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Water and Nonelectrolyte Permeability of Plant Cell Membranes after Short Term Application of Amino Acids and Phosphorylated Amino Acids

Yong-Fang Li[1], Yu-Fen Zhao[1], In-Sup So[3], and Ok Young Lee-Stadelmann[2†]*

[1]Department of Chemistry, Tsing hua University, Beijing 100084, China
[2]Department of Biology, Cheju National University, Cheju City, Korea
[3]Department of Horticulture, Cheju National University, Cheju City, Korea

The effects of amino acids (aa) and N-(diisopropyloxyphosphoryl)-amino acids (DIPP-aa) on cell membranes were investigated by evaluating water and methyl urea permeability. Permeability coefficients P_f and P_s were determined by standard osmotic methods for cells of *Pisum sativum* stem base epidermis after 20 min exposure to a 5 mM solution of each aa and DIPP-aa. The P_f value of *P. sativum* epidermal cells (untreated controls) was $1.3 \pm 0.4 \times 10^{-3}$ μm s^{-1}. Treat ments with the diisopropyl-oxyphosphoryl derivatives of three one charged and three polar amino acids (serine, threonine, asparagine, and aspartic acid) and unsubstituted (free) serine and threonine increased water permeability up to about two fold of the control value. Serine and threonine and their DIPP-derivatives increased methyl urea permeability (controls $1.03 \pm 0.09 \times 10^{-3}$ μm s^{-1}) 30 to 80 percent. Other amino acids and their DIPP-derivatives caused small or insignificant changes of water permeability. Only certain polar amino acids and their DIPP-derivatives increased the osmotic water and methyl urea permeation through the plasma membrane. The specificity of these molecules on plasma membranes suggests that the active amino acids (serine and threonine) and their DIPP-derivatives interact with charged membrane molecules. The relatively small changes in water and methyl urea permeability may indicate that the effective aa's and their DIPP-derivatives interact with phospholipids rather than aquaporin. A concurring alteration of water channel proteins, however, cannot excluded.

Keywords: Permeability coefficient, (Plasmamembrane), serine, solute permeability, threonine, water transport

Phosphorylation of proteins which occurs in biological membranes plays an important role in many biological functions such as enzyme reactions and signal transduction. Recently Li et al. (1993) selected diisopropyl oxyphosphoryl amino acids (DIPP-aa) as model molecules for phosphopeptides and observed significantly increased fluidity in the human erythrocyte membrane; the polar part of the membrane phospholipids became less ordered, and the spacing of the fatty acid chains became wider while the ends of the chains became more ordered.

The aim of this study was to further analyze the DIPP-aa effect on cell membranes by testing 20 DIPP-aa's and free aa's for their effectiveness in altering membrane fluidity by measuring membrane permeability.

Membrane fluidity is important for biological functions (Aloia and Boggs, 1985) especially under suboptimal temperatures. Lipid composition, particularly the content of unsaturated fatty acid and phospholipid packing density, influences membrane fluidity which controls membrane transport properties and protein lipid interaction (In't Veld et al., 1991). Water and solute permeability have been often proposed as indicators for membrane fluidity (Pietras and Wright, 1974; Van Zoelen et al., 1978; Verkman and Ives, 1986; Worman et al., 1986).

Human and animal erythrocytes and ghosts were most frequently used model systems for membrane water transport studies. Erythrocyte membranes are different from many other cell membrane types, however, and osmotic water transport operates mainly through water channel proteins (aquaporins), where membrane lipids contribute only little to water permeation (Verkman, 1992; Benga et al., 1993). Osmotic water permeability coefficients for membranes with water channels are high (e.g. for erythrocytes 200 μm s^{-1}, Finkelstein, 1987; for *Nitella* 102 μm s^{-1}, Wayne and Tazawa, 1990).

Water channel proteins were recently found in the cell membrane of a higher plant (*Arabidopsis thaliana*; Kammerloher and Schäffner, 1993) and may occur also in other higher plant cell species. The contribution to water permeation through this second water pathway parallel to the membrane lipids, however,

*Corresponding author; fax 1-612-624-4941
email stade002@tc.umn.edu
†Present address: Department of Horticultural Science, University of Minnesota, St. Paul, MN 55108, USA

may be less important in higher plant cell membranes than in erythrocytes or Characean cells; water permeability coefficients measured so far for higher plant cell membranes are in the range of 1 to 15 $\mu m\ s^{-1}$ (Stadelmann, 1969), and thus are at the lower side of the values for water permeation through artificial phospholipid membranes without water channels (about 10-40 $\mu m\ s^{-1}$; Fettiplace and Hydon, 1980). This fact and the high activation energy for water permeation through cell membranes (e.g. Chen et al., 1991) suggests that in higher plant cells water permeates through unaltered cell membranes mostly by solution and diffusion through the phospholipid bilayer. Protein channels, if present, seem to be under normal conditions, inactive.

Aquaporins may be important, however, for the often observed considerable increase of water permeability after a variety of treatments or environmental conditions; such factors may activate water channels so that their contribution to the water permeation through the cell membranes becomes substantial.

The model membrane systems selected here were fully differentiated plant cells surrounded by a cell membrane (plasmalemma), containing a large central vacuole limited by the tonoplast, the permeability of which is considerably higher than for the plasmalemma.

The permeability for water and methyl urea was determined after treatment with DIPP-aa and compared with the permeability of the same material pretreated with the free amino acid and of untreated controls. To avoid interference of possible urea channels in the cell membrane (Hasegawa et al., 1992), methyl urea was selected as permeator for which no channels are known. The higher lipophilicity of methyl urea also shortens the time needed for each experiment.

MATERIAL AND METHODS

Plant Material

Internodal epidermis of *Pisum sativum* var. 'Alaska' seedlings were used. Pea seeds (Jordan Seed Inc. Woodbury, Minnesota, USA) were washed with tap water and distilled water and soaked in distilled water in the dark for 6 h. Next, 20 seeds were placed on a round filter paper wetted with 10 mL distilled water in a 9 cm diameter petridish. Seeds were germinated for 24 h in the dark. The germinating seeds were planted in pots (top diameter 11 cm, height 8 cm; 10 seeds per pot) and transferred into a growth chamber with a day/night period 16/8 h at 17°C with 82 $\mu mol\ m^{-2}\ s^{-1}$ irradiation (400-700 nm). Pots were watered daily. All experiments were performed at 20 ± 1°C.

Chemicals

Amino acids (Gly, Ala, Val, Leu, Ile, Met, Phe, Trp, Pro, Ser, Thr, Cys, Tyr, Asn, Asp, Glu, Lys, Arg, His, all chromatography grade) were obtained from Sino American Biotechnology Co. (Beijing, China), D-mannitol (HPLC grade) from Fluka, (Buchs, Switzerland), D-sorbitol (extra pure) from Junsei Chemical Co. (Japan), $CaCl_2 \cdot 2H_2O$ (99%) from Sigma (St. Louis. MO, USA) and KCl (first grade, 99%) from Shinyo (Pure Chemicals Co, Japan). Methyl urea (purity > 97%, Fluka) was further purified by two recrystallization steps in ethanol. Amino acids were phosphorylated to N-(o,o-diisopropyl) phosphoryl amino acids: $([(CH_3)_2 \cdot CHO]_2 \cdot P(O) \cdot R$; R = amino acid residue) as described earlier (Ma and Zhao, 1989; Li et al., 1993). These compounds hydrolyzed only negligibly during the duration of the experiment.

Solutions

Solutions were prepared in a balanced salt medium obtained either by dissolving 3.7 mM $CaCl_2$ and 25 mM KCl in 1 L double distilled water, or by diluting stock solutions of KCl and $CaCl_2$ solution (9 parts 1 molar KCl solution and 1 part 1 molar $CaCl_2$ solution) as described elsewhere (Lee-Stadelmann and Stadelmann, 1989). The final plasmolyzing solution (0.55 osm mannitol) was prepared by dissolving 0.6 mol mannitol in 1 L of the balanced salt medium. The osmolarities were checked with a vapor pressure osmometer (Wescor, Model No 1000). As concentrations for intermediary plasmolysis steps 0.20 and 0.40 osmolal mixtures of mannitol and sorbitol were prepared. The solutions for deplasmolysis were 0.20 or 0.35 osm mannitol.

Tissue Preparation

Ten mm long segments of the lowest internodes above ground (morphologically first or second) of 9 day old seedlings were used. Next the internode segments were vacuum infiltrated in the balanced salt medium for 1 to 1.5 min to remove air from intercellular spaces and facilitate separation of the tissue.

After infiltration, shallow transversal incisions were made on each side of the internode, the tissue care-

fully stripped off with a fine forceps and placed in fresh balanced salt medium. The strips generally contain epidermis, subepidermis and parenchyma cell layers. Three tissue strips were used for each experiment.

Pretreatment

To minimize the osmotic shock, the tissues were pretreated (plasmolyzed) in two intermediary concentrations (0.20 and 0.40 osm) of mannitol or sorbitol for 10 min in each.

Treatment Time and Concentration

Treatment time and concentration were tested for their dffect on water permeability changes with DIPP-asparagine on pea cells under otherwise equal experimental conditions. Treatment of 5 min showed first decreases in deplasmolysis time. Ten, 20, 30, and 40 min of treatment showed no significant difference. Likewise 0.1, 1.0, and 5.0 mM DIPP-aa had about the same effect on deplasmolysis time. For assuring sufficient effectivity 20 min and 5.0 mM DIPP-aa were chosen as treatment.

Application of DIPP-aa and aa

From the 0.4 osm pretreatment solution one section each was transferred to the final plasmolyzing solution (0.55 osm mannitol) containing either 5 mM DIPP-aa or their corresponding unsubstituted aa. The 3rd section (control) was transferred into the final plasmolyzing solution without treatment with either DIPP-aa or free aa. The solutions were adjusted to pH 5.5 ± 0.5 with drops of 1 M KOH solution. To obtain the same K^+ concentrations in each solution drops of 1 M KCl were added when needed. After 20 min the sections were rinsed and transferred into a dish with fresh plasmolyzing solution.

Cell Solute Potential

Cell solute potential was determined in preliminary experiments to select optimal concentrations for plasmolysis and deplasmolysis. Solute potential of at least 10 individual cells with nearly cylindrical shape was determined by the plasmometric method for each test (Lee-Stadelmann and Stadelmann, 1989).

Permeability Determinations

Two experimental protocols were followed. In the first protocol (deplasmolysis time method) twenty DIPP-aa and unsubstituted amino acids were screened semi-quantitatively for their effect on water permeability of the *Pisum* epidermis cells. The plasmolyzed tissue was perfused with a deplasmolyzing solution (0.20 or 0.35 osm) and the time was measured from contact of the solution with the tissue until deplasmolysis (the protoplast ends touch the transversal cell wall) occurred in about 90% of the cells (deplasmolysis time). The deplasmolysis time (between 10 and 20 min) was accurately determined from a series of photomicrographs evaluating 30 to 40 cells per frame. It is a relative indicator for water permeability in experiments using the same cell material and osmotic driving force.

Second protocol (determination of P_f, and P_s). For aa and DIPP-aa treatments which showed a noticeable effect on water permeability, the permeability coefficients for water and methyl urea (P_f, and P_s, respectively) were calculated from deplasmolysis time, cell length and width and protoplast length as described earlier (Stadelmann, 1969; Lee-Stadelmann and Stadelmann, 1989; Stadelmann and Lee-Stadelmann, 1989).

In both protocols, 3 test runs [non treated (control), unsubstituted aa, and DIPP-aa] were performed. For water permeability the test runs were arranged sequentially with 10 min intervals. In each test run one tissue strip was transferred from the final plasmolyzing solution into a droplet of the same solution in a perfusion chamber mounted on the microscope stage. The chamber was closed with a cover glass tightly sealed on the edges with vaseline. Since the dilatation of the protoplast in methyl urea permeability experiments was relatively slow the three sections were placed together next to each other into the same perfusion chamber and observed simultaneously. All observations were made under 400 x total magnification.

Absence of Cell Damage

Absence of cell damage from DIPP-aa and aa treatment was tested by measuring the volume of the plasmolyzed protoplast: damaged cell membranes are leaky for vacuolar solutes, thus increasing the degree of protoplast contraction with time.

Statistics

For each DIPP-aa treatment at least three experiments were done. Standard deviation in deplasmoly-

sis time was about 15% of the mean. The standard deviation for the permeability constant was either directly derived from its values or from the average standard deviation of the input values.

RESULTS

Water Permeability

DIPP-serine, DIPP-threonine, and DIPP-Asparagine treatment increased water permeability by shortening deplasmolysis time to about half the control value. Free threonine and serine caused a similar increase in water permeability. Among the electrically charged amino acids, only DIPP-histidine and DIPP-aspartic acid treatment decreased deplasmolysis time. The free amino acids had no clear effect. None of the non-polar amino acids and their phosphorylated derivatives caused a significant increase in water permeability (Table 1).

For the five DIPP-aa treatments which significantly affected the deplasmolysis time the water permeability coefficient P_f was calculated (Table 2).

The P_f values (Table 2) generally correspond well to the deplasmolysis time differences. DIPP-serine treatment increased P_f about 1.5 fold while the free amino acid yielded an increase of only about 50%.

DIPP-asparagine increased P_f to almost twice the control value while treatment with the free amino acid showed no effect on P_f. For threonine, conversely, the free amino acid had a stronger effect than DIPP-threonine and increased water permeability to about 2.5 fold of the control value. DIPP-aspartic acid increased water permeability about 50 percent while the free amino acid had no significant effect.

Table 1. Relative water permeability of stem epidermal cells of *P. sativum* seedlings after 20 min treatment with unsubstituted (aa) and diisopropyl oxyphosphorylated amino acids (DIPP-aa).

	mean deplasmolysis time (min) of untreated control	deplasmolysis time ratios	
		DIPP-AA	AA
		Control	Control[2]
Nonpolar amino acids			
Glycine(3)[1]	232±174[3]	1.19±0.22[4]	1.23±0.26
Alanine(4)	227±77	1.16±0.41	1.11±0.25
Valine(2)	340, 180	0.94, 1.00	1.01, 138
Leucine(3)	417±210	0.74±0.39	0.789±0.11
Isoleucine(2)	303, 315	1.14, 1.42	0.84, 1.60
Methionine(2)	340, 330	0.87, 1.45	0.94, 0.77
Phenylalanine(2)	215, 230	1.07, 1.57	1.13, 1.20
Tryptophan(2)	235, 220	0.81, 0.89	0.85, 0.68
Proline(2)	360, 320	0.97, 0.96	1.13, 0.75
Polar amino acids			
Serine(4)	458±361	0.47±0.08	0.556±0.10
Threonine(2)	590, 270	0.63, 0.54	0.56, 0.81
Cysteine(4)	208±58	1.26±0.23	1.34±0.66
Tyrosine(2)	160, 250	0.79, 1.12	1.0, 1.24
Asparagine(3)	310±58	0.528±0.09	0.808±0.015
Electrically charged amino acids			
Aspartic acid(2)	388, 328	0.65, 0.80	1.60, 0.64
Glutamic acid(2)	318, 320	0.97, 0.75	1.79, 1.69
Lysine(2)	195, 165	1.57, 0.45	1.0, 0.79
Arginine(4)	397±160	0.839±0.057	0.846±0.045
Histidine(2)	358, 410	0.67, 0.47	0.90, 0.90

[1]number of experiments suitable for evaluation.
[2]Control: Tissue without treatment with neither amino acid nor DIPP-aa. The ratios of DIPP-aa/Control and aa/Control indicate the effect of DIPP-aa and free aa on water permeability: the ratio higher than 1.0 indicates iecrease and a ratio smaller than 1.0 indicates increase of water permeability.
[3]The high SD reflects the difference between individual experiments. The deplasmolysis time ratios for an individual experiment do not contain these fluctuations.
[4]Standard deviation.

Table 2. Water permeability coefficient P_f for *P. sativum* stem base epidermis treated with DIPP-aa and free amino acids (mean values of 20-30 cells).

aa	P_f (μm s^{-1})			DIPP-aa	free aa
	Control	Free aa	DIPP-aa	control	control
Serine	1.10	1.60	2.82	2.57	1.46
Asparagine	1.13	1.07	2.08	1.84	0.95
Threonine	1.21	3.00	2.15	1.77	2.48
Aspartic acid	1.10	1.14	1.69	1.54	1.04
Histidine	2.12	1.26	1.68	0.80	0.59

P_f : permeability coefficient for water; aa: amino acid, DIPP-aa: diisopropyl oxyphosphoryl amino acid. The ratios of DIPP-aa/control and aa/control indicate the effects of DIPP-aa and free-aa respectively. Value higher than 1.0: increasing effect of the treatment. Amino acids arranged in decreasing order of ratio DIPP-aa/control.

Glutamine had no effect either as free amino acid or as DIPP-derivative. The only discrepancy with the results of the deplasmolysis time method was found in the histidine dxperiments: deplasmolysis time significantly decreased after DIPP-histidine treatment, while the calculated P_f value was lower than in controls. The cause for this discrepancy may be sample variability, and has not been investigated further.

The water permeability coefficients for the untreated epidermal cells of the *P. sativum* stem basis ranged from 1.10 to 2.12 μm s^{-1}. Treatment with DIPP-amino acids raised these limits to 1.68 to 2.82 μm s^{-1} while with treatment with free amino acids this range is from 1.07 to 3.00 μm s^{-1} (Table 2).

Solute Permeability

Serine and threonine treatment, which were most effective for changing water permeability were tested for their effects on methyl urea permeation. The permeability coefficients P_s (control values) ranged from 6.01 to 11.30 10^{-4} μm s^{-1}, as expected, about 1,000 times lower than P_f. Both DIPP and free amino acids increased the permeability (between 23 to 83%; Table 3). The effect of DIPP-aa was similar to that of the free amino acid.

Table 3. Methyl urea permeability coefficient P_s. Mean values of 10 to 20 cells. Treatment with DIPP-serine, DIPP-threonine, and their free amino acids.

	$P_s \times 10^{-4}$ (μm s^{-1})			DIPP-aa	free aa
	Control	Free aa	DIPP-aa	control	control
Serine					
Exp.1	9.50	11.69	12.39	1.30	1.23
Exp.2	9.99	14.93	15.40	1.54	1.49
Threonine					
Exp. 1	11.30	13.88	16.41	1.45	1.23
Exp. 2	6.01	9.68	10.98	1.83	1.61

Control : untreated; aa: amino acid, DIPP-aa: diisopropyl oxyphosphoryl amino acid. The ratios of DIPP-aa/control and aa/control indicate the effect of DIPP-aa and free aa respectively.

Table 4. Osmotic solute content in the cell sap of *A. cepa* inner epidermal cells of the 3rd bulb scale after 20 min treatment with serine (5 mM) and DIPP-serine (5 mM).

Concentration of osmotically active solutes (osm) in the cell sap			DIPP-a	Free aa
Control	Free aa	DIPP-aa	control	control
0.460	0.468	0.473	1.02	1.01
0.446	0.433	0.460	1.03	0.97
0.402	0.376	0.422	1.05	0.94

Each value is the mean of 5 cells.

The cell damage test was performed with *Allium cepa* bulb scale epidermal cells which were also used for preliminary permeability experiments (data not shown). Free amino acid and DIPP-aa treatment did not cause any leakage of the solutes from the vacuole, indicating no damage by the treatment (Table 4). This also excludes effects of possible breakdown products of DIPP-aa during the relatively short experimental time.

DISCUSSION

The classic theory concerning the transport mechanism of water and small molecular nonelectrolytes (e.g. methyl urea) into plant cells postulates transport through the lipid bilayer of the membranes: the molecules permeate by their relative solubility in the lipid phase. The higher the solubility in the lipid phase (i.e., higher partition coefficient in olive oil/water), the higher is the expected permeation rate. Collander as early as 1933 already observed that water permeation into the vacuole was higher than expected from its solubility (Collander and Bärlund, 1933), but an explanation for this discrepancy was not available. Recently, an increasing number of reports suggest that water transport takes place in addition to the phospholipid way largely via water channel proteins (aquaporins) embedded in the phospholipid bilayer of the membrane. Most data are based on studies from developing frog oocytes and animal red blood cells. In plant cells where transport occurs mainly through aquaporins, experimental evidence for the existence of aquaporins was first proven for Charac-

ean cells and for the vacuolar membranes of growing tissues (Maurel, 1997). Little is known about factors regulating these proteins and the mechanisms of water transport through these channels.

Treatments of pea stem base cells with 19 amino acids and their DIPP derivatives reveal a remarkable diversity in their effects on water permeability, reflecting a specificity of the reaction of the membrane to the individual aa species. The absence of any significant effect of apolar aa and their DIPP derivatives suggests polarity or electrical charge as a requirement for the activity on cell membranes. The polar aa's (serine and threonine) caused the most significant increase in water permeability with or without the attached DIPP-moiety. Cysteine and tyrosine had no effect, while for asparagine, only the DIPP derivatives showed a clear effect.

Free amino acids and their DIPP derivatives most likely do not penetrate through the lipid layer of the cell membrane because they are charged molecules at the experimental pH. Also the treatment time is too short to expect appreciable active transport. This implies that their reaction must occur on the outer surface where breaking of H-bonds may be required to penetrate through the ordered water layers at the membrane surface (Drost-Hansen, 1985). Diisopropyl oxyphosphorylation of the amino acids leads to no uniform results; it considerably increased water permeability compared to the free aa for serine and asparagine, slightly increased for aspartic acid and histidine, but reduced water permeability for threonine and had no clear effect with all other aa tested.

The specificity of amino acids and DIPP-aa appears to suggest that membrane proteins, possibly aquaporins, may be involved in the observed changes of water permeability whereby the effective aa and DIPP-aa may function as regulators for the activity of the aquaporins. It has been reported that water permeation through aquaporins can be one order of magnitude higher than through the lipid bilayer, at least with animal cells.

Whether the changes observed in this study can be explained by the involvement of aquaporins needs to clarified. The molecular mechanisms of the permeability changes by aas and their DIPP-aa can not be concluded from our data.

Li et al. (1993) suggested that the net negative charges of the applied amino acids at the experimental pH makes the membrane phospholipids less orderly and therefore more fluid. This hypothesis is reasonable, but cannot explain the specificity of aa's observed in this study.

The molecular structure of the aa may be a decisive factor for specific interactions with membrane molecules. Hydrogen bonding can be pivotal in the processes involved in water permeation and its alteration by aa's and DIPP-aa. The OH-group in serine and threonine may be a more efficient spacer for phospholipids which leads to higher permeability. There is also the possibility that the binding of the effective aa's to lipids is similar to protein lipidation (Casey, 1995).

Experiments with methyl urea as a typical test substance for the lipid way of permeation suggest that serine and threonine specifically induce higher permeability perhaps by increasing greater membrane fluidity or by changing the spacing between neighboring phospholipid molecules.

Since serine and threonine and their DIPP-derivatives increased methyl urea permeability to a similar degree (Table 3), their effectiveness must be attributed to the aa rather than to the DIPP moiety.

It can be visualized that the aa interacts directly with the polar heads of the phospholipid molecule and/or with water channel proteins. In the latter case these proteins not only increase water permeation by the assumed activation of the water channels but the aa's also induce alteration of membrane lipids resulting in the higher permeability observed for methyl urea. An increase in membrane water permeability just by insertion of proteins into the lipid bilayer has already been reported (Zeidel et al., 1992) and may play a role for water permeability increase, especially by DIPP-aa's. Further experiments are needed to clarify these seminal observations and to determine if or to what degree if any, the aquaporins are involved in the molecular mechanisms for the permeability changes induced by aa and DIPP-aa.

Water present in the hydrophobic membrane core (Meier et al., 1990; Kavanau, 1994) and on the membrane surface ("ordered water" Drost-Hansen, 1985; Venable et al., 1993) as well as hydrogen belts and polar zones in the membrane (Brockerhoff, 1974) may have to also be considered.

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