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Frankia-Derived Antibiotic Demethyl (C-11) Cezomycin, (DC-11C), and Its Mechanism of Inhibition of Ca^{2+} Transport in Plant Mitochondria

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Abstract

The effect of *Frankia* AiPs1 derived demethyl (C-11) cezomycin, (DC-11C), an antibiotic compound belonging to a class of calcimycins, on calcium transport was studied in wheat root mitochondria. An inhibitory effect in nanomolar concentrations of DC-11C on the rate of Ca^{2+} uptake by plant mitochondria was detected. Based on the structural similarity of DC-11C to the electroneutral ionophore A23187, a mechanism of DC-11C action on mitochondrial calcium transport, similar to the mechanism of A23187, is suggested. Comparison of the effects of the electroneutral A23187 and electrophoretic ETH-129 ionophores to that of DC-11C on Ca^{2+} loaded plant mitochondria, strongly supports the proposed mechanism where DC-11C controls mitochondrial calcium transport by exchanging Ca^{2+} ions to H^+ across the mitochondrial inner membrane leading to dissipation of the Ca^{2+} gradient, and hence, not inhibiting the mitochondrial Ca^{2+} transporter as such. Implications for signal transfers between the microsymbiont and the host cells are discussed.

Keywords: *Frankia*, demethyl (C-11) cezomycin, DC-11C, plant mitochondria, A23187, ETH-129

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1. Introduction

Very recently, Haansuu et al. (2001) described the biological activities of frankiamide, an antibiotic that was purified from the culture broth of the symbiotic *Frankia* strain AiPs1. This compound inhibits the growth of several Gram-positive bacteria and plant pathogenic fungi. The original structural elucidation of frankiamide (Klika et al., 2001) has been revised based on the results of single-crystal X-ray analysis (Klika et al., 2003) and frankiamide relocated to the calcimycin class of antibiotics and pyrrolether ionophores. In light of this and since the new structure is particularly close to cezomycin (Albrecht-Gary et al., 1994), frankiamide has been renamed demethyl (C-11) cezomycin, DC-11C (Fig. 1).

In earlier studies, several *Frankia* culture broth extracts were shown to contain compounds that considerably inhibited Ca^{2+} fluxes in clonal rat pituitary GH_4C_1 tumor cells (Haansuu et al., 1999). Later, DC-11C was reported to strongly inhibit Ca^{2+} influx in GH_4C_1 cells (Haansuu et al., 2001).

Calcimycins comprise a small group of natural antibiotic compounds capable of transporting mono- and divalent metal cations across biological membranes. Complexation of Na^+ and K^+ ions by DC-11C is reported by Klika et al. (2001, 2003). By far the most studied of calcimycins is calcium ionophore A23187, which was originally isolated from *Streptomyces chartreusis* strain NRRL 3882 (Reed and Lardy, 1972). A23187 accomplishes an electroneutral exchange of one Ca^{2+} for two H^+ across biological membranes. After releasing the cation A23187 transports two H^+ back across the membrane. Two molecules of the ionophore as carboxylic anions can complex and carry one Ca^{2+} ion across the membrane (2:1 stoichiometry). Formation of a A23187 complex with Mg^{2+}, Mn^{2+} or Be^{2+} (Reed and Lardy, 1972), complexation with divalent transition metal ions and heavy metal cations, such as Cd^{2+} and Pb^{2+} (Chapman et al., 1990) has also been detected.

Since A23187 regulates the cytoplasmic calcium concentration by complexing calcium and transporting it from the extracellular matrix, vacuole, and mitochondria into the cytoplasm, this molecule has been used in numerous studies investigating the effect of calcium influx on different physiological phenomena. In plant cells A23187 has been used to mimic the effects of enhancement of calcium influx in host cells caused by plant pathogen-derived elicitors, leading to synthesis of phytoalexins (Stäb and Ebel, 1987) and callose (Waldmann et al., 1988), or induction of a hypersensitive reaction (Tavernier et al., 1995). A23187 has been also implicated in plant abiotic (e.g. cold) stress studies to induce calcium-triggered cold acclimation (Sangwan et al., 2001).

We have previously shown that wheat root mitochondria are able to actively transport Ca^{2+} from the surrounding medium and the matrix localisation of Ca^{2+} was verified by addition of the ionophore, A23187

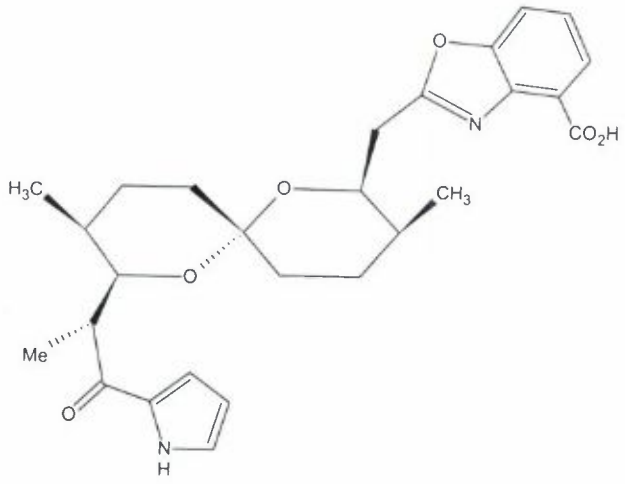

Figure 1. The chemical structure of demethyl (C-11) cezomycin (DC-11C).

(Virolainen et al., 2002). The accumulation of Ca^{2+} ions is sensitive to ruthenium red, an inhibitor of Ca^{2+} uniporter in mammalian mitochondria, and insensitive to verapamil in micromolar range (unpublished results). In a study by Rugolo et al. (1990) similar action of A23187 was shown in plant mitochondria. In addition, in a study by Curtis and Wolpert (2002) A23187 was used to load oat mitochondrial matrices with calcium in order to induce permeability transition.

In this investigation wheat root mitochondria were chosen as a model because the Ca^{2+} transport activity and physiological responses due to Ca^{2+} accumulation have been characterised previously (Virolainen et al., 2002). The present study shows that DC-11C, similarly to A23187, is able to transport Ca^{2+} ions by an electroneutral mechanism across the inner mitochondrial membrane. This finding indicates that DC-11C is able to complex alkaline earth metal ions, such as Ca^{2+}. Inhibitory effect of DC-11C on the mitochondrial Ca^{2+} transport is due to DC-11C-induced dissipation of Ca^{2+} gradient but not due to true inhibition of the transporter.

2. Materials and Methods

The Frankia strain AiPs1

The *Frankia* strain AiPs1 used in this study was isolated from a stand of Finnish Scots pines (*Pinus sylvestris* L.) by inoculating axenic gray alder (*Alnus*

incana L.) roots with soil suspensions and isolating the strain from the root nodules. On the basis of sequence similarity the strain AiPs1 has been classified as part of the *Alnus* host infection subgroup IIIb (Maunuksela et al., 1999). To facilitate the extraction of DC-11C the strain was cultivated in PC broth (K_2HPO_4 300 mg l^{-1}, $NaH_2PO_2 \cdot 2H_2O$ 260 mg l^{-1}, $MgSO_4 \cdot 7H_2O$ 200 mg l^{-1}, $CaCl_2 \cdot 2H_2O$ 10 mg l^{-1}, NH_4Cl 100 mg l^{-1}, Na-FeEDTA 10 mg l^{-1}, biotin 2 mg l^{-1}, casamino acids 500 mg l^{-1}, Na-propionate 800 mg l^{-1}, 1 ml l^{-1} micro nutrient solution: $CoCl_2$ 250 mg l^{-1}, $CuSO_4 \cdot 5H_2O$ 800 mg l^{-1}, H_3BO_3 28.6 g l^{-1}, $MnCl_2 \cdot 4H_2O$ 18.1 g l^{-1}, $NaMoO_4 \cdot 2H_2O$ 250 mg l^{-1}, $ZnSO_4 \cdot 7H_2O$ 2200 mg l^{-1}, pH 6.7) without shaking at +28°C for approximately 8 weeks (Smolander and Sarsa, 1990; Weber et al., 1988).

Purification of DC-11C from Frankia culture broth

DC-11C was isolated from the *Frankia* culture broth according to Haansuu et al. (2001). After ethyl acetate extraction of the culture broth, DC-11C was purified from crude ethyl acetate extract using techniques based on normal- and reversed-phase thin layer chromatography (TLC; normal-phase silica gel 60 F_{254} TLC plates, Merck, Germany; water-saturated chloroform, and reversed-phase RP-18 $WF_{254}S$ TLC plates, Merck, Germany, mobile phase: 46.2% methanol, 37.5% acetonitrile and 16.4% water). The final purification was accomplished using high-performance liquid chromatography (HPLC; 3.9 × 150 mm Nova-Pak® column, C_{18}, 4 µm, pore size 60 Å). The RP-TLC mobile phase was used for elution, except that 2.5 mM Na_2HPO_4 was added in order to enhance the chromatographic performance of DC-11C.

Plant material

Etiolated wheat seedlings (*Triticum aestivum* cv. Leningradka) were grown in Knop's nutrient medium for 6–7 d at a constant temperature (+23°C) in the dark with continuous aeration of the medium.

Isolation of wheat root mitochondria

Mitochondria were isolated from the roots of wheat seedlings by differential centrifugation essentially according to Douce et al. (1987). Mitochondrial fraction was further purified by self-generating Percoll density gradient centrifugation. The integrity of outer mitochondrial membrane was 94% as estimated by succinate:cytochrome c oxidoreductase activity determination (Gualberto et al., 1995).

Determination of Ca^{2+} transport across the inner mitochondrial membrane

Ca^{2+} transport of mitochondria was assayed with a metallochromic indicator Arsenazo III (Scarpa, 1979) using the wavelength pair 665 and 685 nm as measuring and reference wavelengths, respectively, and recorded by a dual beam UV-Visible spectrophotometer (Hewlet-Packard 8452A). Measurements were carried out at +25°C in medium (1 ml) containing 0.25 M sucrose, 10 µM EGTA (to chelate Ca^{2+} originating from impurities in the reagents, the concentration was estimated by back-titration of Arsenazo III signal with EGTA), 25 µM Arsenazo III, 10 mM HEPES-Tris (pH 7.4) and 0.2 mg mitochondrial protein.

Determination of membrane potential changes ($\Delta\Psi$)

Membrane potential was measured with safranine O as an indicator of membrane potential changes ($\Delta\Psi$) recorded by a dual beam UV-Visible spectrophotometer (Hewlet-Packard 8452A) using the wavelength pair 511 and 533 nm as measuring and reference wavelength, respectively (Moore and Bonner, 1982). Measurements were carried out at +25°C in 1 ml medium containing 0.25 M sucrose, 10 µM EGTA, 5.3 µM safranine O, 10 mM HEPES-Tris (pH 7.4) and 0.2 mg mitochondrial protein.

Protein assay

Mitochondrial protein was determined with the Bradford method (Bradford, 1976) using the Bio-Rad Protein Assay (Bio-Rad Laboratories) with bovine serum albumin as a standard.

3. Results and Discussion

The mechanism of DC-11C action on Ca^{2+} transport by plant mitochondria

An inhibitory effect of DC-11C in nanomolar concentrations on the rate of Ca^{2+} uptake by wheat root mitochondria was observed (Fig. 2). Addition of DC-11C prior to initiation of Ca^{2+} uptake caused significant inhibition of Ca^{2+} transport in a concentration-dependent manner. Addition of 10 µM DC-11C completely inhibited Ca^{2+} uptake by mitochondria (data not shown). Since DC-11C bears structural similarity to the electroneutral ionophore A23187, the resemblance in DC-11C action on Ca^{2+} transport was tested. This was accomplished by measuring DC-11C induced Ca^{2+} transport across the inner mitochondrial membrane and determining the electroneutrality of such a

transfer. According to the theory the ionophore exchanges free Ca^{2+} for $2H^+$ across a hydrophobic phase (a biological membrane). Therefore, the direction of transport in such a system is dependent on the Ca^{2+} concentration gradient and on the membrane potential (Bernardi, 1999). The inhibition by DC-11C may represent i) a true inhibition of Ca^{2+} channel, ii) an indication of outward directed Ca^{2+} transport induced by the ionophore (i.e. an exchange mechanism).

To distinguish between these two possibilities, the effects of calcium ionophores with different properties on Ca^{2+} loaded mitochondria were investigated. The following ionophores were used: A23187 – an electroneutral ionophore (Chapman et al., 1990), ETH-129 – an electrogenic ionophore (Wang et al., 2001) and DC-11C. Electroneutral ionophores transport uncharged (zwitterionic) complexes and exchange a cation for H^+ or for another cation (Chapman et al., 1990). The electrogenic ionophore ETH-129 transports Ca^{2+} ions electrophoretically, meaning that the ionophore bears a positive charge after compexation with a cation and thus dissipates the membrane potential (Wang et al., 2001). Another basic difference between electroneutral and electrogenic ionophores, such as A23187 and ETH-129, respectively, is that transport of a cation carried by ETH-129 is dependent on membrane potential while in the case of A23187, it is influenced by transmembrane pH gradients (Wang et al., 2001).

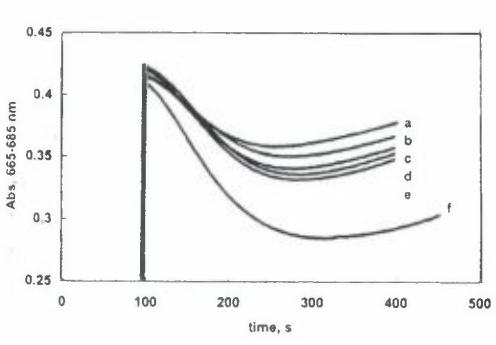

Fig. 2.

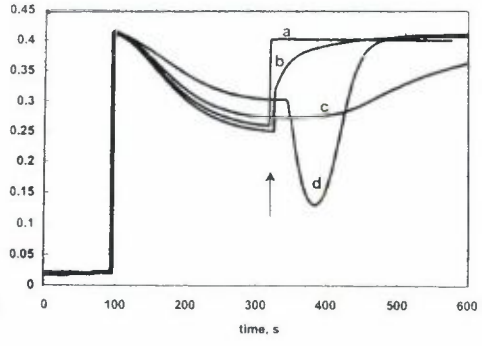

Fig. 3.

Figure 2. DC-11C-induced inhibition of Ca^{2+} uptake by wheat root mitochondria. Mitochondria were incubated with DC-11C ca. 2 min prior to initiation of Ca^{2+} uptake with NADH. DC-11C concentration: line a - 100 nM; b - 75 nM; c - 50 nM; d - 15 nM; e - 25 nM. Line f- control, no DC-11C present. Incubation medium: 10 mM Hepes-Tris pH 7.4, 10 µM EGTA, 0.25 M sucrose, 5 mM inorganic phosphate, 100 µM $CaCl_2$.

Figure 3. Effect of calcium ionophores: electrogenic ETH-129, electroneutral A23187 and DC-11C, on Ca^{2+} transport in Ca^{2+}-loaded mitochondria. The arrow indicates time of ionophore addition: a - 10 µM A23187; b - 10 µM DC-11C; c - no ionophore addition; d - 5 µM ETH-129.

In our experiments Ca^{2+} ions were partially sequestered inside mitochondria and partially available in the medium surrounding the mitochondria. Ionophores were introduced into the system when Ca^{2+} uptake was far from cessation (Fig. 3). The results ruled out DC-11C inhibition of the Ca^{2+} transporter. Indeed, in this case the addition of the compound should stop (inhibit) Ca^{2+} uptake (as ruthenium red, an inhibitor of Ca^{2+} uptake does), but would not induce an acute release of the cation as shown in Fig. 3b. The same effect of the structurally similar A23187 on Ca^{2+} uptake supports Ca^{2+}/H^{+} exchange mechanism for DC-11C, although the release of Ca^{2+} induced by A23187 was more rapid (Fig. 3a). Addition of ruthenium red, an inhibitor of the transporter, prior to DC-11C did not have any effect on DC-11C-induced Ca^{2+} release (data not shown), thus confirming that the release did not occur through the reversal of Ca^{2+} transporter. In contrast, addition of the electrogenic ionophore ETH-129 first enhanced Ca^{2+} transport, due to high membrane potential, which first favoured electrophoretic Ca^{2+} uptake and then induced a release (Fig. 3d). Application of the Ca^{2+} chelator EGTA (two sequential additions, 30 μM) completely abolished DC-11C-induced Ca^{2+} release confirming the results. A similar effect was observed when DC-11C was substituted with 10 μM A23187 (data not shown).

Effect of DC-11C, A23187 and ETH-129 on inner mitochondrial membrane potential

Orientation of the inner mitochondrial membrane potential (inside negative) electrogenically favours cation transport into mitochondria. Divalent cation transport (Ca^{2+} and Mn^{2+}) catalyzed by carboxylic ionophores has been shown to decrease on depolarisation of the plasma membrane potential in different cell models and in artificial liposomes (Fasolato and Pozzan, 1989). Positive correlation between membrane potential and the transport rate of A23187-La^{3+} complex has been shown in phospholipid vesicles (Wang et al., 1998). Plant cells are fully capable of controlling plasma membrane potential and pH. *In vivo* it implies the possibility to regulate DC-11C -facilitated cation flux via changes in plasma membrane potential and /or extracellular pH.

Monitoring mitochondrial membrane potential in a medium with high Ca^{2+} concentration together with either DC-11C or electrogenic ionophore ETH-129, confirmed the electroneutrality of DC-11C-induced Ca^{2+} transport. The coordinated changes in Ca^{2+} transport and membrane potential are presented in Fig. 4. The electrophoretic Ca^{2+} uptake induced by 5 μM ETH-129 was accompanied by partial dissipation of the membrane potential (Fig. 4b). In contrast, 10 μM DC-11C did not cause significant changes in membrane potential (Fig. 4a) thus supporting the electroneutral nature of DC-11C functioning.

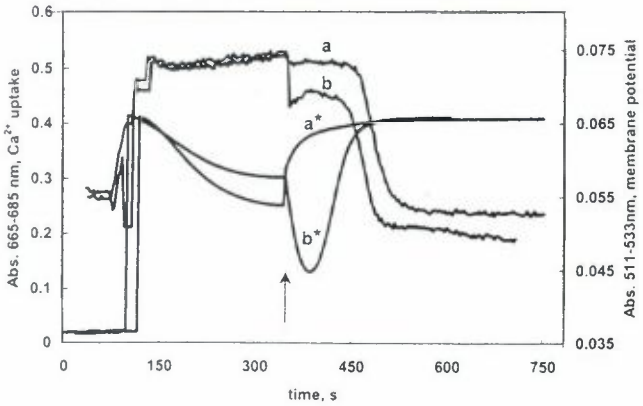

Figure 4. Coordinated changes in mitochondrial membrane potential and Ca^{2+} transport induced by calcium ionophore ETH-129 and DC-11C. The arrow indicates time of ionophore/DC-11C addition: 10 μM electroneutral DC-11C (lines a and a*) or 5 μM electrogenic ETH-129 (lines b and b*). Membrane potential - lines a and b; Ca^{2+} transport - lines a* and b*. Downward deflection of the traces indicates inner membrane depolarisation (upper lines) and a decrease of Ca^{2+} concentration in the incubation medium (lower lines).

Comparison of the traces of Ca^{2+} uptake (Fig. 4a*, b*) with the traces of inner membrane potential (Fig. 4a, b) suggests that changes in membrane potential were due to Ca^{2+} transport across the membrane.

Ca^{2+} ion is an important intracellular signal in plant cells, with regulatory roles in a variety of physiological and biochemical processes (reviewed in White and Broadley, 2003), including gene expression (Thain and Wildon, 1996). Ca^{2+} is also recognized to have some special roles in plant-microbe interactions. In infected host plant cells, the actions of both symbiotic and pathogenic microbes are known to activate a signal transduction cascade that involves Ca^{2+} influx in the plant cells (Cárdenas et al., 1999; Kaile et al., 1991; Miller et al., 2000; Salzer and Boller, 2000).

The proposed mechanism, where calcium ions are complexed and transferred through cell membranes by DC-11C, makes this compound highly fascinating. From the early events of infection to the formation of the symbiotic nitrogen-fixing nodule, *Frankia* is in close interaction with the host plant cells through a host-derived pectic capsule that surrounds the growing bacterial hyphae. Nutrient exchange and other signal transfers between the microsymbiont and the host cells are mediated via this symbiotic interface (Berg 1990, Liu and Berry, 1991a, Liu and Berry, 1991b). In the capsule, demethyl (C-11) cezomycin may have a regulatory function by controlling Ca^{2+} fluxes through host cell membranes, thus affecting the physiology of the host cells e.g. by suppressing the host defence reactions.

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