Mechanisms by Which Elevated Intracellular Calcium Induces S49 Cell Membranes to Become Susceptible to the Action of Secretory Phospholipase A2*

(Received for publication, September 21, 1998, and in revised form, February 1, 1999)

Heather A. Wilson‡, Jacqueline B. Waldrip‡, Kelli H. Nielson‡, Allan M. Judf‡, Sang Kyou Han§, Wonhwa Cho§, Peter J. Sims¶, and John D. Bell¶

From the ‡Department of Zoology, Brigham Young University, Provo, Utah 84602, the §Department of Chemistry, the University of Illinois, Chicago, Illinois 60607-7061, and the ¶Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

Exposure of S49 lymphoma cells to exogenous group IIA or V secretory phospholipase A2 (sPLA2) caused an initial release of fatty acid followed by resistance to further hydrolysis by the enzyme. This refractoriness was overcome by exposing cells to palmitoyl lysolecithin. This effect was specific in terms of lysophospholipid structure. Induction of membrane susceptibility by lysolecithin involved an increase in cytosolic calcium and was duplicated by incubating the cells with calcium ionophores such as ionomycin. Lysolecithin also activated cytosolic phospholipase A2 (cPLA2). Inhibition of this enzyme attenuated the ability of lysolecithin (but not ionomycin) to induce susceptibility to sPLA2. Lysolecithin or ionomycin caused concurrent hydrolysis of both phosphatidylethanolamine and phosphatidylcholine implying that transbilayer movement of phosphatidylethanolamine occurred upon exposure to these agents but that susceptibility is not simply due to exposure of a preferred substrate (i.e. phosphatidylethanolamine) to the enzyme. Microvesicles were apparently released from the cells upon addition of lysolecithin or ionomycin. Both these vesicles and the remnant cell membranes were susceptible to sPLA2. Together these data suggest that lysolecithin induces susceptibility through both cPLA2-dependent and -independent pathways. Whereas elevated cytosolic calcium was required for both pathways, it was sufficient only for the cPLA2-independent pathway. This cPLA2-independent pathway involved changes in cell membrane structure associated with transbilayer phospholipid migration and microvesicle release.

Much of the research into the structure, function, and biophysics of lipid membranes has focused on the use of artificial bilayers such as vesicles. These studies are easily justified by the need to obtain simple models for which all variables can be controlled or measured. However, an important goal of these investigations is to attempt to apply them to biological systems. Accordingly, our recent efforts have focused on identifying means whereby studies of the relationship between membrane structure and protein function can be applied to cellular systems.

One system that appears amenable to this task is the control of membrane susceptibility to the action of secretory phospholipase A2 (sPLA2). Normally, both cell membranes and large vesicles composed of saturated phosphatidylcholines resist catalysis by sPLA2 (1–3). However, they become susceptible under certain conditions including exposure to specific molecules (1–5), upon oxidation of the phospholipids (6, 7), or, in the case of cells, possibly during pathological conditions such as inflammation, sepsis, or ischemia (reviewed in Ref. 8).

Most of the experimental work with artificial bilayers has focused on vesicles of defined composition and sPLA2 purified from snake venoms (groups I and IIA) or mammalian pancreas (group I). In such systems, the induction of susceptibility appears to involve the acquisition of specific membrane physical properties (1, 4, 5, 9, 10). One molecule that confers susceptibility on artificial membranes is palmitoyl-lysolecithin (lyso-PPC) which is thought to cause specific perturbations that increase the access of membrane phospholipids to the enzyme-active site (1, 9).

The possibility that lyso-PPC might also cause the membranes of living cells to become susceptible to the action of sPLA2 has not been considered previously. Nevertheless, many recent investigations have explored potential pathological and physiological effects of the lipid such as participation in certain inflammatory conditions (8, 11, 12), atherosclerosis (13, 14), ischemia (15), and regulation of smooth muscle (16, 17). On a biochemical scale, lysolecithin has been reported to regulate protein kinase C (18) and phospholipase D activities (19), cellular calcium concentration (20, 21), gene expression (22, 23), hormone secretion (24), and cation currents (25, 26).

The mechanisms by which susceptibility to sPLA2 is determined in living cells are not yet known. Generally, induction of susceptibility requires an increase in the intracellular calcium concentration (2, 6, 27, 28). We have considered three hypotheses for this effect of calcium as follows. Hypothesis 1, suscep-

The abbreviations used are: sPLA2, secretory phospholipase A2; lyso-PPC, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; ADI-FAB, acrylodan-derivatized fatty acid binding protein; laurdan, 6-decyl-2,3-dimethylaminonaphthalene; lyso-PPG, 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-rac(1-glycerol); lyso-OPC, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine; lyso-PHA, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphatidylethanolamine; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; cPLA2, cytosolic calcium-dependent phospholipase A2; MAPF, methyl arachidonyl fluorophosphonate; AAOCCF2, arachidonyl trifluoromethyl ketone; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

*This work was supported by U. S. Public Health Service Grants GM49710 (to J. D. B.), HL36946 (to P. J. S.), and GM52598 (to W. C.) from the National Institutes of Health and by funds from Brigham Young University (to J. D. B.) and the Arthritis Foundation (to W. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Dept. of Zoology, Brigham Young University, Provo, UT 84602. Tel.: 801-378-8160; Fax: 801-378-7499; john_bell@byu.edu.
Cell Membrane Susceptibility to Phospholipase A<sub>2</sub>

S49 lymphoma cells, calcium ionophores, and groups IIA and V sPLA<sub>2</sub> as an experimental system to examine these hypotheses. We have also determined whether lysospholipids such as lyso-PPC induce susceptibility and whether they share common mechanisms with calcium ionophores. The results of these experiments have led us to the novel conclusion that these cells are naturally susceptible rather than resistant to the enzyme and that brief exposure to sPLA<sub>2</sub> causes them to become refractory to further hydrolysis. Elevated intracellular calcium appears to circumvent the refractoriness causing persistent susceptibility.

EXPERIMENTAL PROCEDURES

Materials—S49 lymphoma cells were grown as described (2). The various sPLA<sub>2</sub> enzymes were expressed and/or isolated according to published procedures (monomeric aspartate 49 (Asp-49) and lysine 49 (Lys-49) enzymes from the venom of Agkistrodon piscivorus piscivorus, Ref. 32; human group IIA, Ref. 33; human group V, Ref. 34). Bee venom sPLA<sub>2</sub> (group III) was obtained from Sigma. The final concentrations sPLA<sub>2</sub> in all experiments were 0.5 to 5 μg/ml depending on the specific activity of the individual preparation except as specified in Fig. 1. Lysospholipids were purchased from Avanti (Birmingham, AL) and suspended in methanol (10 mM) and diluted in 50 mM KCl and 3 mM NaN<sub>3</sub> to yield a 1 or 2 mM stock for use in experiments. The amount of lyso-PPC partitioned into the cell membrane in experiments was assessed by thin layer chromatography. Under conditions where an upper limit would be expected (i.e. conditions of Table 1), the cellular lysoleicithin content increased from about 1.6% of the total phospholipid to about 3.8%. Pharmacological agents were dissolved in appropriate solvents (dimethyl sulfoxide, methyl acetate, or ethanol). Control experiments demonstrated that these solvents did not have effects on the experimental data at the concentrations used.

Fluorescence Spectroscopy—Cells were harvested, washed, and suspended to a final density of about 1–2 x 10<sup>6</sup> cells/ml in a balanced salt solution as described (2). Measurements with fluorescent probes were obtained at 37 °C using a Fluoromax (Spex Industries) photon-counting spectrophotometer (2). Release of fatty acids from cells was assayed with an acrylodan-labeled intestinal fatty acid-binding protein (ADI-FAB) (−0.2 μM final, excitation = 390 nm, emission = 432 and 505 nm; Refs. 2 and 35), and results were quantified by calculation of the generalization polarization (2, 36). The amount of phospholipid hydrolyzed was assessed (35, 37) for oleate and arachidonate to give a lower and upper estimate of the quantity of fatty acid produced. Propidium iodide (37 μM final, excitation = 536 nm, emission = 617) was used to assess general cell membrane permeability. Due to their aqueous solubility, neither ADIFAB nor propidium iodide required previous equilibration with the sample; thus, both were added immediately prior to other experimental agents. Laurdan (0.05 μM final, excitation = 350 nm, used to measure bilayer polarity, Ref. 36) and indo-1 (3.75 μM final, excitation = 350 nm, emission = 405 and 480 nm, used to measure intracellular calcium) were incorporated into cells and assayed as described (2). Calcium concentration was determined by calculating the generalized polarization and comparison to a calibration curve. BAPTA (90 μM) was equilibrated with cells for 2 h at 37 °C in cell culture medium without serum.

Light Scattering—Cells were harvested as described above, suspended at a density of approximately 5 x 10<sup>6</sup> cells/ml, and incubated at 37 °C in a shaking water bath. At various time intervals following addition of experimental agents, 1 ml of cells was removed and centrifuged 10 s at 13,000 rpm. The supernatant was immediately removed and transferred to liquid nitrogen to quench the reaction. An aliquot (500 μl) of the supernatant was added to 2 ml of aqueous solution, and the intensity of scattered light (excitation = 500 nm, emission = 510 nm) was assayed.

Flow Cytometry—To detect phosphatidylserine exposure in the outer leaflet of the plasma membrane induced by ionomycin, cells were stained with fluorescent-labeled annexin V and propidium iodide for 15 min at 25 °C according to instructions provided with an Apoptosis Detection Kit purchased from R & D Systems (Minneapolis, MN). Flow cytometry data were collected and analyzed using a Coulter Epics XL flow cytometer and the associated software.

Phospholipid Extraction and Thin Layer Chromatography—Cells were prepared, incubated, and centrifuged, and pellets and supernatants were frozen in liquid nitrogen as described for light scattering experiments. Samples were quickly thawed, and lipids were extracted with chloroform and methanol (38). Phospholipids and lysosphospholipids were separated by thin layer chromatography in 6.5:2.5:1 (v/v) chloroform:methanol:acetic acid and were developed in the presence of iodine crystals. Spots were identified using standards. Lipid content in each spot was quantified either by densitometry or by phosphate assay (39).

RESULTS

Incubation of S49 lymphoma cells with the Asp-49 snake venom sPLA<sub>2</sub> caused a small transient release of fatty acid followed by a return of extracellular fatty acid levels to near base line (Fig. 1, panel A, curve a). Similar phenomena were observed when the enzyme was added to a variety of human white blood cell lines (Raji, HL-60, and MOLT-4 cells) suggesting that these phenomena are not unique to S49 cells. Likewise, this behavior was not limited to snake venom group IIA sPLA<sub>2</sub> since recombinant human group V sPLA<sub>2</sub> produced a similar effect (Fig. 1, panel A, curve b). No hydrolysis was detectable with this assay upon addition of human recombinant group IIA enzyme (Fig. 1, panel A, curve c).

Refractoriness—The fatty acid released in the time courses of Fig. 1, panel A, represented a small fraction of the cellular phospholipid (−2–5%; see legend to Fig. 1). Therefore, the transient nature of the time course could reflect a limitation in the availability of substrate for hydrolysis or some inhibitory mechanism preventing further hydrolysis. To consider these possibilities, we repeated the time courses shown in Fig. 1, panel A, with a second addition of sPLA<sub>2</sub> following the return of fatty acid levels to base line. The second application of sPLA<sub>2</sub> resulted in no release of fatty acid (Fig. 1, panel B, curve a). Reduction of the initial concentration of enzyme by a factor of 10 (from 0.1 to 0.01 μg/ml) still caused the cells to be refractory to a second addition of the higher concentration of the enzyme (i.e. 1 μg/ml, curve b). Further reduction in the initial sPLA<sub>2</sub> concentration allowed hydrolysis to be accomplished upon the second addition (curve c). Control experiments revealed that the phenomenon was not an effect of incubation time or the solvent in which the enzyme was dissolved.

This ability of an initial dose of sPLA<sub>2</sub> to cause resistance to hydrolysis by subsequent enzyme was independent of the amount of initial bilayer hydrolysis and was nonspecific with respect to the type of sPLA<sub>2</sub>. As shown in Fig. 1, panel B, incubation of the cells with human group V sPLA<sub>2</sub> caused refractoriness to a second addition of either the same enzyme (curve d) or the snake venom enzyme (curve e). The less-related sPLA<sub>2</sub> from bee venom (group III, curve f) also produced the same effect. Importantly, addition of human group IIA sPLA<sub>2</sub> reduced the ability of the group V enzyme to hydrolyze the bilayer even though no detectable hydrolysis was observed during the initial incubation (curve g). This independence from hydrolysis was verified by incubating the cells with snake venom sPLA<sub>2</sub>, which is catalytically inactive due to a substitution of the amino acid lysine for aspartic acid at position 49 (Lys-49 sPLA<sub>2</sub>; curve h).

Effect of Lysosphospholipids—Fig. 2 demonstrates that this refractoriness was reversed (panel A, curve a) or prevented (panel B, curve a) by incubation with 5 μM lyso-PPC. The increase in base-line slope upon addition of lyso-PPC (Fig. 2, panel B, curve a) appears to reflect the activity of cPLA<sub>2</sub> as explained below in the description of Fig. 6. As shown in panel C, the ability of lyso-PPC to induce susceptibility to sPLA<sub>2</sub> was applicable also to the human enzymes. This effect of lyso-PPC...
Cell Membrane Susceptibility to Phospholipase A₂

**Fig. 1. Effect of sPLA₂ incubation on membrane hydrolysis.** Panel A, S49 cells were incubated with 1 µg/ml snake venom Asp-49 (curve a), human group V (curve b), or human group IIA sPLA₂ (curve c), and hydrolysis was assessed with ADIFAB as described under “Experimental Procedures.” Enzyme was added at the dashed line. All curves are plotted on the same relative scale and are displaced along the ordinate for clarity of presentation. The maximum amount of hydrolysis in curve a was estimated as 0.2–0.5 µm/10⁶ cells (see “Experimental Procedures”). Panel B, the effect of the second application of sPLA₂ (arrow) with cells already exposed to one of the three types of sPLA₂ (dashed line). Curve a, 1 µg/ml snake venom Asp-49 sPLA₂, and then 1 µg/ml snake venom Asp-49 sPLA₂; curve b, 3 ng/ml snake venom Asp-49 sPLA₂, and then 1 µg/ml snake venom Asp-49 sPLA₂; curve c, 3 ng/ml snake venom Asp-49 sPLA₂, and then 1 µg/ml snake venom Asp-49 sPLA₂; curve d, 1 µg/ml human group V sPLA₂, and then 1 µg/ml human group V sPLA₂; curve e, 3 µg/ml human group V sPLA₂, and then 1 µg/ml snake venom Asp-49 sPLA₂; curve f, 1 µg/ml human group IIA sPLA₂, and then 1 µg/ml snake venom Asp-49 sPLA₂; curve g, 1 µg/ml human group IIA sPLA₂, and then 1 µg/ml human group V sPLA₂; curve h, 1 µg/ml snake venom Lys-49 sPLA₂, and then 1 µg/ml snake venom Asp-49 sPLA₂. Panel C, theoretical curves were generated using Equation 5 (see “Appendix”). Enzyme concentrations were the same as in the corresponding curves (a–c) of panel B. Other parameter values were constant and are listed under the “Appendix.”

was concentration-dependent up to about 7–10 µM (Fig. 3, panel A). As expected based on previous studies (2), the calcium ionophores, A23187 and ionomycin, each produced the same effect as lyso-PPC (see Fig. 2, panel B, curve b, for example). Similar results were obtained with Raji, HL-60, MOLT-4, and K-562 cells. HeLa (epithelial) cells, however, did not respond to ionophore or lyso-PPC.

We tested other lysophospholipids to determine whether the induction of membrane susceptibility by lyso-PPC was specific for the structure of the lipid. Lyso-PPG (Fig. 3, panel B, curve a) behaved similarly to lyso-PPC. The relative potency of the other lipids was as follows: lyso-PPG > Lyso-OPC > lyso-PPA > lyso-PPE (no detectable effect). 1-Alkyl-2-hydroxy-sn-glycero-3-phosphocholine had the same action as lyso-PPC suggesting that the effect was not due to metabolites resulting from S₁ hydrolysis.

The dependence on lysophospholipid structure illustrated in Fig. 3, panel B, suggested the possibility that lyso-PPC acts at specific binding sites and that its effect involves distinct biochemical pathway(s). Neither isoproterenol, which leads to activation of the G-protein Gₛ and elevates cAMP levels in S49 cells, nor phorbol myristate acetate, which activates protein kinase C, enhanced the ability of sPLA₂ to hydrolyze S49 cells. Furthermore, the following inhibitors pertussis toxin (other G-proteins), wortmannin (phosphatidylinositol 3-kinase), and K-252 (several protein kinases) did not reduce the ability of lyso-PPC to render the cell membrane susceptible to sPLA₂. In contrast, cobalt, a general blocker of transmembrane calcium transport, greatly attenuated the response (Fig. 4, panel A, curve b) suggesting that increases in cytosolic calcium could be responsible for the action of lyso-PPC on membrane hydrolysis as is the case for calcium ionophores. Nickel (0.5 mM) produced a similar effect (not shown). This observation was validated by experiments demonstrating that the intracellular calcium chelator BAPTA also attenuated the response to lyso-PPC (Fig. 4, panel B, curve b).
hydrolysis upon exposure to sPLA2 and the indicated concentrations of
lyso-PPC. The open square contained control diluent equivalent to 5 μM
lyso-PPC. Panel B, various lysophospholipids (10 μM) were added at the
arrow to cells incubated with snake venom sPLA2: curve a, lyso-PPG;
curve b, lyso-OPC; curve c, lyso-PPA; curve d, lyso-PPE. See Fig. 2 for
explanation of the ordinate scale.

Accordingly, we used indo-1 to monitor the effect of lyso-PPC
on cytosolic calcium. As shown in Fig. 5, panel A (curve a), the
addition of lyso-PPC caused an immediate rise in intracellular
calcium. This effect was dependent on lyso-PPC concentration
and reached a maximum at 7–10 μM reminiscent of the concentra-
tion dependence displayed in Fig. 3, panel A, for induction of
susceptibility. Experiments with extracellular EDTA demonstrated
that this elevation of intracellular calcium represented an influx of the ion from the cell exterior.

Even though it appeared that lyso-PPC and calcium ionophore
induced membrane susceptibility by common mechanisms, important differences were found between these agents.
First, the size of the calcium influx required for ionomycin to
induce susceptibility was larger and more persistent than that
achieved with lyso-PPC (Fig. 5, panel A, curve a). Second,
although both lyso-PPC and ionophore caused immediate in-
creases in cytosolic calcium, about 100–200 s were required for
complete realization of membrane susceptibility with iono-
phore, whereas the effect of lyso-PPC was more rapid in onset
(Fig. 5, panel B, triangles).

Calmodulin—We examined the possible involvement of cal-
modulin in the process of inducing membrane susceptibility to
sPLA2 using several calmodulin inhibitors as follows: W-7,
calmidazolium, and ophiobolin A. In initial experiments,
ophiobolin A alone was found to induce susceptibility of S49
cells to sPLA2 and therefore was not used in additional exper-
iments. W-7 significantly reduced the rate of hydrolysis in cells
incubated with lyso-PPC and sPLA2, increasing the half-time
for complete hydrolysis by a factor of 100. A less profound effect
was obtained with ionomycin as the stimulant of susceptibility;
W-7 increased the half-time for membrane hydrolysis from 97.4
to 287.6 s. This effect of W-7 was dose-dependent (EC50
about 170 nM). Calmidazolium had little or no reproducible effect.

Hypothesis 1—As stated in the Introduction, three hypothe-
ses for the effect of calcium to cause membranes to become
susceptible to sPLA2 were considered. To test hypothesis 1, a
specific inhibitor of cPLA2 (MAFP) was used (40). As shown in
Fig. 6, panel A, the release of fatty acid from the membrane
normally observed upon the addition of lyso-PPC alone (5 μM;
curve a) was blocked by MAFP (curve b) consistent with a
previous observation that lysolecithin can activate cPLA2 (41).
Also, the rate of hydrolysis upon addition of human sPLA2 was
reduced in cells treated with MAFP (Fig. 6, panel B, curve b)
com pared with control cells (curve a). In contrast to lyso-PPC,
ionomycin did not stimulate measurable fatty acid release by
cPLA2 unless the incubation was prolonged (>1000–1500 s, not
shown). Accordingly, MAFP had no detectable effect on the
time course of S49 cell hydrolysis in the presence of ionomycin.
These effects were not confined to the human enzyme since
they were reproducible with the snake venom sPLA2 (Fig. 6,
panel C). A second inhibitor of cPLA2 (AACOCF3; Ref. 42) also
decreased the initial rate of hydrolysis in the presence of lyso-
PPC and sPLA2. However, in contrast to MAFP, AACOCF3 also
reduced the rate of hydrolysis in the presence of ionomycin.
That these inhibitory effects of AACOCF3 were probably due to
a nonspecific action of the agent was demonstrated by the
observation that an alcohol analog of AACOCF3 (AAC(OH)CF3)
that does not inhibit cPLA₂ produced the same effects on lyso-
PC and ionomycin-stimulated susceptibility to sPLA₂.

Hypothesis 2—We first addressed whether significant expo-
sure of phosphatidylserine occurred upon incubation with ino-
mycin on a time scale relevant to the induction of susceptibility.
Cells were incubated for 15 min at 25°C with or without ino-
mycin in the presence of propidium iodide and fluorescent-
labeled annexin V (a phosphatidylserine binding protein, Ref.
43). The percentage of cells binding annexin V (i.e. those with
phosphatidylserine exposed to the cell exterior) was then quan-
tified by flow cytometry. Incubation with ionomycin increased
the percentage of cells binding annexin from 2 to 87%. Parallel
control experiments without annexin V verified that the cells
were susceptible to sPLA₂ under the same conditions.

Because of limitations in the time resolution of the annexin
binding assay, we investigated hypothesis 2 further by moni-
toring the time course of hydrolysis of the major classes of
phospholipid. We reasoned that if translocation were slower
and not necessary for membrane susceptibility, phos-
phatidylcholine would be hydrolyzed first and that subsequent
hydrolysis of other phospholipids would reflect delayed trans-
location. Conversely, hydrolysis of phospholipids from the in-
ner leaflet (phosphatidylethanolamine and/or phosphatidyl-
serine) concurrent with or prior to phosphatidylcholine would
reflect translocation of those lipids. Table I displays the initial
rate of hydrolysis of the two most abundant phospholipids in
S49 cell membranes, phosphatidylcholine and phosphatidy-
lethanolamine, in the presence of ionomycin or lyso-PPC (at
20 μM because of the higher cell density required in the exper-
iment). Initial hydrolysis of both lipids was rapid and concur-
rent for either the ionophore or lyso-PPC. When sPLA₂ was
present alone, the reaction was transient as was observed in
the continuous fluorescence assay of total fatty acid release
(Fig. 1). Control experiments revealed that the observed hy-
drolysis of phosphatidylethanolamine was due to sPLA₂ rather
than cPLA₂. Reproducible hydrolysis of both phosphatidylcholi-
ne and phosphatidylethanolamine was also observed with the
groups IIA and V human sPLA₂, and differences among the
three enzymes (human and snake venom) reflected apparent
overall rates of hydrolysis rather than preferential hydrolysis
of specific phospholipids (Table I). Detailed investigations into
the mechanisms of differences among the human enzymes are
presented elsewhere.²

² W. Cho and M. H. Gelb, manuscript in preparation.

Table I

| Conditions                  | PC    | PE    |
|-----------------------------|-------|-------|
| Initial rate (%/s for first 90 s) |       |       |
| Ionomycin (300 nM), snake venom sPLA₂ | 0.8 ± 0.1 | 0.7 ± 0.1 |
| Lyso-PPC (20 μM), snake venom sPLA₂ | 0.7 ± 0.1 | 0.8 ± 0.2 |
| % PC or PE hydrolyzed after 1200 s (300 nA ionomycin) |       |       |
| Human group IIA | 19 ± 10 | 8 ± 4 |
| Human group V | 39 ± 8 | 28 ± 14 |
| Snake venom | 91 ± 7 | 89 ± 3 |

Hypothesis 3—Light scattering was used to detect the pres-
eence of vesicles shed from cells. After exposing S49 cells to
ionomycin or lyso-PPC for various times, cells were separated
from any microvesicles by centrifugation, and the intensity of
light scattered by the supernatant was then assessed. As
shown in Fig. 7, panel A, a time-dependent increase in the light
scattering was observed upon exposure of the cells to either
ionomycin (squares) or lyso-PPC (circles) suggesting that par-
ticles had been released from the cells. Extraction of the super-
natant with chloroform and methanol followed by thin layer
chromatography revealed that these particles contained phos-
pholipid (phosphatidylethanolamine > phosphatidylcholine).
Isolation of the particles and subsequent exposure to sPLA₂
demonstrated that they were susceptible to hydrolysis by the
enzyme (≥40% of vesicle phospholipid hydrolyzed, estimated by
ADIFAB).

Comparison of the data shown in Fig. 7, panel A, for iono-
mycin and lyso-PPC reveals that the release of the particles
(presumably microvesicles) was slower for ionomycin than for
lyso-PPC. Furthermore, the time course was typically biphasic
in the case of lyso-PPC. To test whether the release of mi-
rovesicles stimulated by lyso-PPC was calcium-dependent, we
repeated the experiment of panel A in the presence or absence

Fig. 6. Effect of MAFP on membrane hydrolysis by sPLA₂. Cells were incubated 900 s with 10 μM MAFP (curves b) or control solvent (curves a). Panel A, 5 μM lyso-PPC was added at the arrow. Panel B, the time courses shown in panel A were continued. For curve a (control), human group V sPLA₂ was added at the onset of the data shown. Curve b displays the data from the sample treated with MAFP (also in the presence of group V sPLA₂) beginning with the time point at which the amount of intact phospholipid remaining in the cell membrane was the same as that at the onset of curve a (i.e. to account for contribution of cPLA₂). Panel C, the experiment was repeated and displayed exactly as described for panel B with snake venom Asp-49 sPLA₂ instead of the human enzyme. See Fig. 2 for explanation of the ordinate scale.
of cobalt and found that the initial (but not the prolonged) phase of the time course was blocked (panel B, squares). Finally, we compared other lysophospholipids to determine whether there was a relationship between the specificity for microvesicle release and the specificity for induction of susceptibility to sPLA$_2$ (i.e., lyso-PPC) caused the same changes to the laurdan emission spectrum as lyso-PPC (shown in Fig. 8, panel C). As shown in Fig. 7, panel C, the same relationship among the various lysophospholipids was observed (lyso-PPC = lyso-PPG > lyso-OPC > lyso-PPE > lyso-PE).

Previous work suggested that induction of membrane susceptibility to sPLA$_2$ may involve changes in physical properties of the plasma membrane detectable by laurdan and propidium iodide fluorescence. As shown in Fig. 8, panels A and B, incubation of S49 cells with ionomycin did not affect membrane permeability to propidium iodide and caused only minor changes to the emission spectrum of membrane-bound laurdan. In contrast, larger changes in the fluorescence of both probes were observed with lyso-PPC (panels C and D). In the presence of lyso-PPC, the propidium iodide intensity increased substantially and the laurdan emission spectrum shifted to shorter wavelengths. Cobalt inhibited the effect of lyso-PPC on propidium iodide fluorescence (panel E) but had little or no effect on the ability of lyso-PPC to shift the laurdan spectrum (panel F). Control experiments testing whether lyso-PPC was toxic to the cells revealed that cells exposed to lyso-PPC continued to grow in culture similar to non-exposed cells. Consistent with their effect on susceptibility, lyso-PPG and lyso-OPC (but not lyso-PPE or lyso-PE) increased propidium iodide uptake by the cells to about 0.3–0.5 of the extent shown for lyso-PPC in Fig. 8, panel C. However, all lysophospholipids tested except lyso-OPC caused the same changes to the laurdan emission spectrum as lyso-PPC (shown in Fig. 8, panel D).

**DISCUSSION**

These studies revealed three novel general observations. First, S49 lymphoma cells are naturally susceptible to sPLA$_2$ but quickly become resistant upon initial exposure to the enzyme. Second, lyso-PPC can reverse or prevent this development of resistance and thus render the cell membranes persistently susceptible to the enzyme. Third, this effect of lyso-PPC is calcium-dependent and displays many similarities with calcium ionophore treatment on vulnerability of S49 cells to sPLA$_2$. Importantly, these observations were found to be relevant to a variety (but not all) of human cell lines and for human groups IIA and V sPLA$_2$ in addition to snake venom group IIA enzyme. Thus, these findings are general phenomena (at least for certain leukocytes) rather than isolated experimental curiosities.

**Refractoriness**—The observation that initial exposure to sPLA$_2$ causes the cell membranes to become refractory to further hydrolysis by the enzyme was surprising. The mechanism for this phenomenon is not yet known, but several possibilities can be excluded based on the data of Fig. 1. For example, both the Lys-49 sPLA$_2$ from snake venom and the recombinant human group IIA which did not cause detectable hydrolysis of the cell membrane were capable of protecting the membrane...
from hydrolysis by a more active enzyme such as the snake venom Asp-49 or the human group V sPLA₂. Therefore, membrane hydrolysis is not required for the effect, and hypotheses such as physical changes to the membrane structure during hydrolysis or involvement of reaction products can be excluded as explanations for the refractoriness. The results of Fig. 1 also address whether the inactivity of the second dose of enzyme is due to limited substrate or saturation of binding sites on the membrane. This hypothesis assumes that the enzyme-binding sites responsible for refractoriness and the sites of hydrolysis are identical and that those enzymes that do not catalyze initial hydrolysis (human group IIA and venom Lys-49) still adsorb to and saturate these binding sites. If this hypothesis were true, reduction in the initial concentration of sPLA₂ to the point that there is less hydrolysis (such as in *curve b* of Fig. 1, *panel B*) would correspond to a condition at which these “binding sites” would not be saturated and hydrolysis would thus occur when a second dose of sPLA₂ is added. However, the contrary was observed (*curve b*, Fig. 1, *panel B*) eliminating this “saturation of binding sites” hypothesis.

A likely explanation for the data is that membrane hydrolysis and induction of refractoriness represent two separate effects of sPLA₂ that involve independent binding sites with different affinities for the enzyme (the site responsible for refractoriness would have higher affinity). To consider this possibility quantitatively, we generated a mathematical model (see “Appendix”) that incorporates three separate events as follows: 1) binding of enzyme to and hydrolysis of the cell membrane, 2) binding of enzyme to a distinct high affinity site that results in time-dependent inhibition of the first event, and 3) slower removal of product. As shown in Fig. 1, *panel C*, this model was capable of reproducing all of the results observed experimentally (Fig. 1, *panel B*, *curves a–c*) at the various doses of sPLA₂ tested. Therefore, this hypothesis is a viable explanation for the data. Although an attractive candidate for the putative additional binding site is the 180-kDa M-type sPLA₂ receptor (44), the observation that bee venom sPLA₂ also induced refractoriness argues against this possibility since the bee venom enzyme does not bind to the M-type receptor (44).

Nevertheless, other receptors for sPLA₂ have been described, and it is reasonable to propose that sPLA₂ induces refractoriness through an action at one such site.

**Effects of Lyso-PPC**—The presence of lyso-PPC both prevented and reversed the refractoriness of the S49 and various human cells to hydrolysis by sPLA₂. This effect is reminiscent of the ability of the lyposphospholipid to render phosphatidylcholine bilayers susceptible to the enzyme (1, 9). In the latter case, the induction of susceptibility is a biophysical effect involving structural alterations to the bilayer making it easier for phospholipids to migrate into the active site of enzyme adsorbed to the bilayer surface (9). This similarity between cells and artificial bilayers raises the question of whether the effect of lyso-PPC on cells is biophysical or biochemical.

Several observations suggest that the ability of lyso-PPC to make cells susceptible to sPLA₂ involves specific biochemical pathways rather than direct biophysical perturbation of the bilayer. First, the concentration of lyso-PPC required for the effect was similar to or lower than that required for a variety of biochemical and physiological effects of the lipid reported for other cells and tissues (15–26). Second, as is true for other reported biochemical effects of lyso-PPC (18, 24), there was a structural specificity for the phenomenon (see Fig. 3, *panel B*). Third, the effect of lyso-PPC to cause the cells to be susceptible to sPLA₂ was dependent on calcium influx through cobalt-sensitive pathways. Had the lipid simply been creating nonspecific holes in the membrane, it is unlikely that such would have been inhibited by the presence of cobalt or nickel. Control experiments verified that the inhibitory effect of cobalt was not due to direct inhibition of sPLA₂. Also, experiments with indo-1 demonstrated that cobalt blocked the influx of calcium into the cell induced by lyso-PPC (not shown). Fourth, lyso-PPC did not cause susceptibility to sPLA₂ in all cell types (*e.g.* HeLa cells did not respond unless damaged by excessive trypsin treatment). Also, P388D₁ macrophages appear not to respond to lysophospholipids (29). Had the action of lyso-PPC been a generic nonspecific effect, one would expect it not to depend on cell type. The fact that the HeLa cells also did not respond to calcium ionophore suggests that the deficiency in their ability to respond to lyso-PPC involves one or more downstream events. Future investigations with HeLa cells may therefore help to identify further the nature of the mechanisms that govern susceptibility or resistance to sPLA₂.

Notwithstanding this evidence that lyso-PPC may function through specific biochemical pathways, it is clear that structural changes to the membrane do occur upon incubation with lyso-PPC. These include apparent transbilayer migration of phospholipids (Table I) and release of microvesicles (Fig. 7) as well as changes detected by the fluorescent probes propidium iodide and laurdan (Fig. 8). Experiments using fluorescence microscopy (not shown) revealed that the enhancement of propidium iodide fluorescence reflected increased permeability of the cells to the probe allowing it access to DNA (45). This result raises the question of whether lyso-PPC could be toxic to the cells since dead cells are permeable to the dye. This question is difficult to answer because some of the uptake of propidium iodide by the cells was not accompanied by a proportional increase in permeability to trypan blue. Furthermore, in a few cases, cells that stained with trypan blue were impermeable to propidium iodide. The ultimate test of cell viability is whether the cells continue to multiply. As stated under “Results,” cells exposed to reasonable concentrations (similar to those of the figures) of lyso-PPC continued to divide at normal rates. Accordingly, it was clear from our results that cells death is not a prerequisite to sPLA₂ susceptibility induced by lyso-PPC or ionomycin. Obviously, as suggested by the data in Table I, the presence of lyso-PPC and sPLA₂ together (especially snake venom enzyme) can be lethal to the cells due to extensive membrane hydrolysis.

Although it appeared that cell death was not a prerequisite for susceptibility to sPLA₂, we note that high concentrations of lyso-PPC alone can induce cell death as well as a latent nonspecific susceptibility to sPLA₂ that is not sensitive to inhibitors such as cobalt. This latter observation reveals a necessary caution for interpreting investigations into the effects of lyso-PPC since many studies have been done at these higher concentrations that appeared toxic in this system. Based on our experience, we recommend that the effects of lyso-PPC on cellular biochemistry and physiology be studied at concentrations below ~5 µM/10⁶ cells.

The membrane alteration reflected by the cobalt-sensitive increase in permeability to propidium iodide is not an absolute requirement for the induction of susceptibility to sPLA₂ since ionophore treatment did not cause a similar increase in permeability. Nevertheless, the alteration may be ancillary in causing membrane susceptibility since lyso-PPC enhanced the ability of sPLA₂ to hydrolyze the cells faster and at lower calcium concentration than ionophore (Fig. 5). That this and/or other effects of the lipid promote membrane hydrolysis was also seen by the observation that lyso-PPC was much more effective than ionophore at promoting membrane degradation by cPLA₂ (Fig. 6).

The data with laurdan (Fig. 8) suggested that lyso-PPC
caused a decrease in the interaction of water with the bilayer since the laurdan emission spectrum is sensitive to the amount and mobility of water molecules in the region of the phospholipid glycerol backbone (46, 47). Importantly, this effect of lysophosphatidylethanolamine is a better substrate for sPLA2 than phosphatidylcholine, phosphatidylethanolamine, or neither exist in the literature (e.g. Ref. 52–55). One plausible explanation is that some other calcium-binding protein involved in the induction of susceptibility such as the enzymes described below shares sufficient structural similarity to calmodulin than W-7 cross-reacts with that protein. We note, for example, that W-7 also inhibited the activity of cPLA2 stimulated by lysophosphatidylcholine. Based on the results obtained in this study, we are reluctant to conclude that calmodulin plays a large role in the effect of intracellular calcium to promote cell membrane hydrolysis by sPLA2.

Hypothesis 1, cPLA2—Data published recently by Balsinde and Dennis (29) demonstrated that blocking the activity of cPLA2 also caused a 75% reduction in the activity of extracellular sPLA2 toward P388D1 macrophages. These results suggested that membrane hydrolysis by sPLA2 is subsequent to activation of cPLA2. Similar conclusions have been reached by other investigators (30, 31). The hypothesis is that the activation of cPLA2 perturbs the cell membrane in ways that make it susceptible to sPLA2 (29).

Lyso-PPC appeared to induce susceptibility by dual mechanisms. One of these mechanisms involved the apparent activation of cPLA2. This activation of cPLA2 was calcium-dependent since cobalt, nickel, and BAPTA all prevented fatty acid release in the presence of lyso-PPC alone (Fig. 4). A stimulatory effect of lyso-PPC on cPLA2 has also been reported previously (41), and the coupling of this event to hydrolysis by sPLA2 was consistent with the proposal of Balsinde and Dennis (29). The second mechanism, also requiring calcium, was independent of the action of cPLA2 and appeared to involve membrane perturbations such as transbilayer migration of phospholipids and production of microvesicles (see below). Elevation of intracellular calcium alone (i.e. through ionophore) was sufficient to mimic the latter but not the former effect of lyso-PPC. This latter mechanism could be analogous to the membrane perturbations downstream of the activation of cPLA2 leading to susceptibility to sPLA2. Alternatively, it may represent a separate mechanism by which susceptibility can be induced without requirement for cPLA2.

The same structural specificity of lysophospholipid species identified for the induction of sPLA2 susceptibility (Fig. 3, panel B) was observed for the activation of cPLA2 and probably reflects the ability of each species to promote calcium entry into the cells. This structural specificity is important for preventing possible positive feedback. If lysophosphatidylethanolamine were capable of stimulating calcium uptake and activation of cPLA2, positive feedback would result from the hydrolysis of phosphatidylethanolamine on the interior of the cell membrane. Likewise, the natural membrane asymmetry protects phosphatidylcholine from attack by cPLA2 and the cell from the positive feedback that would result from that event.

Hypothesis 2, Transbilayer Migration of Phospholipids—One of the effects of increased intracellular calcium is translocation of phospholipids normally found on the extracellular face (such as phosphatidylethanolamine) to the interior of the cell membrane and export of phospholipids normally found on the intracellular face (phosphatidylethanolamine and phosphatidylserine) to the extracellular face (reviewed in Ref. 50). These transbilayer migrations may involve at least two calcium-dependent events. First, the enzyme normally responsible for maintaining bilayer asymmetry, aminophospholipid translocase, is inhibited by elevated intracellular calcium that results in a progressive loss of membrane phospholipid asymmetry (50). A second enzyme, scramblase, is activated by a rise in cytosolic calcium and responds by catalyzing migration of phospholipids across the membrane (50, 51). It has been hypothesized that phosphatidylethanolamine is a better substrate for sPLA2 than phosphatidylcholine and that the simple act of exposing the aminophospholipid to the exterior might be sufficient to account for the effect of intracellular calcium on membrane hydrolysis by extracellular sPLA2 (3). However, the specificity of sPLA2 for phospholipid head groups appears controversial. Reports of preference for phosphatidylcholine, phosphatidylethanolamine, or neither exist in the literature (e.g. Ref. 52–55). One possible resolution to the controversy is that specificity is determined not by the structure of the head group per se, but by the physical state of the phospholipids within the bilayer and that an altered distribution of phospholipids across the bilayer could affect that physical state (52). Another explanation for the controversy may lie in differential specificity of the various types of sPLA2 (venom versus mammalian, group II versus group V; see Ref. 34).

Measurements of annexin binding illustrated that exposure of phosphatidylserine on the outer leaflet of S49 cell membranes occurs at a dose of ionomycin and time scale consistent with the induction of susceptibility. Also, rapid transbilayer movement of phospholipids stimulated by calcium (i.e. in the range of minutes or less) has been observed (56). Therefore, this hypothesis appears feasible. The data of Table I also supported rapid phospholipid translocation since the initial rate of phosphatidylethanolamine hydrolysis was comparable to that of phosphatidylcholine. Moreover, these data do not support the idea that translocation of a preferred substrate is the mechanism by which calcium induces susceptibility. Nevertheless, translocation of phospholipids may be important for other physical changes to the bilayer that result in susceptibility (see below). Also, since sPLA2 binding to bilayers is promoted by negative charge (4, 5), the exposure of phosphatidylserine may help recruit enzyme to the bilayer surface for catalysis.

Hypothesis 3, Membrane Vesiculation—Several cells have been shown to shed vesicles upon stimulation with ionophores (27, 56, 57), and these vesicles appear to be susceptible to sPLA2 (27). This shedding of microvesicles relates to calcium levels by two pathways. First, it is promoted by the loss of membrane asymmetry described in the previous section (56, 57). Second, it also involves hydrolysis of cytoskeletal elements by calcium-dependent proteases (58). Fig. 7 suggests that an increase in intracellular calcium causes S49 cells to shed vesicles. Two lines of evidence support the idea that the release of these vesicles is associated with the induction of susceptibility to sPLA2 in S49 cells. First, vesicle release was more rapid...
when induced by lyso-PPC than by ionophore (Fig. 7, panel A). This time dependence coincided with the relative time dependence for the two agents to induce susceptibility (Fig. 5, panel B). Second, the same dependence on lysophospholipid structure for release of the vesicles was found as for induction of susceptibility (Fig. 3, panel B).

Interestingly, cobalt blocked rapid release of microvesicles stimulated by lyso-PPC but did not block slower subsequent release (Fig. 7, panel B). The rapid, cobalt-sensitive phase also appeared to coincide with the rapid, transient rise in intracellular calcium observed with lyso-PPC (Fig. 5, panel A). The reason why the intensity of light scattering during the rapid phase of vesicle release decreased is also not known but would be consistent with a time-dependent reduction in vesicle size. The origin of the slower phase of vesicle release is not known, but we did observe in some experiments at excessive lyso-PPC concentrations a cobalt-insensitive induction of susceptibility that was much slower in onset consistent with slower vesicle release.

These correlations between vesicle release and susceptibility suggest that the vesicles could be the source of membrane hydrolysis as has been proposed (27). These vesicles would have high curvature (the reported size of vesicles shed from the plasma membrane of cells is around 50-200 nm in diameter; Ref. 59) which is known from studies in artificial membranes to promote increased hydrolysis by sPLA$_2$ (60). Indeed, these vesicles were susceptible when isolated and incubated alone with sPLA$_2$. However, further experiments revealed that vesicle hydrolysis alone cannot account for all of the phospholipid catalysis observed when cells were made susceptible. In thin layer chromatography experiments such as those described in Table I, cells were separated from microvesicles by centrifugation. Although a significant proportion of the cellular pellet could contain vesicles, nearly complete hydrolysis of extractable lipids was observed suggesting that both cells and vesicles were substrates of the enzyme and that changes in cellular membrane structure per se must be part of the induction of susceptibility.

Sphingomyelin and Re-esterification—One other hypothesis to explain promotion of susceptibility is that sphingomyelin inhibits the action of sPLA$_2$ and that susceptibility could be induced by removing sphingomyelin from the outer leaflet by translocation across the membrane, by hydrolysis, or by sequestration into domains segregated from sPLA$_2$ substrate. Indeed, some cells appear to be more readily hydrolyzed by sPLA$_2$ following pre-hydrolysis of the plasma membrane with sphingomyelinase (27, 55, 61). We examined this hypothesis briefly by incubating S49 cells with 1 unit/ml sphingomyelinase for various times at 37 °C and then assaying the time course of membrane hydrolysis in the presence of sPLA$_2$. Regardless of the length of treatment with sphingomyelinase (up to 90 min), there was no evidence that it increased the amount of membrane hydrolyzed upon subsequent exposure to sPLA$_2$. However, the time course of restoration of fatty acid levels to baseline normally observed with sPLA$_2$ alone (Fig. 1) was absent or reduced in several of the experiments. This result may explain the previous reports of promotion of membrane hydrolysis by sPLA$_2$ after treatment with sphingomyelinase since those data were collected following lengthy incubation with sPLA$_2$ after which the restoration to baseline after initial hydrolysis would already be completed.

It is likely that this restoration to baseline that occurs in the absence of ionophore represents re-esterification of phospholipid after hydrolysis catalyzed by sPLA$_2$ has ceased. Thin layer chromatography experiments supported this interpretation since it was observed that lysophospholipid levels fall and phospholipid levels rise over the same time course as those shown in Fig. 1. The results with sphingomyelinase described above, then, raises the possibility that the re-esterification reaction is regulated by sphingomyelin and/or its hydrolysis product (ceramide).

Concluding Remarks—In a biological setting, excess lysolecithin is produced as a consequence of hydrolysis of oxidized lipoproteins (13, 14). A broad range of responses to this lipid have been explored in an effort to understand the relationship between it and the pathology of diseases such as atherosclerosis (8, 11-17). Based on this study, a potential action of lysolecithin to render cells vulnerable to extracellular sPLA$_2$ must now be added to the list. The relationship of these observations to long term prostaglandin synthesis induced by certain proinflammatory agents is more difficult to predict (31). It appears likely that the action of cPLA$_2$ is involved in priming cells for the action of sPLA$_2$ during long term prostaglandin synthesis (29–31) as it was in the response to lyso-PPC reported here. Nevertheless, the additional calcium-dependent and cPLA$_2$-independent effects of the lysophospholipid appear more substantial in promoting membrane hydrolysis by sPLA$_2$ compared with stimulation by agents such as platelet-activating factor and lipopolysaccharide (29). Consequently, the response to lyso-PPC probably relates more to pathological conditions that result in larger scale membrane hydrolysis and tissue damage. We emphasize that this response is relevant to other investigations attempting to understand the action of lysolecithin since it occurs at concentrations similar to or less than other biological and pathological effects of the lipid.

In summary, we propose the following as a testable hypothesis for this action of lysolecithin and sPLA$_2$ on mammalian white blood cells (Fig. 9). First, cells are initially susceptible to the enzyme but respond by quickly becoming resistant. This phenomenon is a response to the enzyme involving binding to sites separate from the loci of membrane hydrolysis. Lyso-PPC supersedes this resistance by elevating intracellular calcium. Part of the effect involves a combined action of calcium and lyso-PPC to activate cPLA$_2$ which then leads to susceptibility to sPLA$_2$ as proposed by Balsinde and Dennis (29). The remainder of the effect of lyso-PPC requires only the elevation of intracellular calcium. The calcium then acts on enzymes such as scramblase, aminophospholipid translocase, and specific proteases that lead to phospholipid translocation across the bilayer and release of microvesicles from the plasma membrane into the extracellular fluid. Changes associated with the
The disposition of fatty acid during the initial interaction of sPLA₂ is described by three processes as follows: production through hydrolysis of phospholipids, removal of fatty acid, and time-dependent inhibition of the production dependent on sPLA₂ binding to a separate site with higher affinity for the enzyme than the loci of hydrolysis. The rate of change of fatty sPLA₂ binding to a separate site with higher affinity for the enzyme than the loci of hydrolysis. The rate of change of fatty acid concentration ($\alpha$) is a constant rate for fatty acid formation; $\beta$ is the rate constant for fatty acid removal. The time-dependent inhibition is described by the accumulation of an inhibitory process ($I_{(i)}$) that alters the value of $\alpha$ as described in Equation 2.

$$I_{(i)} = \gamma (1 - I_{(0)}) - \gamma e^{-k_{\text{cat}} t}$$

Integration of Equation 3, rearrangement assuming an excess of enzyme concentration relative to binding sites, and normalization of $I_{(i)}$ as a fraction of the total number of binding sites yields Equation 4.

$$\frac{dI_{(i)}}{dt} = k_{\text{cat}} E_{(i)} (S_{(i)} - k_{d} I_{(i)})$$

Integration of Equation 1 with substitution of Equations 2 and 4 gives the final solution as shown in Equation 5.

$$P = \frac{k_{\text{cat}} E_{(i)} (1 - \gamma) (1 - e^{-k_{d} t})}{\beta} - \frac{k_{d} E_{(i)} (1 - \gamma)}{\beta} (e^{-k_{d} t} - e^{-\beta t})$$

All parameter values were held constant in the simulations except for $E_{(i)}$ which was adjusted for each simulation according to the experimental conditions described in Figure 1, panel B (curves a–c). $K$ was set at $0.007 \text{s}^{-1}$ based on observation of the experimental enzyme concentration dependence of the initial rate of hydrolysis. The ratio $k_{\text{cat}}/k_{d}$ was fixed at $200 \text{ml} / \text{g}$ to reflect the assumption of higher affinity for the binding site responsible for repressor kinetics. $I_{(0)}$ was assumed to be zero for the first addition of enzyme. For the second addition, $I_{(0)}$ was set to the value of $I_{(i)}$, at $t = 500 \text{s}$ from the first addition (arrow in Fig. 1, panel C). The values of the rate constants $k_{d} (0.03 \text{s}^{-1})$ and $\beta (0.007 \text{s}^{-1})$ were set based on the time dependence of the experimental results, and $k_{\text{cat}}$ was set at $1 \text{s}^{-1}$ since fatty acid accumulation was relative.
Cell Membrane Susceptibility to Phospholipase A_2

52. Gelb, M. H., Jain, M. K., Hanel, A. M., and Berg, O. G. (1995) *Annu. Rev. Biochem.*, 64, 653–688
53. Murakami, M., Kudo, I., and Inoue, K. (1991) *FEBS Lett.*, 294, 247–251
54. Kikuchi-Yanoshita, R., Yanoshita, R., Kudo, I., Arai, H., Takamura T., Nomoto, K., and Inoue, K. (1993) *J. Biochem. (Tokyo)* 114, 33–38
55. Koumanov, K., Wolf, C., and Bereziat, G. (1997) *Biochem. J.*, 326, 227–233
56. Chang, C. P., Zhao, J., Wiedmer, T., and Sims, P. J. (1993) *J. Biol. Chem.*, 268, 7171–7178
57. Comfurius, P., Senden, J. M. G., Tilly, R. H. J., Schroit, A. J., Bevers, E. M., Zwaal, R. F. A. (1990) *Biochim. Biophys. Acta*, 1026, 153–160
58. Basse, F., Gaffet, P., and Bienvenue, A. (1994) *Biochim. Biophys. Acta*, 1190, 217–224
59. Dumaswala, U. J., and Greenwalt, T. J. (1984) *Transfusion* 24, 490–492
60. Gheriani-Gruszka, N., Almog, S., Biltonen, R. L., and Lichtenberg, D. (1988) *J. Biol. Chem.*, 263, 11808–11813
61. Chap, H. J., Zwaal, R. F. A., and Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta*, 467, 146–164