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AN EXTRACELLULAR INOSITOL PHOSPHOLIPID-SPECIFIC PHOSPHOLIPASE C IS RELEASED BY CULTURED SWISS 3T3 CELLS

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Summary: A phosphatidylinositol-cleaving phospholipase C (PI-PLC) activity is released into the extracellular environment by intact Swiss 3T3 cell cultures. The activity is found in both serum-containing and serum-free defined culture medium. The cells remain attached and intact by Trypan Blue exclusion and lactate dehydrogenase assays. The activity is specific for phosphoinositides as no cleavage of phosphatidylcholine is observed. The activity is a phospholipase C rather than D since the water soluble products formed from cleavage of [^{3}H] phosphatidylinositol were inositol phosphates and not inositol. Analysis of the inositol phosphate products showed a variation in composition with the pH of the assay, the ratio of noncyclic:cyclic forms being 60:40 at pH 7.5 and 40:60 at pH 5.5. This external phospholipase C resembles the well-characterized intracellular isozymes in that it is calcium-dependent and has a pH optimum between 5 and 6. From membrane filter assays the molecular weight of the native enzyme is estimated to be between 50 and 100 kDa.

The role of mammalian phosphatidylinositide-specific phospholipase C (PI-PLC) in the PI-dependent pathway of signal transduction has been extensively documented (1-3). The mammalian PI-PLC is actually a family of intracellular isozymes (2,4), which depending on the type, can be found associated with membranes, the cytoskeleton, in the cytosol or nucleus (5-7). Recent work has revealed the existence of a less well-characterized but very likely distinct member of the PI-PLC family which occurs on the cell surface of a number of mouse and rat cell types. This enzyme (ecto-PLC) is capable of cleaving both an exogenously added fluorescent PI analogue [NBD-PI (8,9)] and

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Abbreviations: PI-PLC: phosphatidylinositide-specific phospholipase C; PIP_2: phosphatidylinositol 4,5-bisphosphate; PI: phosphatidylinositol; [^{3}H]-PI: [inositol-2-^{3}H]-phosphatidylinositol; ecto-PLC: cell surface phosphatidylinositide-specific phospholipase C; [^{14}C]-PC: [choline-methyl-^{14}C]-dipalmitoylphosphatidylcholine; [^{3}H]-lysoPI: [inositol-2-^{3}H]-lysophosphatidylinositol; buffer A: 10 mM HEPES, 4 mM KCl, 126 mM NaCl, pH 7.4; IP: inositol monophosphate; GPI: glycosylphosphatidylinositol; NBD-PI: 1,2-(N-(6-[(7-nitrobenz-2-oxa-1,3-diazole-4-yl)aminocaproyl]),oleoyl)-phosphatidylinositol.

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radiolabeled lysophosphatidylinositol [lysoPI (10,11)]. In the process of further characterization of this extracellular membrane-bound ecto-PLC, we have found that a PI-PLC activity is present in conditioned medium from Swiss 3T3 cells. To our knowledge, this is the first report of a PI-PLC activity released from cultured mammalian cells. In this study, we characterize the activity of the free extracellular PI-PLC from Swiss 3T3 fibroblasts.

MATERIALS AND METHODS

Cells and materials: The mouse fibroblast cell line Swiss 3T3 (CCL 92), was obtained from the American Type Culture Collection. PI (bovine liver) was from Avanti Polar Lipids. [^{3}H]-PI and [^{14}C]-PC were from Du Pont NEN. [^{3}H]-lysoPI was prepared from [^{3}H]-PI as described previously (10).

Cell culture: Cells were seeded into either 100 or 150 mm Corning tissue culture dishes in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Celox) containing 10% iron-supplemented bovine calf serum (Intergen). Serum-free medium alone was not tolerated well by Swiss 3T3 cells for more than a few hr, and serum-containing medium made the ultrafiltration steps slow and the resulting enzyme recovery highly variable. Therefore, once the cell cultures were confluent they were switched to a serum-free medium with a defined supplement (TCH from Celox) at least one day (typically 2-3 days) prior to use. All cultures were checked frequently for mycoplasma contamination.

PI-PLC assay: To prepare conditioned buffer, culture dishes were washed three times with ice-cold filter-sterilized buffer A (10 mM HEPES, 4 mM KCl, 126 mM NaCl, pH 7.4). After the last wash, 2.5 ml of cold buffer A was added containing two protease inhibitors: 20 μg/ml leupeptin (Sigma) and 24 μg/ml aprotinin (Sigma). Unless specified otherwise, the dishes were rocked in a cold box at 7°C for 30 min. At the end of this time, and up to 60 min, the cells were intact as assayed by Trypan Blue exclusion and assay for lactate dehydrogenase (kit from Sigma) and could be returned to the incubator in culture medium with no evidence of stress. The conditioned buffer was centrifuged briefly to remove any floating cells. 0.5 ml aliquots of the buffer were transferred into glass culture tubes (previously treated with Sigmacote, Sigma) and either $CaCl_2$ or EGTA was added to a final concentration (except where indicated) of 1 mM. To prepare conditioned medium, 150 mm cultures were rinsed once with TCH supplemented serum-free medium, placed in 5-10 ml of this medium containing 20 μg/ml leupeptin and 25 μg/ml aprotinin, and rocked in an incubator at 37°C for 30 min up to 18 hr. The medium was removed, centrifuged as above, an additional 20 μg/ml leupeptin and 25 μg/ml aprotinin was added, and the medium transferred to two Centricon-50 microconcentrators (Amicon) for centrifugation at 5,000 x g for 30 min. The concentrate was resuspended in an additional 2 ml each of buffer A containing 0.5% PEG-600 and the concentration step was repeated. The final concentrate was brought up in the desired volume of buffer A containing PEG, transferred to microfuge tubes and centrifuged again to remove any aggregates, and aliquoted for assay in the presence of either $CaCl_2$ or EGTA as above. In some experiments the 0.5 ml aliquots received 30 μl 0.5 M Tris-0.5 M maleic acid buffer, pH 4.8 which was sufficient to decrease the pH from 7.4 to 5.5. Effects of dithiothreitol (DTT) on activity were tested by adding DTT at 1 mM and 5 mM to the assay buffer at pH 5.5 and 7.4, in the presence of $CaCl_2$.

The assays were initiated by addition of a 1:20 dilution from a stock of 500 uM radiolabeled (6-7 mCi/mmol for the PI) lipid which had been prepared by drying down appropriate ratios of the hot and cold lipids under N_2 and bath sonicating on ice in buffer A until clear. Assay tubes were immediately vortexed and placed in a 37°C water bath (typically for 30 min) followed by sequential chloroform/methanol/HCl and chloroform extractions (10) and liquid scintillation counting of the water-soluble phase.

For the determination of pH dependence of the released PI-PLC activity, Swiss 3T3 conditioned cold buffer was prepared and concentrated in Centricon-50 microconcentrators in the presence of additional protease inhibitors as described above. Buffer (50 mM Tris, 50 mM maleic acid, 50 mM NaCl) of appropriate pH was added to aliquots of the concentrate to produce a final volume of 0.5 ml, followed by addition of either $CaCl_2$ or EGTA (final concentration: 1 mM, except where indicated) and [^{3}H]-PI substrate and assay as described above.

Analysis of inositol phosphate products: samples to be analyzed by ion-exchange chromatography were prepared as above except that HCl was omitted from the first extraction step (chloroform/methanol). 0.5 ml of the final aqueous phase from the chloroform extraction was loaded on 1 ml Dowex AG1-X8 (formate form; BioRad) columns in glass Pasteur pipettes. Fractions of 3 ml were collected as the columns were eluted sequentially with the following: 2.5 ml H_2O, 3 ml 50 mM ammonium formate, 3 x 3 ml 150 mM ammonium formate and 3 ml 1 M ammonium formate. The fractions were mixed with 15 ml of EcoLume (ICN) for scintillation counting. Columns were calibrated with standard samples consisting of [^{3}H]-lysoPI, *sn*-glycero-3-phospho-D-1-*myo*-inositol (Sigma), DL-*myo*-inositol 1-monophosphate (Sigma) and DL-*myo*-inositol 1,2-cyclic monophosphate (Sigma).

RESULTS AND DISCUSSION

A phospholipase activity assay based on the generation of water-soluble radioactive products from ([^{3}H]-PI) was used to examine extracellular enzymatic activity present in Swiss 3T3 cell cultures. By this assay, PI-cleaving activity can be measured in both conditioned culture medium and in conditioned isotonic buffer. Fig. 1 shows that a PI-cleaving activity is present in isotonic buffer conditioned by exposure to intact Swiss 3T3 cells for 30 min at 7°C. In the presence of 1 mM Ca^{2+}, the amount of radioactive products present in the aqueous fraction is much higher than in the presence of EGTA, strongly suggesting the existence of a calcium-dependent enzymatic cleaving activity released by the cells. Use of [^{14}C]-PC as a substrate resulted in only background levels of water soluble radiolabeled products (data not shown), indicating that this activity is specific for inositol-containing phospholipids.

The extracellular PI-cleaving activity was also strongly pH dependent (Fig. 2). The activity increased from low levels at pH 4.5 to a maximum near pH 5.5, then rapidly fell off to lower but still measurable levels at pH 7.5. At both the acidic peak and at pH 7.5, there was a pronounced calcium dependence (shown in Fig. 2 for pH 5.5 only).

Separation of the water-soluble products on Dowex AG1-X8 anion exchange columns (Fig. 3) indicated that these products were a mixture of cyclic and noncyclic inositol phosphate; one product comigrated with *myo*-inositol 1:2-cyclic phosphate (cyclic IP), and the other with *myo*-inositol 1-phosphate. Generation of inositol phosphates from PI is indicative of a phospholipase C activity. No significant amount of free *myo*-inositol was observed, which rules out a phospholipase D activity. This information together with the lack of activity with [^{14}C]-PC as substrate indicates that the extracellular phospholipid cleaving activity observed in Swiss 3T3 conditioned buffer is that of a PI-specific phospholipase C.

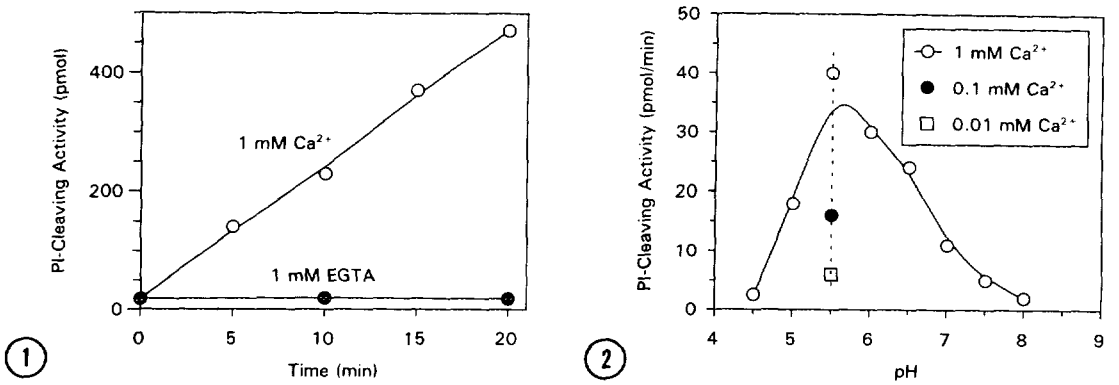

Fig. 1. Time course of PI-cleaving activity. A 150 mm dish of confluent, quiescent Swiss 3T3 cells was gently rocked in 3.5 ml buffer A (pH 7.4) at 7°C for 0.5 hr in the presence of protease inhibitors and aliquots of this conditioned buffer assayed for PI-cleaving activity in the presence of either 1 mM $CaCl_2$ or EGTA. The water-soluble radioactive products of PI cleavage are shown as a function of assay time at 37°C.

Fig. 2. pH dependence and calcium dependence of PI-cleaving activity released from Swiss 3T3 cells. Conditioned buffer was concentrated in Centricon-50 devices in the presence of protease inhibitors and aliquots assayed in Tris-maleate-saline at the pHs and $CaCl_2$ concentrations indicated.

The ratio of cyclic to noncyclic IP formed by the extracellular PI-PLC was dependent upon the pH of the buffer in which the assay was carried out. In assays carried out at pH 7.4 (the unadjusted pH of the conditioned buffer) approximately 40-45% of the inositol phosphate was generated in the cyclic form (not shown). When the conditioned buffer was adjusted to pH 5.5 prior to the assay, cyclic IP was the predominant product (approximately 60%; Fig. 3). Control experiments showed that the chromatography profile of the inositol phosphate products was a function of the pH of the enzyme assay in which the products were generated, rather than merely due to an effect of the buffer pH on the elution profile of the products.

Many studies of secreted cellular enzymes make use of culture medium conditioned for 1-2 days as a source of material; however we find that recovery of the extracellular PI-PLC at 37°C is better accomplished in 1-2 hr, rather than overnight. This may be due to proteolytic activity accumulating in the culture medium during longer conditioning times. Addition of the protease inhibitors aprotinin and leupeptin was essential to optimal enzyme activity recovery following the centrifugal ultrafiltration steps required to change from culture medium to assay buffer, indicating that extracellular proteases are indeed present. Although extracellular PI-PLC activity was present in subconfluent actively growing cell cultures, there was substantially more activity found in fully confluent quiescent Swiss 3T3 cultures.

Convenient and reproducible recovery of extracellular PI-PLC activity occurred when washed confluent cultures were incubated with isotonic assay buffer containing protease inhibitors at 7°C for

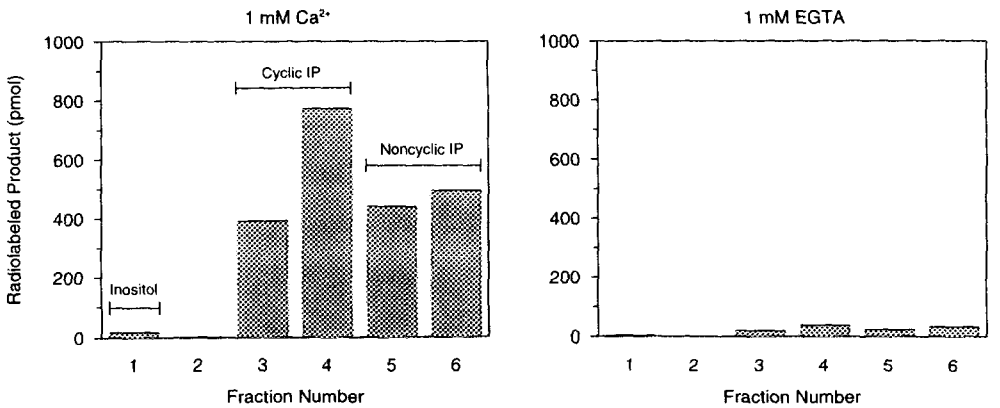

Fig. 3. Analysis of the water-soluble products. Elution profile on 1 ml Dowex AG1-X8 anion exchange columns of water-soluble radiolabeled products obtained from extracellular PI-PLC activity acting on [^{3}H]-PI. Swiss 3T3 conditioned buffer was assayed at pH 5.5 in the presence of either 1 mM Ca^{2+} or 1 mM EGTA. Fractions 1, 2, 3-5, and 6 consisted of H_2O, 50 mM, 150 mM, and 1 M ammonium formate, respectively. With this type of column, inositol cyclic phosphate appears in fractions 3 and 4, and non-cyclic inositol monophosphate is found in fractions 5 and 6.

short times (20-30 min). The low temperature minimizes proteolytic activity, and centrifugal ultrafiltration to change from culture medium to assay buffer becomes unnecessary. The time course of release of the extracellular activity was examined by successively exposing the cells to three aliquots of buffer A for twenty minutes each. The third aliquot contained only about half the activity of the first aliquot. Thus, the release of extracellular activity decreases with time at low temperatures. This observation is consistent with enzyme secretion or enzymatic release from the cell surface rather than cell leakage; i.e. the cells exposed for longer times in the buffer would be expected to exhibit increased leakage. Trypan Blue exclusion and lactate dehydrogenase assay showed that the cells remain intact up to at least 1 hr in isotonic buffer at 7°C, which provides independent evidence that the extracellular PI-PLC is not an artifact of cellular leakage.

An extracellular membrane-bound lysoPI- (and NBD-PI-) cleaving PI-PLC activity has previously been reported associated with the external cell surface of a number of cultured cell lines, including Swiss 3T3 (8-11). However, it is unlikely that the extracellular PI-PLC present in conditioned medium from cultured cells is derived from cell surface PLC (ecto-PLC) because we observe the extracellular PI-PLC in the external medium of actively growing Swiss 3T3 cells which are negative for ecto-PLC expression until the onset of quiescence. In addition, in preliminary experiments we find the extracellular PI-PLC activity with a variety of other cell types including transformed mouse fibroblasts and human fibroblasts which do not express the ecto-PLC. We also find that the activity of the ecto-PLC has a broad maximum between pH 7 and 8, while that of the extracellular soluble PI-PLC has a relatively sharp peak of maximal activity between 5 and 6 (Fig. 2). On the other hand, the released PI-PLC activity described here has certain characteristics in common

with the intracellular PI-PLCs, particularly the pH and calcium ion dependence (4). Intracellular PI-PLC is known to consist of at least three principal types of isozymes, and each of these types contains several distinct subtypes with molecular weights of the different isozymes ranging between 85 and 150 kDa (2,12). The molecular weight of the extracellular PI-PLC from cultured cells is not yet known; however, the activity was retained on 50,000 mw cut-off Centricon filters, but largely passed through 100,000 mw cut-off filters, suggesting a molecular weight of between 50 and 100 kDa for this enzyme. At this point we cannot rule out the possibility that the extracellular PI-PLC is identical to all or part of an intracellular isozyme. Exposure of the extracellular PI-PLC to dithiothreitol did not abolish the activity, as would be expected if the enzyme contained disulfide bonds, commonly found in extracellular proteins. However, the ecto-PLC activity was not abolished by dithiothreitol either, and this enzyme is known to exist on the outside of the cell membrane. The immunological relationship of the released PI-PLC to the well-characterized intracellular counterparts can be investigated through the use of isozyme-specific antibodies, and this effort is currently in progress in this laboratory.

Several related enzymes have been reported to be present in conditioned medium from cultured cells, including phospholipase A_2 (13), which is known to mediate inflammatory processes, and a glycosylphosphatidylinositol (GPI)-specific phospholipase D, which may regulate the surface expression of GPI-anchored proteins (14). The present study provides evidence that PI-PLC activity is released from cultured cells as an active extracellular enzyme. Although the function is unknown, a released PI-PLC could play a role in regulation of cell surface phospholipids and intercellular communication.

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