Phospholipase A_2 Action on Planar Lipid Bilayers Generates a Small, Transitory Current That Is Voltage Independent

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ABSTRACT Addition of either bee venom or *Trimeresurus flavoviridis* phospholipase A_2 (PLA_2) to the solution bathing the front side of a voltage-clamped, planar lipid bilayer consistently produced a transitory current lasting ~100 s. This current is consistent with anions moving through the membrane to the rear side. The peak current is independent of holding potential. PLA_2 activity on phospholipid membranes not only produced a current but also led to membrane rupture within 300 s. The current depends on Ca^{2+} and lipid type. Addition of PLA_2 in the absence of Ca^{2+} or to membranes made of nonsubstrate lipids (e.g., glycerol monooleate or lysophosphatidylcholine) produced no current and did not break the bilayer. Peak current height, signal decay time, and time to membrane rupture all depended on PLA_2 dose, whereas total charge produced was constant. This current does not flow through ion channels because there are no channels present and the current is not voltage dependent. The evidence is consistent with the hypothesis that the current is generated by the movement of ionized fatty acid produced by PLA_2 action. These results demonstrate a simple method to measure enzyme activity in the presence of different substrates and varied environmental conditions.

INTRODUCTION

Phospholipases are a family of proteins that hydrolyze phospholipids. The products of this hydrolytic breakdown depend on which bond a particular class of phospholipase attacks (Fig. 1). Phospholipase A_2 (PLA_2), used in our experiments, cleaves target phospholipids at the *sn*-2-acyl chain, forming lysophospholipid and fatty acid (FA). Phospholipase D, on the other hand, hydrolyzes the bond between the headgroup and the phosphate group, yielding a free headgroup and a phosphatidic acid. Because phospholipases are associated with a number of physiologic (Dennis et al., 1991; Exton, 1994; Gips et al., 1994) and pathophysiologic states (Anderson et al., 1994; Navab et al., 1995; O'Regan et al., 1995; Bazan et al., 1995) in the human body, considerable interest has been generated in the mechanism of action of this enzyme family.

A great deal of research, using several different experimental systems, has been done to elucidate the catalytic activities of PLA_2 (Verheij et al., 1981; Dennis, 1983; Cherny et al., 1993; Jain et al., 1995; Sheffield et al., 1995). We previously showed that bee venom PLA_2 disrupts planar lipid membranes when it is applied to one side of the membrane at concentrations 10 times lower than those used in experimental cerebral ischemia systems (O'Regan et al., 1995). During this set of experiments we made the serendipitous observation that PLA_2 action was associated with a small, transitory current. The current flow was from the enzyme side to the other side of the membrane via the ammeter (voltage-to-current converter) connected between the chambers. In most experiments the current had not returned to zero when the membrane ruptured. It was concluded that this PLA_2-associated current could represent a simple, direct, and continuous record of PLA_2 activity. Therefore, we investigated the origin of the current to establish the relationship between PLA_2 activity and current generation.

Two hypotheses of signal origin were formulated: 1) The products of PLA_2 (or PLA_2 itself) form ion channels through the membrane, allowing current to flow, and 2) the enzymatic activity of the PLA_2 liberates products that generate a current. We present the results of experiments designed to distinguish between these hypotheses. Our results rule out ion channel formation and are consistent with the hypothesis that the current results from the movement of ionized FAs split from phospholipids by PLA_2.

Received for publication 10 June 1996 and in final form 13 November 1996.

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0006-3495/97/01/247/07 $2.00

MATERIALS AND METHODS

Formation of bilayers

Planar lipid bilayers were formed according to standard methods (Hanke and Schule, 1993; Kelly and Woodbury, 1996). Usually, membranes were composed of brain phosphatidylethanolamine and brain phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster, AL) mixed in a 7:3 weight ratio and dissolved in decane (20–40 mg/ml). We formed bilayers across a small hole (~200 μm) in a plastic cup by brushing the hole with a phospholipid–decane bubble on the tip of a pipette. Both sides of the cup hole were bathed in standard solution (40–50 mM NaCl, 8 mM HEPES, pH 7.2, and 0.1 mM $CaCl_2$, unless noted). The side to which PLA_2 was added is called the front chamber, and it has a volume of 1.0 ml. In some experiments 0.25 mM ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) was added to the standard solution.

Measurement of membrane current and voltage

Electrical connections were made between the solutions bathing the membrane by Ag–AgCl electrodes. In most experiments membrane current was

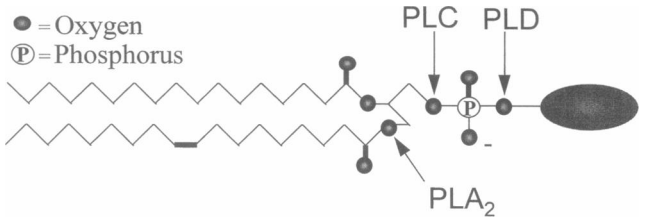

FIGURE 1 Specific hydrolytic cleavage sites of three members of the phospholipase family, phospholipase A_2 (*PLA_2*), phospholipase D (*PLD*), and phospholipase C (*PLC*), on a typical phospholipid. The reaction splits water, leaving an —OH group on each end of the cleavage site. The products of PLA_2 action on a phospholipid are FA and lysophospholipid.

measured under standard voltage-clamp conditions with the current-to-voltage converter (ammeter) attached to the back electrode. Voltages were applied between ground and the front electrode.

In some experiments we measured membrane voltage by replacing the ammeter with a voltage follower (voltmeter input resistance $\sim 10^{13}\ \Omega$) and clamping current at zero. The voltage generated by charge separation, such as that which is due to ionization of FA following PLA_2 addition, decays with a time constant equal to the product of the membrane resistance and the membrane capacitance (*RC*). Therefore, only membranes that had time constants of >10 s were used for experiments done with this configuration (typically $C = 150$ pf and $1/R < 15$ pS). The final voltage was estimated from values obtained soon after addition of PLA_2.

PLA_2 dose-response experiments

For all experiments, bee venom PLA_2 (1200 units/mg; Sigma Chemical Co., St. Louis, MO) was added to the front chamber at concentrations ranging from 0.125 to 1.25 μg/ml. The front chamber solution was stirred, after 30 s, by a magnetic flea. Membrane current was recorded with a chart recorder. Voltage-independence and Ca^{2+}-dependence experiments were repeated with PLA_2 isolated from *Trimeresurus flavoviridis* (1200 units/mg; Calbiochem Biochemicals, La Jolla, CA)

Four quantities were measured on each bee venom PLA_2 current record: the maximum (peak) current, the half-life of signal decay, the time to membrane rupture, and the total charge (area under signal). Measurements were made on a minimum of four membranes at each dose. The maximum current following addition of PLA_2 is proportional to cup hole size. Therefore, we normalized peak current by dividing the maximum current by the cup hole surface area, resulting in units of pA/cm^2. Signal half-life was defined as the length of time for the current to fall to half of its maximum value ($T_{1/2}$). In several experiments the membrane broke shortly before $T_{1/2}$. In these cases $T_{1/2}$ was determined by linear extrapolation (for the rare case when the membrane broke before a definite decay began, the data were rejected). Time to membrane rupture, previously described by O'Regan et al. (1996), was defined as the time between the beginning of the positive current and membrane rupture (as indicated by a sudden, large increase in membrane conductance). Total charge movement was defined as the quantity of charge produced by PLA_2 action on the planar bilayer. Normally, one can estimate total charge by measuring the area under the entire curve (i.e., from initial positive rise to end baseline value). Unfortunately, most of the membranes broke before baseline was attained. However, inasmuch as the decay is exponential in nature, we estimated the total charge by multiplying peak current by τ, the exponential decay time ($\tau = 1.44 \times T_{1/2}$).

Statistics

We performed statistical analysis by using a one-way analysis of variance followed by Student Newman Keul's or Sheffe's post hoc tests. A $p < 0.05$ was accepted as denoting a significant difference.

Substrate specificity experiments

Thin membranes (bilayers, described above) were used in all experiments, except in substrate specificity analysis. Measurements of substrate specificity were done on thick "membranes" because of the difficulty of forming thin membranes from some lipids. Thick membranes can easily be made by addition of slightly more lipid to the cup hole and by discontinuing brushing as soon as the hole is plugged with the lipid–decane solution. Thick membranes probably resemble two monolayers separated by a thick lipid–decane sheet. The capacitance of a thick membrane is low (12–16 pF), usually <5% of the maximum calculated capacitance for a given cup hole diameter. No significant difference in bee venom PLA_2 current size and shape was observed between thick and thin membranes composed of standard lipid. As mentioned above, current size depends on cup hole area. Several of the less-decane-soluble phospholipids tested for PLA_2 specificity were mixed 1:1 with 1,2-diphytanoyl phosphatidylcholine (DphyPC) to increase miscibility.

RESULTS

Voltage independence of peak current

Fig. 2 shows records of current versus time measured at holding potentials of +60, 0, and −60 mV after application of 0.375 μg/ml bee venom PLA_2. Five membranes were tested at each voltage. It can be seen that current flow is always in the same direction and that the size and shape are similar in all three records. Application of 0.375 μg/ml *Trimeresurus flavoviridis* PLA_2 also led to a voltage-independent current with size and shape comparable with those produced by the bee venom enzyme (data not shown).

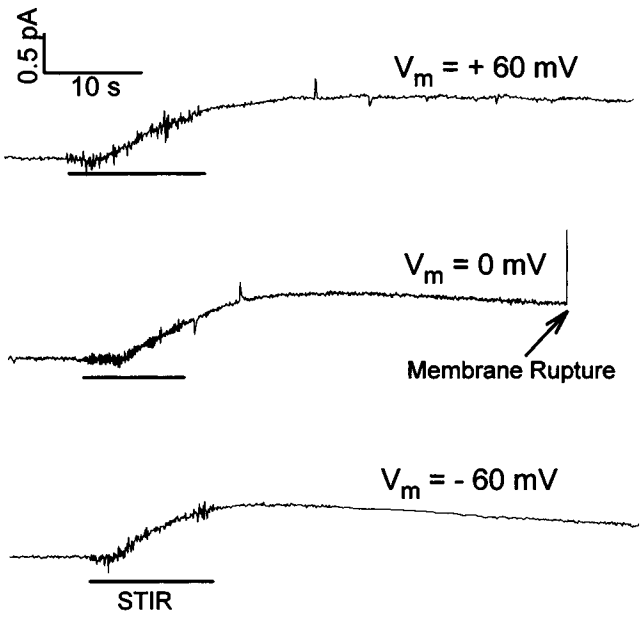

FIGURE 2 Current as a function of time following addition of PLA_2 in the front chamber with holding potentials of +60, 0, and −60 mV. Note that direction of current flow, size, and shape of the signals are similar at all voltages. Cup diameter and membrane capacitance: 278 μm, 173 pF; 281 μm, 185 pF; 276 μm, 180 pF at $V_m = +60$, 0, and −60 mV, respectively. All experiments were done with standard solution, except that $[Ca^{2+}] = 2$ mM.

The IV (peak-current-versus-voltage) curves given in Fig. 3 show the difference between an experimentally determined IV curve for the bee venom PLA$_2$-associated current and a theoretical IV curve of a simple ion channel based on Ohm's law.

Calcium dependence

Either bee venom or *Trimeresurus flavoviridis* PLA$_2$ can be delivered to an artificial membrane in an active or an inactive state, depending on the concentration of free calcium ions. Addition of PLA$_2$ to the front chamber containing standard solution (50 mM NaCl, 8 mM HEPES, 0.1 mM $CaCl_2$, pH 7.2), followed by stirring, delivers active enzyme to the phospholipid surface. The current starts rising shortly after stirring begins (Fig. 2). As shown in Fig. 4, the result is much different if EGTA (0.25 mM) is added to the standard solution before the addition of PLA$_2$. No signal is observed until excess Ca^{2+} is added to the solution and stirring begins. It is important to note that a large quantity of Ca^{2+} (i.e., 2.5 mM) was added to ensure Ca^{2+} levels well in excess of EGTA, not because higher levels of Ca^{2+} are necessary to activate PLA$_2$. No differences in current size and shape were observed between 0.1 and 2.5 mM Ca^{2+}. The signal following Ca^{2+} addition has much steeper rising and falling phases; however, there is still a delay of ~90 s before the membrane ruptures. Addition of 2.5 mM Ca^{2+} in the absence of PLA$_2$ produced a small negative transient current and never resulted in membrane disruption.

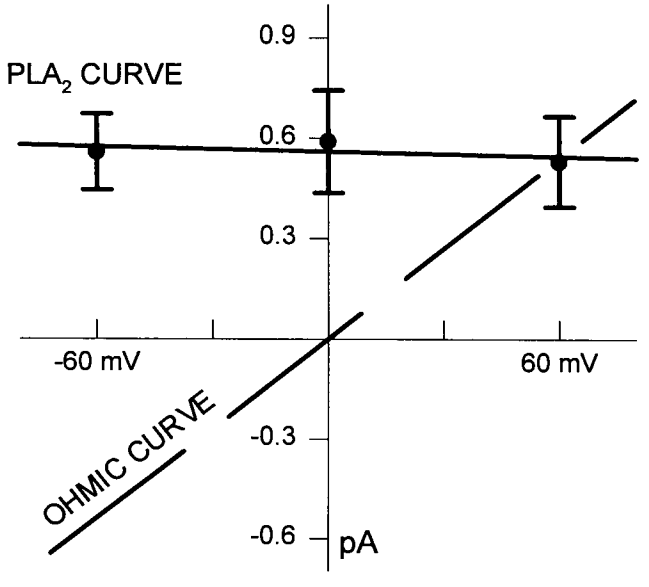

FIGURE 3 IV curves of a hypothetical ion channel, based on Ohm's law (*OHMIC CURVE*) and experimental data for the maximum current produced by PLA$_2$ activity (*PLA$_2$ CURVE*) on lipid membranes held at +60, 0, and −60 mV. Clearly, the PLA$_2$-induced current is not due to formation of holes or channels through the membrane. Error bars are 95% confidence limits based on five (including those shown in Fig. 2) membranes at each voltage.

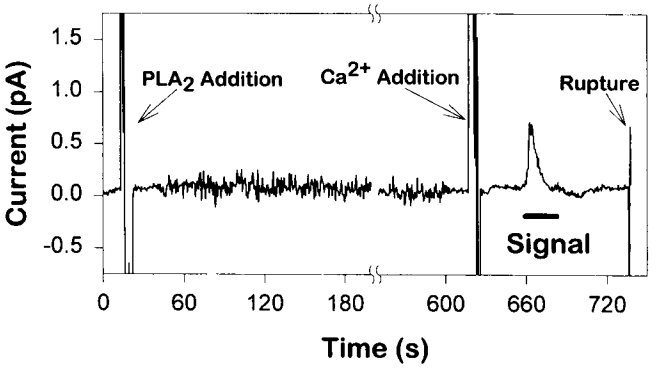

FIGURE 4 Ca^{2+} is necessary for PLA$_2$ activity on a bilayer. 0.125 μg/ml PLA$_2$ was added to the front chamber solution containing 0.25 mM EGTA at 20 s. The solution was then stirred for 9 min, resulting in the additional signal noise observed soon after addition of PLA$_2$. However, no current signal is visible. Ca^{2+} (2.5 mM) was then added to the solution at 625 s and, 30 s later, stirring was resumed for 5 s, leading to a current with a steep rise and an exponential decay. The current transient is over in less than 20 s. Note that the current transient is much shorter in duration than those shown in Fig. 2 but that the time to membrane rupture is approximately the same (90 s). Cup diameter, 207 μm; membrane capacitance, 93 pF.

Effect of PLA$_2$ dose on signal size and duration

As defined in Materials and Methods, four quantities were measured from each bee venom PLA$_2$-induced current: 1) maximum (peak) current, 2) half-life of signal decay, 3) time to membrane rupture, and 4) total charge movement (area under signal). A summary of all the results is shown in Fig. 5. There was a significant dose-dependent change in all the measured parameters (Fig. 5 *A–C*) except total charge movement, which remained constant with dose (Fig. 5 *D*).

Effect of PLA$_2$ on membrane voltage

It would be expected that the currents that are measured in voltage-clamp configuration would generate a voltage across the membrane under open-circuit conditions. To test this prediction we performed current-clamp studies at $I = 0$ to measure the size of the membrane potential generated by bee venom PLA$_2$ action on a phospholipid bilayer. Addition of 1.25 μg/ml led to a large, rapid increase in membrane potential to a maximum of 60–100 mV, followed by a slow decline, until the membrane ruptured within 90 s (results not shown). These results are consistent with previous measurements (by a similar technique) of the voltage change across black lipid membranes as the result of PLA$_2$ activity (Cherny et al., 1993). These voltage increases, by themselves, are not sufficient to rupture bilayer membranes but could modulate enzyme activity.

Specificity of PLA$_2$

It has been determined experimentally that bee venom PLA$_2$ cleaves many different phospholipids, independently of

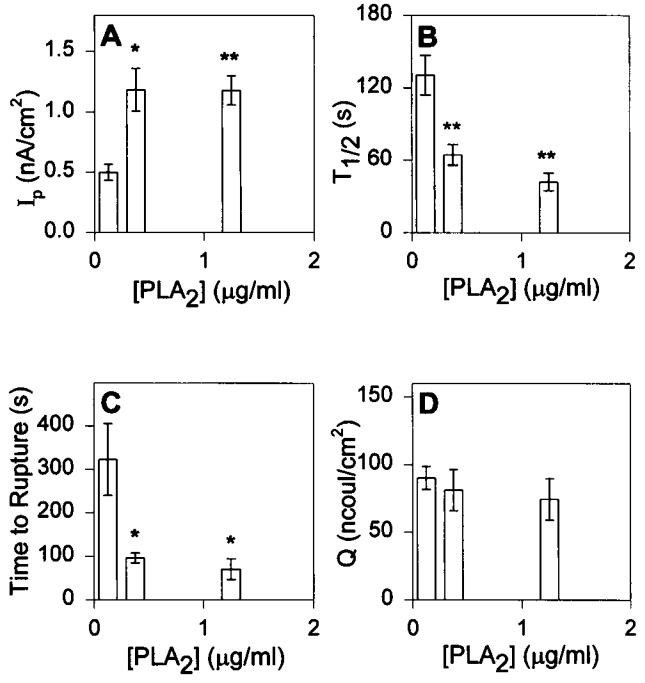

FIGURE 5 Effects of PLA_2 concentration on four measured parameters of the current signal. Error bars are ± 1 SE. Statistical significance is indicated as follows: *, $p < 0.05$, **, $p < 0.01$ compared with the lowest dose. A minimum of four membranes was tested at each dose. *A*, Relationship between dose and peak current (corrected for membrane area). The maximum current, I_p, is significantly higher at doses of both 0.375 and 1.25 μg/ml than at 0.125 μg/ml. *B*, Relationship between PLA_2 dose and signal decay. $T_{1/2}$ is defined as the time it takes for the current to decay to half of its maximum value. There is a significant difference between signal decay times at the two higher doses and the lowest dose. This suggests that lipid hydrolysis continues for longer periods when a lower dose of PLA_2 is used. *C*, Relationship between PLA_2 dose and time until membrane breakage. There is a significant difference between the lowest dose and the two higher doses. *D*, Total charge flow is the same at all three doses. The ordinate is an estimate of the area under the IV curve calculated from $Q = 1.44 \times I_p \times T_{[1/2]}$. Approximately the same amounts of substrate are consumed at all the PLA_2 doses.

headgroup (Pluckthun and Dennis, 1985) or of FA chain length (Op Den Kamp et al., 1974; Raykova and Blagoev, 1986). We performed a series of experiments to corroborate these findings, using the PLA_2-induced current as a detector of activity. In this set of experiments 1.25 μg/ml PLA_2 was added to thick membranes (see Materials and Methods). Thick membranes were used because stable thin bilayers could not be formed with some types of phospholipid used. Table 1 summarizes the results obtained for all phospholipids tested. Note that no signal was observed with thick or thin membranes formed from pure glycerol monoolein (GMO) or lysophosphatidylcholine (lysoPC), neither of which can be a substrate for PLA_2.

A typical current signal was observed regardless of length, saturation status, or type of double bond (*cis–trans*) of the FA occupying the *sn*-2 position of phosphatidylethanolamine or PC. The one exception was membranes made of 100% DphyPC; no signal was observed. Nor did a signal

TABLE 1 Bee venom PLA_2 specificity

Phospholipid Headgroup	Fatty Acid Tails	Signal?
Ethanolamine	1-Palmitoyl 16:0 2-Oleoyl 18:1 (*cis*)	Yes
Choline (PC)	1-Palmitoyl 16:0 2-Oleoyl 18:1 (*cis*)	Yes
PC	1-Oleoyl 18:1 (*cis*) 2-Palmitoyl 16:0	Yes
PC	1-Palmitoyl 16:0 2-Arachidonoyl 20:4	Yes
PC	1,2-Dipalmitoyl 16:0	Yes
PC	1,2-Dielaidoyl 18:1 (*trans*)	Yes
Glycerol	Mixture: 18:1 (31%), 16:0 (34%), other (35%)	Yes
LysoPC	Monooleoyl 18:1 (*cis*)	No
Glycerol (GMO)	Monooleoyl 18:1 (*cis*)	No
Inositol (phosphatidylinositol)	Mixture: 16:0 (30%), 18:2 (47%), other (23%)	No
PC	1, 2-Diphytanoyl 16:0 [$(CH_3)_4$]	No

or rupture occur in thin membranes after 15 min of exposure to PLA_2. These two results provide strong evidence that DphyPC is not a substrate for bee venom PLA_2. Interestingly, no signal was observed with thick membranes made of 100% phosphatidylinositol.

DISCUSSION

The two primary topics of discussion are 1) the evidence that the current is generated by enzymatic activity and does not flow through ion channels and 2) the probable nature of the mechanisms that generate the current.

Ion channels are not the source of the current

The evidence that the current is not due to ion flow through channels is compelling: 1) There are no concentration gradients that could drive ions through a channel at $V_m = 0$ mV. 2) The IV curve is approximately horizontal (Fig. 3), indicating that the mechanism that generates the current is not much affected by membrane voltage. 3) The current through a channel would be steady, not slowly rising and falling. 4) It is not clear what could form an ion channel; if PLA_2 formed channels when it bound to the membrane or if the breakdown of phospholipids permitted the formation of lipid channels (Woodbury, 1989) the IV curve would go through zero, and the conductance would steadily increase with time. These four points eliminate ion channel formation as a practical hypothesis for the observed current, and therefore it will not be considered further.

Melittin is a major contaminant of bee venom PLA_2 and is known to interact with bilayer membranes (Hermetter and Lakowicz, 1986; Alder et al., 1991; Ohki et al., 1994). Therefore, the possibility exists that the PLA_2-associated signal is caused by melittin or by another contaminant. There are three lines of evidence that this is not the case: 1) Melittin is reported to cause membrane leakage (Ohki et al.,

1994). However, as explained above, the source of the current is not consistent with a leak current. 2) Melittin-induced leakage is inhibited by Ca^{2+} (Alder et al., 1991), whereas the current that we observed requires the presence of Ca^{2+} consistent with the activation of PLA_2 by Ca^{2+}. 3) A similar PLA_2-associated current is elicited after addition of *Trimeresurus flavoviridis* PLA_2, which would not be expected to be contaminated by melittin. Thus, we conclude that melittin or any other contaminant that causes leaks in membranes is not the cause of the current signal.

Does the current reflect PLA_2 activity?

Figs. 4 and 5 and Table 1 provide strong evidence that current is generated only when phospholipids are being cleaved to lysophospholipid and FA. The relationship between peak current and PLA_2 concentration shown in Fig. 5 *A* is accurately described by an equilibrium between PLA_2 in the solution and a fixed number of binding sites on the membrane. That is, the peak current is proportional to bound PLA_2. Also, no current is generated when the lipid is not split by PLA_2 (Table 1: GMO and lysoPC).

The Ca^{2+} dependence of PLA_2-induced current (Fig. 4) offers additional support to the hypothesis that the current observed after addition of PLA_2 to an artificial membrane is due to the hydrolytic action of PLA_2 on phospholipids. It was previously determined that PLA_2 molecules from many sources, including bee venom, require Ca^{2+} for activity (Dennis, 1994). When Ca^{2+} was chelated with EGTA, no PLA_2 current was observed. However, the enzyme must already have been bound to the membrane, because when Ca^{2+} was added to the solution a faster-rising current was generated (Fig. 4). These results suggest that the rising phase in the normal signal (Fig. 2) most likely represents binding of PLA_2 to the bilayer. Furthermore, these results support the finding of Menashe et al. (1986) in that PLA_2 does not need Ca^{2+} to bind to the phospholipid membrane and that Ca^{2+} is necessary for the hydrolysis of the phospholipid.

Some lipids are not substrates for bee venom PLA_2

Control experiments using thick membranes made from 100% nonsubstrate lipid revealed no current after application of PLA_2. Two separate lipids were used for these experimental controls: lysoPC, which is a reaction product of PLA_2 activity, and GMO, which resembles lysoPC.

Bee venom PLA_2 has proved to be nonspecific in regard to the length (Op Den Kamp et al., 1974; Raykova and Blagoev, 1986) of the FA that occupies the *sn*-2 position of a phospholipid, unlike some PLA_2s associated with intracellular signaling (Dennis et al., 1991; Dennis, 1994). However, no current was observed after PLA_2 was added to thick or thin membranes composed entirely of DphyPC. Furthermore, the thin membranes did not rupture on addition of PLA_2. These two results (no signal and no membrane rupture) indicate that DphyPC is not a substrate for PLA_2. Phytanoyl is a 16 carbon, saturated FA with methyl groups attached to carbons 3, 7, 11, and 15. It is likely that one or more of these methyl groups inhibit the interaction of the PLA_2 catalytic domain with the FA chain. All other phospholipids tested, except for phosphatidylinositol, yielded a current on addition of PLA_2, independently of chain length, type of double bond, or saturation status of the FA that occupies the *sn*-2 position (Table 1). Inositol is a relatively bulky headgroup compared with the other phospholipid headgroups tested and, therefore, could sterically exclude PLA_2 from interacting with it.

Mechanism of current generation by PLA_2 action

Fig. 6 is a cartoon of the proposed chain of events that show how the breakdown products of PLA_2 action are the source of the observed current. In the electronic configuration employed for these experiments, positive charges moving through the membrane toward the front chamber, negative charges flowing toward the back chamber, or both could lead to the initial positive current. As shown in Fig. 6 (*step (2)*), the only charged molecule produced by PLA_2 activity on artificial membranes is the deprotonated FA. It was previously established that a majority of the FA released by PLA_2 remains in the membrane after it has been cleaved from the phospholipid (Kupferberg et al., 1981). FAs in the membrane can generate a positive current by either of two mechanisms. First, when FA on the enzyme side of the membrane deprotonates (*step (3)*), a positive charge (H^+) will move toward the front chamber electrode (*step (4)*), whereas a negative charge (the FA), remains in the membrane. Alternatively, the negatively charged FA may flip to the other side of the membrane (*step (5)*). In either case a

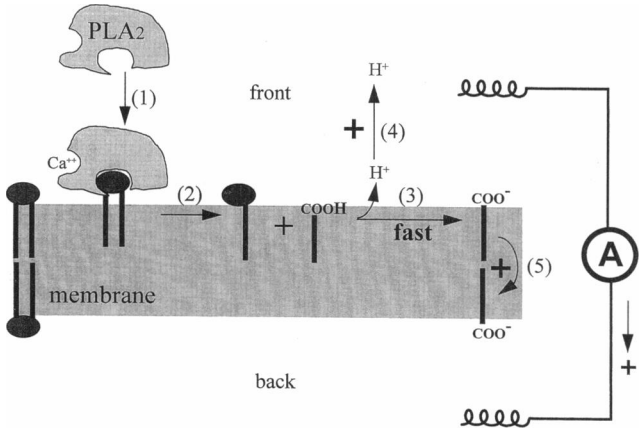

FIGURE 6 Cartoon of PLA_2 action and current generation. The five steps represent (*1*) binding of PLA_2 to the planar membrane; (*2*) enzymatic production of lysophospholipid and FA; (*3*), (*4*) deprotonation of FA; and (*5*) flip-flop of the negatively charged FA across the membrane. Both steps (*4*) and (*5*) could produce a positive current as measured by the ammeter, *A*, placed across the membrane. The downward arrow below the ammeter defines the direction of positive current flow.

positive current is generated, driven by the concentration gradient of FA molecules. It was previously established that charged FAs can flip rapidly across protein-free phospholipid membranes (Gutknecht, 1988).

The transitory nature of the current naturally follows from the fact that there is only a finite amount of phospholipid in the membrane that can be cleaved. The immediate questions raised by this model are the following: 1) Is there enough FA produced to supply the total amount of charge (Fig. 5 *D*) that flows? 2) If so, can PLA_2 cleave phospholipid at a rate sufficient to supply the measured peak current (Figs. 2 and 4)?

The amount of available charge is easily calculated from the area occupied by each phospholipid molecule, ~72 $Å^2$ (Huang and Mason, 1978; Cornell and Separovic, 1983), in the membrane. The result is 22,000 nC/cm^2. Fig. 5 *D* shows a total charge of less than 100 nC/cm^2. Therefore, only a small percentage of the phospholipid must be cleaved to yield adequate FA to produce the observed current. Alternatively, if we assume that all the phospholipid is hydrolyzed (see below), then only a small fraction of FA produces a current detected by the electrodes. This is possible because approximately half of the FA released will be in the protonated state (pK ~7; Miyazaki et al., 1992), and opposing (negative) currents could be generated after flipping and ionization of neutral FAs.

The maximum rate of FA production in membranes can be estimated from the average peak current of 0.6 pA. As one unit of PLA_2 produces 1 μmol FA/min, 3.7×10^{-10} units of PLA_2 are needed to generate FA at the required rate. This is orders of magnitude less PLA_2 than what is added to the system (~1 unit) and implies that most of the PLA_2 is not bound to the membrane.

Mechanism of signal decline

The mechanism described above predicts that a continuous current should be produced following addition of PLA_2; however, all experiments are characterized by a peak current followed by a decline toward zero. Theoretically, there are two possible reasons for the decline in current: a decrease in turnover of the enzyme or the initiation of a process that generates an opposing (negative) current. A decrease in enzyme turnover could be due to product inhibition or to a decrease in lipid substrate. Product inhibition was previously observed with bee venom PLA_2 (Lawrence, 1975) and could account for signal decline in this system. As noted above, substrate depletion is reasonable because of the large quantity of PLA_2 relative to phospholipid. However, if all the lipid were hydrolyzed and every molecule of FA contributed a positive charge, a much larger signal would be produced than is observed. Therefore, some process that generates an opposing current would have to occur. Two simple processes that could generate a negative current are 1) the loss of charged FA from the membrane into the front solution and 2) flip-flop of neutral FA (Kamp et al., 1995) to the back side followed by deprotonation. Further investigation will be necessary to elucidate the exact mechanism of current decline.

SUMMARY

Our intention here has been to describe the current generated after PLA_2 is applied to planar lipid bilayers. We have shown that a transient (~100 s), voltage-independent current is generated when PLA_2 is added to voltage-clamped, planar membranes. This current is a direct consequence of the activity of PLA_2, as demonstrated in the Ca^{2+}-dependence experiments, and is probably generated by the ionization of FAs produced by the cleavage of phospholipids, the flipping of ionized FA from the enzyme side to the other side, or both. Our experiments show that bee venom PLA_2 is nonselective with regard to the FA attached at the *sn*-2 acyl position, which is consistent with previously published data.

The system described here has the potential to expand our knowledge of phospholipase A_2 action on lipid membranes, mainly because the environmental conditions surrounding both sides of the membrane can easily be manipulated. Furthermore, the fact that the mass action of a hydrolytic enzyme can produce a voltage-independent current could have interesting ramifications, especially when one is considering membrane threshold phenomena. Although further investigation is necessary if we are to understand completely the currents produced by PLA_2, the potential for experimental manipulation is obvious, as demonstrated by the broad range of lipids used in Table 1. This technique also allows for continuous time analysis, an advantage not offered by other methods of PLA_2 analysis (for a review see Verheij et al., 1981; Jain et al., 1995).

Special thanks to Dr. M. O'Regan and Dr. D. Yingst for carefully reading the manuscript and to Dr. J. W. Woodbury for extensive conversation, criticism, and insights. D. J. Woodbury is the recipient of National Institutes of Health Grant R29 MH50003.

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