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Identification of chiro-inositol-containing phospholipids and changes in their metabolism upon salt stress in soybean seedlings

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Abstract

We report here the presence of chiro-inositol-containing phospholipids in addition to the common myo-inositol-containing phospholipids in soybean seedlings. Using sequential TLC and HPTLC, eleven inositol phospholipids were isolated from soybean seedlings and analyzed after 6 N HCl hydrolysis for their inositol and fatty acid compositions by anion exchange Dionex HPLC and GC, respectively. Upon analysis, we have identified three species of chiro-inositol-containing phospholipids and further observed that the chiro-inositol-containing phospholipids and their fatty acid composition changed in response to 1 and 24 h 150 mM NaCl treatment compared to the control (no NaCl treatment). In addition, we found the different tissue distribution and metabolic changes of the chiro-inositol-containing phospholipids in apical, elongating and mature parts of hypocotyls and roots of soybean seedlings upon salt stress.

Keywords: *Glycine max* L. cv Williams; Leguminosae; chiro-Inositol; chiro-Inositol phospholipid; Salt stress

1. Introduction

Inositols are a family of nine stereoisomeric hexahydroxycyclohexanes. myo-Inositol or 'inositol' is the most widely distributed isomer in plants and animal tissues. In nature, chiro-inositol and derivatives are present chiefly in plants (Drew, 1983), mold antibiotics (Umezawa et al., 1965) and insects (Hipps, Holland, & Sherman, 1972). Formation of chiro-inositol from myo-inositol by inversion of C3 hydroxyl group of myo-inositol by an oxidoreductive epimerization mechanism has been demonstrated in the cockroach fat body (Hipps et al., 1972) and in algae (Wober, Ruis, & Hoffmann-Ostenhoff, 1971). However, this enzymatic mechanism has not been shown to produce chiro-inositol in animal tissues (Hipps, Segal, Holland, & Sherman, 1973). Previously we identified and characterized chiro-inositol-containing phospholipids and chiro-inositol galactosamine-containing glycosylphosphatidylinositols (GPIs) from bovine and rat liver (Pak & Larner, 1992). Recently, we established that conversion of myo-[^{3}H]inositol to chiro-[^{3}H]inositol occurred specifically in the myo-[^{3}H]inositol-containing phospholipids, since no change was detected in the free inositols or inositol phosphates in rat fibroblast (HIRc-B) cells (Pak, Paule, Bao, Huang, & Larner, 1993), Sprague–Dawley rat (Pak, Huang, Lilley, & Larner, 1992) and Wistar rat tissues (Pak et al., 1998). Further our study with spontaneous non-obese insulin-resistant type II diabetic Goto–Kakizaki (G.K.) rats (Pak et al., 1998) demonstrated a severe defect in conversion of myo-[^{3}H]inositol to chiro-[^{3}H]inositol at the inositol phospholipid level of insulin-sensitive tissues of the G.K. rat and the existence of a pathway with increased activity for incorporation of chiro-[^{3}H]inositol into tissue phospholipids in the G.K. rats (Pak et al., 1998). Thus the latter finding suggested a bypass pathway of the biosynthetic myo-inositol to chiro-inositol conversion defect at the inositol phos-

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pholipids (i.e. direct incorporation of chiro-inositol or chiro-inositol phospholipid to inositol phospholipids) in the diabetic G.K. rats by administering chiro-inositol or chiro-inositol phospholipid as a nutritional or therapeutic supplement.

Studies with diabetic mammalian systems showed that chiro-inositol administration decreased blood glucose levels, thus mimicking the action of insulin and urinary chiro-inositol excretion which was closely correlated to insulin resistance (Ortmeyer, Bodkin, Lilley, Larner, & Hansen, 1993; Kennington et al., 1990; Suzuki et al., 1994; Craig, Larner, & Asplin, 1994). Therefore these studies suggested that chiro-inositol as a metabolic index of insulin sensitivity in diabetes (Pak et al., 1998; Ortmeyer et al., 1993; Kennington et al., 1990; Suzuki et al., 1994; Craig et al., 1994). Accordingly, we have been investigating which foodstuffs contain large amounts of chiro-inositol by surveying vegetables, edible plants and other staples in an effort to explore the nutritional supplements of chiro-inositol. In the course of our investigation, we found that soybean and soybean seedlings have high chiro-inositol levels. Interestingly, soybean seedlings are one of the most popular vegetables in Korea and are consumed almost daily in Korean families. Furthermore, soybean seedling is also one of the agronomically and economically important vegetables in Korea.

In the present study, we have isolated inositol phospholipids from soybean seedlings using two sequential TLC systems and analyzed their inositol and fatty acid compositions by anion exchange Dionex HPLC and GC, respectively. From these analyses, we have identified chiro-inositol-containing inositol phospholipids and further observed that the chiro-inositol-containing phospholipids and their fatty acid composition changed in response to 1 and 24 h NaCl treatment compared to control with no NaCl treatment. In addition, we found different tissue distributions and metabolic changes of chiro-inositol-containing phospholipids in various tissues of soybean seedlings upon the salt stress.

2. Results and discussion

2.1. Identification of chiro-inositol-containing phospholipids and changes in their metabolism in response to 150 mM NaCl stress

As shown in Fig. 1, the second HPTLC from the two sequential TLC profiles, as described in the Experimental, routinely resolved a total of 11 species of inositol phspholipids from the whole seedlings. Upon the inositol analysis of each acid hydrolysate from the individual inositol phospholipids by Dionex HPLC, band 1 (migrating at PIP_2 standard), band 6

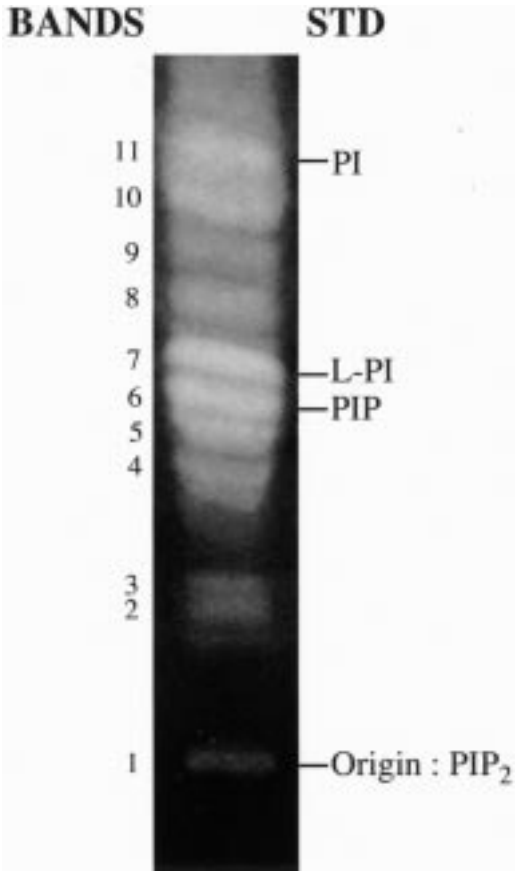

Fig. 1. HPTLC analysis of inositol-containing phospholipids from soybean seedlings. Eluates of the inositol-containing phospholipids from the first TLC in an acid solvent system of $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (40:15:18:12:10, v/v/v/v/v) were rechromatographed in a base solvent system of $CHCl_3/CH_3OH/NH_4OH/H_2O$ (86:76:6:16, v/v/v/v) for further purification with authentic standards as shown. Each purified species was visualized, eluted, pooled and analyzed for chemical composition as described in Experimental. PI, phosphatidylinositol; L-PI, lysophosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

(migrating at PIP standard), band 7 (migrating at L-PI standard), bands 10 and 11 (migrating near the area of PI standard) and bands 5, 8 and 9 contained predominantly myo-inositol (78–100%). However, bands 2, 3 and 4 were proven to be chiro-inositol-containing phospholipids having predominantly chiro-inositol ranging from 87–100%. Interestingly, when we subjected soybean seedlings to 150 mM NaCl stress for 1 and 24 h, the 3 species of chiro-inositol-containing phospholipids responded differently. As shown in Table 1 and Fig. 2, band 2 contained 37.8 nmol chiro-inositol/g tissue in the control without salt stress and dramatically decreased to 0.3 and 0.5 nmol chiro-inositol/g tissue upon 1 h and 24 h incubation with 150 mM NaCl, respectively. In the case of band 3, however, the undetected chiro-inositol in the control increased to 9.6 after 1 h and 9.4 nmol chiro-inositol/g tissue by 24 h salt stress (Table 1 and Fig. 3). Band 4 of the control

Table 1
Changes of chiro-inositol-containing inositol phospholipids upon 1 and 24 h 150 mM NaCl-stressed soybean seedlings (nmol chiro-inositol/g tissue). The values are the mean ± S.D. from three independent experiments

chiro-Inositol phospholipid	chiro-Inositol/g tissue (nmol)		
	Control	1 h	24 h
Band 4	0.8 ± 0.1	1.2 ± 0.1	–[a]
Band 3	–	9.6 ± 0.4	9.4 ± 0.3
Band 2	37.8 ± 1.4	0.3 ± 0.1	0.5 ± 0.1
Total[b]	38.6	11.1	9.9

[a] Not detected.
[b] Total sum of all chiro-inositol-containing phospholipids.

(0.8 nmol chiro-inositol/g tissue) increased 1.6 fold (1.2 nmol chiro-inositol/g tissue) at the 1 h salt stress and was not detected after 24 h salt stress (Table 1 and Fig. 4). When the total sum of all chiro-inositol phospholipids was calculated, 38.6 nmol chiro-inositol/g tissue in the control gradually decreased to 11.1

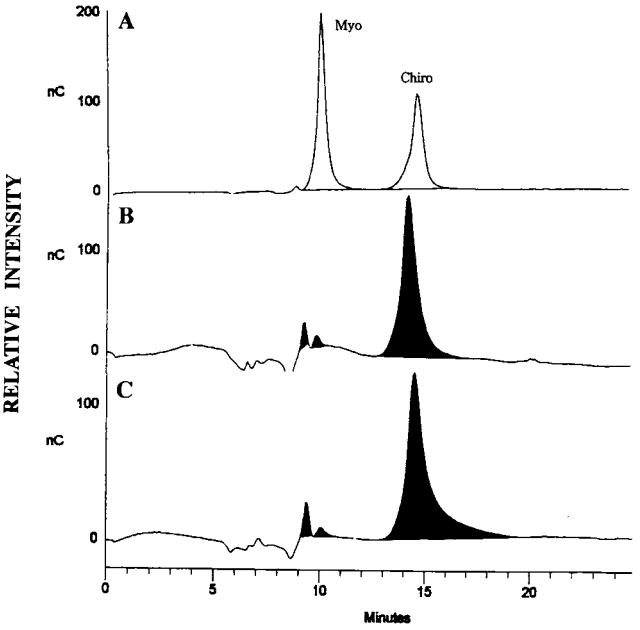

Figure 3. Dionex HPLC analysis of band 3 and its change in response to 150 mM NaCl stress. Band 3 is designated as in Fig. 1(A) HPLC elution profile of myo- and chiro-inositol standards; (B) 1 h 150 mM NaCl stress; (C) 24 h 150 mM NaCl stress.

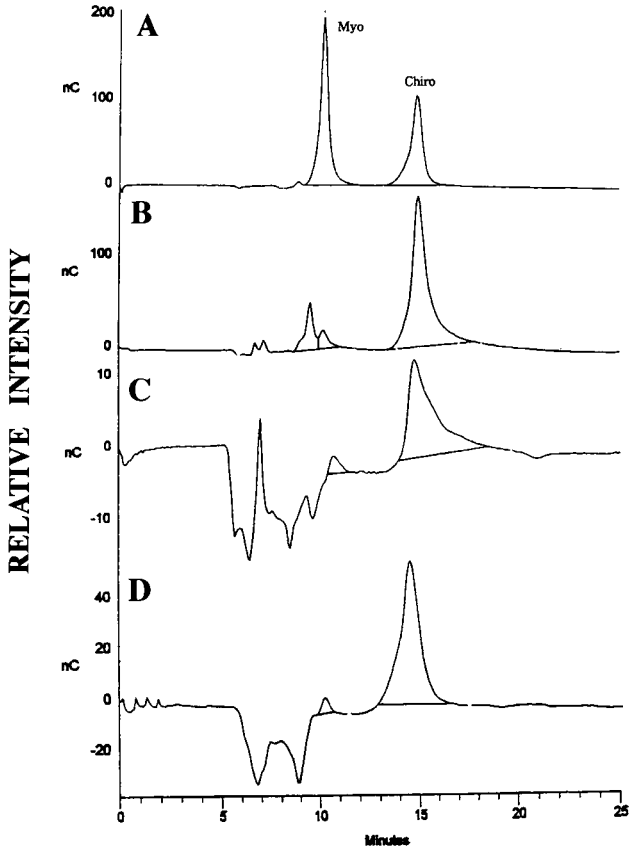

Figure 2. Dionex HPLC analysis of band 2 and its change in response to 150 mM NaCl stress. Band 2 is designated as in Fig. 1(A) HPLC elution profile of myo- and chiro-inositol standards; (B) Control without 150 mM NaCl stress; (C) 1 h 150 mM NaCl stress; (D) 24 h 150 mM NaCl stress.

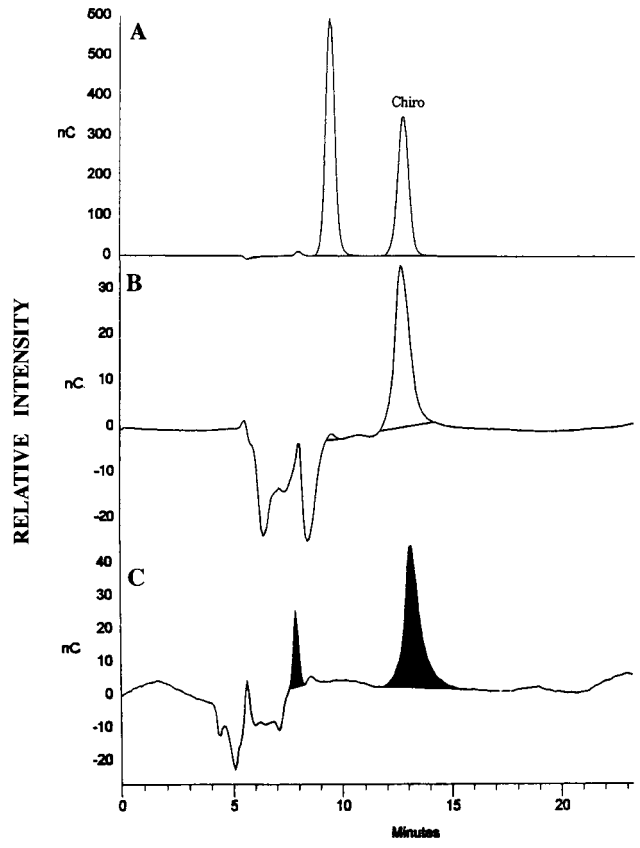

Figure 4. Dionex HPLC analysis of band 4 and its change in response to 150 mM NaCl stress. Band 4 is designated as in Fig. 1(A) HPLC elution profile of myo- and chiro-inositol standards; (B) Control without 150 mM NaCl stress; (C) 1 h 150 mM NaCl stress.

and 9.9 nmol chiro-inositol/g tissue upon 1 and 24 h incubation with 150 mM NaCl, respectively (Table 1).

2.2. Changes in fatty acid composition of chiro-inositol-containing phospholipids in response to 150 mM NaCl stress

Fatty acid analysis of the chiro-inositol-containing phospholipids by GC (Table 2) showed an almost 2 fold increase of 16:1 and 18:3 and a 2 fold decrease of 18:0 in band 2 of the 1 h and 24 h 150 mM NaCl-stressed seedlings compared to the control, respectively. In band 3, 16:0, t16:1, 16:1 and 18:0 of 1 h-stressed seedlings decreased 1.3, 1.4, 4.4 and 3.9 fold at 24 h stress, respectively. In contrast, 18:1, 18:2 and 18:3 of the 1 h salt-stressed seedlings increased 2.3, 4.4 and 3.9 fold in 24 h-stressed seedlings, respectively. Unlike the increase of the polyunsaturated fatty acids of bands 2 and 3 upon salt stress, 18:3 of band 4 in control was markedly decreased 3.6 fold and 18:0, 18:1 and 18:2 increased 1.4, 1.3 and 1.6 fold at 1 h-stressed seedlings.

2.3. Tissue distribution and metabolic changes of chiro-inositol-containing phospholipids in response to 150 mM NaCl stress

As shown in Table 3, among the various tissues examined, the roots have the highest content of chiro-inositol-containing phospholipids (16.7 ± 1.0 nmol chiro-inositol/g tissue); on the other hand, the apical hypocotyls have the lowest (4.3 ± 0.3 nmol chiro-inositol/g tissue) with the mature and elongating parts of the hypocotyls being in between in the control with no NaCl treatment. In response to salt stress, the content of chiro-inositol-containing phospholipids from all tissues decreased at 1 and 24 h stress compared to the control. Interestingly, while the roots showed the highest content of chiro-inositol-containing phospholipids with a 6.8 fold decrease in mature hypocotyls at 1 h salt stress, the mature part of the hypocotyls showed the largest accumulation at 24 h salt stress with a 4.3 fold increase compared to 1 h salt stress among the tissues (Table 3).

Recently, we suggested a bypass pathway of the biosynthetic myo-inositol to chiro-inositol conversion defect at the inositol phospholipids in the diabetic G.K. rats by administering chiro-inositol or chiro-inositol phospholipid as a nutritional or therapeutic supplement (Pak et al., 1998). Accordingly, in an effort to explore the nutritional supplements of chiro-inositol and chiro-inositol phospholipid, we examined the chiro-inositol contents by surveying vegetables, edible plants and other staple foodstuffs. In the course of our investigation, we found that soybean and soybean seedlings had the highest levels of chiro-inositol and chiro-inositol phospholipids (Hong, Y. and Pak, Y., unpublished data). We were therefore prompted to investigate the metabolism of chiro-inositol-containing phospholipids in the soybean seedlings. As shown in Table 1, we identified 3 species of chiro-inositol phospholipids with a total content of 38.6 nmol chiro-inositol/g tissue of the soybean seedlings. Further we observed that the three chiro-inositol phospholipids responded differently to salt stress in terms of changes in chiro-inositol phospholipid content and their fatty acid compositions (Tables 1, 2). In addition, we found the different tissue distribution and metabolic changes of the chiro-inositol-containing phospholipids among apical, elongating and mature parts of hypocotyls and

Table 2
Changes in fatty acid composition of chiro-inositol-containing inositol phospholipids in 1 and 24 h 150 mM NaCl-stressed soybean seedlings (mol%, total fatty acid). The values are the mean from three independent experiments. The deviation of values was < 3% in every case

Fatty acid	Total fatty acid (mol%)						
	Band 4		Band 3		Band 2		
	Control	1 h	1 h	24 h	Control	1 h	24 h
16:0[a]	33.4	32.8	23.0	17.2	27.1	23.7	24.9
t16:1	–[b]	–	2.6	1.8	–	–	–
16:1	9.8	8.2	7.9	1.8	10.9	21.3	18.0
18:0	20.4	27.5	47.0	12.2	21.4	11.3	10.7
18:1	11.4	14.1	7.8	17.8	15.9	16.9	16.1
18:2	7.7	12.5	8.4	36.5	21.3	20.0	24.5
18:3	7.4	4.8	3.2	12.6	3.4	6.9	5.8

[a] 16:0, palmitic acid; t16:1, 3-trans-hexadecenoic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid. The number preceding the colon represents the number of carbon atoms in the fatty acid and the number following the colon indicates the number of double bonds present.

[b] Not detected.

Table 3
Tissue distribution and metabolic changes of chiro-inositol-containing inositol phospholipids in response to 1 and 24 h 150 mM NaCl stress in various tissues of soybean seedlings (nmol chiro-inositol/g tissue). The values are the mean ± S.D. from three independent experiments

Tissue	chiro-Inositol/g tissue (nmol)		
	Control	1 h	24 h
Apical	4.3 ± 0.3	–[a]	–
Elongating	5.2 ± 0.4	2.8 ± 0.3	0.3 ± 0.1
Mature	10.2 ± 1.0	1.5 ± 0.2	6.4 ± 0.6
Root	16.7 ± 1.0	6.0 ± 0.8	2.9 ± 0.3

[a] Not detected.

roots of soybean seedlings in response to salt stress (Table 3).

Although scyllo-[^{3}H]inositol-containing phospholipid was characterized from myo-[^{3}H]inositol-labeled barley (Kinnard, Narasimhan, Pliska-Matyshak, & Murthy, 1995), to our knowledge, our study is the first report of chiro-inositol phospholipids in plants. Thus our finding suggests that soybean seedling is a good system to investigate the metabolism of chiro-inositol phospholipids, serves as well as a good nutritional supplement of chiro-inositol and chiro-inositol phospholipid for diabetic patients.

Our data suggest that chiro-inositol-containing phospholipid metabolism might be regulated by salt stress in the soybean seedlings as evidenced by the changes of the chiro-inositol phospholipids and fatty acid composition in response to salt stress. However, possible biological roles of the chiro-inositol phospholipids in soybean seedlings experiencing salt stress remain to be established. Further work on the novel enzyme(s) regulating the metabolism of chiro-inositol phospholipids and the precise structure of these phospholipids will be necessary to determine the biological function of chiro-inositol phospholipids. For these reasons, we are actively investigating the biosynthesis of chiro-inositol phospholipid and its enzymatic regulation in parallel with the structural characterization of novel chiro-inositol-containing phospholipids.

3. Experimental

3.1. Plant materials and treatments

Soybean seeds (*Glycine max* L. cv. Williams) were surface-sterilized with 10% chlorox, rinsed and soaked in distilled water for 3–4 h. The seeds were sown in moist vermiculite which was kept constantly moist with tap water and seedlings were grown for 4 d in darkness at 25°C (Harryson, Morre, & Sandelius, 1996). After 4 d of growth, seedlings (5–50 g) were incubated further for 1 and 24 h under the condition of 150 mM NaCl in darkness at 25°C. In parallel, seedlings with no NaCl treatment were incubated with tap water for 1 and 24 h for comparison. Whole seedlings and various tissues (apical, elongating and mature parts of hypocotyls and roots) were examined for chiro-inositol-containing phospholipids following the 1 and 24 h time course, this being compared to controls with no NaCl treatment.

3.2. Extraction and separation of inositol phospholipids

After incubation at each time point, whole seedlings or various tissues were frozen in liquid N_2, powdered in a liquid N_2-cooled mortar and pestle and extracted for total lipids using $CHCl_3/CH_3OH$/12 N HCl (200:100:1.5, v/v/v) (Pak et al., 1993). Individual inositol phospholipids were separated from total lipid extracts by two sequential TLC systems on precoated silica gel plates (Kieselgel 60, Merck) as described (Pak & Larner, 1992; Pak et al., 1993, 1992, 1998). Briefly, total lipid extracts were separated into individual phospholipids developing in an acid solvent system of $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (40:15:18:12:10, v/v/v/v/v) for the first TLC. Each inositol-containing phospholipid as well as authentic phospholipid standards were visualized under UV light after spraying with primuline (Pak & Larner, 1992; Pak et al., 1993, 1992, 1998). Inositol-containing phospholipids were then removed by scraping and elution with $CHCl_3/CH_3OH$ (2:1, v/v) and $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v/v). Inositol-containing phospholipids from the various tissues were next hydrolyzed in 6 N HCl and analyzed for chiro-inositol by Dionex HPLC. For whole seedlings, eluates from the first TLC were subjected to further purification by rechromatography in a solvent system of $CHCl_3/CH_3OH/NH_4OH/H_2O$ (86:76:6:16, v/v/v/v) for the second HPTLC (Merck) (Pak & Larner, 1992; Pak et al., 1992, 1993, 1998). Each species was then visualized, scraped, eluted as described and pooled separately for the identification and quantification of chiro-inositol after acid hydrolysis by Dionex HPLC and for fatty acid analysis after methanolysis by GC as described below.

3.3. Identification and quantification of chiro-inositol-containing phospholipids by Dionex HPLC

To verify the presence of chiro-inositol and to quantify chiro-inositol from the total inositol-containing phospholipids and the individual inositol phospholipids isolated from first TLC and second HPTLC, respectively, samples were analyzed by an anion exchange Dionex HPLC (Pak & Larner, 1992; Pak et al., 1992, 1993, 1998). Briefly, each sample was eluted as described above from the TLC plates and hydrolyzed with 6 N HCl at 110°C for 48 h. Hydrolysates were lyophilized, dissolved in H_2O and microcentrifuged through nylon membranes (0.2 μm, Costar Spin-X 8169, Cambridge, MA). Samples were dried in a SpeedVac, resuspended in 25 μl of H_2O and analyzed by a Dionex CarboPac MA-1 column with MA-1 guard column on a Dionex DX-300 in a LCM-3 Gradient System using an isocratic system of 80 mM NaOH. All samples were analyzed at a flow rate of 0.4 ml/min and an elution time of 25 min by a Pulsed Electrochemical Detector as described (Pak & Larner, 1992; Pak et al., 1992, 1993, 1998). Typically, a baseline separation between the two inositols was achieved with a 4–5 min difference in their retention time. Each peak with the same retention times as standard myo-

inositol and chiro-inositol was identified and quantified. All samples were analyzed at least three times and data presented is a representative of the three analyses.

3.4. Fatty acid analysis of chiro-inositol-containing phospholipids by GC

The individual chiro-inositol-containing phospholipids identified from Dionex HPLC analysis were subjected to methanolysis by incubating in 5% HCl–CH_3OH solution at 80°C for 2 h. The transmethylated fatty acid methyl esters (FAME) were extracted in hexane and analyzed by GC using a 30 m × 0.25 mm SP-2330 fused silica capillary column (Supelco). Briefly, the oven temperature was maintained at 170°C and head pressure of the carrier gas (N_2 and He) was 60 psi. A split injection was used. Injector and detector temperatures were maintained at 230 and 250°C, respectively as described (Lynch & Thompson, 1984).

Acknowledgements

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References

Craig, J. W., Larner, J., & Asplin, C. M. (1994). In B. Draznin, & D. Le Roith, *Molecular biology of diabetes* (p. 343). Totowa, NJ: Humana Press.

Drew, E. A. (1983). In D. H. Lewis, *Physiology and biochemistry of storage carbohydrates in vascular plants*. Cambridge, UK: Cambridge University Press.

Harryson, P., Morre, D. J., & Sandelius, A. S. (1996). *Plant Physiol.*, *110*, 631–637.

Hipps, P. P., Holland, W. H., & Sherman, W. R. (1972). *Biochem. Biophys. Res. Commun.*, *46*, 1903–1908.

Hipps, P. P., Segal, R. K., Holland, W. H., & Sherman, W. R. (1973). *Biochemistry*, *12*, 4705–4712.

Kennington, A. S., Hill, C. R., Craig, J., Borgardus, C., Raz, I., Ortmeyer, H. K., Hansen, B. C., Romero, G., & Larner, J. (1990). *New Eng. J. Med.*, *323*, 373–378.

Kinnard, R. L., Narasimhan, B., Pliska-Matyshak, G., & Murthy, P. P. N. (1995). *Biochem. Biophys. Res. Commun.*, *210*, 549–555.

Lynch, D. V., & Thompson Jr, G. A. (1984). *Plant Physiol.*, *74*, 193–197.

Ortmeyer, H. K., Bodkin, N. L., Lilley, K., Larner, J., & Hansen, B. C. (1993). *Endocrinol.*, *132*, 640–645.

Pak, Y., & Larner, J. (1992). *Biochem. Biophys. Res. Commun.*, *184*, 1042–1047.

Pak, Y., Hong, Y., Kim, S., Piccariello, T., Farese, R. V., & Larner, J. (1998). *Mol. Cells*, *8*, 301–309.

Pak, Y., Huang, L. C., Lilley, K. J., & Larner, J. (1992). *J. Biol. Chem.*, *267*, 16904–16910.

Pak, Y., Paule, C. R., Bao, Y. D., Huang, L. C., & Larner, J. (1993). *Proc. Natl. Acad. Sci. USA*, *90*, 7759–7763.

Suzuki, S., Kawasaki, H., Satoh, Y., Ohtomo, M., Hirai, A., Hirai, S., Onoda, M., Matsumoto, M., Hinokio, Y., Akai, Y., Craig, J., Larner, J., & Toyota, T. (1994). *Diabetes Care*, *17*, 1465–1468.

Umezawa, H., Okami, Y., Hashimoto, T., Suhara, Y., Hamada, M., & Takeuchi, T. (1965). *J. Antibiot. (Tokyo) Ser. A*, *18*, 101–104.

Wober, G., Ruis, H., & Hoffmann-Ostenhoff, O. (1971). *Monatshefte fur Chemie*, *102*, 459–464.