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Induction of Transient Ion Channel-Like Pores in a Cancer Cell by Antibiotic Peptide

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The anticancer activity of anti-bacterial cecropins makes them potentially useful as peptide anti-cancer drugs. We used the cell-attached patch to study the effect of cecropin B (CB; having one hydrophobic and one amphipathic α-helix) and its derivative, cecropin B3 (CB3; having two hydrophobic α-helices) on the membrane of Ags cancer cells. Application of 10–60 μM CB onto the membrane of the cancer cell produces short outward currents. Comparative study with CB3, which induces no outward currents, shows that the amphipathic group of CB is necessary for the pore formation. The results provide a rationale to study the cell-killing activity of antimicrobial peptides at the single cancer cell level.

Key words: antibiotic peptides; cancer cell; cecropin B; membranes; patch clamp technique; pore formation.

Abbreviations: CB, cecropin B; CB3, cecropin B3; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TEA, tetraethylammonium chloride.

Membrane lysis is one of the cell-breaking pathways causing cell death. This pathway involves physical rather than genetic factors, and cell-lysing procedures are therefore specific rather than general. Antimicrobial peptides isolated from insects, amphibians and mammals selectively lyse the membranes of cells such as bacteria and tumor cells. The killing activity results mainly from the interactions of peptides with lipids of the membranes rather than the membrane proteins (*1–3*). Many studies have been performed on bacterial membrane lysis induced by antimicrobial peptides via particular killing mechanisms (*4–7*). The "barrel stave" model for transmembrane channel or pore formation (*8*) and the "carpet" model for membrane disruption (*9*, *10*) are two major killing pathways induced by most antimicrobial peptides (*11*). Based on our previous observations (*12–23*), the interacting motif of peptides with lipids on the surface of the cell membrane is a factor determining these breaking pathways. In general, amphipathic peptides perform the killing function by forming pores in the membranes, and hydrophobic peptides break the cell through the flip-flop mechanism.

Cecropins and magainins have been reported to show cytotoxic activity toward cancer cells that parallels their antimicrobial activity (*24–26*). These antimicrobial peptides that are relatively non-toxic to normal cells have therapeutic value for treatment of cancers (*26*). However, more detailed investigations on the lysis of tumor cells by lytic peptides are essential at this infant stage. Patch-clamp is a powerful electrophysiological technique, which has been widely used to study the mechanism of pore formation in membranes. For example, membrane channel formation by the lyphocyte pore-forming protein was confirmed by using whole-cell recording (*27*). The mechanism of cell death induced by *Actinobacillus actinomycetemcomitans* leukotoxin (LTX) in cultured HL60 cells was investigated by patch electrode recording (*28*), showing that LTX-induced pores in susceptible cells overwhelm the ability of the cell to maintain osmotic homeostasis, causing cell death. Cationic currents caused by enterotoxin in whole-cell recording indicated that cation-permeant pores were formed in the apical membrane of human intestinal CaCO-2 cells (*29*). In this paper, cecropin B (CB, KWKVFKKIEK-MGRNIRNGIVKAGP-AIAV-LGEAKAL; a natural amphipathic peptide having one hydrophobic and one amphipathic α-helix) and its analog cecropin B3 (CB3, AIAVLGEAKAL-MGRNIRNGIVK-AGP-AIAVLGEAKAL; a custom-made hydrophobic peptide having two hydrophobic α-helices) (*13*) were used to explore the channel formation of these peptides on single cancer cells by using the patch-clamp technique. The results of the transient channel-like pore formation induced by CB were observed. This proves that the cell-attached patch is a useful technique in the study of the interactions between the lytic peptides and a cancer cell and consequently, useful in the developments of peptide anti-cancer agents in the future.

MATERIALS AND METHODS

Cell Culture—The stomach carcinoma cell line Ags (No. 1739-CRL) was purchased from American Type Culture Collection (ATCC), USA. A monolayer cell line

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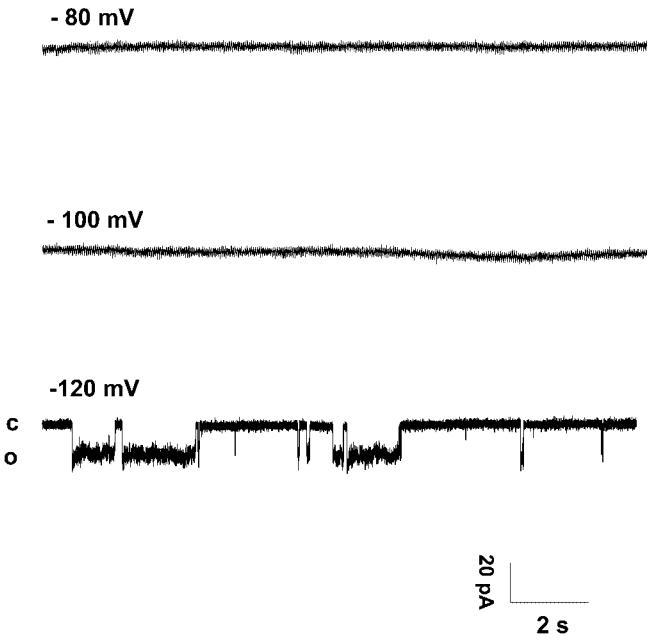

Fig. 1. **Single-channel activity of Ags cancer cell.** Currents were recorded from cell-attached patches at various holding potentials in symmetric physiological saline (5 mM K^+) in the bath and in the microelectrode. The channel closed and open levels are indicated by "C" and "O."

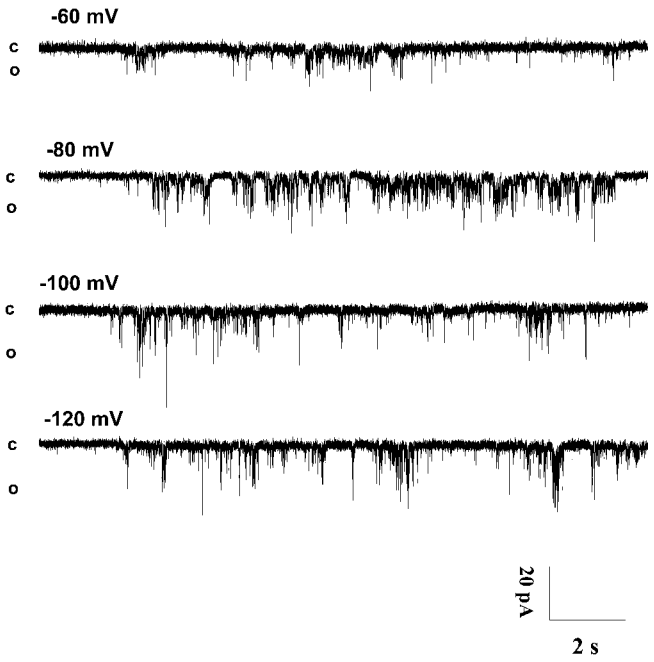

Fig. 2. **Example traces of current recorded from cell-attached patches at different holding potentials with 40 μM CB in the microelectrode.** Other conditions are the same as in Fig. 1.

grown in F-12 nutrient mixture (Ham) medium (Gibco, USA) was maintained (*12*) by seeding 5×10^5 cell/ml (Ags) in a 37°C incubator (5% CO_2, 95% air). Before the electrophysiological study, cells were washed with extracellular fluid three times.

Electrophysiological Recordings and Data Analysis—Patch-clamp experiments were performed at room temperature (~24°C). Cell-attached patches (*30*) were utilized for this study. Patch pipettes with resistances of 10–15 MΩ when filled with extracellular fluid were fabricated from borosilicate glass on a pipette puller (Model P-97, Sutter Instrument Co., USA). Single-channel recordings were made with a patch-clamp amplifier (EPC-9, HEKA electronik GmbH, Germany). Data was filtered at 1 kHz with an eight-pole Bessel filter (3 dB) and was sampled at 250 μs. Physiological salt solution used to bathe the cells during seal formation and cell-attached recording contained (mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) with pH 7.4. The solution was used to wash the cells before experiments. CB and its analog CB3 were synthesized, purified and characterized as reported (*12*). Both CB and CB3 peptides were dissolved in the physiological solution and then filled into the microelectrodes for recording. The effects of tetraethylammonium chloride (TEA, from Calbiochem) were tested by diluting 1 M TEA stock solutions in physiological solution as required.

Statistical Analysis—Values used are means ± SE; n is the number of cells examined. Changes in mean amplitude of single-channel current and NP_0 of channels were analyzed using paired *t*-test. In all comparisons, $p < 0.05$ was considered significant. Single-channel recordings were analyzed off-line with a Macintosh 8500 computer and TAC software. NP_0 was determined from the samples of 30- to 60-s duration and defined as $NP_0 = \Sigma\,(t_1 + 2t_2 + 3t_3 + \ldots + nt_n)$, where N is channel number, P_0 is open probability, and t_1, t_2, t_n are the ratios of open time to total time of measurement for each channel, at each of the current levels.

RESULTS

Single-Channel Activity of Cancer Cells by Cell-Attached Recordings—Cell-attached recording was used to study the characterization of channel activity of the Ags cancer cell without peptide in the physiological solution. With symmetric physiological saline (5 mM K^+) in the bath and in the microelectrode, single-channel activity current could only be recorded at –120 mV as illustrated in Fig. 1. At the applied pipette potential from 0 mV to –100 mV, no channel activity was observed (Fig. 1, top and second traces). In marked contrast, when the pipette potential of –120 mV was applied, cell-attached recordings of the cancer cell exhibited single channel activity (11 of 15 patches) as shown in the last trace of Fig. 1. In 5 patches, the NP_0 at –120 mV was 0.17 ± 0.02 with mean amplitude of 23 ± 1.2 pA. The current may be due to the opening of K^+ channels as it can be partly reduced by TEA (data not shown), a K^+ channel blocker.

Pore Formation by CB on the Cancer Cell Membrane—CB has been reported to be able to lyse cancer cells (*12*, *15*), making it potentially useful as a peptide anti-cancer drug. Morphological changes of cancer cells and bacterial membranes treated with CB and its analogs have been studied by scanning electron microscopy (SEM) and

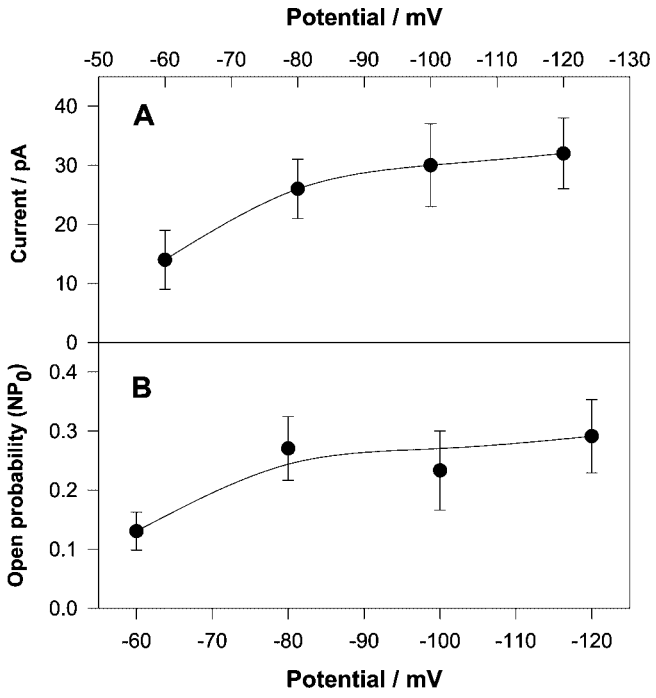

Fig. 3. **(A) The current–voltage relationship and (B) open probability in the cell-attached patches with 40 μM CB in the microelectrode under the conditions described in Fig. 2.**

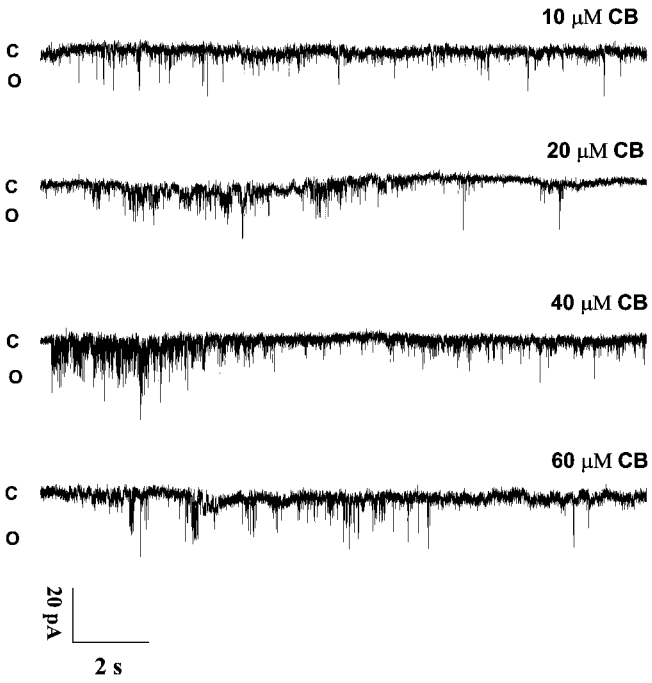

Fig. 4. **Example traces of current in cell-attached patches in the presence of various concentrations of CB in the microelectrode at a holding potential of –80 mV.** The channel closed and open levels are indicated by "C" and "O."

transmission electron microscopy (TEM) (*14*). The primary results showed that the surface of Ags cancer cells is covered with abundant clusters of cilia and bulbous projections. After treatment with CB, the CB pre-binds with the cell surface. However, the killing of cancer cell by CB has been an unresolved issue (*31*). Therefore, we first tested the effect of CB on the membrane of cancer cells by cell-attached recording to examine the possibility of pore formation.

Application of 40 μM CB to a cancer cell with cell-attached membrane patches resulted in a burst of outward current over a wide range of applied potentials (Fig. 2). The current caused by CB is voltage-dependent, with only brief channel openings (NP_0 is 0.1304 ± 0.0320 with amplitude of 14.0 ± 5.0 pA at –60 mV), whereas considerable channel activity is apparent from –80 mV to –120 mV with amplitude increasing from 26.0 ± 5.0 pA to 32.0 ± 6.0 pA as shown in Fig. 3A. Compared to the characterization of channel activity without CB in Fig. 1, it can be concluded that CB has a strong influence on the membrane, possibly forming a pore that allows the outward flow of ions across the membrane.

It is also found that the open probability in the presence of CB increases as the applied potential increases from –60 mV to –80 mV. However, in the range of –80 mV to –120 mV, no significant difference with regards to the open probability is observed (Fig. 3B), showing that ions can pass through the pore formed by CB over a wide range of potential.

Effect of Concentration of CB—In view of the observed differences of the channel activity with and without CB described in this study, the effect of CB concentration from 10 to 60 μM was tested using cell-attached patches at the holding potential of –80 mV. As shown in Fig. 4, the addition of CB within the range of 10~60 μM in the pipette can produce outward currents. It suggests that even 10 μM CB might form pores in the cancer cell membrane. However, it was found that when the concentration of CB increases to 80 μM, the giga-ohm sealing of the pipette becomes very difficult. This may be due to the strong interaction of CB with the microvilli, which cover the cell surface (*14*, *15*).

Cecropins are believed to be important as anti-cancer drugs because of their potential to attack transformed cells. It has also been shown that cecropins with different characteristics can produce different morphological changes in the membrane of bacterial and cancer cells (*14*). This indicates that the potency of the cecropins on the cell membrane is dependent on their helical characteristics. CB has a strong effect on bacteria and cancer cells, while CB3 has little effect on either. The interesting question about the relationship of the anti-cancer potency with the cecropin structure remains. The mechanism of the pore formation in the cancer cell membrane may answer this question.

To examine whether the outward current produced by cecropin is related to its structure, CB3 (formed by replacing the N-terminal 1–10 segment of CB with the C-terminal sequence of CB) was used. Figure 5 shows typical recordings at –80 mV with different concentrations of CB3. No outward current was observed with the concentration of CB3 ranging from 10 to 50 μM. This indicates that CB3 cannot form the pore in the cancer cell membrane. As CB3 has two hydrophobic α-helices, whereas CB contains one amphipathic and one hydrophobic α-helix, the primary result suggests that the amphipathic

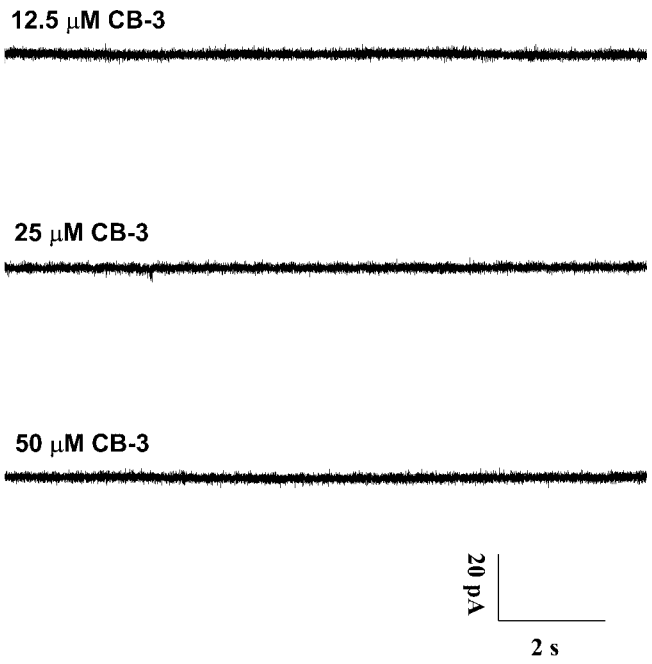

Fig. 5. **Example traces of current in cell-attached patches in the presence of various concentrations of CB3 in the microelectrode at a holding potential of –80 mV.**

group of CB may interact and bind with the cilia and microvilli in the surface of the cancer cell. A positive correlation was evident between the pore-forming ability and the potency of anti-cancer activity. These data may explain the experimental result that CB3 has little effect on cancer cells, while CB has strong anti-cancer potency (*12*, *14*).

DISCUSSION

The detailed cell-killing mechanism of CB on Ags cells remains unclear. The structure, sequence, and net charge might play roles in the lysis of cell membranes. Srisailam *et al*. (*18*, *21*) have studied the NMR structures of CB derivatives cecropin B1 (CB1) and cecropin B3 (CB3). Their results suggest that cecropins have common configurations with two α-helices bridged with a linker, Ala-Gly-Pro. Recent findings show that upon approaching a membrane, linear CB forms two segments of amphipathic and hydrophobic helices (*16*) and imposes them to generate a positive curvature strain on the membrane and form a toroidal pore. In contrast, if negative curvature-inducing lipids are present, a large amount of peptides such as CB3 would be accumulated on the cell membrane surface, leading to the irreversible membrane disruption. In this study, application of CB to the membrane of Ags cancer cells produced an outward current in a cell-attached patch with the holding potential from –60 mV to –120 mV. CB in the range of 10 to 60 μM had a similar effect, producing an outward current. We assume this current is the result of the pore formation by CB in the membrane of the cancer cells. Comparative study done by replacing CB with CB3 shows that the amphipathic group of CB, which reacts with the microvilli in the surface of cancer cell, is necessary for the pore formation. This study supports the assumption that the killing mechanism of anti-cancer activity by cecropins is pore formation (*32–34*). The detailed scenario of the killing pathway of CB on single Ags cells will be as follows. Peptides are initially assembled in the surface of the cell membrane with the N-terminal segment paralleling the outward lipids and the C-terminal segment vertically going around the surface. Subsequently, the C-terminal inserts into the lipid bilayers to form a pore in the membrane by tetramers (observed from the computer simulation, unpublished data). These transient pores exist until the imbalance of living materials in the cell causes cell death.

In this study, the patch-clamp technique was used to study the possible pore formation in the cancer cell membrane by cecropins. The direct observation of outward currents suggests that pores are formed in the membrane by CB. In bulk solution, the IC_{50}, *i.e*., the concentration of the peptide killing 50% of the cells, of CB on Ags cells is higher than 50 μM, whereas the IC_{50} of CB3 is not detectable (unpublished data). This implies that the patch-clamp measurement on single Ags cells is very sensitive and may become a useful tool for IC_{50} investigation as compared to the conventional cell viability test in the culture system. However, the relationship between the outward currents of patch clamp and the potency of peptides on a single cell is not clear yet (even based on the present study). In the present study, we directly use a cancer cell line, Ags cell, to compare the killing activity of two different characteristics of CB and CB3. Hence instead of using the normal cell as an experimental control, CB3 peptide was used to compare the results derived from CB because CB3 produces no effect on cell-killing activity.

However, direct *in situ* observation of the pore formation can be further investigated by atomic force microscopy (*35*). Recent development has been reported to combine both scanning probe (SP) and patch-clamp techniques to improve the preparation of membranes by holding whole cells or membrane patches at the tip of a pipette (*36*). In this way, the membrane is stabilized by cellular structures and by the attachment to the supporting glass rim of the pipette, and this can be verified by measuring whether a giga-ohm seal is formed. With this advance in the development of scanning probe instruments, one can investigate the dynamics of biological processes of cell membranes under physiological conditions.

The development described above suggests that the combination of scanning microscopy with the patch-clamp techniques is potentially useful for characterizing cell membranes. Further studies such as real-time resolution of pore-formation by cecropins in the cancer cell membrane can be planned. The new techniques are also able to measure the influx of ions across the cell membrane simultaneously with the patch-clamp.

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