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Intracellular Mediators of Granulysin-Induced Cell Death[1]

Satoshi Okada,[2]* Qing Li,[2]*‡ John C. Whitin,† Carol Clayberger,* and Alan M. Krensky[3]*

Granulysin, a molecule present in the granules of CTL and NK cells, is cytolytic against microbes and tumors. Granulysin induces apoptosis of mammalian cells by damaging mitochondria and causing the release of cytochrome *c* and apoptosis-inducing factor, resulting in DNA fragmentation. Here we show that Ca^{2+} and K^+ channels as well as reactive oxygen species are involved in granulysin-mediated Jurkat cell death. The Ca^{2+} channel blockers, nickel and econazole, and the K^+ channel blockers, tetraethylammonium chloride, apamin, and charybdotoxin, inhibit the granulysin-induced increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), the decrease in intracellular K^+, and apoptosis. Thapsigargin, which releases Ca^{2+} from the endoplasmic reticulum, prevents a subsequent granulysin-induced increase in $[Ca^{2+}]_i$ in Jurkat cells, indicating that the initial increase in $[Ca^{2+}]_i$ is from intracellular stores. The rise in $[Ca^{2+}]_i$ precedes a decrease in intracellular K^+, and elevated extracellular K^+ prevents granulysin-mediated cell death. In granulysin-treated cells, electron transport is uncoupled, and reactive oxygen species are generated. Finally, an increase in intracellular glutathione protects target cells from granulysin-induced lysis, indicating the importance of the redox state in granulysin-mediated cell death. ***The Journal of Immunology*, 2003, 171: 2556–2562.**

Granulysin, a cytolytic molecule expressed by human CTL and NK cells, is colocalized in granules with perforin and granzymes and is active against tumors and a variety of microbes, including *Mycobacterium tuberculosis* (1, 2). Using the human T cell tumor Jurkat, we previously showed that granulysin binds to the cell surface based upon charge and causes increased intracellular Ca^{2+} ($[Ca^{2+}]_i$)[4] (3). In granulysin-treated cells, mitochondria are damaged, with a loss of electrostatic potential and release of cytochrome *c* (3) and apoptosis-inducing factor (AIF) (4). Although cytochrome *c* is released, caspase 9 is not activated; other caspases, however, are involved in granulysin-mediated cell death (3). These studies indicate that the cell membrane and mitochondria are important targets of granulysin. Outstanding issues include identification of the mediators between the cell surface and mitochondria, the mechanism of mitochondrial damage, and downstream events giving rise to DNA degradation.

We show here that an increase in $[Ca^{2+}]_i$ and a decrease in $[K^+]_i$ are required for granulysin-mediated apoptosis. In granulysin-treated cells, both cytosolic and intramitochondrial calcium levels rise, followed by a decrease in intracellular potassium ($[K^+]_i$) levels. Granulysin-mediated cell death is prevented by both calcium and potassium channel blockers. Granulysin gains entry into the cytoplasm and directly damages mitochondria in the presence of calcium. Inhibitors of electron transport or an increase in cellular glutathione protect Jurkat cells from granulysin-mediated death. Taken together, these findings indicate that mitochondria are the major targets of granulysin and that several different pathways contribute to mitochondrial damage and subsequent cell death.

Materials and Methods

Reagents

Reagents used include the channel blockers, econazole (Sigma-Aldrich, St. Louis, MO); tetraethylammonium chloride, apamin, charybdotoxin, MTT, and thapsigargin (Calbiochem, San Diego, CA); propidium iodide (PI), Fura-2/AM, K^+-binding benzofuran isophtalate (PBFI), C2938-AM (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester), and Rhod-2/AM (Molecular Probes, Eugene, OR); mitochondrial electron transport inhibitors, antimycin A, rotenone, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and potassium cyanide (KCN; Sigma-Aldrich); HRP-conjugated secondary Abs and ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ); anti-cytochrome *c* Ab (BD PharMingen, San Diego, CA); anti-AIF Ab (Santa Cruz Biotechnology, Santa Cruz, CA); anti-actin Ab (Oncogene, San Diego, CA); and FITC-dUTP and TdT (BD PharMingen).

Cells

Jurkat cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% FBS (HyClone, Logan, UT), 200 U/ml penicillin, 200 μg/ml streptomycin, and 2 mM L-glutamine.

Granulysin and granulysin-derived peptides

Recombinant granulysin (9 kDa) was expressed and purified as previously described (2). Granulysin peptides G8 and G9 were synthesized as previously described (5). G8 and G9 include residues 23–51 of granulysin and are identical, except that cysteine was replaced by serine at positions 34 and 45 in G9 (5).

Cytotoxicity assays

Jurkat cells were seeded in 96-well plates at a density of 8×10^5/ml (100 μl/well) in RPMI 1640 supplemented with 0.1% FCS. Cells were cultured for 3 h after the addition of granulysin. Cell death was measured by PI uptake (6), DNA fragmentation (3), or MTT assay (7).

$[Ca^{2+}]_i$ and $[K^+]_i$ measurements

To measure $[Ca^{2+}]_i$ or $[K^+]_i$, Jurkat cells were loaded with Fura-2/AM or PBFI/AM, respectively, in a 1/1 (v/v) cell suspension at 37°C for 60 min.

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[4] Abbreviations used in this paper: $[Ca^{2+}]_i$, intracellular Ca^{2+}; AIF, apoptosis-inducing factor; BSO, buthione sulfoximine; $[Ca^{2+}]_m$, mitochondrial calcium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\psi_m$, mitochondrial transmembrane potential; $[K^+]_i$, intracellular K^+; KCN, potassium cyanide; OTC, 2-oxo-4-thiazolidine carboxylic acid; PBFI, potassium binding benzofuran isophtalate; PI, propidium iodide; ROS, reactive oxygen species; SOC, store-operated calcium channel.

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The cells were then washed twice with PBS and incubated for an additional 30 min for complete de-esterification. $[Ca^{2+}]_i$ and $[K^+]_i$ were monitored using an Attofluor digital fluorescence microscope (Atto Instruments, Rockville, MD). Briefly, a cell suspension was loaded into chambers, and after 50 s the test agent was added. The Ca^{2+}- or K^+-dependent fluorescence signal was obtained using dual excitation at 334 and 380 nm and ratioing fluorescence intensities detected at 510 nm. In some experiments high K^+ modified RPMI 1640 medium containing 140 mM KCl and 5 mM NaCl was used as previously described (8).

DNA fragmentation

Cells treated with granulysin or control were washed in saline, fixed with 1% paraformaldehyde for 15 min at 4°C, and suspended in 70% ethanol for 30 min at 4°C. The cells were then washed in saline and mixed with 50 μl of a mixture containing 2 M potassium cacodylate, 25 mM Tris-HCl, 2.5 mM cobalt chloride, and FITC-dUTP in the presence of 100 μg/ml of TdT. After two washes with saline, the cells were resuspended in 500 μl of PBS containing 10 μg/ml PI plus 10 μg/ml RNase, incubated for 30 min at room temperature in the dark, and analyzed by flow cytometry using a FACScan (BD Biosciences, Mountain View, CA). Nonclumped cells were gated based on a display with DNA area and DNA width, and a second gated dual parameter display with FITC-dUTP and DNA area was generated. Cells without DNA fragmentation were gated based on FITC-dUTP fluorescence in control cells.

Electron microscopy

Jurkat cells (5×10^5/ml) were suspended in RPMI 1640 without FCS and incubated with 50 μM granulysin for 20 min at 37°C. The cells were washed twice in 0.1 M phosphate buffer and resuspended in 0.2 ml of fixation buffer (2% formaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer) on ice for 15 min. The samples were centrifuged, the supernatant was removed, 0.2 ml of fixation buffer was added, and the cells were incubated on ice for another 20 min. Cells were washed twice in cold 0.1 M phosphate buffer and processed for electron microscopy. Sections were stained with rabbit anti-human granulysin Ab (9), followed by Immunogold-conjugated goat anti-rabbit IgG (1/10).

Subcellular fractionation and Western blot

Mitochondrial and cytosolic (S100) fractions were prepared as previously described (3) by resuspending Jurkat cells in 0.8 ml of ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml PMSF, 8 μg/ml aprotinin, and 2 μg/ml leupeptin (pH 7.4)). Cell membranes were disrupted by repeatedly forcing cells through a 30-gauge needle. Unlysed cells and nuclei were pelleted at $750 \times g$ for 10 min, and the supernatant was centrifuged at $10{,}000 \times g$ for 25 min. The pellet (representing the mitochondria-enriched fraction) was resuspended in lysis buffer; the supernatant was subjected to further centrifugation at $100{,}000 \times g$ for 1 h. The supernatant from the final centrifugation represents the S100 fraction. Mitochondrial (5 μg protein/lane) and S100 (10 μg protein/lane) fractions were subjected to Western blot analysis as previously described (3). Blots were incubated with mAb specific for cytochrome *c*, AIF, or actin and were diluted in Tris-buffered saline supplemented with 0.2% Tween 20. Primary Abs were detected by peroxidase-labeled secondary Ab and were developed using ECL. In experiments to determine the effects of granulysin on isolated mitochondria, mitochondria were resuspended in regenerating buffer (100 mM Tris-HCl (pH 7.4), 10 mM sodium citrate, 2 mM NAD^+, and 1 mM $MgCl_2$) as indicated.

Mitochondrial calcium

Mitochondrial calcium was measured using Rhod-2/AM. Jurkat cells were cultured in a poly-L-lysine-coated chamber and were incubated with 1 μM Rhod-2/AM (10) for 30 min. Cells were washed with PBS and then further incubated overnight in RPMI 1640 supplemented with 10% FCS to eliminate extracellular dye. Immediately before use, cells were washed again and resuspended in RPMI 1640 supplemented with 0.1% FCS. Rhod-2 fluorescence was monitored using a confocal microscope.

Reactive oxygen species (ROS)

Jurkat cells were incubated with granulysin or medium for 1 h. The cells were then washed and incubated with 0.5 μM C2938 for 1 h, washed, and resuspended in PBS. Cells were incubated with PI (3 μg/ml) for an additional 30 min and washed, and two-color fluorescence was analyzed by FACScan (BD Biosciences, San Jose, CA).

Treatment with 2-oxo-4-thiazolidine carboxylic acid (OTC) or buthionine sulfoximine (BSO)

Jurkat cells were incubated with 10 mM OTC (a cysteine prodrug that is metabolized to cysteine intracellularly, and thus can contribute to glutathione synthesis) (11, 12) or 100 μM BSO (a specific inhibitor of glutathione synthesis) (13) for 24 h. After washing, apoptosis and glutathione content (14) were determined.

Results

An increase in $[Ca^{2+}]_i$ is required for both granulysin- and granulysin peptide-induced apoptosis

We previously reported that granulysin induces an increase in $[Ca^{2+}]_i$ in Jurkat cells (3). In the present study we asked whether this increase in $[Ca^{2+}]_i$ was required for apoptosis induced by granulysin or its derivative peptides, G8 and G9. Peptide G8 corresponds to residues 23–51 and lyses Jurkat cells. Peptide G9 is identical with G8, except that the cysteine residues at positions 34 and 45 were replaced with serine; G9 does not lyse Jurkat cells (5). Granulysin and peptide G8 cause an increase in $[Ca^{2+}]_i$ within 200 s after treatment of Jurkat cells with 50 μM granulysin or 25 μM G8 (Fig. 1, *A* and *D*). Peptide G9 does not increase $[Ca^{2+}]_i$ (Fig. 1*G*). In lymphocytes, ligation of the Ag receptor leads to a release of Ca^{2+} from intracellular stores, including the endoplasmic reticulum and mitochondria, which, in turn, causes the activation of Ca^{2+} channels in the plasma membrane. The latter channels are therefore referred to as store-operated Ca^{2+} channels

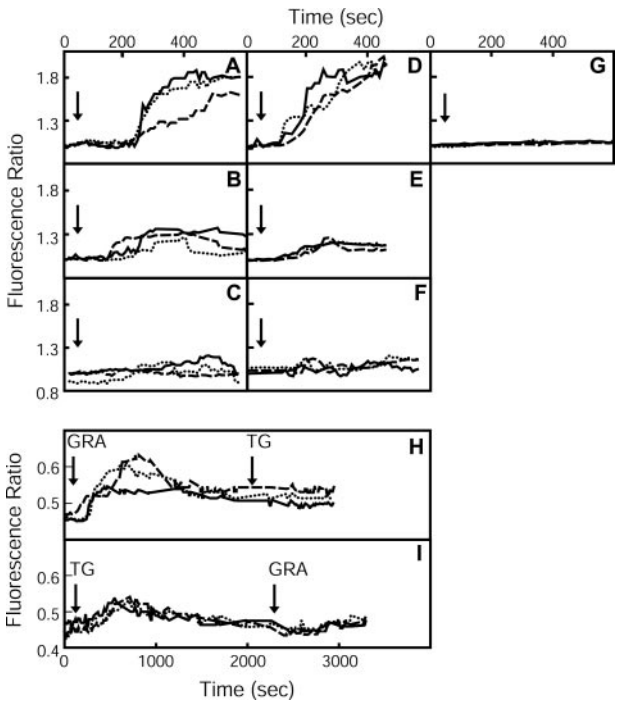

FIGURE 1. Ca^{2+} channel blockers prevent granulysin-induced $[Ca^{2+}]_i$ increase. Jurkat cells were pretreated with medium (*A*, *D*, and *G*), 1 mM nickel (*B* and *E*), or 1 μM econazole (*C* and *F*) for 30 min and loaded into a chambered cover glass. After a stable fluorescence baseline was obtained, 50 μM granulysin (*A–C*), 25 μM G8 (*D–F*), or 25 μM G9 (*G*) were added, as indicated by arrows. The 340/380 nm fluorescence ratio was observed using an Attofluor digital fluorescence microscope. Data from three representative cells in each experimental condition are shown. All experiments were performed a minimum of three times. *H* and *I*, Release of intracellular Ca^{2+} from the endoplasmic reticulum inhibits granulysin-induced $[Ca^{2+}]_i$ increase. Jurkat cells were labeled with Fura-2 AM and resuspended in low Ca^{2+} HBS. Granulysin (GRA; 50 μM) or thapsigargin (TG; 500 nM) was added as indicated by the arrows.

(SOC) (15). To determine whether SOC were involved in the granulysin-induced rise in $[Ca^{2+}]_i$, Jurkat cells were then preincubated with the SOC blockers nickel and econazole before addition of G8, G9, or granulysin. Both nickel and econazole prevented the granulysin or G8-induced increase in $[Ca^{2+}]_i$ (Fig. 1, *B*, *C*, *E*, and *F*) and inhibited granulysin- or G8-induced apoptosis (Table I). These data indicate that an increase in $[Ca^{2+}]_i$ is necessary for granulysin- or G8-induced apoptosis of Jurkat cells.

$[Ca^{2+}]_i$ mediates granulysin-induced apoptosis

The observation that SOC channel blockers prevent the granulysin-mediated increase in $[Ca^{2+}]_i$ suggests that $[Ca^{2+}]_i$ stores are released by granulysin. Therefore, we tested the effect of thapsigargin, a potent and selective sarco/endoplasmic calcium ATPase inhibitor, on changes $[Ca^{2+}]_i$ mediated by granulysin. The pattern of $[Ca^{2+}]_i$ increase induced by thapsigargin is similar to that induced by granulysin (Fig. 1, *H* and *I*). Furthermore, there was no additional $[Ca^{2+}]_i$ increase in cells treated first with granulysin and then with thapsigargin or visa versa, confirming the involvement of $[Ca^{2+}]_i$ stores.

Granulysin decreases $[K^+]_i$

Potassium is another important ion implicated in apoptosis (9, 16, 17). Treatment of Jurkat cells with granulysin or G8 decreased $[K^+]_i$ levels (Fig. 2, *A* and *G*), while peptide G9 did not alter $[K^+]_i$ (Fig. 2M). The granulysin-mediated decrease in $[K^+]_i$ was prevented by the inclusion of potassium channel blockers (Fig. 2, *B–D* and *H–J*). Granulysin decreased $[K^+]_i$ from ~120 to <60 mM within 15 min (Fig. 2*N*). Increasing $[Ca^{2+}]_i$ by treatment with either ionomycin (Fig. 2*F*) or thapsigargin (Fig. 2*L*) caused similar $[K^+]_i$ decreases in Jurkat cells. Both granulysin- and G8-induced apoptosis were inhibited by potassium channel blockers (Table I).

The observation that the decrease in $[K^+]_i$ is more gradual and occurs later than the granulysin-induced rise in $[Ca^{2+}]_i$ (compare Figs. 1 and 2) suggested that the increase in $[Ca^{2+}]_i$ may trigger the decrease in $[K^+]_i$. To test this, Jurkat cells were treated with the calcium channel blocker nickel, and granulysin- or G8-induced changes in $[K^+]_i$ were measured (Fig. 2, *E* and *K*). No decrease in $[K^+]_i$ was observed, indicating that an increase in $[Ca^{2+}]_i$ is required for the granulysin-induced decrease in $[K^+]_i$.

Granulysin enters cells and increases mitochondrial calcium

To determine whether granulysin remained associated with the plasma membrane, Jurkat cells were treated with 50 μM granulysin for 20 min, washed, fixed, and processed for electron microscopy. Immunogold staining throughout the cell, including mitochondria, endoplasmic reticulum, Golgi, nucleus, and cytosol, was evident by 20 min (Fig. 3), indicating that granulysin rapidly enters target cells and does not localize to a particular organelle. Cells incubated with granulysin for 1–2 min before processing for electron microscopy showed no staining (not shown).

Next, we evaluated whether granulysin could alter mitochondrial calcium ($[Ca^{2+}]_m$; Fig. 4). Jurkat cells were pretreated with the $[Ca^{2+}]_m$-specific indicator Rhod-2, and then 50 μM granulysin, 1 μM ionomycin, or 1 μM thapsigargin was added. $[Ca^{2+}]_m$ was elevated within 30 min after granulysin was added, while

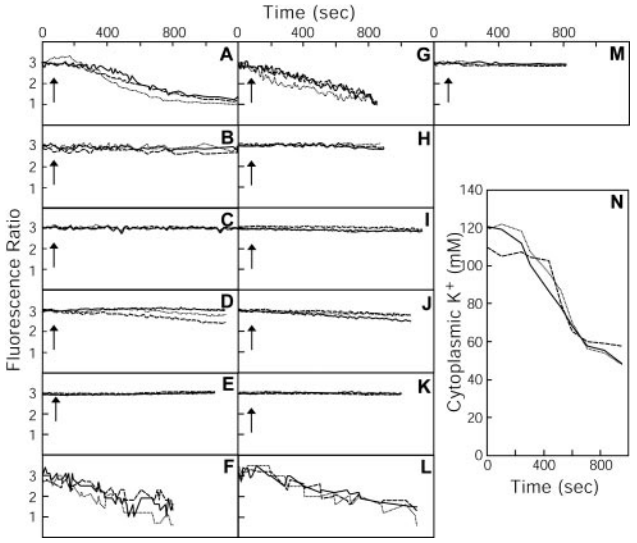

FIGURE 2. Granulysin causes a decrease in $[K^+]_i$. Jurkat cells were pretreated for 30 min with medium (*A*, *G*, and *M*), 40 mM tetraethylammonium (*B* and *H*), 62.5 pM apamin (*C* and *I*), 1 μM charybdotoxin (*D* and *J*), or 1 mM nickel (*E* and *K*). Granulysin (*A–E*; 50 μM), 25 μM G8 (*G–K*), or 25 μM G9 (*M*) was added (indicated by the arrow), and fluorescence was measured as described in Fig. 1. Ionomycin (F) and thapsigargin (L) cause similar decreases in $[K^+]_i$. Cytoplasmic K^+ was determined by calibration of PBFI using the potassium ionophore valinomycin (*N*) as previously described (46). All experiments were performed a minimum of three times.

Table I. *Ca^{2+} and K^+ channel blockers inhibit granulysin- and peptide G8-induced DNA fragmentation in Jurkat cells*[a]

	% FITC-dUTP$^+$ Cells	
	Granulysin	Peptide G8
Calcium channel blockers		
Medium	65	80
Nickel (1 mM)	6	14
Econazole (1 μM)	1	15
Potassium channel blockers		
Medium	76	90
High K^+ buffer	10	16
TEA (50 mM)	32	27
Apamin (62.5 pM)	35	16

[a] Jurkat cells were pretreated with the indicated compound for 30 min prior to the addition of granulysin (50 μM) or G8 (20 μM). DNA fragmentation was measured as described in *Materials and Methods*. Data shown are representative of at least three similar experiments.

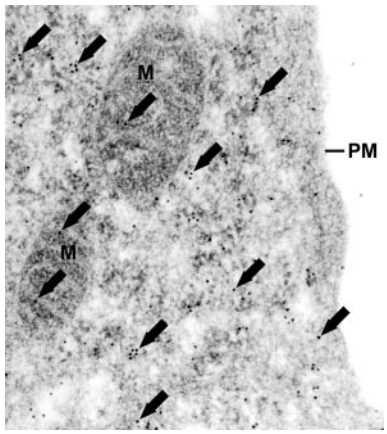

FIGURE 3. Granulysin rapidly enters Jurkat cells. Cells were treated with 50 μM granulysin for 20 min and processed for electron microscopy (magnification, ×40,000). Arrows indicate representative Immunogold staining. There was no Immunogold staining in cells treated identically but without treatment with granulysin. M, mitochondrion; PM, plasma membrane.

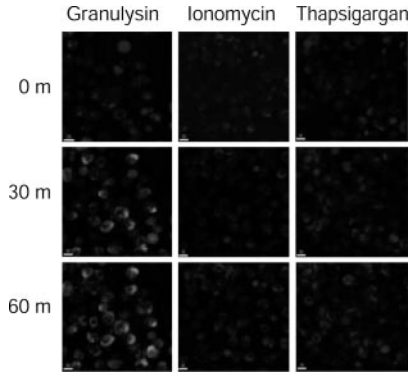

FIGURE 4. Granulysin increases mitochondrial calcium. Rhod-2/AM-loaded Jurkat cells were treated with 50 μM granulysin (GRA), 1 μM ionomycin (IM), or 1 μM thapsigargin (TG), and fluorescence was observed using a confocal microscope.

$[Ca^{2+}]_m$ did not change in cells treated with ionomycin and thapsigargin. Thus, granulysin, but not ionomycin or thapsigargin, induced a large increase in $[Ca^{2+}]_m$ in Jurkat cells.

Granulysin damages isolated mitochondria in the presence of calcium

We previously reported that recombinant granulysin did not damage isolated mitochondria, suggesting that other molecules acted as intermediaries between the plasma membrane and mitochondria (3). However, these experiments were performed in medium that lacked calcium. Our results, indicating a major role of calcium in granulysin-mediated apoptosis, prompted us to re-examine this finding in medium supplemented with calcium (Fig. 5). In the presence of calcium and regenerating buffer, isolated mitochondria were damaged by recombinant granulysin, releasing both AIF and cytochrome *c*.

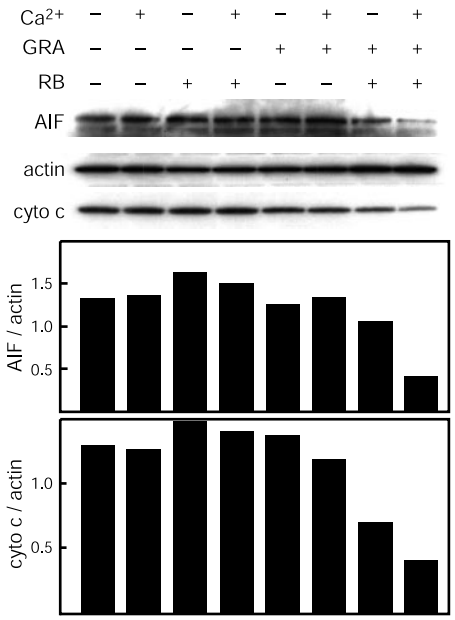

FIGURE 5. Granulysin damages isolated mitochondria in the presence of calcium and regenerating buffer. *Upper panel*, Western blot of AIF, actin, and cytochrome *c* (cyto c) from isolated mitochondria incubated as indicated with 50 μM granulysin (GRA), 1 μM calcium, or regenerating buffer (RB). *Lower panels*, Ratio of intensity of the indicated bands as determined by densitometry.

Granulysin induces ROS production

Because granulysin damages mitochondria directly, we investigated ROS generation in granulysin-treated Jurkat cells. ROS increased in granulysin-treated Jurkat cells (Fig. 6*A*), and this increase was inhibited when cells were incubated in medium containing EGTA, suggesting that the granulysin-induced ROS increase is calcium dependent. Granulysin-induced ROS is also inhibited by preincubation of Jurkat cells with rotenone (Fig. 6*B*). This observation suggested that mitochondrial electron transport was involved.

Therefore, we tested the effects of granulysin on Jurkat cells preincubated with electron transport complex inhibitors (Fig. 7, *A–F*). Rotenone, antimycin, and KCN all inhibited granulysin-induced cell death. Next, the effect of electron transport inhibitors on granulysin-induced mitochondrial damage was studied. Rotenone inhibited the release of cytochrome *c* and AIF from mitochondria by granulysin (Fig. 7, *G* and *H*).

Rotenone and CCCP had no significant effect on the granulysin-induced increase in $[Ca^{2+}]_i$ (Fig. 8, *A–C*). In contrast, both rotenone and CCCP inhibited the granulysin-induced $[Ca^{2+}]_m$ increase (Fig. 8*D*). These data indicate that electron transport is involved in calcium uptake into mitochondria and that inhibition of this uptake blocks granulysin-mediated cell death.

Glutathione affects granulysin-mediated cell death

Taken together, these findings implicate the redox state in granulysin-mediated cell death and suggest that changing intracellular glutathione levels may affect granulysin-mediated apoptosis. To test this hypothesis, Jurkat cells were preincubated with OTC or BSO. The glutathione level in cells treated with OTC, which is transported into cells and metabolized into cysteine by 5′-oxoprolinase (18), increased 200%, while the glutathione level in cells

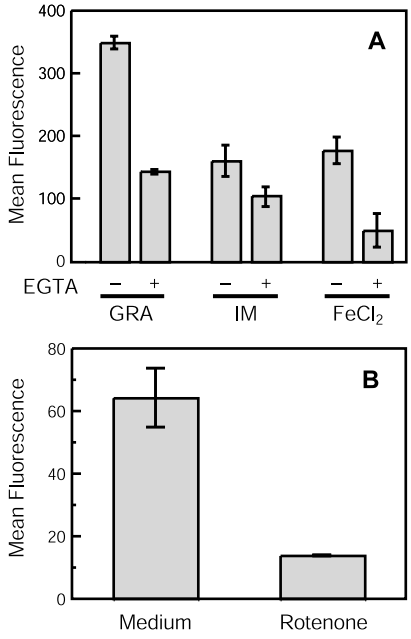

FIGURE 6. ROS increase in granulysin-treated Jurkat cells. *A*, ROS levels in Jurkat cells treated with 50 μM granulysin (GRA), 1 μM ionomycin (IM), or 30 μM $FeCl_2$ for 1 h in the presence or the absence of 5 mM EGTA. Baseline ROS levels were 81 $\pm$ 6.3 without EGTA and 62 $\pm$ 5.4 in the presence of EGTA. *B*, Jurkat cells were preincubated with 1 μM rotenone or medium for 30 min, followed by addition of 50 μM granulysin. ROS were determined after an additional 60 min. In the absence of granulysin or rotenone, baseline ROS was 40 $\pm$ 3.

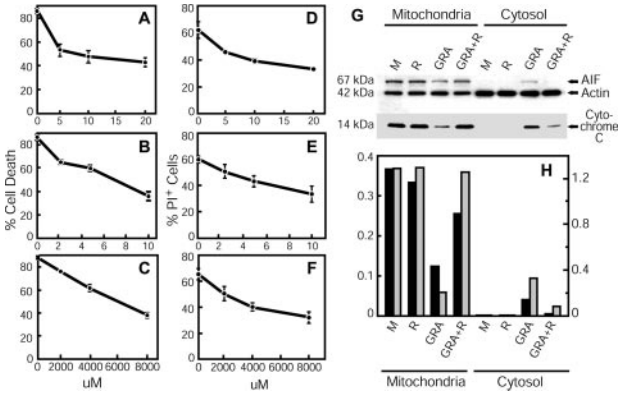

FIGURE 7. Electron transport complex inhibitors block granulysin-induced apoptosis. Jurkat cells were preincubated with the electron transport complex I inhibitor rotenone (*A* and *D*), the complex III inhibitor antimycin A (*B* and *E*), or the complex IV inhibitor KCN (*C* and *F*) for 30 min. Granulysin-mediated cell death was assessed by MTT assay (*A–C*) or PI uptake (*D–F*). No cytotoxicity was observed in cells incubated with the rotenone, antimycin A, or KCN alone. *G*, Jurkat cells were incubated with medium (M), 20 μM rotenone (R), or 50 μM granulysin (GRA) or were preincubated with 20 μM rotenone, followed 50 μM granulysin (GRA+R). After a 3-h incubation, mitochondrial and cytosolic fractions were prepared and subjected to Western blot analysis with mAb specific for AIF, actin, or cytochrome *c*. *H*, Densitometry of *G* (■, ratio of AIF/actin; ▨, ratio of cytochrome *c*/actin).

treated with BSO, an irreversible inhibitor of γ-glutamylcysteine synthetase (18), decreased by >90% (data not shown). Granulysin-induced apoptosis was significantly inhibited in OTC-treated Jurkat cells (Fig. 9*A*), while only a minimal effect was observed in BSO-treated cells (Fig. 9*B*). Thus, the redox state affects susceptibility to granulysin-mediated cell death.

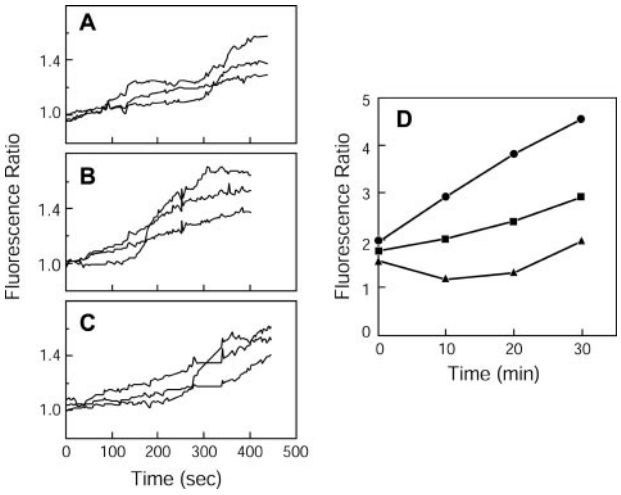

FIGURE 8. Electron transport complex inhibitors do not affect the granulysin-induced increase in $[Ca^{2+}]_i$, but block the increase in $[Ca^{2+}]_m$. Jurkat cells loaded with Fura-2/AM to measure $[Ca^{2+}]_i$ were preincubated with medium (*A*), 1 μM rotenone (30 min; *B*), or 1 μM CCCP (5 min; *C*). Granulysin (50 μM) was added, and $[Ca^{2+}]_i$ was determined by fluorescence microscopy. *D*, Jurkat cells loaded with Rhod-2/AM to measure $[Ca^{2+}]_m$ were pretreated with medium (●), 1 μM rotenone (30 min; ■), or 1 μM CCCP (5 min; ▲). Granulysin (50 μM) was added, and Rhod-2 fluorescence was determined. Each value represents the mean fluorescence value from 30–50 cells in each experiment.

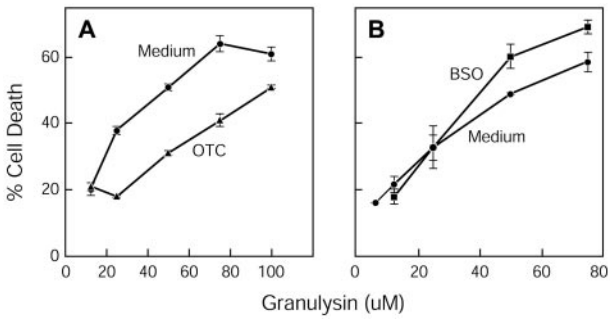

FIGURE 9. Granulysin-induced cell death is modulated by glutathione. Jurkat cells were incubated with the indicated agents for 24 h, granulysin was added, and cell death was determined by MTT assay.

Discussion

In this study we demonstrate that one of the earliest events following the interaction of granulysin with cells is a store-operated Ca^{2+} channel-dependent release of Ca^{2+} from the endoplasmic reticulum. Remarkably, this Ca^{2+} release occurs before measurable breakdown of the plasma membrane and appears to be an almost immediate event (3). The increase in $[Ca^{2+}]_i$ leads to a decrease in $[K^+]_i$, presumably by opening a calcium-dependent potassium channel. Cells treated with granulysin exhibit an increase in $[Ca^{2+}]_m$ that is blocked by inhibitors of electron transport. Granulysin does not remain associated with the plasma membrane; rather, it enters the cell, where it may inflict further injury. Recombinant granulysin damages isolated mitochondria in the presence of calcium and ATP-regenerating buffer. Once damaged, mitochondria release AIF and cytochrome *c* and generate ROS, further potentiating apoptosis. Taken together, these data add to our understanding of the pathways from the cell surface to the mitochondria and implicate calcium, potassium, redox state, ROS, and oxidative metabolism in granulysin-mediated apoptosis.

Ca^{2+} is a common signal in many different cell death pathways, and there is an intricate relationship among mitochondrial function, ROS production, Ca^{2+}, and cell death (19). Depletion of the endoplasmic reticulum Ca^{2+} pool can lead to apoptosis, while loss of mitochondrial function causes an increase in cytosolic Ca^{2+}. Overload of $[Ca^{2+}]_i$ has long been implicated in the final common pathway in cell death (20). Recently, it has become clear that large increases in $[Ca^{2+}]_i$ are associated with necrosis, whereas less dramatic increases are associated with apoptosis. Our observation that granulysin induces a 2- to 3-fold increase in $[Ca^{2+}]_i$ in Jurkat cells that go on to apoptosis is consistent with findings using other apoptosis-inducing agents in other cell types (21–23). Ca^{2+} release from the endoplasmic reticulum and/or capacitative Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels induces apoptosis (24, 25). Again, our finding that granulysin causes initial release of Ca^{2+} from intracellular stores/endoplasmic reticulum is consistent with Ca^{2+}-induced apoptosis. It has been proposed that release of Ca^{2+} from intracellular stores may trigger apoptosis due to the proximity of mitochondria (26). Mitochondria take up this Ca^{2+}, and the increase in mitochondrial Ca^{2+} may trigger the opening of transition pores and the release of cytochrome *c* and AIF. We previously reported that granulysin caused a rapid increase in $[Ca^{2+}]_i$ (3) and the release of AIF from mitochondria (4), consistent with these hypotheses. We show here that granulysin induces a much larger increase in $[Ca^{2+}]_m$ than either ionomycin or thapsigargin, and the increase in $[Ca^{2+}]_m$ is abrogated by the electron transport inhibitors CCCP and rotenone.

A decrease in $[K^+]_i$ has previously been associated with apoptosis (6, 27). Physiologic levels of $[K^+]_i$ play a key role in maintaining caspases in their inactive proforms (28). Activated caspases, especially caspase 3, orchestrate DNA fragmentation, nuclear condensation, and membrane blebbing as well as damage to mitochondria (29). We previously demonstrated that caspase 3 is activated in granulysin-treated cells (3). Activation of caspase 3 may result from the decrease in $[K^+]_i$ or from mitochondrial damage and loss of components that form the caspase-activating apoptosome. Loss of K^+ also results in cell shrinkage that is associated with apoptosis.

Disruption of mitochondrial transmembrane potential ($\Delta\psi_m$) is thought to be the point of no return in apoptotic signaling (30, 31). We showed earlier that $\Delta\psi_m$ is decreased in granulysin-treated Jurkat cells (3). ROS levels are partially inhibited in *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-treated Jurkat cells activated by Fas, suggesting that caspase 3 activation, perhaps through damage of mitochondrial membrane integrity, contributes to ROS production and serves as a positive feedback loop at later stages of the apoptotic process. Green and co-workers (29) showed that both the rapid loss of $\Delta\psi_m$ and the generation of ROS are due to the effects of activated caspases on mitochondrial electron transport complexes I and II. Caspase 3 disrupts oxygen consumption induced by complex I and II substrates, but not that induced by electron transfer to complex IV. These findings indicate that after cytochrome *c* release the activation of caspases feeds back on the permeabilized mitochondria to damage mitochondrial function (loss of $\Delta\psi_m$) and generate ROS through effects of caspases on complex I and II in the electron transport chain. Intracellular levels of ROS are controlled by a balance of mitochondrial ROS production and the concentration of reducing equivalents, such as glutathione. Increased production of ROS was demonstrated in TNF- (32–34) and Fas-mediated (35–37) cell death. We report here that ROS are increased in granulysin-treated cells.

Mitochondrial $\Delta\psi_m$ is generated by the electron transport system that consumes O_2 and pumps protons across the mitochondrial inner membrane to produce ATP. Rotenone, antimycin A, and KCN hamper this process by disturbing complex I, III, or IV of the electron transport chain, respectively. We found that these inhibitors reduced granulysin-induced apoptosis. Rotenone prevented the release of AIF and cytochrome *c* from granulysin-treated cells. The inhibitor CCCP is a protonophore that disrupts the mitochondrial proton gradient. Collapsing this gradient abrogates mitochondrial membrane potential and thereby disrupts the gradient driving Ca^{2+} into mitochondria (38, 39). This explains in part why rotenone and CCCP inhibit the granulysin-mediated increase in $[Ca^{2+}]_m$.

The permeability of the mitochondrial membrane is regulated by an oxidation-reduction equilibrium of ROS, pyridine nucleotides, and glutathione levels. Glutathione is the most important intracellular reducing compound in cells, making up >90% of the intracellular nonprotein thiols that are sequestered in subcellular components. It is the major determinant of the intracellular redox potential (40) and is an important determinant of cellular apoptotic potential (41). We show here that increasing the intracellular glutathione level in OTC-treated cells protected from granulysin induced apoptosis, while decreasing the intracellular glutathione level in cells treated with BSO had minimal effects. We expected that decreasing intracellular glutathione by >90% would render cells more susceptible to the effects of granulysin. However, this was not the case, suggesting that the residual glutathione was sufficient to protect cells from some of the effects of granulysin. We were unable to reduce the intracellular glutathione levels further, as treatment of cells with >100 μM BSO resulted in increased cell death in the absence of added granulysin.

Several lines of evidence point to mitochondrial calcium uptake as a triggering point for ROS generation (42–44). Three dehydrogenases of the Krebs cycle (pyruvate, isocitrate, and oxoglutarate dehydrogenase) are activated by $[Ca^{2+}]_m$, which, in turn, leads to increased availability of NADH to the electron transport system. Eventually these electrons are transferred from NADH and $FADH_2$ to oxygen. This can also result in oxygen free radical generation, since there is a tendency for electrons to transfer to oxygen directly rather than to the next component in the chain. Our observations that 1) rotenone prevents the release of cytochrome *c* induced by granulysin; 2) rotenone decreases the production of ROS in granulysin-treated cells; and 3) ROS generation occurs after the granulysin-induced $[Ca^{2+}]_m$ increase are consistent with the increase in $[Ca^{2+}]_m$ as the initial trigger for granulysin-induced apoptosis.

Mitochondria appear to be the major targets of granulysin-mediated apoptosis of Jurkat tumor T cells. Several different pathways converge on the energy-generating machinery of the cell. Here we identify several mediators of this process, including $[Ca^{2+}]_i$, $[K^+]_i$, and granulysin itself. Preliminary results with microarrays of *Mycobacteria tuberculosis* treated with granulysin show similar pathways of membrane damage, ion fluxes, and interference with the redox state and oxidative metabolism. It is likely that the tumoricidal activity of granulysin is a byproduct of its evolved function as an antimicrobial peptide. Of note, however, it is possible to separate these activities with synthetic peptides (5), offering the opportunity to design antimicrobial therapies with limited toxicity to mammalian cells. On the other hand, granulysin toxicity to tumor cells far exceeds its activity against normal cells, such as macrophages and dendritic cells (1), suggesting that granulysin may prove a potent antitumor agent as well (45).

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