The signals and the molecular machinery mediating release of dense matrix granules from pathogenic protozoan parasites are unknown. We compared the secretion of the endogenous dense granule marker GRA3 in Toxoplasma gondii with the release of a stably transfected foreign reporter, β-lactamase, that localizes to parasite dense granules. Both proteins were released constitutively in a calcium-independent fashion, as shown using both intact and streptolysin O-permeabilized parasites. N-Ethylmaleimide and recombinant bovine Rab-guanine dissociation inhibitor inhibited β-lactamase secretion in permeabilized parasites, whereas recombinant hamster N-ethylmaleimide-sensitive fusion protein and bovine α-SNAP augmented release. Guanosine 5′-3-O-(thio)triphosphate, but not cAMP, augmented secretion in the presence but not in the absence of ATP. The T. gondii NSF/SNAP/SNARE/Rab machinery participates in dense granule release using parasite protein components that can interact functionally with their mammalian homologues.

Toxoplasma gondii is an obligate intracellular parasite that survives in all nucleated cells by creating a unique membrane bounded compartment called the parasitophorous vacuole (reviewed in Ref. 1). Like all Apicomplexan parasites, T. gondii contains three morphologically distinct secretory organelles: rhoptries, micronemes, and dense granules (DG).1 The protein content of these organelles, the temporal regulation of their secretion, and the eventual localization within the infected cell of proteins secreted from the organelles are all different. T. gondii, therefore, represents an interesting model for analysis of the differential control of secretion from morphologically distinct secretory compartments.

DG in T. gondii contain a homogeneous dense core and are similar in morphology to dense matrix granules in exocrine and neuroendocrine cells (reviewed in Ref. 2). DG exocytosis involves a putative fusion event with either the parasite plasma membrane or the inner membrane complex (a patchwork of closely opposed flattened vesicles located beneath the plasma membrane). In contrast to micronemes and rhoptries, which discharge their contents only at the time of cell invasion (3–5), the kinetics of DG exocytosis are less well defined. The tacit assumption (based largely on their morphology) has been that DG release is also a regulated process. We and others have described a burst of DG exocytosis shortly after invasion (3, 6), but exocytosis of DG proteins also continues during the prolonged intracellular residence of the parasite (6–8). Spontaneous release of DG proteins from extracellular parasites is also observed (9), and this release may be augmented by the addition of heat-inactivated serum (10). Thus, based on the limited information available, DG exocytosis exhibits characteristics of both a regulated and a constitutive process.

Several common themes operate for dense matrix granule secretion in most higher eukaryotic cells. A rise in intracellular calcium generally triggers the final fusion event with the plasma membrane (11). An ATP-dependent priming step precedes the calcium-dependent event (12, 13), reflecting at least in part ATP hydrolysis by N-ethylmaleimide-sensitive factor (NSF) (14, 15). Additional proteins participating in regulated, calcium-dependent exocytosis of dense core granules include components of the 20 S vesicle docking complex (16), synaptobrevin/VAMP (17), syntaxin, SNAP 23/25 (18–20), NSF (21), and SNAPs (22–24). The monomeric GTPase Rab3 (25) and proteins with calcium binding domains, most notably synaptotagmin (26), also participate.

Phylogenetically, components of the NSF/SNAP/SNARE/Rab machinery are broadly conserved across species barriers in higher eukaryotes (27). To date, however, the identification of these molecules in pathogenic protozoa has been largely limited to monomeric GTPases of the Rab family. Functional analyses of their participation in granule exocytosis has not been reported. Experiments in the nonpathogenic ciliate, Paramecium (28, 29), have identified Rab proteins on secretory vesicles and a SNARE-like molecule that complements a secretion mutant (30).

We recently reported that foreign soluble reporter proteins (Escherichia coli β-lactamase (BLA) and alkaline phosphatase)
stably expressed in *T. gondii* are routed quantitatively to DG (31), suggesting that DG are a constitutive rather than regulated secretory organelle. Our observation raised several key questions. Is DG release constitutive or regulated? If DG release is regulated, what are the triggers for release? What is the protein machinery controlling DG docking and fusion? Is the protein secretory machinery conserved between *T. gondii* and higher eukaryotes?

We have addressed these questions by following the release of both an endogenous DG marker (GRA3) and the secreted recombinant reporter protein BLA in intact and streptolysin O-permeabilized extracellular parasites. Our results demonstrate that DG markers are released constitutively in a calcium-independent fashion, via the participation of the NSF/SNAP/SNARE/Rab machinery. While calcium-independent constitutive release of soluble proteins from a dense matrix granule is unusual, the protein machinery mediating release in *T. gondii* is broadly conserved with that in higher eukaryotic cells.

**MATERIALS AND METHODS**

**Buffers and Reagents**

The following buffers were used. Hanks' balanced salt solution (HBSS) was supplemented with 1 mg/ml bovine serum albumin (BSA) and 10 mM Hepes buffer, pH 7.4. AISS, an intracellular buffer, contained 120 mM KCl, 20 mM NaCl, 1 mM MgCl₂, 10 mM glucose, and 20 mM Hepes, pH 7.2. AISS plus buffer consisted of AISS supplemented with 1× minimum essential medium essential and nonessential amino acids, 1× minimum essential medium vitamins, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate. Both buffers contained leupeptin, aprotonin, pepstatin, chymostatin, and phenylmethylsulfonyl fluoride at 10 mM, 100 µM, and 100 µg/ml, respectively. PBS/Tween 20/BSA contained 0.15M NaCl, 0.05% Tween 20, and 10 mM HEPES, pH 7.2. All chemicals were obtained from Sigma unless stated otherwise. Paranitrophenyl phosphate was used as a substrate 0.25% BSA, pH 7.2. All chemicals were obtained from Sigma unless stated otherwise. Paranitrophenyl phosphate was used as a substrate for alkaline phosphatase, and nitrocefin was used for β-lactamase (Becton Dickinson Co., Cockeysville, MD). Streptolysin O was obtained from stated otherwise. Paranitrophenyl phosphate was used as a substrate for alkaline phosphatase, and nitrocefin was used for β-lactamase (Becton Dickinson Co., Cockeysville, MD). Streptolysin O was obtained from Murex Diagnostic Ltd. (Dartford, United Kingdom). Recombinant bovine Rab-GDI (stock of 2 mg/ml in HEPES-KOH, pH 6.0, 100 mM KCl, 8 mM MgCl₂, 2 mM EDTA, 0.5% CHAPS (Fierce), and 50% glycerol (v/v)) was a gift from J. F. Dubremetz (Villeneuve d'Ascq, France). (T34A7), and rabbit anti-ROP2,3 antiserum described previously (33) were kind gifts from J. F. Dubremetz (Villeneuve d’Ascq, France). (T62H11), rabbit polyclonal anti-ROP2,3,4 monoclonal antibody to GRA3 (T62H11), rabbit polyclonal anti-ROP2,3 monoclonal antibody GRA3 in both the 7000×g supernatant and 7000×g pellets, and the OD for GRA3 (data not shown).

**Buffers and Reagents**

To measure GRA3 released by intact parasites, supernatant derived from 10³ parasites/ml was diluted 1:5 with PBS/Tween 20/BSA, and 100 µl (2×10⁹ parasite equivalents) was added to each well and incubated at room temperature for 3 h. The plates were washed six times, and 100 µl of rabbit anti-GRA3 or a control antiserum (1:2000) was added to each well and incubated at room temperature for 2 h. Plates were washed, incubated for an additional 2 h with 100 µl of rabbit anti-goat IgG (Fc)-alkaline phosphatase (Promega) (1:4000 in PBS/Tween 20/BSA), washed again, and developed with 200 µl of p-nitrophenyl phosphate for 45 min followed by the addition of 50 µl of 3 M NaOH. Plates were read at 550 nm in a Titertek Multiskan ELISA reader, and OD values for each sample were calculated by subtracting the average of triplicate wells with rabbit anti-GRA3 from the average of triplicate wells that received normal rabbit serum.

**Enzyme Assays for BLA and β-Galactosidase**

BLA in the parasite supernatant as well as in the pellet was determined by the method described earlier (38) with minor modifications. Parasite supernatant (4×10³ parasites/ml) diluted 2-fold with AISS buffer and 20 µl was added to each well of a 96-well plate. The reaction was developed by adding 180 µl of nitrocefin mix (0.2 mM nitrocefin, 0.25 mg/ml BSA, 50 mM potassium phosphate, 0.5% MeSO₄, pH 7.0) for 30 min at 25 °C followed by the addition of 50 µl of 3 M NaOH. Plates were read at 492 nm in an ELISA reader, and the OD values for each sample were calculated by subtracting the average of duplicate wells with supernatant derived from RH parasites and wells with substrate only.

**Secretion Assays**

Secretion by extracellular parasites was determined by incubating 0.4–1×10⁸ parasites/ml (total of 3 ml) in various buffers at 37°C. Glucose was essential for maximum secretion (secretion was reduced by 50% less in buffer without glucose; data not shown).

All assays were performed in prechilled Eppendorf tubes previously coated with BSA (1 mg/ml) to prevent parasites adhesion. Before transferring parasite suspensions from ice to 37°C for the secretion assay, 500 µl of parasite suspension was removed and processed as the time 0 reading. The rest of the parasite suspension was incubated in a water bath. After 15, 30, 60, and 120 min of incubation, 500 µl of the parasite suspension was removed and centrifuged at 760×g for 10 min at 4°C to pellet intact parasites. The pellet was set aside, and the supernatant was further centrifuged at 7000×g for 10 min at 4°C. GRAs in both the 7000×g supernatant and the pellet were determined by antigen capture sandwich ELISA (see below) and by immunoblot, as described previously (37). BLA activity was measured as described below.

**Quantitative ELISA for GRA3 and ROP2,3**

An ELISA was developed to measure GRA3 and ROP2,3 released from extracellular parasites in secretion assays. Plates (Linbro/Titer-trek) were coated with 50 µl of monoclonal anti-GRA3 (T62H11) diluted 1:1000 in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed four times with PBS/Tween 20/BSA. In initial experiments, whole parasite lysates were tested to validate the assay. A dose-response relationship was observed between the lysis from the increasing number of parasites and OD for GRA3 (data not shown). To measure GRA3 released by intact parasites, supernatant derived from 10³ parasites/ml was diluted 1:5 with PBS/Tween 20/BSA, and 100 µl (2×10⁹ parasite equivalents) was added to each well and incubated at room temperature for 3 h. The plates were washed six times, and 100 µl of rabbit anti-GRA3 or a control antiserum (1:2000) was added to each well and incubated at room temperature for 2 h. Plates were washed, incubated for an additional 2 h with 100 µl of rabbit anti-goat IgG (Fc)-alkaline phosphatase (Promega) (1:4000 in PBS/Tween 20/BSA), washed again, and developed with 200 µl of p-nitrophenyl phosphate for 45 min followed by the addition of 50 µl of 3 M NaOH. Plates were read at 550 nm in a Titertek Multiskan ELISA reader, and OD values for each sample were calculated by subtracting the average of triplicate wells with rabbit anti-GRA3 from the average of triplicate wells that received normal rabbit serum.

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**In preliminary experiments, a linear relationship was observed between BLA release and parasite number (data not shown). The enzymatic activity of released BLA was not affected by treatment with dithiothreitol, indicating that the oxidation-reduction state of the enzyme did not influence BLA activity (39). BLA activity in the pellet was substantially lower than in the supernatant, regardless of whether parasites were lysed in TX-100 or in the French press cell. Mixing experiments of pellet and supernatant indicated that there was an inhibitor of BLA activity in the parasite pellet (data not shown). Therefore, total released BLA was determined by immunoblot.

**β-Galactosidase activity in the supernatant as well as in the parasite pellet from β-galactosidase-expressing parasites was determined as described previously (35).**
**Dense Granule Secretion in *T. gondii***

**Influence of Incubation Conditions on DG Secretion in Extracellular Parasites**

Temperature, Energy, and Serum Dependence of Secretion—Secretion was measured during incubation of extracellular parasites at 4 and 15 °C, or with 10 mM sodium azide (Naa₃) at 15 °C for 15 min, followed by incubation at 37 °C for 1 h. The effect of serum on DG secretion by extracellular parasites was determined by adding 10% heat-inactivated fetal bovine serum to AIS buffer. The details of other conditions tested are described in the legend to Table I.

**Parasite Permeabilization with SLO and Cytosol Depletion**

SLO—Permeabilization of extracellular parasites with SLO was done using modifications of a described protocol (40). Extracellular parasites were washed two times with ice cold potassium acetate buffer containing glucose (115 mM potassium acetate, 2.5 mM MgCl₂, 10 mM glucose, and 25 mM HEPES, pH 7.2, with KOH) and then incubated with 100 μl of 1 unit/ml of SLO for 10 min at 4 °C. The suspension was transferred to 37 °C and incubated for 2 min, and then parasites were pelleted at 760 × g for 5 min. Supernatant was removed and saved to measure released BLA during the permeabilization step. The parasite pellet was gently suspended in ice cold potassium acetate buffer, washed two times, and resuspended to 4 × 10⁷ parasites/ml. Permeabilized parasites were immediately used for secretion assays at 37 °C. BLA secretion was also monitored from control parasites, which were treated similarly at every step without the presence of SLO.

**Monitoring Permeabilization—**Parasite permeabilization was assessed by incubating organisms with 4 μg/ml propidium iodide for 5 min at room temperature, and the percentage of parasites positive for nuclear fluorescence was quantitated by fluorescence microscopy.

**Determining Cytosol Leakage—**Release of lactate dehydrogenase as a cytosolic marker from parasites could not be quantitated with accuracy, due to contamination with host cell lactate dehydrogenase. Therefore, a stable transgenic clone of parasites expressing a cytosolic GFP marker (GRA3) was treated with SLO. The percentage loss of GFP fluorescence, immediately after permeabilization and following continued incubation at 15 °C for 1 h, was determined by measuring the fluorescence of parasites at 490 nm and emission of 510 nm in a fluorimeter and compared with total GFP in nonpermeabilized parasites treated in a similar fashion in the absence of SLO. The GFP release from SLO-permeabilized parasites was also assessed by phase and by fluorescence microscopy.

**Effects of Calcium on DG Release from Permeabilized Parasites**

Extracellular parasites were washed two times with cold potassium acetate-EGTA (5 mM) buffer and permeabilized with SLO in the same buffer, and then various concentrations of free calcium (0–100 μM final concentration) were added in the presence or absence of an ATP-regenerating system (ARS; 2 mM NaATP, 20 mM creatine phosphate, and 40 μM creatine phosphokinase). The concentrations of free Mg²⁺ and Ca²⁺ in the buffer were calculated using a software program of Focchi and Warchol (Ulm, Germany). Alternatively, parasites were permeabilized with SLO in potassium acetate buffer containing no EGTA and then preincubated with or without MAPTA-AM (50 μM final concentration) in the presence or the absence of ARS at 0 °C for 15 min followed by incubation at 37 °C for 30 min for secretion to occur.

**Determining the Protein Machinery of DG Secretion Using SLO-permeabilized *T. gondii***

The influence of N-ethylmaleimide on DG secretion was carried out by preincubating both SLO-permeabilized as well as nonpermeabilized parasites with or without 1 mM N-ethylmaleimide (NEM) for 15 min at 0 °C. Excess NEM was removed by washing parasites two times with ice-cold potassium acetate buffer. Following NEM treatment, both nonpermeabilized and permeabilized parasites (treated with or without NEM) were suspended in potassium acetate buffer with or without ARS (2 mM MgATP was used in place of Na ATP in the ARS mixture) and incubated at 37 °C for secretion to occur. The effect of NSF on DG secretion was determined by incubating SLO-permeabilized parasites with 10 μM recombinant hamster NSF, or with the catalytically inactive N domain of NSF (10 μg/ml) at 0 °C for 1 h before switching to 37 °C for secretion to occur. Permeabilization of parasites at 0 °C was used to avoid the large size of full-length NSF (76 kDa). These experiments were conducted only in the presence of ARS, since NSF is unstable in the absence of ATP. Two μM Mg²⁺-ATP was used in place of Na ATP in the ARS mixture.

The involvement of α-SNAP and Rab-GDI on DG secretion was determined by preincubating permeabilized parasites for 15 min at 15 °C with various concentrations of α-SNAP (0.5 to 5 μg/ml) and Rab-GDI (4 μg/ml) in the presence or absence of ARS followed by switching to 37 °C for secretion to occur. DG secretion was also tested by preincubating permeabilized parasites with a 100 μM final concentration of GTPγS, ATPγS, and cGMP in the presence or absence of ARS at 0 °C for 15 min, after which parasites were switched to 37 °C for secretion to occur. All results were expressed as percentage of BLA release, with 100% being the amount of BLA released by nonpermeabilized control parasites.

**Statistical Analysis**

Statistical analysis of the data was performed using InStat software for MacIntosh. Student t test was used for comparison of two groups. Results were considered significant at p values of <0.05.

**RESULTS**

**Parasites Spontaneously Secrete Dense Granule Proteins in an Energy- and Temperature-dependent Fashion**

Extracellular parasites spontaneously secreted both GRA3 and BLA in buffer. When compared with protein in total cell lysates, 11–14% of parasite GRA3 (Fig. 1A and B) and 15–18% of BLA (Fig. 2B) were secreted during a 1-h incubation at 37 °C. Secretion was abolished at 4 °C or 15 °C (Figs. 1A and 2A) or by the addition of 10 mM sodium azide (Fig. 1A) and was neither due to parasite lysis or to a general secretory response of extracellular parasites (Fig. 1A). No inhibition of BLA secretion was observed in cycloheximide-treated parasites (Fig. 2A), a treatment that rapidly and completely inhibits protein synthesis (41). This latter result suggests that BLA secretion is occurring through preformed organelles, confirming previous observations (31).

We therefore asked whether conditions could be identified that might trigger or inhibit DG secretion. Based on suggestions that there is a burst of DG release after parasite entry (3, 6), we reasoned that intracellular buffer conditions might augment release in comparison with extracellular buffer conditions. No difference in GRA3 or BLA release was observed when parasites were incubated in high sodium (HBSS) versus high potassium (AISS) buffer, except at later time points in the incubation for GRA3 (Fig. 1A). Secrecion was not influenced by adding extracellular calcium or by chelating calcium with EGTA (Table I), although chelation of intracellular calcium with MAPTA-AM resulted in some inhibition of DG secretion. The addition of ATP, ADP, or AMP in the buffer had no effect on GRA3 or BLA release, nor was secretion altered by the addition of 10 mM glutathione at varying reduced:oxidized ratios. In contrast to one previous report (10), the addition of heat-inactivated fetal bovine serum in AISS buffer did not further enhance GRA3 or BLA release (Table I). In summary, these results suggest that DG release by extracellular *T. gondii* occurs constitutively.

**Dense Granule Release in Permeabilized Parasites Is ATP-dependent**

To facilitate the identification of signaling molecule(s) and protein machinery involved in DG secretion, we established a permeabilized cell secretion assay with *T. gondii* using streptolysin O as permeabilizing agent. Under the conditions developed for permeabilization, 50–60% of parasites were permeabilized by 1 unit/ml of SLO (assessed by propidium iodide staining of the nucleus, not shown). Approximately one-quarter (see legend to Fig. 3A for calculation) of the cytosolic marker green fluorescent protein, was released from permeabilized transgenic parasites (34) (Fig. 3A), showing that macromolecules are depleted.

Secretion of BLA was inhibited by 49 ± 19% in SLO-permeabilized parasites as compared with untreated controls (Fig.
The extent of BLA release during permeabilization was no greater than in controls incubated at 37 °C without SLO. The addition of an ARS restored secretion to 81% of control. ARS had no effect on BLA secretion from nonpermeabilized parasites, further confirming our results (Table I). Preincubation of permeabilized parasites with an ATP depletion system (195 units/ml of hexokinase 10 mM glucose at 15 °C for 30 min) resulted in a further 15–20% inhibition of secretion in the absence of ATP, in comparison with permeabilized parasites not treated with hexokinase and glucose (data not shown). This is likely to be the base line for secretion in the absence of ATP, since only 40–60% of parasites were permeabilized by SLO. Altogether, these results show that ATP is necessary for DG secretion.

Neither Calcium nor cAMP Triggers DG Secretion from Permeabilized Parasites

An increase in intracellular calcium serves as a signal for secretion of dense matrix granules in many cells (42). In T. gondii, however, the addition of up to 100 μM calcium to SLO-permeabilized parasites had no effect on BLA secretion in either the presence or absence of ARS. Chelation of intracellular...
TABLE I

DG secretion is not altered by extracellular versus intracellular buffer conditions

| Buffer + reagents | GRA3 release | BLA release |
|-------------------|--------------|-------------|
| AISS              | 100%         | 100%        |
| + ATP (2 mM)      | 93 ± 3%      | 91 ± 12%    |
| + ATP+PS (0.1 mM)| 97±%         | 96±%        |
| + ARS             | 93 ± 20%     | 98 ± 7%     |
| + GTP+PS          | NDa          | 86 ± 12%    |
| + glutathione (10 mM, 60:1) | 92 ± 9% | 108±% |
| + glutathione (10 mM, 30:1) | 102 ± 2% | 103±% |
| + glutathione (10 mM, 1:1) | 100±% | 102±% |
| + glutathione (10 mM) + ATP (2 mM) | 96 ± 3% | 99 ± 12% |
| + glutathione (10 mM) + ADP (2 mM) | 107 ± 6% | 99 ± 5% |
| + glutathione (10 mM) + AMP (2 mM) | 95 ± 4% | 111 ± 2% |
| + cAMP (2 mM)     | 92 ± 2%      | 89 ± 2%     |
| + gGMP (2 mM)     | 99 ± 2%      | 104 ± 2%    |
| + CaCl2 (0.1 mM)  | 98 ± 2%      | 99 ± 1%     |
| + EGTA (5 mM)     | 93 ± 4%      | 100 ± 2%    |
| + MAPTA-AM (500 µM) | 69 ± 3% | 65 ± 8% |
| + FBS (10%, HI)   | ND           | 99 ± 4%     |
| + vitamins + amino acids | 68 ± 2** | 63 ± 2** |
| HBSS              | ND           | 97 ± 2%     |

a The data represent the mean ± S.D. of four experiments done in triplicate except in a few cases where only a single experiment was performed.

b ND, not determined.

dense granules reflect an intracellular environment (low Na+, low K+, high Ca++, oxidizing, low ATP, HBSS) and conditions reflecting an intracellular environment (low Na+, high K+, low Ca++, reducing, high ATP, AISS) or with other conditions, including added ARS, ATP+PS, GTP+SNAP, AMP, gGMP, and heat-inactivated fetal bovine serum (FBS). The influence of extracellular calcium on DG secretion was further tested by either adding exogenous Ca++ (2 mM) to AISS buffer or by chelating Ca++ with either 5 mM EGTA or 500 µM MAPTA-AM in AISS buffer. The influence of the oxidation/reduction state was tested by adding 10 mM glutathione at reduced to oxidized ratios of 60:1, 30:1, and 1:1 to AISS buffer. The released DG proteins were measured at 0 and 60 min postincubation by ELISA for GRA3 and by enzyme assay for BLA. The results are expressed as percentage of release and calculated with 100% being the amount of DG proteins released in AISS buffer. In most cases, there was no significant alteration in release of either GRA3 or BLA in comparison with AISS buffer. MAPTA-AM inhibited secretion of both BLA and GRA3 by 35 ± 5% and 34 ± 3%, respectively (*, p < 0.05). DG secretion was also significantly lower (**, p < 0.01) in the presence of vitamins and amino acids. The results of GRA3 release in HBSS are shown in Fig. 1 and therefore not included here. Unlike DG secretion, release of the rhoptry protein ROP2 from nonpermeabilized parasites was negligible (<1%) in any of the buffer conditions tested (not shown).

The Involvement of NSF, α-SNAP, and GTP-binding Proteins in DG Exocytosis in T. gondii

We next explored the protein machinery mediating DG release in T. gondii. In other eukaryotic systems, NSF, the SNAP-SNARE machinery, and the Rabs have been shown to play important roles in exocytosis of regulated secretory organelles (14, 17, 18, 21–25). Involvement of NSF—Treatment with 1 mM of the alkylating agent, NEM, resulted in 80 ± 4% inhibition of BLA secretion from nonpermeabilized parasites and 65 ± 3% inhibition of BLA secretion from permeabilized parasites in the presence of ARS (Fig. 5A). Although use of NEM at 1 mM has been previously used to inactivate NSF, NEM may have other effects. We therefore tested the effects of adding purified recombinant hamster NSF to permeabilized parasites. When parasites were incubated with recombinant hamster NSF and ARS, DG re-
buffer containing 5 mM EGTA with or without ARS and with or withoutabilized parasites. SLO-permeabilized parasites were incubated in addition of 100
involvement of GTP-binding proteins in DG secretion. The
ery to control DG docking and/or fusion.
hibited secretion by 18%.
6
6
1
6
6
AWS. Values represent the means ± S.D. from two experiments performed in triplicate.

**Table II**
GTP-γS stimulates while MAPTA-AM inhibits DG secretion in permeabilized T. gondii

Parasites were permeabilized with SLO by the usual procedure. GTP-γS (100 μM), MAPTA-AM (50 μM), and cAMP (2 mM) were added to the indicated final concentration. Parasites were preincubated with these reagents at 0 °C for 15 min and thereafter switched to 37 °C for secretion to occur. GTP-γS significantly augmented BLA secretion (the data are the mean ± S.D. of three experiments done in triplicate. *, p < 0.05 compared with parasites with ARS alone), while MAPTA-AM inhibited secretion by 18%.

| Buffer + reagents (mM concentration) | BLA release (30 min postincubation) |
|-------------------------------------|------------------------------------|
| Nonpermeabilized                    | % |
| Buffer alone                        | 100 |
| Permeabilized                       | % |
| Buffer alone                        | 53 ± 7 |
| Buffer + ARS (mixture 1)            | 87 ± 14 |
| Mixture 1 + cAMP (2 mM)             | 103 ± 11 |
| Mixture 1 + GTP-γS (0.1 mM)         | 135 ± 4* |
| Mixture 1 + MAPTA-AM (0.005 mM)     | 82 |

le was augmented in comparison with ARS alone (Fig. 5B). In contrast, incubation of parasites with the N domain of NSF, a fragment devoid of ATPase activity, did not augment BLA secretion from permeabilized parasites, arguing for the specificity of the NSF effect (Fig. 5B).

**Involvement of α-SNAP**—Treatment of permeabilized parasites with recombinant bovine α-SNAP resulted in stimulation of BLA secretion. Maximum secretion occurred at 2 μg/ml of α-SNAP, in the presence of ARS (Fig. 6A). Based on this dose-response curve, 2 μg/ml α-SNAP was used in subsequent experiments. Secretion was augmented by α-SNAP in the presence of ARS (Fig. 6B). α-SNAP alone, in the absence of ARS, had no effect on DG secretion. These results suggest that DG secretion is regulated by α-SNAP and that heterologous α-SNAP proteins can interact with the SNARE/SNAP machinery to control DG docking and/or fusion.

**Involvement of GTP-binding Proteins**—Finally, we tested the involvement of GTP-binding proteins in DG secretion. The addition of 100 μM GTP-γS resulted in significant enhancement of BLA secretion (38 ± 4%) from permeabilized parasites in the presence of ARS (Table II), suggesting that GTP-binding proteins regulate DG exocytosis in T. gondii. To specifically explore the involvement of Rabs in DG secretion, SLO-permeabilized parasites were incubated with 4 μg/ml recombinant bovine Rab-GDI. This resulted in 11 ± 4% inhibition of DG secretion (data not shown). This difference, while small, was reproducible and is similar to the levels reported in other systems.

**DISCUSSION**

The molecular machinery controlling secretion of dense core granules is broadly conserved evolutionarily. Nonetheless, un-
meabilized parasites with ARS alone. Bovine recombinant bovine a-SNAP was added to permeabilized parasites at the indicated final concentration. a-SNAP at 2 µg/ml was optimum for BLA release from permeabilized parasites. The data are representative of two separate experiments. B, augmentation of BLA secretion by a-SNAP. Recombinant bovine a-SNAP (2 µg/ml final concentration) significantly augmented secretion by 27 ± 9% from permeabilized parasites in the presence of ARS. Results represent mean ± S.D. of four experiments done in triplicate. *, p ≤ 0.05 compared with BLA released from permeabilized parasites with ARS alone.

Fig. 6. Bovine a-SNAP stimulates DG secretion in SLO-permeabilized T. gondii. A, dose-response curve for a-SNAP. Recombinant bovine a-SNAP was added to permeabilized parasites at the indicated final concentration. a-SNAP at 2 µg/ml was optimum for BLA release from permeabilized parasites. B, augmentation of BLA secretion by a-SNAP. Recombinant bovine a-SNAP (2 µg/ml final concentration) significantly augmented secretion by 27 ± 9% from permeabilized parasites in the presence of ARS. Results represent mean ± S.D. of four experiments done in triplicate. *, p ≤ 0.05 compared with BLA released from permeabilized parasites with ARS alone.

Until this study, there has been no direct evidence that this machinery functions in pathogenic unicellular protozoan parasites, either for constitutive or regulated secretion. In contrast to this conserved aspect of dense granule release, secretion of T. gondii dense granules is calcium-independent, reflecting the unusual nature of the protein sorting machinery within the organism (31).

In most systems, secretion from dense matrix granules is regulated by changes in intracellular calcium concentration (11), while constitutive transport between the trans-Golgi network and plasma membrane is calcium-independent (46, 47). Preliminary experiments indicated that treatment of extracellular parasites with i) the calcium ionophore A23187, ii) the ER CaATPase inhibitor thapsigargin, or iii) NH4Cl, (under conditions reported to increase intracellular calcium levels in T. gondii; Ref. 48) had no effects on secretion of GRA3 and BLA (data not shown). Theoretical considerations also support the notion that increased intracellular calcium is unlikely to be the trigger for continuous exocytosis of dense granules from intracellular T. gondii, since the parasite resides in a low calcium environment and would not be capable of rapidly replenishing intracellular calcium by opening plasma membrane calcium channels (reviewed in Ref. 49). In order to examine this question further and more precisely control intracellular calcium levels, we developed a permeabilized cell system and unequivocally demonstrated that increases in intracellular calcium do not trigger DG release.

Spontaneous release of “regulated proteins” in the absence of a stimulus is generally ascribed to either low level release of dense core granules or simultaneous delivery of the regulated proteins to both regulated and constitutive secretory vesicles (50, 51). The distinction between basal and constitutive release of dense core vesicles depends on the capacity to identify triggers (typically a rise in intracellular Ca2+) for granule release in the former instance (46). The results shown above demonstrate that DG release in T. gondii is not triggered by an increase in intracellular Ca2+, precluding the use of this signal to make the distinction between basal and constitutive release. Based on our previous results with brefeldin A (31), showing quantitative delivery of GRA3 and BLA to DG, and results here showing that short term treatment of extracellular parasites with cycloheximide had no effect on DG release, it is unlikely that DG proteins are simultaneously delivered to DG and constitutive secretory vesicles. An alternative possibility is that the DG in Toxoplasma are analogous to the immature secretory granules in endocrine and exocrine cells (52–58) and that secretion occurs via budding of small constitutive secretory vesicles from the DG. Vesicle budding from immature secretory granules in mammalian cells is brefeldin A-sensitive (59, 60), arguing against this explanation for constitutive secretion of dense granule proteins in T. gondii. Moreover, neither we nor others have observed structures compatible with immature secretory granules in the organism. Taken in total, the results are most consistent with the notion that DG secretion is constitutive in T. gondii.

The discovery that hamster NSF and bovine a-SNAP augment DG secretion provides the first functional demonstration that the NSF/SNAP/SNARE system operates in a pathogenic protozoan parasite. Although we do not yet have direct evidence for the involvement of endogenous T. gondii components, the function of hamster and bovine proteins in the parasite system suggests that the T. gondii homologues will be structurally similar with their mammalian counterparts (reviewed in Ref. 27).

Numerous Rabs have been identified in Toxoplasma (61), but the specific molecules involved in regulating DG secretion are not known. Since Rab-GDI exhibits broad substrate specificity, even across species barriers (reviewed in Refs. 62 and 63), the activity of bovine protein in the parasite is structurally similar with their mammalian counterparts (reviewed in Ref. 27).

T. gondii-like active light chain of tetanus toxin, which cleaves synaptobrevin/VAMP (66) and inhibits both calcium-dependent (66) and calcium-independent (67) granule secretion, had no effect on BLA release (data not shown). Unfortunately, interpretation of this experiment must await identification of T. gondii VAMP homolog, because not all VAMP/synaptobrevins are cleaved by TeTx (66).

The molecular basis for the GTPγS-mediated enhancement of DG secretion is unknown. Calcium-independent GTPγS-augmented secretion is also observed in mast cells and neutrophils (68, 69), where it is mediated by ARF1 activation of phospho-

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lipase D to generate phosphatidylinositol bisphosphate (70). This is the most likely explanation for the GTPγS-mediated enhancement of DG exocytosis in T. gondii, where GTPγS is revealing the components involved in the constitutively occurring process. Alternatively, it is possible that a parasite G-protein-coupled plasma membrane receptor could be linked to DG exocytosis. Heterotrimeric GTP-binding proteins have been identified in T. gondii, and AIF is reported to increase the extrusion of filamentous structures from the parasite surface (71), although in our hands BLA release was altered by NaF alone, making interpretation of these results difficult. By identifying the mechanism for the GTPγS-augmented secretion, the possible contribution of a regulated component to DG exocytosis (3, 6) in T. gondii may also be defined.

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