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A Unique Phosphatidylinositol Bearing a Novel Branched-Chain Fatty Acid from *Rhodococcus equi* Binds to Influenza Virus Hemagglutinin and Inhibits the Infection of Cells[1]

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From the aquatic bacterium *Rhodococcus equi* strain S_{420}, we isolated a substance that strongly binds to influenza viruses. Structural analyses revealed that it is a unique type of phosphatidylinositol (PtdIns) bearing a branched-chain fatty acid (14-methyloctadecanoic acid). In a TLC/virus-binding immunostaining assay, this PtdIns bound to all subtypes of hemagglutinin (HA) of influenza A viruses tested, isolated from humans, ducks and swine, and also to human influenza B viruses. Furthermore, the PtdIns significantly prevented the infection of MDCK cells by influenza viruses, and also inhibited the virus-mediated hemagglutination and low pH–induced hemolysis of human erythrocytes, which represents the fusogenic activities of the viral HA. We also used purified hemagglutinin instead of virions to examine the interaction between viral HA and PtdIns, showing that the PtdIns binds to hemagglutinin. These findings indicate that the inhibitory mechanism of PtdIns on the influenza virus infection may be through its binding to viral HA spikes and host cell endosomal/lysosomal membranes, which are mediated by the function of viral HA.

Key words: aquatic bacterium, branched-chain fatty acid, hemagglutinin, influenza virus, phosphatidylinositol.

Influenza virus initiates infection through a receptor–ligand interaction and subsequent receptor-mediated endocytosis (*1*, *2*). After entry, the components of the viral particle are disassembled in the endosome where a low pH induces the irreversible conformational change of hemagglutinin (HA), resulting in fusion of the viral and endosomal/lysosomal membranes (*3–6*). The viral genome is then released from the endosome and transported into the nucleus, where replication occurs (*7*). After replication, viral genomes leave the nucleus and progeny virus particles assemble and bud from the cellular membrane of the infected cells (*6*, *8*).

Viral proteins, which perform essential functions during the life cycle of the influenza virus, are potential targets for the development of antiviral agents. Several agents involved in the infectious cycle have already been documented (*9–14*). It is known that the sialic acid–containing glycoproteins or glycolipids on cell surfaces are receptors for influenza viruses (*15–20*). Two major virus glycoproteins, HA and sialidase, mediate the interaction between influenza viruses and cellular receptors. They are responsible for the attachment to target cells and for release of progeny viruses from the surface of infected cells, respectively (*1*, *6*). Therefore, many inhibitors of influenza viruses have been developed against virus HA or/and sialidase (*14*, *21–24*). Several studies reported that neoglycoproteins or synthetic co-polymers containing sialic acid residue strongly inhibited the HA and sialidase activities, resulting in prevention of influenza virus infection (*24–30*). However, the effects of most inhibitors are usually restricted to HA subtypes of influenza virus isolates, and their stability has been influenced by the hydrolysis of sialic acid–linkage by viral sialidase. Influenza virus vaccines are also dependent on viral-specific antigens (*31*).

Recent studies have shown that a non-sialylated glycolipid, sulfatide, also has receptor-like activity (*32*) and

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Abbreviations: HA, hemagglutinin; MDCK, Madin Darby canine kidney; Neu5AcnLc4Cer, Neu5Ac-Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide; PtdIns, phosphatidylinositol; TLC, thin layer chromatography; YMPG medium, 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone, pH 7.0; YSGG medium, 1% glucose, 2% glycerol, 0.4% yeast extract, 0.1% soy flour, 0.1% K_2HPO_4, 0.1% $MgSO_4$–$7H_2O$, 0.2% NaCl, 0.2% $CaCO_3$.

that two non-sialylated glycoglycerolipids from *Coryneform bacteria* have binding and neutralizing activities against influenza viruses (*33*). Since desialylated glycolipids are not substrates for viral sialidase (*34*, *35*), which is a receptor-destroying enzyme and is integrated into the influenza virus envelope, they might be useful as anti-influenza drugs.

Coryneform bacteria are Gram-positive microorganisms, which are widely distributed in the environment. The aquatic bacterium *Rhodococcus equi*, a strictly aerobic Gram-positive co-bacillus belonging to the Corynebacterium family, is a facultative intracellular bacterium that can cause pneumonia in both young horses and immunocompromised patients (*36*, *37*). We purified a unique phosphatidylinositol (PtdIns) bearing a novel branched-chain fatty acid from an *R. equi* strain S_{420}. This PtdIns bound to all subtypes of influenza virus HA tested and was demonstrated to significantly inhibit virus-mediated hemolysis and viral growth in tissue culture.

EXPERIMENTAL PROCEDURES

Preparation of Lipids from the R. equi Strain S_{420}—From 2,000 aquatic soil samples taken in Japan, 257 bacteria were isolated that produced detectable levels of glycolipids, of which 27 strains were selected because of their high productivity as described previously (*33*). In *R. equi* strain S_{420} (Table I), we identified a virus-binding substance upon screening by a thin layer chromatography (TLC)/virus-binding immunostaining method as described previously (*24*).

R. equi strain S_{420} was cultivated in a 250-ml Erlenmeyer flask containing 30 ml of YMPG medium [0.3% yeast extract (Difco), 0.3% malt extract (Difco), 0.5% polypepton (Nihon Pharmaceutical), 1% glucose, pH 7.0] for 20 h at 28°C on a rotary shaker (220 rpm with a 50-mm stroke). An aliquot (0.7 ml) was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of YSGG medium [0.4% yeast extract, 0.1% soy flour, 1% glucose, 2% glycerol, 0.1% K_2HPO_4, 0.1% $MgSO_4$–$7H_2O$, 0.2% NaCl, 0.2% $CaCO_3$] and cultivated for 96 h at 28°C. The culture was extracted with an equal volume of a chloroform/methanol (1:1, v/v) solvent. The extract was used for this experiment.

TABLE I. **Microbiological properties of the *R. equi* strain S_{420}.**

	Strain S_{420}
Colony	
Shape	Flat and round
Tone	Buttery, opaque, and beige
Cell shape	
Shape	Long-rod
Width	0.8 μm
Moving	−
Gram-staining	+
Spore formation	−
Enzyme properties	
Pyrazinamidase	+
Pyrrolidonearylamidase	−
Alkaline phosphatase	+
Catalase	+
β-Glucuronidase	−
β-Galactosidase	−
β-Glucosidase	+
N-Acetyl-β-glucosaminidase	−
Urease	+
Esculin hydrolysis	+
Gelatin hydrolysis	−
Nitrate reduction	−
Fermentation ability	
Glucose	−
Ribose	−
Xylose	−
Mannitol	−
Maltose	−
Lactose	−
Sucrose	−
Glycogen	−

Total lipids from strain S_{420} were fractionated by Q-Sepharose column chromatography (*38*). The developing solvent was a mixture of chloroform/methanol/water (60:35:8, v/v/v). Lipids were separated on a TLC plate. The virus-binding substance was purified by LH-20 column chromatography (*39*).

Identification of the Virus-Binding Substance—^1H and ^{13}C NMR spectra were recorded on a JEOL GSX500 (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR). FABMS and HRFABMS were obtained on a JEOL SX102A spectrometer (glycerol as matrix). Optical rotation was measured on a JASCO DIP-370 digital polarimeter.

Fatty acid analysis: 10% sodium methylate (0.6 m) was added to the sample (0.1 mg) in 0.2 ml of benzene and the reaction mixture was held at 60°C for 20 min. After the addition of a few drops of 1 N AcOH to stop the reaction followed by 2 ml of distilled water, the reaction mixture was extracted with 2 ml of *n*-hexane. The *n*-hexane extract was evaporated to dryness, and the residue was subjected to fatty acid analysis by gas chromatographic mass spectrometry (GC-MS). The conditions for GC were: column, J & W Scientific DB-1 (0.25 mm by 15 m); column temperature, 100–270°C, raised at 20°C/min; injection temperature, 280°C; carrier gas, He, 1.2 kg/cm.

Viruses and Antibodies—Influenza viruses from human isolates [A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2), A/Aichi/2/68 (H3N2), B/Lee/40, and B/Bangkok/163/90 strains], swine isolates [A/swine/Hokkaido/2/81 (H1N1) and A/swine/Italy/309/83 (H3N2) strains] and avian isolates [A/duck/HK/36/76 (H1N1), A/duck/HK/273/78 (H2N2), A/duck/HK/24/76 (H3N2), A/duck/HK/849/80 (H4N1) A/duck/HK/313/78 (H5N3) A/duck/HK/13/76 (H6N1) A/duck/HK/47/76 (H7N2), A/duck/HK/86/76 (H9N2), A/duck/HK/33/76

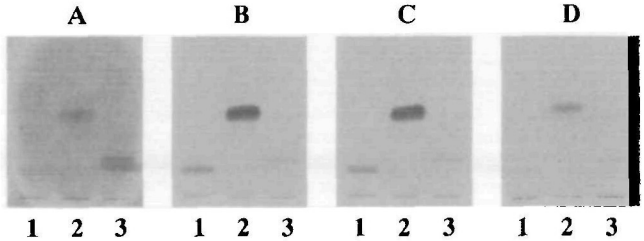

Fig. 1. Binding specificity of the purified PtdIns to human influenza A viruses. The binding specificity of the PtdIns isolated from the aquatic bacterium *R. equi* strain S_{420} (2 nmol) (lane 2) to human influenza A viruses [A/PR/8/34 (H1N1) (A), A/Singapore/1/57 (H2N2) (B), and A/Aichi/2/68 (H3N2) (C)] was determined by TLC/virus-binding immunostaining assay as described under "EXPERIMENTAL PROCEDURES." TLC plate was visualized with orcinol-H_2SO_4 reagent (D). IV^6Neu5AcnLc4Cer (lane 1) and IV^3Neu5AcnLc4Cer (lane 3) (1 nmol) were used as positive controls.

(H10N1), A/duck/HK/44/76 (H11N3) and A/duck/HK/862/80 (H12N5) strains] were used for this study. Virus strains were propagated in the allantoic cavity of 11-day-old chicken eggs for 48 h at 35°C and purified by sucrose density gradient centrifugation (*24*, *40*, *41*). To obtain bromelain-cleaved HA (BHA), purified virus [A/Aichi/2/68 (H3N2) strain] was digested with bromelain and purified as described previously (*42*, *43*). Rabbit anti-influenza virus antibodies were raised by immunization with various strains grown in eggs as described previously (*18*, *44*). Monoclonal anti-HA (H3 subtype) antibody (MAb 2E10) was propagated as described previously (*45*).

Phospholipids and Glycolipids—L-α-PtdIns [1,2-diacyl-*sn*-glycero-3-phospho-(1-D-myo-inositol)] from bovine liver (Sigma P-8443) or from soybean (Sigma P-5954), L-α-glycerophospho-D-myo-inositol from soybean (Sigma G-1891), and sialylphosphatidylethanolamine derivative (Neu5Ac-PE) (*24*) were used this study as controls. IV^{3}Neu5AcnLc4Cer (Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal-β1-4Glcβ1-ceramide) and IV^{6}Neu-5AcnLc4Cer (Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide) were prepared from human erythrocytes (*46*) and from human meconium (*47*), respectively.

Virus-Binding Immunostaining Assay—Lipids (1–2 nmol) were spotted on silica gel plastic plates (Polygram Sil G; Macherey-Nagel, Germany). Immunochemical detection of virions or BHA on the TLC plates was performed as described previously (*18*, *24*). The binding of lipids to influenza virus was carried out at 4°C by incubation overnight (over 12 h). In the inhibition assay for virus binding, viruses were preincubated with inhibitors for 1 h at 4°C and then submitted to the binding assay.

Hemagglutination and Hemolysis Inhibition Assays—Hemagglutination and hemolysis inhibition assays were performed as described previously (*24*, *41*, *48*, *49*).

Neutralization Assay—The neutralization of the PtdIns on human influenza A virus infection to Madin Darby canine kidney (MDCK) cells was detected as described previously (*50*, *51*).

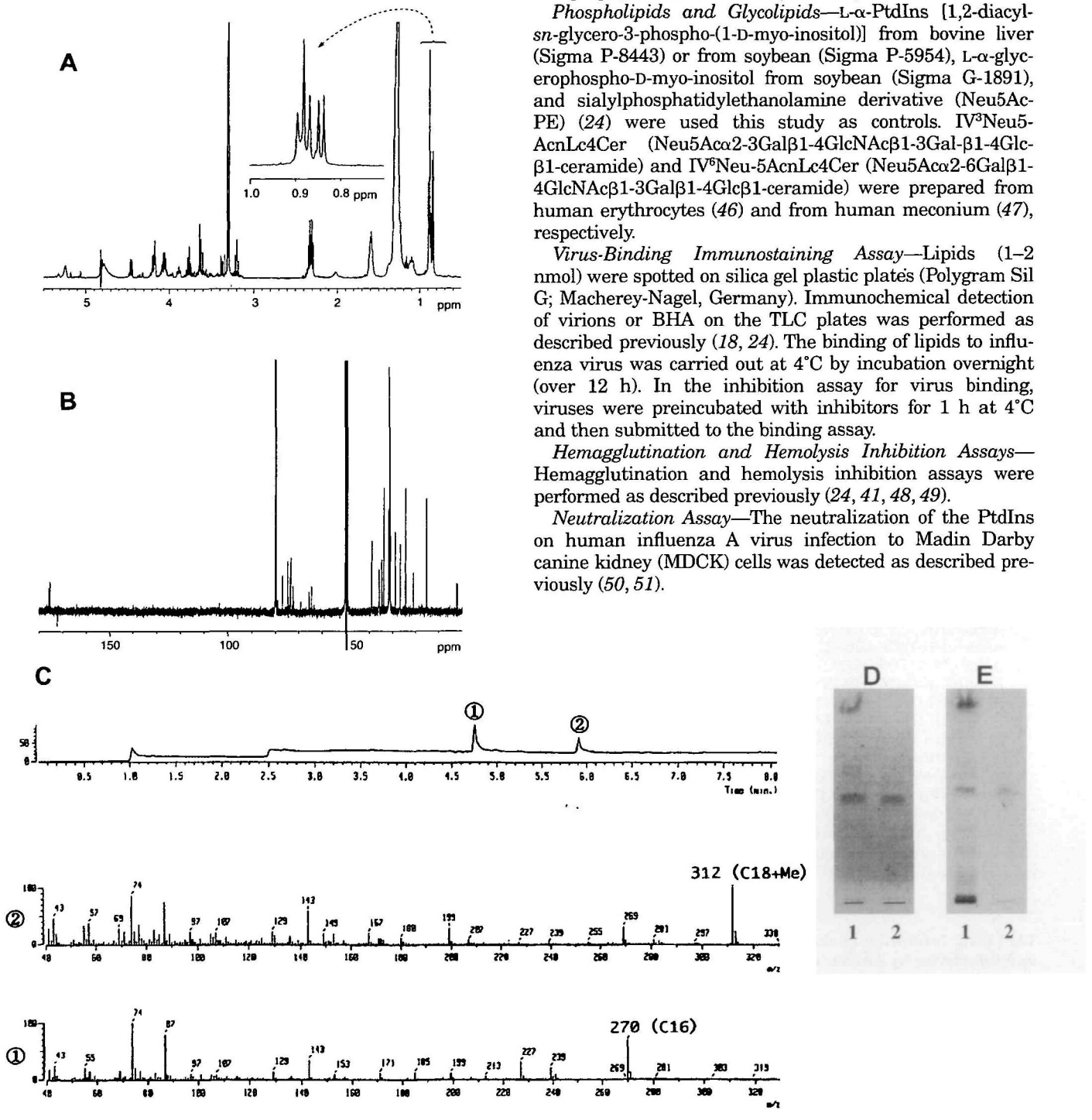

Fig. 2. **A: An ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectrum of the PtdIns from the *R. equi* strain S_{420}. B: A ^{13}C-detected multiple-bond heteronuclear multiple quantum coherence spectrum of the PtdIns from the *R. equi* strain S_{420}. C: A gas chromatography–electron impact mass spectrum of the PtdIns from the *R. equi* strain S_{420}. D and E: Profile of phospholipids and total glycolipids from the *R. equi* strain S_{420}.** Profiles of phospholipids (plate D) and glycolipids (plate E) were visualized with Dittmer's reagent and orcinol-H_2SO_4 reagent, respectively. Lane 1 is total lipids and lane 2 is the purified PtdIns (2 nmol).

RESULTS

Presence of a Substance That Binds to Influenza Viruses in the R. equi Strain S_{420}—From 2,000 aquatic soil samples taken in Japan, 257 bacteria were isolated that produced detectable levels of glycolipids, of which 27 strains were selected because of their high productivity. The TLC-separated glycolipids were assayed for binding activity to human influenza viruses. *R. equi* strain S_{420} showed one active spot on all H1 (A/PR/8/34 strain), H2 (A/Singapore/1/57 strain), and H3 (A/Aichi/2/68 strain) subtypes of human influenza A viruses (Fig. 1).

Structure of the Virus-Binding Substance—The virus-binding substance was positive in Dittmer's test (*50*), indicating the presence of a phosphate group. Figure 2A shows ^{1}H NMR ($CD_3OD/CDCl_3$, 10:1, v/v): δ_H 0.84 (3H, d, J = 6.4 Hz, CH_3), 0.88 (6H, t, J = 6.9 Hz, $CH_3 \times 2$), 1.10 (2H, m, CH_2), 1.27 (41H, m, CH and $CH_2 \times 20$), 1.30 (2H, m, CH_2), 1.35 (4H, m, $CH_2 \times 2$), 1.59 (4H, m, $CH_2 \times 2$), 2.30 (2H, t, J = 7.3 Hz, CH_2-C=O), 2.33 (2H, t, J = 7.3 Hz, CH_2-C=O), 3.20 (1H, t, J = 9.4 Hz, 5′-H), 3.37 (1H, dd, J = 9.4 and 3.0 Hz, 3′-H), 3.62 (1H, t, J = 9.4 Hz, 4′-H), 3.76 (1H, t, J = 9.4 Hz, 6′-H), 3.89 (1H, Br, t, J = 9.4 Hz, 1′-H), 4.06 (2H, m, 3-H_2), 4.17 (1H, t, J = 3.0 Hz, 2′-H), 4.20 (1H, dd, J = 12.2 and 7.0 Hz, 1-H), 4.45 (1H, dd, J = 12.2 and 3.1 Hz, 1-H), 5.24 (1H, m, 2-H). Figure 2B shows ^{13}C NMR (CD_3OD/

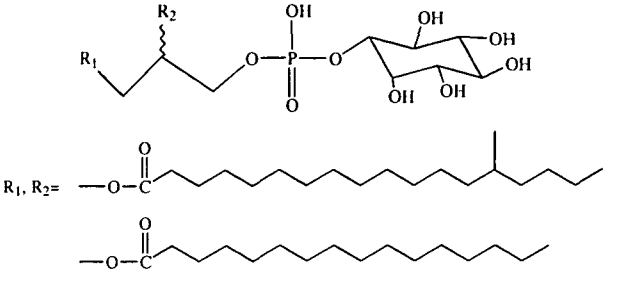

Fig. 3. **Structure of the PtdIns from the *R. equi* strain S_{420}.**

TABLE II. **Binding reactivities of human, avian, and swine isolates of influenza virus to the purified PtdIns from the *R. equi* strain S_{420}.**

Influenza virus	Relative binding reactivity (%)		
	IV^{3}Neu5AcnLc4Cer	IV^{6}Neu5AcnLc4Cer	PtdIns
Human isolates			
A/PR/8/34 (H1N1)	100	12 ± 6	26 ± 6
A/Singapore/1/57 (H2N2)	21 ± 4	100	43 ± 5
A/Aichi/2/68 (H3N2)	26 ± 5	100	48 ± 7
B/Lee/40	16 ± 2	100	28 ± 5
B/Bangkok/163/90	18 ± 8	100	31 ± 6
Avian isolates			
A/duck/HK/36/76 (H1N1)	100	42 ± 8	51 ± 8
A/duck/HK/273/78 (H2N2)	100	13 ± 5	19 ± 6
A/duck/HK/24/76 (H3N2)	100	10 ± 3	38 ± 6
A/duck/HK/849/76 (H4N1)	100	16 ± 8	34 ± 7
A/duck/HK/313/76 (H5N3)	100	56 ± 6	31 ± 6
A/duck/HK/13/76 (H6N1)	100	15 ± 6	36 ± 5
A/duck/HK/47/76 (H7N2)	100	22 ± 9	33 ± 7
A/duck/HK/86/76 (H9N2)	100	32 ± 6	40 ± 5
A/duck/HK/33/76 (H10N1)	100	28 ± 5	33 ± 5
A/duck/HK/44/76 (H11N3)	100	21 ± 4	30 ± 8
A/duck/HK/862/80 (H12N5)	100	14 ± 2	37 ± 7
Swine isolates			
A/swine/hokkaido/2/81 (H1N1)	13 ± 5	100	33 ± 5
A/swine/Italy/309/83 (H3N2)	15 ± 3	100	31 ± 7

The binding activities of human, avian, and swine isolates of influenza virus to purified PtdIns (1 nmol) were determined using a TLC/virus-binding assay as described under "EXPERIMENTAL PROCEDURES." IV^{3}Neu5AcnLc4Cer (1 nmol) and IV^{6}Neu5AcnLc4Cer (1 nmol) were used as positive controls. The values are expressed as the mean ± SD of three independent experiments and as a percentage of that of IV^{3}Neu5AcnLc4Cer and IV^{6}Neu5AcnLc4Cer.

TABLE III. **Inhibition of the purified PtdIns from the *R. equi* strain S_{420} on viral hemagglutination, virus-induced hemolysis, and infectivity by human influenza A viruses.**

Viruses	Inhibition activity of purified PtdIns			Inhibition activity of Neu5Ac-PE		
	HAI	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)	HAI	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)
A/PR/8/34 (H1N1)	2	50.3 ± 7.2	20.1 ± 2.3	<2	>1,000	>1,000
A/Singapore/1/57 (H2N2)	2	42.4 ± 5.6	14.7 ± 3.2	32	158.5 ± 7.4	94.7 ± 5.2
A/Aichi/2/68 (H3N2)	4	38.9 ± 4.9	10.4 ± 4.1	256	142.3 ± 8.2	64.3 ± 7.8

Inhibition activities of the purified PtdIns on viral hemagglutination, virus-induced hemolysis and infectivity of human influenza A viruses were detected as described under "EXPERIMENTAL PROCEDURES." The maximum dilution of the PtdIns (starting concentration, 1 mM) showing complete inhibition of hemagglutination (HAI) is defined as the hemagglutination inhibition titer. The inhibition activity of the PtdIns against viral hemolysis or infection of MDCK cells by viruses is expressed as IC_{50} (the concentration at which the hemolysis or the infection was inhibited by 50 or 50% of infection cells were inhibited). The values are expressed as the mean ± SD of three independent experiments. Each experiment was carried out in duplicate. Neu5Ac-PE, which is a sialylphosphatidylethanolamine derivative (*24*), was used as a control.

TABLE IV. **Influence of fatty acid residues of PtdIns to viral hemagglutination, virus-induced hemolysis, and influenza virus infection.**

Phosphatidylinositols	Fatty acid residue	Binding activities	Inhibition activity		
			HAI	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)
PtdIns from strain S_{420}	14-Methyloctadecanoic acid and palmitic acid	43 ± 5	2	38.9 ± 4.9	10.4 ± 4.1
PtdIns from bovine liver	Stearic acid and arachidonic acid	41 ± 6	2	578.4 ± 5.6	360.7 ± 5.2
PtdIns from soybean	Palmitic acid and linoleic acid	39 ± 7	2	616.4 ± 9.7	396.1 ± 8.3
Glycerophosphatidylinositol from soybean	No fatty acid residue	NB*	<2	>1,000	>1,000

Inhibition activities of PtdIns containing different fatty acid residues on viral hemagglutination, virus-induced hemolysis and infection of MDCK cells by human influenza A virus (A/Aichi/2/68 strain) to MDCK cells were detected as described under "EXPERIMENTAL PROCEDURES." The maximum dilutions of samples (starting concentration, 1 mM) showing complete inhibition of hemagglutination (HAI) are defined as the hemagglutination inhibition titer. The inhibitory activities of samples against viral hemolysis or infection of MDCK cells are expressed as IC_{50} (the concentration at which the hemolysis or the infection was inhibited by 50%, or 50% of infected cells were inhibited). The values are expressed as the mean ± SD of three independent experiments. Each experiment was carried out in duplicate. *NB, no bound.

$CDCl_3$, 10:1, v/v): δ_C 15.3 (2C), 21.0, 24.4 (2C), 26.7 (2C), 30.9–31.8 (19C), 33.8 (2C), 34.6, 35.9 (2C), 38.9 (2C), 64.5 (C-1), 65.6 ($J_{C\text{-}P}$ = 5.7 Hz, C-3), 72.6 ($J_{C\text{-}P}$ = 7.6 Hz, C-2), 73.5 (C-3′), 73.7 (br, C-2′), 73.9 ($J_{C\text{-}P}$ = 5.7 Hz, C-6′), 74.7 (C-4′), 76.9 (C-5′), 79.0 (br, C-1′), 175.4, 175.7. (+)FABMS *m/z*: 875 $[M+Na]^+$, 593. (−)FABMS *m/z*: 851 $[M-H]^-$, 689. (−)HRFABMS *m/z* 851.5666 $[M-H]^-$ (Calcd. for $C_{44}H_{84}O_{13}P$: 851.5650).

The compound showed a pseudomolecular ion peak at *m/z* 851, matching $C_{44}H_{85}O_{13}P$ (HRFABMS: *m/z* 851.5666 $[M-H]^-$, calcd. for $C_{44}H_{85}O_{13}P$: 851.5650). Both ^1H and ^{13}C NMR spectra (Fig. 2, A and B), together with a DEPT spectrum, revealed that the substance contains two long-chain acyl groups, two oxygenated methylenes, and seven oxygenated methines. The ^1H-^1H COSY and 1D homonuclear Hartman-Hahn (HOHAHA) spectra indicated the presence of a cyclitol moiety and a glycerol unit. The cyclitol was shown to be *myo*-inositol on the basis of the ^1H vicinal coupling constants of H-1′ to H-6′ [J(1′/2′) = 3.0 Hz, J(2′/3′) = 3.0 Hz, J(3′/4′) = 9.4 Hz, J(4′/5′) = 9.4 Hz, J(5′/6′) = 9.4 Hz, J(6′/1′) = 9.4 Hz]. The phosphate was determined to be attached at C-3 and C-1′ on the basis of the ^{13}C-P coupling constants of C-2 (7.6 Hz), C-3 (5.7 Hz), C-1′ (<2 Hz), C-2′ (<2 Hz), C-6′ (5.7 Hz). The ^1H chemical shifts of the H_2-1 (δ 4.45 and 4.20) and H-2 (δ 5.24) signals indicated that both the C-1 and C-2 positions of the glycerol moiety were acylated.

Treatment of the compound with MeOH/MeONa afforded 16:0 and 19:0 fatty acid methyl esters which showed molecular ion peaks at *m/z* 270 and 312, respectively, in the gas chromatography-electron impact mass spectrum (GC-EIMS) (Fig. 2C). The 16:0 fatty acid methyl ester showed GLC retention time and mass spectrum identical to those of authentic palmitic acid methyl esters. The absence of an ion peak at *m/z* 241, corresponding to the ion $[M-C_5H_{11}]^+$, in the EIMS of the 19:0 fatty acid methyl ester indicated that the 19:0 fatty acid was 14-methyloctadecanoic acid. Thus, the structure of the virus-binding substance was determined to be a PtdIns bearing a branched-chain fatty acid (Fig. 3).

Chemical staining analyses (*52*, *53*) showed that the purified PtdIns was the main lipid (16.5% of total lipids) and was also present as a major phospholipid (about 55% of phospholipids) in the *R. equi* strain S_{420} (Fig. 2, D and E). The purification rate of PtdIns was approximately 60%.

The Binding Specificity of the Purified PtdIns to Influenza Viruses—The binding specificity of the purified PtdIns to influenza viruses was determined using a TLC/virus-binding immunostaining assay. As shown in Table II, all the isolates tested from human (A and B types) and animal (avian and swine) species bound to the purified PtdIns. The binding specificity of various influenza viruses to the purified PtdIns was different from their viral sialic acid–linkage specificity. These findings indicate that the binding of purified PtdIns to viruses was not dependent on the isolate or HA type of the influenza viruses.

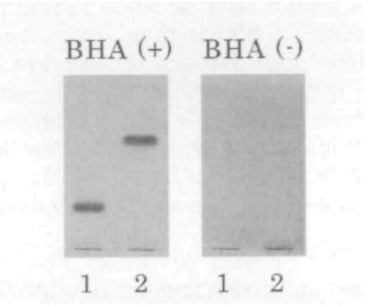

Fig. 4. **Binding of the purified PtdIns to bromelain released ectodomain of influenza hemagglutinin from the A/Aichi/2/68 (H3N2) strain.** The binding of purified PtdIns (2 nmol) (lane 2) to the bromelain-released ectodomain of influenza HA (BHA) from the A/Aichi/2/68 (H3N2) strain was determined using a TLC/HA-binding immunostaining assay as described under "EXPERIMENTAL PROCEDURES." IV^{6}Neu5AcnLc4Cer (1 nmol) (lane 1) was used as a positive control.

Inhibition Activity of the Purified PtdIns on the Virus-Mediated Hemolysis and the Cell Infection by Influenza Viruses—To determine the anti-viral activity of the purified PtdIns, we carried out hemagglutination inhibition, hemolysis inhibition and neutralization assays. As shown in Table III, the purified PtdIns potently reduced the releasing LDH activity of virus-infected MDCK cells in a dose-dependent manner. The IC_{50}s of the purified PtdIns (the concentration inhibiting infection by 50%) for A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2), and A/Aichi/2/68 (H3N2) strains were 20.1 ± 2.3, 14.7 ± 3.2, and 10.4 ± 4.1 μM (n = 3 experiments), respectively. The inhibitory activity of purified PtdIns was about 6-fold stronger than that of Neu5Ac-PE, which was previously indicated as a potent inhibitor against human influenza virus (*24*). Similarly, the inhibitory activity of the purified PtdIns toward virus-mediated hemolysis of erythrocytes was also stronger than that

of Neu5Ac-PE. However, the purified PtdIns exhibited weaker inhibition of viral hemagglutination than Neu5Ac-PE. These findings showed that the purified PtdIns significantly prevented both virus-mediated hemolysis and infection by human influenza A virus *in vitro*, and was effective against influenza A viruses regardless of subtype.

Inhibition Activities of PtdInses Containing Different Fatty Acid Residues—To examine the influence of species of fatty acid residues on viral infection, we tested PtdInses containing three types of fatty acid residues as inhibitors of influenza viruses. As shown in Table IV, the inhibitory activities of PtdInses were significantly different from each other. The effect of the PtdIns from *R. equi* strain S_{420} on virus-mediated hemolysis and cell infection by the influenza virus *in vitro* were 15-fold and 40-fold stronger than those of PtdInses from bovine liver and soybean, respectively. We also used a fatty acid-free PtdIns [*sn*-glycero-3-phosphatidylinositol (GPI)] in these experiments. Although the GPI inhibited viral hemagglutination at a concentration of 1 mM, it did not inhibit either the virus-mediated hemolysis or viral infection of MDCK cells even at a concentration of 2 mM (data not shown). These findings indicated that the fatty acid residue on PtdIns might be indispensable for its inhibition of viral hemolysis and viral replication.

Binding of the Purified PtdIns to Bromelain-Cleaved Hemagglutinin—The above findings indicate that the purified PtdIns prevents influenza virus infection. To clarify the mechanism of its anti-influenza activity, we used the bromelain-cleaved hemagglutinin (BHA) of A/Aichi/2/68 (H3N2) virus instead of virus particles for TLC/virus-binding assay. The binding specificity of PtdIns to BHA on plates was examined using mouse monoclonal anti-HA (H3) antibody and HRP-conjugated goat anti-mouse IgG+IgM (H+L) (Jackson Immunoresearch Laboratories). As shown in Fig. 4, the BHA clearly bound to the purified PtdIns as well as IV^6Neu5AcnLc4Cer. This indicates that HA of influenza virus is involved in the binding to purified PtdIns.

DISCUSSION

In this study, we isolated and purified a unique type of phosphatidylinositol (PtdIns) from *R. equi*, bearing a branched-chain fatty acid (14-methyloctadecanoic acid). This PtdIns was found to inhibit strongly the virus-induced hemolysis and the infection of influenza virus *in vitro*, and thus it represents a new type of anti-influenza compound in microorganisms. In addition, it was shown that PtdIns in the *R. equi* strain S_{420} was the main phospholipid and accounted for about 55% of the total phospholipids, because the proportion of PtdIns in *Corynebacteria* is usually lower than 10% of total phospholipids (*54*, *55*). This creates favorable conditions for opening up new agents against influenza.

The binding of influenza viruses to sialic acid has been shown to depend on the molecular species of the sialic acid, the sialic acid–Gal linkage and the carbohydrate core structure of ganglioside (*19*). Therefore, the anti-influenza effects of the sialic acid-containing inhibitors are usually restricted by these factors. It is a novel finding that the PtdIns from the *R. equi* strain S_{420} binds to influenza viruses and that it significantly inhibits the influenza virus infection *in vitro*. The inhibitory activity of the purified PtdIns on influenza viruses is not restricted to animal species or types (or subtypes) of viral HA. The purified PtdIns from strain S_{420} may be considered a new type of anti-influenza virus agent, widely effective against human and other animal influenza A viruses.

Influenza viruses enter host cells by endocytosis, followed by fusion between the endosomal/lysosomal and the viral membrane. Fusion is mediated by the trimeric integral membrane protein HA and is triggered by the low endosomal/lysosomal pH, which induces a conformational change in the protein (*3–6*). The finding shows that purified PtdIns from strain S_{420} strongly inhibited the virus-mediated hemolysis in comparison with sialic acid–containing inhibitors, or other PtdIns from different materials. We also examined the influence of the purified PtdIns on the binding between virus and sialylparaglobosides, which have been indicated to bind to a sialic acid pocket on the head of influenza viral HA (data not shown). Combine analysis of from the data in Fig. 4, we think that the purified PtdIns attached to viral HA and that the binding position of PtdIns differs from the sialic acid-binding pocket and may be close to the pocket on the head of the HA trimer. Regarding the anti-influenza virus mechanism of the purified PtdIns, one possibility is that PtdIns adheres to the viral HA and then enters the endosome together with the virion, resulting in interference in the fusion of viral and endosomal/lysosomal membranes.

We also used other phospholipids in the same experiment to compare their effects against influenza viruses (Table V). Bovine liver PtdIns, phosphatidylserine (PS,

TABLE V. **Comparison of the effect of various phospholipids on the binding activity to virus, viral hemagglutination, and virus-induced hemolysis.**

Phospholipids	Binding activities (%)*	Inhibition activity	
		Hemagglutination (HAI)**	Hemolysis (IC_{50}, μM)***
PtdIns from bovine liver	89	2	750
PS from bovine brain	56	2	1,000
PE from bovine brain	48	<2	>2.000
PC from bovine liver	NB****	<2	>2,000
SM from bovine brain	NB	<2	>2,000

*Relative binding activities of various phospholipids (1 nmol) to influenza A virus A/Aichi/2/68 (H3N2) were detected using a TLC/virus-binding assay. Their binding activities are expressed as a percentage of the binding activity of the bacterial PtdIns. **Inhibition activities of various phospholipids (starting concentration, 2 mM) on the viral hemagglutination were determined as described under "EXPERIMENTAL PROCEDURES." The maximum dilutions of samples showing complete inhibition of the hemagglutination (HAI) are defined as the hemagglutination titer. ***Inhibition activities of various phospholipids against the virus-induced hemolysis were expressed as IC_{50} (the concentration at which the hemolysis was inhibited by 50%). ****NB, not bound.

from bovine brain, Sigma P-7769) and phosphatidylethanolamine (PE, from bovine brain, Sigma P-7693), all of which carry a negative charge, bound to influenza viruses, but neutral glycophospholipids such as phosphatidylcholine (PC, from bovine liver, Sigma P-6638) and sphingomyelin (SM, from bovine brain, Sigma S-7004) did not. None of PC, PE, and SM showed inhibitory activities toward virus-induced hemolysis at a concentration of 2 mM. Both bovine liver PtdIns and PS did show inhibitory activities, but their effects were much lower than that of the bacterial PtdIns. Adhesion of phospholipids to viral HA may be an indispensable step in preventing influenza virus infection. It is noteworthy that the virus-binding activity of bovine liver PtdIns to influenza viruses was similar to that of purified PtdIns, but the inhibitory activity of bovine liver PtdIns on the influenza virus-mediated fusion was lower than that of the bacterial PtdIns. The structural difference between the bacterial PtdIns and bovine liver PtdIns is that the former bears a branched-chain fatty acid. L-α-Glycerophospho-D-myo-inositol from soybean, which has no fatty acid residue, showed no inhibitory activity of virus-mediated hemagglutination and hemolysis. These findings indicated that the phosphatidylinositol and branched fatty acid residue may be important for the binding of viral HA and for interfering with membrane fusion, respectively, leading to inhibition of infection by influenza viruses. The mechanism of the methyl-branched fatty acid is unclear. One possibility is that it mediates to influence the covalent attachment of fatty acid and to interfere with the palmitoylation of the cysteine residues of viral hemagglutinin, because the cysteine residues in the cytoplasmic tail of hemagglutinin are important for infectious particle formation (*56*).

Some sialic acid-containing derivatives usually inhibit the attachment of viral HA to target cells and/or the enzymatic activity of viral sialidase, thus preventing infection by influenza virus. In contrast, the purified PtdIns from *R. equi* preferentially targets virus-mediated cellular membrane fusion, and the results show the possibility that the bacterial PtdIns binds near the fusion loop of viral HA spikes, resulting in interference with the fusogenic function of viral HA, thus preventing the growth of human influenza A virus.

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