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Magainin-induced cytotoxicity in eukaryotic cells: kinetics, dose–response and channel characteristics

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Abstract

Magainin 1 and magainin 2 are broad-spectrum antimicrobial and antifungal peptides initially purified from *Xenopus laevis* skin glands. The mechanism of cytotoxicity of the naturally occurring magainin 2 and a potent all-D amino acid analogue, MSI-238, was examined for eukaryotic cells using flow cytometric analysis with propidium iodide (PI). Exposure to MSI-238 resulted in cell death within seconds to minutes, depending on the concentration of the peptide. Several cell types were examined including a mouse fibroblast cell line Balb/3T3 and a Rous sarcoma virus Balb/3T3-transformed cell line, SRD/3T3, primary chick embryo fibroblasts and cells derived from a human ovarian carcinoma, OVCA-3. The $K_{0.5}$ values determined from 5 min exposures ranged from 24 to 80 μg/ml for MSI-238 and ~ 600 μg/ml for magainin 2. Molecular properties of MSI-238 induced channels were studied in excised membrane patch recordings from Balb/3T3 and SRD/3T3 cells. At low concentrations of 0.1 μg/ml, occasional, brief, multiple-level current fluctuations were seen suggesting channels with multiple, rapidly changing conductance levels. At 5 or 10 μg/ml of MSI-238, the current fluctuations were larger in magnitude and occurred more frequently producing a general disruption of the membrane similar to the effects of melittin on membranes.

Keywords: Magainin peptide; Ion channel; Cytolysis; Eukaryotic cell; Flow cytometry; Propidium iodide; Anti-microbial peptide

1. Introduction

The closely related peptides magainin 1 and magainin 2 were isolated from the skin of the African clawed frog *Xenopus laevis* on the basis of their anti microbial activity [1,2]. These peptides, postulated to play a defensive role against microbial invasion during wound healing in *Xenopus laevis*, produce rapid depolarization of the *Escherichia coli* membrane suggesting that cell death is caused by collapse of the ionic gradients across the membrane [3–5]. The magainin family of related peptides, including recently synthesized all-D analogues [6,7], are members of a large, structurally-diverse family of host-defense peptides distributed in the animal kingdom, which include cecropins, defensins, and bactenecins (reviewed in [3,8,9]). Magainin 2 and related synthetic analogue peptides have also been shown to kill tumor-derived eukaryotic cells at concentrations producing minimal toxicity to peripheral blood lymphocytes [10–12]. The anti-tumor potential of the magainin peptides suggested by in vitro experiments led to a series of in vivo experiments designed to investigate the therapeutic potential of magainins [10]. Mice bearing several types of tumors were i.p. injected with magainin 2 and an all-D amino acid analogue, MSI-238 both of which demonstrated activity against P388 leukemia, S180 ascites, and a spontaneous ovarian tumor. MSI-238 showed somewhat greater activity, presumably because of the reduced susceptibility to proteolysis.

Molecular details of the mechanism of magainin-induced cell kill remain unclear although a number of physical studies have provided evidence for a direct membrane–peptide interaction rather than a membrane-receptor mediated mechanism [13–22]. The current models show that magainin 2, a positively charged 23 amino acid peptide, forms a α-helix upon contact with a trifluoro-

Abbreviations: PI, propidium iodide; $K_{0.5}$, 50% cytotoxic concentration; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

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ethanol/water mixture or a hydrophobic surface [6,20,21,23]. At low concentrations, the peptide lies on the surface of a membrane bilayer at the membrane/water interface. As the peptide concentration increases, peptide monomers associate and the aggregate forms a trans-membrane structure which disrupts the lipid packing and forms an aqueous pathway through which ions can enter or leave the cell. Studies with artificial bilayers have shown that the addition of magainin peptides lead to increases in the membrane conductance and, presumably, these conductance changes are the initial event leading to cell death [14,24].

In order to better understand how magainins interact with cell membranes, we investigated the kinetics and concentration dependence of magainin-induced cell kill using flow cytometric analysis with propidium iodide. The results show that exposure to magainin peptides induces cell death within seconds to minutes, depending on the magainin concentration. A comparison of several types of transformed and non-transformed cells showed similar dose–response relationships for MSI-238 on all cell types tested; magainin 2 was less effective than MSI-238 but the concentration range did not vary significantly among the cell types tested. Magainin-induced membrane activity was also explored in eukaryotic cells using patch electrode recording. This technique allows currents from small patches of cell membrane to be measured [25] and ion channels, both constitutive and those produced by pore-forming peptides, can then be studied directly. Membrane patches excised from susceptible cells were exposed to MSI-238 at concentrations ranging from 0.1 to 10 μg/ml. At low concentrations, occasional, brief, multiple-level current fluctuations were seen suggesting channels with multiple, rapidly changing conductance levels. At higher concentrations, the current fluctuations occurred more frequently eventually resulting in disruption of the membrane. These results are consistent with the hypothesis of peptide aggregates forming at the membrane surface which insert into the membrane producing large conductance, oligomeric structures which lead to membrane disruption [15,18,19].

2. Materials and methods

2.1. Magainin peptides

The peptides were provided by Dr. M. Zasloff. Magainin 2 and MSI-238 were synthesized by the solid-phase procedure and purified as previously described [10]. MSI-238 was synthesized from all-D amino acids.

2.2. Target cells

Mouse Balb/3T3 and 3T3 cells stably transformed with the Schmidt Ruppin D strain of Rous Sarcoma Virus (SRD/3T3) were grown in high glucose DMEM plus 10% calf serum [26]. Chicken embryo fibroblasts were obtained from 11-day-old virus-free minus embryos (SPAFAS, Norwich, CT) and grown at 41°C in high glucose DMEM plus 1% chicken serum (Gibco) and 5% calf serum (J.R. Scientific). The NIH:OVCAR-3 cell line (ATCC HTB 161) was derived from the ascites of a patient with an adenocarcinoma of the ovary [27]. Cells were grown in RPMI medium supplemented with 10 μg/ml insulin and 10% FCS.

2.3. Analysis of magainins effects using flow cytometric analysis

Cells propagated at sub-confluency levels were washed with PBS, and detached with trypsin-EDTA (0.05% and 0.02% w/v, respectively) solution. The suspension of harvested cells was diluted with 10 volumes of growth medium, sedimented at $300 \times g$ for 4 min and resuspended in Solution A containing 140 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose and 25 mM Hepes, pH 7.4 at a density of $2 \cdot 10^6$ cells/ml.

Cytometric analysis were performed on an Epics Elite Flow Cytometer (Coulter, Hialeah, FL) [28]. At the beginning of each experiment, propidium iodide (PI; 10 μg/ml) was added to an aliquot of cells to determine the fraction of viable cells. Two channels were selected in order to determine the fraction of PI positive cells. Channel B was set to include all cells sorted at each sampling and Channel C was set at a higher intensity which excluded $> 95\%$ of viable cells in the control. For comparisons among experiments, data were normalized to the number of viable cells in the presence of PI.

Propidium iodide produced cytotoxic effects only after long exposure (> 60 min) resulting in $\sim 10\%$ positive cells. To minimize this problem, PI was added to each sample just prior to sorting. The time points indicated in the figures reflect time after addition of the magainin peptides, not the addition of PI.

2.4. Phase contrast

To examine the magainin effects on cell morphology, adherent cells were cultured in 35 mm tissue culture dishes at low confluency, washed three times with phosphate buffered saline (PBS) and incubated with 0.25 ml of PBS containing PI (10 μg/ml) or PI plus the MSI-238 at 10 μg/ml for the time indicated at 22°C. The monolayer was then rapidly washed three times with PBS, and the cells were fixed with 1% formaldehyde in PBS for 4 min. The cultures were washed again three times with PBS and mounted with Elvanol. Cells were examined using a $100 \times$ Nikon PlanApo objective, N.A. 1.4 (Nikon, Microphot-FXA, Garden City, CA).

2.5. Patch electrode voltage clamp

BALB/3T3 cells or SRD/3T3 cells were grown on small, round coverslips under conditions described above. To record from the cells, the coverslip was placed on the bottom of an experimental chamber constructed from a glass coverslip and fixed to the stage of an inverted microscope as described previously [29]. Solutions were introduced into the chamber through a small bore polyethylene tubing attached to a selectable solution reservoir. Solution flow to the membrane patch was maintained throughout the duration of the patch exposure to the peptide. Since the patch experiments required a large amount of the peptide for each measurement, MSI-238 with a lower $K_{0.5}$ was used in order to conserve the total amount of peptide required for these experiments.

Excised patches were studied in both inside/out and outside/out configurations. The electrodes were pulled from Corning 0010 glass with resistances of 10–15 MΩ in the bath containing 160 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM KCl and 10 mM Hepes, pH 7.4 (Solution B). Recording electrodes were connected to the headstage of a Dagan 8900 patch clamp amplifier (Dagan, Minneapolis, MN); Ag/AgCl electrodes in 1 M KCl agar bridges were inserted into a standard polystyrene electrode holder (E.W. Wright, Guilford, CT). Similar agar bridges were used for both the ground electrode and a bath reference amplifier. Currents were filtered at 1 kHz with an eight-pole Bessel filter and recorded in two ways. Current–voltage relations (I–V) were generated with a linear voltage ramp, digitized by an IBM AT (5 kHz, 12 bit A/D, Labmaster, Scientific Solutions, Solon, OH) and stored to hard disc. To follow the time course of the entire experiment, the holding potential and currents were continuously recorded on VCR tape using a Pulse Code Modulation and video recorder as described previously [30].

It was important to use stable patches for these experiments in order to distinguish the effects of magainin from patch rupture. Generally, patches with low resistance seals of < 1 GΩ were unstable and ruptured within the first 5 min. Routinely, the seal resistance was checked during the initial 5 min after obtaining the seal and if the resistance decreased during this period, the patch was discarded before control recordings were initiated. When patches were pre-screened in this manner the stable seals lasted for times as long as an hour in the absence of peptide or at low concentrations (0.1 μg/ml) of peptide. Throughout these experiments we never had a patch rupture during exposure to low concentrations of peptide before the addition of the higher (5 or 10 μg/ml) concentrations.

3. Results

3.1. Kinetics of MSI-238 induced cell death

Flow cytometric analysis with propidium iodide was employed to resolve the kinetics of MSI-238 induced cell

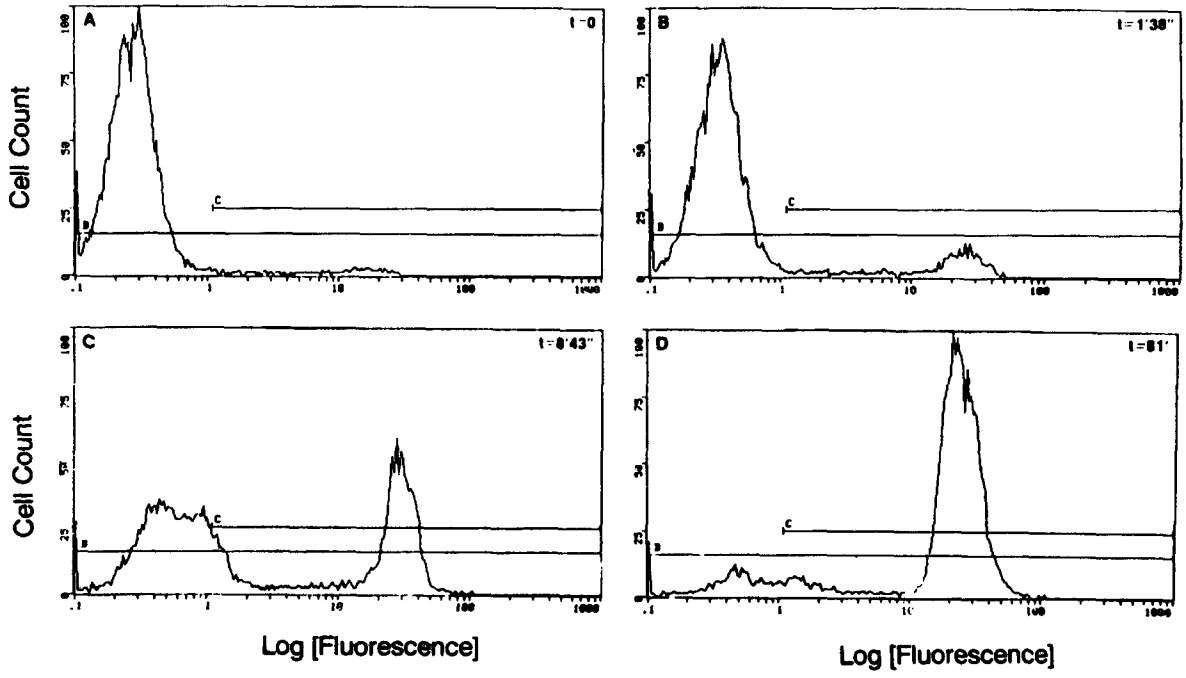

Fig. 1. Fluorescence emission profile of Balb/3T3 cells incubated with propidium iodide (PI) is altered by the magainin peptide, MSI-238. The fluorescence emission profile of Balb/3T3 cells incubated in the presence of PI was monitored with flow cytometry as described in Section 2. Panel A shows the cell count plotted against the log of the fluorescence emission intensity profile after the addition of 10 μg/ml PI but before exposure to the peptide. MSI-238 at 20 μg/ml was added to the cells and the time after addition is shown at the top of each panel. An increase in the number of PI positive cells, sorting with a higher fluorescence intensity (Channel C) was seen with increasing time. The fraction of PI positive cells in panel A is 4%, Panel B is 13%, Panel C is 50% and Panel D is 89%. All analysis was done between 22 and 24°C. A, $t = 0$; B, $t = 98$ s; C, $t = 8$ min 43 s; D, $t = 81$ min.

death on suspensions of cultured cells. Normally, cells exclude PI but once the cell membrane is permeabilized, PI interacts with cellular DNA and RNA to form a highly fluorescent conjugate [31]. The time-course of MSI-238 induced cell death on a suspension of cultured Balb/3T3 is shown in Fig. 1 at four representative times after exposure to 20 μg/ml of the peptide. The initial peak seen in Fig. 1A reflects the low fluorescence level of cells prior to magainin exposure. The appearance of cells permeable to PI, sorting at higher fluorescence intensity in Channel C, is seen as early as 98 s after exposure of the suspension to MSI-238, Fig. 1B. With time, the number of cells displaying high fluorescence levels increases, indicating that a larger fraction of cells has been permeabilized. By 81 min 89% of the cells are PI positive (Fig. 1D). At this concentration of MSI-238, 50% of the cells were PI positive within 5 min at 22°C.

The kinetics of peptide-induced cell kill was measured at varying concentrations of MSI-238 ranging from 10 to 50 μg/ml and the results are shown in Fig. 2A. The experiment shows that the magainin derivative acts rapidly, in a dose-dependent manner, to kill cells and that all cells are susceptible to the peptide at concentrations greater than 10 μg/ml. In other experiments (data not shown), cell suspensions exposed to 5 μg/ml of MSI-238 showed only a small increase in the number of cells killed over the control. At the highest concentration of the peptide employed, 100 μg/ml, the kinetics were too fast to resolve with flow cytometry.

3.2. Comparison of magainin effects on several cell types

The question of target cell specificity was explored by investigating the cytotoxicity of magainin 2 and MSI-238 on two closely related cell lines, Balb/3T3 cells and a transformed cell line derived from the Balb/3T3 cells by infection with Rous sarcoma virus, SRD/3T3 [26]. The concentration dependence of the MSI-238 effect, shown in Fig. 2B, was determined by selecting a 5-min time window and plotting the fraction of PI positive cells at that time point vs the concentration of the magainin derivative. These data, fitted to a non-linear fitting function, showed similar dose response curves for the two cell types with $K_{0.5}$ values of 27 μg/ml and 24 μg/ml and Hill coefficients, N_h, of 2.6 and 2.4 for Balb/3T3 cells and SRD/3T3 cells, respectively. The Hill coefficient of ~ 2.5 suggests cooperative interactions among the peptides, however it should be noted that our experiments do not measure the actual binding of magainin but events subsequent to binding. Under these conditions, the Hill coefficient is not easily interpreted. The assumption that multiple peptide molecules interact at the cell surface is, nevertheless, consistent with the idea that monomer aggregation is required for the formation of current-conducting complexes.

The results with Balb/3T3 and SRD/3T3 cells did not show a difference in sensitivity to MSI-238 which was

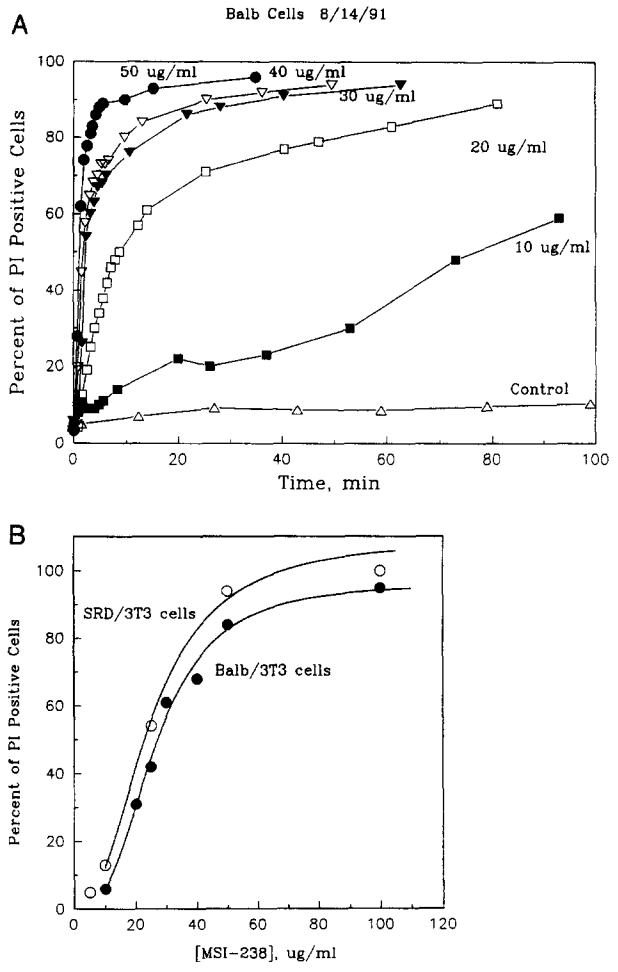

Fig. 2. (A) Kinetics of MSI-238 induced cell kill. Balb/3T3 cells were exposed to increasing concentrations of MSI-238 peptide and examined by flow cytometric analysis as described in Fig. 1. At each time point, the fraction of PI positive cells was determined from the fraction of cells in channel C. The effect of the magainin on cell viability was dose dependent. At magainin concentrations higher than 30 μg/ml, the half-time for 100% cell death was less than 2.5 min. The kinetics of cell kill were slowed at concentrations lower than 20 μg/ml but all the cells died eventually after prolonged exposure to MSI-238. 24°C. (1814BM). (B) Dose–response of Balb/3T3 and Rous sarcoma virus transformed Balb/3T3 (SRD/3T3) cell kill to MSI-238. Balb/3T3 (●) and SRD/3T3 (○) cells were incubated with various concentrations of MSI-238 for 5 min as described in Section 2. The percent of PI positive cells was determined and plotted against the concentration. Data were fitted to the Hill equation with

$$\text{Fraction of cells} = 1/\left(1+(K_{0.5}/L)^{N_h}\right)$$

a Levenberg-Marquardt algorithm for non-linear, weighted, least-squares fitting where L is the concentration of MSI-238. The best fit for Balb/3T3 cells gave a $K_{0.5}$ of 27 μg/ml and a cooperativity index, N_h, of 2.6; SRD/3T3 data were fitted with a $K_{0.5}$ of 24 μg/ml and an N_h of 2.4. Thus, both cell lines shown a similar sensitivity to MSI-238.

expected based on the previous studies with transformed cells [10–12]. Several factors were considered which might have contributed to the loss of target selectivity in our experiments and the results are shown in Fig. 3. One possibility is that the all-D amino acid derivative used in our study has lost the property of cellular specificity. We

explored this hypothesis by examining the cytotoxic activity of the naturally occurring magainin 2 on the same cell types. Although the native peptide was ~ 10-fold less potent than the synthetic peptide, the $K_{0.5}$ values of ~ 600 μg/ml measured at 5 min for SRD/3T3 cells and ~ 800 μg/ml for Balb/3T3 cells do not reveal a significant difference in magainin sensitivity between these cell types. We next considered whether primary cultured cells might be more resistant to magainins than the cell lines, Balb/3T3 and SRD/3T3, continuously propagated in culture. Chicken embryo fibroblasts passed only twice in culture showed an approximate 2-fold decrease in sensitivity to MSI-238, as compared to SRD/3T3 cells, and little difference in sensitivity to magainin 2. We also examined OVCA3 cells, derived from a multi-drug resistant ovarian carcinoma; these cells were less sensitive than the chick primary cells to MSI-238.

To determine whether magainin resistance might be a property of adherent cells which is lost when the cells are suspended, monolayers of cells cultured for 24 h were washed free of growth medium and exposed to PI alone or PI plus 10 μg/ml MSI-238. As shown in Fig. 4B, no fluorescence was detected when cells were exposed to PI alone. In contrast, fluorescent cells were detected after exposure to MSI-238 for 1 or 5 min, Fig. 4D and F, respectively. By 5 min, more than 75% of the cells displayed some fluorescence, varying from weak nuclear staining to strong PI staining throughout the cell. These results suggest that initial cell damage might occur at faster rates than determined by flow cytometry analysis which might detect a relatively late event in the sequence of magainin-induced cell death. Cells exposed to higher concentrations of MSI-238 detached immediately preventing further analysis. Similar results were observed for Balb/3T3 cells (data not shown) indicating that the sensitivity of Balb/3T3 and SRD/3T3 cells to MSI-238 is a property of both suspended and adherent cells.

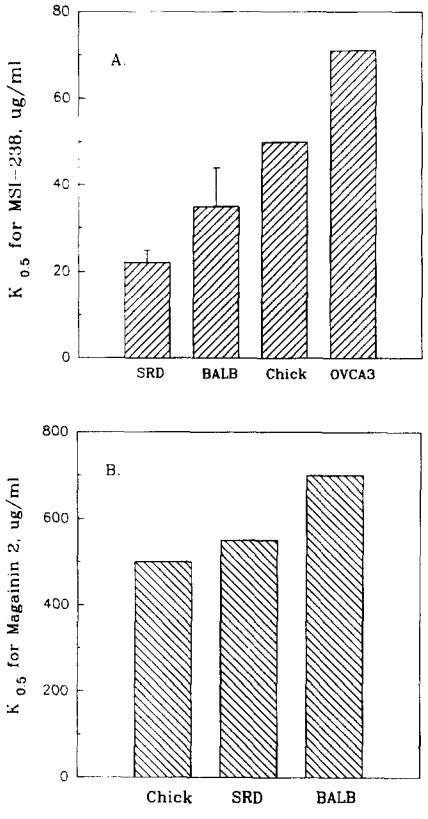

Fig. 3. Dose-response of primary and immortalized cells to MSI-238 and magainin 2. Flow cytometric analysis was performed on trypsinized cell suspensions at various concentrations of MSI-238 (A) and magainin 2 (B) and the fraction of PI positive cells was determined as discussed in Section 2. MSI-238 concentration dependence was determined as described in Fig. 2B. For Balb/3T3 and SRD/3T3 cells, results from three experiments were averaged and the mean and standard deviations are shown.

3.3. Excised membrane patch recording

Although early work with bacteria suggested that magainins exert their lethal effects by permeabilizing the cell membrane, leading to a collapse of the ionic gradients resulting in cell death, few details are available about the mechanism of the underlying membrane permeability changes. To directly observe the changes in membrane permeability, electrophysiological recording methods which detect individual ion channel activity in small patches of cell membranes are used. In these patches, both constitutive membrane channel activity and toxin-inserted conductance activity can be observed. Patches in both inside/out and outside/out configurations were excised from Balb/3T3 or SRD/3T3 cells. Control current recordings in the absence of MSI-238 often showed constitutive ion channel activity in the membrane patch. To characterize the channel activity in a patch before exposure to MSI-238, a voltage ramp was applied to the membrane and the current was recorded. Long segments of the membrane current at fixed potentials were also recorded on VCR tape before application of MSI-238.

A series of current vs. voltage, I–V, relations for three representative patches, before and at various times after the application of MSI-238, is shown in Fig. 5. I–V plots from an SRD/3T3 cell inside-out patch are presented in Fig. 5A. The initial I–V trace in the left panel shows constitutive membrane channels opening and closing during the ramp. The control I–V in the right panel, measured directly after initiating the flow of 5 μg/ml of MSI-238 into the chamber, shows little change from the initial trace (note change in current scale). Less than 1 min after applying MSI-238, the conductance in the patch increased dramatically as shown by the large pipette currents which saturated the amplifier at applied potentials smaller than 10 mV indicating a general disruption of the membrane.

The I–V plots from a Balb/3T3 cell outside/out patch are shown in Fig. 5B. This patch also displayed constitu-

tive channel activity in the control trace shown on the left. In the right panel, the $I-V$ plots at various times (see legend) after addition of MSI-238 to the bath show a monotonic increase in current with time. A general disruption of the membrane similar to that seen in Fig. 5A was observed shortly after recording the largest $I-V$. A third patch also recorded from a Balb/3T3 cell shows a similar sequence of events occurring over a 3 min interval following MSI-238 application, Fig. 5C.

Long channel diaries at several potentials are shown for two patches in Fig. 6. Constitutive channel activity is apparent in both patches. In the top trace in Fig. 6A, some channel activity is apparent for the first few seconds of the recording with channels opening at negative potentials. Following several seconds of activity during the application of MSI-238, the membrane becomes relatively quiet; the constitutive channels make slow excursions throughout the record. The channel opens occasionally but for brief times in the next record which was taken at 6 min following application of 5 μg/ml. In the bottom trace of Fig. 6A, typical magainin-induced membrane activity is seen with multiple conductance levels interspersed with relatively quiet periods. Abruptly the membrane conductance increases as shown at the right-hand edge of the trace, and the current saturates the recording electronics at this gain. Once the general membrane disruption occurs, the membrane does not recover.

The recording from a Balb/3T3 cell shown in Fig. 6B has a similar pattern. The first trace, before MSI-238 is added, shows that several channels are active. Rapid bursts of activity are seen at 40 mV with an occasional return to the baseline. At negative potentials, the channels undergo slow transitions from closed to open. No obvious effect of 0.1 μg/ml of MSI-238 is seen. At 6 min, the concentration of the peptide is increased to 5 μg/ml. At 12 min, there is a clear increase in the membrane activity with the membrane returning to a quiet state intermittently. At the end of this recording, the activity increases rapidly and as seen in the previous patch, the current saturates the amplifier. Although more apparent in the Fig. 5 where the gain of the amplifier was adjusted to show the increased current, the large current increases suggest a general membrane disruption characteristic of magainin-induced activity.

The pattern shown in Figs. 5 and 6 was seen with most of the cells examined. A total of 17 stable patches were

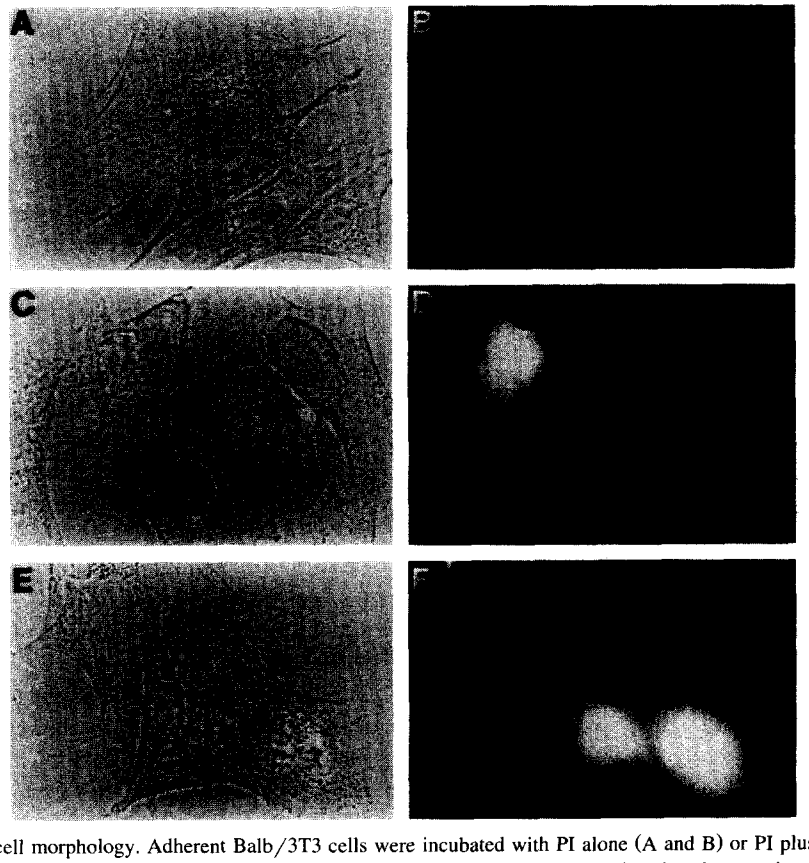

Fig. 4. Effect of MSI-238 on cell morphology. Adherent Balb/3T3 cells were incubated with PI alone (A and B) or PI plus MSI-238 at 10 μg/ml for 1 min (C and D) and 5 min (E and F) as described in Section 2. The left panels show cells examined under phase optics and the right panels show the complimentary fluorescent image. No fluorescence was detected when cells were exposed to PI alone, Panel B. In contrast, more than 75% of the cells were fluorescent after 5 min of exposure to the magainin peptide, panel F.

obtained from Balb/3T3 (9) and SRD/3T3 (8) cells. All but 5 of the patches had constitutive channel activity and these 12 patches showed the same general membrane disruption at varying times following the addition of the MSI-238. Of the five patches without constitutive channel activity before the addition of MSI-238, three had no response to the peptide. It is possible that the non-responsive patches were sealed membrane vesicles or plugs which would explain their lack of response. The presence of constitutive channels in a patch was therefore a useful indirect signature of patch integrity.

The major variability in patches exposed to MSI-238 was the time from application of the peptide to the general membrane disruption. This delay time was related to the concentration of peptide applied to the bath. In initial experiments, the membrane was stable for as long as 40 min following the application of 0.1 μg/ml of peptide and membrane disruption only occurred after increasing the concentration of magainin to 5 or 10 μg/ml. Following these initial experiments, the peptide concentration was increased in order to decrease the recording time following exposure to the peptide. The mean recording time before the addition of the peptide was 10.5 min (n = 10; 5.2 min S.D.). Once the initial recordings were completed, peptide was added to the bath. The mean lifetime of the patches following the addition of 5 or 10 μg/ml of peptide was 2.1 min (n = 10; 0.84 min S.D.) until large current fluctuations appeared. It should be noted that the initial mean of 10.5 min represents only the time before addition of higher concentrations of the peptide and not the lifetime of the patch in the absence of peptide. As described in Section 2, patches in the absence of any peptide or at very low concentrations of peptide were stable for long times. No differences in the magainin effects were noted between

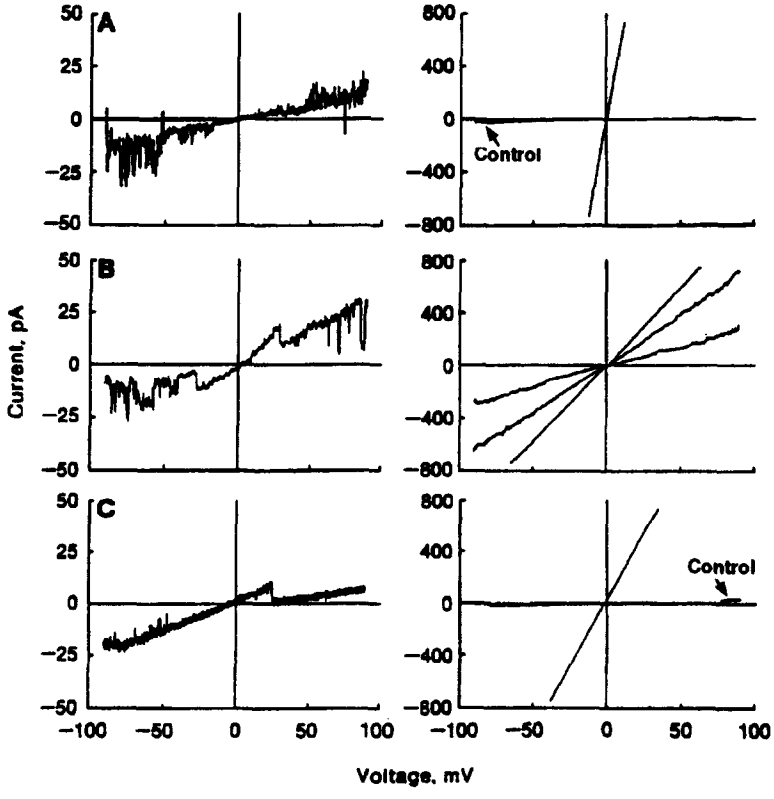

Fig. 5. The effects of MSI-238 on currents recorded from excised patches. (A) Currents were recorded in response to a linear voltage ramp from −90 mV to 90 mV on an excised inside-out patch from an SRD/3T3 cell. The left panel is the I–V relation before application of MSI-238 showing the presence of channel activity in the patch. The solutions, detailed in Section 2, were the same for both sides of the membrane. Immediately following this I–V recording, 5 μg/ml of MSI-238 was perfused into the chamber bath. The control I–V in the right panel was taken about 20 s after the initiation of the flow and little change is seen in the I–V. The final trace showing the loss of the membrane seal was recorded less than 60 s after the application of the peptide (Patch 21095). (B) The left panel shows an I–V response from an outside/out patch from a Balb/3T3 cell before the application of MSI-238. This patch shows the presence of constitutive channel activity in the left panel. The right panel shows a series of I–V plots taken after adding 10 μg/ml of MSI-238 The currents increase monotonically with the times of exposure which were (a) immediately after switching to the MSI-238 solution, (b) about 30 s later and (c) about 2 min after the initial trace. The seal broke immediately after the largest I–V was recorded (Patch 2422). (C) Current–voltage relations from an inside-out patch from a Balb/3T3 cell. Channel activity is seen in the patch before the addition of the magainin derivative, left panel. The control current trace in the left panel was taken ~ 1 min after exposing the patch to 0.1 μg/ml MSI-238. Little change was seen in the current at this low concentration of peptide. The final trace was taken less than 3 min after adding 5 μg/ml of MSI-238 to the bath (Patch 2109).

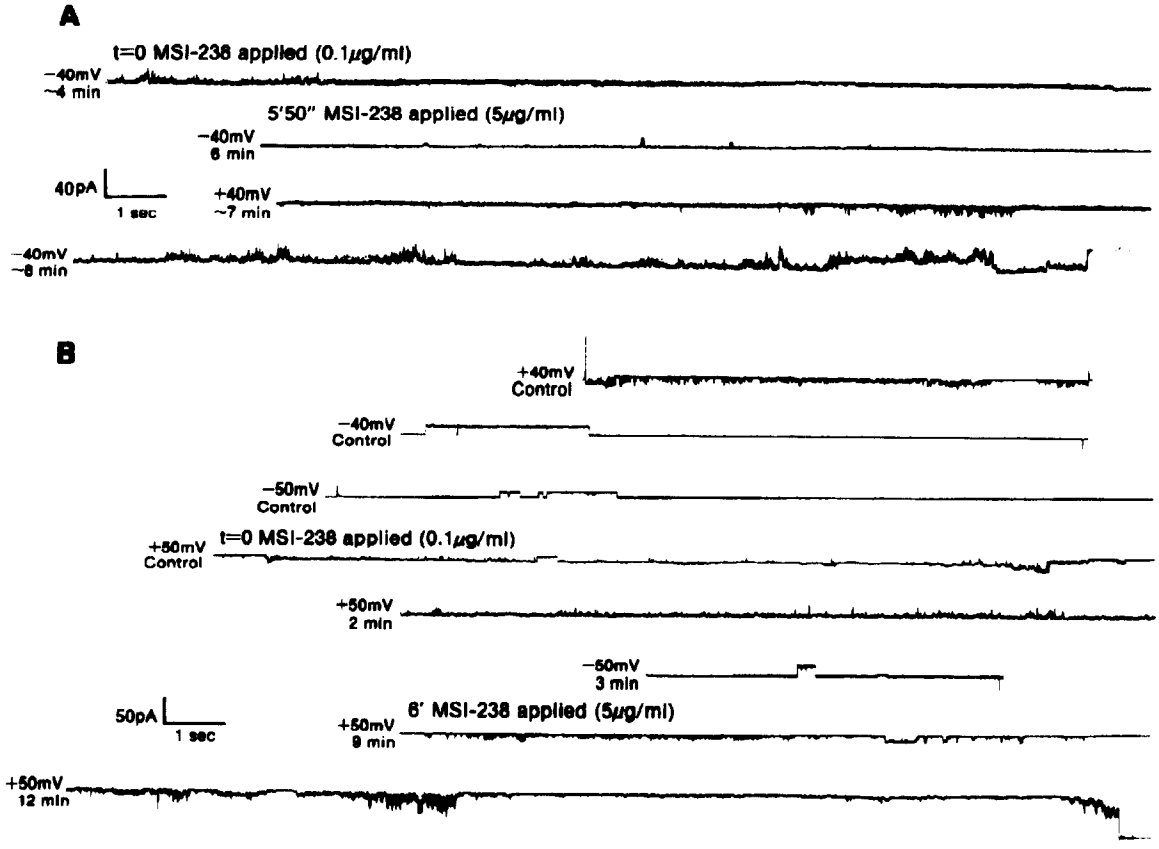

Fig. 6. Selected voltage-clamped current recordings from two patches. (A) Selected current recordings from an inside-out SRD/3T3 cell. This patch had some channel activity before the addition of MSI-238 and little increase in the membrane conductance was seen after the addition of 0.1 μg/ml. The membrane potentials and times after adding magainin are shown on the records. At negative potentials the channels open upward. 5 μg/ml of magainin derivative was added at 5 min 50 s and the increased conductance was seen within 90 seconds. In the final trace, large fluctuations in the current are seen with intermittent returns to the baseline. The continuous final recording shows periods of quiet followed by periods of increasing levels of current activity eventually leading to very large conductance fluctuations which are off-scale at the gain of this recording. (B) Selected recordings from a Balb/3T3 cell inside-out patch. This patch shows constitutive channel activity before the application of the magainin derivative. Little change is seen with the addition of 0.1 μg/ml MSI-238. At 6 min, 5 μg/ml was added to the bath. In the final trace, the current increased briefly but returned to baseline activity before a large increase in the conductance which saturated the amplifier at the recording gain.

inside/out and outside/out patches suggesting that the peptides can act from either membrane face.

4. Discussion

Cytolytic peptides and proteins are commonly used as defensive strategies against invading microorganisms and foreign cells [3,9]. One of the ways these molecules effect cell kill is through the formation of ion channels in the target cell membrane which dissipate the electrochemical gradient across the membrane [32]. Our results from flow cytometry and fluorescence microscopy demonstrate that magainin peptides act rapidly to produce cell death in eukaryotic cells in culture with $K_{0.5}$ concentrations measured at 5 min ranging from 24–80 μg/ml for the all-D peptide MSI-238 and ~ 600 μg/ml for magainin 2. These concentrations are comparable to those used in previous experiments with different assays. In a report by Cruciani et al. [11], hematopoietic tumor cell lines were briefly exposed (~ 5 min) to magainin 2 or magainin G and cell viability was determined by trypan blue exclusion after three days. IC_{50} values ranged from 12 to 53 μg/ml with magainin analogues A, B, and G; the efficacy of magainin 2 was > 150 μg/ml. In experiments by Ohaski et al. [12], the synthetic magainins A and G were added to cells in culture and cell survival was measured on day four by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The average IC_{50} values for both analogues on the small cell lung cancer cells were ~ 8 μg/ml. Baker et al. [10] also used the MTT assay to detect cell survival 48 h after exposure to various concentrations of magainin peptides. Murine Ehrlich ascites tumor cells showed < 10% of cells exposed to concentrations ≥ 10 μg/ml of MSI-238 survived and exposure of these cells to magainin 2 resulted in > 50% cell survival at concentrations as high as ~ 800 μg/ml. Results were similar with human lung A549 cells exposed to MSI-238 whereas exposure to magainin 2 resulted in 100% cell kill at ~ 800 μg/ml.

It is well known that membrane lytic toxins are not equally active on all cell types. Work by Cruciani et al. [11] and Ohaski et al. [12] showed that tumor cells were more sensitive to magainin than peripheral blood lymphocytes and polymorphonuclear neutrophils suggesting a possible tumor cell receptor for magainins. Our comparison of untransformed Balb/3T3 and transformed SRD/3T3 cells of close clonal relationship failed to detect a difference in the sensitivity of the two cell types to either MSI-238 or magainin 2. We also found no difference in MSI-238 sensitivity between a human ovarian tumor cell line (OVCA3) and embryonic fibroblasts passed in culture only twice. Our results on magainin-induced eukaryotic cytotoxicity are consistent with the idea that a brief exposure to magainin peptides kills many tumor derived cell types but our studies do not support the idea of a tumor-specific receptor for magainin peptides. It is possible that in vivo non-tumor cells are protected from magainin attack and this is suggested by the experiments by Baker et al. [10]. It is also possible that certain conditions or factors which are important in regulating the cytotoxic response operate differently under in vivo conditions.

4.1. Channel properties

The high-frequency current fluctuations seen in excised membrane patches from cells can be compared to the events previously seen in artificial lipid bilayers exposed to magainin peptides. Results from Duchohier et al. [24] showed short-lived, flickering conductance levels varying from 0 to 680 pS and a larger population with conductances ranging from 1200 to 1900 pS in uncharged lipid bilayers exposed to magainin 1. In another bilayer study, Cruciani et al. [14] saw large increases in the bilayer conductance following the addition of magainin 1 or magainin 2. The magainin-induced conductance properties were influenced by the type of lipid in the bilayer consistent with a requirement for negatively charged lipids. Small bilayers, formed on the tip of micropipettes, showed activity suggestive of channels with high-frequency fluctuations (Fig. 5D, [14]), similar to those seen in membrane patches from cells.

The current fluctuations seen in MSI-238 exposed patches resemble more closely the bilayer activity seen with melittin [25,33,34] than the long-lived stable channels formed by hemolysin or sarcotoxin (reviewed in [32]), *Actinobacillus actinomycetemcomitans* leukotoxin [35] and certain of the model α-helical peptides [30,36]. The melittin-induced bilayer activity is suggestive of a population of conductance units that are heterogeneous both in their conductance properties and in their lifetimes [33]. In a study of model leucine (L) and serine (S) peptide channels [30], the sequence of $(LSSLLSL)_2$ produced erratic increases in the bilayer current (see Fig. 2B, [30] which resemble the fluctuations induced by the magainin peptides. The longer $(LSSLLSL)_3$ peptide with the same unit sequence produced stable channels which had discrete long-lived open and closed states. The work on model peptide channels illustrates that drastically different channel properties are observed with model leucine and serine peptides having minor changes in the amino acid sequence.

Electrophysiological experiments can not directly address changes in the secondary structure of the peptides, however, the current fluctuations can be considered in light of physical biochemical studies on secondary structure of the peptides. Current models for magainin–membrane interactions have been derived from a number of physical studies which show that at low concentrations, the peptide is α-helical and lies parallel to the water/membrane interface. With increasing concentrations of peptide, an aggregate structure forms which is perpendicular to the membrane plane, disrupting the lipid packing and forming an aqueous pathway [15–19,22]. The observations reported here, including the index of cooperativity in our dose–response studies and the concentration-dependent activity seen in the excised patches, support the idea of an oligomeric magainin peptide assembly which transverses the membrane. In addition, the high-frequency current fluctuations seen in membrane patches suggest that the transmembrane α-helices do not form long-lived, stable transmembrane structures. It seems likely that the small fluctuations at low concentrations of MSI-238 represent the membrane disruption due to infrequent peptide insertion and at higher peptide concentrations a general disruption of the membrane occurs which is lethal. Future structure–function studies might reveal both underlying principles governing α-helical peptide organization in lipid bilayers and cell membranes and factors which oppose or enhance structural organization that play a role in determining both channel properties and functional specificity.

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