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Selectivity of phospholipase C isozymes in growth factor signaling

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Xenopus laevis oocytes were injected with mRNA extracted from growth factor-responsive CCL39, Chinese hamster lung fibroblasts. The expression of functional growth factor receptors on the oocytes was demonstrated by growth factor-induced $^{45}Ca^{2+}$ efflux. To determine the isozyme(s) of phospholipase C (PLC) coupled to growth factor receptors, growth factor-induced $^{45}Ca^{2+}$ efflux were measured following coinjection of mRNA from CCL39 cells with PLC antibodies. PLC-γ1 antibody did not lead to loss of $^{45}Ca^{2+}$ efflux induced by thrombin but resulted in loss of that induced by platelet-derived growth factor (PDGF). In contrast, PLC-δ1 antibody did not block PDGF-induced $^{45}Ca^{2+}$ efflux but led to inhibition of thrombin-induced $^{45}Ca^{2+}$ efflux. PLC-β1 antibody did not affect Ca^{2+} efflux by the treatment of either thrombin or PDGF. These results suggest that these growth factor receptors are coupled to specific effectors, i.e. thrombin receptor to PLC-δ1 and PDGF receptor to PLC-γ1.

Growth factor; Phospholipase C isozyme; Thrombin receptor; Ca^{2+} mobilization; *Xenopus* oocyte

1. INTRODUCTION

Stimulation of a variety of cell surface receptors results in the activation of inositol phospholipid-specific phospholipase C (PLC). Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol-1,4,5 trisphosphate (IP_3) and diacylglycerol (DG) [1]. IP_3 releases Ca^{2+} from intracellular stores, whereas DG activates protein kinase C (PKC) [2,3]. The PLC enzymes are subdivided on the basis of cDNA cloning and deduced amino acid sequence similarity into at least three well defined groups: PLC -β, -γ, and -δ [1].

Growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) stimulate cell growth via multiple signal transduction pathways that include PLC-mediated hydrolysis of PIP_2. EGF and PDGF lead to an increase in the phosphorylation of PLC-γ, but not PLC-β or PLC-δ [4–6]. This phosphorylation is mediated by the intrinsic tyrosine kinase activity of the receptors for EGF and PDGF [7]. CCL39, a line of Chinese hamster lung fibroblasts is a good model system for studing signal transducing mechanisms involved in growth factor activation of cell division. Thrombin is a potent mitogen for CCL39 cells and one of the post-receptor events stimulated by thrombin is phosphoinositide hydrolysis [8].

To identify which PLC isozyme is responsible for growth factor-induced PIP_2 hydrolysis, we have used *Xenopus* oocytes which could easily be injected mRNA from CCL39 cell or antibodies to PLC isozyme. In mRNA-injected oocytes PIP_2 hydrolysis resulting from application of growth factor was indirectly demonstrated by mobilization of $^{45}Ca^{2+}$ [8–10]. Here we demonstrate that receptors for two growth factors, thrombin and PDGF, in CCL39 cells are specifically linked to different PLC isozymes, PLC-δ1 and PLC-γ1, respectively.

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2. MATERIALS AND METHODS

2.1. *Materials*

Thrombin, collagenase, soybean trypsin inhibitor were purchased from Sigma. PDGF (BB) was obtained from Promega. PLC-β1, PLC-γ1 and PLC-δ1 antibodies were generously provided by Dr. S.G. Rhee (NHLBI, NIH, USA). $^{45}Ca^{2+}$ was obtained from Amersham. [^{3}H]PIP_2 was purchased from New England Nuclear.

2.2. *Cells*

CCL39, Chinese hamster lung fibroblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) contained in 75 cm^2 stock flasks under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.3. *RNA preparation*

Total RNA was extracted from CCL39 cells by the guanidium thiocyanate/cecium chloride gradient method of Chirgwin et al. [11], and mRNA was purified by oligo d(T)-cellulose affinity chromatography. mRNA for microinjection was resuspended in filtered diethylpyrocarbonate-treated H_2O at a concentration of 2 mg/ml.

2.4. *Microinjection*

Ovarian fragments were surgically removed from adult female *Xenopus laevis* (Xenopus I, Ann Arbor) and follicular cells surrounding the oocyte were removed by treatment for 2 h at room temperature with 2 mg/ml collagenase and 0.4 mg/ml soybean trypsin inhibitor in modified Barths' saline (MBS) (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 15.0 mM HEPES pH. 7.6, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.82 mM $MgSO_4$) containing penicillin (50 units/ml) and streptomycin (50 μg/ml). Dumont stage V and VI oocytes were microinjected with approximately 50 nl RNA solution. After injection, oocytes were stored at 20°C in MBS; this medium was changed daily.

2.5. *Immunoprecipitation and immunoblotting*

CCL39 cells or oocytes were homogenized in 1 ml of ice-cold lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM phenylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) by 10 strokes with a Kontes Pellet Pestle Mortar. After centrifugation, the soluble extracts were treated with one of the mixture of monoclonal antibodies to PLC isozymes for 4 h and then with 10 of the heat-inactivated, precoated *S. aureus* cells [12]. The immunoprecipitates were washed with a washing buffer (1.0% Triton X-100, 1.0% deoxycholate, 0.1% SDS, 150.0 mM NaCl, and 50.0 mM Tris, pH 8.5). Immunoprecipitated proteins were released by the addition of 20.0 μl Laemmli buffer and heating for 5 min at 95°C. SDS-polyacylamide gel by the method of Laemmli [13], and the separated proteins were then transferred to nitrocellulose for 2 h at 50 V. After blocking with 2% bovine serum albumin, the filters were probed with a mixture of monoclonal antibodies of each PLC isozyme. Immune complexes were detected with alkaline phosphatase-conjugated antibody to mouse IgG and a phosphatase substrate system.

2.6. *$^{45}Ca^{2+}$-efflux assay*

24 h after mRNA injection when the growth factor-responses as well as the gross-appearances of the oocytes were best, oocytes were incubated for 2.5 h at 20°C in MBS containing 50 μCi/ml $^{45}Ca^{2+}$. Following $^{45}Ca^{2+}$ loading oocytes were rinsed three times with MBS at room temperature and placed in individual perfusion chambers, each containing 0.5 ml of MBS plus soybean trypsin inhibitor (1 mg/ml). This medium was collected for determination of radioactivity at the indicated time.

3. RESULTS

3.1. *Expression of PLC isozymes in CCL39 cells and mRNA-injected* Xenopus *oocytes*

We immunoprecipitated PLC isozymes from lysates of CCL39 cells and oocytes which had been injected with mRNA from CCL39 cell, and immunoblotted using PLC isozyme-specific antibodies. CCL39 cells expressed at least three PLC isozymes, PLC-β1, PLC-γ1, and PLC-δ1, that exhibit apparent molecular masses of 150, 145, and 85 kDa, respectively, on SDS-polyacylamide gels (Fig. 1). *Xenopus* oocytes have only PLC-γ1 as an intrinsic PLC enzyme, but expressed all three PLC isozymes, as in CCL39 cells, after injected with mRNA (Fig. 2). These results suggest that *Xenopus* oocytes expression system works well for studying growth factor-induced signaling mechanism in CCL39 cells.

3.2. *Growth factor responses in mRNA-injected* Xenopus *oocytes*

To evaluate whether the mRNA-injected oocytes are functionally intact we measured $^{45}Ca^{2+}$ efflux resulting from application of thrombin or PDGF. Non-injected oocytes did not show any response to the treatment of thrombin or PDGF (Fig. 3a). This result suggested that the oocytes used in these experiments did not have endogenous thrombin or PDGF receptors, and/or the relevant signaling system. However, in oocytes which had been injected with mRNA from CCL39 cells, $^{45}Ca^{2+}$ efflux was induced by treatment with either thrombin or PDGF (Fig. 3b). Taken together with the data from expression of the three PLC isozymes in oocytes injected with mRNA from CCL39 cells, these results establish

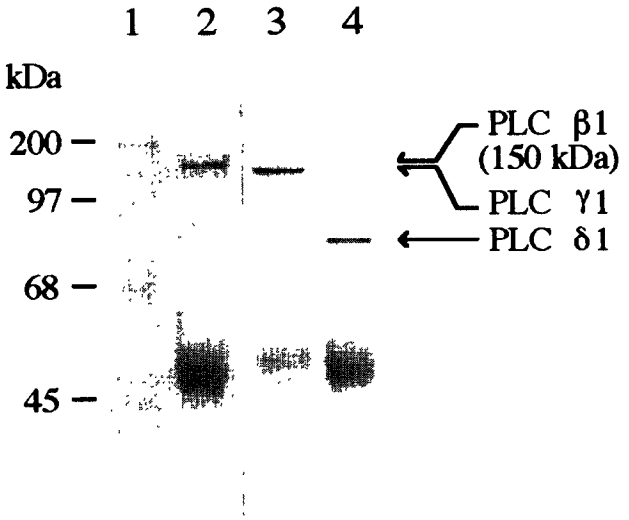

Fig. 1. Protein immunoblot showing the expression in CCL39 cells. Three isoforms of PLC were immunoprecipitated individually by using monoclonal antibodies specific to PLC-β1, PLC-γ1 and PLC-δ1. The immunoprecipitated proteins were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with PLC-β1, -γ1 and -δ1 antibodies, respectively. The protein bands were visualized with alkaline phosphatase substrate system.

that the full repertoire of signaling machinery for growth factors was expressed in oocytes by mRNA injection.

3.3. *Effects of PLC isozyme antibodies on $^{45}Ca^{2+}$ efflux stimulated by growth factors*

To elucidate which isoform of PLC might be coupled

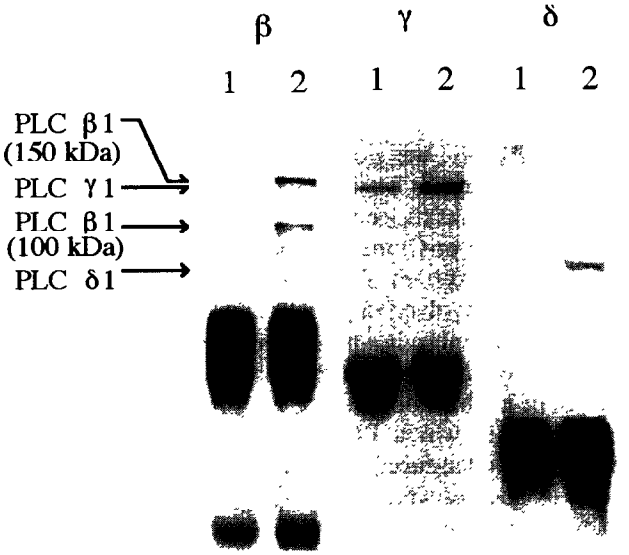

Fig. 2. Expression of PLC isozymes in *Xenopus* oocytes. Intact oocytes (1) or oocytes which have been injected with mRNA from CCL39 cells (2) were lysed, immunoprecipitated, and immunoblotted as described in Fig. 1.

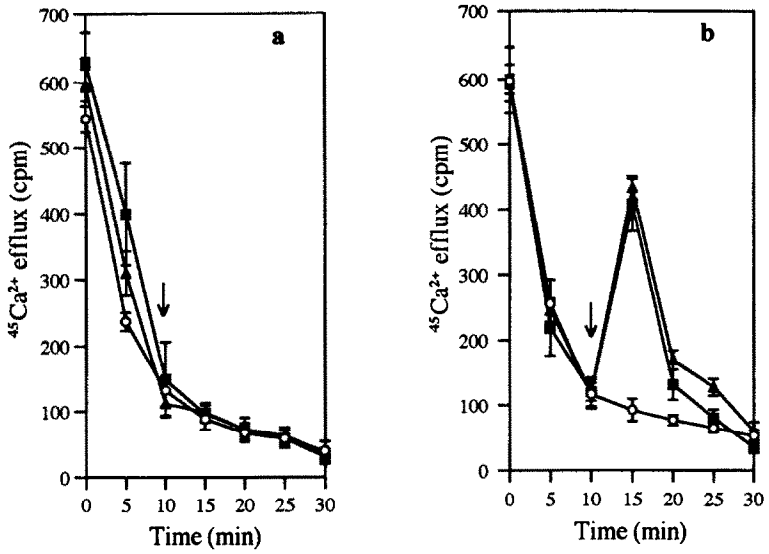

Fig. 3. $^{45}Ca^{2+}$ efflux stimulated by growth factors. $^{45}Ca^{2+}$ efflux was determined on single oocytes either non-injected (a) or microinjected with mRNA from CCL39 cells (b). Thrombin (1 U/ml) (■) or PDGF (200 ng/ml) (▲) or vehicle (○) were added where indicated by arrows. The medium (100 μl) was collected and fresh medium were replaced every 5 min. The results are expressed as means ± S.E.M.

to each growth factor receptor, we measured $^{45}Ca^{2+}$ efflux in oocytes which had been coinjected with mRNA from CCL39 cells and antibodies to each specific PLC isozymes. In the case of oocytes coinjected with PLC-β1 antibody, $^{45}Ca^{2+}$ efflux induced by thrombin or PDGF was unaffected (Fig. 4a). In contrast, in oocytes which had been coinjected with mRNA from CCL39 cells and PLC-γ1 antibody $^{45}Ca^{2+}$ efflux induced by PDGF was completely blocked, although thrombin was still effective (Fig. 4b). On the other hand, in oocytes which had been coinjected with mRNA from CCL39 cells and PLC-δ1 antibody, PDGF induced $^{45}Ca^{2+}$ efflux but thrombin had no effect (Fig. 4c).

4. DISCUSSION

In this study, CCL39 cells have been found to express at least three PLC isozymes, PLC-β1, -γ1, and -δ1. These PLC isozymes could be expressed by microinjecting mRNA from CCL39 cells into oocytes, which have only endogenous PLC-γ1. PLC-β1 expressed in injected oocytes was MW 100 kDa in addition to the

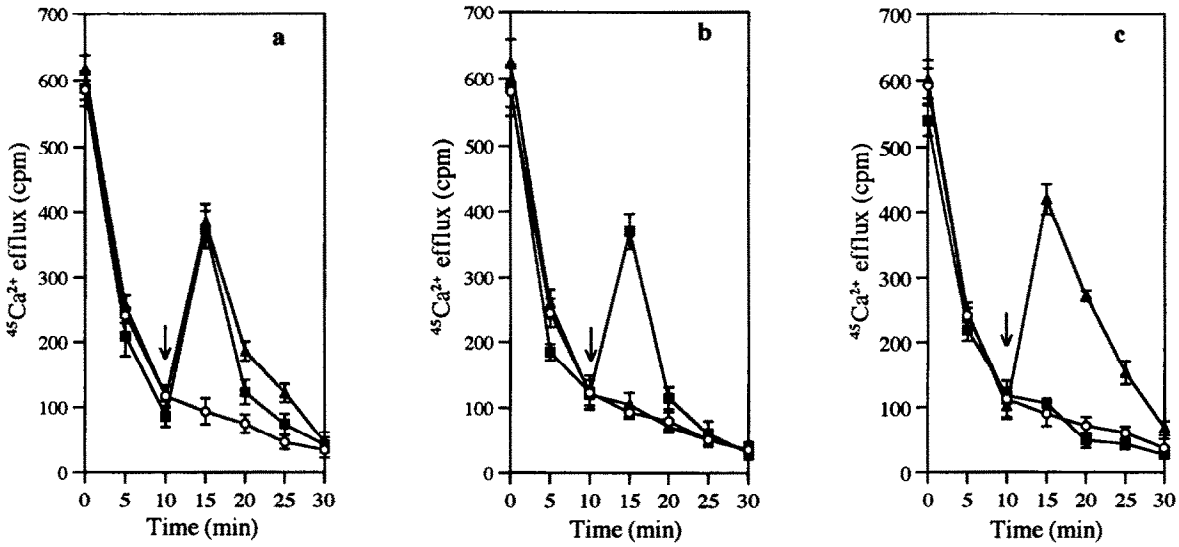

Fig. 4. Effects of PLC isozyme antibodies on $^{45}Ca^{2+}$ efflux stimulated by growth factors. $^{45}Ca^{2+}$ efflux was determined on single oocytes coinjected mRNA from CCL39 cells with PLC-β1 antibody (a), PLC-γ1 antibody (b), or PLC-δ1 antibody (c). Thrombin (1 U/ml) (■) or PDGF (200 ng/ml) (▲) or vehicle (○) were added where indicated by arrows. The medium (100 μl) was collected and fresh medium were replaced every 5 min. The results are expressed as means ± S.E.M.

authentic, MW 150 kDa in CCL39 cells. PLC-β1 has been known to be highly susceptible to proteolysis and contain amino acid sequences which are specific sites for the $^{45}Ca^{2+}$-dependent protease, calpain [14]. Binding of growth factors, such as PDGF, to their receptors, intrinsic tyrosine kinase is activated and this phosphorylates several target proteins including PLC-γ1, which hydrolyzes PIP_2 [4–6]. Although *Xenopus* oocytes have endogenous PLC-γ1, PDGF could not induce $^{45}Ca^{2+}$ efflux (Fig. 3a). This result suggests that oocytes used in this experiment did not have endogenous PDGF receptors. The fact that thrombin also could not induce $^{45}Ca^{2+}$ efflux in oocytes agrees with a previous report that endogenous thrombin receptors are depleted in oocytes following long treatment (> 15 min) with collagenase [8]. However, mRNA-injected oocytes showed $^{45}Ca^{2+}$ efflux in response to application of either thrombin or PDGF (Fig. 3b). The $^{45}Ca^{2+}$ effluxes in oocytes induced by these two growth factors had very similar patterns in response time and amplitude.

Coinjection of PLC-β1 antibody into oocytes had no effect on the $^{45}Ca^{2+}$ efflux induced by the treatment of either thrombin or PDGF (Fig. 4a). This finding suggests that intracellular antibody injection itself has no effect on growth factor signaling. The results showing that PLC-γ1 antibody completely blocked PDGF-induced $^{45}Ca^{2+}$ efflux but PLC-β1 or PLC-δ1 antibodies had no effect are consistent with the view that the PDGF receptor is coupled exclusively to PLC-γ1 [6]. In contrast, the PLC-δ1 antibody completely blocked the thrombin-induced $^{45}Ca^{2+}$ efflux (Fig. 4c). This result was confirmed by PLC assays; the PLC-δ1 antibody completely inhibited thrombin-induced PIP_2 hydrolysis to the basal level, whereas PLC-β1 antibody or PLC-γ1 antibody only slightly inhibited thrombin-induced PIP_2 hydrolysis (data not shown). These results suggested that the blockage of thrombin-induced Ca^{2+} efflux by the PLC-δ1 antibody resulted from inhibition of the PLC-δ1-catalyzed hydrolysis of PIP_2. In a previous study, a mutant of CCL39 cells, which had no detectable PLC-δ1 was found to be defective in thrombin-induced mutagenesis [15]. However, a definitive conclusion could not be made because PLC-γ from mutant extracts showed altered catalytic behavior in contrast to PLC-γ from wild type extracts.

In conclusion, we would consider the *Xenopus* oocytes expression system as ideal for studying growth factor-activated signalling pathways. Furthermore, antibodies to specific PLC isozymes could be used to selectively block the growth factor-induced signals so as to dissect the signalling pathways.

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