

Eosinophil leukocyte degranulation in response to serum-opsonized beads: C5a and platelet-activating factor enhance ECP release, with roles for protein kinases A and C

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Background Eosinophils have a typical content of granule-bound, cytotoxic, cationic proteins which may, when released to the external milieu, play roles in diseases such as asthma and parasitic infestation. Therefore, we have investigated possible mechanisms by which their release is regulated in eosinophils.

Methods The enzyme-linked immunosorbent assay (ELISA) was used to detect released eosinophil cationic protein (ECP). Release of ECP was induced by serum-opsonized, nonphagocytosable Sephadex beads (SOS).

Results The complement fragment C5a and platelet-activating factor (PAF) were found to enhance ECP release in response to SOS in a dose-dependent fashion, and, contrary to previous reports, they were not found to act as secretagogues themselves on eosinophils in suspension. The role of protein kinase C (PKC) in eosinophil degranulation has been controversial. We found that ECP release induced by SOS was inhibited by the PKC inhibitors staurosporine and calphostin C. Activation of protein kinase A (PKA), by raising cAMP, also inhibited ECP release. Furthermore, pertussis toxin decreased ECP release on opsonized beads, indicating the involvement of pertussis-toxin-sensitive G proteins.

Conclusions C5a, and PAF enhance granule release, rather than acting as secretagogues themselves. PKC and PKA have opposing roles in the regulation of ECP release in response to SOS.

Key words: C5a; eosinophils; platelet-activating factor; protein kinase A; protein kinase C.

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Eosinophils are effector cells in the allergic inflammatory response seen in, for example, asthma and helminthic infestation (1). Eosinophils have a characteristic content of basic proteins stored in cytoplasmic granules (1), and one of these, eosinophil cationic protein (ECP), seems to be unique to eosinophils (2). The actions of this protein when released to the exterior, e.g., at a site of inflammation, may explain some roles of the eosinophil. Therefore, it is important to determine mechanisms involved in the regulation of eosinophil degranulation.

Binding of eosinophils to a surface by comple-

ment receptors induces a strong signal for degranulation and release of granule contents (3, 4). This binding involves complement receptors 1 (CR1/CD35) and 3 (CR3/CD11b/CD18), and the low-affinity receptor for IgG (CD32, Fc-gamma-RIII) (5, 6). Therefore, serum-opsonized beads can be used for attachment of eosinophils and investigation of degranulation (3, 7).

Cytokines, such as GM-CSF, interleukin (IL)-3, IL-5, and tumor necrosis factor- α (TNF- α), enhance eosinophil cytotoxicity (8–11), but they do not alone induce significant degranulation (4, 6, 12, 13). However, a short incubation with GM-CSF,

IL-3, IL-5, or TNF- α is followed by an increased response to a degranulation stimulus such as immunoglobulin-coated Sepharose (12) or opsonized Sephadex (6, 7, 13). This effect is termed "priming". The enhanced capability of eosinophils to bind opsonized particles after preincubation with the cytokines mentioned is inhibitable by antibodies to CR3 (14), indicating that CR3 is important in cytokine-primed binding of eosinophils to serum-opsonized particles. Priming may also partly be explained by an increased number of the complement receptors (15), leading to an increased ability of eosinophils to bind opsonized particles.

Unlike the cytokines mentioned above, other soluble agents with receptors on eosinophils have been reported to have secretory effects by themselves. Among these are platelet-activating factor (PAF) (16), which is produced by endothelial cells and eosinophils (17). PAF is a potent chemotactic factor (18), and is also capable of making eosinophils respond with an increased respiratory burst upon adherence to opsonized particles (19). One possible explanation of the priming effect may be an increased affinity for the ligand, enhancing the binding of opsonized particles (20). Eosinophils also have receptors for the complement fragment C5a (21), which acts as a secretagogue on cytochalasin B-treated eosinophils (22). It is also a potent chemotactic factor (23).

The protein kinase C (PKC) activator phorbol myristate acetate (PMA) was found to promote ECP release in single adherent eosinophils (4). As PMA bypasses steps involving membrane receptors, the physiologic relevance of these findings is unclear. The role of PKC in eosinophil degranulation is unclear. PKC inhibitors have actually been reported to enhance eosinophil degranulation (22). Activation of protein kinase A (PKA) by cAMP inhibits eosinophil degranulation induced by immunoglobulin-coated beads (24).

The role of pertussis-toxin-sensitive G proteins in eosinophil degranulation has been investigated. Pertussis toxin was found to block degranulation almost completely on immunoglobulin-coated beads (25). However, for immobilized IgG, the inhibition was only temporary, while release induced by immobilized IgA was permanently blocked during the period of observation. This indicates that different secretory signals involve different G proteins (25).

In the present work, the release of granule-bound ECP was investigated in eosinophils attached to serum-opsonized Sephadex. C5a and PAF were investigated for enhancing effects in this system. The roles of PKC and PKA were also investigated.

Material and methods

Special reagents

Recombinant human GM-CSF was obtained from British Biotechnology Ltd, Abingdon, UK. Recombinant human C5a, IBMX, pertussis toxin, and dibutyryl-cAMP were from Sigma, St Louis, MO, USA. PAF (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphoryl-choline)-C16 was from Bachem, Saffron Walden, UK. Pentoxifylline was a generous gift from Swedish Hoechst AB, Stockholm, Sweden. Staurosporine and calphostin C were supplied by Calbiochem, San Diego, CA, USA.

Isolation of eosinophils

Eosinophils were purified by negative selection by immunomagnetic beads, as previously described (26). In short, blood was collected in heparinized tubes from healthy volunteers after informed consent. After dextran sedimentation, leukocytes were washed twice in Hanks' balanced salt solution (HBSS) (Flow Lab., Irvinshire, UK) containing 1% fetal bovine serum (FBS) and 10 mg/ml DNase (Sigma, St Louis, MO, USA). Percoll (Pharmacia, Uppsala, Sweden) was used to prepare a two-layer, discontinuous gradient consisting of a lower solution of density 1.094 g/ml (4 ml) and an upper solution of density 1.082 g/ml (4 ml). Leukocytes (2 ml, 4×10^7 cells/ml) were applied and centrifuged (400 g) for 30 min at room temperature. Granulocytes in the lower interface were collected, washed twice, and resuspended in HBSS-FBS-DNase. Sterile magnetic polymer beads (Dynabeads M-450, Dynal, Oslo, Norway) coated with covalently bound, affinity-purified sheep antimouse IgG were mixed with a monoclonal antibody, mouse anti-CD16 (Immunotech SA, Marseille, France), which recognizes the Fc-gamma-III receptor on neutrophils. The mixture was incubated overnight at 4°C on a rotary mixer in order to attach the anti-CD16 antibody. Coated beads were retrieved with a magnet (Dynal magnetic particle concentrator, MPC 1, Dynal, Oslo, Norway), washed, and mixed with the granulocytes in a final volume of approximately 300 μ l, coated beads to granulocytes (10^7 cells/ml) at a ratio of 1:4 (cells/beads). The mixture was incubated for 25 min at 4°C on a rotary mixer to bind the neutrophils to the beads. HBSS-FBS-DNase was added to a total volume of 5 ml, and neutrophil-coated beads were removed magnetically. Erythrocytes were lysed with sterile water, and the remaining cells consisting of more than 95% eosinophils were resuspended in HBSS-FBS-DNase and counted in a Bürker chamber.

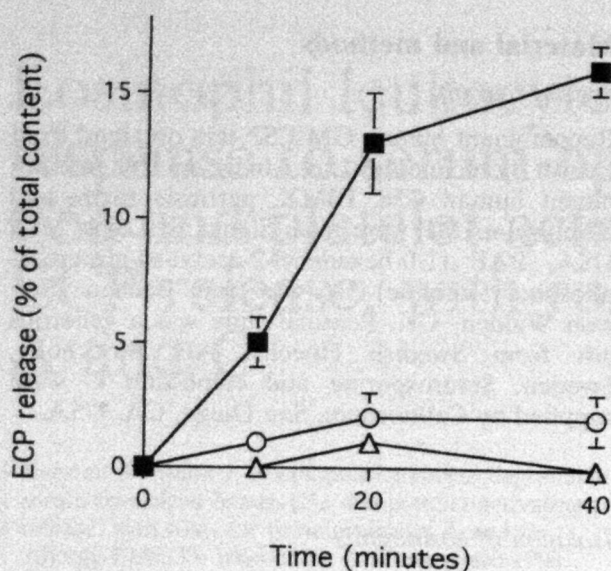


Fig. 1. Time-response curve for ECP release in response to SOS (■), nonopsonized Sephadex (○), or medium alone (△).

Contaminating cells were predominantly mononuclear cells.

ELISA for ECP

ECP in cell supernatants was measured by ELISA, as previously described (6, 27). The range of measurements using this ELISA was 0.1–50 ng ECP/ml.

Eosinophil degranulation

Sephadex G15 beads (Pharmacia) at 150 mg/ml were allowed to swell for 15 min in HBSS, and were collected after spontaneous sedimentation. A volume of 1 ml of freshly frozen human serum was added per 150 mg Sephadex (dry weight), and the mixture was incubated during rotation for 25 min at 37°C. Opsonized beads were washed three times and resuspended in HBSS to the initial volume. After agitation, 50 µl of the opsonized Sephadex beads was mixed with 150 µl of eosinophils (3.5×10^5 cells/ml). After incubation with various stimuli at 37°C, the reaction was stopped by centrifugation for 15 s in a Beckman Microfuge (Beckman Instruments, Palo Alto, CA, USA) at 11500 rpm (8370 g). The supernatants were removed and stored at -20°C until used for ELISA.

For determination of the total amount of ECP present in the cells at $t=0$, the cells were extracted in 0.2% hexadecyl-trimethyl-ammonium bromide (CTAB) for 5 min and centrifuged at 8370 g, and the resulting supernatants were analyzed.

Results

Incubation of eosinophils with serum-opsonized Sephadex resulted in extracellular release of approximately 16% of total cellular content of ECP within 40 min (Fig. 1). Incubation with nonopsonized Sephadex or medium alone caused little or no ECP release.

Enhancement of ECP release by C5a, PAF, and GM-CSF

In these experiments, eosinophils were preincubated with each agent followed by addition of serum-opsonized Sephadex particles (SOS). The time-course was determined for the preincubation and the dose-response relationship with a fixed preincubation time (60 min). None of these agents induced any detectable ECP release during preincubation of eosinophils in suspension, indicating an absence of any secretagogue effect (Fig. 2). C5a and PAF both showed dose-dependent enhancement of ECP release in response to SOS ranging from 137% to 166% (Fig. 2a and c). For comparison, GM-CSF at a concentration of 0.1 nmol/l, an established primer of eosinophil degranulation (7), caused ECP release of 147% ($\text{SEM} \pm 7\%$, $n=4$) in parallel experiments (not shown). The results from time-course experiments are more difficult to examine because a priming effect can also occur during the subsequent coincubation for 40 min of the agents with opsonized beads (Fig. 2b and d). To some extent, the latter will hide the effect of preincubation. In experiments where C5a or PAF was washed out after the preincubation, no enhancement of ECP release could be detected (data not shown). This indicates that the continuous presence of C5a or PAF is necessary for the enhancement in this system.

Thus, both C5a and PAF have enhancing effects on ECP release that are in the same order as the enhancement induced by GM-CSF. Neither C5a nor PAF induces significant ECP release by itself in this system.

Effect of PKC inhibitors

To investigate the role of PKC in eosinophil secretion, we used the PKC inhibitors staurosporine (28) and calphostin C (29). Fig. 3 shows that both inhibitors reduce ECP release in a dose-dependent fashion. This suggests a role for PKC in the mediation of eosinophil secretion on serum-opsonized particles.

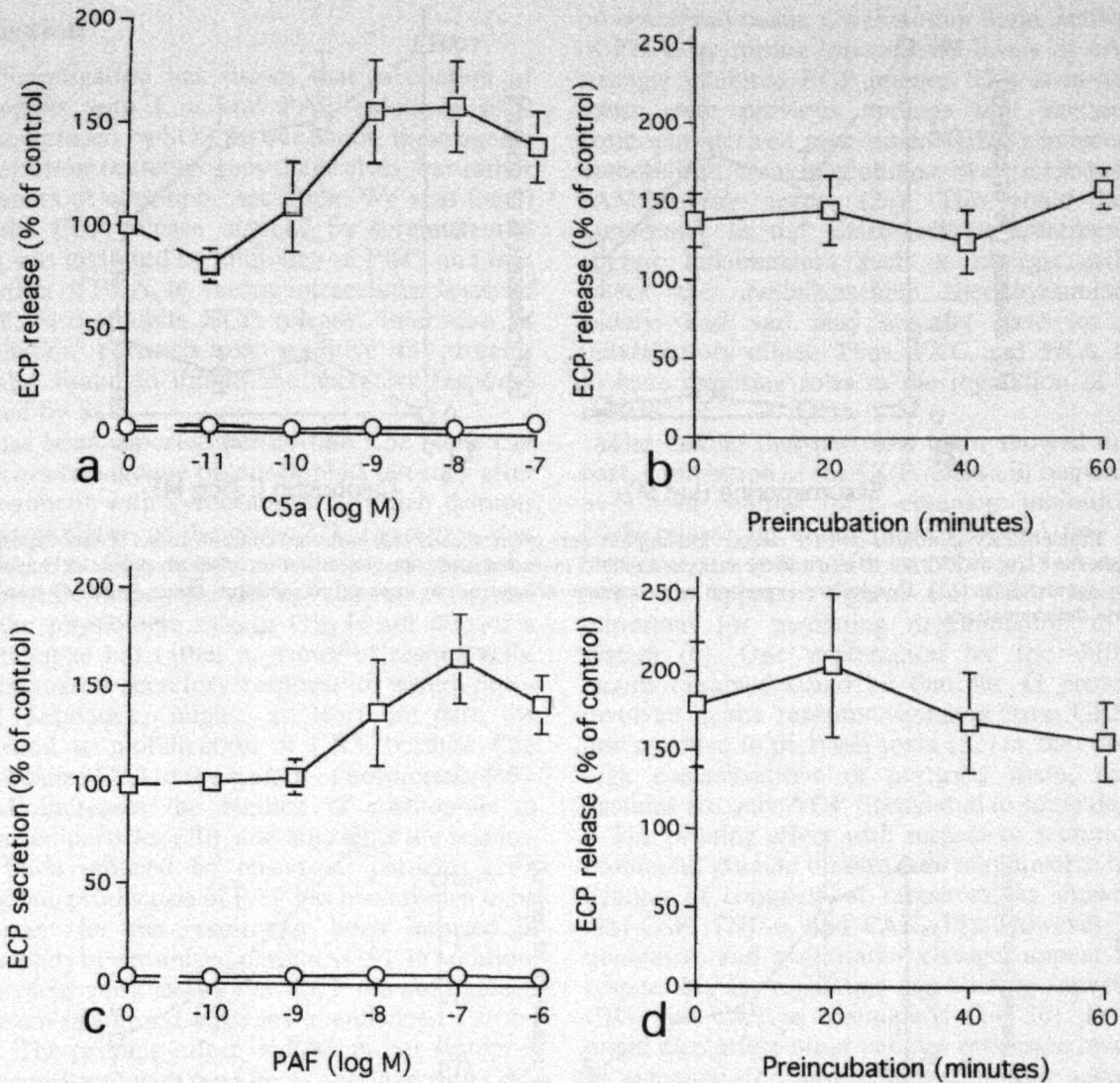


Fig. 2. Effect of various concentrations of C5a on ECP release induced by SOS. Cells were preincubated with C5a (a) or PAF (c) for 60 min and then incubated with SOS for 40 min in continued presence of agents at same concentration (□) or agents alone (○). Time course for enhancing effects of C5a (b) and PAF (d) on ECP secretion induced by SOS is also shown. Cells were preincubated with PAF (100 nmol/l) or C5a (10 nmol/l) for 0, 20, 40, or 60 min. Preincubation time 0 corresponds to results from exposure to opsonized Sephadex for 40 min without preincubation. Values given are expressed as percentage of control, preincubated with medium alone without agents, and then exposed to SOS. Mean ($\pm$ SEM) from three to five separate experiments.

Effect of cAMP-raising agents

IBMX and pentoxifylline are methylxanthines that indirectly cause increased cAMP levels by inhibiting the degradative enzyme, cAMP-phosphodiesterase. Eosinophils were preincubated with phosphodiesterase inhibitor before being exposed to SOS in the continued presence of phosphodiesterase inhibitor. Phosphodiesterase inhibitors reduced ECP release in a dose-dependent manner (Fig. 4a and b). In addition, cells were preincubated with dbcAMP for 15 min and mixed with SOS, in the continued presence of dbcAMP. The ECP release was inhibited in a dose-dependent

manner (Fig. 4c). Thus, cAMP elevations in eosinophils inhibit degranulation.

Effect of pertussis toxin

G proteins that mediate the signal from receptor to phospholipase C are uncoupled by pertussis toxin through ADP ribosylation. Cells incubated with pertussis toxin reduced their ECP release to $56 \pm 4\%$ (mean $\pm$ SEM from three separate experiments). This indicates that receptors with pertussis-sensitive G protein are involved in ECP release induced by SOS.

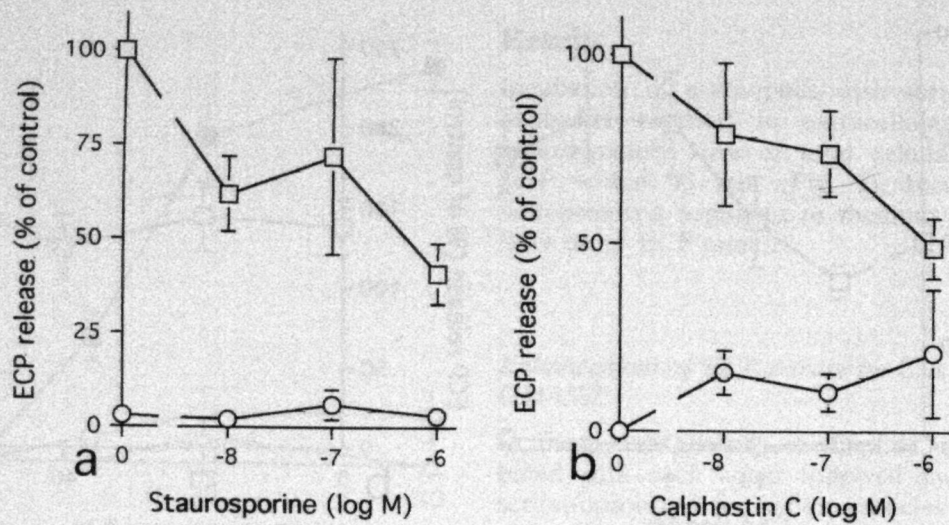


Fig. 3. Effect of PKC inhibitors on ECP release. Eosinophils were preincubated with indicated concentrations of staurosporine (a) or calphostin C (b) at 37°C for 15 min and then exposed to SOS in presence of same concentration of staurosporine or calphostin C (□) or agents alone (○). Results are expressed as percentage of control not exposed to inhibitor, mean ($\pm$ SEM) from three separate determinations.

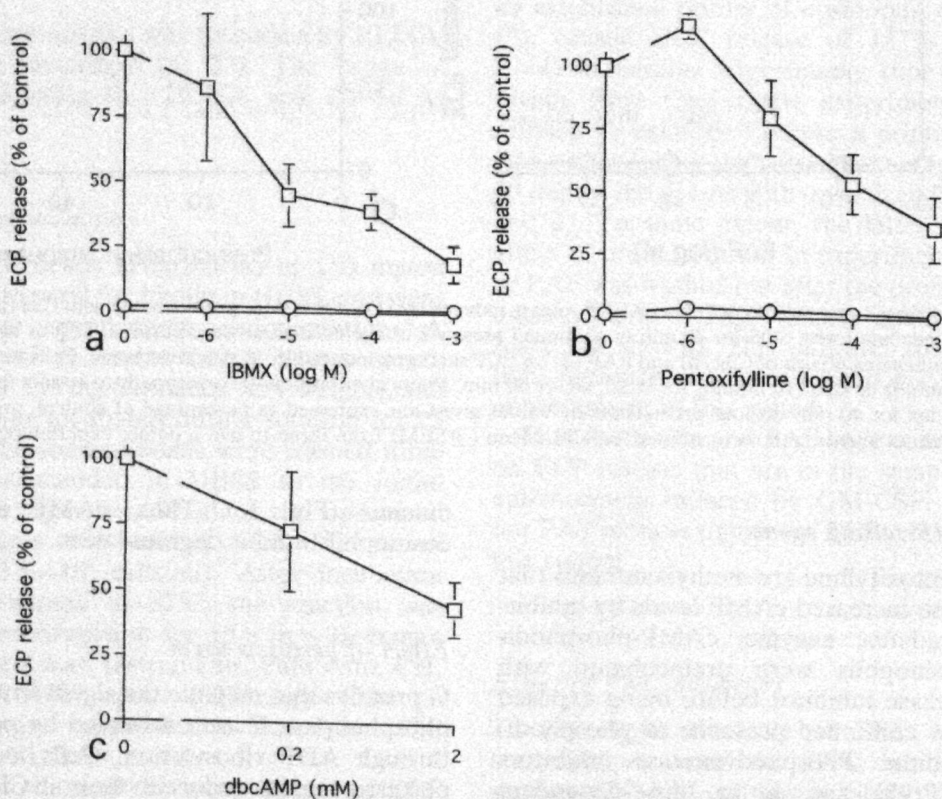


Fig. 4. Effect of cAMP-raising agents on ECP release induced by SOS. Cells were preincubated with IBMX (a) and pentoxifylline (b) for 5 min, and with dbcAMP (c) for 15 min at concentrations indicated, and then incubated with beads for 40 min in continued presence of these agents at unchanged concentrations (□) or agents alone (○). Results given are from three separate determinations, and are expressed as percentage of control not incubated with phosphodiesterase inhibitor, mean ($\pm$ SEM).

Discussion

This investigation has shown that incubation of eosinophils with C5a and PAF enhances ECP release induced by SOS. In our hands, these agents did not act as secretagogues themselves, but rather as primers of eosinophil secretion. We also found that the ECP release induced by serum-treated beads was inhibited by inhibitors of PKC, and that activation of PKA, by raising intracellular levels of cAMP, also inhibits ECP release. Inhibition of stimulatory, pertussis-toxin-sensitive G proteins was also found to inhibit the secretory response induced by SOS.

It has been reported before that C5a alone can act as a secretagogue on eosinophils but only after pretreatment with cytochalasin B, which disrupts the cytoskeleton of the cells (22). Therefore, it is possible that C5a, apart from having chemotactic properties, enhances several cellular functions, and that the physiologic role of C5a is not that of a secretagogue but rather a primer of resting cells. The increased secretory response to serum-opsonized Sephadex, might, at least in part, be attributed to mobilization of CR3, because C5a can mobilize CR3 to the surface of eosinophils (15).

PAF increases the binding of eosinophils to opsonized particles (20), and augments the respiratory burst induced by opsonized particles (19). Autocrine production of PAF has been shown to be important for the respiratory burst induced in eosinophils by opsonized particles (19). In addition, the present work shows that ECP release induced by serum-opsonized particles is enhanced also by PAF. The priming effect of PAF in our system is quite consistent with the effects obtained with GM-CSF and C5a. While we did not observe secretion by PAF alone of eosinophils in suspension, results from a previous study indicated that PAF can act as a secretagogue for eosinophils on its own (16). One possible explanation may be the use of different materials in test tubes. If a surface is supplied that promotes cellular adhesion, such as polystyrene, an increased binding via CR3 could cause degranulation. We used polypropylene tubes that may counteract adhesion.

We have shown before that activation of PKC by PMA caused ECP secretion in single adherent eosinophils (4). However, the role of PKC in eosinophil granule release has been questioned. In cytochalasin B-treated eosinophils, release of eosinophil peroxidase (EPO) was not inhibited by the PKC inhibitor staurosporine (22). Our present results show that the PKC inhibitors staurosporine and calphostin C both inhibit ECP release induced by opsonized beads. These results indicate that PKC can be involved in eosinophil degranulation

on opsonized beads. On the other hand, activation of PKA, by raising intracellular levels of cAMP, strongly inhibited ECP release. This is in accordance with previous findings that release of eosinophil-derived neurotoxin (EDN) induced by immobilized immunoglobulins was inhibited by cAMP-raising agents (24). This could be of importance in the pharmacologic treatment of allergic inflammation such as allergic asthma, where the methylxanthine theophyllamine is widely used and may actually have an anti-inflammatory effect. Thus, PKC and PKA seem to have opposing roles in the regulation of ECP release.

The finding that pertussis toxin showed only a partial inhibition of the ECP release in response to SOS is in contrast to its complete inhibition of EDN release on immobilized IgA and IgG (25). However, the system with immobilized IgA or IgG lacks an apparent ligand for CR3, which is important for mediating degranulation in our system (6). One explanation for the different results obtained could be that the G protein(s) involved in the receptor signaling from CR3 are less sensitive to pertussis toxin (32) or that despite high concentrations of pertussis toxin, the G proteins are only ADP ribosylated to some degree.

The priming effect with respect to secretion of eosinophil granule constituents may involve mobilization of complement receptors, as shown for GM-CSF, TNF- α , and PAF (15). However, both qualitative and quantitative changes appear to be responsible for regulating the binding capacity of CR1 and CR3 in eosinophils (14, 20). Priming might also affect other cellular processes involved in signaling for and effectuating the secretory response.

It is possible that C5a and PAF, which may be involved in the recruitment of eosinophils to sites of inflammation, are not secretagogues themselves, but rather primers of cellular functions. Neutrophils, which are inflammatory cells related to eosinophils, have several granule populations – for example, secretory vesicles and specific granules – which can be mobilized by soluble secretagogues (33). Among other things, these granules contain receptors of importance for effector functions. Azurophil granules of neutrophils contain cytotoxic proteins, and after neutrophil binding to microbes through complement receptors and Fc-receptors and subsequent phagocytosis, the content of these granules is released into the phagosome. To obtain release to the external milieu of azurophil granules, cytochalasin B is required in an experimental situation *in vitro* (34). In contrast, the specific granules of eosinophils can be released both to the external milieu and into phagosomes,

depending on the size of the target, after binding of the cell to the target by complement receptors and Fc-receptors (6). While the cationic proteins of eosinophils require high local concentrations to be cytotoxic (35), a close contact with the target is necessary, providing possibilities for the eosinophil to release its granule contents onto the surface of, for example, a parasite. Therefore, in the case of eosinophils, it seems doubtful that there are any soluble secretagogues of physiologic significance. To become secretagogues, most soluble substances that have been used *in vitro* require pretreatment of eosinophils with cytochalasin B (22, 24). Thus, several substances produced at the inflammatory event, such as C5a and PAF, enhance granule release and activate the cell, rather than acting as secretagogues themselves.

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