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Phospholipase-Induced Maturation of *Xenopus laevis* Oocytes: Mitogenic Activity of Generated Metabolites

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Abstract Signal transduction induced by generation of second messengers from membrane phopholipids is considered a major regulatory mechanism in control of cell proliferation. We report here that in the *Xenopus laevis* oocytes model, microinjection of the three most relevant types of phospholipases acting on membrane phospholipids (A2, C, and D) are capable of inducing oocyte maturation with similar efficiencies. This effect is mediated by the generation of known second messengers such as lyso-phospholipids, arachidonic acid, diacylglycerol, and phosphatidic acid. Specific inhibitors of protein kinase C made it possible to identify alternative independent signalling pathways for induction of oocyte maturation. Our results indicate that while phospholipase C seems to be dependent on protein kinase C (PKC), phospholipase A2, and phospholipase D are completely independent of protein kinase C function. Thus, the oocyte system is a powerful tool for the analysis of the potential mitogenic activity of lipid metabolites. It is also an excellent tool for unravelling the different routes involved in the regulation of cell growth. © 1993 Wiley-Liss, Inc.

Key words: oocyte maturation, phospholipases, second messengers, cell proliferation, mitogenic activity

Membrane phospholipids are essential structural components of all eukaryotic cells whose composition and distribution may have a role in physical properties such as fluidity and permeability. Besides this structural role, a great deal of information has been accumulated for the past two decades indicating that phospholipids also play an important role in the control of cellular responses such as regulation of cell proliferation, secretion, platelet aggregation, macrophage function, or fertilization [Pelech and Vance, 1989; Billah and Anthes, 1990; Nishizuka, 1992]. This notion is based on abundant information suggesting that hydrolysis of phosphatidyl inositol 1,4 bisphosphate (PIP2) by phosphoinositide specific phospholipase C (PI-PLC) is one of the most relevant events associated to regulation of cell proliferation. This concept has been linked to the generation of two important second messengers by PI-PLC: diacylglycerol (DAG) which is the alosteric activator of protein kinase C (PKC) and inositol 1,4,5 trisphosphate (IP3) which when binding to specific receptors located at reticular membranes releases calcium from intracellular stores. This phospholipid pathway is activated by most factors interacting with receptor tyrosine kinases including growth initiating factors such as PDGF or basic FGF, progression factors such as insulin or IGF-1 and neurotrofic factors such as NGF [Berridge, 1984; Hokin, 1985; Berridge, 1987; Cockcroft and Thomas, 1992].

Recently, other nonclassical routes have also been implicated in the regulation of cell growth. Thus, it has been shown that type D phospholipase (PLD) is activated by mitogenic signals such as those initiated by receptor tyrosine kinases [Plevin et al., 1991; Wright et al., 1992] and PKC activators [Cook et al., 1991; Hii et al., 1991]. Moreover, hydrolysis of phosphatidylcholine (PC) by PLD with the generation of phosphatidic acid (PA) may be a common response to mitogens which also induce hydrolysis of inositol lipids in fibroblasts [Cook and Wakelam, 1991]. On this regard, PA has been shown to behave as a second messenger with potent mitogenic activity in fibroblasts [Moolenaar et al., 1986]. Finally, similar mitogenic activity has been reported for the products derived from PLA2-mediated phospholipid hydrolysis, such as Lyso-PC, Lyso-PI, and Lyso-PA [van Corven et al., 1989].

On the other hand, several phospholipid metabolites are capable of triggering critical cell

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responses other than cell proliferation. This is the case for Lyso-PC, which increases activation of human T-lymphocytes induced by a membrane permeant DAGs and ionomycin [Asaoka et al., 1992]. Moreover, Lyso-PC also enhances cellular differentiation of HL-60 cells into macrophages and chemotaxis [Nishizuka, 1992].

The possible involvement of phospholipid metabolism regulation during malignant transformation has been proposed [Cuadrado et al., submitted]. Indeed, a number of oncogenes have been shown to increase constitutively the levels of lipid metabolites such as inositol phosphates, DAG and phosphorylcholine (*P*Cho) [Preiss et al., 1986; Lacal et al., 1987; Wolfman and Macara, 1987]. Oncogenes such as *src* and *ras* have been shown to induce the constitutive activation of a type D phospholipase [Song et al. 1991; Cuadrado et al., submitted]. Finally, *ras* proteins have been shown to activate a type A2 phospholipase in PC12 [Bar-Sagi and Feramisco, 1986] and fibroblasts [Sharma, 1992].

Despite the abundant information available demonstrating the involvement of phospholipid metabolism in the regulation of cellular processes in eucaryotic cells, very little is known regarding the implication of this process in the oocytes system. This latter system constitutes an excellent model to investigate the potential mitogenic activity of lipid metabolites since it allows biochemical analysis in individual cells. It is well established that DAG and phorbol esters can induce germinal vesicle breakdown (GVBD), a process similar to mitogenic induction of mammalian cells [Varnold and Smith, 1990; Stith and Proctor, 1989].

We show here evidence that the three most relevant phospholipases involved in the regulation of proliferation in mammalian cells (A2, C, and D) are efficient mitogens in the oocyte system. Furthermore, we have identified the specific metabolites generated by these phospholipases with biological activity. Moreover, using specific PKC inhibitors we have been able to dissect different pathways for oocyte maturation induced by these mitogenic agents.

METHODS

Oocyte Maturation and Microinjection

Stage VI oocytes were selected as previously described by manual dissection [Lacal, 1990]. Series of 30–50 oocytes were either treated for hormonal induction of germinal vesicle breakdown (GVBD) with 1 μg/ml progesterone, 50 μg/ml insulin, and 50 to 200 nM tetradecanoyl phorbol acetate (TPA) in Ringer's buffer (100 mM NaCl, 1.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 4 mM $NaHCO_3$, pH 7.8). GVBD was assessed following 16 h incubation at room temperature by the appearance of a white spot in the animal pole. Oocyte microinjection was performed as previously described [Lacal, 1990]. Visual verification of nuclear vesicle breakdown was performed by splitting the oocytes after treatment with 16% trichloracetic acid. Maturation by microinjected enzymes was followed in a similar manner using 25 nl per oocyte of purified enzyme solutions containing 1 unit/ml of each enzyme in 20 mM MES buffer, pH 7.0.

MPF Assays

Analysis of oocytes maturation were performed by determination of the in vitro MPF activity essentially as follows: series of 20 oocytes were treated or microinjected with indicated compounds and then homogenized in a buffer containing 20 mM Hepes pH 7.0, 10 mM β-glycerophosphate, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 2 mM DTT, 100 μg/ml leupeptine, and 100 μM PMSF. Following centrifugation at 13,000*g* for 15 min, extracts were assayed for 10 min at 30°C in a final reaction volume of 50 μl containing 20 mM Hepes, pH 7.0, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 10 μM γ-^{32}P-ATP (2–5 dpm-fmol), 0.2 μg of PKA inhibitor, and 1 mg/ml of type III-S calf thymus histone (Sigma Chemical Co., St. Louis, MO). Reactions were stopped by addition of PAGE-sample buffer, boiling, and then run in a 15% polyacrylamide gel electrophoresis (PAGE). Gels were dried and exposed 5 h at −70°C to an autoradiographic film.

Assays for Phosphatidylcholine and Phosphatidylinositol Phospholipases

The biochemical activity of commercially available enzymes (Sigma Chemical Co., St. Louis, MO) were tested by microinjection into oocytes previously labeled in the presence of N-methyl-(^{14}C) choline or (^{3}H)myo-inositol. After microinjection with the indicated enzymes, oocytes were incubated for 30 min at room temperature, the reactions stopped by lysis, and samples kept on ice. Organic phases were extracted as previously indicated [Lacal, 1990], lyophilized, and resuspended in 50 μl of chloroform. Choline derivatives were resolved by TLC in chloroform: acetone: methanol: acetic acid: H_2O (40:15:13:12:7,

TABLE I. *Xenopus laevis* Oocyte Maturation Induced by Hormone Treatment or Microinjection of Phospholipases*

Enzyme	% GVBD Exp. 1	% GVBD Exp. 2
Control (untreated)	10	5
BSA microinjected	11	10
Insulin	35	40
Progesterone	65	65
TPA	100	100
PLA_2 (from *Apis mellifera*)	94	91
PC-PLD		
Type I (from cabbage)	68	nt
Type II (from peanut)	85	100
Type V (from cabbage)	64	nt
Type VI (from Streptomyces)	95	90
Type VII (from Streptomyces sp.)	90	100
PC-PLC		
Type III (from *B. cereus*)	50	40
Type IX (from *C. perfringens*)	35	30
Type XI (from *B. cereus*)	nt	87

*Oocytes were prepared as described in Experimental Procedures; 25 μu of enzyme were microinjected per oocyte in a volume of 25 nl of 20 mM MES buffer. Treatments were carried out with 10 μg/ml insulin, 1 μg/ml progesterone, and 200 nM TPA. GVBD was analyzed 18 h after microinjection by fixing in 10% TCA. nt, Not tested.

V:V:V:V:V). Phosphatidyl-inositols were resolved by TLC on PEI plates pretreated with potassium oxalate (1%) in chloroform: methanol: ammonium hydroxide: H_2O (45: 35: 2.5: 7.5). Plates were exposed to an X-ray film and the radioactive bands were quantitated by scratching the plate and performing scintillation counting.

Note that all the experiments were performed at least three times with similar results to those included in this article.

RESULTS

Phospholipids Breakdown Induce GVBD

A number of phospholipid metabolites have been proven capable of triggering off mitogenic signals in eukaryotic cells. In order to analyze whether phospholipid metabolites are capable of inducing a mitogenic response in oocytes, we have first microinjected several phospholipases (PLs) from different origins and followed their ability to induce germinal vesicle breakdown (GVBD). Table I summarizes the results of such microinjection experiments using PLA2, PLC, and PLD enzymes. All phospholipases analyzed were able to induce oocyte maturation as determined by the dissappearance of the germinal

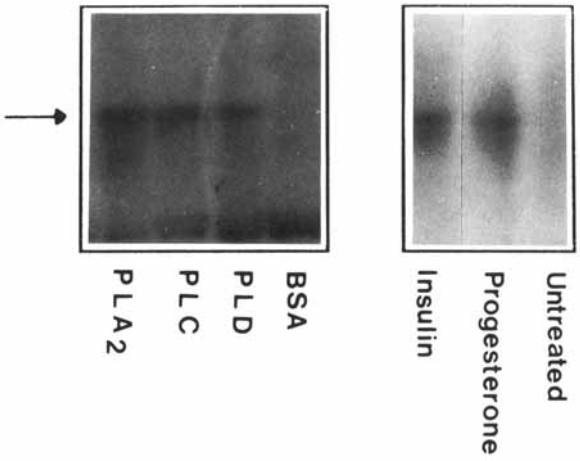

Fig. 1. Activation of the MPF-kinase complex by microinjection of phospholipases into *X. laevis* oocytes. Oocytes were selected and prepared as described in Methods and microinjected with 25 μu/oocyte of each phospholipase, or 25 nl of 1 mg/ml BSA as control. MPF assay was performed as described in Methods and run in a 15% PAGE. Arrow indicates the type III-S calf thymus histone (Sigma Chemical Co., St. Louis, MO).

vesicle, with similar activities, although PLC was the less efficient for the amounts analyzed (25 μu/oocyte). As a negative control, microinjected bovine serum albumin (BSA) was used in the assay, while treatment with well characterized inducers of oocyte maturation such as insulin, phorbol esters, or progesterone were all positive controls. Although direct comparison between these two systems, microinjection and treatment, cannot be made, a clear biological activity was observed.

In order to determine the specificity of the maturating signal and to validate our results with its biochemical analysis, phospholipases-induced maturation was also determined by activation of the MPF through its ability to phosphorylate in vitro histone Type III as described in Methods. As shown in Figure 1, the three phospholipases activated MPF kinase activity indicating that microinjection of phospholipases into oocytes induced efficiently GVBD through MPF in a similar way as other well-characterized mitogenic factors.

Phospholipase-Generated Metabolites Are Also Inducers of Oocytes Maturation

The above results demonstrated that phospholipases (A2, C, and D) are biologically active when microinjected into oocytes. The most reasonable explanation to account for these results is that PLs induced oocytes maturation by generating known products of their respective enzy-

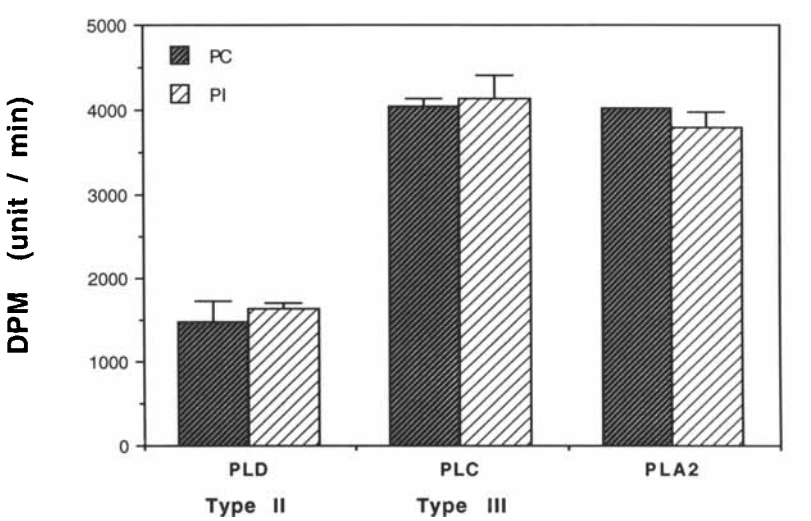

Fig. 2. In vivo specificity of PLA2, PLC, and PLD on PI and PC. Oocytes were microinjected with either (^{3}H)myo-inositol or (^{14}C)choline and incubated for 18–20 h at room temperature. Then, oocytes were microinjected with 25 μu of PLA2, PLC, or PLD and incubated for 30 min at 20°C. At this time, oocytes were lyzed and processed for estimation of hydrolysis of PI or PC. Data represent dpm per microinjected unit of each enzyme.

matic activities. In order to analyze specific metabolites generated by the hydrolysis of those phospholipids most likely involved in this process, we analyzed the specificity of each enzyme for either PI or PC, the two most likely candidates for generation of mitogenic signals. Oocytes were labeled in the presence of either (^{3}H)myo-inositol or (^{14}C)choline for several hours, and then microinjected with 25 μu of selected PLA2, PLC, or PLD enzymes. Figure 2 shows that none of the injected enzymes were specific for PI or PC, and therefore they generated known metabolites indistintively from either PI or PC.

Abundant literature has previously identified the products of each of the phospholipases investigated in our study, such as lyso-PC, lyso-PI, and unsaturated fatty acids for PLA2 activity; *P*Cho, inositol trisphosphates, and DAG for the PLC enzymes; and choline, inositol, and phosphatidic acid (PA) as the PLD products [Pelech and Vance, 1989; Billah and Anthes, 1990; Exton, 1990]. All known products generated by the PLs used in our study were independently microinjected into oocytes, and their biological activity scored after 20 h of injection. Series of 30–50 oocytes were either microinjected or treated with the known metabolites for each enzyme. Table II shows the results of these experiments, indicating that not all metabolites were active.

Regarding the products of PLC and PLD, choline and PCho were inactive, suggesting that these metabolites may not have any relevant mitogenic function in this system. Similarly, inositol phosphates have been extensively investigated by others with negative results [Stith and Maller, 1987; Stith and Proctor, 1989] and were not further investigated in our study. Saturated fatty acids were also completely inactive, but unsaturated fatty acids were weakly active, indicating that most fatty acids released by PLA2 do not seem to be either important signaling mediators. However, arachidonic acid (AA) showed a potent biological activity, as well as phosphatidic acid (PA) and lyso-PC. In contrast, lyso-PI showed a weaker but still significant activity. OAG, a diacylglycerol (DAG) analogue capable of efficiently activating in vitro and in vivo protein kinase C [Nishizuka, 1992] showed a weak but significant activity which correlates with its biological activity in other systems such as stimulation of DNA-synthesis in murine fibroblasts. Other DAGs such as dipalmitoil-, dimirystoil-, and dihexanoil-glycerol showed different degrees of activity. As expected from previous studies, oocytes treated with TPA, a more stable PKC activator, showed a complete induction of GVBD in treated oocytes.

Finally, the fact that lyso-PC and lyso-PI were also capable of efficiently inducing GVBD prompted us to investigate whether these metabolites were themselves biologically active or they required further modification to generate the actual active compounds. Both glycerophos-

TABLE II. Induction of *Xenopus laevis* Oocyte Maturation After Microinjection of the Products of Phospholipases-Mediated Phospholipid Hydrolysis†

Reagent	% GVBD 100 ng	% GVBD 200 ng
Control (untreated)	10	10
BSA	13	11
PLA_2 products		
Lyso-PC	66	74
Lyso-PI	28	50
Unsat. fatty acids[a]	27	20
Sat. fatty acids[b]	5	10
Arachidonic acid	80	90
PC-PLC products		
Phosphorylcholine	7	14
OAG	39	33
Dipalmitoil-glygerol	—	67
Dimirystoil-glygerol	—	40
Dihexanoil-glygerol	—	80
TPA[c]	80*	100**
PC-PLD products		
Choline[d]	15#	15##
Phosphatidic acid	39	80
Others		
Lyso-PE	—	15
Glycerophosphocholine	21	17

†Oocytes were prepared as described and microinjected with indicated amounts of each metabolite.
[a]Unsaturated fatty acids contained equimolar amounts of oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3).
[b]Saturated fatty acids contained equimolar concentrations of palmitic acid (16:0), stearic acid (18:9), arachidic acid (20:0), and behenic acid (22:0).
[c]TPA treatment correspond to 100 nM (*) or 200 nM (**).
[d]Choline was tested at 10 mM (#) or 100 mM (##).

phorylcholine and saturated fatty acids, the products of an attack by a putative phospholipase B (PLB) on lyso-PC, do not induce GVBD. Moreover, lysophosphatidylethanolamine (lyso-PE) was also inactive, indicating a rather specific activity for the PC- and PI-generated lysophospholipids and further suggesting that lyso-PC and lyso-PI may be the actual active metabolites.

Further characterization of the mitogenic activity of these metabolites was performed by analysis of their ability to induce MPF phosphorylation. As shown in Figure 3, all the active compounds were able to activate MPF kinase activity, supporting their role as important second messengers in the regulation of cell proliferation. Thus, the above results demonstrate that at least one of the two directly generated products from the phosphodiesteric attack on PC or PI by either PLC, PLD, or PLA2 is a potent inducer of *X. laevis* oocytes maturation.

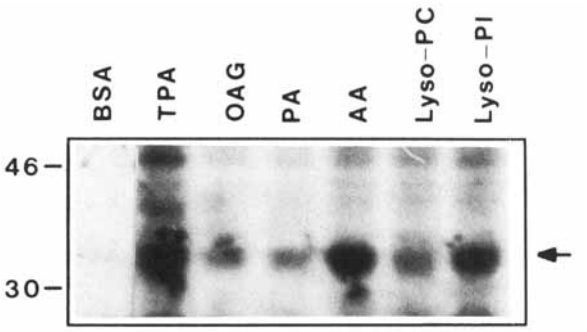

Fig. 3. Activation of the MPF-kinase complex by phospholipid metabolites capable of inducing GVBD. Oocytes were microinjected with 200 ng/oocyte each of phosphatidic acid (PA), arachidonic acid (AA), lyso-phosphatidyl-choline (lyso-PC), lyso-phosphatidyl-inositol (lyso-PI), or oleyl-acetyl-glycerol (OAG). After 12 h, oocytes were processed for MPF-kinase assay as described in Methods. Samples were run in a 15% PAGE, dried, and exposed. Arrow at the right indicates the type III-S calf thymus histone (Sigma Chemical Co., St. Louis, MO).

Protein Kinase C Dependency of Mitogenic Activity of Phospholipases

DAG as well as AA are potent activators of protein kinase C (PKC) either in vitro or in vivo [Nishizuka, 1992]. Since DAG are critical central metabolites in the anabolic and catabolic pathways of most phospholipids, it was possible that the activity shown by the phospholipases-generated metabolites may be mediated by activation of PKC. Thus we analysed the putative requirement of PKC for PLA2, PLC, and PLD mitogenic activity in the oocyte system.

We first characterized the inhibitory effect of three different PKC inhibitors on the biological activity of TPA and several DAGs. Table III shows that staurosporine was able to reduce the mitogenic activity of TPA, OAG, dipalmitoil-, dimirystoil-, and dihexanoil-glycerol to 43–60% of control oocytes treated with the inducers alone. GF 109203 X, a staurosporine analogue whose inhibitory action is mediated by its competition with the ATP-binding site [Toullec et al., 1991], was even more powerful with a reduction of 80–90% of their activity. Finally a synthetic peptide, which is a PKC-pseudosubstrate broadly utilized as a PKC inhibitor, was as efficient as staurosporine to block TPA and OAG activity. Thus known PKC inhibitors were able to interfere with oocyte maturation induced by either TPA or DAGs with similar efficiencies.

TABLE III. Maturation of *X. laevis* Oocytes Induced by Phorbol Esters and DAGs in the Presence of PKC Inhibitors*

Product	Staurosporine	Peptide	GF109203X
TPA	50	58	20
OAG	40	35	20
DiPalm.	57	NT	14
DiMir.	NT	NT	14
DiHex.	42	NT	NT
PA	89	92	100
AA	118	100	130
LPC	135	100	100
LPI	NT	94	NT

*Oocytes were selected as described and microinjected with either 10 nM staurosporine or the PKC pseudosubstrate (0.2 μg/oocyte), or treated with 10 μM GF 109203 X. Oocytes were then microinjected with 200 ng of each of the indicated compounds or treated with 100 nM TPA. DiPalm, dipalmitoil-glycerol; DiMir., dimirystoil-glycerol; DiHex., dihexanoil-glycerol; PA, phosphatidic acid; AA, araquidonic acid; LPC, Lyso-phosphatidylcholine; LPI, Lyso-phosphatidylinositol. Data represents percentage of control, untreated oocytes, or oocytes microinjected with BSA. NT, not tested.

These PKC inhibitors, allowed us to analyze the putative involvement of PKC in the biological activity of each phospholipase. Figure 4 shows that PLC was inhibited at a rate similar to that observed for TPA and synthetic DAGs. In contrast, the mitogenic activity of PLA2 or PLD was unaffected by the three inhibitors analyzed. When the active products of type A2 or D phospholipases were analyzed (i.e., PA, lyso-PC, lyso-PI, or AA) they were insensitive to the action of PKC inhibitors (Table III). These results indicate that PLC is dependent on PKC function, while PLA2 and PLD are completely independent of this enzymatic activity.

Ionic Implications of Phospholipase Function in *Xenopus laevis* Oocytes

Differential modulation of ion transport by mitogens or differentiating factors leads to changes in the intracellular ionic concentrations. This effect has been postulated as an important early event in the regulation of cell growth and differentiation [Geering, 1986]. In a variety of experimental systems, the Na^+-K^+/ATPase are largely responsible for the maintenance of cellular Na^+ and K^+ homeostasis [Jorgensen and Anderssen, 1988]. Examples for the developmental modulation of Na^+, K^+, and Ca^{2+} currents via modulation of the Na^+-K^+/ATPase have been described in *Xenopus laevis* oocytes [O'Connor et al., 1977]. Also, a functional correlation between PKC activation and regulation of the homeostasis of ionic fluxes has been established through regulation of the Na^+-K^+/ATPase [Bertorello et al., 1991]. Following the above reasoning, we have investigated the putative requirement of ionic fluxes for oocyte maturation induced by activation of PKC. In agreement with the above results, we observed that removal of K^+ ions from the incubation medium drastically reduces the efficiency of oocyte matu-

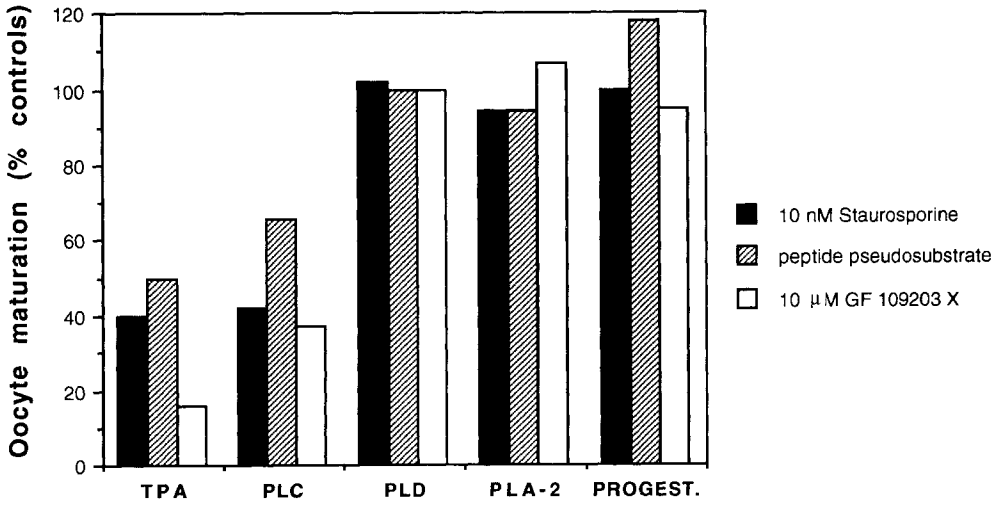

Fig. 4. Effect of PKC inhibitors on oocyte maturation by phospholipases. Oocytes were treated with GF 109203 X, or microinjected with staurosporine or the PKC-pseudosubstrate as indicated in Methods. After 30 min, oocytes were either microinjected with 25 μu of PLA2, PLC, or PLD, or treated with 100 nM TPA. GVBD was determined after overnight incubation at 20°C. Data represent percentage of control, untreated oocytes from one representative experiment.

TABLE IV. Maturation of *X. laevis* Oocytes by Different Phospholipases in the Absence of Potassium Ions*

	Ringer buffer		
	Control	$-K^+$	% Inhibition
BSA	4	5	—
200 nM TPA	100	30	70
PLC	31	5	80
PLD	100	100	0
PLA 2	86	92	−5

*Oocytes were treated with 200 nM TPA or microinjected with 25 μu of phospholipases or BSA (25 nl of 1 mg/ml solution in Mes 20 mM pH 7.0) in Ringer's buffer with (control) or without potassium. Oocytes were evaluated after 18 h of incubation at 20°C by treatment with 16% TCA.

ration induced by TPA (Table IV). As expected from previous studies indicating a functional substitution of K^+ by Rb^+ [Ledbetter and Lubin, 1977], Rb^+ addition to K^+-depleted medium, completely reversed the inhibitory effect due to the absence of K^+ (data not shown) Under these conditions of K^+ removal, GVBD induced by microinjection of PLC was drastically reduced with an efficiency similar to that observed for treatment with TPA. In contrast, oocytes microinjected with either PLA2 or PLD showed no reduction on their activity and were independent of K^+. These results support the hypothesis that PLC requires PKC for its biological activity in this system, and that PLA2 and PLD-dependent signals are not mediated by PKC.

DISCUSSION

Phospholipases are key enzymes responsible for the generation of important intracellular lipid metabolites and second messengers involved in the regulation of most relevant signalling processes in a large variety of cells. While PLC specific for phosphatidylinositol has been exhaustively investigated, much less information is available on the regulation and function of other phospholipases such as PLD and PLA2. We provide here evidence for the first time that the latter enzymes are also powerful biological effectors in the *Xenopus laevis* oocyte system, since microinjection of either PLA2, PLC, or PLD generated potent mitogenic signals which induce both activation of MPF and GVBD.

Considering their lack of specificity, we were able to identify the biologically active compounds responsible for the mitogenic activity in each case. DAG seems to be the only direct PLC-generated metabolite with biological activity, since phosphorylcholine was completely inactive, and inositol phosphates have been previously shown noncapable to induce GVBD [Stith and Maller, 1987; Stith and Proctor, 1989]. This result also implies that Ca^{2+} may not have a role at all in the biological activity of microinjected PLC.

PA is the only PLD-derived active metabolite, since choline and inositol are also negative. In contrast, PLA2 seems to be capable of generating a variety of potent biologically active metabolites such as lysophospholipids and unsaturated fatty acids. Lyso-PC or lyso-PI were quite active, although lyso-PC seems to be much more powerful. Also, lyso-PA showed an important biological activity in this system (data not shown), which is reminiscent of its mitogenic effects on fibroblasts [van Corven et al., 1989]. On the other hand, some of the fatty acids released by PLA2, such as AA, may also mediate important mitogenic signals.

There is abundant evidence indicating the importance of DAG as a second messenger involved in the activation of PKC and the regulation of a large number of cell functions including cell growth and differentiation [Nishizuka, 1988, 1992]. Recent evidence strongly supports as well the putative role of PA in cell proliferation [Moolenaar et al., 1986]. However, while DAG has a well-characterized mechanism of action as a PKC activator, it is not entirely understood how PA functions. One of the possibilities is that PA is further converted to DAG by a specific PA-hydrolase, and therefore its biological function would be mediated also by activation of PKC [Billah and Anthes, 1990]. Our results indicating that PLC and DAG depend on PKC function, but that PA is independent of this enzyme, suggest the existence of alternative pathways for PLC and PLD, and by extension of DAG and PA, respectively. However, we can not rule out entirely the possibility of some side effects of the inhibitors used on other intracellular kinases. Also, some cross-talk among these pathways can exist, especially among those activated by PLD and PLA2. In any case, our results are consistent with the implication of PLC and PLD in regulatory events of intracellular mitogenic signalling.

Regarding the products of PLA2 action, very little is known about the biological function of lyso-PC or lyso-PI. Recently, it has been proven that lyso-PA may have specific receptors located

at the cell membrane with tyrosine-kinase activity [van der Bend, 1992]. Thus, it is reasonable to speculate that lyso-PI and/or lyso-PC may follow a similar mecanism. On the other hand, arachidonic acid (AA) is the substrate for further convertion into thromboxanes, prostaglandins, and leukotrienes, all of them known powerful inducers of biological responses. Since AA can be generated from either PI or PC molecules when hydrolyzed by PLA2 [Irvine, 1982], it could be possible that the biological function of PLA2 after microinjection into oocytes could be related to AA production. Our results clearly indicate that both kinds of metabolites generated by PLA2 (lyso-PLs and AA) are highly active. Thus, further research to clarify their precise mechanism of action will be an interesting issue to pursue.

Microinjection of similar amounts of PLA2 or PLD to those of PLC were more efficient for the induction of GVBD. This may be a reflection of a more powerful intacellular signaling cascade, or different efficiencies in their biochemical activities after microinjection. However the latter seems not to be the case, since in vivo PLC was even more powerful than PLD for the hydrolysis of PI and PC under similar conditions. The more efficient induction of GVBD by PLA2 and PLD was confirmed by the relative efficiency of their respective biologically active products since PA, AA, and lyso-PC are stronger maturating inducers than several DAGs analyzed. The lower mitogenicity of PLC or its only biologically active product, DAG, probably reflects the rapid intracellular metabolization of DAG, and therefore its fast inactivation.

It is interesting to observe that both PLC and all DAGs analyzed are efficiently inhibited by specific PKC inhibitors. Thus, their action seems to be mediated by PKC. As expected, a more stable PKC activator such as TPA, showed a complete induction of GVBD, supporting the above hypothesis. As previously reported by others [García de Herreros et al., 1991], microinjection of PC specific PLC is capable of inducing oocyte maturation. As far as we are concerned, PLC was nonspecific for PI or PC, even when the same source of enzyme was used.

We report that the action of PLC seems to be dependent on endogenous PKC activity, since treatment with saturosporine, GF 109203 X, or a PKC-pseudosubstrate showed a 40–65% reduction of PLC-induced maturation, an inhibition similar to that obtained on TPA. We have also provided evidence that activation of PKC by TPA requires K^+ ions since incubation of the oocytes in the absence of K^+ ions reduced the biological activity of both TPA, DAGs, and PLC microinjection. Thus, these results are consistent with the notion that PLC uses the PKC pathway through generation of DAG. In contrast, none of the PKC inhibitors that efficiently inhibited TPA, DAGs, or PLC affected PLA2 or PLD activity. PLA2 and PLD were also equally efficient in the presence or absence of K^+ ions, suggesting that they follow an independent pathway to that used by PKC.

The results showed in this study clearly demonstrate that PLC, PLA2, and PLD may have also an important regulatory role in the control of cell proliferation since the products generated by their bochemical activity are powerful inducers of GVBD. While PLC requires PKC for its activity, PLA2 and PLD follow PKC-independent mechanisms. Thus, the oocyte system is a powerful tool for the characterization of these enzymes and for the study of their possible implication in the alternative mitogenic pathways found in eukaryotic cells.

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REFERENCES

Asaoka Y, Oka M, Yoshida K, Sasaki Y, Nishizuka Y (1992): Role of lysophosphatidylcholine in T-lymphocyte activation: Involvement of phospholipase A2 in signal transduction through protein kinase C. Proc Natl Acad Sci USA 89:6447–6451.

Bar-Sagi DD, Feramisco JR (1986): Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science 233:1061–1068.

Berridge MJ (1984): Inositol triphosphate and diacylglycerol as second messengers. Biochem J 220:345–360.

Berridge MJ (1987): Inositol triphosphate and diacylglycerol: Two interacting second messengers. Annu Rev Biochem 56:159–194.

Bertorello AM, Aperia A, Walaas SI, Nairn AC (1991): Phosphorylation of the catalytic subunit of Na^+, K^+-ATPase inhibits the activity of the enzyme. Proc Natl Acad Sci USA 88:11359–11362.

Billah MM, Anthes JC (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis Biochem J 269 281–191

Cockroft S, Thomas GMH (1992) Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors Biochem J 288 1–14

Cook SJ, Wakelam MJO (1991) Hydrolysis of phosphatidylcholine by phospholipase D is a common response to mitogens which stimulate inositol lipid hydrolysis in Swiss 3T3 fibroblast Biochem Biophys A 1092 265–272

Cook SJ, Briscoe CP, Wakelam MJO (1991) The regulation of phospholipase D activity and its role in sn-1,2-diacylglycerol formation in bombesin- and phorbol-12-myristate-13-acetate-stimulated Swiss 3T3 cells Biochem J 280 431–438

Cuadrado A, Carnero A, Lacal JC Distinct activation of type D phospholipase after serum stimulation and ras-induced transformation (submitted)

Cuadrado A, Carnero A, Lacal JC (1993) *ras* p21 proteins Switch devices for signal transduction In Lacal JC, McCormick F (eds) "The *ras* Superfamily of GTPases " New York CRC Press

Exton JH (1990) Signaling through phosphatidylcholine breakdown J Biol Chem 265 1–4

Garcia de Herreros A, Dominguez I, Diaz Meco MT, Graziani G, Cornet ME, Guddal PH, Johansen T, Moscat J (1991) Requirement of phospholipase C-catalyzed hydrolysis of phosphatidylcholine for maturation of *Xenopus laevis* oocytes in response to insulin and ras p21 J Biol Chem 266 6825–6829

Geering K (1986) Intracellular ionic changes and cell activation Regulation of DNA, RNA, and protein synthesis Curr Topics Membr Transp 27 221–259

Hii CST, Edwards YS, Murray AV (1991) Phosbol ester-stimulated hydrolysis of phosphatidylcholine and phosphatidylethanolamine by phospholipase D in HeLa cells J Biol Chem 266 20238–20243

Hokin LE (1985) Receptors and phosphoinositide-generated second messengers Annu Rev Biochem 54 205–235

Irvine RF (1982) How is the level of free arachidonic acid controlled in mammalian cells? Biochem J 204 3–16

Jorgensen PL, Anderssen JP (1988) Structural basis for E1–E2 conformational transitions in Na,K-pump and Ca-pump proteins J Membr Biol 103 95–120

Lacal JC (1990) Diacylglycerol production in *Xenopus laevis* oocytes after microinjection of p21-ras proteins is a consecuence of activation of phosphatidylcholine metabolism Mol Cell Biol 10 333–340

Lacal JC, Moscat J, Aaronson SA (1987) Novel source of 1, 2-diacylglycerol elevated in cells transformed by Ha-ras oncogene Nature 330 269–271

Ledbetter MLS, Lubin M (1977) Control of protein synthesis in human fibroblast by intracellular potassium Exp Cell Res 105 223–236

Moolenaar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ, de Laat SW (1986) Growth factor-like action of phosphatidic acid Nature 323 171–173

Nishizuka Y (1988) Molecular heterogeneity of protein kinase C and its implications for cellular regulation Nature 334 661–665

Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C Science 258 607–614

O'Connor CM, Robinson KR, Smith LD (1977) Calcium, potassium, and sodium exanche by full-grown and maturing *Xenopus laevis* oocytes Dev Biol 61 28–40

Pelech SL, Vance DE (1989) Signal transduction via phosphatidylcholine cycles TIBS 14 28–30

Plevin R, Cook SJ, Palmer S, Wakelam MJO (1991) Multiple sources of sn-1,2-diacylglycerol in platelet-derived-growth-factor-stimulated Swiss 3T3 fibroblast Evidence for activation of phosphoinositidase C and a phosphatidylcholine-specific phospholipase D Biochem J 279 559–565

Preiss J, Loomis CR, Bishop WR, Stein R, Niedel JE, Bell RM (1986) Quantitative measurement of sn-1,2-diacylglycerols present in platelets, Hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells J Biol Chem 261 8597–8600

Sharma SV (1992) Melilin resistance A counterselection for ras transformation Oncogene 7 193–201

Song J, Pfeffer LM, Foster DA (1991) v-src increases diacylglycerol levels via type D phospholipase-mediated hydrolysis of phosphatidylcholine Mol Cell Biol 11, 4903–4908

Stith BJ, Maller JL (1987) Induction of meiotic maturation in Xenopus oocytes by 12-*O*-Tetradecanoylphorbol-13-acetate Exp Cell Res 169 514–523

Stith BJ, Proctor WR (1989) Microinjection of inositol 1,2-(cyclic)-4,5-trisphosphate, Inositol 1,3,4,5,-tetratrisphosphate, and Inositol 1,4,5-trisphosphate into intact Xenopus oocytes can induce membrane currents independent of extracellular calcium J Cell Biochem 40 321–330

Toullec D, Pianetti P, Coste H, Belleverge P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Lolliore F, Duhamel L, Charon D, Kirilovsky J (1991) The bisindolymaleimide GF 109203X is a potent and selective inhibitor of protein kinase C J Biol Chem 266 15771–15781

van Corven EJ, Groenink A, Jalink K, Eichholtz T, Moolenaar WH (1989) Lysophosphatidate-induced cell proliferation Identification and dissection of signaling pathways mediated by G proteins Cell 59 45–54

van der Bend RL, Brunner J, Jalink K, van Corven EJ, Moolenaar WH, van Blitterswijk WJ (1992) Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid Embo J 11 2495–2501

Varnold RL, Smith LD (1990) Protein kinase C and progesterone-induced maturation in Xenopus oocytes Development 109 597–604

Wolfman A, Macara IG (1987) Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in ras transformed fibroblast Nature 325 359–362

Wright TM, Willenberger S, Raben DM (1992) Activation of phospholipase D by α-thrombin or epidermal growth factor contributes to the formation of phosphatidic acid, but not to observed increases in 1,2-diacylglycerol Biochem J 285 395–400