁻¹³⁾. The signal transduction unit including G protein transforms the agonist's transmembrane signals and couples them with effectors such as adenylate cyclase^{16,17)}, cGMP phosphodiesterase¹⁸⁾, phospholipase-C¹⁸⁾, -A_2 ¹¹⁾, K^+ -channel¹⁹⁾ or Ca^{2+} -channel²⁰⁾. IAP is a powerful probe to test the participation of G protein in the cellular response²¹⁾. IAP, internalized within the cell to ADP-ribosylate the G protein^{22,23)}, uncouples the receptor-G protein connection, although it requires a considerable amount of time to accomplish this action. In the present study, an approximately 120-minute incubation time after IAP pretreatment was required to inhibit anaphylactic contractions. The eventual inhibition, however, suggests the participation of G protein in the signal transduction pathway which induces anaphylactic contractions.

Neither an increase nor a decrease of cyclic nucleotides during anaphylactic contraction was demonstrated, which indicated an extremely low possibility that adenylate cyclase and cGMP phosphodiesterase were the effectors in the signal transduction unit. G_i and G_o proteins, which represent a subclass related to these 2 effectors^{24,25)}, were also unlikely candidates as effectors for this signal transduction unit.

Phospholipase-C (PL-C) and -A_2 (PL- A_2) have been proposed as effectors for the signal transduction unit^{11,26)}. PL-C activation stimulates Ca^{2+} -release from the intracellular Ca^{2+} store via IP_3 production²⁷⁾, inducing smooth muscle cell contraction. PL- A_2 activation is also related to the contraction via lipoxygenase activation^{7,28)}. Neomycin²⁹⁾ and quercetin³⁰⁾, which inhibit these two effectors, inhibited anaphylactic contraction in the present study (Fig. 5), which suggests that they may be the effectors in the signal transduction unit for anaphylactic contraction. Though we studied PL- A_2 inhibitory effect of quercetin on anaphylactic contraction based on the report³⁰⁾, some reports indicate other actions of quercetin such as suppression of ATPase activity³¹⁾ and phosphodiesterase activity^{32,33)}. It is unlikely that quercetin causes the inhibition of anaphylactic contraction via the suppression of PL- A_2 activity

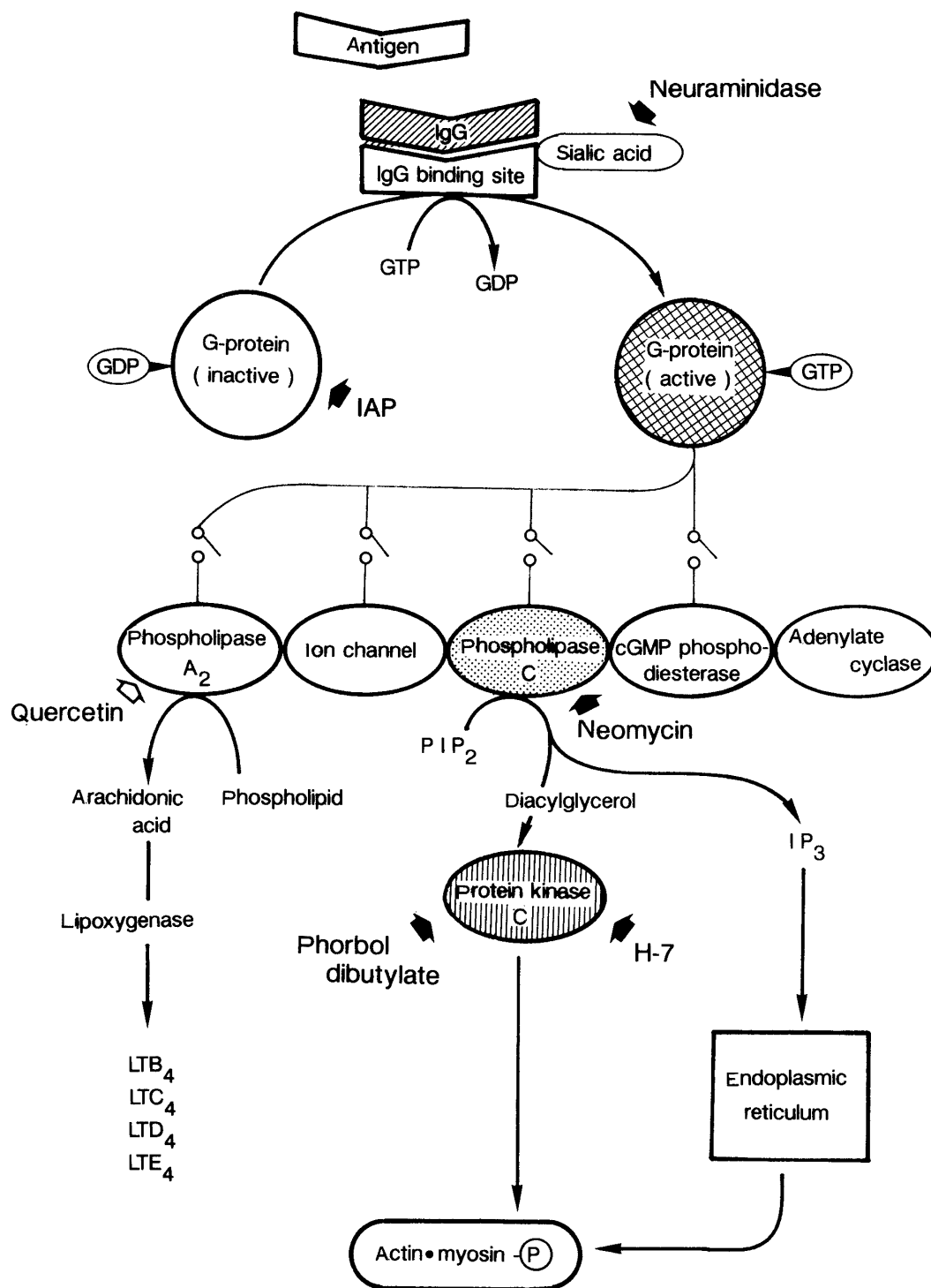


Fig. 8. Possible pathways for intracellular signals of anaphylactic contraction. The sites of action of neuraminidase, IAP, neomycin, quercetin, H-7 and phorbol dibutyrate are indicated by the arrows. See text for explanations.

alone. Considering IAP inhibition of anaphylactic contraction (Fig. 2), the signal transduction pathway for the contraction may be constructed from the signal based on antigen-IgG interaction, G protein-PL-C and G protein-PL-A₂ (Fig. 8). Since

protein kinase C participates in smooth muscle contraction via contractile protein phosphorylation, protein kinase C inhibited by H-7³⁴⁾ and activated by PDBu³⁵⁾, which may be a part of the anaphylactic contraction signal transduction

pathway with reference to PL-C, even though protein kinase C is not an effector of the intracellular signal transduction unit.

K⁺- and Ca²⁺-channels also may represent effectors for the transduction unit¹⁴⁾¹⁹⁾²⁰⁾. Anaphylactic contraction sensitive to the K⁺ concentration³⁶⁾ in the external fluid and facilitated by Ca²⁺ turnover³⁷⁾ may require the presence of G protein to activate ion-channel effectors. Data suggesting such a sequence of events are currently not available and further study is necessary. The mode by which IgG binds to the smooth muscle cell membrane and forms the transmembrane signal triggering anaphylactic contractions remains an interesting question.

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単離平滑筋細胞の IgG を介したアナフィラキシー性収縮 における細胞内情報伝達

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anti-egg albumin IgG で被動性に感作したモルモットの盲腸紐から分散調整した単離平滑筋細胞を用い、アナフィラキシー性収縮 (A 収縮) 時における細胞内情報転換系について検討した。

^{125}I -IgG は単離平滑筋細胞に結合し、IgG で感作した平滑筋細胞は抗原処置により短縮を示した (A 収縮)。 ^{125}I -IgG の細胞への結合は neuraminidase で抑制され、A 収縮も抑制された。また、islet-activating protein (IAP), neomycin, quercetin, H-7 の前処置によって A 収縮は抑制され、phorbol 12,13-dibutyrate によって A 収縮は増強された。

以上の結果から、盲腸紐単離平滑筋細胞に結合した IgG と抗原との反応で発生したシグナルは、細胞膜の G 蛋白質の情報転換機能を介して細胞内収縮要素に伝達されることが示唆された。また、phospholipase-C, -A_2 は A 収縮における細胞内情報転換系のエフェクターである可能性があり、phospholipase-C に連関して活性化される C キナーゼも A 収縮に関与すると推定された。
