

Induction of granule release by intracellular application of calcium and guanosine-5'-O-(3-thiotriphosphate) in human eosinophils

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The roles of Ca and G proteins in granule release from human eosinophils were examined by use of a patch-clamp technique in single cells. The morphologic changes and the release of eosinophil peroxidase (EPO) from single cells were simultaneously observed. In addition, the expression of small molecular weight guanine nucleotide binding protein (small G protein) mRNA (smg p25A [rab 3] and smg p21 [rab 1]) was investigated. The intracellular application of Ca, 10 $\mu\text{mol/L}$, and guanosine-5'-O-(3-thiotriphosphate) (GTP- γ -S), 100 $\mu\text{mol/L}$, induced fusion of EPO containing granules with the surface membrane, which was associated with a marked increase in membrane capacitance. Ca alone caused a rapid granule release at an early stage of cell dialysis, but most granules still remained in a cluster. GTP- γ -S alone caused a gradual degranulation. Northern blot analysis revealed the definite expression of smg p21 mRNA with no appreciable expression of smg p25A. These results provide direct evidence of granule fusion by intracellular application of Ca and GTP- γ -S. In addition, Ca dependent proteins and G proteins act cooperatively in granule release, and these proteins likely regulate the different processes of degranulation. Furthermore, a protein, encoded by smg p21, may be involved in the granule release process in human eosinophils. (J ALLERGY CLIN IMMUNOL 1992;90:789-95.)

Key words: Human eosinophils, patch-clamp technique, degranulation, guanine nucleotide binding proteins, bronchial asthma

Human eosinophil is characterized by its content of eosin staining granules containing the four main proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN). Some of these proteins are found in the bronchial epithelial layer and bronchoalveolar lavage fluid as well as sputa from asthmatic patients. They indicate eosinophil accumulation and successive activation in the inflammatory lesion of the disease. In addition, these granule proteins have considerable potential toxicity to cells

Abbreviations used

MBP:	Major basic protein
ECP:	Eosinophil cationic protein
EPO:	Eosinophil peroxidase
EDN:	Eosinophil-derived neurotoxin
PAF:	Platelet-activating factor
LTB ₄ :	Leukotriene B ₄
GTP- γ -S:	Guanosine-5'-O-(3-thiotriphosphate)
Small G proteins:	Small molecular weight guanine nucleotide binding proteins
EGTA:	Ethyleneglycol bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid)
PIPES:	Piperazine-N,N'-bis(2-ethanesulfonic acid)
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic [acid]
DAB:	3,3'-diaminobenzidine tetrahydrochloride
SSC:	Sodium chloride and sodium citrate solution

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and tissues. Therefore the eosinophil granules are now regarded as potent proinflammatory substances that may have causal roles in the development of diseases such as asthma.¹⁻³

Since the regulation of granule release from eosinophils is clinically useful in asthma therapy, extensive studies have been made to investigate the releasing mechanism of granules.^{4,5} The increase in cytoplasmic Ca has been reported in guinea pig eosinophils stimulated with platelet-activating factor (PAF) and leukotriene B₄ (LTB₄).⁴ In addition, it has been reported that intracellular application of guanosine-5'-O-(3-thiotriphosphate) (GTP- γ -S) induces exocytotic granule fusion in guinea pig eosinophils.⁵ Therefore Ca-dependent processes and G proteins play major roles in granule release from eosinophils. However, the role of Ca and G proteins in the secretory process in human eosinophils is still not clear. The aim of this study is to investigate how Ca and G proteins regulate granule release in human eosinophils. For this purpose we continually observed morphologic changes and membrane fusion, using a light microscope and the capacitance measurement, respectively. In addition, we explored mRNA expression of small G proteins, which have been suggested to be important in the secretory process of mammalian cells.⁶⁻⁸

MATERIAL AND METHODS

Subjects

Fifteen patients with extrinsic asthma (eight men and seven women) participated in this study. All patients had a history of episodic dyspnea with wheezing and bronchial hyperresponsiveness to inhaled methacholine.⁹ All asthmatic patients underwent intradermal skin tests for 26 individual allergens and were sensitive to at least two allergens. The subjects received bronchodilator therapy (theophylline derivatives and β -sympathomimetic agents) but no treatment with steroids. None had had respiratory tract infections for at least 4 weeks before blood sampling. Informed consent was obtained from all patients.

Preparation of polymorphonuclear leukocytes

By use of a dextran sedimentation method, the leukocyte-enriched preparation was collected and washed three times with piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) solution containing (mmol/L): NaCl, 120; KCl, 5; PIPES, 25; and 0.03% human albumin (pH 7.3).

Elutriator centrifugation of eosinophils

The cell suspension was injected into an elutriator centrifugation system (J21C; Beckmann Instruments, Inc., Palo Alto, Calif.) with PIPES solution containing 13 mmol/L trisodium citrate.¹⁰ To separate the different cell populations, the rotor speed and flow rate were kept at 2000 rpm and 12 ml/min, respectively, for 20 minutes. Then the flow rate was increased to 17 ml/min. The procedure yielded 150 ml of a mononuclear cell suspension. Then another 150 ml of

a granulocyte-enriched cell preparation was obtained. The granulocyte-rich specimen was washed twice and resuspended in 3 ml of Percoll (Pharmacia, Uppsala, Sweden) solution (1.070 gm/ml) at a concentration of 7 to 12 $\times 10^7$ /ml.

Purification of eosinophils

The procedure was as described previously.¹¹ In brief, Percoll solutions of 1.112, 1.094, 1.088, and 1.076 gm/ml were prepared and layered successively into a 25 ml polycarbonate tube with use of a peristaltic pump. The tube was then centrifuged at 900g for 15 minutes (18°C), resulting in the separation of the suspension into three distinct bands. The lowest band was collected, washed three times with a PIPES solution, and resuspended in a RPMI 1640 solution at a concentration of 1 $\times 10^7$ /ml. The purity of specimens was evaluated by Wright-Giemsa staining, and it ranged from 90% to 98% (mean, 95%). The viability of eosinophils was greater than 95% by the trypan blue exclusion method. These eosinophils were normal density eosinophils.¹ Some neutrophils were clearly distinguished from eosinophils by their shape under a $\times 600$ or $\times 1500$ magnified light microscope.

Patch-clamp experiments

Patch-clamp experiments were performed as previously described.¹² In brief, 10 μ l of a cell suspension was transferred into a Petri dish with coverglass bottom. After a few minutes the dish was perfused with the standard external solution, containing (mmol/L): NaCl, 150; KCl, 4; Camethanesulfonate, 2; Mg-methanesulfonate, 1; N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 10; and glucose, 5.6 (pH 7.4). The whole-cell configuration of the patch-clamp technique was used to perform cell dialysis, with internal solutions containing (mmol/L): K-aspartate, 150; KCl, 4; NaCl, 10; MgATP, 1 (final concentration); HEPES, 10 with or without 100 μ mol/L GTP- γ -S (pH 7.2). Calcium ion was buffered from no Ca to 10 μ mol/L in the presence of a total of 4 mmol/L ethylene glycol bis(β -aminoethyl ether)-N,N',N'-tetra acetic acid (EGTA) as previously described,¹³ and magnesium ion was set at 2 mmol/L. A glass pipette with a tip resistance of 5 to 8 M Ω was coated with Sylgard (Dow Corning, Kana-gawa, Japan). All experiments were done at 37°C.

Morphologic examinations

Morphologic changes of eosinophils after cell dialysis were studied under a light microscope. We magnified specimens $\times 600$ in our initial experiments, and we later observed the morphologic changes at a magnification of $\times 1500$. At least three experiments of each condition were photographed before and after cell dialysis. Some samples were continuously photographed during the granule release. These pictures were analyzed after the experiments, and reproducible changes were described in the results.

Measurement of EPO activities from single eosinophils

The EPO activities were measured by use of a colorimetric assay, originally described by Strath et al.¹⁴ Dialysis



FIG. 1. Morphologic changes of human eosinophils induced by intracellular application of Ca and/or GTP- γ -S. Photomicrographs taken at indicated time after cell dialysis. The last pictures of each panel taken 3 minutes after application of a solution for an EPO assay. **A**, An eosinophil applied 10 μ mol/L Ca and 100 μ mol/L GTP- γ -S. **B**, An eosinophil dialyzed with 10 μ mol/L Ca alone without GTP- γ -S. **C**, An eosinophil applied 100 μ mol/L GTP- γ -S alone with no Ca. Bar corresponds to 10 μ m.

was performed on single eosinophils with various solutions. After morphologic changes and electrophysiologic examinations were simultaneously studied, bathing solutions were changed to a solution for the EPO assay, containing 0.2 mmol/L 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 2 mmol/L hydrogen peroxide in the standard external solution. Photomicrographs were taken 3, 5, and 10 minutes after exchanging the solution. The EPO activities were detected by the development of brown color either on the cell surface or in the cytoplasm. Preliminary experiments revealed that only extracellular EPO activities were detected at 3 minutes, with no appreciable development of intracellular granules. Therefore we evaluated EPO release from pictures taken at 3 minutes.

Capacitance measurement

To examine granule fusion and concomitant capacitance changes of the plasma membrane, time-resolved patch-clamp capacitance measurements were done according to the method described by Lindau and Neher.¹⁵ In brief, 20 consecutive voltage pulses of -5 mV and 2 msec duration were given every 30 seconds via the command input of a patch-clamp amplifier (EPC-7; List Electronics, Germany).

The liquid junctional potential was corrected before each experiment. The current responses were sampled at intervals of 10 μ sec after low-pass filtering at 30 kHz with a 48 dB/oct Bessel filter (P-85, NF Company, Tokyo, Japan). Membrane capacitance was determined from a single exponential fit of the current response, with use of a nonlinear least squares fitting program.

In our preliminary studies, the average initial capacitance of guinea pig eosinophils was 2.13 ± 0.15 pF (mean $\pm$ SE, $n = 7$), which was not appreciably different from that of normal guinea pig eosinophils reported previously (2.40 ± 0.17 pF).⁵ This indicates that our measurement is comparable with that of previous work.⁵

RNA preparation and Northern blot analysis

RNA was isolated as described elsewhere.^{16, 17} In brief, total RNA, isolated with RNazol method, was subjected to electrophoresis in a 1% agarose/2.2 mol/L formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham Japan, Japan), and hybridized to ³²P-labeled bovine *smg* p25A and *smg* p21 probes. In addition, human β -actin probe was hybridized as a positive control. The hybridization reaction was carried out for 16 to 24 hours at 42°C.

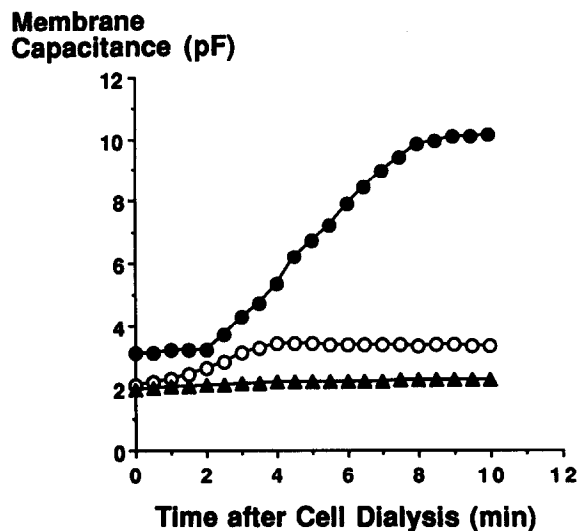


FIG. 2. Representative time course of membrane capacitance change induced by Ca and/or GTP- γ -S. Data obtained from the same samples as shown in Fig. 1. In *closed circles* (●), an eosinophil was dialyzed with 10 μ mol/L Ca and 100 μ mol/L GTP- γ -S. In *open circles* (○), a solution at a Ca concentration of 10 μ mol/L with no GTP- γ -S was applied intracellularly. In *closed triangles* (▲), a solution containing 100 μ mol/L GTP- γ -S with no Ca was applied.

in 25% (vol/vol) formamide, 2 $\times$ sodium chloride and sodium citrate solution (SSC: sodium chloride, 150 mmol/L; sodium citrate, 15 mmol/L), 1 $\times$ Denhardt's solution, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 200 μ g/ml, salmon sperm DNA. After hybridization, the membrane was washed twice with 2 $\times$ SSC at room temperature for 5 minutes and once in 0.1 $\times$ SSC at 60° C for 30 minutes. Autoradiography was performed at -70° C with an intensifying screen, by use of an x-ray film (RX, Fuji, Japan). Human brain samples were obtained by autopsy in accordance with the guideline of the Tohoku University ethics committee.

Statistical analyses

Values were tested by Kruskal-Wallis test and Mann-Whitney U test, considering $p < 0.05$ as significant.

RESULTS

In Fig. 1 the morphologic changes of eosinophils induced by intracellular application of Ca and/or GTP- γ -S are illustrated. When an eosinophil was dialyzed with a pipette solution containing both 10 μ mol/L Ca and GTP- γ -S, the morphologic change of the cell was very remarkable. The cell increased in size rapidly within 1 or 2 minutes. Then intracellular granules dispersed toward the cell membrane. These granules attached to the cell membrane, remaining for 10 to 30 seconds. The granules then fused with the cell membrane and disappeared (6 observations). These changes were associated with considerable loss

of eosinophil granules, resulting in a translucent cytoplasm (Fig. 1, A). To confirm that these changes were due to granule membrane fusion, we changed the bathing solution to one for a colorimetric assay of EPO activities. As illustrated in the last panel of Fig. 1, A, EPO activities were already developed on the cell surface by 3 minutes (3 observations). Since the development of the EPO activities from intracellular granules was considerably delayed, we concluded that EPO activities on the cell surface came from released enzymes. Therefore these results definitely indicate that intracellular application of Ca and GTP- γ -S causes granule release through membrane fusion in human eosinophils.

We next examined the effect of Ca and GTP- γ -S separately. When 10 μ mol/L Ca alone was applied, the cytoplasm close to the pipette initially became translucent because of granule membrane fusion to cell membrane. The cell increased in size initially at a slow speed and then at a somewhat faster speed in 5 to 7 minutes, but most of the granules remained in a cluster (6 observations, Fig. 1, B). The EPO activities were, however, not so prominent as those induced by Ca and GTP- γ -S (3 observations). When GTP- γ -S, 100 μ mol/L, alone was applied to an eosinophil through a pipette, the eosinophil increased gradually in size and intracellular granules dispersed slowly and almost uniformly for several minutes after the rupture of the patch membrane (Fig. 1, C). However, neither granule fusion (6 observations) nor EPO activities (3 observations) on the cell surface was appreciably detected.

Although the morphologic observations and colorimetric assays provided evidence of granule fusion by Ca and GTP- γ -S, these methods were not, however, highly sensitive to granule fusion. Since the membrane capacitance corresponds to the surface area of the cell membrane, the measurement of the membrane capacitance change is very sensitive to detect granule fusion.¹⁵ Therefore we measured membrane capacitance changes to quantify further the effects of Ca and GTP- γ -S on granule fusion. In Fig. 2 membrane capacitance changes obtained from the same specimens as in Fig. 1 are illustrated. When both 10 μ mol/L Ca and 100 μ mol/L GTP- γ -S are applied, the membrane capacitance is increased gradually for 1 to 2 minutes. The capacitance then increases threefold in 8 to 10 minutes in Fig. 2. Ca alone causes initial rapid increase, but the change soon reached a maximum. By contrast, the membrane capacitance gradually increases by application of GTP- γ -S, and the capacitance continues to increase for 8 to 10 minutes in Fig. 2. These results further confirm that intracellular application of Ca and GTP- γ -S can cause marked granule fusion. In addition, either Ca or GTP-

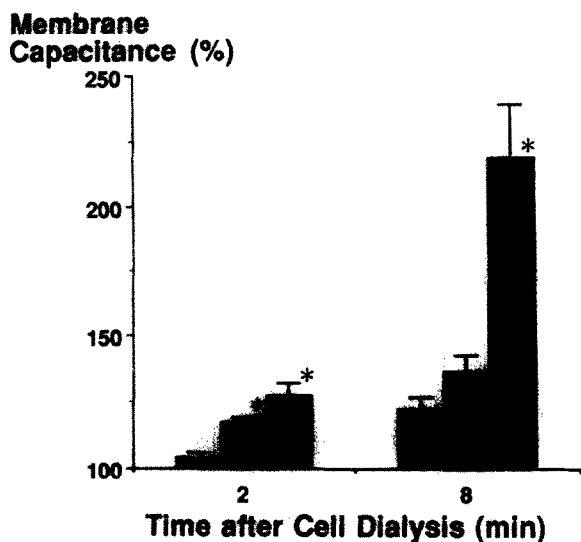


FIG. 3. Time dependent change of membrane capacitance, induced 10 $\mu\text{mol/L}$ Ca and/or 100 $\mu\text{mol/L}$ GTP- γ -S. *Closed columns* (shortest): GTP- γ -S alone ($n = 6$). *Hatched columns* (middle): Ca alone ($n = 8$). *Shaded columns* (tallest): Ca and GTP- γ -S ($n = 8$). Data were obtained 2 minutes (*left*) and 8 minutes (*right*) after cell dialysis. Each column is expressed as mean $\pm$ SE. * $p < 0.05$ as compared with GTP- γ -S alone.

γ -S alone induced certain granule fusion, but the time courses of granule fusion are different from each other. To examine further the time course of granule release, we applied either GTP- γ -S or Ca and measured the membrane capacitance at early (2 minute) and late (8 minute) stages of cell dialysis. When GTP- γ -S alone was applied, the initial change was very slight (Fig. 3). By contrast, the average membrane capacitances increased initially 17% and 28% with Ca alone and Ca + GTP- γ -S, respectively. Therefore these changes (at 2 minutes) were significantly larger than the average value induced by GTP- γ -S alone. At the late stage, GTP- γ -S evoked an additional increase in membrane capacitance, whereas Ca alone did not induce further increase in membrane capacitance.

Therefore final capacitance (at 8 minutes) was not appreciably different between GTP- γ -S alone and Ca alone (Fig. 3). These results indicate that Ca causes early rapid granule fusion, whereas GTP- γ -S induces late gradual increase in membrane capacitance.

We then applied various concentrations of Ca with or without GTP- γ -S and systematically studied the effects of these two compounds on membrane capacitance changes. The results of maximum capacitance change are summarized in Fig. 4. When dialysis was performed on eosinophils with a solution in the absence of Ca or GTP- γ -S, the granules showed initial vigorous movement, followed by a quiescent state (6 observations). The membrane capacitance decreased

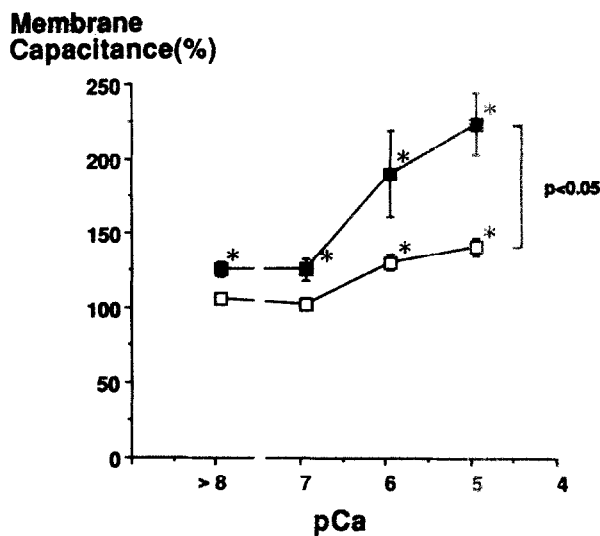


FIG. 4. Effects of Ca and GTP- γ -S on membrane capacitance change of eosinophils. Membrane capacitance changes, obtained in the presence or absence of GTP- γ -S expressed as a function of negative logarithmic of Ca (pCa). Data of no Ca are plotted at pCa more than 8. Data are plotted as percent change from initial membrane capacitance (100%). In *closed symbols* (■), 100 $\mu\text{mol/L}$ GTP- γ -S was applied together with indicated concentrations of Ca. In *open symbols* (□), no GTP- γ -S was applied. All data were taken 8 minutes after establishment of cell dialysis. Data are expressed as mean $\pm$ SE from 5 to 8 experiments. * $p < 0.05$ as compared with initial membrane capacitance (100%). Membrane capacitance changes in the presence or absence of GTP- γ -S at the same concentrations of Ca are compared, and the statistical differences are expressed in the figure.

in some eosinophils after cell dialysis (2 out of 6 experiments). By contrast, the capacitance was invariably increased by raising Ca concentration above 0.1 $\mu\text{mol/L}$, although the change was not so marked. The application of GTP- γ -S alone caused a slight increase in membrane capacitance. The addition of 0.1 $\mu\text{mol/L}$ Ca with GTP- γ -S did not have any further effect. The application of 1 $\mu\text{mol/L}$ Ca with GTP- γ -S caused variable effects. The membrane capacitance of most eosinophils increased very remarkably (5 experiments), but some eosinophils showed low responses (3 specimens). Some other eosinophils, however, ruptured within 2 to 3 minutes because of rapid increase in membrane capacitance (3 specimens). Since eosinophils from the same samples reacted differently, the variable responses were not due to the difference of subjects. The membrane capacitance increased strikingly when GTP- γ -S and 10 $\mu\text{mol/L}$ Ca were applied. The increase was considerably larger than the additive change induced by either GTP- γ -S or Ca alone. These results indicate that Ca and GTP- γ -S act synergistically on granule release.

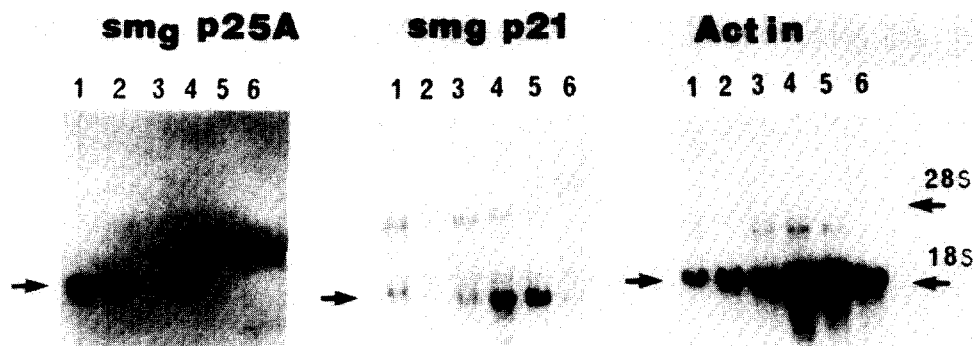


FIG. 5. Northern blot analysis of *smg p25A* and *smg p21* mRNAs. Electrophoresed RNAs were hybridized with the radiolabelled *smg p25A* (left panel) or *smg p21* (middle) cDNA. In the right panel the expression of β -actin mRNA is shown as a positive control. Lane 1, rat brain; lane 2, human brain; lane 3, human lung; lane 4, human eosinophils; lane 5, human eosinophils stimulated with PAF (1 μ mol/L); lane 6, guinea pig eosinophils. The 18s and 28s indicate ribosomal RNAs, used as internal size markers.

Recently small G proteins have been implicated in the pathway of exocytosis.¹⁸ Since *smg p25A* and *smg p21* are supposed to be involved in exocytosis in mammalian cells,⁶⁻⁸ we performed Northern blot analysis to ascertain whether mRNA of *smg p25A* or *smg p21* was expressed in human eosinophils. As described by Sano et al.⁷ *smg p25A* was strongly expressed in bovine brain. The *smg p25A* was not, however, appreciably expressed in human eosinophils, although the probe reacted with human brain samples. By contrast *smg p21* was strongly expressed in human eosinophils (Fig. 5).

DISCUSSION

Since eosinophil granules have been implicated as having a causal role in bronchial asthma, the regulatory mechanism of granule release has recently been intensively investigated.¹⁻⁵ When an eosinophil receives a stimulation with an activator such as PAF, an increase in cytoplasmic Ca is induced through activation of phospholipase C, followed by subsequent inositol triphosphate formation via G proteins.¹⁹ This indicates the involvement of Ca-dependent mechanisms in the secreting process.⁴ In several other cell types, however, Ca, depending on the conditions, is not mandatory for the secretory process, and the involvement of G protein (GE) in the secretory pathway has been emphasized.²⁰ Since intracellular application of GTP- γ -S can induce granule release, the primary role of GE protein in the secretory mechanism in eosinophils has recently been emphasized.⁵ The role of GE in exocytosis is, however, still controversial.²¹ In addition, it is still not clear how and at which stage Ca and G proteins play roles in the secretory process of eosinophils. To study these questions we applied

Ca and GTP- γ -S separately or simultaneously through patch pipettes and continually observed the morphologic changes as well as granule membrane fusion. In addition, we studied mRNA expression of small G proteins, which were regarded as important molecules in the secretory processes in mammalian cells.^{6-8, 18}

Findings in the present study were as follows: first, Ca and GTP- γ -S induced distinct granule membrane fusion, which was associated with marked increase in membrane capacitance. Second, either Ca alone or GTP- γ -S alone induced certain granule release, and their effects were synergistic. Third, Ca induced rapid granule release, whereas GTP- γ -S had rather gradual effects. Finally, mRNA of *smg p21* was definitely expressed in human eosinophils, but *smg p25A* mRNA was not found in appreciable amounts.

These results provide strong evidence of granule fusion induced by Ca and GTP- γ -S. In addition, both Ca and G protein-dependent mechanisms are likely involved in the granule release process in human eosinophils. Furthermore, the present results indicate reciprocal action between Ca and G protein-dependent mechanisms, because marked and synergistic granule release was observed with simultaneous applications of both GTP- γ -S and a high concentration of Ca. Therefore Ca and G protein-dependent mechanisms cooperatively work on granule release in human eosinophils. These results were characteristically different from those in guinea pig eosinophils, where prominent granule release can be induced by GTP- γ -S and therefore appears to be less Ca dependent.⁵

Continuous morphologic examinations, together with simultaneous measurement of membrane capacitance change, suggested additional aspects of Ca and G protein-dependent mechanisms in the granule re-

lease pathway. When a high concentration of Ca was applied in the absence of GTP- γ -S, initial rapid granule fusion was observed, whereas GTP- γ -S induced very gradual granule release. These morphologic observations were consistent with capacitance measurement where the initial capacitance change caused by Ca was considerably larger than that evoked by GTP- γ -S. In addition, granules tended to remain in a cluster after application of Ca, whereas granule translocation was marked when GTP- γ -S was applied. These results indicate that Ca and G proteins primarily act on different processes of granule release, although they work cooperatively on the releasing mechanism.

The role of GE protein has still not been clear but it is assumed to have an important role in exocytosis.¹⁷ Small G proteins have been recently implicated in the regulation of a variety of cell functions.⁶⁻⁸ The *smg* p21 has very homologous sequences with a protooncogene *ras* p21. In addition, *smg* p25A has been found in a variety of secretory cells such as the pancreas and nerve terminals.⁶⁻⁸ Therefore we explored the expression of these mRNAs in human eosinophils. We found that mRNA of *smg* p21 was definitely expressed in human eosinophils, but *smg* p25A was not appreciably observed. Although the physiologic function of *smg* p21 was not determined in the present experiments, the encoded molecule may play an important role in the cell activity of human eosinophils.

Present results indicate that a Ca dependent process and G proteins work cooperatively in secretory pathways, and each molecule likely plays a different role in the course of exocytosis. Since the granule proteins have a considerable pathogenic role in bronchial asthma, the regulation of granule release is clinically important in asthma therapy. We hope that further studies will elucidate the precise roles of Ca and G proteins in the secretory processes of human eosinophils and will provide new strategies for bronchial asthma therapy in the near future.

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