Quercetin modulates activities of Taiwan cobra phospholipase A_2 via its effects on membrane structure and membrane-bound mode of phospholipase A_2

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The goal of the present study is to elucidate the mechanism of quercetin on modulating *Naja naja atra* phospholipase A_2 (PLA_2) activities. Sphingomyelin inhibited PLA_2 enzymatic activity and membrane-damaging activity against egg yolk phosphatidylcholine (EYPC), while cholesterol and quercetin abrogated the sphingomeyelin inhibitory effect. Quercetin incorporation led to a reduction in PLA_2 enzymatic activity and membrane-damaging activity toward EYPC/sphingomyelin/cholesterol vesicles. Both cholesterol and quercetin increased detergent resistance and reduced membrane fluidity of EYPC/sphingomyelin vesicles. Quercetin reduced detergent insolubility but increased ordered lipid packing of EYPC/sphingomyelin/cholesterol vesicles. Acrylamide quenching studies and trinitrophenylation of Lys residues revealed that quercetin altered the membrane-bound mode of PLA_2 differently upon absorption onto the membrane bilayers of different lipid compositions. However, 8-anilinonaphthalene sulphonate-binding assay revealed that quercetin marginally affected the interaction between active site of PLA_2 with phospholipid vesicles. Collectively, our data indicate that membrane-inserted quercetin modulates PLA_2 interfacial activity and membrane-damaging activity via its effects on membrane structure and membrane-bound mode of PLA_2.

[Chiou Y-L, Lin S-R, Hu W-P and Chang L-S 2012 Quercetin modulates activities of Taiwan cobra phospholipase A_2 via its effects on membrane structure and membrane-bound mode of phospholipase A_2. *J. Biosci.* **37** 277-287] **DOI** 10.1007/s12038-012-9198-2

1. Introduction

Flavonoid compounds in vegetable-based diets bring a significant contribution to the role of fruits and vegetables as heath-promoting foods (Boots *et al.* 2008). A number of studies have shown that flavonoid compounds exhibit antioxidant, antimicrobial and anticancer properties (Tsuchiya *et al.* 2002). The effects of flavonoids have been explained by their binding to, or interference with enzymes, receptors, transporters and signal transduction systems, events that frequently occur in lipid membrane environments (Sang *et al.* 2005; Lee *et al.* 2007). Therefore, the bioactivity of flavonoids may be attributed to their capacity to interfere with the membrane physical properties such as membrane fluidity or the formation of lipid raft (Tarahovsky *et al.* 2008; Ulrih *et al.* 2010).

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavanone) is a flavonoid that is largely present in green tea, fruits and vegetables. Previous studies have shown that quercetin increases membrane fluidity of human peripheral blood mononuclear cells (Mihaela *et al.* 2009). Nevertheless, Tsuchiya *et al.* (2002) found that the effect of quercetin (Qct) on membrane fluidity depends on the Qct concentration and lipid compositions. Pawlikowska-Pawlega *et al.* (2003) proposed that Qct modified the properties of erythrocyte membrane, and altered membrane protein–protein and protein–lipid interaction. Moreover, Qct enhanced TRAIL-induced apoptosis by causing the redistribution of death receptors in lipid rafts (Psahoulia *et al.* 2007). Accordingly, it is conceivable that that intercalation of Qct into membrane bilayers perturbs the protein–lipid interaction.

Keywords. Cholesterol; interfacial activity; phospholipase A_2; quercetin; sphingomyelin

Published online: 25 April 2012

The enzyme phospholipase A_2 (PLA_2) catalyses specifically the hydrolysis of fatty acid bonds at position 2 of 1,2-diacyl-*sn*-phosphoglycerides in the presence of Ca^{2+} (Kini 1997). The binding of PLA_2 to the surface of aggregated substrate, such as membranes or micelles, increases substantially the enzyme activity, an effect known as interfacial activation (Gelb *et al.* 1995; Yu *et al.* 2000). Moreover, PLA_2 activity and PLA_2–lipid interaction are regulated by membrane phospholipid compositions, membrane fluidity and membrane-bound mode of PLA_2 (Tatulian 2003; Pande *et al.* 2006; Ray *et al.* 2007; Chiou *et al.* 2010, 2011a, b). Thus, Qct incorporation into lipid bilayers should inevitably alter the interactions of lipids with PLA_2. Several studies showed that Qct protects erythrocytes from free radical- and cigarette tar extract-induced hemolysis via incorporation into the membrane (Begum and Terao 2002; Kitagawa *et al.* 2004). The cholesterol (Chol) content was found to reduce the protective ability of Qct against oxidative stress in erythrocytes (Sanchez-Gallego *et al.* 2010). It appears that phospholipid compositions regulate the biological effect of Qct. The major constituent of erythrocyte membrane is phosphatidylcholine/ sphingomyelin/Chol (Yawata 2003). Our previous studies revealed that incorporation of 30 mol% cholesterol into phosphatidylcholine/sphingomyelin (60/40, mol/mol) induced the lipid domain formation, and that the modulated effect of sphingomyelin and Chol on PLA_2 enzymatic activity was associated with lipid domain formation (Chiou *et al.* 2010, 2011a). To elucidate the Qct effect on PLA_2 interfacial activation, studies on the interaction of *Naja naja atra* (Taiwan cobra) PLA_2 with Qct-containing phosphatidylcholine/sphingomyelin (60/40, mol/mol) and phosphatidylcholine/sphingomyelin/Chol (42/28/30, mol/mol/mol) vesicles were conducted in this study.

2. Materials and methods

PLA_2 from the venom of *N. naja atra* (Taiwan cobra) was isolated as previously described (Chang *et al.* 1998). Qct was purchased from Alexis Biochemicals. Calcein, 8-anilinonaphthalene sulphonate (ANS), egg yolk phosphatidylcholine (EYPC), egg yolk sphingomyelin (EYSM), Chol and trinitrobenzene sulfonate (TNBS) were purchased from Sigma-Aldrich Inc., and dansyl-phosphatidylethanolamine (DPE), 1,2-bis-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (bisPy-PC) and 6-dodecanoyl-2-dimethyl-aminonaphthalene (Laurdan) were products of Molecular Probes. Sepharose 6B was obtained from Amersham Biosciences. All other reagents were products of analytical grade. Without specific indication, EYPC/EYSM (60/40, mol/mol) and EYPC/EYSM/Chol (42/28/30, mol/mol/mol) vesicles with or without 10 mol% Qct were used in this study.

2.1 *PLA_2 activity assay*

PLA_2 activity was measured by a fluorescence assay, using vesicles that contained 5 mol% bisPy-PC. The samples were dissolved in 10 mM Tris–HCl-0.1 M NaCl (pH7.5) containing 5 mM Ca^{2+}. To examine the effect of Chol or Qct on PLA_2 activity, indicated concentrations of Chol (0–30 mol%) or Qct (0–10 mol%) were incorporated into EYPC/EYSM or EYPC/EYSM/Chol vesicles. The vesicles were prepared by extrusion through 100 nm polycarbonate membrane. A fluorescence spectrum of vesicles with bisPy-PC was measured between 360 and 500 nm using an excitation wavelength at 347 nm. All measurements were conducted under constant stirring. The emission spectrum of monomeric pyrene contains two peaks around 378 and 396 nm, whereas the proximity of pyrene moieties results in a strong excimer peak around 470 nm (Ray *et al.* 2007). Lipid hydrolysis by PLA_2 caused a decrease in the excimer signal at 470 nm and increase in the monomer signal at 378 nm and 396 nm due to the two pyrene moieties separated from each other. The activity was calculated as R_t/R_0-1, where R_t is the ratio of fluorescence intensities at 378 and 470 nm at time t, $R_t=(F_{378}/F_{470})_t$, and R_0 is R_t before addition of PLA_2. The initial catalytic rates of PLA_2 at each concentration were obtained by averaging the first two values of $(Rt/R_0-1)/t$, which give the changes in Rt/R_0-1 per minute immediately following combination of PLA_2 with lipid vesicles.

2.2 *Release of entrapped fluorescent markers from liposomes*

The membrane-damaging activity was determined by measuring the release of the liposome-entrapped, self-quenching fluorescent dye calcein according to the procedure described by Chiou *et al.* (2010). Loss of liposome membrane integrity results in dilution of the fluorophore, with a consequent increase in the fluorescence signal. Lipid mixtures with indicated compositions (the same as in section 2.1) were dissolved in chloroform/methanol (v/v, 2:1) and dried by evaporation. Buffer (10 mM Tris–HCl, 100 mM NaCl, 5 mM Ca^{2+}, pH 7.5) containing 50 mM calcein was added to the film of lipids, and after hydration the suspension was shaken vigorously. The multilamellar vesicles obtained in this way were extruded 10 times, above the transition temperature, through a 100 nm polycarbonate filter and applied to a Sepharose 6B column (2×15 cm) to separate the liposome from the free calcein. Leakage was induced by adding aliquots of PLA_2 to a vesicle suspension directly in the cuvette used for fluorescence determination at 30°C. The kinetics of membrane damage was monitored by the increase in fluorescence with emission at 520 nm and excitation at 490 nm, and the signal was expressed as percentage of total calcein release after the addition of 0.2% Triton X-100.

2.3 *Fluorescence energy transfer between Trp residues and DPE*

Binding affinity of PLA_2 for phospholipid vesicle was measured according to changes in fluorescence energy transfer from Trp residues of PLA_2 to DPE. DPE was incorporated at 10 mol% into phospholipid vesicles, which were prepared by extrusion through the 100 nm polycarbonate membrane. The spectrum was excited at 284 nm and fluorescence emission bands around 350 and 510 nm were generated by the Trp and dansyl groups, respectively. Binding of PLA_2 resulted in fluorescence enhancement of DPE due to energy transfer from the donor (the indole group of Trp) to the acceptor (the dansyl group of DPE). A double reciprocal plot of changes in DPE fluorescence intensity (ΔF) versus protein concentration gave lines with a slope corresponding to the dissociation constant (K_d) of PLA_2 for phospholipid vesicles (Bagshaw and Harris 1987).

2.4 *Measurement of lipid phase transition*

Lipid phase transition was determined by measuring the generalized polarization (GP) of Laurdan according to the procedure described by Ray *et al.* (2007). The experiment was performed in 10 mM Tris–HCl-0.1 M NaCl (pH7.5) containing 5 mM Ca^{2+}. Phospholipid vesicles containing 1 mol% Laurdan was excited at 360 nm, and emission spectra were recorded between 380 and 560 nm. Tight packing of lipids minimizes water penetration into the membrane, resulting in a nonrelaxed fluorescence emission peak of Laurdan around 440 nm, and increase in membrane fluidity leads to a rise in the solvent-relaxed peak around 475 nm arising from water penetration. Values of GP were calculated using the equation $GP=(F_{440}-F_{475})/(F_{440}+F_{475})$, where F_{440} and F_{475} are the fluorescence emission intensities at the respective wavelengths.

2.5 *Solubilization of multilamellar lipid vesicles by Triton X-100*

Multilamellar lipid vesicles containing 400 nmol of total lipid were prepared in 800 µl of 10 mM Tris–HCl-100 mM NaCl (pH 7.5) containing 5 mM Ca^{2+} and dispersed in the buffer at 80°C and then cooled to room temperature. For measuring solubilization by the loss of light scattering, the optical density of these samples was measured at 400 nm. Then 42.1 µl of 10% (v/v) Triton X-100/10 mM Tris–HCl-100 mM NaCl (pH 7.5) was added. After mixing and incubation at room temperature for suitable time intervals, the optical density was measured. The ratio of optical density (%OD) after Triton X-100 incubation to that before the addition of Triton X-100 was then calculated.

2.6 *TNBS modification*

PLA_2 (0.71 nmol) in 200 µl of 10 mM Hepes (pH 7.5) containing 5 mM Ca^{2+} or 5 mM EDTA were modified with 10-fold molar excess of TNBS in the absence or presence of 16 nmol phospholipid vesicles. Incorporation of trinitrophenyl group into PLA_2 caused an increase in the absorbance at 345 nm (Chang *et al.* 1997b), and the extent of trnitrophenylation was determined spectrophotometrically based on the molar absorption coefficient of 11,500 M^{-1} cm^{-1} at 345 nm (Chang *et al.* 1997b).

2.7 *Binding of ANS to PLA_2*

All measurements were performed in a total volume of 2 ml of 10 mM Tris–HCl-0.1 M NaCl (pH 7.5) containing 5 mM Ca^{2+} or 5 mM EDTA. An excitation wavelength at 360 nm was used, and fluorescence measurement was monitored at 450 nm. Titrations with ANS were made manually by the addition of small aliquots of a concentrated ANS solution to a solution of PLA_2 or the mixtures of PLA_2 and phospholipid vesicles.

2.8 *Acrylamide quenching*

Quenching experiments were performed at an excitation wavelength of 295 nm to ensure selective excitation of the Trp residues. PLA_2 (2.16 µM) incubated with lipid vesicles (48.84 µM) in 10 mM Tris–HCl-0.1 M NaCl (pH7.5) containing 5 mM Ca^{2+} or 5 mM EDTA The fluorescence intensity was monitored at the emission maximum, and quenched by the progressive addition of small aliquots of stock quenching solution (5 M acrylamide) to the 1 cm fluorescence cuvettes. For a simple, single fluorophore or homogeneous system, fluorescence quenching was analysed according to the Stern–Volmer equation: $F_o/F=1+K_{sv}[Q]$ where F_o is the fluorescence in the absence of quencher, F is the fluorescence at molar quencher concentration [Q], and K_{sv} is the Stern–Volmer quenching constant obtained from the slope of a plot of F_o/F versus [Q] (Lakowicz, 1999). A modified Stern–Volmer equation $F_o/(F_o-F)=1/[Q]faK_{sv}+1/fa$ was used to obtain the value of *fa*, the fraction accessible fluorescence.

2.9 *Statistical analysis*

All data are presented as mean±SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $P<0.05$ was taken as an indication of statistical significance.

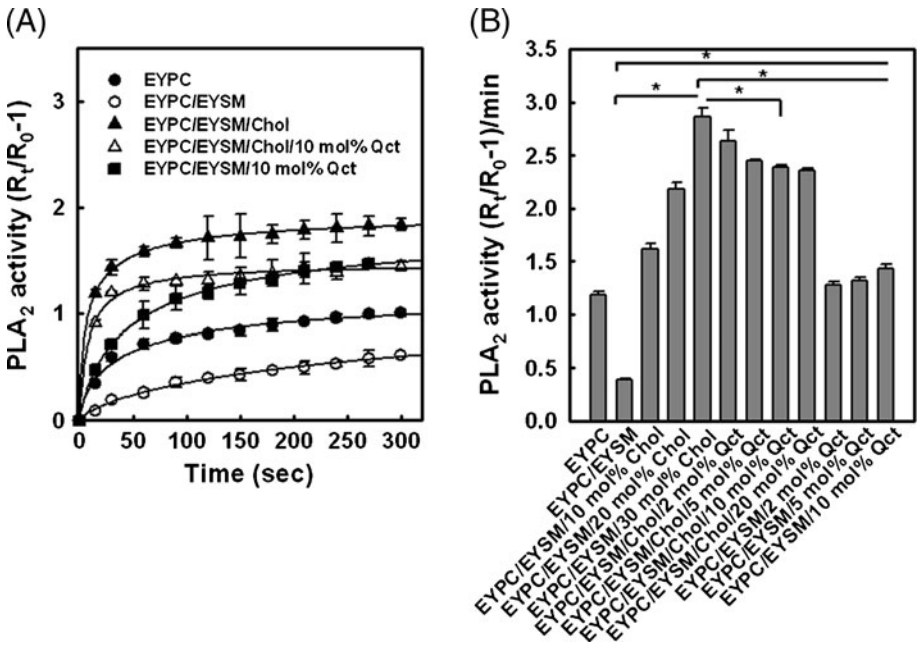

Figure 1. Effect of Qct on enzymatic activity of PLA_2. PLA_2 (0.36 μM) were incubated with bisPy-PC-containing phospholipid vesicles (8.14 μM) for indicated time periods. PLA_2 activity was determined according to the procedure described in the section of Materials and methods. (**A**) PLA_2 hydrolysed time-dependently pyrene-labelled substrate, bisPy-PC. (**B**) Effect of Qct on PLA_2 activity toward EYPC/EYSM and EYPC/EYSM/Chol vesicles. All data are presented as mean±SD (*P<0.05).

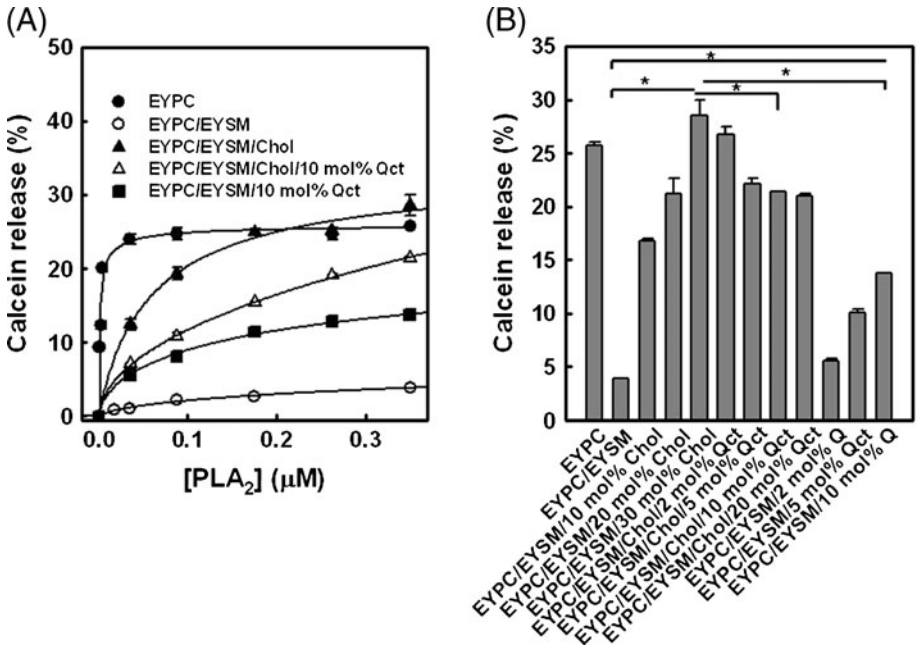

Figure 2. Membrane-damaging activity of PLA_2. (**A**) PLA_2 induced calcein release from phospholipid vesicles in a concentration-dependent manner. The used concentration of lipid vesicles was 8.14 μM. (**B**) Effect of Qct on membrane-damaging activity of PLA_2 toward EYPC/EYSM and EYPC/EYSM/Chol vesicles. The used concentrations of protein and lipid vesicles were 0.36 μM and 8.14 μM, respectively. All data are presented as mean±SD (*P<0.05).

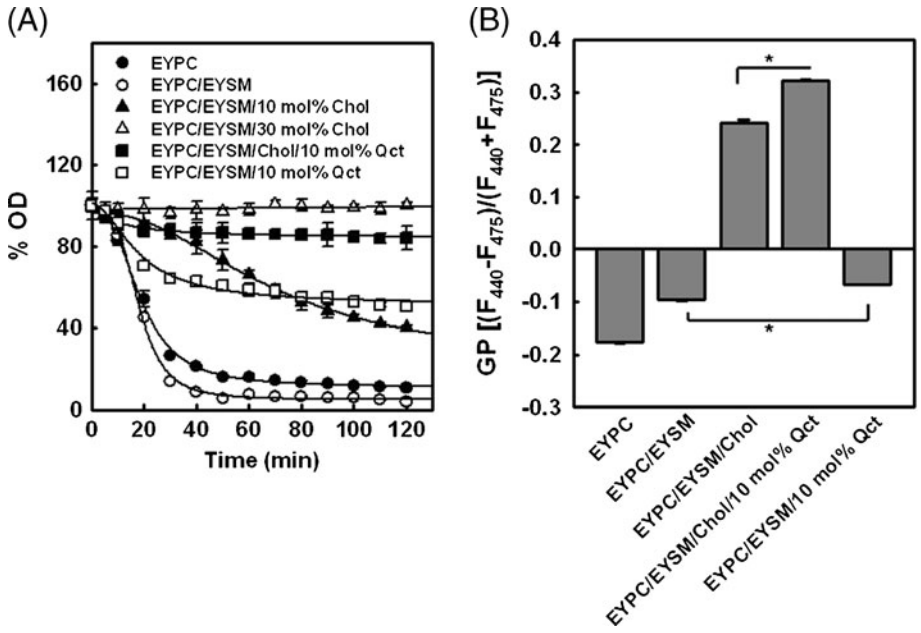

Figure 3. Effect of Qct on detergent resistance and membrane fluidity of EYPC/EYSM and EYPC/EYSM/Chol vesicles. (**A**) Solubilization of lipid mixtures by Triton X-100 as detected by optical density (%OD). Total lipid concentration was 0.475 mM and Triton X-100 concentration was 0.5% (v/v). (**B**) Effect of Qct on membrane fluidity of EYPC/EYSM and EYPC/EYSM/Chol vesicles. Laurdan was excited at 360 nm, and emission spectra were recorded between 380 and 560 nm. Values of GP were calculated as GP=$(F_{440}-F_{475})/(F_{440}+F_{475})$, where F_{440} and F_{475} are the fluorescence emission intensities at the respective wavelengths. All data are presented as mean±SD (*$P<0.05$).

3. Results and discussion

As shown in figure 1A, a plot of the change in PLA_2 activity (R_t/R_0-1) versus time revealed that PLA_2 displayed enzymatic activity toward bisPy-PC in lipid vesicles, and the curve were linear within 30 s after the addition of PLA_2 into lipid vesicles. The initial rate of PLA_2 activity toward bisPy-PC was presented as the change in R_t/R_0-1 per minute. Figure 1B shows that the suppressed effect of sphingomyelin-inhibited PLA_2 activity was relieved by

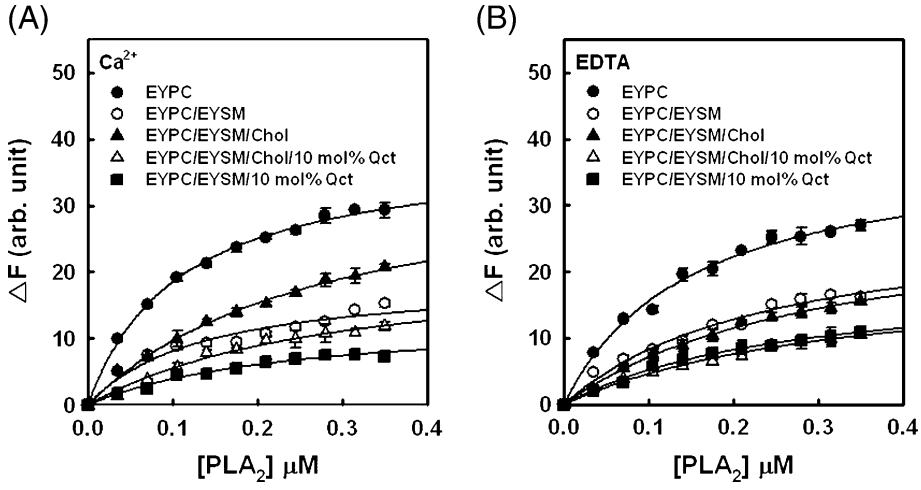

Figure 4. The binding capability of Ca^{2+}-bound and metal-free PLA_2 with lipid vesicles. The experiments were performed in 10 mM Tris–HCl-0.1 M NaCl (pH 7.5) containing (**A**) 5 mM Ca^{2+} or (**B**) 5 mM EDTA. Binding of PLA_2 with phospholipid vesicles enhanced the fluorescence intensity of DPE. The used protein and lipid concentrations were 0.36 μM and 8.14 μM, respectively. Fluorescence emission intensity at 510 nm was recorded. All data are presented as mean±SD.

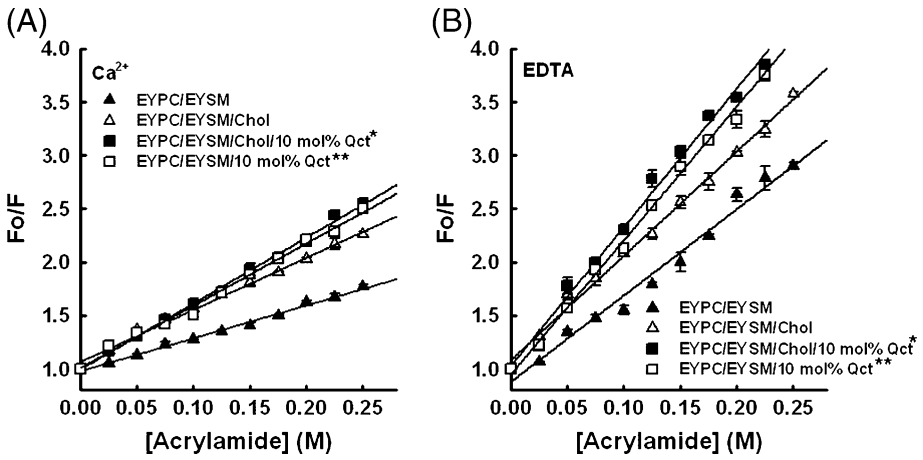

Figure 5. The susceptibility of Trp residues in PLA_2 and membrane-bound PLA_2 for acrylamide quenching. The used protein and lipid concentration was 2.16 μM and 48.84 μM, respectively. The experiments were performed in 10 mM Tris–HCl-0.1 M NaCl (pH 7.5) containing (**A**) 5 mM Ca^{2+} or (**B**) 5 mM EDTA (*P<0.05, EYPC/EYSM/Chol/Qct vesicles vs EYPC/EYSM/Chol vesicles; **P<0.05, EYPC/EYSM/Qct vesicles vs EYPC/EYSM vesicles).

Chol incorporation. Consistently, previous studies suggested that the lipid raft formation by sphingomyelin and Chol abrogated the inhibitory capability of sphingomyelin on PLA_2 activity (Chiou *et al.* 2010). Qct incorporation reduced significantly PLA_2 activity toward EYPC/EYSM/Chol vesicles, and maximal reduction in PLA_2 activity was noted when the Qct concentration was higher than 10 mol% of total lipids. Figure 1B shows that PLA_2 increased bisPy-PC hydrolysis in EYPC/EYSM/Qct vesicles compared with that in EYPC/EYSM vesicles. The PLA_2 enzymatic activity toward EYPC/EYSM/Qct was notably lower than that toward EYPC/EYSM/Chol. Previous studies suggested that the effects exerted by flavonoids on membrane fluidity resemble those of Chol (Hendrich 2006). Moreover, flavonoid molecules potentially initiated the formation of raft-like domains when they were located in the hydrophobic region of the bilayer (Tarahovsky *et al.* 2008). Thus, it was likely that Qct acted as Chol on reducing the PLA_2 activity-inhibitory action of sphingomyelin via the formation of lipid raft-like domains.

As shown in figure 2A, PLA_2 induced membrane leakage of lipid vesicles in a dose-dependent manner and the maximal membrane-damaging activity of PLA_2 was observed at a concentration of 0.36 μM. Figure 2B shows that the maximal calcein release against EYPC and EYPC/EYSM vesicles induced by 0.36 μM NNA-PLA_2 was 25.75±0.43% and 3.89±0.06%, respectively. Chol incorporation into EYPC/EYSM vesicles relieved the EYSM inhibitory effect on PLA_2 membrane-damaging activity. Qct incorporation reduced PLA_2 membrane-damaging activity toward EYPC/EYSM/Chol vesicles. PLA_2 showed a higher membrane-damaging activity on EYPC/EYSM/Qct vesicles compared with that on EYPC/EYSM vesicles. Obviously, Qct exerted different effect on PLA_2 activities toward EYPC/EYSM and EYPC/EYSM/Chol vesicles.

Figure 3A shows that EYPC and EYPC/EYSM phospholipid vesicles were solubilized in the presence of Triton X-100. Chol or Qct incorporation increased detergent resistance of EYPC/EYSM vesicles. The detergent insolubility of EYPC/EYSM/Chol vesicles was indicative of domain formation in an ordered state (Xu and London 2000; Xu *et al.* 2001; Chiou *et al.* 2010). However, Qct incorporation reduced slightly the detergent resistance properties of EYPC/EYSM/Chol vesicles, suggesting that a modulated effect of Qct on physical properties of membrane bilayers depended on the phospholipid compositions.

The membrane fluidity of EYPC, EYPC/EYSM, EYPC/EYSM/Chol, EYPC/EYSM/Chol/Qct and EYPC/EYSM/Qct was examined using Laudran fluorescence. As shown in

Table 1. Acrylamide quenching parameters of Trp fluorescence in PLA_2-phospholipid complexes

	K_{sv} (M^{-1})	f_a
Ca^{2+}-bound PLA_2		
EYPC/EYSM	3.00±0.07	1.07±0.01
EYPC/EYSM/Chol	4.93±0.11	0.99±0.07
EYPC/EYSM/Chol/10 mol% Qct	6.20±0.10	0.97±0.02
EYPC/EYSM/10 mol% Qct	5.93±0.10	0.98±0.05
Metal-free PLA_2		
EYPC/EYSM	8.03±0.26	0.97±0.03
EYPC/EYSM/Chol	9.78±0.20	1.03±0.04
EYPC/EYSM/Chol/10 mol% Qct	13.00±0.21	1.04±0.04
EYPC/EYSM/10 mol% Qct	12.57±0.22	0.99±0.07

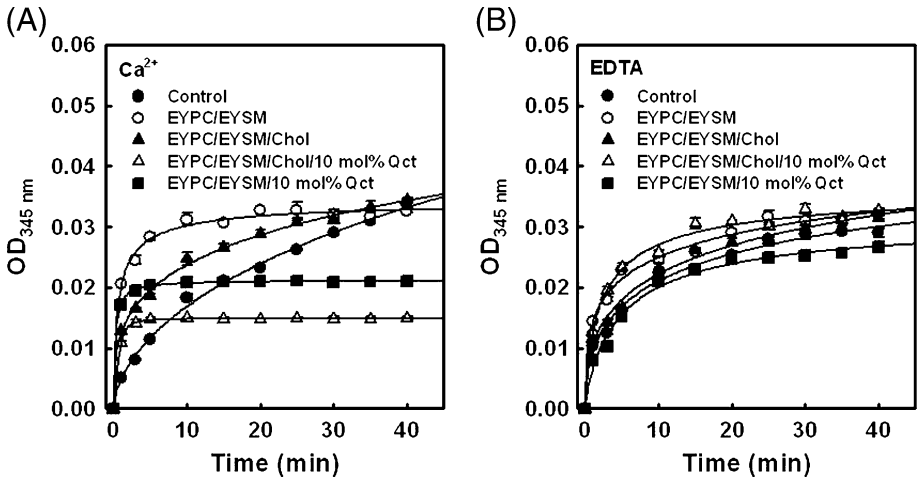

Figure 6. Effect of Qct on trinitrophenylation of membrane-bound PLA_2. PLA_2 (0.71 nmole) was dissolved in 200 µl of 10 mM Hepes (pH 7.5) containing (**A**) 5 mM Ca^{2+} or (**B**) 5 mM EDTA. Modification of PLA_2 with TNBS was conducted in the absence (control) or presence of phospholipid vesicles. The values represent the average of three independent experiments.

figure 3B, membrane fluidity of EYPC, EYPC/EYSM and EYPC/EYSM/Qct vesicles was higher than that of EYPC/EYSM/Chol and EYPC/EYSM/Chol/Qct. Compared with that of EYPC/EYSM/Chol vesicles, Qct incorporation reduced slightly membrane fluidity of EYPC/EYSM vesicles. These findings suggested that Qct-induced ordered lipid packing did not correlate with the differential effect of Qct on PLA_2 activities on EYPC/EYSM and EYPC/EYSM/Chol vesicles.

As shown in figure 4A and B, the binding of PLA_2 with DPE-containing phospholipid vesicles resulted in fluorescence enhancement of the dansyl group in DPE. The binding affinity of PLA_2 for phospholipid vesicles was calculated according to changes in dansyl group fluorescence intensity. The binding affinity (K_d) of Ca^{2+}-bound PLA_2 for EYPC, EYPC/EYSM, EYPC/EYSM/Chol, EYPC/EYSM/Chol/Qct and EYPC/EYSM/Qct vesicles was 0.11±0.01, 0.13±0.04, 0.28±0.03, 0.29±0.06 and 0.23±0.04 µM, respectively; the binding affinity (K_d) of metal-free PLA_2 was 0.15±0.02, 0.24±0.05, 0.31±0.03, 0.34±0.06 and 0.27±0.04 µM, respectively. It appeared that Qct did not greatly affect the binding capacity of Ca^{2+}-bound and metal-free PLA_2 with phospholipid vesicles.

Figure 5A and B shows that the intrinsic fluorescence of EYPC/EYSM/Chol-, EYPC/EYSM/Chol/Qct-, EYPC/EYSM- and EYPC/EYSM/Qct-bound PLA_2 was differently quenched by acrylamide either in the presence or absence of Ca^{2+}. Compared with those of EYPC/EYSM- or EYPC/EYSM/Chol-bound PLA_2, Trp residues of EYPC/EYSM/Qct- or EYPC/EYSM/Chol/Qct-bound PLA_2 showed a higher accessibility for acrylamide regardless of the absence or presence of Ca^{2+}. Using Stern–Volmer plots or modified Stern–Volmer plots, the quenching constant (K_{sv}) and the effective fraction of Trp fluorescence most exposed to acrylamide (*fa*) were obtained (table 1). The accessible class (*fa*) of Trp residues in Ca^{2+}-bound and metal-free PLA_2 was approximately one. Consistently, the linearity of Stern–Volmer plots for Ca^{2+}-bound and metal-free PLA_2 indicated an equal accessibility of all Trp residues in PLA_2 (figure 5A and B). These results suggested the notion that PLA_2 had different membrane-bound mode upon absorption on water–lipid interface of EYPC/EYSM/Chol and EYPC/EYSM vesicles, and Qct further altered the interacted mode of PLA_2 with the phospholipid vesicles.

Previous studies showed that Lys-6, Lys-65 and Lys-115 of PLA_2 were subjected to modification with TNBS (Chang *et al.* 1997b), and conformational changes in PLA_2 induced by the binding of divalent cations could be revealed by the extent of trinitrophenylation (Chang *et al.* 1997a). As shown in figure 6A and B, time-dependent trinitrophenylation of EYPC/EYSM/Chol-, EYPC/EYSM/Chol/Qct-,

Table 2. Effect of phospholipid vesicles on trinitrophenylation of Ca^{2+}-bound or metal-free PLA_2 with TNBS

	No. of incorporated TNP groups in PLA_2	
	*Ca^{2+}	EDTA
Control	1.66±0.05	1.42±0.06
EYPC/EYSM	1.60±0.03	1.57±0.06
EYPC/EYSM/Chol	1.69±0.03	1.54±0.03
EYPC/EYSM/Chol/10 mol% Qct	0.73±0.03	1.60±0.03
EYPC/EYSM/10 mol% Qct	1.03±0.03	1.31±0.08

*Trinitrophenylation of PLA_2 was conducted in 10 mM Hepes (pH 7.5) containing 5 mM Ca^{2+} or 5 mM EDTA

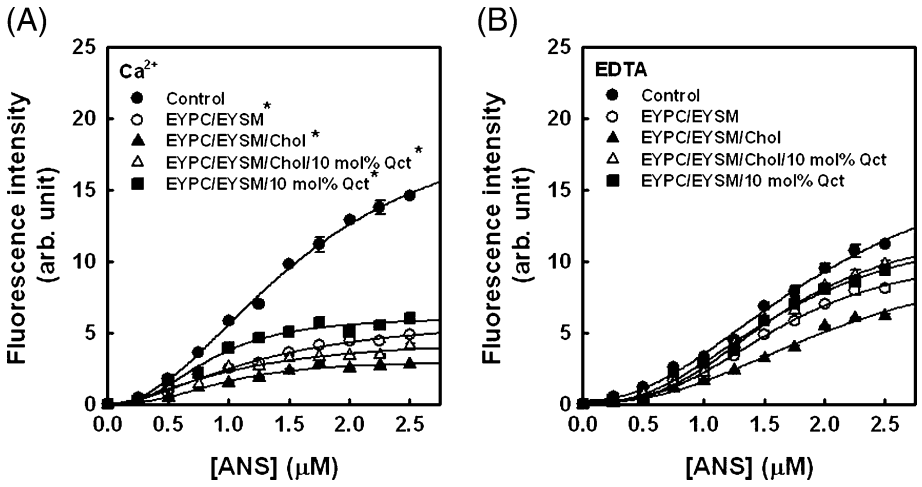

Figure 7. Effect of phospholipid vesicles on the interaction of PLA_2 with ANS. Sample cuvettes contained 1.8 μM PLA_2 in 10 mM Tris–HCl-0.1 M NaCl (pH7.5) containing (**A**) 5 mM Ca^{2+} or (**B**) 5 mM EDTA in the absence (control) or presence of 40.7 μM phospholipid vesicles. The fluorescence emission spectrum was determined at 450 nm in the presence of increasing ANS concentration from 0.25 to 2.5 μM The values represent the average of three independent experiments (*P<0.05, compared with control).

EYPC/EYSM- and EYPC/EYSM/Qct-bound PLA_2 kinetically differed regardless of the absence or presence of Ca^{2+}. These results again suggested that membrane-inserted Qct altered membrane-bound mode of PLA_2. As shown in table 2, the binding of Ca^{2+}-bound PLA_2 with Qct-containing phospholipid vesicles notably reduced the incorporation of

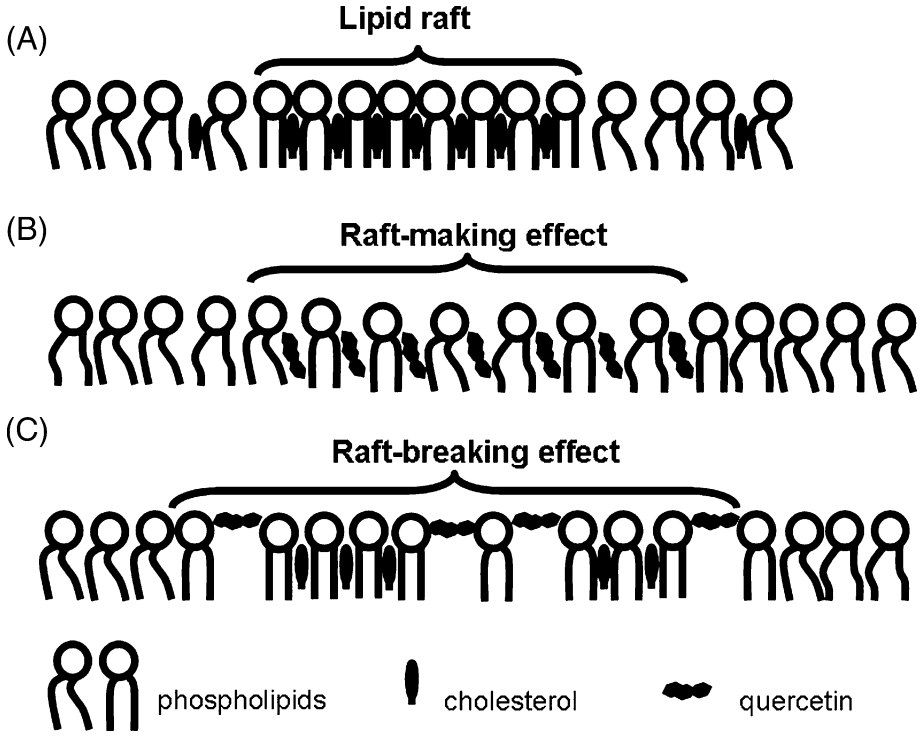

Figure 8. Hypothetical process of Qct on the raft-like domain formation or breaking the lipid raft formed by Chol and phospholipids. (**A**) Lipid raft structure formed by Chol and phospholipids. (**B**) Raft-making effect of Qct. Insertion of Qct molecules into the hydrophobic region of the bilayer forms raft-like domains. (**C**) Qct raft-breaking effect on lipid raft formed by Chol and phospholipids. The absorption of Qct molecules on the polar interface region of the bilayer fluidizes the membranes and shows the raft-breaking effect.

trinitrophenylated (TNP) group into PLA_2, while the number of amino groups in metal-free PLA_2 was similarly modified by TNBS in the presence of either Qct-free or Qct-containing phospholipid vesicles. Obviously, compared with that of metal-free PLA_2, the interfacial binding of Ca^{2+}-bound PLA_2 was notably affected by Qct.

Previous studies revealed that the hydrophobic pocket for binding with ANS was located at the active site of PLA_2, and the binding of ANS with PLA_2 enhanced the fluorescence intensity of ANS (Chiou *et al.* 2008a, b). These findings provide an approach to studying the effect of substrate on the interaction of ANS with PLA_2 enzymes. As shown in figure 7A and B, the enhancing activity of Ca^{2+}-bound PLA_2 on ANS fluorescence was notably reduced in the presence of all tested phospholipid vesicles. Compared with that of Ca^{2+}-bound PLA_2, the binding of ANS with metal-free PLA_2 was less reduced by phospholipid vesicles. The inhibitory capability of EYPC/EYSM/Chol, EYPC/EYSM/Chol/Qct, EYPC/EYSM and EYPC/EYSM/Qct vesicles on ANS binding with either Ca^{2+}-bound PLA_2 or metal-free PLA_2 differed marginally. These findings reflected that Qct did not impede the interaction between the PLA_2 catalytic site and phospholipids.

In this study, our data show that Qct inhibits PLA_2 enzymatic activity and membrane-damaging activity on EYPC/EYSM/Chol vesicles, while Qct and Chol act similarly on relieving the PLA_2 inhibitory activity of sphingomyelin on EYPC/EYSM vesicles. However, the promoted effect of Qct on PLA_2 activity and membrane-damaging activity toward EYPC/EYSM vesicles is weaker than that of Chol. Both Chol and Qct increase detergent resistance and ordered lipid packing of EYPC/EYSM vesicles, but the effect of Qct is smaller than that of Chol. Qct reduced the detergent-resistant properties and membrane fluidity of EYPC/EYSM/Chol vesicles. Together with the finding that Qct suppresses the accumulation of lipid rafts on cell surface (Kaneko *et al.* 2008), our data suggest that Qct perturbs the integrity of lipid-raft structure and affects differently membrane structure of EYPC/EYSM and EYPC/EYSM/Chol vesicles. Tarahovsky *et al.* (2008) suggested that flavonoid molecules located in the hydrophobic region of the bilayer could initiate raft-like domain formation (raft making effect), while molecules located in the polar interface region of the bilayer could fluidize membranes (raft-breaking effect). van Dijk *et al.* (2000) proposed that Qct molecules localized at the boundary between the polar and hydrophobic regions or inside the hydrophobic region of the bilayer and hence interacted with the hydrocarbon chains of lipids. Moreover, membrane-rigidifying effects of Qct depended on the Chol/phosphatidylcholine ratio and the phosphatidylcholine unsaturation degree (Tsuchiya *et al.* 2002). These findings allow us to suggest that the modulated effect of Qct on membrane structure depends on phospholipid compositions. Noticeably, our data indicate that Qct plays different roles in EYPC/EYSM and EYPC/EYSM/cholesterol vesicles (figure 8). Qct may function as Chol in association with EYSM to form raft-like structures in EYPC/EYSM vesicles (figure 8B), while the lipid-breaking effect of Qct distorts the lipid raft in EYPC/EYSM/Chol vesicles (figure 8C). Previous studies showed that Qct interacted directly with snake venom $sPLA_2$ from *Crotalus durissus terrificus* and possessed the ability to inhibit the enzymatic activity and some pharmacological activities of $sPLA_2$ (Cotrim *et al.* 2011). However, Lindahl and Tagesson (1993, 1997) found that Qct selectively inhibited snake venom group II PLA_2, but not group I PLA_2. Given that *Naja naja atra* PLA_2 belongs to group IA PLA_2 family, the observed effect of Qct on the PLA_2 activities should not be related to direct interaction with the active site of the PLA_2 molecule. The finding that Qct did not reduce the interaction between the PLA_2 active site with substrates (figure 7) supports this proposition. Alternatively, our data reveal that Qct affects the membrane-bound mode of PLA_2 as evidenced by acrylamide quenching and TNBS modification (figures 5 and 6). Previous studies showed that the membrane structure and composition affected the interfacial activation of PLA_2 enzymes (Borgstrom 1993; Burack and Biltonen 1994; Oliver *et al.* 1995; Ray *et al.* 2007; Linderoth *et al.* 2008). Collectively, our data suggest that the effects of Qct on membrane structure and membrane-bound mode of PLA_2 modulate interfacial activity and membrane-damaging activity of PLA_2.

Acknowledgements

This work was supported by grant NSC98-2320-B110-002-MY3 from the National Science Council, ROC (to LSC) and grant of the Kaohsiung Medical University Research Foundation.

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MS received 05 January 2012; accepted 12 March 2012

Corresponding editor: AMIT CHATTOPADHYAY