연 구 보 고

WATER AND NONELECTRO -LYTES PERMEABILITY OF PLANT CELL MEMBRANES AFTER SHORT TERM APPLICATION OF PHOSPHORY-LATED AMINO ACIDS

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Interaction of N-(diisopropyloxyphosphoryl)-amino- acids (DIPP-AA) with membrane lipids was analyzed by evaluating their effects on membrane fluidity. Water and methyl urea permeability were used as probes for membrane fluidity. Permeability of epidermal cells of Pisum sativum stem base and the inner epidermis of the Allium cepa bulb scale was measured by standard osmotic method after their exposure to a 5 mM solution of each DIPP-AA for 20 min.

Treatments with the phosphoryl derivates of three polar and one charged amino acids (serine, threonine, asparagine, and histidine) significantly increased water permeability about two fold compared to the untreated controls. Pretreatment with the other amino acids and their derivatives resulted in only small(increase or decrease) or no significant changes in water permeability. The Pf value of Pisum sativum epidermal cells(untreated controls) was $1.3 \pm 0.40 \times 10^{-4}$ cm sec-1.

Free amino acids and their DIPP-derivates equally increased urea and methyl urea permeability but much less than water permeability.

Methyl urea permeability was $10.26 \pm 0.93 \times 10^{-8}$ cm/sec. These results suggest that DIPP-AAs interact with the phospholipid bilayer of the plasmamembrane: maybe the diisopropyl tail of the DIPP-AA anchors the molecule in the acyl part of the membrane while the AA moiety interacts with the polar region of the membrane. These structural fitness of amino acids may lead to AA specific changes in membrane fluidity and packing density.

INTRODUCTION

Phosphorylation of proteins located in biological membranes plays an important role in many biological functions especially in enzyme reactions and signal transductions. Recently Li et al. (1993) using Raman spectroscopy demonstrated that some selected diisopropyl phosphoryl amino acids (DIPP-AA) increased significantly fluidity of 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 end of the chains became more ordered.

Membrane fluidity is important for biological functions especially under suboptimal temperatures. Lipid composition, particularly the content of unsaturated fatty acid and phospholipid packing density influence the membrane fluidity (Fettiplace and Haydon 1980, Finkelstein 1986) which again influences the transport properties of the membrane by affecting the protein lipid interaction (In't Veld et al. 1991). Water and solute permeability has been correlated with membrane fluidity (e. g. Van der goot et al 1989; Worman et al. 1986, Verkman and Ives 1986).

Human and animal erythrocytes and ghosts have been most frequently used model systems to study membrane water transport. Osmotic water transport in red blood cells, however, being different from most other membrane types operates mainly through water channel proteins in the erythrocyte membrane, whereby membrane lipids contribute only little to the water transport(Benga et al. 1993, Verkman 1992). There is a need to study the effect on fluidity of membrane phospholipids using membrane systems where water channels are known to be absent or make only a minor contribution to water transport.

In most plant cells, water and nonelectrolytes such as urea families permeate through membrane bilayer by the solution diffusion mechanism. A contribution to the permeation by water channels as recently found located in the cell membranes of Arabidopsis thaliana can not be significant; the water permeability coefficient of membranes of higher plants is in the range of 1 - 15 x 10^{-4} cm/sec (STADELMANN 1969) and slightly below the range for water permeation in artificial phospholipid membranes without water channels (FETTIPLACE and HYDON 1980).

This study was intended to investigate the effect of phosphorylated amino acids by using the well established model membrane systems of Allium cepa and Pisum sativum epidermal cells for permeability studies of plant cells.. The permeability for water and methyl urea was determined after treatment with DIPP-AA and compared with the permeability of the same material for the free amino acid and untreated controls. Methyl urea was selected instead of the frequently used urea because of its higher lipophily and therefore its faster permeation. The plasmolytic method was used to measure permeability as it is especially suited to determine the permeability for individual cells in a plant tissue (Stadelmann and Lee-Stadelmann 1989). .

MATERIAL AND METHOD

Plant material: Internodal epidermis of Pisum sativum var "Alaska' seedlings and the inner epidermis of Allium cepa bulb scales 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 hrs. Next 20 seeds were placed in a 9 cm diameter petri dish on a round filter paper wetted with 10 ml distilled water. The seeds were germinated for 24 hrs in the dark. The germinating seeds were planted in pots (diameter at the top 11 cm, height 8 cm; 10 seeds per pot) transferred into a growth chamber with a day/night period 16/8 h at 17 oC grown under 82 umol m-2 s-1 irradiation (400-700 nm) during daytime. The pots were watered daily. The onion bulbs were purchased at the local market.

All experiments with Pisum epidermal cells were performed at 20+1 oC. Other temperatures used for the tests with Allium cepa cells is stated in the tables.

Chemicals. Amino acids (Gly, Ala, Val, Leu, Ile, Met, Phe, Trp, Pro, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, (all chromatography grade) were obtained from SinoAmerican Biotechnology Co., Beijing, China, D-mannitol (HPLC grade, Fluka, Buchs, Switzerland), D-sorbitol (extra pure; Junsei Chemical Co., Japan); CaCl2.2H2O (99%, Sigma, St. Louis. MO, USA) and KCl (first grade, 99 %; Shinyo, pure Chemicals Co,

Japan) were used. Methyl urea (>97 %, Fluka) was purified by two time recrystallization in ethanol.

The amino acids were phosphorylated as described earlier (Li et al 1991; Ma and Zhao 1989), resulting in N-(o,o-diisopropyl) phosphoryl amino acids: ((CH3)2CHO)2.P(O).R, where R is the amino acid residue. These compounds hydrolyzed only very slowly so that no appreciable amount of hydrolysis products was present

during the time span of the experiment.

Solutions were prepared in a balanced salt medium which was made either by dissolving 3.7 mM CaCl2 and 25 mM KCl in 1 l double distilled water, or by diluting a stock solution of KCl and CaCl2 solution (9 part 1 molar KCl solution and 1 part 1 molar $CaCl_2$ solution) as described elsewhere (Lee-Stadelmann and Stadelmann 1989). If necessary, the final plasmolyzing solution (0.55 osm) 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 intermediary concentrations 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: Allium cepa. The inner side of the third scale of a dormant onion bulb was incised superficially, for an epidermis piece of about 5 x 10 mm. For Pisum sativum the lowest internodes above ground (morphologically first or second) of 9 day old seedlings 10 mm long segments were used.

Next the incised onion scale and the internode segments were vacuum infiltrated in the balanced salt medium for 1 to 1.5 min to remove air from intercellular spaces and to facilitate separation of the tissue.

After infiltration, the epidermis pieces of inner onion epidermis were pealed off with a forceps. On the pea internode shallow transversal incision were made on each side and the tissue was carefully stripped off with a fine forceps and placed in fresh salt medium. The strips generally contain epidermis, subepidermis and parenchyma cell layers. Three tissue strips were used for the experiment.

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

Treatment with DIP-AA and AA: From the 0.4 osm pretreatment solution one section each was transferred to the final plasmolyzing solution ((0.55 osm) containing either 5 mM DIPP-AA or their corresponding the free AA. The 3rd section as control was transferred into the final plasmolyzing solution and treated neither with 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 to the same value drops of 1 M KCl solution were added where needed. After 20 min the cuttings were rinsed and transferred into a dish with a fresh plasmolyzing solution without the treatment.

The solute potential of the cell was determined in preliminary experiments to select a suitable concentration for the plasmolyzing and deplasmolyzing solution to be used. Since the cells are nearly cylindrical solute potential was determined for a representative number individual cells by the plasmometric method (cf. LEE-STADELMANN and STADELMANN 1989, p. 242).

Permeability determinations. Two experimental steps were designed. At first, twenty DIPP-AA and their free amino acids were screened semi-quantitatively for their effect on water permeability of the Pisum cells. For those DIPP-AA treatments which noticeably affected water permeability, the permeability coefficients for water and methyl urea (Pf, and Ps, respectively) were determined. For all permeability measurements, 3 test runs (non treated, free amino acid and DIPP-AA) were performed sequentially for each experiment with 10 min intervals for water permeability. 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 slow enough the three cuttings were placed together into the perfusion chamber and observed simultaneously. All observations were made under 400 x total magnification.

a) Deplasmolysis time method. The plasmolyzed tissue was perfused with a deplasmolyzing solution (0.20 or 0.35 osm) and the time from contact of the solution to the tissue until deplasmolysis (the protoplast ends touch the transversal cell wall) occurred in about 90 % of the cells.

Deplasmolysis time is a relative indicator for the water permeability for the same osmotic driving force and same cell material.

Water permeability of pea cells for all 20 DIPP-AA and free amino acids was screened by this method. The deplasmolysis time under the driving force used was in the average between 10 to 20 min.

For accurate determination of the deplasmolysis time a series of photomicrographs was made which allows evaluation of 30 to 40 cells per frame.

b) Calculation of the water permeability coefficient Pf. For the selected AA and DIP-AA which showed changes in water permeability, the coefficient Pf was calculated as quantitative measure for water permeability from a formula (2) derived from the general equation for the permeability coefficient. Details and theoretical background of the permeability measurement techniques for water and solutes are described earlier (Stadelmann 1969; Lee-Stadelmann and Stadelmann 1989; Stadelmann and Lee-Stadelmann 1989).

Absence of cell damage by DIP-AA and AA treatment was estimated by measuring any decrease in volume of the plasmolyzed protoplast with time after a treatment: damaged cell membranes become leaky for the solutes in the vacuole increasing with time the degree of protoplast contraction. No such changes were found in cells tested after the experimental procedures used here.

Statistics. For each DIPP-AA treatment at least three experiments were made. Standard deviation in deplasmolysis 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.

RESULT AND DISCUSSION

Water permeability for pea epidermal cells: the effects of all 20 amino acids and their Diisopropyl phosphorylated(DIPP) amino acids tested by the relative deplasmolysis time as described in material and method section are shown in Table 1.

The short term treatment (20 min) of 5 mM of diisopropyl phosphorylated forms of some amino acids increased water permeability both in pea and onion epidermal cells, and the effects were amino acid dependent:among all amino acids tested, DIPP-serine and DIPP-threonine most effectively increased the water permeability up to over 2 folds from the control. Two other polar amino acids, DIPP-asparagine and DIPP-glutamine showed about 50 % increase from the control. Unexpectedly, free amino acids of serine and threonine also increased water permeability, but to a lower extent. The dose-concentration effect was studied for pea cells under the same experimental conditions. The treatment of 10. 20, 30 and 40 min, and the concentrations of 0.01, 0.1, 1.0 and 5.0 mM DIPP-AA showed no significant difference.

Among the electrically charged amino acids group, only DIPP-aspartic acid and histidine showed increased water permeability, about 80% and 200% respectively. None of their free amino acids showed any significant

Table I : Relative water permeability of stem epidermal cells of Pisum sativum seedlings after 20 min treatment with unsubstituted (AA) and diisopropyl phosphorylated amino acids (DIPP-AA).

	Mean deplasmolysis time (min) of untreated control	Number experiment[1]	Ratios AA / Control[2]	Ratios DIPP-AA / Control
Nonpolar amino acids				
Glycine	232±174[3]	3	1.23±0.26	1.19±0.22
Alanine	227±77	4	1.11±0.25	1.16±0.41
Valine	242	2	1.20	0.97
Leucine	417±210	3	0.789+0.11	0.74±0.39
Isoleucine	309	2	1.22	1.28
Methionine	282	2	1.16	0.855
Phenylalanine	223	2	1.17	1.32
Tryptophan	213	2	0.765	0.85
Proline	403	2	0.965	0.94
Polar amino acids				
Serine	355(?)458±361	4	0.556±0.10	0.47±0.08
Threonine	355	2	0.68	0.59
Cystein	208±58	4	1.34±0.66	1.26±0.23
Tyrosine	205	2	1.12	0.96
Asparagine	310±58	3	0.808±0.015	0.528±0.09
Glutamine	410	0		
Electrically charged amino acids				
Aspartic acid	215? 215	2	0.81	0.73
Glutamic acid	319?	2	1.74	0.86
Lysine	180	2	0.89	1.01
Arginine	397±160	4	0.846±0.045	0.839±0.057
Histidine	288	2	0.90	0.568

1) number of experiments which could be evaluated.

2) Control: Tissue without treatment with amino acid or 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 decrease 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 more show these fluctuations.

increase. On the other hand, none of the 9 nonpolar amino acids (whether DIPP or free) showed any effect on water permeability.

The effect of the 4 polar amino acids (serine, threonoine, asparagine and aspartic acid) and 2 electrically charged amino acids (glutamine and histidine) were further tested for water permeability more quantitatively. The water permeability constants(Kw) for the untreated pea cells ranged from 1.10 to 2.12 x 10^{-4} cm/sec, for free amino acids from 1.07 to 3.00 x 10^{-4} cm/sec, and for DIPP-amino acids from 1.68 to 2.82 x 10^{-4} cm/sec (Table II). Again, the most effective amino acid deriatives were serine, threonine. Asparagine and aspartic acids were mildly effective and glutamine and histidine appear to have little effects. The values for free amino acids were similar to those untreated controls. The only discrepancy in water permeability by both methods was with histidine where a significant increase was found with DIPP-histidine by relative method, but rather decrease for both DIPP-histidine and free histidine by the absolute method. The reason for this discrepancy has not been identified.

Water has been known to permeate through the phospholipid layer in most plant cells. The ranges of permeability values found in this study are well in agreement with those found earlier (cf.Lee-Stadelmann and Stadelmann 1989) and within the ranges of lipid way permeability found in animal cells (Jansson and Illsley 1993, Verkman and Masur 1988), and there is no indication that protein channel is involved neither with or without free amino acids and their DIPP-forms. In some specialized animal and human cells (e.g. Benga et. al 1993; Solomon et al 1984; Macey 1984), and renal tubule cell membranes (Meyer and Verkman 1987), it has been reported that water transport is operated through the integral proteins (CHIP or Aquaporin) and therefore their permeability is reported to be orders of magnitude higher than that of lipid way water permeability.

In the osmotic water permeability experiments using living cells like in this study, one of the most critical factors is hether the osmotic driving forces change during the experiment, but not by the treatment of interest or by any other experimental factors. Our data on osmotic solute contents clearly demonstrate that the osmotic driving forces during the measurements were constant (data not shown) which means that the DIPP-aa did not enter the cell nor caused any leakage as result of damage by the treatment. High water permeability has been sometimes observed in damaged membranes (e.g. Lee 1972; Stadelmann 1969). Any by-product effect as a result of decomposition of DIPP during such short experimental time is highly unlikely within such short time scale.

The effect of DIPP-serine and DIPP-threonine on methyl urea permeability was found much less (between 23 to 83%) than on water permeability (Table III). Furthermore, there was no significant differences in permeability ween the free amino acids and their DIPP-amino acid effect. The permeability constants (Ks) for methyl urea ranged from 1.23 to 1.83 x 10 -8 cm/sec, and these values are about 10,000x lower than those for water.

Urea and methyl urea permeability were tested for DIPP-serine and DIPP-histidine and DIPP-serine with onion epidermal cells. The effects were in line with those for pea cells: both solute permeability was increased only slightly, compared with untreated controls. As in pea cells, the effect of DIPP was similar to that of free amino acid, and there was no amino acid specificity. The permeability of onion cells for urea and methyl urea ranged from 4.0 to 8.7 x 10-8 cm/sec and 2.1 to 5.8 x 10-7 cm/sec respectively. The urea permeability for onion cells was at least an order of magnitude higher than for pea cells.

Table II: Water permeability constants of individual cells of Pisum sativum stem epidermis as influenced by some diisopropyl phosphoryl (DIPP) amino acids.

	Kw x 10^{-4} cm/sec				
				Ratios	
AA	Control	Free- aa	DIPP-aa	DIPP-aa / Control	Free aa / Control
Ser	1.10	1.60	2.82	2.57	1.46
Thr	1.21	3.00	2.15	1.77	2.48
Asn	1.13	1.07	2.08	1.84	0.95
Asp	1.10	1.14	1.69	1.54	1.04
Gln	2.02	1.86	2.14	1.06	0.92
His	2.12	1.26	1.68	0.80	0.59

Ser:serine, Thr:threonine, Cys: cystein, Asn: asparagine, Asp: aspartic acid, His:histidine, Val:valine, Glu:glutamic acid, Gln:glutamine, Kw: permeability constant for water in cm/sec. aa: amino acid, Each value is mean of 20 to 30 cells from 2 to 3 independent experiments. Deplasmolysis time ranged from 171 to 511 min. The ratios of DIPP-aa/control and aa/control indicate the effects of DIPP-aa and free-aa respectively: the ratio higher than 1.0 reflects the increasing effect of the treatment. Amino acids arranged in decreasing order of water permeability.

Table III: Methyl urea permeability constant (Ks X 10^{-8} cm/sec) of individual cells of Pisum sativum stem epidermis after the treatment with DIPP-serine and DIPP-threonine.

	Permeability constants in cm/sec				
				Ratios	
				DIPP-aa	Free-aa
	Control	Free-aa	DIPP-ser	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

aa:amino acid, DIPP: diisopropyl phosphoryl, each value is mean of 10 to 20 cells. The ratios of DIPP-aa/control and aa/control indicate the effect of DIPP-aa and free aa respectively.
Experiments done at 20°C

It has been well known that free amino acids do not permeate the phospholipid layers of the biological membrane. No charged molecules can enter the bilayer or very slowly, and therefore phosphorylation of the amino acids are even more impermeable through lipid layer. Diisopropylation of phosphoamino acid on the phosphor group makes at least one part of the amino acid more hydrophobic, thus helps the molecule to slip into the phospholipid easier.

In this regard, a two phase mechanism is proposed to explain the results: the DIPP tail allows the anchoring of the DIPP-AA in the lipid portion of the outer lamella of the cell membrane; significant differences between the DIPP-AA in their effect on water permeability suggest that they interfere in a way specifically dependent upon the structure of the amino acid moiety and their interaction with the polar region at the membrane outside. This results in a different degree of lowering the energy required for breaking the H-H bonds between water molecules needed for passage through the membrane lipid core.

The net charge of the DIPP-aa affects their interaction with membrane molecules and their embedding capability into the bilayer. The net negative charges of the applied amino acids at the experimental pH (Li et.al 1993), tend to make the phospholipids less orderly and therefore more fluid.

No amino acid specificity for methyl urea permeability observed in this study seems to suggest that the increase may be due to the increased hydrophobic environment contributed by the embedded diisopropyl group, rather than the structural effect of amino acid. The molecular sizes or structural differences (steric hindrance or aromatic etc) do not seem have any role in their effect: cystein which has close structural similarity with serine did not show any effect, neither did alanine which has the smallest molecular size. The molecular weight of the 20 amino acids range from 253 to 474.

An involvement of intrinsic proteins in the mechanism for increase or decrease of the water permeability, however, can not be excluded. DIPP-AA could preferable nest in the vicinity of in-trinsic proteins which could provide additional channel-like spaces as pathways for water and small molecular non electrolytes. Further study is needed to verify the extent of involvement of water channels (Preston et al 1992; 1993) by measurment of activation energy and by using channel blockers.

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