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Research article

Unsaturated amino acids derived from isoleucine trigger early membrane effects on plant cells

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ABSTRACT

Unsaturated amino acids (UnsAA) have been shown to affect the activity of various biological processes. However, their mode of action has been investigated poorly thus far. We show in this work that 2-amino-3-methyl-4-pentenoic acid (C2) and 2-amino-3-methyl-4-pentynoic acid (C3) structurally derived from isoleucine (Ile) exhibited a multisite action on plant cells. For one, C2 and C3 induced early modifications at the plasma membrane level, as shown by the hyperpolarization monitored by microelectrode implantation in the pulvinar cells of *Mimosa pudica*, indicating that these compounds are able to modify ionic fluxes. In particular, proton (H^+) fluxes were modified, as shown by the pH rise monitored in the bathing medium of pulvinar tissues. A component of this effect may be linked to the inhibitory effect observed on the proton pumping and the vanadate-sensitive activity of the plasma membrane H^+-ATPase monitored in plasma membrane vesicles (PMVs) purified from pulvinar tissues of *M. pudica* and leaf tissues of *Beta vulgaris*. This effect may explain, in part, the inhibitory effect of the compounds on the uptake capacity of sucrose and valine by *B. vulgaris* leaf tissues. In contrast, an unexpected action was observed in cell reactions, implicating ion fluxes and water movement. Indeed, the osmocontractile reactions of pulvini induced either by a mechanical shock in *M. pudica* or by dark and light signals in *Cassia fasciculata* were increased, indicating that, compared to Ile, these compounds may modify in a specific way the plasma membrane permeability to water and ions.

1. Introduction

Attention towards the “unnatural” amino acids (AAs) has increased since the discovery of their occurrence in organisms. For example, chemical screening of 17 species of *Amanita* resulted in the description of unusual AAs, such as cyclopropyl-derivatives (Yoshimura et al., 1999), chloro-derivatives and unsaturated amino acids (UnsAAs), in particular, 2-amino-5-hexenoic acid and 2-amino-4-hexynoic acid (Li and Oberlies, 2005 and references therein). Different investigations have shown that the UnsAAs exhibit a variety of interesting biological activities, particularly antimicrobial properties (Katagiri et al., 1967; Kirino et al., 1980). For instance, 2-amino-4-methoxy-*trans*-3-butenoic acid (AMB) isolated from a fermentation broth of *Pseudomonas aeruginosa* ATCC-7700 inhibited the growth of *Bacillus* spp. 1283 B (Scannell et al., 1972). Rando (1974) showed that AMB behaved as an irreversible inhibitor of soluble, pyridoxal-linked aspartate amino transferase. The effect of allylglycine (2-amino-4-pentenoic acid) on particular enzymes also has been the subject of extended studies due to its convulsant effect. Studies on the brain have specified that this compound blocked glutamic acid decarboxylase activity, inhibited γ-aminobutyric acid synthesis (Fisher and Davies, 1976) and increased ornithine decarboxylase activity (Laitinen, 1985).

The synthesis of unnatural amino acids (AAs) has been developing widely in recent years for the consideration of their expected

Abbreviations: PMVs, plasma membrane vesicles; MES, 2-(*N*-morpholino)ethanesulfonic acid; UnsAAs, unsaturated amino acids; C2, 2-amino-3-methyl-4-pentenoic acid; C3, 2-amino-3-methyl-4-pentynoic acid; Ile, isoleucine; Val, valine; Suc, sucrose; AA, amino acid.

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technological advances for use in therapeutic applications in relation to the design of synthetic peptides (Pichereau and Allary, 2005). In addition, due to the presence of characteristic functional groups compared to natural AAs, unnatural AAs can be used as probes to obtain a better understanding of biological processes (Perdih and Dolenc, 2011 and references therein). The synthesis of unnatural AAs has expanded greatly due to technical advances in their incorporation into proteins both *in vivo* and *in vitro* (Noren et al., 1989; Dawson and Kent, 2000; Cropp and Schultz, 2004; Hahn and Muir, 2005). Incorporation of selected AA analogs into proteins made *in vivo* has been in use for many years (Fenster and Anker, 1969; Kiick et al., 2002; Bae et al., 2003). As emphasized by Mock et al. (2006), the use of monomers other than the 20 natural AAs leads to introduction of new functionalities into proteins bearing novel physical and chemical properties. Thus, this approach has contributed to the elucidation of details of protein structure and function (Dougherty, 2000) and has expanded knowledges concerning protein–protein and protein–nucleic acids interactions (Hohsaka and Sisido, 2002; Johnson et al., 2010).

Interestingly, non-coded AAs have been found in naturally occurring peptides. In particular, peptides containing dehydro-AAs have been isolated from bacteria, fungi, marine invertebrates and some plants. These peptides show mainly antibiotic, antifungal, antitumor and phytotoxic activities (Siodlak, 2015 and references therein).

The residue Δ−isoleucine (ΔIle) is found in the compound FR225656 which is produced by the fungi *Helicomyces* spp. (Zenkoh et al., 2003) and behaves as an inhibitor of gluconeogenesis (Ohtsu et al., 2003). Similarly, the linear heptapeptide antrimycin contains four unusual AA residues, including (E)- ΔIle. Antrimycin, isolated from the bacterium *Streptomyces xanthocidicus* (Morimoto et al., 1981), shows antibiotic activity against *Mycobacterium smegmatis* (Shimada et al., 1981).

To our knowledge, as described above, the UnsAAs were used widely either in genetic engineering or as intermediates in the synthesis of materials for medicinal and organic chemistry purposes. The main observations regarding their biological effects are restricted to their inhibitory effects on the growth of various microorganisms. Our attention was paid to UnsAAs derived from Ile, namely 2-amino-3-methyl-4-pentenoic acid (C2) and 2-amino-3-methyl-4-pentynoic acid (C3). C3 inhibited the growth of *Saccharomyces cerevisiae* and *Escherichia coli* (Gershon et al., 1954), and C2 has been shown to be a potent antagonist of Ile and valine (Val) to the growth of *Lactobacillus arabinosus* and *E. coli* (Shive and Skinner, 1963). The antimicrobial properties of both analogs also were confirmed later: C2 and C3 did not support cell growth and were toxic to *E. coli* (Michon et al., 2002). However, the mechanisms of their action on cell machinery have been investigated poorly. Primarily, this work was focused on possible effects induced by UnsAAs on plasma membrane functioning.

To achieve our goal, the effects of C2 and C3 were compared to those induced by Ile on bioelectrical membrane potential and on proton fluxes observed in pulvinar cells of *Mimosa pudica*. Furthermore, the effects of C2 and C3 on osmocontractile reactions observed in pulvinar cells of *Mimosa pudica* and *Cassia fasciculata*, which are driven by K^+ and Cl^- fluxes (Samejima and Sibaoka, 1980), may afford interesting data concerning their impact on the ionic migrations taking place at the plasma membrane site. In addition, an investigation into the effects of these compounds on the uptake of sucrose (Suc) and valine (Val) by *Beta vulgaris* leaf cells was also carried out to enlighten whether C2 and C3 may modify the mode of transport of these types of metabolites, taken up by the cells through a H^+-substrate co-transport (Delrot et al., 2001). Finally, using plasma membrane vesicles (PMVs), a direct inhibitory action of these compounds has been shown on the activity of the membrane H^+-ATPase, a key enzyme regulating the studied biological processes.

2. Materials and methods

2.1. Plant material and culture conditions

Seeds of *Mimosa pudica* L., *Cassia fasciculata* Michx. and *Beta vulgaris* L. cv. Aramis were germinated in an organic compost. Seedlings and older plants were grown in this compost watered daily and kept in climate-controlled chambers at 27.5 ± 0.5 °C and 65 ± 5% relative humidity. Illumination was regulated to give 16 h of light (photophase 06.00 h–22.00 h) provided by fluorescent tubes (mixing Osram fluora and Osram day-light types) with a photon fluence rate (400–700 nm) of 80 μmol m^{-2} s^{-1} at the plant apex.

2.2. Electrophysiological measurements

The experiments were done on primary pulvini from mature leaves of 2-month old plants of *M. pudica*, generally the eighth above the cotyledons. A major advantage of the pulvinus model in electrophysiological assays is linked to its size allowing easy handling and also to its particular anatomy characterized by many layers of parenchyma cells surrounding the central cylinder, insuring therefore impalement of a microelectrode in a well-defined kind of cell. The transmembrane potential was measured by the classical electrophysiological method using microelectrodes (tip diameter < 1 μm, tip resistance from 5 to 30 MΩ). For details, see Amborabé et al. (2008). Briefly, leaf was excised from the stem and pulvinus fixed to the bottom of a 4 ml plexiglas chamber filled with a buffered medium (10 mM MES/NaOH, pH 5.2) containing 1 mM NaCl, 0.1 mM KCl, and 0.1 mM $CaCl_2$ (Abe, 1981). The glass microelectrode was impaled into a motor cell of the abaxial ("extensor") half of the organ. Under these conditions, the resting transmembrane potential (Ψ_0) was in the range of −100 to −150 mV. The assays in which Ψ_0 was out of the range −110/−130 mV were discarded since amplitude of the induced electrophysiological modifications may be modulated by the initial resting potential value. Thus, the calculated value from 24 assays was −112 ± 3 mV (SEM).

2.3. Measurement of pH variations

In order to observe H^+ excretion, primary pulvini of *M. pudica* (400 mg) were excised at 10.00 h on 2-month old plants bearing generally 10 leaves. The organs were divided in transverse sections and treated following the same procedure previously described (Amborabé et al., 2008): unbuffered incubation medium composed of 0.50 mM $CaCl_2$, 0.25 mM $MgCl_2$, variations of pH read on a pH-meter provided with combined electrodes (Futura micro-combination, Beckman Coulter) and linked to a potentiometric recorder. Compounds were added as indicated in the figures. In order to quantify the amount of mobilized protons, titration was made 3 h after the application of the compounds on 2 ml of the incubation medium with NaOH or HCl at 5.10^{-3} N. The experiments were repeated at least 4 times.

2.4. Preparation and use of plasma membrane vesicles

Purified plasma membrane vesicles (PMVs) were prepared by phase partitioning of microsomal fractions from the primary pulvini of *M. pudica* and from leaves of *B. vulgaris* according to Lemoine et al. (1991) with some minor modifications. After isolation, PMVs were frozen in liquid nitrogen and stored at −80 °C. They were put in the inside-out configuration by adding 0.05% brij in the assay