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ras-p21 Activates Phospholipase D and A2, But Not Phospholipase C or PKC, in *Xenopus laevis* Oocytes

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Abstract *Xenopus laevis* oocytes are a powerful tool for the characterization of signal transduction pathways leading to the induction of DNA synthesis. Since activation of PLA2, PLC, or PLD has been postulated as a mediator of *ras* function, we have used the oocyte system to study the putative functional relationship between *ras*-p21 and these phospholipases. A rapid generation of PA and DAG was observed after *ras*-p21 microinjection, suggesting the activation of both PLC and PLD enzymes. However, production of DAG was sensitive to inhibition of the PA-hydrolase by propranolol, indicating that PLD is the enzyme responsible for the generation of both PA and DAG. Microinjection of PLD or *ras*-p21 induced the late production of lysophosphatidylcholine on a $p42^{MAPK}$-dependent manner, an indication of the activation of a PLA2. Inhibition of this enzyme by quinacrine does not inhibit PLD- or *ras*-induced GVBD, suggesting that PLA2 activation is not needed for *ras* or PLD function. Contrary to 3T3 fibroblasts, where *ras*-p21 is functionally dependent for its mitogenic activity on TPA- and staurosporine-sensitive PKC isoforms, in *Xenopus* oocytes, induction of GVBD by *ras*-p21 was independent of PKC, while PLC-induced GVBD was sensitive to PKC inhibition. Thus, our results demonstrate the activation of PLD and PLA2 by *ras*-p21 proteins, while no effect on PLC was observed. © 1994 Wiley-Liss, Inc.

Key words: oocyte maturation, *ras* proteins, phospholipases, phosphatidic acid, *Xenopus laevis*

The family of *ras* oncogenes is involved in the generation of a large variety of human cancers [Bos, 1989]. They codify for small GTPases of 21 kDa which function by activation/inactivation cycles mediated by their specific association to GTP or GDP [Bourne et al., 1990]. Overwhelming evidence has implicated *ras* proteins in the regulation of critical steps in cell proliferation and differentiation [reviewed in Barbacid, 1987; Bourne et al., 1991; Lacal and Tronick, 1988]. *ras* proteins can induce a variety of cell responses, such as cell proliferation in fibroblasts, neuronal differentiation in the pheochromocytoma cell line PC-12, and germinal vesicle breakdown (GVBD) in *Xenopus laevis* oocytes.

Elevated levels of phospholipid metabolites have been related to the most critical function of *ras* proteins. While initially a correlation between *ras* transformation and production of inositol phosphates (IPs) supported the hypothesis that *ras* proteins could be regulating a PI-specific phospholipase C (PI-PLC), it is not a universal finding [reviewed in Lacal and Tronick, 1988; Macara, 1989]. However, a good correlation between elevated levels of 1,2-sn-diacilglycerol (DAG) and *ras* function has been reported [Wolfman and Macara, 1987; Lacal et al., 1987a; Lacal, 1990]. Also elevated levels of phosphorylcholine (*P*Cho) correlate with *ras*-transformation [Lacal et al., 1987], supporting the hypothesis of the specific activation of a phosphatidylcholine-specific PLC (PC-PLC) in *ras*-transformed cells.

Another potentially relevant connection between *ras*-p21 and the phospholipid metabolism has been suggested that implies the activation of phospholipase A2, since microinjection of both normal and transforming H-*ras* p21 proteins induced membrane ruffling which correlates with a rapid increase in the levels of lysophosphatidylcholine (Lyso-PC) and lysophosphatidylethanolamine (Lyso-PE) [Bar-Sagi and Feramisco, 1986].

Phospholipase D has also been implicated in the regulation of cell growth since this enzyme is activated by mitogenic signals such as those initiated by receptor tyrosine-kinases [Plevin et al., 1991] and PKC activators [Cook et al., 1991]. Moreover, *ras*-transformed cells have a sustained activation of PLD, while mitogen-treated

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cells show a transient activation of this enzyme [Carnero et al., submitted, a]. We have recently shown that breakdown of phosphatidylcholine (PC) by PLD generates phosphatidic acid (PA) and choline, which are further converted into DAG and *PC*ho by PA-phosphohydrolase and choline kinase, respectively. This latter enzyme has been shown to be constitutively activated in *ras*-transformed cells [Macara, 1989] and after microinjection of *ras*-p21 proteins into *Xenopus* oocytes [Lacal, 1990].

The *Xenopus laevis* oocyte system is a powerful tool for the characterization of mitogenic pathways of eukaryotic cells. Stage VI oocytes are arrested at the G2/M border of the first meiotic division. *ras* proteins, as well as insuline and progesterone, can induce reentry into the cell cycle or GVBD (germinal vesicle breakdown) by a mechanism still not completely understood [Birchmeier et al., 1985]. We have previously shown that the three most relevant types of phospholipases proposed to play a role in signal transduction induced by *ras* proteins (C, D, and A2) induce GVBD in *Xenopus* oocytes, and this effect correlates with the activity of their phospholipid breakdown products. While PLC-induced GVBD seems to be related to activation of protein kinase C (PKC), PLA2- and PLD-mediated GVBD was independent of PKC [Carnero and Lacal, 1993]. In the present study, we have investigated the functional relationship between *ras* proteins and phospholipases and found activation of both PLA2 and PLD, but not PLC. Furthermore, contrary to the mammalian cell system, *ras*-p21 does not require PKC function in this system.

MATERIALS AND METHODS

Oocyte Maturation and Microinjection

Stage VI oocytes were selected for hormonal induction of germinal vesicle breakdown (GVBD) with 3 μg/ml progesterone and 50–200 nM tetradecanoyl phorbol acetate (TPA) in Ringer's buffer (100 mM NaCl, 1.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 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. 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/μl of each enzyme in 20 mM MES buffer, pH 7. Phospholipases used were PLC from B. cereus (Type III from Sigma, St. Louis, MO), PLD from peanut (Type II from Sigma), and PLA2 from bee venom (Sigma).

Purification of *ras*-p21

The v-H-*ras* p21 protein was purified as described previously [Lacal et al., 1986]. After protein induction, 7 M urea extracts were subjected to further purification by chromatography through a Sephadex G-100 column (90 × 2.5 cm) in 7 M urea–20 mM MES, pH 7.0. Fractions of 3 ml were collected and analyzed by SDS-PAGE to estimate purity and by a GTP-binding assay to determine the activity of *ras*-p21. Fractions containing up to 95% purified *ras*-p21 were pooled and dialyzed against 20 mM MES, pH 7.0, and the concentrations were estimated by the Bradford assay system (Bio-Rad Laboratories, S.A., Madrid, Spain).

Analysis of Phosphatidic Acid Release

Oocytes were incubated for 8 h in Ringer's buffer containing 0.5 mCi/ml ^{32}P and rinsed twice to remove unincorporated isotope. Following microinjection with 25 nl of a solution containing either 10 mg/ml bovine seroalbumin (BSA), 1 mg/ml *ras*-p21, or 1 u/μl PLD, the reactions were stopped at indicated times in an ethanol bath kept at −70°C. Phosphatidic acid was extracted with 160 μl water and 0.6 ml chloroform:ethanol (1:2 vol:vol). The organic phase was lyophylized under a nitrogen stream, resuspended in 100 μl chloroform, and resolved in TCL plates with ethyl acetate:trimethyl pentane:acetic acid:H_2O (90:50:20:100, V:V:V:V). TLC plates were expossed to an X-ray film, and the radioactive spots were quantified by scratching and scintillation counting.

Analysis of Lysophosphatidilcholine Production

Oocytes were incubated for 24 h in Ringer's buffer containing 0.1 mCi/ml ^{3}H-methyl-choline and rinsed twice to remove unincorporated isotope. Following microinjection with 25 nl of a solution containing either 10 mg/ml bovine seroalbumin (BSA), 1 mg/ml *ras*-p21, or 1 u/μl PLC, PLD, or PLA2, the reactions were stopped at indicated times in an ethanol bath kept at −70°C. Total lipids were extracted with 125 μl water and 0.5 ml chloroform:methanol (1:1 vol:vol). The organic phase was lyophylized under a nitrogen stream, resuspended in 100 μl chloroform,

and resolved in TCL plates with aceton:chloroform:methanol:acetic acid:H_2O (60:160:52:48:32, V:V:V:V:V). TLC plates were exposed to an X-ray film, and the radioactive spots of Lyso-PC were quantified by scratching and scintillation counting.

Analysis of Diacylglycerol Production

Oocytes were microinjected with 25 nl of 0.1 mCi/ml ^{14}C-glycerol and incubated for 24 h in Ringer's buffer. Following microinjection with 25 nl of a solution containing either 10 mg/ml bovine seroalbumin (BSA) (fatty acid free), 1 mg/ml *ras*-p21, or 1 u/µl of phospholipases C or D, the reactions were stopped at indicated times in an ethanol bath kept at −70°C. Total lipids were extracted with 125 µl water and 0.5 ml chloroform:methanol (1:1 vol:vol). The organic phase was lyophylized under a nitrogen stream, resuspended in 100 µl chloroform, and resolved in silica gel thin layer chromatography (TLC) *60A LK6D* plates (Whatman, Hillsboro, Oregon) using as solvent hexane:ethyl ether:acetic acid (60:40:1). The plates were expossed to an X-ray film, and the radioactive spots of DAG and total lipids were quantified by scratching and scintillation counting.

RESULTS

ras-p21 Does Not Require PKC or PLC in *Xenopus laevis* Oocytes

As has been reported for mitogenic activity in mammalian cells [Lacal et al., 1987b; Pasti et al., 1986], it was possible that *ras*-p21 could be functionally dependent on PKC for its biological activity in *Xenopus laevis* oocytes. Thus we investigated whether well-characterized PKC inhibitors could affect *ras* function. We have used four different inhibitors: staurosporine, GF 109203 X, the PKC pseudosubstrate from α and β isoforms, T^{19}FARKGALRQKNV^{31} (peptide A) which efficiently inhibits in vitro PKC α and β activity [Smith et al., 1990], and the peptide pseudosubstrate from PKC ζ isoform S^{19}IYRRGARRWRKL^{31} (peptide Z) which has been reported to efficiently inhibit in vitro PKC ζ activity [Dominguez et al., 1992].

Maturation induced by treatment of oocytes with phorbol esters, stable PKC activators, was drastically reduced (50–90% inhibition) in the presence of all the PKC inhibitors tested (Table I). We have previously shown that PLC can induce GVBD, most likely through generation of DAG [Carnero and Lacal, 1993]. Staurosporine, GF109203 X, and peptide A reduced PLC function with a similar efficiency to that of TPA (up to 60% inhibition), as expected from a PKC-dependent signalling pathway. However, microinjection of up to 2 ng/oocyte (approximately 30 µM) of peptide Z did not inhibit GVBD induced by any of these mitogens. On the other hand, neither PLA2 nor PLD was affected by PKC inhibition, suggesting a PKC-independent signalling pathway. Finally, neither progesterone nor the *ras*-p21 protein was affected by any of the PKC inhibitors, suggesting that they do not need functional PKC for induction of GVBD. By contrast, and in agreement with previous reports [Lacal et al., 1987b; Lloyd et al., 1989], DNA synthesis in *ras*-transformed NIH 3T3 cells was blocked by these inhibitors at similar concentrations (Carnero and Lacal, unpublished results).

Microinjection of *ras*-p21 into *Xenopus* Oocytes Generates Phosphatidic Acid

It has been previously demonstrated that microinjection of *ras*-p21 proteins into *Xenopus*

TABLE I. *Xenopus laevis* Oocyte Maturation in the Presence of PKC Inhibitors (% Over Maturation Without Inhibitors)

Agent	Staurosporine[a]	GF109203 X	Peptide A	Peptide Z
TPA (100 nM)	50 ± 7	13 ± 3	40 ± 18	130 ± 20
Progesterone	101 ± 1	103 ± 8	100 ± 0	100 ± 2
ras-p21	103 ± 10	100 ± 0	99 ± 3	111 ± 7
PLC	42 ± 16	50 ± 15	66 ± 5	103 ± 3
PLD	100 ± 2	97 ± 2	100 ± 0	113 ± 11
PLA2	106 ± 12	98 ± 3	96 ± 4	100 ± 0

Oocytes were injected with 20 nl of 0.2 µM staurosporine or 0.1 mg/ml peptides A or Z, or treated with 10 µM GF109203 X. After 2 h incubation series of 30–40 oocytes were either microinjected with 25 µU of PLA2, PLC, or PLD or 25 ng of *ras*-p21, or treated with 100 nM TPA or 3 µg/ml progesterone. GVBD was determined after overnight incubation. Data represent the average of percentage of control, untreated oocytes, from two experiments, except ([a]) that represent average of percentage of control, untreated oocytes, from three independent experiments.

oocytes rapidly induces the production of DAG as well as *P*Cho [Lacal, 1990]. These two metabolites could be directly generated by a PC-PLC. Alternatively, DAG could also be generated by hydrolysis of phosphatidic acid (PA) while *P*Cho could be generated by the choline kinase-dependent phosphorylation of choline. Both PA and choline can be released as a consequence of activation of a PC-PLD. Thus we investigated whether *ras*-p21 was functionally connected to a PLD enzymatic activity.

There is evidence that PA can be generated by TPA treatment in fibroblasts, suggesting the involvement of PKC in the regulation of PLD [Cook et al., 1991]. In order to test this possibility in the *Xenopus* oocyte system, $^{32}P_i$-labeled oocytes were stimulated with 200 nM TPA and the levels of PA analyzed. In this system, phorbol esters do not stimulate PA production (Fig. 1). By contrast, microinjection of *ras*-p21 induced a rapid increase in the level of PA. Control oocytes microinjected with BSA showed no increase of PA levels, while those injected with PLD showed a more drastic increase of PA levels. Finally, we have observed that *ras*-induced PA production was not affected by the PKC inhibitor GF 109203 X or the diacylglycerol kinase (DGK) inhibitor R59023 (data not shown), suggesting that PA production depends on PLD.

Propranolol Inhibits *ras*-Induced Diacylglycerol Production

Transformation of fibroblasts or microinjection of oocytes with oncogenic *ras*-p21 induced a rapid increase in DAG levels [Lacal, 1990; Lacal

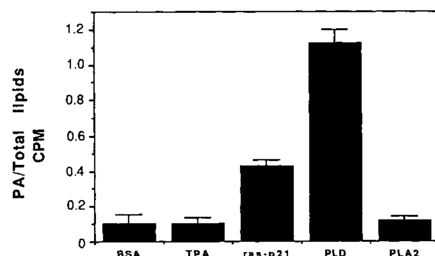

Fig. 1. Phosphatidic acid (PA) production by microinjection of *ras*-p21 into *Xenopus laevis* oocytes. Phosphatidic acid production was estimated in *X. laevis* oocytes labeled with ^{32}P, as indicated in Materials and Methods. Oocytes were microinjected with BSA (50 ng/oocyte), *ras*-p21 (50 ng/oocyte), or PLA2 or PLD (50 microunits/oocyte). Triplicate samples of 10 oocytes/sample were processed separately as described in Materials and Methods 30 min after injection. Data represent phosphatidic acid (PA) production as CPM divided by CPM in total lipids. Bars indicate standart deviation of triplicate samples. The experiment was repeated twice with similar results.

et al., 1987a]. If DAG release after *ras* transformation or microinjection was a consequence of the consecutive activation of PLD and PA-hydrolase, inhibition of this latter enzyme should drastically affect DAG levels. Therefore, we studied whether PA hydrolase was involved in the generation of DAG, by using propranolol, an inhibitor of this enzyme which has been reported to leave PLC or PLD activities unaffected [Billah et al., 1989; Koul and Hauser, 1987]. Oocytes were labeled with [^{14}C]-glycerol for 24 h and then incubated for 30 min in the presence or absence of 300 μM propranolol. Oocytes were microinjected with *ras*-p21, BSA, or PLC and analyzed for DAG production. As has been shown previously, after 30 min of *ras* injection there is an increase of DAG levels over BSA-injected controls (Fig. 2A). However, in the presence of propranolol, generation of DAG induced by *ras*-p21 decreased to basal levels, suggesting that this increase is a consequence of conversion of PA to DAG. However, DAG production induced by PLC was not affected by propranolol. Finally, production of PA generated by PLD was not sensitive to propranolol, and that observed after microinjection of *ras*-p21 was slightly increased (Fig. 2B).

ras-Induced Activation of PLA2

Microinjection of oncogenic *ras*-p21 into fibroblasts induced PLA2 activation determined by the generation of Lyso-PC and Lyso-PE production [Bar-Sagi and Feramisco, 1986]. Thus, we analyzed whether *ras*-p21 was able to activate PLA2 also in the oocyte system and whether this activation was biologically significant. Oocytes were labeled for 24 h with [^{3}H]-choline and then injected with oncogenic *ras*-p21. Injected oocytes were incubated for 1, 2, or 16 h at 18°C in Ringer's buffer and then processed for quantitation of Lyso-PC production. As shown in Figure 3, there is no detectable Lyso-PC production at early times after microinjection of *ras*-p21. However, after 16 h, *ras*-p21 induced a three-to-five fold increase in the levels of Lyso-PC when compared to those of BSA-injected oocytes.

Recently Lin and coworkers [1993] showed that a cytosolic PLA2 is phosphorylated and activated by MAP kinase. Oncogenic *ras*-p21 activates MAP kinase [Leevers and Marshall, 1992; Thomas et al., 1992; Wood et al., 1992; Pomerance et al., 1992; Shibuya et al., 1992], and the late production of a product of PLA2 suggests that *ras* activates PLA2 after MAP

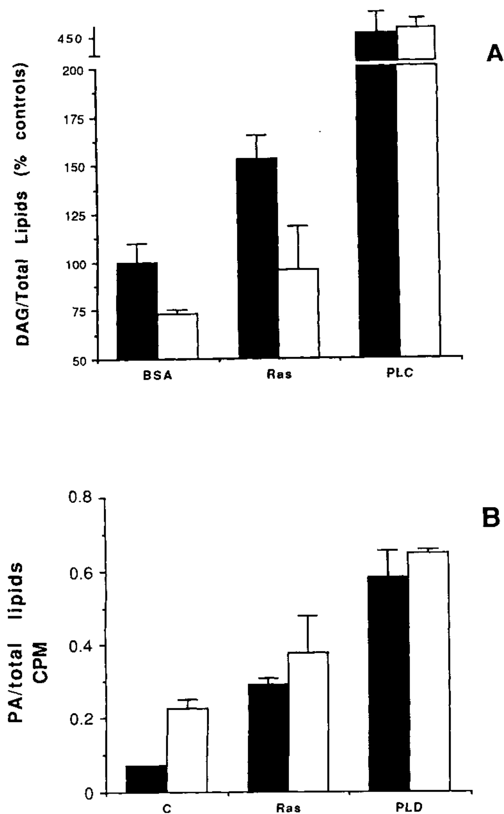

Fig. 2. Effect of propranolol in the *ras*-induced lipid release. **A:** Effect of propranolol in the *ras*-induced DAG production. *Xenopus laevis* oocytes were labeled by microinjecting 25 nl of 0.1 mCi/ml of [^{14}C]-glycerol resuspended in MES 20 mM, pH 7.0. After 24 h incubation in Ringer's buffer at 21°C, oocytes were treated (□) or not (■) with 300 μM propranolol for 30 min and then microinjected with either 25 nl of BSA (10 mg/ml), *ras*-p21 (1 mg/ml), or PLC (1 U/ml). DAG levels were analyzed as indicated in Materials and Methods. **B:** Effect of propranolol on the *ras*-induced generation of PA. Oocytes were labeled by incubation with buffer containing 500 μCi/ml ^{32}P for 4 h and then treated (□) or not (■) with 300 μM propranolol for 30 min. Oocytes were then injected with either 25 nl of BSA (10 mg/ml), *ras*-p21 (1 mg/ml), or PLD (1 U/ml). PA levels were determined as described in Materials and Methods. Data represent phosphatidic acid (PA) or DAG CPM divided by CPM incorporated in total lipids. Results represent mean values and standard deviations of three independent experiments performed in triplicate.

kinase activation. In order to test this hypothesis, oocytes were injected with *ras*-p21 in the presence of the MAP kinase inhibitor 2-aminopurine (2-AP). The purine analog 2-AP has been described as a rather selective inhibitor of certain growth factors-activated protein kinases [Volonté et al., 1989; Tsao and Greene, 1991]. Moreover, Qiu and Green [1992] have recently shown that 2-AP is a potent inhibitor of the myelin basic protein kinase activity of the

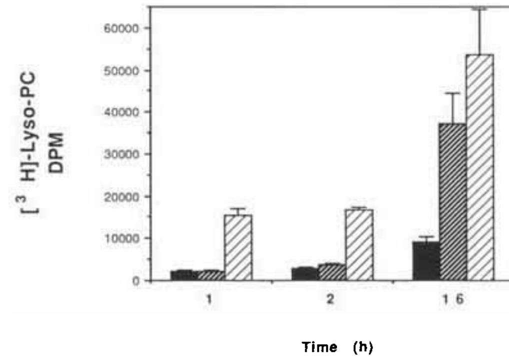

Fig. 3. Lysophosphatidylcholine production after *ras*-p21 microinjection. Oocytes were labeled with [^{3}H]-choline (10 μCi/ml) in Ringer's buffer for 24 h and then washed in the same buffer without the label and microinjected with either 25 nl of BSA (10 mg/ml), *ras*-p21 (1 mg/ml), or PLA2 (1 U/ml). At indicated times, oocytes were processed as described in Materials and Methods for Lysophosphatylcholine production. Data represent mean values of triplicate samples from two independent experiments. ■, BSA; □, *ras*-p21; ▨, PLA2.

p44^{ERK1} in PC12 cells and that inhibition of MAP K activity blocks the neurite outgrowth induced by the oncogenic N-*ras* in the dexametasone-inducible cell line, UR61 [Qiu and Green, 1992]. We have observed that 2-AP blocks both *ras*-induced MAP kinase activation and GVBD in *Xenopus* oocytes. This effect is specific, since 2-AP does not inhibit GVBD induced by PLA2, which does not activate MAP kinase [Carnero et al., submitted, b].

To study whether PLA2 activation was dependent on MAP K, oocytes were labeled for 24 h with [^{3}H]-choline and then injected with either oncogenic *ras*-p21 or phospholipases. Injected oocytes were incubated 16 h at 18°C in Ringer's buffer and then processed for Lyso-PC production in the presence or absence of 2-AP. While PLA2 microinjection was not affected by 2-AP, Lyso-PC production induced by *ras* and PLD was drastically reduced (Fig. 4), suggesting that PLA2 activation mediated by these two proteins is dependent on MAP kinase. By contrast, microinjection of PLC did not induce Lyso-PC production, supporting that PLC signaling uses an alternative pathway to that of *ras*-p21 and PLD.

ras-p21–Induced GVBD Does Not Require PLA2 in *Xenopus* Oocytes

Quinacrine is a potent inhibitor of PLA2 activity in vivo. It has been broadly used in mammalian cell systems to investigate the putative involvement of PLA2 in signaling pathways [Aguila et al., 1990; Schweitzer et al., 1990]. Table II

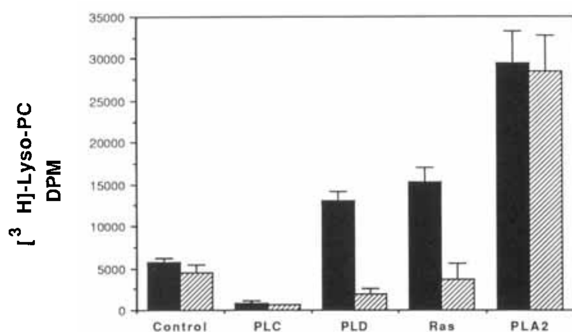

Fig. 4. Effect of 2-AP treatment on Lyso-PC production estimulated by *ras*-p21. Oocytes were labeled as described in the legend to Fig. 3 and then treated (hatched bars) or not (solid bars) with 10 mM 2-AP for 30 min prior to microinjection with either 25 nl of BSA (10 mg/ml), *ras*-p21 (1 mg/ml), or PLC, PLD, or PLA2 (1 U/ml). At 16 h of microinjection, oocytes were lyzed and processed for Lyso-PC determination. Data represent mean values from two independent experiments performed in triplicate.

TABLE II. Inhibition of PLA2-Induced GVBD by Quinacrine*

Agent	Untreated	1 mM Quinacrine
TPA (100 nM)	70 ± 10	70 ± 1
Progesterone	65 ± 10	70 ± 8
ras-p21	90 ± 12	88 ± 11
PLC	40 ± 5	50 ± 16
PLD	100 ± 0	85 ± 10
PLA2	95 ± 5	15 ± 10

*Oocytes were either microinjected with 25 μU of PLA2, PLC, or PLD or 25 ng of *ras*-p21, or treated with 200 nM TPA or 3 μg/ml progesterone. GVBD was determined after overnight incubation. When indicated, oocytes were treated with 1 mM quinacrine during the same period. Data represent the average from two experiments.

shows that treatment of the oocytes with 1 mM quinacrine completely inhibited the biological activity of microinjected PLA2. By contrast, no significant effect was observed when PLC or PLD was injected. Thus, quinacrine was an efficient inhibitor for the biological activity of PLA2 in *Xenopus* oocytes, in agreement with its effect on other biological systems, but allowed the existence of alternative signals.

Since treatment with quinacrine completely blocked PLA2 in vivo, we tested the biological activity of other inducers of oocyte maturation under these conditions. Table II also shows that neither TPA, insuline, nor progesterone was inhibited in the presence of 1 mM quinacrine. Finally, microinjection of transforming *ras*-p21 protein was not affected by quinacrine treatment (Table II). These results indicate that even under conditions of a complete blockage of PLA2 activity, *ras*-p21 is still biologically active to induce *X. laevis* oocyte maturation, indicating that, in this system, *ras* activity is also independent of functional PLA2.

DISCUSSION

There is strong evidence that *ras* proteins induce an altered balance in the intracellular levels of phospholipid metabolites in mammalian cells and *Xenopus* oocytes [Lacal and Tronick, 1988; Carnero et al., submitted, a]. However, it is still a controversial matter how this imbalance is generated and whether it is biologically significant. In this regard, it has been proposed that *ras* proteins are directly involved in the regulation of a PC-PLC enzyme [Lacal et al., 1987a; García de Herreros et al., 1991] with the generation of *P*Cho and DAG. Since in mammalian cells *ras*-p21 requires functional PKC for mitogenic activity [Lacal et al., 1987b], the above hypothesis could explain the relationship between *ras* and PKC. Moreover, it has been shown that *ras*-p21 is able to activate PLA2 [Bar-Sagi and Feramisco, 1986] and PLD [Carnero et al., submitted, a] in fibroblasts. Therefore, we investigated whether *ras*-p21 was functionally dependent on phospholipases type D, A2, or C and PKC in *Xenopus* oocytes.

The oocyte system has proved to be very useful to unveil the biological function of enzymes and lipid metabolites. We have previously shown that PLA2, PLC, and PLD are capable of inducing GVBD [Carnero and Lacal, 1993]. In the case of PLC, it induces GVBD by generation of DAG and activation of PKC [Carnero and Lacal, 1993]. Treatment of oocytes with staurosporine, GF109203 X, and PKC inhibitory peptides indeed revealed a functional requirement of PKC for the biological activity of PLC since these inhibitors drastically reduced the biological activity of PLC, DAG, and phorbol esters. However, PLA2, PLD, progesterone, and *ras*-p21 were unaffected by any of these inhibitors, indicating that they utilize signalling pathways which are independent of that of PLC and PKC. It is of interest to note that in all the experiments performed, the biological activity of PLC and several microinjected DAGs was significantly lower than that of PLD or PLA2 and their active metabolites, and than that of microinjected *ras*-p21 proteins [Carnero and Lacal, 1993].

It has been recently suggested that *ras*-p21 may be functionally related to PKC ζ, a PKC

isoenzyme which is insensitive to TPA downregulation and is not affected by staurosporine or GF 109203 X [Dominguez et al., 1992]. However, there is abundant evidence contrary to this hypothesis. PKC ζ is not activated by phorbol esters or DAGs, but instead by the products of phosphatidylinositol 3 kinase [Nakamishi et al., 1992]. Moreover, in the NIH 3T3 cell system, it has been previously shown that mitogenic activity of *ras* is functionally dependent on PKC isoforms which are downregulated by TPA and inhibited by staurosporine. Finally, we have seen no inhibitory effect on *ras*-induced GVBD by peptide Z, contrary to that shown by Dominguez et al. [1992], while we could detect up to 60% inhibition of the TPA-induced GVBD using peptide A. While the nature of this discrepancy is unknown, the peptide Z used in the present study was an efficient inhibitor for PKC ζ in vitro (data not shown).

The results presented in this study demonstrate that in *Xenopus laevis* oocytes *ras*-p21 seems to be independent of PKC, since its biological function is not blocked by specific inhibitors that efficiently blocked TPA-, DAG-, or PLC-induced maturation. However, in NIH 3T3 cells, it has been shown that *ras* function is dependent on PKC [Lacal et al., 1987b; Lloyd et al., 1989]. A possible explanation for this apparent controversy may rely on the different biological effect that we are analyzing. On somatic cells we are measuring the entry into the S phase from G1, while germinal cells such as oocytes are arrested at the G2/M border. Thus, while *ras*-induced DNA synthesis requires functional PKC, *ras*-induced G2 to M phase transition (measured as GVBD) does not need active PKC. A similar differential PKC requirement for *ras* function has been shown by Lloyd and coworkers [1989] for the NIH 3T3 cell system. While PKC-depleted cells are unable to initiate DNA synthesis in response to activated H-*ras*, they still respond to the morphological changes and increased c-*myc* expression associated with *ras*-induced transformation. Thus, morphological alterations in NIH 3T3 cells induced by *ras* appear to be PKC-independent. However, further research in the functional relationship between *ras* and PKC in different systems would be necessary to reach a complete understanding of the interaction between these two important signalling molecules.

Generation of *P*Cho and DAG after *ras* microinjection could be the result of a PC-PLC activity. However, they could be also obtained by activation of a PC-PLD, which would produce PA and choline, followed by PA-phosphohydrolase and choline kinase activities. Indeed, we have detected a rapid increase of PA and DAG levels within 30 min of *ras*-p21 microinjection. Propranolol, a potent inhibitor of PA-phosphohydrolase, drastically inhibited *ras*-induced DAG production with no reduction in PA levels. Moreover, propranolol did not affect *ras*-p21 GVBD (data not shown), and PA is itself a potent inducer of oocyte maturation [Carnero and Lacal, 1993]. In addition, Lee et al. [1991] showed that the DAGs derived from PC hydrolysis by PLC are poor substrates of DAG-kinase. All these results suggest that *ras*-induced maturation of *Xenopus* oocytes is related to PA generation, most likely by activation of a PLD enzyme. Moreover, we have recently shown that in *ras*-transformed NIH 3T3 fibrobrasts there is a sustained activation of PLD [Carnero et al., submitted, a], further supporting the hypothesis of a functional link between *ras*-p21 and PLD.

It has been shown that *src*-induced transformation may be connected to production of PA in murine fibroblasts [Song et al., 1991; Song and Foster, 1993]. PDGF may be also functioning through generation of PA [Plevin et al., 1991] rather than activation of a PC-PLC. Since microinjection of Y13-259, a neutralizing antibody against *ras*-p21 proteins, efficiently blocks both PDGF and *src* function [Mulcahy et al., 1985], there is a possibility that all these mitogenic signals may have a common mechanism to induce DNA synthesis through generation of PA or a related metabolite.

The observation that there is no activation of PLD in *X. laevis* oocytes in response to phorbol esters suggests the existence of a PLD which is independent of PKC activity. This novel finding is supported by a recent study showing that in *src*-transformed cells there is a PLD activity which is independent of PKC [Song and Foster, 1993]. We have also observed that in *Xenopus* oocytes, the PLD enzyme induced by *ras*-p21 and is not sensible to the PKC inhibitor GF 109203 X (data not shown).

In the present study, we have further investigated the functional relationship between *ras*-p21 and PLA2. Microinjection of *ras*-p21 and PLD, but not PLC, induced PLA2 activation at late times, and this activation was dependent on MAP kinase activity. Recently we have shown

that PLD but not PLC or PLA2 was able to activate p42^{MAPK} [Carnero and Lacal, submitted]. It has been shown that PLA2 was phosphorylated and activated by MAP kinase but not by PKC [Lin et al., 1993]. Thus, the observed activation of PLA2 by *ras*-p21 in NIH 3T3 cells and *X. laevis* oocytes may be a secondary effect of MAP kinase activation by *ras* and PLD. However, using quinacrine, a specific inhibitor for PLA2, we have been able to discriminate signalling pathways among progesterone, phorbol esters, and oncogenic *ras*-p21 protein and found that none of these agents depend on functional PLA2 in oocytes.

All these results suggest the existence of different, alternative pathways to induce oocyte maturation. A linear pathway connecting *ras* and PLD with the activation of MAP kinase can be drawn from the results presented in this study. MAP kinase activates PLA2 and most likely other products necessary for induction of GVBD. An alternative pathway for GVBD induction connects PLC to PKC without MAP kinase or PLA2 activation. Our results clearly indicate that the oncogenic *ras*-p21 protein does not use this second pathway for the induction of GVBD. *ras*-p21 can generate PA and this metabolite is further converted into DAG. However, the generated DAG seems to be insufficient to activate this second pathway, making the *ras*-PLD-PA pathway the only biologically relevant signalling mechanism for *ras* proteins in *Xenopus laevis* oocytes. Future research oriented to the full understanding of the nature and biological activity of the generated PAs and DAGs after microinjection of *ras*-p21 proteins will clarify further the relevance of the *ras*/PLD relationship.

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