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Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation

(antitumor/cytolysis/magainin channels)

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ABSTRACT **Magainins are an ionophoric class of vertebrate peptides with antibiotic activity against various microorganisms. Here we show that magainin 2 and synthetic analogues can rapidly and irreversibly lyse hematopoietic tumor and solid tumor target cells with a relative cytotoxic potency that parallels their antibacterial efficacy and at concentrations that are relatively nontoxic to well-differentiated cells. The cytotoxicity is prevented by cell depolarization. Magainins represent a natural cytolytic agent in vertebrates and may provide another therapeutic strategy for certain tumors.**

Magainins are a naturally occurring ionophoric class of peptides recently isolated from *Xenopus laevis* skin that exhibit antibiotic activity against various microorganisms at concentrations having little, if any, toxicity for differentiated erythrocytes (1). Magainin and synthetic analogues irreversibly depolarize bacteria in direct proportion to their antibiotic activity, which is correlated with the formation of an α-helical configuration in a nonpolar environment (2–4). Magainins are thus thought to be α-helical peptide ionophores that rapidly dissipate ion gradients to lyse bacteria (4). The observation that magainin molecules can polymerize under certain conditions suggests the hypothesis of a multimolecular channel formation (5). Here we show that magainins irreversibly lyse various hematopoietic tumor and solid tumor cells with a relative potency that parallels their antibiotic activity and at concentrations that exhibit relatively little toxicity for peripheral blood lymphocytes (PBLs).

MATERIALS AND METHODS

Cell Culture. *Hematopoietic cell lines.* Cells were maintained in suspension culture using RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) to a density of 4×10^5 cells per ml. PBLs were purified from whole human blood using a Ficoll gradient. Cells were centrifuged at 1200 rpm for 5 min in a Sorvall centrifuge and resuspended in fresh medium. This procedure was repeated four times, after which they were transferred to a 75-ml flask at 10^6 cells per ml and incubated at 37°C and 7% CO_2. To remove monocytes, which readily attach to the walls of the flask, PBLs remaining suspended after 30 min of incubation were carefully transferred to a new flask on four successive occasions and then maintained at 37°C. Tumor cells and PBLs were centrifuged at 1200 rpm for 5 min, resuspended in "fresh medium" containing 140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM Tris·HCl (320 mosM, pH 7.3), and then allowed to recover at 37°C for 30 min. Cells (4×10^5 per ml) were then incubated for 20 min in 96-well flat-bottom plates (Costar) at 37°C in the presence of varying concentrations of magainin peptide analogues. Cytotoxicity was assessed by counting the number of cells stained with trypan blue (TB) (6).

Solid tumor cell lines. Cells were grown in 100-ml flasks. After cell confluence the medium was removed and 0.5 ml of RPMI 1640 medium containing 0.01% trypsin was added to the flask for 30 sec. Three minutes after removing the trypsin 10 ml of fresh medium was added and the cells were resuspended. Cells were counted and cultured at 10^5 cells per ml in 96-well plates (Costar). Forty-eight hours later experiments of the type described above were carried out.

Fluorescence Measurements. Cells were incubated at room temperature (22–24°C) in 200 nM anionic oxonol dye [Di Ba-C4(3); Molecular Probes; excitation, 488; emission 530] until a stable level of fluorescence intensity was recorded in a fluorescence spectrophotometer (Perkin–Elmer LS-5). As cells change potential the dye redistributes between the medium and the cells. More anionic dye concentrates in depolarized cells, which fluoresce with more intensity. The increased fluorescence signal is likely due to interaction between dye molecules and cellular lipid and protein, since in the absence of cells fluorescence intensity is directly proportional to the concentration of natural lipid or protein dissolved in salines containing identical concentrations of dye (N. Hardegen and J.L.B., unpublished observations).

Fluorescence Calibration. Gramicidin peptides have been used to calibrate aggregate oxonol fluorescence of cellular populations in terms of membrane potential with fluorescence spectrophotometry (7). Assuming that 200 nM gramicidin D, which is composed of gramicidins A, B, C, and D, forms β-helical ion channels that are equally selective for Na^+ and K^+ ions in tumor cells, and that the intracellular concentrations of Na^+ and K^+ total about 150 mM and remain constant during the several-minute fluorimetric recording period, then we can estimate the average membrane potential, V_m, from the simple Nernstian formulation $V_m = 59 \log(K_o^+ + Na_o^+)/150$, where Na_o^+ (extracellular Na^+ concentration) was varied from 0 to 145 mM by centrifuging and resuspending cells in saline containing *N*-methylglucamine$^+$ (NMG^+) ions, which are impermeant in gramicidin channels, substituted for Na^+ ions. In the presence of physiological Na^+ concentration the cell membrane potential was close to 0 mV as calculated by the Nernst equation. Varying Na^+ in this manner revealed a linear relationship between fluores-

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Abbreviations: PBL, peripheral blood lymphocyte; TB, trypan blue; PMN, polymorphonuclear neutrophil; NMG, *N*-methylglucamine.

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cence intensity and Na_o^+ over the 0–145 mM range (not shown). At physiological Na_o^+ and K_o^+ (extracellular K^+ concentration) (5 mM), V_m averages −70 mV in these cells.

RESULTS

Magainins Are Cytotoxic for Various Tumor Cells. Cytotoxicity assays were carried out on suspension cultures of various hematopoietic tumor and solid tumor cell lines and normally differentiated circulating lymphocytes using nine magainin peptide analogues. The peptides showed varying degrees of cytolytic activity within minutes against various tumor targets (Fig. 1 *A1* and *A2*; Table 1). The relative antitumor activities of different structures were similar to their relative antibiotic potencies. Synthetic magainins A, B, and G, all of which are amidated and relatively resistant to peptidase digestion (ref. 8 and unpublished observations), were at least 9-fold more potent than the natural magainin 2 structure against 8402 cells, which parallels their relative antibiotic potencies against *Escherichia coli* (3). In terms of absolute concentration the more potent analogues were 5–11 times less effective against these tumor cells than against *E. coli*. Similar degrees of cytotoxic activity were expressed by the magainins against six other erythroleukemia phenotypes as well as several types of solid tumor cells (Table 1). The growth inhibition assay (Fig. 1*A2*) correlated well with the results of acute TB exclusion determination, suggesting a good correspondence between the two assays and demonstrating the inability of tumor cells to recover once viability had been compromised (e.g., TB^+) in the presence of the peptide.

Magainins Are Selectively Cytotoxic for Tumor Cells. The selectivity of the magainins for tumor targets was tested by carrying out cytotoxicity assays on PBLs. Magainin G was the most selective of the compounds tested, having virtually no cytolytic effect on PBLs after 60 min of incubation at a concentration twice that required to lyse 100% of tumor cells in 10 min (Fig. 1*B*). Similar results were observed with polymorphonuclear neutrophils (PMNs) (data not shown). Magainin B, the most potent of the structures tested, exhibited lytic activity against PBLs and PMNs at ≈5 times the effective antitumor concentration (data not shown). Magainin B exhibits the highest degree of α-helical configuration in a nonpolar environment (3), which may account for its cytotoxic effects on PBLs and PMNs. The rest of the structures (e.g., magainin 2; Fig. 1*B*) possessed lytic activity against PBLs and PMNs at concentrations >10 times their antitumor efficacies. Thus, *in vitro* magainin peptides are tumoricidal at concentrations 5–10 times greater than those required for antibiotic effects but 10–20 times less than those toxic to normally differentiated cells.

Magainins Shift Tumor Cell Membrane Potential. Since magainins decrease bacterial membrane potential within minutes (4), presumably by forming ion-conducting α-helical channels in the plasma membrane similar to the ion channels recorded in lipid bilayers (refs. 9 and 10; unpublished observations), we studied the effects of magainins on the membrane potential of tumor cells by staining them with fluorescent potentiometric oxonol dye and then recording fluorescence signals fluorimetrically in response to magainin peptides. We used the well-established peptide ionophore gramicidin D, which creates β-helical channels equally selective for Na^+ and K^+ ions in bilayers (11), to "clamp" tumor cells at membrane potentials over the physiological range (−90 to 0 mV) and thus determine the relationship of aggregate oxonol fluorescence intensity to cellular potential. Addition of gramicidin to oxonol-stained tumor cells incubated in various $[Na^+]_o$ values resulted in clear and reproducible changes in fluorescence intensity (Fig. 2*A*), demonstrating the potentiometric capability of the oxonol dye in fluorimetric

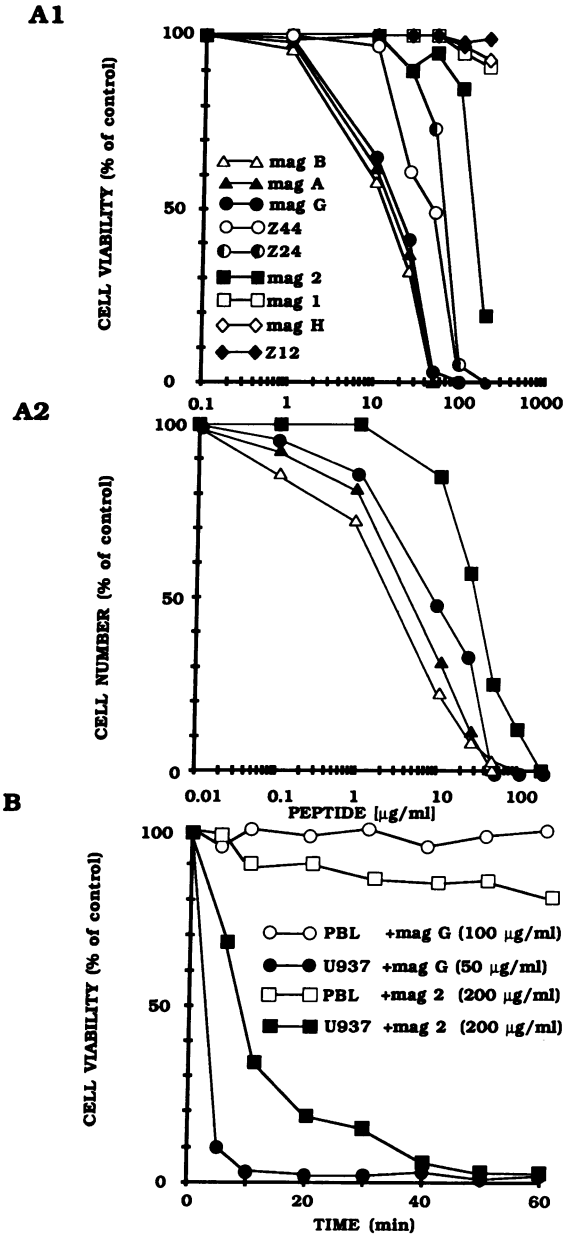

FIG. 1. Magainin peptides are antitumor *in vitro*. (*A1*) Naturally occurring and synthetic magainin peptides are cytotoxic for U937 cells. Varying degrees of cytotoxic activity were recorded among the nine analogues tested. Active peptides exhibited cytotoxicity over relatively narrow ranges of concentration (within 1 logarithmic unit). Some of the amidated analogues (mag A, B, and G) were considerably more potent than the natural magainin 2 peptide. (*A2*) U937 cells were cultured as described in the text. After exposing the cells for 20 min to varying concentrations of the peptides (for control cells we added equivalent volumes of distilled water), 40 ml of fresh medium was added to dilute the peptide. The samples were centrifuged at 1200 rpm for 5 min and then resuspended three times to remove magainin from the extracellular medium. After the last resuspension, 4×10^5 cells per ml were placed in the incubator. Three days later the cells were counted with a Coulter Counter. The results are plotted as a percent of control. Brief exposure to increasing concentrations of the peptides completely eliminated progressively greater numbers of cells. IC_{50} (concentration in μg/ml that lysed 50% of the cells) values paralleled the results from acute exposure assays and appeared to be severalfold lower. These results strongly suggest that TB^+ reflects cellular death. (*B*) Magainin G combines greater potency and selectivity than magainin 2. One hundred percent cytolytic activity was observed at 10 min with half of the concentration of magainin G that did not manifest cytotoxic effects on PBLs at 60 min. Higher concentrations of magainin 2 were less quick to kill and lysed a fraction of PBLs.

Table 1. Antitumor activities of magainin peptides

Peptide	Hematopoietic tumor cell line							Solid tumor cell line		
	U937	8402	SSKT1	Daudi	MLA	Raji	K562	CHP-100	MCF7	PC-3
Magainin B	17 ± 3	12 ± 2	27 ± 3	28 ± 4	30 ± 4	23 ± 3	32 ± 6	32 ± 11	37 ± 7	41 ± 11
Magainin G	19 ± 4	16 ± 3	29 ± 5	38 ± 4	33 ± 7	25 ± 4	37 ± 5	39 ± 12	42 ± 13	47 ± 10
Magainin A	16 ± 5	18 ± 4	23 ± 5	30 ± 5	34 ± 4	20 ± 2	40 ± 5	45 ± 11	49 ± 12	53 ± 12
Z24	97 ± 15	60 ± 12	107 ± 9	115 ± 11	80 ± 14	64 ± 13	98 ± 7	ND	ND	ND
Z44	123 ± 18	83 ± 15	109 ± 11	143 ± 20	102 ± 16	72 ± 11	112 ± 19	ND	ND	ND
Magainin 2	>150	>150	>150	>150	>150	>150	>150	>200	>200	>200

ND, not determined. Cytolytic potencies of magainin 2 and analogues on tumor cells are indicated. Potency is expressed as IC_{50} (μg/ml). The origins of the cell lines are as follows. Hematopoietic cell lines: SSKT1 (human acute B leukemia, provided by Stephen Smith, Univ. of Chicago); 8402 (human acute T leukemia); U937 (human histiocytic lymphoma); Daudi and Raji (human Burkitt lymphoma); K562 (human blastic crisis-chronic myelogenous leukemia); MLA (ape leukemia T, ATCC). Solid tumor cell lines were provided by Leonard Neckers (NCI): MCF7 (human breast tumor), PC-3 (prostatic tumor), CHP-100 (neuroepithelioma). The peptide sequences of Z44 and Z24 are $[ALSK]_6$ and $[AKSK]_6$, respectively. The sequences of the rest of the peptides have been published elsewhere (1, 3).

recordings of these tumor cells. Gramicidin, which depolarizes cells to 0 mV in physiological Na_o^+ and has antibiotic activity (11), was not cytotoxic to the various tumor cells studied at concentrations used to calibrate fluorescence in terms of membrane potential for up to 1 hr. Addition of inactive, noncytotoxic magainins did not alter membrane potential (Fig. 2*B*), whereas sublethal, noncytotoxic concentrations of active magainins elicited variable but detectable changes in oxonol fluorescence (Fig. 2 *C* and *D*), indicative of membrane potential shifts presumably resulting from a low number of magainin channels permeable to ambient ions.

Magainin-Induced Channels Are Permeable to Na^+, K^+, and Cl^- Ions. The ionic mechanisms involved in the changes in potential were evaluated by altering Na_o^+ and Cl_o^- since both ions permeate magainin channels formed in bilayers (ref. 9; unpublished observations). Fluorescence intensity was consistently greater in low Cl_o^- than in physiological Cl_o^-, suggesting that Cl^- ions permeate magainin 2 channels formed in the plasma membrane of the tumor cells (Fig. 2*C*). The depolarization induced by magainin 2 in physiological Na_o^+ disappeared in Na_o^+-free medium and a just-detectable hyperpolarization occurred (Fig. 2*C*), suggesting that Na^+ and K^+ ions also permeate. Low concentrations of the more potent magainin G induced a just-detectable hyperpolarization in physiological Na_o^+ and Na_o^+-free medium but did not apparently change potential in medium containing physiological Na_o^+ and low Cl_o^- (Fig. 2*D*). The hyperpolarizations suggest that magainin G induces channels that may be more K^+-ion selective, whereas the elimination of any potential shift in low Cl_o^- suggests some contribution of Cl^- ions as well in the response. Lowering Cl_o^- shifts the equilibrium potential for Cl^- in a depolarizing direction. Cl^- ion-dependent conductance was mediated by gramicidin channels. The depolarization could neutralize the hyperpolarization recorded in saline containing physiological Cl_o^- concentration. Ten-fold higher, cytotoxic concentrations of active analogs invariably increased fluorescence intensity in physiological saline (Fig. 3*A1*) but this "depolarizing" effect was independent of Na_o^+ (Fig. 3*A2*). The fluorescence increase was related to the cytotoxic effects of the peptides, indexed by the number of TB-stained cells (Fig. 3*B*). Fluorimetric recording of fluorescence changes induced by cytotoxic concentrations of magainins cannot resolve the relationship between the fluorescence changes induced in single cells and their viability. The increase in fluorescence intensity induced by lethal concentrations in Na_o^+-free medium either reflects interaction of the dye with cellular protein and lipid independent of membrane potential in a magainin-permeabilized cell or a decrease in the selectivity of magainin-induced channels such that NMG^+ ions carry net positive charge into the cell leading to apparent depolarization. Results with oxonol fluorescence and TB staining of tumor cells are consistent with the notion that cytotoxic concentrations of magainins form poorly selective ion channels whose appearance closely corresponds to the cytotoxic activities of the peptides against tumor targets.

Depolarization Prevents Cytotoxic Activity of the Magainins. Cells depolarized near 0 mV, either by resuspension in 150 mM KCl or by prior addition of gramicidin in physiological Na_o^+ (Fig. 4), excluded TB in the presence of cytotoxic concentrations of active magainin B. Cells resuspended in NMG^+ and then hyperpolarized by adding gramicidin (Fig. 4) were as sensitive, if not slightly more sensitive, to magainin B; 90% ± 4% ($n = 5$) of the highly fluorescent cells were TB^+. Note that resuspension in NMG led to a modest hyperpolarization of cells relative to resuspension in physiological saline. We estimate in this experiment the resting potential to be about −71 ± 5 mV and the resuspension in Na_o^+-free medium to hyperpolarize cells, after the addition of gramicidin, to be about −87 ± 6 mV. Note also that the aggregate fluorescence intensity of cells in physiological saline depolarized to about 0 mV by adding gramicidin is quite close to the value obtained by resuspending cells in 150 mM KCl (within an estimated 5 mV).

DISCUSSION

Here we have found that various magainin peptides are antitumor *in vitro* at concentrations that parallel their antibiotic activities, that antitumor potency is less than antibiotic potency on a molar basis, and that these peptides are selective for tumor targets over well-differentiated phenotypes, but the degree of selectivity varies among analogues.

The results also indicate that noncytotoxic concentrations of active magainin peptides render tumor cells variably permeable to monovalent cations prevalent on either side of the plasma membrane and to Cl^-, similar to their effects on artificial lipid bilayers (ref. 9; unpublished observations). Alteration in membrane permeability was also observed in vesicles (12). Presumably, noncytolytic concentrations of magainin peptides form insufficient numbers of variably selective α-helical ion channels to compromise cell viability. Since gramicidin (a highly selective Na^+/K^+-permeable ionophore) did not induce cytotoxicity the poor selectivity of magainin peptide channels likely accounts for the rapid lysis at cytotoxic concentrations.

Previous studies have shown that the channel-forming properties of magainin peptides in lipid bilayers are dependent on the lipid composition of the bilayer (unpublished observations) and the presence of an electrical or ionic gradient (9). These physicochemical properties have also been found to be important in the ion-channel-forming behavior of antibiotic cecropin peptides in bilayers (13). We found that the cytotoxic activity of magainin peptides for tumor cells was prevented by eliminating the electrical gradient across the plasma membrane. These results are consistent with the notion that cellular potential is critical for magainin channel formation in native membranes of tumor

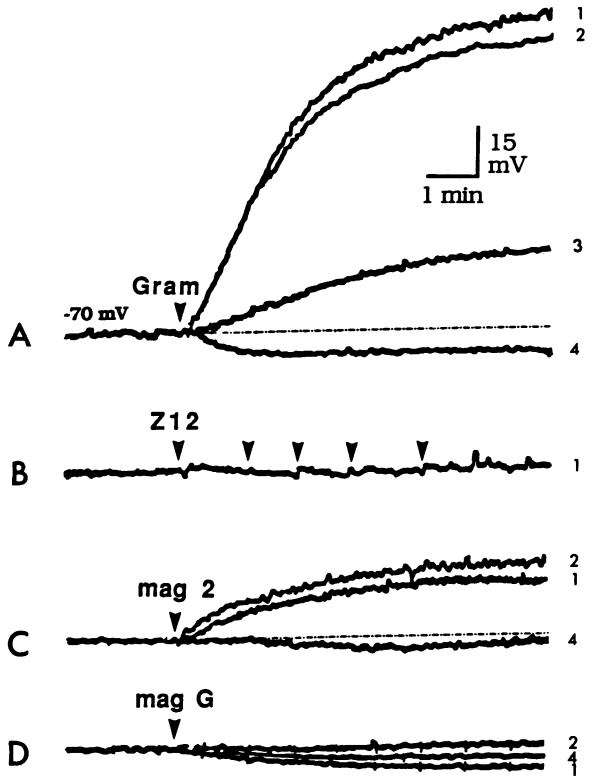

FIG. 2. Ionophoric gramicidin and magainin peptides alter tumor cell membrane potential. U937 erythroleukemia cells (4×10^5 cell/ml) were centrifuged, resuspended in altered electrolytes at room temperature (22–24°C) for 5 min, and then stained with 200 nM oxonol dye for 5 min. After a stable baseline recording of aggregate cellular fluorescence intensity was obtained, either 200 nM gramicidin D (Gram) (*A*), 50 μg (noncytotoxic) of Z12 peptide per ml (*B*), or noncytotoxic levels of magainin 2 (10 μg/ml; *C*) or magainin G (2 μg/ml, *D*) was added to the cuvette. Na_o^+ ions in the physiological saline (traces marked 1; see Fig. 1 legend) were replaced with NMG^+ (traces marked 4) and Cl_o^- was lowered by replacing NaCl with sodium gluconate (traces marked 2). Responses labeled 3 were recorded in salines containing NMG^+ and Na^+ ions in 6:1::NMG^+:Na^+ proportion so as to give a theoretical membrane potential of 45 mV under conditions where cells are equally permeable to Na^+ and K^+ ions (in the presence of gramicidin) and assumed to have a total of intracellular Na^+ and K^+ complement of 150 mM. (*A*) Resuspension in altered Na_o^+ or Cl_o^- does not significantly alter baseline fluorescence, indicating insignificant contributions of Na_o^+ and Cl_o^- to the resting membrane potential. Gramicidin D induces rapid changes in cellular fluorescence in each electrolyte and fluorescence becomes directly and significantly proportional to Na_o^+ ($Y = -0.43x + 30.6$; $r = 0.93$). The estimated resting potential averaged −67 mV ± 7 in 23 experiments. (*B*) Z12 has no detectable effect on resting potential after accumulating to a final concentration of 250 μg/ml. (*C*) Magainin 2 (10 μg/ml) induces potential changes in altered electrolytes that implicate Na^+, K^+, and Cl^- ions. (*D*) Magainin G (2 μg/ml) elicits potential responses suggesting contributions of K^+ and Cl^- ions. Higher noncytotoxic magainin G concentrations evoked potential changes similar to these shown for magainin 2 in *C*, indicating that Na^+ ions are also implicated.

cells as well as in artificial lipid bilayers. Since differentiated PBL cells physiologically exhibit negative membrane potential (14) but remain relatively insensitive to the cytotoxic effects of the peptides, either there may be other physicochemical properties that render tumor cells targets for magainin channel formation and lysis or slight differences in membrane potential may account for critical changes in sensitivity to the cytolytic activity of these peptides.

The cecropins have been reported to present cytolytic activity against tumor cell lines (6). The interpretation of the mechanism of action was believed to be related to alterations

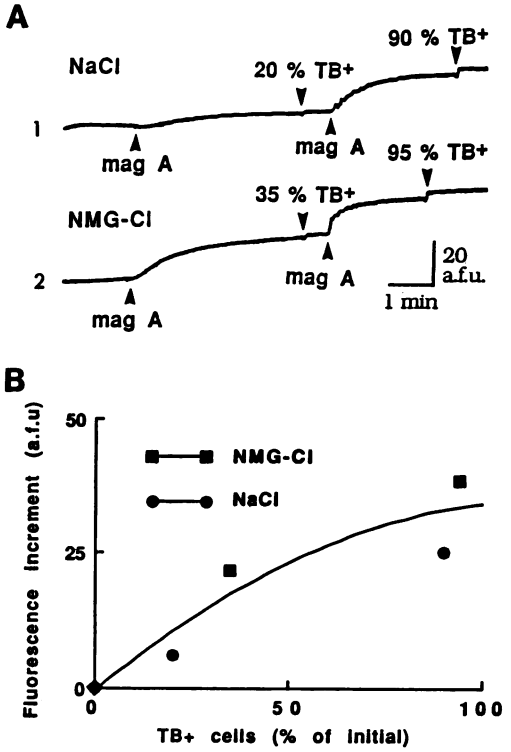

FIG. 3. Magainin induces parallel increases in oxonol fluorescence and cytotoxicity. U937 erythroleukemia cells were stained with 200 nM oxonol and their fluorescence responses to sequential additions of cytolytic concentrations of magainin A (30 μg/ml; upward arrowhead) were recorded fluorimetrically as described in the legend to Fig. 2. Aliquots of cells were assayed for cytotoxicity using TB at the times indicated by downward arrowheads. (*A*) Fluorescence intensity and the percentage of TB^+ cells increase in parallel after exposure to magainin A and these effects occur in physiological saline and Na_o^+-free saline (NMG-Cl). The rate of change in fluorescence signal and the absolute level of emission are greater in Na_o^+-free than in physiological saline. (*B*) The increase in fluorescence intensity is plotted as a function of TB^+ cells and shows that the two parameters are proportional. a.f.u., arbitrary fluorescence units.

in the cell cytoskeleton rather than to channel formation on the cell membrane as we proposed for the magainins (6). In

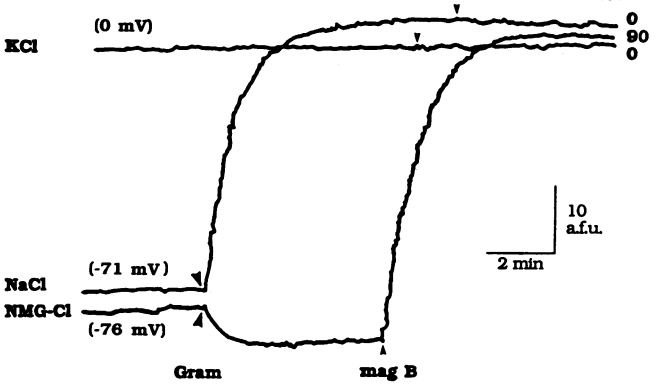

FIG. 4. Depolarization protects tumor cells from magainin cytotoxicity. K562 cells were resuspended in 150 mM KCl, 150 mM NaCl, or 150 mM NMG-Cl and stained with 200 nM oxonol, and their fluorescence responses to sequential additions of 200 nM gramicidin (Gram) (big arrowheads) and 100 μg of magainin B per ml (small arrowheads) were recorded using fluorescence spectrophotometry. Fifteen minutes after addition of gramicidin, cell viability was evaluated with TB. Depolarization of cells near 0 mV in 150 mM K_o^+ or gramicidin in physiological Na_o^+ protects cells from lysis, whereas resuspension in Na_o^+-free medium followed by hyperpolarization in gramicidin does not protect cells.

sum, we have found that, *in vitro*, antibiotic magainin peptides are also tumoricidal, rapidly and irreversibly lysing hematopoietic and solid tumor cells at concentrations having little effect on differentiated PBLs or PMNs. Whether cells in certain stages of the cell cycle are more or less sensitive to these peptides remains to be determined. Of all peptides tested, the most selective magainin G, which is also relatively resistant to hydrolysis by peptidases (8), should be suitable for quickly identifying putative malignant cells and for testing toxicity and antitumor efficacy *in vivo*.

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