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The defense substance allicin from garlic permeabilizes membranes of *Beta vulgaris*, *Rhoeo discolor*, *Chara corallina* and artificial lipid bilayers

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ABSTRACT

Background: Allicin (diallylthiosulfinate) is the major volatile- and antimicrobial substance produced by garlic cells upon wounding. We tested the hypothesis that allicin affects membrane function and investigated 1) betanine pigment leakage from beetroot (*Beta vulgaris*) tissue, 2) the semipermeability of the vacuolar membrane of *Rhoeo discolor* cells, 3) the electrophysiology of plasmalemma and tonoplast of *Chara corallina* and 4) electrical conductivity of artificial lipid bilayers.
Methods: Garlic juice and chemically synthesized allicin were used and betanine loss into the medium was monitored spectrophotometrically. *Rhoeo* cells were studied microscopically and *Chara*- and artificial membranes were patch clamped.
Results: Beet cell membranes were approximately 200-fold more sensitive to allicin on a mol-for-mol basis than to dimethyl sulfoxide (DMSO) and approximately 400-fold more sensitive to allicin than to ethanol. Allicin-treated *Rhoeo discolor* cells lost the ability to plasmolyse in an osmoticum, confirming that their membranes had lost semipermeability after allicin treatment. Furthermore, allicin and garlic juice diluted in artificial pond water caused an immediate strong depolarization, and a decrease in membrane resistance at the plasmalemma of *Chara*, and caused pore formation in the tonoplast and artificial lipid bilayers.
Conclusions: Allicin increases the permeability of membranes.
General significance: Since garlic is a common foodstuff the physiological effects of its constituents are important. Allicin's ability to permeabilize cell membranes may contribute to its antimicrobial activity independently of its activity as a thiol reagent.

1. Introduction

Plants protect themselves from attacks by pathogens and pests by producing defense chemicals upon injury. The chemical nature of such defense substances is very diverse in the Plant Kingdom and in the onion and garlic family (Alliaceae) it is mainly sulfur-containing substances that are involved. The chemistry of the sulfur compounds in garlic, and the various reactions that occur when *Allium* species are wounded, are complex and many of the sulfur-containing volatiles formed are physiologically active [1]. The major antimicrobial agent in fresh garlic juice was identified as allicin by Cavallito and Bailey [2,3], and this has been confirmed subsequently several times [4,5]. For many test organisms, allicin's antimicrobial effectiveness is comparable to that of conventional antibiotics such as penicillin, kanamycin and ampicillin [2,6]. Allicin is a reactive sulfur species (RSS) which oxidizes thiol groups in proteins and glutathione [7]. Allicin has been shown to be able to shift the cellular redox potential and induce apoptosis in yeast and animal cells [8,9]. Because of its activity as a thiol reagent allicin can react with and inactivate key enzymes and this is thought to contribute to its antimicrobial activity [4]. Allicin is active against human pathogens such as *Helicobacter pylori* and bacteria with multiple antibiotic resistances e.g., vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) [10,11] and against many plant pathogenic bacteria and fungi [6,12–14]. Furthermore, the anti-coagulatory effects of the allicin breakdown/follow on product ajoene are reported to have positive effects on the pulmonary system. Thus, since allicin is a physiologically active natural substance, found in a common foodstuff, it has an attractive potential for use in medicine, where approximately 50% of drugs in clinical use are of natural product origin [15], and in organic agriculture where there is a need for fungicides derived from natural products [14]. As with all active substances used in medicine and agriculture it is important to know as much as possible about the physiological effects and mechanisms of action of allicin.

Allicin is taken up readily by cells and has a calculated log$P = 1.29 \pm 0.13$ (http://www.vcclab.org). It has been suggested that this ability to pass through cell membranes easily contributes to its effectiveness

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as an antibiotic [16]. Allicin and the well-known membrane-permeabilizing substance dimethyl sulfoxide (DMSO, calculated log$P$ = -0.51 ± 0.58) have structural similarities and both contain a sulfinyl moiety, that is, an oxidized sulfur atom with the oxygen atom bonded to the sulfur via a dative (dipolar) bond (Fig. 1). By virtue of its physical properties, DMSO forms fluctuating pores in lipid membranes and so increases their permeability [17–19]. This prompted us to test whether allicin might also have permeabilizing effects on plant cell and artificial lipid membranes independently of its chemical reactivity as a thiol reagent. Therefore, we investigated the membrane effects of allicin in garlic juice and chemically synthesized allicin in a number of experimental systems.

Cells of the swollen hypocotyl of beetroot (*Beta vulgaris* subsp. *vulgaris* var. *conditiva* L., Amaranthaceae) contain the red-colored alkaloid pigment betanine in their vacuoles in high concentrations (up to 0.5 g kg^{-1} fresh weight) [20]. If the plasmalemma and tonoplast membranes are permeabilized, for example by chloroform treatment, betanine is subsequently lost from the cells into the bathing medium. Loss of pigment from the cells can be monitored quantitatively by following the increase in A_{537} in the bathing solution spectrophotometrically. This well established method (e.g., [21–23] presents an alternative to using conductivity measurements to quantify membrane leakage.

The behavior of cells in an osmolyticum is also an indicator of membrane integrity. Thus, cells with damaged membranes that have lost their semipermeable properties do not plasmolyse normally in hypertonic solutions [24]. Loss of the ability to plasmolyse is therefore an indicator for membrane damage. Cells of *Rhoeo discolor* L'HER (Syn. *Tradescantia spatacea* SW. Commelinaceae) contain anthocyanin pigments in the vacuole of the lower leaf epidermal cell-layer, and make it a suitable subject to visualize plasmolysis easily under the microscope [25,26].

The green alga *Chara corallina* belongs to the family Characeae and has a simple thallus, comprising nodal regions with initials for lateral internodal and rhizoidal cells. It produces giant internodal cells of about 1 mm in diameter and several centimeters in length. Those cells have been of interest for plant physiologists over decades because they exhibit a simple cylindrical symmetry, a large central vacuole and are very suitable for use in electrophysiological research. Furthermore, it is easy to produce cytoplasmic droplets, delineated by the tonoplast, from *Chara* cells. For example, cytoplasmic droplets have been successfully used in patch clamp studies on the tonoplast as a model membrane to investigate the primary effect of caterpillar regurgitant in forming membrane pores [27]. A survey over the wide field of *Chara* research is given by Tazawa [28]. A further advantage of using charophyte internodes for membrane studies is their close relationship to higher plants. Thus, it is reported that the charophytes and coleochaetales/zygnematales are the predecessors of land plants [29,30].

In this paper we describe the effects of allicin on membranes in three different organisms and artificial lipid bilayers. We show that allicin, on a mol-for-mol basis, is two orders of magnitude more effective at permeabilizing cell membranes than are agents such as ethanol or DMSO and that allicin is capable of building fluctuating pores in artificial lipid bilayers.

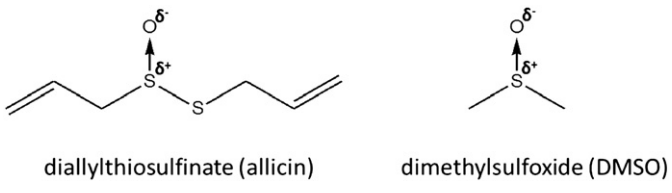

Fig. 1. Structural similarity of diallylthiosulfinate (3-[(prop-2-ene-1-sulfinyl)sulfanyl] prop-1-ene or allicin) with dimethylsulfoxide (methanesulfinylmethane or DMSO).

2. Materials and methods

2.1. Preparation of garlic juice and determination of allicin concentration

Garlic bulbs were purchased from the supermarket and stored at 4 °C in the dark. Garlic juice was extracted using a domestic juicer (TurmixFabr.No.1068; Turmix AG, Jona, Switzerland) after peeling the axillary buds of composite garlic bulb. To remove the pulp from the liquid, the juice was centrifuged at 5000 rpm (Megafuge 1.OR; Heraeus Instruments, Osterode, Germany) in a sterile falcon tube and the floating debris was scooped off before filtering under pressure to eliminate remaining pulp.

To determine allicin-concentration with HPLC garlic juice was diluted 1:10 with water (HPLC-grade) and 1 ml was mixed with 1.5 ml internal standard (0.05 mg ml^{-1} hydroxybenzoic acid in methanol). To protect the column, mixture was filtered through a polyether sulfon membrane (pore size 0.22 μm; Carl Roth, Karlsruhe). Twenty microliters were injected in a Jasco HPLC system using a UV-detector at 254 nm (Jasco Germany, Groß-Umstadt). HPLC was controlled using the HPLC software Chrompass Version. As mobile phase, a mixed gradient of solvent A (30% v/v methanol + 0.1% formic acid) and solvent B (100% v/v methanol) was used [8].

2.2. Synthesis of allicin

Allicin synthesis was performed according to the method described in [31]. Diallyldisulfide (DADS; Sigma-Aldrich, Munich, Germany) was distilled at 1 mbar under vacuum before use. Two grams (= 13 mmol) of freshly distilled DADS was dissolved in 5 ml cold glacial acetic acid in an ice bath and 3 ml of cold hydrogen-peroxide (Merck, Darmstadt, Germany) was added slowly under stirring. After 30 min the temperature was allowed to increase to room temperature and stirring was continued for an additional 2 h.

The reaction was quenched by diluting with 15 ml deionized water (Sartorius pro, 18.2 MΩ cm; Sartorius, Goettingen, Germany). After extracting with 30 ml dichloromethane (DCM; Riedel de Haen, Seelze, Germany), remaining acetic acid was removed by washing the extract with 5% $NaHCO_3$ (Merck, Darmstadt, Germany) several times and finally washed with deionized water to pH 6–7.

The extract was evaporated *in vacuo* and the yellow liquid remaining was dissolved in 200 ml water. Unreacted DADS was removed by double extraction with 0.1 vol. hexane (Merck, Darmstadt, Germany) and allicin was extracted with DCM again. The dichloromethane-phase was dried over anhydrous $MgSO_4$ (Merck, Darmstadt, Germany) and concentrated *in vacuo*.

Purity of allicin was determined by ^{1}H-NMR or HPLC as described previously [8]. Synthetic allicin preparations were >98% pure.

2.3. Betanine release assay from beetroot tissue

The betanine-release assay was adapted from the method of Grunwald [21]. Beetroot (*Beta vulgaris* subsp. *vulgaris* var. *conditiva*) was purchased from a local store. Cylinders of beetroot were cut from the swollen hypocotyl tissue with a No. 7 cork borer (12 mm diameter) and subsequently divided up into 5 mm uniform discs using a razor-blade assembly. The discs were washed in running tap water (approximately 10 min) until no release of betanine from damaged cells at the cut surface into the washing water was observable. The discs were dried lightly with a paper towel and in each case five discs were incubated in 10 ml of test solution for 15 min with gentle shaking. Discs incubated in deionized water served as a negative control.

After incubation in the test solutions discs were washed in deionized water and given into 10 ml sodium phosphate buffer (pH 6). Release of betanine was followed over 1 h using a Beckman DU800 spectrophotometer at 537 nm [32].

2.4. Use of plasmolysis in Rhoeo discolor to demonstrate membrane integrity

To demonstrate that the test compounds have a membrane-damaging effect, lower epidermal cell layer of *Rhoeo discolor* leaves was peeled and incubated for 15 min in test solutions and subsequently for 15 min in 0.85 M KNO_3 solution to facilitate plasmolysis [24]. Microscopic pictures were taken in light-microscopy modus using a Leica DM RBE (Leica, Wetzlar, Germany) and documented with a digital camera (KY-F75U; JVC Germany GmbH, Friedberg, Germany).

2.5. Electrophysiology of biological membranes (Chara) and artificial lipid bilayers

2.5.1. Experimental chamber

The apparatus was modified from Shimmen et al. [33]. The perspex chamber consisted of three rows of compartments (Fig. 2). The lowermost row holds the internodal *Chara* cell, the compartments of the middle row and the connecting channels contain artificial pondwater (APW, see Bathing solutions) solidified with agar (2% w/v), and the top row compartments 1 M KCl as the electrode solution. From each compartment of the top row, membrane voltage could be recorded by integrated Ag/AgCl-electrodes. In this way, large electrode offset voltages, due to different Cl^--concentrations between the 110 mM KCl compartment and an APW compartment, could be avoided. Built-in electrodes in the lower compartments allowed current injection into the cell. Both the current and the voltage circuits were independent of each other thus excluding interference.

2.5.2. Bathing solutions

While the leftmost chamber of the lowermost row was filled with a 110 mM KCl solution, the other compartments contained artificial pond water (APW, 0.1 mM KCl, NaCl, and $CaCl_2$ in 5 mM Tris/HCl buffer pH 7.5). To balance the osmotic difference to the 110 mM KCl compartment, APW was made 180 mM with sorbitol. An overnight pre-incubation of isolated internodal cells in sorbitol-free APW permitted recordings of stable membrane voltages and resistances.

2.5.3. Recording conditions

Chara internodal cells of about 100 mm length and 0.8 mm in diameter were placed into a groove that joined several compartments. The groove between the compartments was sealed with Vaseline, thus dividing the internodal cell into equal 10 mm segments. The Vaseline provided an extracellular electrical insulation while the protoplast formed a continuum throughout the whole cell. The Vaseline cover also protected the cell segments between the compartments from drying out. After positioning the cell, compartments were filled with their respective solutions. Membrane voltage was recorded by a home-made amplifier (input impedance 10^{12} Ω), applied current pulses were controlled via serial resistances, at least 100-fold greater than the reported specific membrane resistance of the *Chara* plasmalemma [34]. Rectangular pulses of electrical current were delivered from a battery-powered generator (UTG 100, ELV Elektronik AG, Leer, Germany) with 5 V output, ranging at 0.15 μA depolarizing current amplitude (33 MΩ pre-resistor), with a cycle of 1 s (on) and 4 s (off). To find out effects of extracted diluted garlic juice or synthesized allicin, salt solutions of APW-compartments were replaced by identical solutions with added garlic substances.

2.5.4. Patch clamp recording of pore-like current events

To detect possible effects of allicin at the molecular level we used cytoplasmic droplets from *Chara* internodal cells and artificial lipid bilayers. Cytoplasmic droplets form in an aqueous solution (about 300 mOsm) from effused cytoplasm after cutting one cell end off the slightly wilted cell. Generally, droplets are delineated by the original tonoplast [35,36]. Cytoplasmic vesicles were bathed in a solution containing either 120 mM LiCl, or NaCl, or 84.5 mM $CaCl_2$ (i.e., isoosmotic to the monovalent salts), buffered to pH 7.5 by 5 mM Tris/HCl. The patch pipette contained a symmetrical solution and additionally either diluted garlic juice or synthetic allicin). In order to examine a possible effect on the native maxi-K channel in the presence of allicin, we used asymmetric ionic solutions of equal concentrations (e.g., 120 mM KCl/120 mM NaCl) in the bath and the pipette, respectively, to observe a K^+ current through the maxi-K channel which should be blocked beyond the reversal voltage by Na^+, where no current fluctuations should occur except those through novel nonselective pathways.

The lipid used for artificial membrane formation was L-α-lecithin (Azolectin, Sigma type IV-S). Vesicles of artificial lipid membranes were produced by fast extrusion of dissolved lipid (50 mg L-α-lecithin ml^{-1} hexane) through a disposable 26-gauge needle into a solution made of 120 mM KCl and 5 mM Tris/HCl pH adjusted to 7.5 with additional garlic juice diluted to 1:300 or 100 μM allicin, respectively. Multilamellar vesicles or unilamellar vesicles greater than 50 μm in diameter developed within 1 h at room temperature (approximately

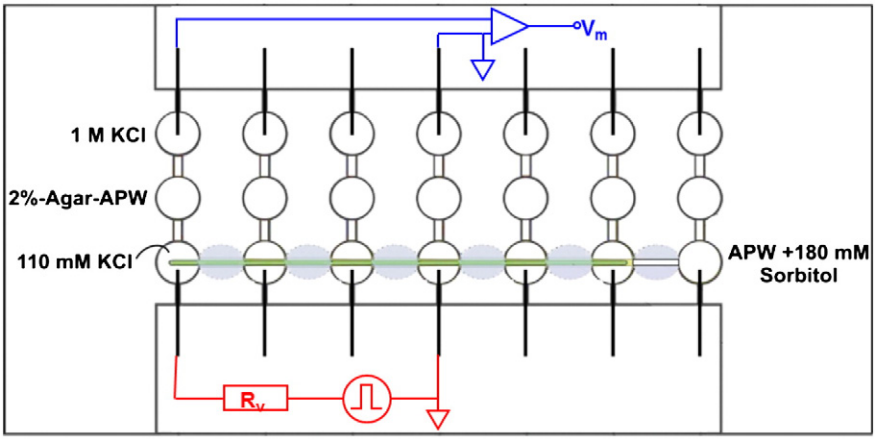

Fig. 2. Perspex chamber for K^+-anesthesia experiments on intact *Chara* internodal cells. Membrane potential differences (V_m) between cellular segments, separated by Vaseline bridges from each other, were recorded from top row 1 M KCl compartments that were electrically connected to different cellular segments by the high K^+-concentration, the cellular segment in the 110 mM KCl compartment is completely depolarized and the external electrode can be regarded as being located intracellularly, whereas the electrode connected to an APW compartment is a true extracellular electrode. Hence, membrane voltage is detected by the virtual intracellular electrode through the cytoplasm across the membrane segment of an APW compartment. Independent of the voltage recording circuit, current pulses could be applied via a pre-resistor (R_v) to cell segments in the lowermost row of compartments (see section Materials and methods for details).

21 °C) and were patched with micro-pipettes containing the same solution as the bath. Current fluctuations were recorded from excised patches or in the cell-attached configuration.

Artificial planar lipid membranes were produced by spreading lipid solution (50 mg ml^{-1} decane) by the monolayer folding technique [37] across a 60 μm hole in a Teflon® membrane (polytetrafluoroethylene), submersed in a salt solution identical to that used for vesicles of artificial membranes. After ascertaining electrical silence while allicin was absent, the substance was added at the *cis*-side of the membrane, the applied potential at this side was referred to the *trans*-side set to ground.

Patch clamp equipment was composed of a Multiclamp 700A amplifier, a digitizer Digidata 1440A, and the controlling and analyzing software pCLAMP 10 (all Axon/Molecular Devices, Sunnyvale, CA). Patch electrodes were pulled on a DMZ puller (Zeitz Instr., Munic, Germany) to pipette diameters of less than 1 μm; they were positioned by an electrically controlled micromanipulator (SM I, Luigs & Neumann, Ratingen, Germany).

Signals were conditioned by a Bessel low-pass set to 2 kHz corner frequency and stored directly on computer hard disc.

3. Results

3.1. Allicin in garlic juice triggers release of betanine from beetroot vacuoles

To test whether garlic juice has an effect on biomembranes, discs of swollen beetroot hypocotyl were incubated both in garlic juice and synthetic allicin for 15 min and subsequently in phosphate buffer for 1 h. Cells with enhanced membrane permeability show an accelerated release of betanine from the vacuoles. The loss of betanine into the medium was greater in the samples treated with garlic juice and allicin than the water-incubated control (Fig. 3). Pigment loss from beetroot discs treated with chloroform to completely permeabilize the cell membranes is shown for comparison (Fig. 3).

3.2. Kinetics of betanine-leakage from beet tissue after treatment with allicin, ethanol or DMSO

DMSO and ethanol (EtOH) are both known to permeabilize cell membranes and were compared with allicin as positive controls. The release of betanine from beetroot discs after treatment with different concentrations of synthetic allicin, ethanol or DMSO was followed at 537 nm in the spectrophotometer. The changes in absorption (ΔA_{537}) against time are shown in Fig. 4. Whereas incubation of the beetroot discs in 2 M ethanol or 1 M DMSO for 15 min resulted in no increase in the rate of betanine loss compared to water treatment, exposure to as little as 10 mM allicin caused substantial subsequent pigment loss (Fig. 4). Comparative rates of pigment loss were caused by 4 M ethanol or 2 M DMSO, respectively. The latter translates into an approximately

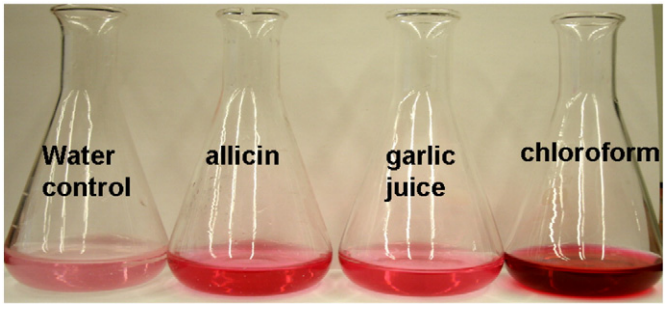

Fig. 3. Allicin permeabilizes beet cell membranes. Ten discs of beetroot tissue were incubated for 15 min either in water (negative control), 10 mM allicin solution, 10 mM allicin in garlic juice, or chloroform (positive control). The discs were washed in de-ionized water and then incubated for 1 h in 25 ml sodium phosphate buffer (pH 6).

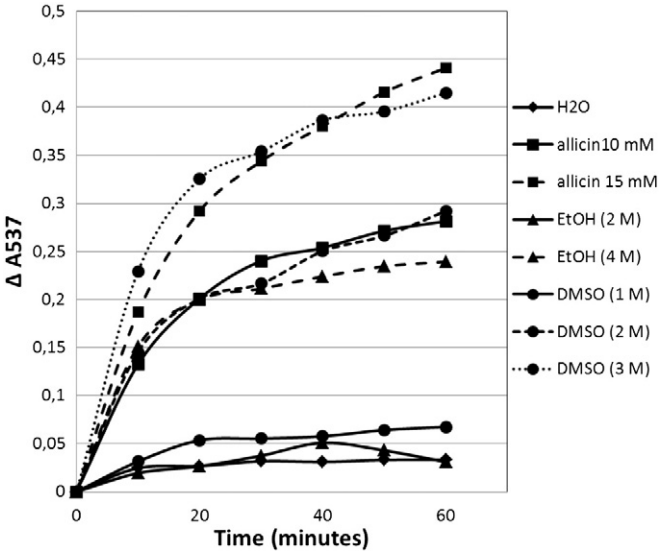

Fig 4. Kinetics of betanine loss from beetroot tissue pre-incubated in allicin, EtOH or DMSO. Beetroot discs (10) were bathed for 15 min in the test solutions, washed extensively in tap water, then incubated for 1 h in 10 ml sodium phosphate buffer (pH 6). The change in absorbance (ΔA_{537}) was measured spectrophotometrically. A representative data set from one experiment is shown. The experiment was repeated 3 times with similar trends.

400 and 200 fold greater effectivity on a mol-for-mol basis in membrane permeabilization by allicin over ethanol or DMSO, respectively. This relationship was maintained at the higher concentration of allicin (15 mM) which caused pigment loss at a similar rate to 3 M DMSO.

We have performed membrane-permeability experiments with beets very many times over several years and although the trends are always similar the absolute rates of pigment loss for a given treatment vary between individual swollen hypocotyls. Thus, it is important for the sake of reproducibility and consistency to use discs from the same beetroot hypocotyl in a single experiment. It should be remembered that the beetroot is purchased fresh from the supermarket, when in season, and that age, time of storage and storage conditions of the beetroots will vary between samples and this probably results in the variability seen. Thus, the results show the kinetics for pigment loss in a single experiment with discs from a single beetroot and are not from pooled replicates. Hence there is no statistical analysis.

3.3. Synergism between allicin/EtOH and allicin/DMSO

Beetroot discs treated with either 2 M EtOH or 1 M DMSO did not show an enhanced rate of betanine leakage compared to water-treated controls (Figs. 4, 5 and 6). Similarly, 2 mM or 4 mM allicin did not enhance betanine leakage. Interestingly however, in combination, ineffective concentrations of EtOH or DMSO with allicin apparently showed a synergistic action (Figs. 5 and 6).

3.4. Effect of allicin and DMSO on the plasmolysis of Rhoeo discolor protoplasts

The red anthocyanin pigments of *Rhoeo discolor* are localized in the central vacuoles. In an osmoticum such as 0.85 M KNO_3 water loss via osmosis leads to shrinkage of the protoplast which rounds up to a spherical body within the cell (Fig. 7 control). After treatment with 1 mM allicin, either synthetic or in garlic juice, or with 500 mM DMSO as a positive control known to permeabilize cell membranes, the cells lose their ability to plasmolyse normally and angular shaped protoplasts result (Fig. 7).

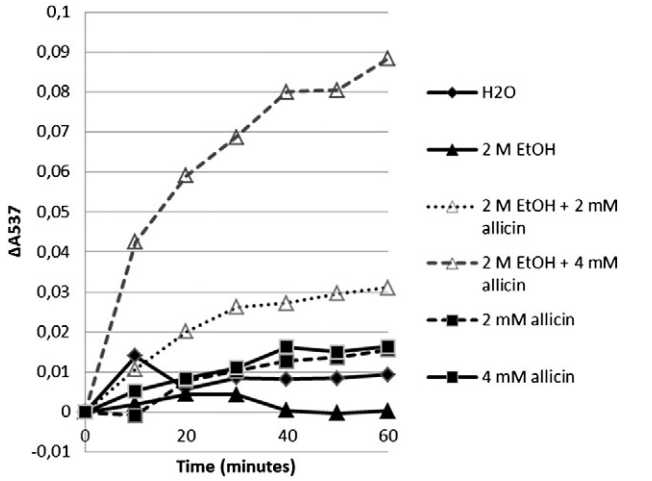

Fig. 5. Synergy between allicin and EtOH in permeabilizing beetroot cell membranes. Beetroot discs were bathed for 15 min in the test solutions, washed extensively in tap water, then incubated for 1 h in 10 ml sodium phosphate buffer (pH 6). The change in absorbance (ΔA_{537}) was measured spectrophotometrically. A representative data set from one experiment is shown. The experiment was repeated twice with similar trends.

3.5. Effects of allicin on the electrophysiology of Chara internodal cells

The resting voltage of the *C. corallina* plasma membrane under the conditions of the experiment was approximately −240 mV. Exposure of the giant internodal *Chara corallina* cell to diluted garlic juice or synthetic allicin caused an immediate depolarization and decrease in membrane resistance (Fig. 8). Garlic juice diluted to $1{:}10^4$ with APW (~3 μM allicin) caused a small but clear membrane depolarization of about 10 mV and a decrease in membrane resistance (R_m) of about 40% (Fig. 8A). At ~10 μM allicin a depolarization of ~75 mV and a drop in R_m of about 60% from an initial 130 kΩ down to ~50 kΩ was observed. This effect was similar over the dilution range of garlic juice containing ~30, 100 and 300 μM allicin (Fig. 8). Washing with APW to remove allicin allowed a rapid recovery of membrane potential and resistance. Confirmatory experiments with synthetic allicin added to APW (100 μM) gave rise to a similar membrane voltage response (recordings not shown).

3.6. Effects of allicin on the electrophysiology of Chara cytoplasmic vesicles bounded by the tonoplast

Patch clamp experiments were carried out on *C. corallina* cytoplasmic droplets as a tonoplast model system to observe an immediate

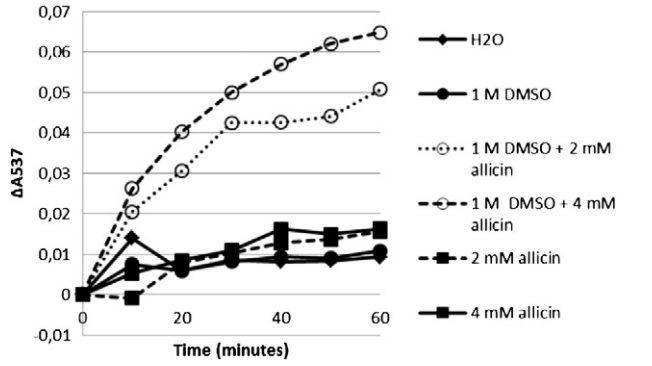

Fig. 6. Synergy between allicin and DMSO in permeabilizing beetroot cell membranes. Beetroot discs were bathed for 15 min in the test solutions, washed extensively in tap water, then incubated for 1 h in 10 ml sodium phosphate buffer (pH 6). The change in absorbance (ΔA_{537}) was measured spectrophotometrically. A representative data set from one experiment is shown. The experiment was repeated twice with similar trends.

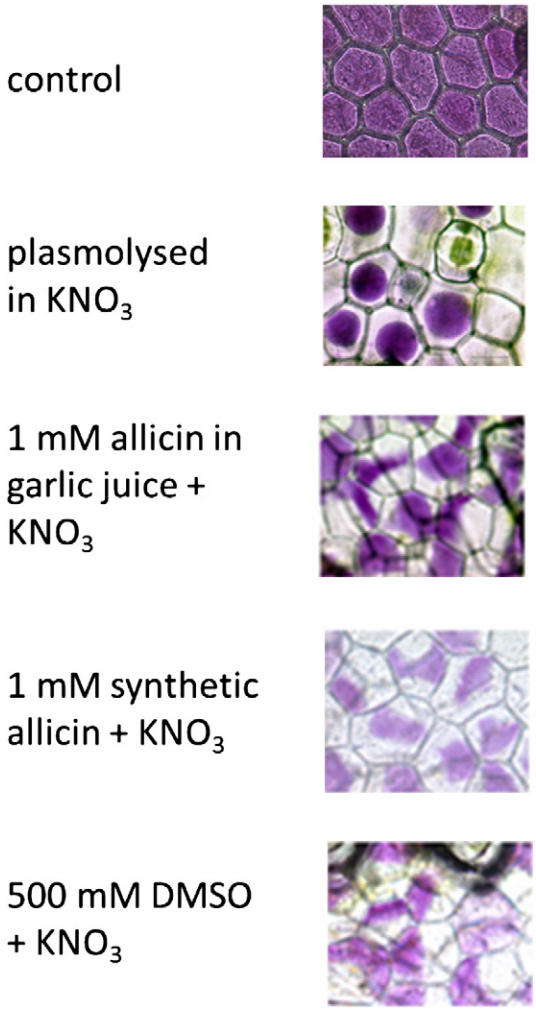

Fig. 7. The effect of treatment with allicin in garlic juice (1 mM) or synthesized allicin (1 mM) or DMSO (500 mM, positive control) on the ability of protoplasts of *Rhoeo discolor* to plasmolyse in KNO_3 solution.

effect of diluted garlic extract and allicin, respectively, on biological membranes. To test the functionality of the maxi-K channel under the experimental conditions, asymmetric solutions of KCl and NaCl at equimolar concentration (120 mM) were used on either side of the membrane. The advantage of these conditions is that voltage-dependent current responses of the well-known maxi-K channel confirm an intact membrane, while signals arising from added membrane-active substances, in this case allicin, can be classified as authentic membrane responses. A linear relationship for the amplitude of the current flow due to K^+ (I_K) was observed with increasing applied voltage (Fig. 9A and B). Measured in an outside-out patch configuration, the native maxi-K channel in the tonoplast of *C. corallina* was not affected by allicin, but was blocked by Na^+ ion beyond the reversal voltage of about +60 mV. The reversal potential of +60 mV reflects the relative affinity of the maxi-K channel for K^+ and Na^+ ions, respectively. Thus, it was shown that there was no effect of allicin on the native maxi-K channel which performed as expected at the applied voltages.

Fig. 10 depicts current fluctuations elicited by allicin in the pipette solution at a positive clamp voltage of +140 mV. Since, at positive pipette potential beyond the reversal voltage, Na^+ ions are driven through the membrane patch, the current pathway clearly arose from a direct allicin effect on the membrane.

In Figs. 11 and 12 it is shown that both diluted garlic extract and pure allicin induce current fluctuations through the *Chara* cytoplasmic droplet membranes. In these experiments, symmetrical solutions were used

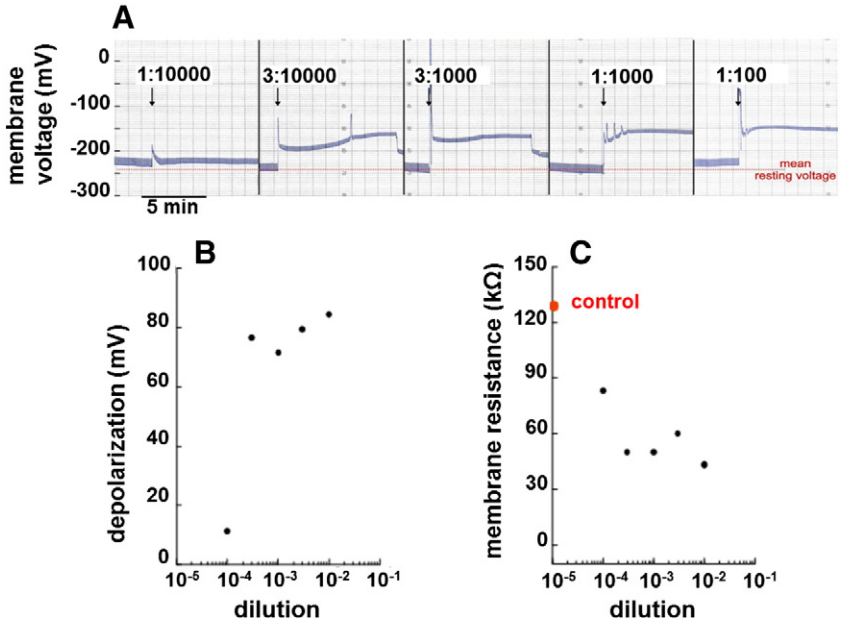

Fig. 8. Membrane voltage (V_m) and resistance (R_m) of a *Chara corallina* internodal cell strongly responded to garlic juice diluted in artificial pond water (APW, measured by the K^+-anesthesia method). A. Changes in membrane voltage upon extracellular application of diluted garlic sap (dilution factor and start of application are given on top of traces, 1:10,000 = ~3 μM, 3:10,000 = ~10 μm; 1:1000 = 30 μM, 3:1000 = ~100 μM and 1:100 = ~300 μM allicin, respectively). Small voltage deflections in depolarizing direction are responses to injected constant current pulses. Note the recovery of membrane voltage and resistance after washing the garlic sap-treated cell segment with APW. Segmented graphics show consecutive applications on the same cell. B. While garlic juice at a 10^{-4} dilution (~3 μM allicin) has only a small effect on V_m, over the range from ~10 to 300 μM allicin shifted V_m into a saturated state of approximately −150 mV (a depolarization of ~75 mV). C. The 10^{-4} diluted garlic juice decreased R_m significantly to about 65% of its initial value, while the higher concentrations of allicin tested lowered R_m to a final level of about 50 kΩ (~40% of the initial value), which represents a specific membrane resistance of 1.2 Ω m^2 (compared to 4 Ω m^2 exhibited in the absence of garlic sap).

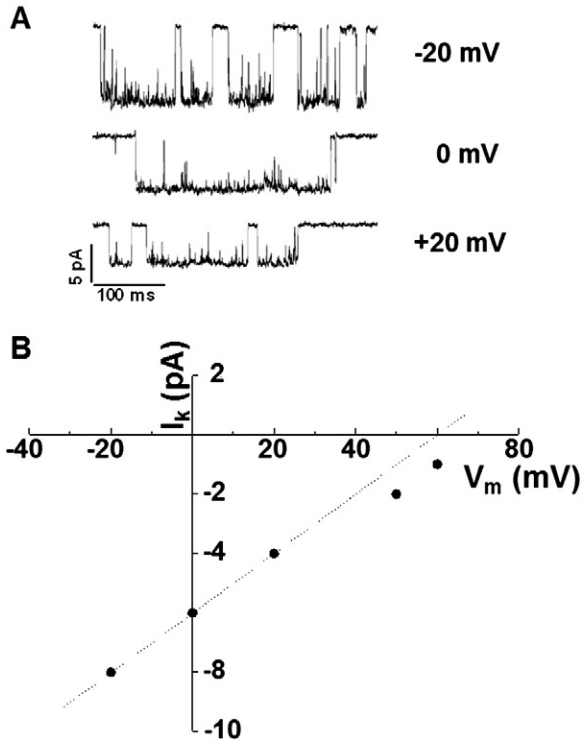

Fig. 9. Effects of allicin on the native tonoplast K^+ channel. Measured in an outside-out patch configuration, the native maxi-K channel in the tonoplast of *C. corallina* was not affected by allicin, but was blocked by Na^+ ion beyond the reversal voltage of about +60 mV. Bathing solution contained 120 mM KCl, the pipette solution 120 mM NaCl, 300 μM allicin. A. Traces recorded at −20, 0, and +20 mV clamp voltage revealed a conductance of the open maxi-K channel of about 133 pS, current through the open maxi-K channel appears as downward fluctuations. B. At voltages more positive than +70 mV, beyond the reversal voltage of the bi-ionic system, an inward Na^+ current through the K^+ specific channel did not occur.

in bath and pipette, containing either 120 mM LiCl (Fig. 11) or 84.5 mM $CaCl_2$ (isotonic to 120 mM LiCl, Fig. 12). All these employed ions do not permeate the highly specific native maxi-K channel but block it. Thus, it can be concluded that the recorded current fluctuations were allicin-induced.

3.7. Effects of allicin on artificial lipid bilayers

In order to confirm that the allicin-induced currents observed in the patch clamp experiments with *Chara* cytoplasmic vesicles (Figs. 8–12) were due to the effects of allicin on the lipid component of the membrane, experiments were carried out on artificial membranes made of L-α-lecithin. On pressure injection of a lecithin solution (50 mg ml^{-1} hexane) pure lecithin vesicles formed in salt solutions within 1 h. Patch clamp technique could be applied on excised patches. Pipette and bathing solutions were identical to those used with cytoplasmic vesicles and contained either diluted garlic extract (Fig. 13) or allicin (Figs. 14–15). Clearly, channel-like current fluctuations appeared on

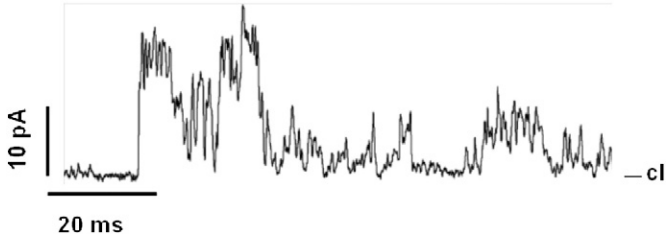

Fig. 10. Na^+ current fluctuations through allicin-induced pores in the *Chara* tonoplast. Na^+ current fluctuations through allicin-induced pores could be observed at clamp voltages greater than the reversal voltage for K^+ under bi-ionic conditions, here, +140 mV (cf. Fig. 9). Data were obtained recording outside-out patches drawn from the membrane delineating cytoplasmic droplets. Variable pore size and quantity probably contribute to the irregular current flow observed. Bathing solution contained 120 mM KCl, pipette solution 120 mM NaCl, and 300 μM allicin. The non-conducting level is indicated at the right-hand side (cl).

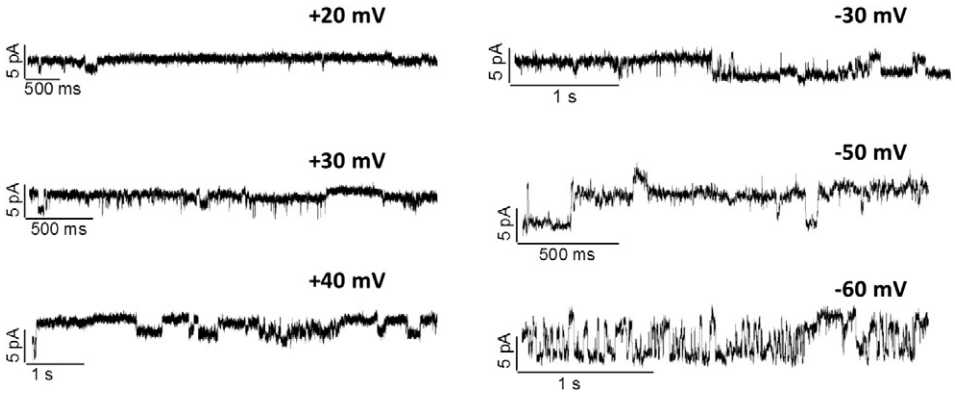

Fig. 11. Recording on a *Chara* cytoplasmic droplet in the inside-out excised patch conformation with diluted garlic juice. Symmetrical solutions were used in bath and pipette containing 120 mM LiCl and 5 mM Tris/HCl, pH 7.5. The pipette contained diluted garlic juice 1:100 (~300 μM allicin).

voltage applications of either polarity. A further confirmation of these results was obtained on planar lipid bilayers also made of L-α-lecithin (50 mg ml^{-1} decane). Fig. 16 depicts those recordings.

4. Discussion

Our results show clearly that both synthetic allicin, and allicin in garlic juice, even at high dilutions, can increase the permeability of biological and artificial membranes. While chloroform (trichloromethane) is known to disrupt biomembranes, dimethyl sulfoxide (DMSO), a common solvent in chemistry and pharmacy, permeabilizes membranes by pore formation [18]. This mechanism is well understood for DMSO at the molecular level since atomic simulation modeling recapitulates the processes involved [17,19]. Because of a superficial structural similarity between DMSO and allicin (Fig. 1) we were motivated to test whether allicin might also be able to permeabilize cell membranes. We observed not only that both allicin in garlic juice and synthetic allicin were able to do this (Fig. 3), but to our surprise, we found that allicin was approximately 200-fold more active on a mol-for-mol basis than DMSO at permeabilizing beetroot membranes in a standard pigment-leakage assay procedure (Fig. 4). Ethanol is also active in the beetroot assay and in this case allicin was observed to be approximately 400-fold more active on a mol-for-mol basis (Fig. 4). This observation is of potential relevance to allicin's antimicrobial activity. Thus, some antibiotics like Gramicidin S kill bacteria by depolarization of the bacterial membrane system [38]. In eukaryotes, depolarization of the mitochondrial membrane is correlated with induction of apoptosis via cytochrome c release [39]. In this context it has been shown that allicin is able to induce apoptosis in a number of different organisms [8,9,40].

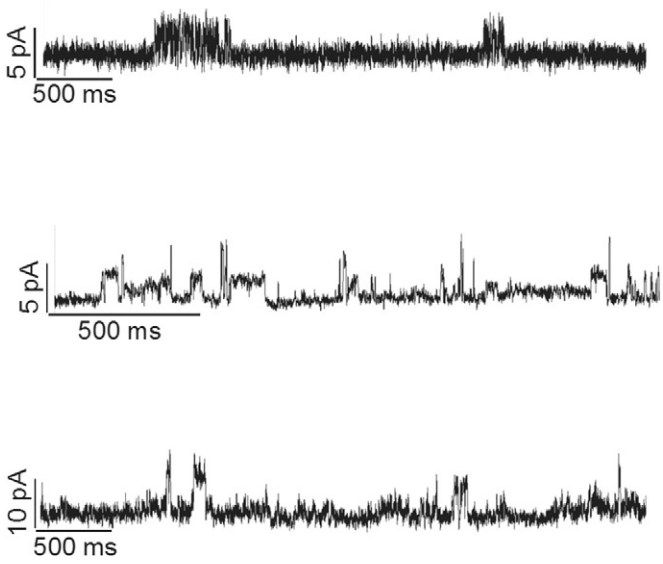

Fig. 12. Recording on a *Chara* cytoplasmic droplet in the inside-out excised patch configuration with synthetic allicin. Symmetrical solutions were used in bath and pipette, containing 84.5 mM $CaCl_2$ (isotonic to 120 mM LiCl, see Fig. 11) and 5 mM Tris/HCl, pH 7.5. The pipette contained 300 μM allicin. Clamped voltage +100 mV.

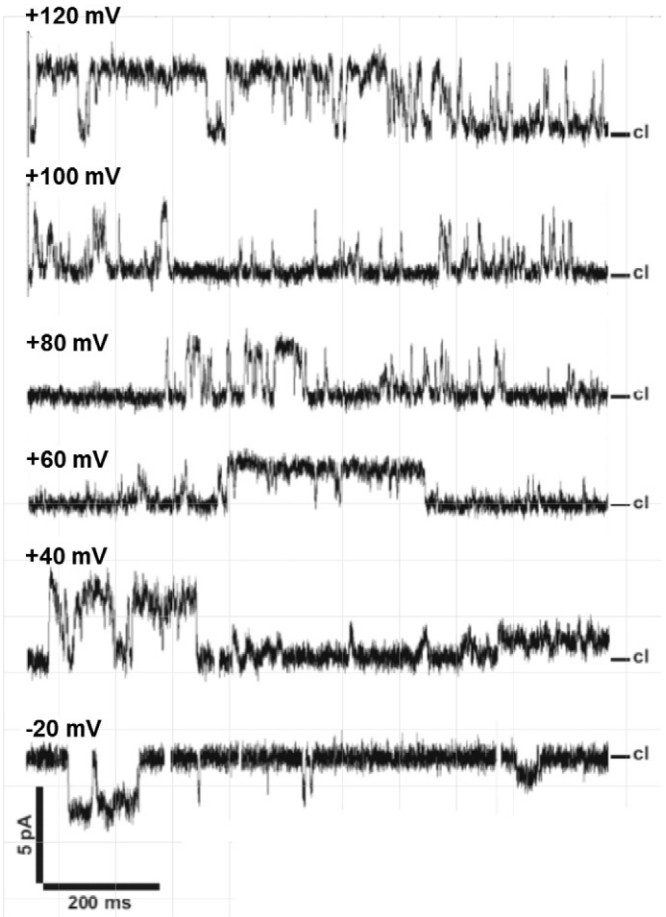

Fig. 13. Current fluctuations through pores in artificial lipid membrane vesicles induced by garlic juice. Vesicles made of L-α-lecithin were bathed in a solution containing 120 mM KCl and 5 mM Tris/HCl, pH adjusted to 7.5, and garlic sap at a 1:300 dilution (~100 μM allicin). With glass-micropipettes (filled with identical solution), patches were voltage-clamped in a vesicle-attached configuration by step commands to constant values, from −20 mV to +120 mV. The closed state of a pore is indicated by a bar right-hand side of either trace (cl).

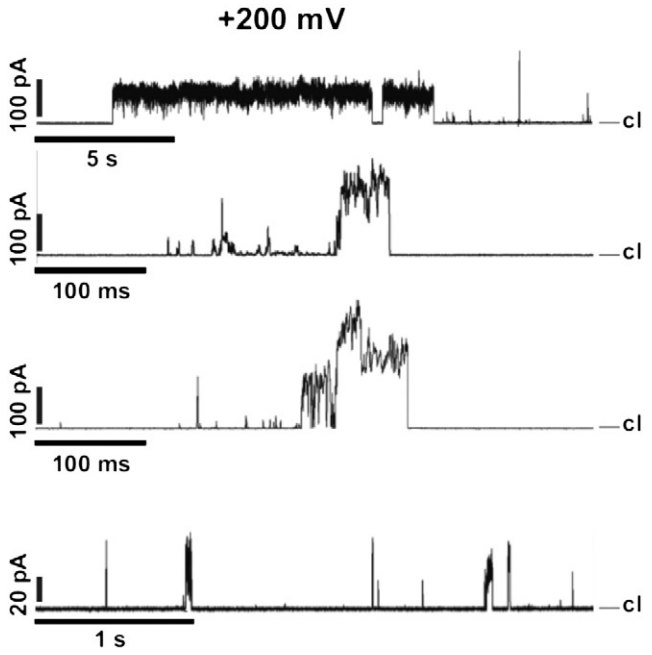

Fig. 14. Current fluctuations through pores in artificial lipid membrane induced by allicin. Vesicles made of L-α-lecithin (50 mg ml^{-1} hexane) were bathed in a solution containing 120 mM KCl and 5 mM Tris/HCl, pH adjusted to 7.5, additionally 100 μM allicin. With glass-micropipettes (filled with identical solution), excised patches were drawn from the membrane delineating vesicles and voltage clamped to +200 mV. Traces display differently conducting states of allicin-induced pores.

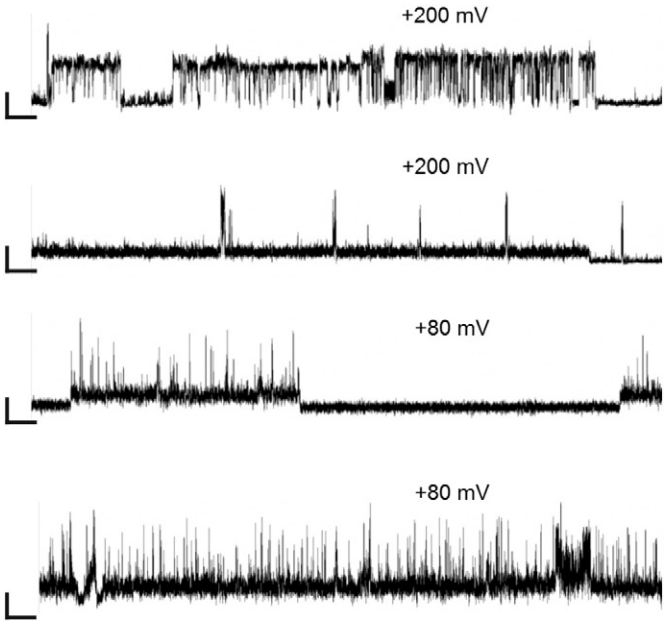

Fig. 16. Current fluctuations through pores in artificial planar lipid membrane induced by allicin. Planar membranes made of L-α-lecithin (50 mg ml^{-1} decane) were bathed in a solution containing symmetrically 120 mM KCl and 5 mM Tris/HCl, pH adjusted to 7.5, additionally 100 μM allicin (*cis*-face). Voltage was clamped to +200 and +80 mV, respectively. Calibrations: bars at the left-hand side define 50 pA and 500 ms, respectively, for upper two traces at +200 mV, 5 pA and 300 ms for the lower traces at +80 mV.

In yeast the induction of apoptosis also depended upon allicin's chemical reactivity in depleting and oxidizing the reduced glutathione pool [8] but in light of our present results, membrane depolarization effects of allicin in apoptosis induction must also be considered.

We tested whether allicin might act synergistically with ethanol and DMSO in permeabilizing beet cell membranes and found this was indeed the case (Figs. 5 and 6). In light of this observation it is interesting to note that the antifungal activity of the antibiotics amphotericin-B and polymixin-B was shown to be enhanced by allicin [41,42]. Amphotericin B is a polyene antibiotic known to interact with ergosterol in fungal membranes and to induce pore formation [43] whereas polymixin B, usually employed against bacteria, is a cyclic peptide with a hydrophobic tail which binds to lipopolysaccharide (LPS) and disrupts bacterial membranes [44]. This is also interesting in the context of our observation of synergy between allicin and two independent membrane-active substances, DMSO and ethanol, and might be indicative of the mechanism of synergy for allicin with these antibiotics. While this synergistic effect may be relevant *in vitro*, it seems unlikely that allicin, which reacts very readily with glutathione and cysteine residues in proteins, could be used *in vivo* with antibiotics because its effective concentration would be rapidly reduced by the high glutathione content of blood and tissues.

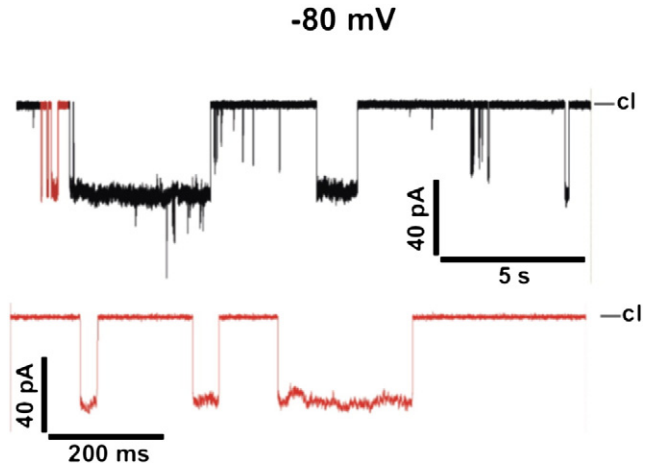

Fig. 15. Current fluctuations through pores in artificial lipid membrane vesicles induced by allicin. Vesicles made of L-α-lecithin (50 mg ml^{-1} hexane) were bathed in a solution containing 120 mM KCl and 5 mM Tris/HCl, pH adjusted to 7.5, additionally 100 μM allicin. With glass-micropipettes (filled with identical solution), excised patches were drawn from the membrane delineating vesicles and voltage clamped to −80 mV. Closed state is indicated right-hand side of traces (cl). Traces display stable conducting states of allicin-induced pores of long open dwell-times. The red colored part of the upper trace is expanded in the lower trace to show the allicin-induced current fluctuations more clearly.

Allicin was also shown to be membrane-active in another plant species, namely *Rhoeo discolor*, where it caused treated cells to lose their ability to plasmolyse properly (Fig. 7). This confirms that the membrane activity of allicin might be a general phenomenon and not limited to beetroot cells.

To investigate allicin-induced effects on the plasma membrane of the integral internodal cell of *Chara corallina*, the so-called K^+-anesthesia technique was used to measure membrane potential and resistance. The technique replaces the conventional procedure of intracellular glass-microelectrode insertion [33]. The resting membrane voltage (V_m) of *Chara* cells under the conditions employed was measured at approximately −240 mV (Fig. 8A). The dramatic effect of allicin on *Chara* cells was illustrated by the observation that garlic juice pressed directly from a single clove with a kitchen garlic press and diluted 1:10,000 with artificial pond water (APW) caused a measurable immediate depolarization of ~10 mV and decreased the membrane resistance (R_m) to approximately 70% of its initial value. Dilutions of garlic juice in the range between 1:3000 and 1:100 resulted in a depolarization of ~80 mV and decreased the membrane resistance from ~130 kΩ to ~50 kΩ (Fig. 8B and C). Similar results were obtained with pure synthetic allicin, confirming that allicin is the membrane-active substance in garlic juice. Washing the test cell rapidly restored the membrane to its original state (Fig. 8A) and this suggests that allicin is only effective at the membrane surface and not submerged in the hydrophobic core of

the membrane from where an elution with aqueous solutions would hardly be possible.

However, the very rapid depolarization of V_m and reduction of R_m could be explained either by an effect of allicin on integral membrane proteins, e.g., by forcing them into an open conducting state, or, it is possible that allicin caused rapid pore formation in the lipid phase of the *Chara* membrane. Patch clamp techniques provide a suitable experimental approach to address these questions. Appropriate experiments were performed on tonoplast-delineated cytoplasmic vesicles prepared from *Chara* internodal cells. In this way, possible direct effects of allicin on the well-known maxi-K channel in this membrane were explored. In addition, the potential ability of allicin to induce new pores into the tonoplast was investigated after complete blockage of the known ion channels by other cations. As can be seen in Figs. 9–12 the functioning of the native maxi-K channel was not affected by allicin or garlic extract. Thus, in the presence of allicin the maxi-K channel could be shown to keep its known characteristics and be blocked by other cations. Hence, it can be concluded that, temporarily, allicin produced pore-like modifications of the membrane structure. Interestingly, there is a recent report of a specific inhibitory effect of allicin on the human epithelial sodium channel (ENaC) which was dependent upon allicin's thiol-reactivity [45]. Thus, further effects of allicin on *Chara* ion channels other than the maxi-K channel cannot be ruled out without further experimentation. Nevertheless, effects on anion channels, for example Cl^-, can be ruled out on theoretical grounds, because any increase in ion traffic would cause membrane voltage to rise to positive values because of the ion gradients involved [46].

Experiments on artificial lipid membranes confirm that allicin is not exerting its effects via integral membrane ion channels but on the lipid bilayer itself. Current fluctuations were observed on artificial lipid vesicles (Figs. 14 and 15) and planar membranes (Fig. 16) which fluctuated in electrical conductance amplitude and time dependency over short intervals after allicin treatment. This indicates that the conducting pores, once established, were not steady-state structures during a single event, but were subject to spontaneous, dynamic changes.

Since the small size and chemical structure of the allicin molecule do not allow the formation of a trans-membrane pathway by a single molecule (like, e.g., the 20-amino-acid peptide alamethicin), the induction of electrically visible pores is more likely due to a deformation of the membrane resulting in a toroidal opening of the plane, or a step by step multimerization of inserted stress molecules eventually forming arcs into the membrane [47–51] as has been modeled for DMSO [17].

Previous reports have shown that allicin enhances the formation of lipid-peroxides in the model system *Saccharomyces cerevisiae* but the release of K^+ ions and UV-light absorbing substances from yeast cells was reported not to be altered [42,46]. Lipid peroxidation often is associated with an increase of the membrane permeability, but formation of lipid peroxides does not necessarily cause enhanced membrane-permeability [52]. However, in contrast to the results reported here, Miron and co-workers have reported that allicin did not increase the permeability of an artificial lipid-bilayer-system [16]. It has, however, been reported that related sulfur-containing garlic substances diallyldisulfide (DADS) and diallyltrisulfide (DATS) modified membrane fluidity [53]

We believe that our data, showing a very high membrane activity of allicin, a natural product in a common foodstuff which has potential medicinal value, contribute an important new facet to the consideration of allicin's physiological activities independent of its proven reactivity as a thiol reagent.

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