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Exocytosis of a complement component C3-like protein by tunicate hemocytes

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Abstract

This study investigates the exocytic responses of invertebrate hemocytes to pathogen-associated antigens. It demonstrates that a homologue of complement component C3, a key defensive protein of the innate immune system, is expressed by phagocytic hemocytes (non-refractile vacuolated cells) of the tunicate, *Styela plicata*. C3-like molecules are localized in subcellular vesicles and are rapidly exocytosed after stimulation with bacterial, fungal or algal cell surface molecules. Signal transduction analysis indicated that the induced secretion of C3-like molecules is mediated by a G-protein dependent signaling pathway, which modulates tubulin microtubules. All of this evidence indicates that hemocytes can contribute to host defense responses by rapidly exocytosing C3-like proteins at sites of infection.

Keywords: Tunicate; Ascidian; Complement; Exocytosis; Signal transduction

1. Introduction

It has been apparent since the pioneering experiments of Metchnikov at the end of the 19th century that mobile defensive cells in invertebrates migrate to sites of infection or wounding in order to establish inflammation-like responses [1]. For instance, hemocytes in the tunicate, *Styela plicata*, aggregate around, and migrate into, allogeneic tissue transplants [2]. Once within transplanted tissue those hemocytes undertake the cytolytic destruction of allogeneic cells. Similarly, the injection of particulate material (erythrocytes, oocytes, stromata or colloidal carbon) or soluble molecules (bovine serum albumin, hemoglobin or hemocyanin) into the epidermal tissue of another tunicate (*Ciona intestinalis*) leads to the active infiltration of macrophage-like hemocytes. Infiltrating hemocytes phagocytose or encapsulate the injected material. They also undergo rapid degranulation, presumably releasing a variety of defense-related molecules from intracellular stores

Abbreviations: anti-IgG-AP, alkaline phosphatase-conjugated anti-goat/sheep IgG; cAMP, cyclic adenosine mono-phosphate; FSW, sterile-filtered sea water; IP_3, inositol-1,4,5-triphosphate; LPS, lipopolysaccharide; MAC, marine anti-coagulant buffer; NHS, normal horse serum; NRVC, non-refractile vacuolated cells; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbol 13-myristate 12-acetate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TTCM, tunicate tissue culture medium.

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[3–6]. Observations of this type are common in studies of invertebrate host defense responses. However, cytophilic molecules that might direct the migration of defensive cells to sites of inflammation have only recently become apparent.

Complement-like proteins are among the obvious candidates for this role as pro-inflammatory mediators [7–12]. Homologues of C3, the central component of the complement cascade, have been characterized in the purple sea urchin, *Strongylocentrotus purpuratus* [7], and in the tunicates, *Halocynthia roretzi*, *S. plicata* and *C. intestinalis* [9,11,13]. Like their vertebrate counterparts, C3-like proteins from these deuterostome invertebrates are heterodimers comprising α and β chains. Their α chains incorporate thiolester bonds, which allow them to covalently bind foreign targets, like yeast cell walls [9,10,14,15]. In tunicates, activation of thiolester bonds is stimulated by the lectin-mediated recognition of pathogen-associated antigens [15,16]. Recognition results in the proteolytic cleavage of the α-chain by lectin-associated serine proteases, and the concomitant activation of thiolester bonds [16,17]. Sequence comparisons with vertebrate C3α chains suggest that the proteolysis of tunicate C3-like proteins forms two fragments—a large C3b-like molecule that mediates opsonization and a small (approximately 9 kDa) peptide akin to the vertebrate anaphylatoxin, C3a [9,16].

Recent experiments have shown that a C3a-like peptide from the tunicate, *Pyura stolonifera*, is a pro-inflammatory mediator [18]. It acts as a powerful chemotaxin that stimulates the migration of hemocytes. This suggests that C3a-like peptides generated by induced proteolysis in infected tissues may contribute to the recruitment of defensive hemocytes during inflammatory responses.

The current study demonstrates that the pro-inflammatory activity of tunicate C3-like proteins may be part of a positive feedback system, which establishes rapidly amplified and localized defensive responses. The experiments reported here show that tunicate phagocytes, of the type that are likely to be recruited to inflamed tissues by C3a-mediated chemotaxis, themselves contain C3-like proteins that can be actively exocytosed in response to pathogen-associated antigens.

2. Materials and methods

2.1. Antibodies and tunicate C3-like protein

S. plicata C3-like protein was detected with polyclonal goat anti-human C3 (anti-C3, Sigma Aldrich, Sydney, NSW). This antibody has previously been used to immunoaffinity purify C3-like protein from *S. plicata*, and can detect both the intact and reduced forms of the protein in Western blots. The identity of the *S. plicata* protein detected by anti-C3 has been confirmed by amino acid sequence and amino acid composition analysis, and by serological cross reactivity with C3-like proteins from another tunicate species, *H. roretzi* [11,15].

Normal goat immunoglobulin (normal goat Ig, Sigma-Aldrich) was used as an irrelevant primary antibody (negative control) in immunohistochemistry and ELISA. Purified human C3 was purchased from Sigma-Aldrich. *S. plicata* C3-like protein was immunoaffinity purified from hemolymph by the method of Raftos et al. [11].

2.2. Tunicates

S. plicata were collected from boat moorings and shark proof netting in Sydney Harbor with the permission of New South Wales Fisheries (Marine Research Permit #95067). The tunicates were kept in 40 l aerated aquaria (15 °C) and fed every second day with Marine IVF Invertebrate Diet (New Wave, Sydney, NSW).

Hemolymph was collected from single incisions in the buccal siphon into a 3-fold excess of ice-cold marine anti-coagulant buffer (MAC; 0.45 M sodium chloride, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 12.7 mM ethylenediamine tetraacetic acid, pH 7.0) and centrifuged (200*g*, 5 min, 4 °C). Individual tunicates were bled once, and then discarded. After centrifugation, supernatants were removed and the hemocytes re-suspended in MAC or sterile-filtered sea water (FSW; 0.45 μm filter). To obtain hemolymph serum for ELISA, whole hemolymph was collected in the absence of MAC and immediately centrifuged so that the supernatant (serum) could be decanted.

2.3. Immunohistochemistry

Hemocytes (30 μl, 1×10^6 cells/ml in MAC) were cytocentrifuged onto glass microscope slides at room temperature for 5 min (200*g*). Slides were then removed from the sample chambers and allowed to air dry before being fixed with 100% methanol at 4 °C for 20 min.

A Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA.) was used to immunohistochemically stain cytocentrifuged hemocytes according to the manufacturer's instructions. All incubations were carried out in a humidified chamber at room temperature and slides were washed for 5 min with phosphate buffered saline (PBS; 130 mM sodium chloride, 2 mM potassium chloride, 10 mM sodium hydrogen orthophosphate, 1.7 mM potassium dihydrogen orthophosphate, pH 7.2) between each step. Cytocentrifuged hemocytes were incubated with 0.3% (v/v) hydrogen peroxide in methanol (30 min), and blocked for 20 min with PBS containing 1.5% (v/v) heat-inactivated normal horse serum (PBS-NHS). PBS-NHS was removed before the cells were incubated with anti-C3 (1:1000 in PBS) for 30 min. Slides were incubated for 30 min with biotinylated rabbit anti-goat IgG (Vector Laboratories), and then for another 30 min with Vectastain Elite ABC Reagent. Cells were then stained with 3, 3′-diaminobenzidine for 5 min before being rinsed in tap water and counterstained with hematoxylin.

Bright field and differential interference contrast microscopy were used to examine immunohistochemically stained cells. Negative controls (no primary antibody or normal goat Ig as primary antibody) were included in all experiments to confirm the specificity of anti-C3 immunostaining.

2.4. Transmission electron microscopy

Hemocytes (5×10^6 in 1 ml MAC) were centrifuged (12,000*g*, 8 s) and resuspended in 4% (w/v) glutaraldehyde in FSW for 1 h. Fixed cells were then centrifuged (12,000*g*, 8 s) and the resulting pellet was mounted in 1% (v/v) molten, cooled agar in distilled water. The mounted pellet was fixed in osmium tetroxide and ultrathin sections were inspected with a Philips CM10 transmission electron microscope.

2.5. Exocytosis assay

Hemocytes were centrifuged (12,000*g*, 8 s) and resuspended at 4×10^6 cells/ml in tunicate tissue culture medium (TTCM; 20 ml RPMI 1640 containing 20% (w/v) sodium chloride in 180 ml FSW pH 7.0) [19]. This cell density is equivalent to that of whole hemolymph. A variety of microbial surface molecules were added to 200 μl of resuspended cells and incubated at room temperature for 1–15 min. Those molecules (5–50 μg/ml) were lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6, mannan from *Saccharomyces cerevisiae*, carrageenan λ from *Gigartina aciculaire* and *Gigartina pistillata*, and carrageenan κ from *Eucheuma cottonii*. All of these molecules were supplied by Sigma-Aldrich. After incubation, hemocyte cultures were centrifuged at 12,000*g* for 8 s so that culture supernatants could be analyzed by ELISA or Western blotting.

2.6. Signal transduction analysis

A range of pharmacological reagents that are known to either inhibit or stimulate G-protein-mediated exocytic signal transduction systems were used to determine if the release of C3-like molecules from stimulated hemocytes resulted from an inducible secretory pathway. Inhibitory reagents included those that are known to interfere with G-proteins (10 ng/ml cholera toxin, [20]), protein kinase C (PKC; 2 μM chelerythrine, [21]; 0.1 μM staurosporine, [22]), tubulin microtubule formation (0.25 mM colchicine, [23]) and F-actin polymerization (2.5 μg/ml cytochalasin D, [24]). Stimulatory reagents included ones that enhance the activity of adenylate cyclase (5 μM forskolin, [25]), PKC (1 μM phorbol 13-myristate 12-acetate, PMA, [26]) and intracellular calcium mobilization (5 μM calcimycin, [27]; 1 μM thapsigargin, [28]). All of these reagents were supplied by Sigma-Aldrich.

To test the effects of these reagents on the release of C3-like molecules, hemocytes were resuspended in TTCM at 4×10^6 cells/ml. Inhibitory reagents (cholera toxin, chelerythrine, staurosporine,

colchicine and cytochalasin D) were incubated with 200 μl of resuspended cells at room temperature for 15 min. Mannan (50 μg/ml) was then added for a further for 15 min. Activators of signaling pathways (forskolin, PMA, calcimycin, and thapsigargin) were also incubated with 200 μl hemocytes (4×10^6 cells/ml) for 15 min, but mannan was not added. In both cases, hemocytes were removed from incubates by centrifugation at 12,000*g* for 8 s and the remaining culture supernatants were assayed for C3-like protein by ELISA.

2.7. Anti-C3 ELISA

Hemocyte culture supernatants or hemolymph serum samples were aliquoted (100 μl/well) into EIA/RIA plates (96-well, flat bottom, polystyrene, high binding; Costar, Cambridge, MA) and proteins were allowed to adsorb overnight at 4 °C. In all subsequent steps, plates were washed three times with Tris-buffered saline (TBS; 10 mM Tris base, 150 mM sodium chloride, pH 7.5) and incubations were performed on a 96-well plate shaker. The plates were blocked with bovine serum albumin (5%, w/v in TBS; Roche Diagnostics) for 1 h before being incubated for 90 min with 100 μl/well of 1:2000 anti-C3 in TBS containing 0.2% (v/v) Tween 20 (TBS/Tween). Plates were then incubated for 90 min with 100 μl/well of alkaline phosphatase-conjugated anti-goat/sheep IgG (anti-IgG-AP, Sigma-Aldrich; diluted 1/10,000 v/v in TBS/Tween). Color development was achieved by adding 100 μl/well of 1 mg/ml 4-nitrophenyl phosphate (in 0.1 M glycine, 1 mM magnesium chloride, 1 mM zinc chloride, pH 10.4), and was quantified at 415 nm using an automated microplate reader. In all experiments, negative controls included wells without culture supernatant, and wells in which anti-C3 was omitted or replaced with normal goat Ig.

2.8. Western blotting

To confirm the identity of the anti-C3 reactive proteins detected by ELISA, supernatants from hemocytes stimulated for 5 min with LPS, mannan or carrageenan λ were subjected to western blot analysis. SDS-PAGE was performed according to the method of Laemmli [29]. Samples were prepared by heating to 100 °C for 5 min in SDS-PAGE sample buffer containing 10 mg/ml dithiothreitol. Electrophoresed proteins were blotted onto nitrocellulose (0.2 μm; Trans-Blot transfer medium, Bio-Rad, Sydney) at 12 V for 45 min using the semi-dry method of Khyse-Andersen [30].

After blotting, nitrocellulose membranes were washed with milli-Q water for 5 min. All subsequent incubations were carried out at room temperature on a rocker platform. Membranes were washed (3 × 5 min) with TBS between each step. Blots were first incubated in BSA (5%, w/v in TBS) for 2 h. Membranes were then incubated overnight with anti-C3 (1:2000 in TBS-Tween) before being incubated for 2 h with anti-IgG-AP (1:10,000 in TBS-Tween). Membranes were developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) according to the manufacturer's instructions.

2.9. Hemocytes viability assay

Hemocytes from exocytosis or signal transduction assays were suspended in 500 μl FSW after the final 12,000*g* centrifugation step from which culture supernatants were harvested. Two microliters ethidium bromide (Sigma-Aldrich) was added to the cell suspensions immediately before they were analyzed on a FACScan flow cytometer (Becton Dickenson, North Ryde, NSW). The frequency of dead cells (i.e. those that had taken up ethidium bromide) was determined in fluorescence histograms. Flow cytometry (forward vs. 90° light scatter plots) was also used to confirm that hemocyte culture supernatants did not contain residual cellular material.

2.10. Statistical analysis

The statistical significance of differences between mean values was determined by two-tailed Students *t*-tests for populations with unequal variance using the SPSS software package (SPSS Inc., Chicago, Ill). In cases where results are presented as percentages of control values, *t*-tests were preformed on the raw data (optical density units at 415 nm) before it was transformed to percentages.

3. Results

3.1. S. plicata C3 is localized in the vesicles of non-refractile vacuolated hemocytes

Immunohistochemistry revealed that C3-like proteins were located exclusively in the sub-cellular vesicles of hemocytes identified as non-refractile vacuolated cells (NRVC) by the classification scheme of Radford et al. [31] (Fig. 1A and B). Anti-C3-positive vesicles were often in close proximity to the plasmalemma of these cells, even though they were excluded from areas of the peripheral cytoplasm associated with pseudopodia (Fig. 1A and B). Transmission electron microscopy of NRVC revealed numerous electron dense vesicles within the peripheral cytoplasm (Fig. 1C). These vesicles were 0.63 ± 0.13 μm ($n = 168$) in diameter and often numbered more than 30 per hemocyte. They were enclosed in a bi-layer membrane and could be distinguished from the larger organelles evident in this cell type by the density and homogeneity of their inclusions.

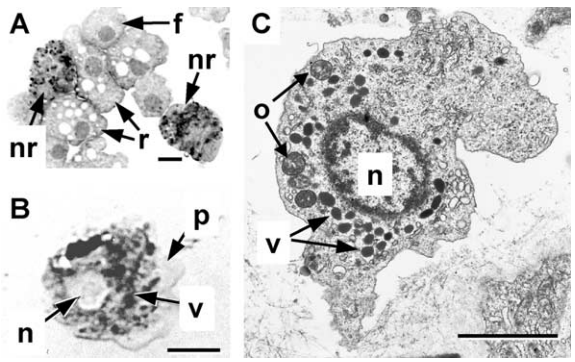

Fig. 1. (A) Bright field micrograph of hemocytes immunohistochemically stained with anti-C3 and counterstained with hematoxylin. Anti-C3-positive NRVC (*nr*) display granular cytoplasmic immunostaining. The other cells in the field include refractile vacuolated cells (*r*) and fine granular (*f*) hemocytes. Bar = 10 μm; (B) differential interference micrograph of a non-refractile vacuolated cell immunohistochemically stained with anti-C3. *v*, anti-C3 positive vesicles, *n*, nucleus, *p*, pseudopodium. Bar = 10 μm; (C) transmission electron micrograph of a non-refractile vacuolated cell showing numerous electron-dense vesicles and less frequent large organelles in the cytoplasm. *v*, electron-dense vesicles, *n*, nucleus, *o*, large organelles. Bar = 10 μm.

3.2. C3 secretion from hemocytes

Significantly more anti-C3 reactivity, relative to non-stimulated controls, could be detected by ELISA of culture supernatants from hemocytes that had been stimulated with LPS, carrageenans or mannan ($p < 0.05$ for all concentrations, except 10 μg/ml carrageenan-λ, vs. non-stimulated controls; Fig. 2). Responses to all of the molecules reached plateaus at concentrations of 50 μg/ml. Mannan was the most effective elicitor. It could stimulate the release of 5 times more C3-like protein than was evident in non-stimulated controls. Carrageenan-κ and LPS increased the secretion of C3-like molecules by three-fold, whilst maximal responses to carrageenan-λ were approximately two-fold those of controls. Flow cytometry of antigen-stimulated hemocytes that had been incubated with ethidium bromide showed that none of the stimulatory molecules significantly altered hemocyte viability, relative to non-stimulated controls, at any of the concentrations shown in Fig. 2 ($p > 0.05$; Table 1). Moreover, flow cytometric forward vs. 90° light scatter plots did not detect

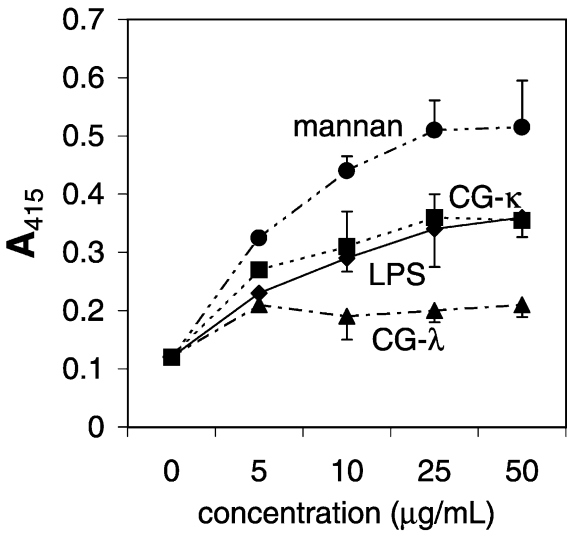

Fig. 2. Effect of different concentrations of mannan, LPS, carrageenan κ (CG-κ) and carrageenan λ (CG-λ) on the secretion of anti-C3 reactive proteins from cultured hemocytes. Culture supernatants were collected 15 min after the addition of the stimulators. The concentrations of anti-C3 reactive proteins were determined by ELISA and are shown as absorbance at 415 nm (A_{415}). Bars (where shown) = SEM, $n = 4$.

Table 1
Percentage of ethidium bromide positive hemocytes present in hemocyte cultures after 15 min incubations with various concentrations of LPS, mannan, carrageenan-κ (CG-κ) or carrageenan-λ (CG-λ) (mean ± SEM, $n = 4$)

Concentration (μg/ml)	Ethidium bromide positive hemocytes (%)			
	Mannan	LPS	CG-κ	CG-λ
0	5.9 ± 1.2	4.3 ± 1.1	4.6 ± 1.3	4.5 ± 1.2
5	4.9 ± 1.6	3.8 ± 1.7	4.4 ± 1.4	5.1 ± 1.2
10	5.0 ± 1.2	4.6 ± 1.3	2.9 ± 1.3	6.3 ± 1.5
25	4.2 ± 1.1	5.8 ± 1.1	3.9 ± 1.8	4.4 ± 0.9
50	5.2 ± 0.9	6.5 ± 1.5	4.8 ± 0.7	5.1 ± 0.8

residual cellular material in hemocyte culture supernatants (data not shown).

All of the culture supernatants tested contained substantially more C3-like protein than hemolymph serum samples. Fourteen times more C3-like protein was detected by ELISA in hemocyte culture supernatants stimulated with 50 μg/ml mannan ($A_{415} = 0.51 \pm 0.08$) than in serum ($A_{415} = 0.04 \pm 0.01$, $n = 10$).

The release of anti-C3 reactive proteins from hemocytes occurred rapidly after stimulation with LPS (Fig. 3). Within 1 min, more than twice as much

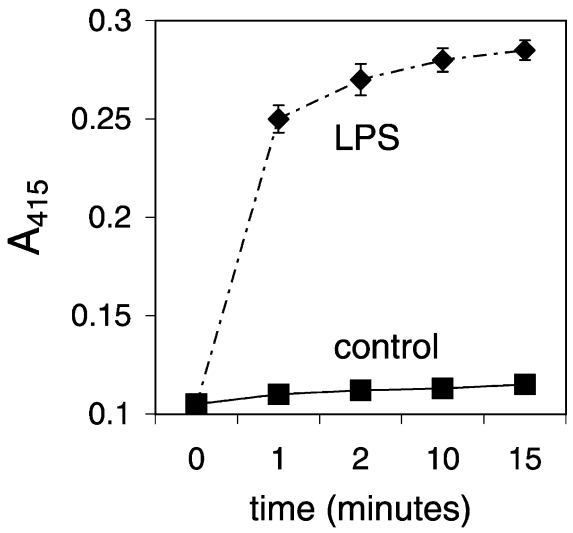

Fig. 3. Anti-C3 reactive protein content of supernatants removed from hemocytes at various times after the addition of 25 μg/ml LPS. LPS was not added to control hemocytes. The concentrations of anti-C3 reactive proteins were determined by ELISA and are shown as absorbance at 415 nm (A_{415}). Bars = SEM, $n = 4$.

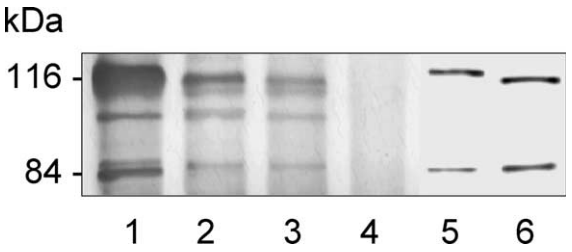

Fig. 4. Anti-C3 immunostained western blots of supernatants from hemocytes stimulated with 25 μg/ml mannan (lane 1), carrageenan κ (lane 2) or LPS (lane 3). Also shown are supernatant from unstimulated hemocytes (lane 4), purified human C3 (lane 5) and purified *S. plicata* C3-like protein (lane 6). The position of molecular weight markers is shown on the left.

anti-C3 reactivity was detected in culture supernatants from LPS-stimulated hemocytes compared to non-stimulated controls ($p < 0.05$). After this initial increase, the anti-C3 reactivity of culture supernatants increased more gradually over the remainder of the time course. It appeared to be approaching an asymptote within 15 min.

The identity of the anti-C3 reactive proteins detected by ELISA was confirmed by Western blot analysis of culture supernatants from hemocytes stimulated with mannan, LPS or carrageenan κ (Fig. 4). Western blots identified a number of anti-C3 reactive bands. Double bands were often evident at approximately 116 and 80 kDa and a single band appeared at 105 kDa. The bands at approximately 116 and 80 kDa corresponded in molecular weight to those in affinity purified human and *S. plicata* C3 preparations (Fig. 4). The identity of the 105 kDa band is unclear, but it may represent a proteolytic activation product of the 116 kDa polypeptide. The appearance of double bands at 116 and 80 kDa may also reflect limited proteolysis. In accordance with ELISA data, the anti-C3 reactive bands were most intense in mannan-stimulated supernatants. No anti-C3 staining could be detected in supernatants from non-stimulated hemocytes.

3.3. Effect of signal transduction modulators on C3 secretion

The release of anti-C3 reactive proteins from hemocytes was significantly suppressed ($p < 0.05$) by pharmacological reagents that are known to inhibit

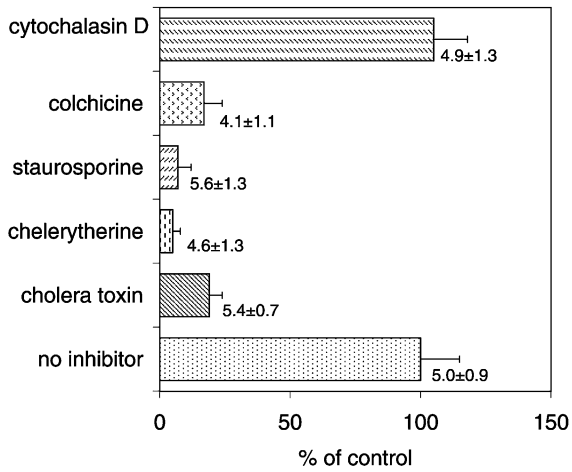

Fig. 5. The effects of signal transduction inhibitors on the release of anti-C3 reactive proteins from *S. plicata* hemocytes stimulated with 25 μg/ml mannan. The concentrations of anti-C3 reactive proteins were determined by ELISA and data are presented as percentages relative to the level of anti-C3 reactivity released from hemocytes that were stimulated with 25 μg/ml mannan in the absence of inhibitors (no inhibitor). Bars = SEM, $n = 6$. The numbers shown with each reagent represent the percentage of ethidium bromide-positive (non-viable) hemocytes present after incubation (mean ± SEM).

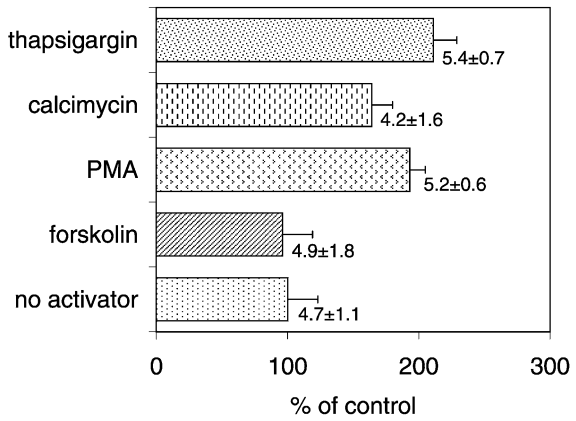

Fig. 6. The effects of signal transduction activators on the release of anti-C3 reactive proteins from *S. plicata* hemocytes. The concentrations of anti-C3 reactive proteins were determined by ELISA and data are presented as percentages relative to the level of anti-C3 reactivity released from hemocytes in the absence of activators (no activator). Bars = SEM, $n = 6$. The numbers shown with each reagent represent the percentage of ethidium bromide-positive (non-viable) hemocytes present after incubation (mean ± SEM).

G-proteins (cholera toxin), PKC (chelerythrine and staurosporine) and tubulin microtubule assembly (colchicine) (Fig. 5). All of these reagents decreased the release of anti-C3 reactive proteins to less than 25% of that evident among hemocytes stimulated with mannan in the absence of inhibitors. In contrast, incubation of hemocytes with an inhibitor of actin microfilament assembly (cytochalasin D) had no significant effect on the release of *S. plicata* C3 when compared to untreated controls ($p > 0.05$).

A similar pattern emerged when non-stimulated hemocytes (without added mannan) were incubated with various activators of exocytic signal transduction cascades (Fig. 6). Reagents that activate PKC (PMA) and intracellular calcium release (calcimycin and thapsigargin) significantly increased the secretion of anti-C3 reactive proteins relative to untreated controls ($p < 0.05$). An activator of adenylate cyclases (forskolin) had no effect ($p > 0.05$ vs. untreated controls). None of the reagents described above significantly altered the viability of hemocytes relative to untreated controls ($p > 0.05$; Figs. 5 and 6).

4. Discussion

Gnathostomes are the only animals that can mount adaptive immune reactions involving immunoglobulin antibodies [32,33]. However, this does not mean that other inducible components of host defense have equally restricted phylogenetic distributions. Defensive systems like the inflammatory response seem to have evolved long before antibodies. The current study has investigated the molecular mechanisms that control the exocytosis of tunicate C3-like molecules that are likely to contribute to such inducible inflammatory reactions.

We have shown that hemocytes from *S. plicata* secrete C3-like proteins when exposed to pathogen-associated antigens. Hemocytes stimulated with bacterial (LPS), fungal (mannan) or algal (carrageenans) cell surface molecules were found to release up to 6 times more C3-like protein than unstimulated hemocytes, and up to 14 times more C3-like protein than was evident in hemolymph serum. Only limited constitutive secretion could be detected from non-stimulated cells, and responses to stimulatory molecules were dose dependent. These results suggest that the release of C3-like molecules into culture supernatants is an actively induced response. None of

the stimulatory molecules used here decreased hemocyte viability, indicating that the release of C3-like protein was not simply due to hemocyte death and subsequent lysis. Similarly, flow cytometry could not detect any cellular material remaining in hemocyte culture supernatants, confirming that the ELISA data do not simply reflect residual cellular contamination of supernatants.

The active induction of an exocytic pathway is also indicated by signal transduction analysis. Treatment of hemocytes with a variety of pharmacological reagents showed that the release of C3-like molecules is initiated by a G-protein dependent signaling cascade of the type that is often associated with exocytosis. In other animals, receptor-associated G-proteins induce exocytosis by activating either adenylate cyclases to produce cAMP, or phospholipases to produce inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. Both the phospholipase and cAMP pathways initiate divalent cation (Ca^{2+} or Mg^{2+}) release from the endoplasmic reticulum and stimulate protein kinases like PKC. This ultimately results in exocytic cytoskeletal activity, most often involving the translocation of secretory vesicles along tubulin microtubules rather than F-actin microfilaments [34–37].

The sensitivity of *S. plicata* hemocytes to reagents that modulate G-proteins (cholera toxin), PKC (chelerythrine, staurosporine and PMA), calcium mobilization (calcimycin and thapsigargin) and microtubule assembly (colchicine)-and their insensitivity to an adenylate cyclase activator (forskolin)-suggests that a typical G-protein activated IP_3 signaling pathway initiates the exocytosis of C3-like molecules. This conclusion is based on the premise that the reagents used here have similar pharmacological effects in *S. plicata* to those in other species. That assumption is supported by numerous studies of other invertebrates (including tunicates), which have confirmed the efficacy and specificity of pharmacological reagents that target signal transduction pathways [37–39].

Signal transduction analyses in other invertebrates reveal broad similarities with the sub-cellular pathway used to activate exocytosis in *S. plicata*. For instance, G-protein inhibitors such as cholera toxin down-regulate IP_3-mediated degranulation of *Limulus polyphemus* amoebocytes stimulated with LPS [37,40], and inhibit cecropin synthesis in *Drosophila* cells [41]. Similarly, degranulation of crayfish (*Pascifastacus leniusculus*) coelomocytes after receptor-binding by an endogenous defensive molecule involves a PKC-dependent pathway, albeit one that initiates the production of cAMP rather than IP_3 [35].

The exocytic system of *S. plicata* also resembles those of other invertebrates in its rapidity. Secretion of C3-like molecules by *S. plicata* hemocytes occurs within 1 min of stimulation, a time scale comparable to that for induced degranulation by other invertebrate cells [35,37]. The speed with which these responses occur suggests that hemocytes are releasing pre-existing stores of protein, rather than initiating biosynthesis. The existence of such intracellular pools is supported by immunohistochemistry, which suggested that intracellular *S. plicata* C3-like protein is localized in the membrane-bound vesicles of NRVC. This hemocyte type, and its counterparts in other tunicates, are avidly phagocytic—to the extent that they have been referred to as macrophages [42]. They have also been directly implicated in inflammatory responses. NRVC, which comprise 28% of the cell population in the hemolymph of normal *S. plicata*, are among a sub-set of hemocytes that have been shown to actively infiltrate inflamed tissues in which cellular degranulation occurs [4,5,31,42,43].

The identification of a controlled exocytic system in NRVC that is responsive to pathogen-associated antigens suggests that these hemocytes can contribute to localized inflammatory reactions. More importantly, the recent identification of a C3a-like chemotaxin in another tunicate (*P. stolonifera*) suggests that NRVC may be involved in a positive feedback system that rapidly amplifies localized defensive responses [18]. Even though exocytosis from hemocytes may increase the systemic concentration of C3-like proteins in circulating hemolymph, we envisage a system in which pathogen-associated antigens induce the proteolysis of C3-like proteins to form pro-inflammatory, C3a-like, molecules specifically at the site of infection. Those C3a-like peptides have the capacity to recruit NRVC to the infected area by directed chemotaxis. Once in those sites, infiltrating NRVC are known to degranulate, probably by the mechanism described in this study. The exocytosis and subsequent activation of C3-like proteins secreted by NRVC would contribute opsonic effector molecules, and

more pro-inflammatory C3a-like peptides with the capacity to further amplify inflammatory responses. In this context, it is noteworthy that collectin-like proteins, which may contribute to the activation C3-like molecules, are also secreted by *S. plicata* hemocytes in response to inflammatory stimuli [15,44].

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