Activation of Intracellular Kinases in \textit{Xenopus} Oocytes by p21\textsuperscript{ras} and Phospholipases: a Comparative Study

AMANCIO CÁRNERO AND JUAN CARLOS LACAL

Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, 28029 Madrid, Spain

Received 26 July 1994/Returned for modification 1 September 1994/Accepted 1 November 1994

Signal transduction induced by generations of second messengers from membrane phospholipids is a major regulatory mechanism in the control of cell proliferation. Indeed, oncogenic p21\textsuperscript{ras} alters the intracellular levels of phospholipid metabolites in both mammalian cells and \textit{Xenopus} oocytes. However, it is still controversial whether this alteration is biologically significant. We have analyzed the ras-induced signal transduction pathway in \textit{Xenopus} oocytes and have correlated its mechanism of activation with that of the three most relevant phospholipases (PLs). After microinjection, ras-p21 induces a rapid PLD activation followed by a late PLA\textsubscript{2} activation. By contrast, phosphatidylincholine-specific PLC was not activated under similar conditions. When each of these PLs was studied for its ability to activate intracellular signalling kinases, all of them were found to activate maturation-promoting factor efficiently. However, only PLD was able to activate MAP kinase and S6 kinase II, a similar pattern to that induced by p21\textsuperscript{ras} proteins. Thus, the comparison of activated enzymes after microinjection of p21\textsuperscript{ras} or PLs indicated that only PLD microinjection mimetized p21\textsuperscript{ras} signalling. Finally, inhibition of the endogenous PLD activity by neomycin substantially reduced the biological activity of p21\textsuperscript{ras}. All these results suggest that PLD activation may constitute a relevant step in ras-induced germinal vesicle breakdown in \textit{Xenopus} oocytes.

The ras family is highly conserved in evolution, being present in organisms ranging from yeasts to humans (5). Ras proteins activated by point mutations are found in a significant fraction of human and carcinogen-induced animal tumors (2, 27). A number of previous studies have demonstrated alterations in the phospholipid metabolism induced by p21\textsuperscript{ras} proteins (reviewed in reference 22). Ras-transformed cells show a significant increase in the basal levels of diacylglycerol (DAG) and phosphorylcholine (PCho) (21). We have recently shown that these metabolites are generated by a complex pathway involving phospholipase D (PLD) activation followed by choline kinase and phosphatidic acid (PA) hydrolase (6). Furthermore, no significant activation of a PC-specific PLC (PC-PLC) was observed. While a constitutively activated PC-PLD enzyme and a twofold increase in the basal levels of PA were observed in ras-transformed cells, very small alterations of these parameters were detected at late times after serum stimulation of quiescent cells (6). Therefore, cell proliferation induced by ras oncogenes in fibroblasts may be functionally linked to activation of a PC-PLD enzyme.

Cell proliferation in eukaryotic cells is triggered by events initiated at the plasma membrane that control reentry into the cell cycle. The subsequent biochemical pathways activated actually direct the process of cell division itself. Both of these aspects of cell growth regulation can be studied in \textit{Xenopus} oocytes undergoing meiotic maturation in response to mitogenic stimulation by hormones and mitogens (18, 31). Xenopus oocytes are arrested at the G2/M border of the first meiotic prophase. Several signals can induce reentry into the cell cycle, which can be followed by the activation of different kinases, used as biochemical markers of meiotic maturation. Several inducers of oocyte maturation can activate a preexisting factor, the pre-MPF (maturation-promoting factor) which is an heterodimer of cyclin B and the p34\textsuperscript{cdc2} protein kinase. Activation of pre-MPF promotes reentry into the cell cycle (reviewed in references 18, 31, and 37). As a consequence of MPF activation, there is a burst of phosphorylation of intracellular substrates 30 to 60 min before the germinal vesicle breakdown (GVBD). After the stimulation with hormones, MAP kinase (MAP K) is activated during the M-phase transition under the control of MPF, and it may constitute part of the kinase cascade downstream of MPF (13, 15, 31). However, while p21\textsuperscript{ras} induces MAP K activation without MPF activation (8, 34), MAP K activation still seems to be necessary for GVBD induced by progesterone and p21\textsuperscript{ras} (8, 20). Moreover, p42MAPK phosphorylates and activates S6 kinase II (KII) (48), the \textit{Xenopus} homolog of mammalian pp90\textsuperscript{raf} (11). Thus, MPF, MAP K, and S6 KII can be used as biochemical markers for Ras and PL pathways in \textit{Xenopus} oocytes.

Phospholipids play an important role in the control of cellular responses such as secretion, platelet aggregation, macrophage function, and fertilization (4, 36, 40). They also play an important role in signal transduction, since generation of second messengers from membrane phospholipids is a major regulatory mechanism in control of cell proliferation. We have reported that in the \textit{Xenopus laevis} oocytes model, microinjection of the three most relevant types of PLs acting on membrane phospholipids (PLA\textsubscript{2}, PLC, and PLD) are capable of inducing oocyte maturation (9). This effect is mediated by the generation of known second messengers such as lysophospholipids, arachidonic acid, DAG, and PA. Furthermore, using specific inhibitors of protein kinase C (PKC), we have identified alternative independent signalling pathways for the induction of oocyte maturation. Our results indicate that while PLC seems to be dependent on PKC, PLA\textsubscript{2} and PLD are completely independent of PKC function (9). In this study, we further investigated the functional relationship between p21\textsuperscript{ras} and PLs in the \textit{X. laevis} oocyte system. Our results suggest that PLD is an important component of the ras signalling pathway in this system, in keeping with our previous findings that ras

**Corresponding author. Mailing address: Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid, Spain. Phone: (34-1) 585.4607. Fax: (34-1) 585.4587.**
FIG. 1. Activation of PLD by p21ras. (A) Oocytes were prepared as described in Materials and Methods. After 18 h of incubation with 0.5 μCi of [32P] per ml at room temperature, oocytes were washed and incubated for 2 h in the presence of 10 μM GF109203X (C1), 100 μM (final concentration) R59022 (microinjected) (II), or buffer alone (I). Oocytes were further given microinjections of 50 ng of purified p21ras or BSA in 25 μl of PLD 0.1 h of incubation, PA was analyzed as described in Materials and Methods. (B) Oocytes were given microinjections of 100 μM R59022 (II) or buffer alone (I). After 2 h of incubation, the oocytes were given microinjections of 25 nCi of [α-32P]ATP per oocyte and 50 μl of BLO buffer containing 40 μM MgCl2, 2 mM dithiothreitol, 50 μM NaF, 0.3 mM EDTA, 150 mM NaCl, 1.8 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 4 mM NaHCO3 [pH 7.3]. Microinjection of 25 μl of each PL, or 25 nCi of the p21ras protein was performed as previously described (21). After overnight incubation at 18 to 20°C in Ringer’s buffer, oocytes were lysed for biochemical characterization or fixed in 16% trichloroacetic acid (TCA). Visual verification of nuclear vesicle breakdown was performed by splitting the oocytes open after fixation. (C) PLs were obtained from Sigma. Their respective origins are as follows: bee venom for PLα, Baccillus cereus type I for PLc, and peanut type II for PLD.

Analysis of PA and phosphatidylbutanol production. Oocytes were incubated for 8 h in Ringer’s buffer containing 0.5 μCi of [32P] per ml and rinsed twice to remove unincorporated isotope. Following microinjection of 50 nl of a solution containing 1 mg bovine serum albumin (BSA) per ml, 1 ng of p21ras per ml, or 0.5 μl of PC-PLD per ml, the reactions were stopped at the indicated times in an ethanol bath kept at ~70°C. PA and phosphatidylbutanol were extracted with 160 μl of water and 0.6 ml of chloroform-methanol (1:1, vol/vol). The organic phase was lyophilized under a nitrogen stream, resuspended in 100 μl of chloroform and resolved on thin-layer chromatography plates with the upper phase (100 μl) containing a mixture of ethyl acetate, trimethyl pentane, acetic acid, and H2O (90:50:20:100, by volume), to which 1 μl of acetic acid was added. The thin-layer chromatography plates were exposed to an X ray film, and the radioactive spots were quantified by scratching and scintillation counting.

MPF assays. MPF assays were carried out with total extracts from series of 10 oocytes treated with progesterone or given microinjections of p21ras or PLs. After incubation for 18 to 20 h, oocytes were homogenized in BLO buffer containing 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.0), 10 mM β-glycerophosphate, 5 mM ethylene glycol-bis-(aminoethyl ether)-N,N’,N”-N”-tetraacetic acid EGTA, 5 mM MgCl2, 50 mM NaCl, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 25 μg of aprotinin per ml, and 100 μM phenylmethylsulfonyl fluoride. Following centrifugation at 13,000 × g for 15 min, extracts were assayed for 15 min at 30°C in a final reaction volume of 50 ml containing 20 mM HEPES (pH 7.0), 5 mM β-mercaptoethanol, 10 mM MgCl2, 100 μM [α-32P]ATP (2 to 5 dpm/nmol), 0.2 μg of PKA inhibitor, and 1 μg of type IIS calf thymus histone (Sigma) per ml. Reactions were stopped by addition of PAGE sample buffer and boiling for 5 min. The samples were subjected to PAGE (15% polyacrylamide), dried, and exposed at ~70°C. The band corresponding to H1 was excised from the gel and quantitated by a scintillation counter.

RESULTS

Activation of PLD by microinjection of p21ras. We have recently shown that in ras-transformed fibroblasts there is a sustained activation of PLD activity, resulting in constitutive elevation of PA levels (6). This could be generated as a consequence of direct or indirect activation of PLD mediated by Ras protein. To investigate whether p21ras induces the early activation of PLD, we analyzed PA production after microinjection of p21ras into X. laevis oocytes. Oocytes were labeled with 0.5 μCi of [32P] per ml at room temperature for 18 h, washed extensively, and given microinjections of 25 μU of PLD, 50 ng of the purified p21ras protein, or equivalent amounts of BSA. PA levels were analyzed 30 min after injection. As shown in Fig. 1A, microinjection of either p21ras or PLD induced a rapid increase in the level of PA.

There are alternative mechanisms for the generation of PA, such as direct activation of a PKC-independent PLD, activation of a PLD by a PKC-dependent pathway, or activation of a DAG kinase (DGK). Therefore, the levels of PA were analyzed in oocytes pretreated with the PKC inhibitor bisindolylmaleimide or the DGK inhibitor R59022. Ras- and PLD-induced production of PA was not affected by the PKC inhibitor (Fig. 1A), suggesting that p21ras can induce PA production in

and PLD lie within the same signalling pathway in the NIH 3T3 system.

MATERIALS AND METHODS

Oocyte maturation and microinjection. Stage VI oocytes were selected by manual dissection. Series of 30 to 50 oocytes were treated for hormonal induction of maturation with 1 μg of progesterone per ml in Ringer’s buffer (100 mM NaCl, 1.8 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 4 mM NaHCO3 [pH 7.3]). Microinjection of 25 μl of each PL, or 25 nCi of the p21ras protein was performed as previously described (21). After overnight incubation at 18 to 20°C in Ringer’s buffer, oocytes were lysed for biochemical characterization or fixed in 16% trichloroacetic acid (TCA). Visual verification of nuclear vesicle breakdown was performed by splitting the oocytes open after fixation.

Protein purification. The v-H-ras p21 ras protein was purified as described previously (25). After protein induction, 7 M urea extracts were subjected to further purification by chromatography through a Sephadex G-100 column (90 by 2.5 cm) in 7 M urea–20 mM morpholineethanesulfonic acid (MES; pH 7.0). Fractions containing up to 95% pure p21ras were pooled and dialyzed against 20 mM MES (pH 7.0), and the concentrations were estimated by the Bradford assay system (Bio-Rad).

PLs were obtained from Sigma. Their respective origins are as follows: bee venom for PLα, Baccillus cereus type I for PLc, and peanut type II for PLD.
a PKC-independent form. Moreover, the PA production induced by p21\textsuperscript{ras} is not affected by R59022, suggesting that PA was generated by a mechanism involving PLD activation rather than by activation of DGK.

To further confirm this conclusion, we have analyzed PA production under conditions where it can proceed only from DGK activity. Oocytes were given microinjections of 100 \textmu M R59022 or buffer alone. After 2 h of incubation, oocytes were given microinjections of 25 nCi of [γ\textsuperscript{32}P]ATP per oocyte and 50 ng of BSA or p21\textsuperscript{ras} per oocyte, or 25 nl of purified DGK. After 30 min of incubation at room temperature, the oocytes were processed for PA production as described in Materials and Methods. Under these conditions, the observed labeled PA would originate from DGK activity since insufficient radioactivity is incorporated into phospholipids (data not shown). As shown in Fig. 1B, microinjection of DGK induced a 33-fold increase in the PA levels, and this increase was inhibited by R59022, an indication of its generation through the DGK enzyme. However, under the same conditions, p21\textsuperscript{ras} was unable to induce PA generation, indicating that ras-induced PA originated directly from phospholipid hydrolysis by PLD activation. These results were confirmed when we analyzed the ras-induced PLD activity directly by its specific transphosphatidylating activity. Oocytes were labeled with 0.5 mCi of \textsuperscript{32}P per ml at room temperature for 18 h, washed extensively, and then given microinjections of 50 ng of purified p21\textsuperscript{ras} protein or BSA. Oocytes were further incubated for 1 h in the presence or absence of 0.5% n-butanol, and the \textsuperscript{32}P phosphatidylbutanol levels were analyzed. Microinjection of p21\textsuperscript{ras} induced a rapid increase in the transphosphatidylating activity (Fig. 1C), confirming that the ras-induced PA production is due to activation of a PLD enzyme.

### Inhibition of p21\textsuperscript{ras}-induced GVBD

Microinjection of p21\textsuperscript{ras} protein into X. laevis oocytes induces the production of DAG (21, 23). It has been suggested that this DAG production is a consequence of PC-PLC activation (10, 14, 30) and that this activation is necessary for ras-induced GVBD. We have previously demonstrated that microinjection of a B. cereus PLC is able to induce GVBD and this effect is dependent on PKC, since PKC inhibitors efficiently block PLC-, DAG-, and tetradecanoyl phorbol acetate-induced GVBD (9). Moreover, GVBD induced by PLD and PLA\textsubscript{2} seems to be independent of the PKC pathway. To study whether p21\textsuperscript{ras} requires a functional PC-PLC enzyme, we have microinjected p21\textsuperscript{ras} in the presence of different concentrations of bisindolylmaleimide (GF109203X). As shown in Fig. 2A, this PKC inhibitor efficiently blocks tetradecanoyl phorbol acetate-induced GVBD but does not interfere with the GVBD induced by progesterone or p21\textsuperscript{ras}. This result indicates that ras-induced GVBD is independent of the PLC-PKC pathway and that the ras-induced DAG production is most probably not necessary for oocyte maturation.

In NIH 3T3 cells, transformation by activated p21\textsuperscript{ras} induces PLD activation, with a significant increase in the PA levels (6). Thus, we have analyzed whether PLD activation is necessary for the GVBD induced by Ras proteins. Neomycin has been reported to inhibit the PC-PLD activity with a 50% inhibitory concentration of 65 \textmu M in vitro, and it is able to block the PC-PLD activity in vivo at concentrations near to 1 mM (29). Therefore, we investigated whether PLD activity was necessary for ras function in this system. Neomycin was able to block the p21\textsuperscript{ras}-induced GVBD in a dose-dependent manner (Fig. 2B) and with comparable results to those shown by others (38). This effect was parallel with a drastic reduction in ras-induced PLD activation (data not shown). Thus, the ras-induced GVBD seems to be independent of PC-PLC activity but dependent on PC-PLD activity. However, the lack of specificity of this drug made it necessary to explore other approaches to the problem. Therefore, we analyzed the PLD-induced biochemical pathway in the X. laevis oocytes and compared it with that of p21\textsuperscript{ras} and the other PLs (PLC and PLA\textsubscript{2}).

MPF activity associated with PL-induced GVBD. A number of phospholipid metabolites have been proven capable of triggering mitogenic signals in eukaryotic cells. Microinjection of PLA\textsubscript{2}, PLC, or PLD into X. laevis oocytes induces the G\textsubscript{2}-to-M phase transition through the generation of second messengers generated from phospholipid breakdown, since DAGs, PA, arachidonic acid, lyso-PC, and lysophosphatidylinositol each induce the same effects (9). This effect is comparable to the biological activity of progesterone, phorbol esters, or p21\textsuperscript{ras}.

Mitogenic stimulation of X. laevis oocytes can be monitored by measuring posttranslational modification of the key enzymes involved in the process. Quiescent stage VI oocytes contain inactive MPF. Upon stimulation with progesterone or the oncogenic p21\textsuperscript{ras} protein, the MPF complex is activated. When we measured the p34\textsuperscript{cdc2} kinase activity in extracts of PL-injected oocytes, we observed a similar H1 kinase activity in all of them when compared with that observed in extracts from oocytes treated with progesterone or given microinjections of p21\textsuperscript{ras} proteins (results not shown). A more detailed analysis of the time course of MPF activa-
tions show that progesterone induced the H1 phosphorylation at least 2 h before the GVBD took place, correlating with the results reported by other groups (reviewed in reference 31). However, p21ras induced MPF activity only after a significant level of GVBD was reached, indicating that ras-induced MPF activation does not precede GVBD. When MPF activation was analyzed after microinjection of PLs (Fig. 3) and compared with the GVBD, we observed that PL2, induced a rapid GVBD (almost 100% at 4 h). However, PLD and PLC induced GVBD more slowly. Activation of MPF by PL2 was rapid but did not precede the GVBD, since it was maximal at 6 h, 2 h after complete GVBD induction was observed. PLD also showed a late MPF activation but only after GVBD was observed. Finally, PLC showed a partial activation of MPF that preceded GVBD. Thus, a comparison between ras- and PL-induced activation of MPF showed that ras and PLD had similar kinetics.

MAP K is activated by PLD but not by PLC or PL2. Mammalian MAP K is a serine/threonine kinase whose activation and phosphorylation on Tyr and Thr residues are rapidly induced by a variety of mitogens (16, 17, 26, 43–45). This kinase is considered to have a critical role in a network of protein kinases in mitogenic signal transduction (see references 32, 35, and 39 for reviews). Xenopus p42MAPK is also a Ser/Thr kinase closely related to the mammalian MAP K. It is phosphorylated on Tyr and Thr residues, a mechanism for its activation which takes place during entry into the M phase in the cell cycle of the Xenopus oocyte.

We have investigated the activation of MAP K by microinjection of PLs. As shown in Fig. 4A, PLD was able to induce MAP K activation, as indicated by a net shift in electrophoretic mobility observed on SDS-PAGE. By contrast, neither PL2 nor PLC was capable of activating this enzyme. A similar effect on MAP K activation was also observed after microinjection of 1-stearoyl-2-arachidonoyl-PA, strengthening our conclusion that PLD induces activation of MAP K.

To further demonstrate the activation of MAP K after PLD microinjection, an alternative assay was performed as shown in Fig. 4B. After microinjection of either Ras, PLD, or BSA into oocytes or after treatment of oocytes with progesterone, the oocytes were incubated for 9 h in Ringer’s buffer. Then the oocytes were lyzed in BLO buffer and samples were immunoprecipitated with an anti-phosphotyrosine antibody. The resulting immunoprecipitates were then resolved by PAGE (10% polyacrylamide), transferred to nitrocellulose, and Western immunoblotted with an anti-MAP K antibody. Either progesterone, Ras, or PLD treatment was sufficient to induce tyrosine phosphorylation of MAP K. These results suggest that the three PLs follow different pathways for induction of GVBD and that only PLD activates p42MAPK like progesterone or p21ras does.

S6 KII activation is induced by PLD but not by PLC or PL2. Phosphorylation of the 40S ribosomal protein S6 is common to all known oocyte maturation inducers. This process is one of the last known events in the kinase pathway that leads to reentry into mitosis in oocytes (31). In X. laevis oocytes, the S6 protein phosphorylation is mostly mediated by S6 KII, a member of the pp90raf family (11). We have analyzed the time course of S6 KII activation induced by microinjection of PLs with a specific peptide substrate for this family. As expected
from the results of MAP K activation, PLD was able to induce activation of this kinase with a similar kinetics to that of Ras or progesterone (Fig. 5). By contrast, neither PLC nor PLA₂ was able to significantly activate S6KII. Finally, PA alone was also able to induce S6 KII activation at 16 h after injection, with a similar efficiency to that of PLD, Ras, or progesterone, suggesting that PLD-mediated activation of S6 KII is mediated by the generation of PA.

Since cycloheximide(CHX) was able to block MPF-dependent signals (8), we used this inhibitor to study whether S6 KII activation in progesterone-, PLD- and ras-induced oocytes follows similar pathways. As shown in Fig. 6A, when oocytes were treated with CHX and then stimulated with progesterone, no activation of the S6 KII enzyme could be detected, indicating that S6 KII activation requires protein synthesis. In contrast, when the oocytes were given microinjections of the p21ras protein or PLD, the S6 KII enzymatic activity could be detected at a similar rate to that in control CHX-untreated oocytes. Moreover, microinjection of 200 ng of PA per oocyte resulted in a similar pattern of phosphorylation to that observed by microinjection of p21ras or PLD (data not shown). These results indicate that S6 KII activation induced by ras, PLD, or PA follows routes that do not require protein synthesis.

2-Aminopurine (2-AP) is a purine analog that has been described as a rather selective inhibitor of protein kinases activated by certain growth factors (49,50). In particular, 2-AP blocks ras-induced activation of p42MAPK in PC12 cells (42) and in X. laevis oocytes (8). Therefore, we studied the effects of 2-AP treatment on the intracellular signals generated by progesterone and PLs and compared them with those induced by progesterone or PLs.
TABLE 1. Effect of CHX or 2-AP treatment on Xenopus oocyte maturation

<table>
<thead>
<tr>
<th>Oocyte stimulation</th>
<th>GVBD after treatment with:</th>
<th>CHX</th>
<th>2-AP</th>
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</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td></td>
<td>15 ± 1</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>PL A</td>
<td></td>
<td>111 ± 12</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>PLC</td>
<td></td>
<td>91 ± 10</td>
<td>70 ± 20</td>
</tr>
<tr>
<td>PLD</td>
<td></td>
<td>100 ± 2</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>p21ras</td>
<td></td>
<td>100 ± 7</td>
<td>12 ± 4</td>
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</tbody>
</table>

*Oocytes were prepared as described in Materials and Methods. A 25-μM portion of each PL was microinjected per oocyte in a volume of 25 nl in MES buffer. BSA (25 ng) as negative control or p21ras (50 ng) as a positive control were microinjected per oocyte in a volume of 25 nl in 20 mM MES buffer. Treatments were carried out with 10 μg of progesterone per ml. Induction of GVBD was analyzed 18 h after microinjection or treatment by fixing in 10% TCA.

**Data represent the mean and standard deviation of two independent experiments and are shown as a percentage of the value for untreated oocytes.

p21ras proteins. When oocytes were treated with 2-AP and then stimulated with progesterone, a complete inhibition of S6 KII activity was observed (Fig. 6B), indicating that S6 KII activation by progesterone depends on MAP K activity. Similar results were also observed in PLD- and ras-microinjected oo-
cytes, indicating that PLD- and ras-induced activation of S6 KII may also be mediated by activation of the MAP K. Finally, when we analyzed the effect of 2-AP treatment on PA-induced S6 KII activation, we found that it was also blocked (data not shown), supporting the functional connection of p21ras, PLD, and PA in this system.

Protein synthesis and MAP K requirement for PL-induced GVBD. GVBD induced by the three PLs analyzed is independent of CHX (Table 1), as has been previously shown for ras (1). By contrast, GVBD induced by progesterone is dependent on protein synthesis. Inhibition of GVBD by 2-AP seems to be related to the requirement of MAP K, since only inducers that activate MAP K are blocked by this drug: progesterone, PLD, and p21ras (Table 1). In contrast, PL A and PLC can bypass the 2-AP effect, indicating that they follow a different pathway from that of MAP K activation. The fact that PL A and PLC can bypass the drug effect indicates that inhibition of the biological activity of PLD, ras, and progesterone by 2-AP treatment is not a result of a nonspecific effect or toxicity.

PL microinjection can induce GVBD through generation of their respective known metabolites (9). Therefore, we analyzed whether active lipid metabolites were sensitive to CHX and 2-AP inhibition. As shown in Table 2, none of the metabolites generated by PL A, PLC, or PLD were significantly sensitive to CHX. By contrast, PA, the only biologically active metabolite in X. laevis oocytes generated by PLD, was significantly sensitive to 2-AP treatment, as shown for PLD microinjection.

**DISCUSSION**

PLs are key enzymes responsible for the generation of important intracellular lipid metabolites and second messengers involved in the regulation of the most relevant signaling processes in a large variety of cells (3, 12, 36). While the PL-induced processes in somatic cells have been extensively studied, much less information is available about the regulation of germinal cell cycles by PLs. We provide evidence that PL A, PLC, and PLD are also powerful biological effectors in the Xenopus oocyte system and activate intracellular signalling pathways.

PL A, PLC, and PLD were able to activate p34cdc2 kinase as did p21ras. However, the time course of p34cdc2 kinase activation showed clear differences among PLs. While PLC and PL A showed a rapid activation, PLD, like p21ras, showed a similar delayed H1 kinase activation. Moreover, none of the PLs studied were inhibited by CHX in their biological activity, suggesting that, like p21ras proteins, PLs do not need protein synthesis for oocyte maturation. p42MAPK is activated by progesterone and p21ras, and this activation is necessary for oocyte maturation (8, 20). These results are in agreement with previous observations indicating that oocyte maturation requires MPF in progesterone- but not ras-induced GVBD (34). PLD, like progesterone and ras, activates p42MAPK. In contrast, PLC and PL A do not, indicating that ras and PLD follow a different pathway from PLC and PL A. Thus, both MPF and MAP K seems to be sufficient elements to distinguish among signalling routes in oocytes, clearly indicating that ras is not related to PLC.

Cytosolic PL A is phosphorylated and activated by MAP K (28). This suggests that PL A is located downstream of MAP K in the pathway and can explain the lack of detected activation of MAP K by PL A injection. Indeed, when oocytes were treated with 2-AP, a MAP K inhibitor, the activation of PL A by p21ras or PLD microinjection was abolished, indicating that PL A is located downstream of MAP K activation (7). Finally, since PLC was unable to activate p42MAPK and PLD, in oocytes, it must be located within an alternative pathway to MAP K activation for the induction of GVBD in Xenopus oocytes.

MAP K phosphorylates and activates S6 KII (48). Therefore, inducers that activate MAP K should also activate S6 KII. Progesterone and oncogenic p21ras activate S6 KII through activation of MAP K (8). We show that PLD, which activates p42MAPK, also activates S6 KII. By contrast, in X. laevis oocytes, PL A and PLC failed to activate pp90erks, another indication that p21ras and PLD follow different pathways from PLC in this system.

We have also analyzed the dependency of S6 KII activation on protein synthesis and MAP K activity. Oocytes stimulated with progesterone showed S6 KII activation which was sensitive to CHX treatment, indicating that its activation is dependent on protein synthesis. In contrast, PLD- or ras-induced oocytes showed S6 KII activation and GVBD independent of protein synthesis. These results are consistent with the previous studies suggesting that although progesterone needs protein synthesis for p42MAPK activation, ras-induced p42MAPK

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**TABLE 2. Effect of CHX or 2-AP treatment on the induction of Xenopus oocyte maturation by PLs**

<table>
<thead>
<tr>
<th>Oocyte stimulation</th>
<th>GVBD after treatment with:</th>
<th>CHX</th>
<th>2-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL A metabolites</td>
<td></td>
<td>93 ± 7</td>
<td>87 ± 14</td>
</tr>
<tr>
<td>Lyso-PI</td>
<td></td>
<td>103 ± 2</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>Lyso-PC</td>
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<td>89 ± 8</td>
<td>111 ± 8</td>
</tr>
<tr>
<td>AA</td>
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<tr>
<td>PLC metabolite</td>
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<td>100 ± 7</td>
<td>117 ± 12</td>
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<tr>
<td>S-A-Gly</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>P L D metabolite</td>
<td></td>
<td>99 ± 3</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

*Oocytes were prepared as described in Materials and Methods. Each metabolite (200 ng) was microinjected per oocyte in a volume of 25 nl in MES buffer.

**Abbreviations:** Lyso-PC, lysophosphatidylcholine; Lyso-PI, lysophosphatidyl-

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activation is independent of protein synthesis (8, 46). We report that PLD is also insensitive to CHX for S6 KII activation, again a good correlation to the function of Ras proteins.

2-AP, a reported protein kinase inhibitor, was able to block the S6 KII activation by ras and progesterone. We have observed that 2-AP blocks ras- and progesterone-induced activation of MAP K in oocytes (8), suggesting that MAP K is necessary for the induction of S6 KII activation. Our results indicate that PLD-induced activation of S6 KII is also sensitive to 2-AP.

It has been suggested that p21ras is functionally dependent on a PC-PLC activity (10, 14, 30). However, analysis of the biochemical pathways of cell cycle-dependent kinases followed by the three more important PLs compared with the ras pathway indicates that this hypothesis does not appear to be correct. p21ras activates p42MAPK and S6 KII. In contrast, a nonspecific PLC that is able to hydrolyze both PC and phosphatidylinositol (9) does not activate p42MAPK and S6 KII. PLD, as well as p21ras, activates p42MAPK and S6 KII, and its ability to induce GVBD is independent of protein synthesis. We also show in this study that PLD but not PLA2- or PLC-induced GVBD was sensitive to 2-AP treatment. Moreover, the PKC inhibitor bisindolylmaleimide inhibits the PLC- and DAG-induced GVBD (7) but not the ras-induced GVBD (Fig. 2A). Therefore, our results suggest that p21ras does not activate PLC but may be functionally related to PLD, since both proteins show a similar pattern of signalling during GVBD induction.

When we analyzed the functional relationship between these two proteins, we found that oncogenic p21ras was able to induce the PLD activity with a net PA production, which would be sufficient to induce GVBD (9). Moreover, treatment with propanoIol, a well-known inhibitor of the PA-phosphohydrolase, inhibits DAG production but does not alter ras-induced GVBD (results not shown), indicating that this biological effect is not mediated by DAG. Also, we have recently shown that in ras-transformed NIH 3T3 fibroblasts there is a sustained activation of PLD (6), further supporting the hypothesis of a functional link between p21ras and PLD.

It has been shown that src-induced transformation may be connected to activation of PLD in murine fibroblasts through a GTP-binding protein (19, 47). PDGF may be also functioning through activation of PLD (41) rather than activation of a PC-PLC. Since microinjection of Y13-259, a neutralizing antibody against p21ras proteins, efficiently blocks both PDGF and src function (33), it is reasonable to postulate that all these mitogenic signals may have a common mechanism that implies the generation of PA. However, the exact implication of this metabolite in the ras-induced pathways in different systems is still unknown; further research is required to elucidate this.

A clear connection between insulin and ras has been reported for both mammalian cells and Xenopus oocytes (reviewed in reference 22). We have also observed that insulin-induced GVBD in oocytes is associated with a rapid production of PA, similar to that observed after p21ras microinjection (data not shown). These results suggest the putative involvement of p21ras as the coupling system of insulin receptor and PLD; this deserves further investigation.

In summary, in X. laevis oocytes, microinjection of purified oncogenic p21ras protein induces activation of PLD, and inhibition of this enzyme by neomycin blocks the ras-induced GVBD. Moreover, the pathway activated by p21ras that leads to GVBD correlates with the PLD-induced pathway. Thus, activation of PLD by p21ras proteins may be an essential step in the pathway that leads to DNA synthesis in X. laevis oocytes, as we have previously demonstrated in the NIH 3T3 cell system.

ACKNOWLEDGMENTS

We thank B. Jiménez and L. del Peso for generation of the MAP kinase antibody, and we acknowledge the expert technical assistance of M. A. Ramos.

This work was supported by grant 93/0293, Fonduo de Investigación Sanitaria (FIS), from the Spanish Department of Health and Fundación Salud 2000. A.C. is a Fellow of FIFIO.

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