Membrane permeabilization induced by discodermin A, a novel marine bioactive peptide

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Abstract

The effects of discodermin A (DC-A), a novel marine bioactive peptide extracted from sea sponge Discodermia kiiensis, on the vascular smooth muscle cells and tissues were examined. Analysis with a confocal laser microscope showed that DC-A (0.1–30 \(\mu\)M) permeabilized the plasma membrane of A10 cells to the non-permeable fluorescent agents, ethidium homodimer-1 (MW = 857) and calcein (MW = 623), in a concentration-dependent manner. In the vascular tissue treated with 30 \(\mu\)M DC-A, addition of a micromolar concentration of Ca\(^{2+}\) evoked a sustained contraction in the presence of ATP, suggesting that DC-A increased the permeability of the membrane to Ca\(^{2+}\) and ATP. DC-A at higher concentrations (30 \(\mu\)M) significantly increased the leakage of lactate dehydrogenase (140 kD) from the vascular tissue. These results suggest that DC-A has a permeabilizing effect on the plasma membrane possibly by interacting with plasma membrane phospholipids with its six successive hydrophobic amino acid residues at N-terminal. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Discodermin A (DC-A) is a novel polypeptide isolated from a sea sponge, Discodermia kiiensis, for its antimicrobial effect (Matsunaga et al., 1984, 1985). DC-A consists of 14 peptides with two \(\tau\)-Leu residues and several \(d\)-amino acids (Fig. 1). However, the effect of DC-A on mammalian cells and the mechanism of DC-A have not been studied. Since DC-A has six successive lipophilic amino acid residues in its N-terminal (Fig. 1), it is possible that DC-A may have effects on plasma membrane. In the present study, we investigated the mechanism of the biological action of DC-A using vascular smooth muscle cells and tissues.

2. Methods

2.1. Cultured vascular smooth muscle cells

Rat aortic smooth muscle cell line (A10) cells were
obtained at passage 12 from American Type Culture Collection (Rockville, MD, USA). They were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and were then subcultured as described by Korbmacher et al. (1988). For confocal microscopic imaging, A10 cells were used about two to three days after seeding (subconfluent stage). Cells used in this study were from passages 22–41.

2.2. Leakage of fluorescent probes from the vascular smooth muscle cells

Calcein-AM is a virtually nonfluorescent cell-permeant dye that is converted to the intensely fluorescent calcein by intracellular esterase. Non-permeable calcein stays within living cells, producing an intense uniform green (about 530 nm) fluorescence by 488 nm excitation light. Ethidium homodimer-1 (EthD-1), a non-permeable fluorescent agent, enters a cell only when the membrane is damaged, and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby, producing a bright red fluorescence (>600 nm). Sub-confluent A10 cells cultured on round cover glass were treated with calcein-AM (2 μM) and EthD-1 (4 μM) in normal HEPES solution for 30 min at 37°C (Hauser et al., 1994; Kirshenbaum and Schneider, 1995). The cells were then placed in a bath with normal HEPES solution (in mM: NaCl 125.4, KCl 5.9, MgCl2 1.2, CaCl2 1.5, glucose 11.5, HEPES 10.0) that contained only EthD-1 (4 μM) in normal HEPES solution for 30 min at 37°C (Hauser et al., 1994; Kirshenbaum and Schneider, 1995). The cells were then placed in a bath with normal HEPES solution (in mM: NaCl 125.4, KCl 5.9, MgCl2 1.2, CaCl2 1.5, glucose 11.5, HEPES 10.0) that contained only EthD-1 (4 μM). Observations were made with a confocal laser microscope (Insight Plus, Meridian Instruments, Okemos, MI, USA). The acquisition and analysis of data were done with Insight Plus IQ software (Meridian). To prevent phototoxicity and photobleaching of the fluorescent dyes, the cells were exposed to the exciting light (λ = 488 nm) for only 30 ms per image. The light emitted from the preparation was collected through a 530 nm or a 605 nm bandpass filter. Images were obtained every 30 s for 45 min with a 40× objective lens (Olympus Splan APO 40; NA 0.95, Olympus, Tokyo, Japan). DC-A (0.1–30 μM) was added 5 min after the imaging started at room temperature.

2.3. Transmembrane passage of Ca2+ and ATP in the vascular smooth muscle tissues

A small ring of the rabbit mesenteric artery (0.2 mm wide) was prepared and mounted in a small chamber filled with 250 μl of normal HEPES solution at room temperature (23–25°C) under a resting tension of 1 mN. After measuring the high-K+ (72.7 mM)-induced contraction, the solution was changed with a relaxing solution containing (in mM): Mg-methansulphonate 7.1, PIPES 20, K-methansulphonate 108, EGTA 2, adenosin-5’-triphosphate (ATP) 5.88, creatine phosphate 2, creatine phosphokinase 10 U/ml, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) 1 μM, E-64 1.0 mg/l at pH 6.8 and 25°C. The free Ca2+ concentration was changed by adding an appropriate amount of CaCl2 to EGTA (Ca2+-EGTA buffer). The apparent binding constant of EGTA for Ca2+ was considered to be 10−6 M at pH 6.8 (Harafuji and Ogawa, 1980).

2.4. Leakage of lactate dehydrogenase from the vascular smooth muscle tissues

A rabbit portal vein strip (2 mm wide and 6 mm long) was treated with 0.5 ml phosphate-buffered saline PBS, (in mM: NaCl 138, KCl 2.7, Na2HPO4.12H2O 8.1, KH2PO4 1.2, pH 7.4) with or without DC-A (1 or 30 μM) at room temperature (23–25°C) for 60 min. Every 15 min, the solution was changed and collected. After the final incubation period, the strip was quickly frozen in liquid nitrogen and then homogenized with PBS, centrifuged at 2000 × g for 20 min and supernatant was collected to assess the re-

![Chemical structure of discodermin A (DC-A) isolated from sea sponge Discodermia kiiensis (Ryu et al., 1994).](image-url)
residual lactate dehydrogenase (LDH). Each sample was quickly frozen in liquid nitrogen and stored at −80°C until the LDH measurements. LDH activity was assayed using a lactate dehydrogenase CII test kit with a modified version of the method described by Iizuka et al. (1994). The percentage of the residual LDH in tissue was normalized to the total tissue LDH (sum of all the LDH leaked out of the tissue and supernatant collected).

2.5. Chemicals

DC-A was purified from sea sponge (*Discodermia kiiensis*) as described by Matsunaga et al. (1984). Other chemicals used were: ATP (Yamasa Shoyu, Tokyo, Japan), EDTA, EGTA, HEPES, PIPES (Dojindo, Kumamoto, Japan), creatine phosphate, creatine phosphokinase, FCCP, E-64 (Sigma Chemicals, St. Louis MO, USA), calcein-AM, EthD-1 (Molecular Probes, Eugene OR, USA), DMEM powder (Nissui Pharmaceutical, Tokyo, Japan), norepinephrine bitartrate, lactate dehydrogenase CII test kit (Wako Pure Chemicals, Osaka, Japan), penicillin–streptomycin (Gibco BRL, Rockville MD, USA) and fetal bovine serum (HyClone, Logan UT, USA).

2.6. Statistics

Data are expressed as mean ± S.E.M. Statistical

![Image](image-url)

Fig. 2. Effect of DC-A on the transmembrane passage of calcein (green) and EthD-1 (red) in A10 cells. The confocal imaging of the changes in each fluorescence shown at 5 min before the addition (control), 8 min after the addition, and 43 min after the addition of DC-A (0.1–30 μM).
analysis was performed by unpaired Student's t-test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Transmembrane passage of fluorescent dyes

After treatment with calcein-AM and ethidium homodimer-1 (EthD-1) for 30 min, all A10 cells showed green fluorescence when excited by the 488 nm light (calcein fluorescence; Fig. 2). The intensity of calcein fluorescence did not change in the controlled medium for more than 45 min (data not shown). Addition of 0.1 μM DC-A induced a gradual decrease in calcein fluorescence, and an increase in red fluorescence (EthD-1 fluorescence) which were observed in approximately 50% of the A10 cells within the image scans when excited by the 488 nm light (Figs. 2 and 3). Addition of 1 μM DC-A enhanced both the rate of the fluorescent shift and the number of the cells affected to approximately 90%. Higher concentration of DC-A (30 μM) further enhanced the rate of fluorescent shift that was observed in all the cells within the image scans (Figs. 2 and 3).

3.2. Transmembrane passage of Ca$^{2+}$ and ATP

In the intact rabbit mesenteric artery, application of high-K$^+$ solution caused a rapid increase followed by a small sustained increase in muscle tension in the presence of 1.5 mM extracellular Ca$^{2+}$ (Fig. 4). After treatment of tissue with a Ca$^{2+}$ free solution with 2.0 mM EGTA and 5.88 mM ATP for 20 min, application of 10 μM Ca$^{2+}$ induced no perceptible contraction. However, in the presence of 5.88 mM ATP and 10 μM Ca$^{2+}$, addition of DC-A (30 μM) induced a slow and sustained contraction. After 30 min, removal of both DC-A and Ca$^{2+}$ decreased the contraction to the resting level. Re-application of 10 μM Ca$^{2+}$ in the absence of DC-A induced a sustained contraction in the presence of ATP, suggesting that the effect of DC-A is irreversible.

3.3. Leakage of lactate dehydrogenase induced by DC-A

To determine whether the intracellular proteins are leaked out from vascular tissues by the application of DC-A, we measured the leakage of lactate dehydrogenase (LDH). In the control rabbit portal vein tissue, LDH slowly leaked out in a time-dependent manner (Fig. 5). Application of 1 μM DC-A caused no appreciable changes in the amount of leakage of LDH. However, 30 μM DC-A significantly increased the leakage of LDH only at 45 and 60 min in the incubation period.

4. Discussion

To examine whether or not DC-A changes membrane permeability, we measured the transmembrane passage of two different fluorescent dyes, calcein and EthD-1, in vascular smooth muscle A10 cells. When a hydrophobic (membrane-permeable) form of calcein (acetoxymethyl ester of calcein; calcein-AM) was applied to cultured A10 cells, it entered the cells and was changed by endogenous esterases to membrane-impermeable free calcein. The free form of calcein is well retained within the cells and exhibited green fluorescence (Fig. 2A–C, left). Addition of DC-A (0.1–30 μM) rapidly decreased the calcein-green fluorescence, suggesting that DC-A increased membrane permeability to leak calcein out of the cell. On the other hand, it has been shown that EthD-1 is an impermeable dye and, therefore, enters the cells only when membrane is damaged. In cytoplasm, it produces a bright red fluorescence upon binding to nucleic acids (Hauser et al., 1994). In fact, we did not observe the
EthD-1 fluorescence when EthD-1 was applied to A10 cells in the absence of DC-A. After treatment of A10 cells with DC-A, however, a red fluorescence was observed at cell nuclei (Figs. 2 and 3). These results suggest that the effect of DC-A on the membrane was to permeabilize for fluorescent dyes, calcein and EthD-1, in the vascular smooth muscle cells.

To examine whether or not DC-A changes membrane permeability also in tissues, we used rabbit mesenteric artery. It has been shown that the increase in cytosolic Ca\(^{2+}\) concentration elicits contraction of arteries in the presence of ATP (Saida, 1982; Nishimura et al., 1988). However, in the present experiment, addition of Ca\(^{2+}\) and ATP to the extracellular medium did not elicit contraction because the membrane was impermeable to these agents. After treatment of the artery with 30 \(\mu\)M DC-A, it was found that addition of Ca\(^{2+}\) and ATP to the extracellular medium elicited contraction (Fig. 4). This result suggests that DC-A increases membrane permeability to both Ca\(^{2+}\) and ATP in the arterial tissue. It is also suggested that at least a part of the contractile proteins needed for arterial contraction was not lost during the DC-A treatment.

To further examine the effects of DC-A on intact tissue, we then measured the leakage of LDH. Results indicated that a higher concentration (30 \(\mu\)M) of DC-A significantly increased the LDH leakage only after 45 and 60 min in the incubation period. It has been reported that permeabilization with \(\beta\)-escin allows the transmembrane passage of LDH, whereas the contractile proteins are still retained in the cell (Iizuka et al., 1994). This may be the reason why the permeabilized vascular cells still responded to Ca\(^{2+}\) and ATP to elicit contraction.

It is known that peptides, which have hydrophobic amino acid residues, are able to associate with plasma membrane phospholipids. For example, Gramicidin A, a biosynthetic product from *Bacillus brevis*, is a hydrophobic peptide consisting 15 amino acids with alternating L and D configurations (Veatch et al., 1974). Gramicidin A incorporates into membranes and forms ion channels (Hladky and Haydon, 1970). Since DC-A also has hydrophobic amino acid residues, it is possible that DC-A interacts with plasma membrane phospholipids.

In summary, DC-A seemed to have a permeabilizing effect on the plasma membrane possibly by interacting with plasma membrane phospholipids with its six successive hydrophobic amino residues at N-terminal.

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References


