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ROLE OF PHOSPHOLIPID HYDROLYSIS IN THE MECHANISM OF TOXIC CELL DEATH BY CALCIUM AND IONOPHORE A23187

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SUMMARY: Manganese chloride inhibits the hydrolysis of arachidonate-containing phospholipids stimulated in 3T3 mouse fibroblasts by ionophore A23187 in the presence of extracellular calcium. The inhibition is reduced by increasing extracellular calcium concentrations. Stimulation by A23187 of this phospholipid hydrolysis and cell killing are inhibited at similiar concentrations by (i) manganese chloride or (ii) reduced extracellular calcium. These results indicate an important role for the phospholipid hydrolysis in the mechanism of cell killing by A23187 plus calcium. Analysis of the rates of the two processes indicates that phospholipid hydrolysis triggers cell killing, but it is not itself the membrane permeabilizing step.

It has been proposed(1,2) that cell killing by a variety of toxic agents involves at least two steps, (a) a disruption of the integrity of the plasma membrane by any of a variety of mechanisms followed by (b) a common final pathway to toxic cell death involving an alteration in intracellular calcium concentrations, most likely an influx of calcium across the damaged plasma membrane down a steep concentration gradient. Although the generality of this mechanism has been questioned (3), it does provide a plausible toxic mechanism under conditions achieved in a variety of circumstances. However, it leaves unanswered the question of how altered intracellular calcium concentrations effect the alterations in membrane permeability used to define cell death in these studies. The role of Ca^{2+} in cell death is conveniently studied by using the divalent cation ionophore A23187, which is known (4) to kill cells and to induce Ca^{2+} fluxes across membranes. A23187 has been shown to stimulate phospholipid hydrolysis in a variety of cell types (5-7). Stimulated phospholipid hydrolysis has been associated with ischemic cell injury (8,9), and with the mechanism of action of a variety of cytolytic toxins (10,11). In the present paper we introduce the use of manganese chloride as a tool to study the role of stimulated phospholipid hydrolysis in cell killing. The results are consistent with stimulation of hydrolysis of arachidonate-containing phospholipids being an important step in the mechanism by which altered intracellular Ca^{2+} causes cell killing, but the results indicate that it is not the step which results in the membrane permeabilization used to monitor cell death.

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MATERIALS AND METHODS: Ionophore A23187 was purchased from Calbiochem Corp. (San Diego, CA). [5,6,8,9,11,12,14,15(n)-^{3}H]Arachidonic acid (83.7 Ci/mmole) was obtained from New England Nuclear (Boston, MA). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Swiss mouse 3T3 fibroblasts (clone 3J) were a subclone of cells obtained from R.W. Holley (Salk Institute) and maintained at 37° in a humidified 15% CO_2:85% air atmosphere in Dulbecco's modified Eagle's medium (DME) and 10% calf serum (Grand Island Biological Co, Grand Island, NY). Trypsin (0.05% v/v) was used to subculture cells. The cells were free of mycoplasma as judged by autoradiography using Kodak nuclear track emulsion type NTB-2 after tritiated thymidine incorporation.

Phospholipid hydrolysis assay conditions. Phospholipid hydrolysis assays were carried in triplicate as described previously (12,13) with 3T3 cells cultured at 1.2 x 10^5 cells per 34-mm Falcon plastic dishes for 24h in 2 ml of medium containing 0.25% calf serum and 0.5 μCi of tritiated arachidonic acid. Under these conditions the label is incorporated predominantly into phospholipids (~94%) with ~3% as triglycerides and ~3% as free fatty acid. For assay the cells were washed three times on the dish with 2 ml medium and incubated under the usual culture conditions for the indicated times with 0.6 ml of medium plus A23187 added in sufficient dimethyl-sulfoxide to give a final concentration of 0.05% (V/V). Control cultures contained the same amount of dimethylsulfoxide. The radioactivity released into the culture medium has been shown to reflect hydrolysis of labeled phospholipids in the cells (12,13). The medium from the cultures was extracted and analysed for lipid composition of radioactivity as described. The radioactivity released from the cells as free arachidonic acid plus known metabolites was calculated as a percent of total incorporated radioactivity in the cells at the beginning of the assay. The data are presented as the mean ± SEM.

Analysis of the phospholipid distribution of radioactivity biosynthetically incorporated into the cells was carried out on lipids extracted from cells on plastic dishes using a modified Folch extraction procedure (14). The extracts were evaporated *in vacuo* and fractionated by thin layer chromatography on silica gel (Macherey-Nagel Co., Postfach, Germany) with the solvent system chloroform:methanol:water:acetic acid 25:15:2:4 at 4°. Authentic lipid standards were co-applied. Radioactivity co-migrating with the standards was determined as described for phospholipid hydrolysis assays.

The effectiveness of 1 μM indomethacin and 50 μM phenidone (1-phenyl-3-pyrazolidone) (15) at inhibition of prostaglandin E_2 synthesis was demonstrated by monitoring radioactivity comigrating with authentic prostaglandin E_2 in the phospholipid hydrolysis assay described above. Inhibition of lipoxygenase activity by 50 μM phenidone to below background levels was demonstrated by using the high performance liquid chromatography assay system of Eling, *et al.* (16) with 15-hydroxy-5,8,11,13-eicosatetraenoic acid (17) as standard.

Assay of cell killing. 3T3 fibroblasts were cultured as described for the assay of phospholipid hydrolysis except that no tritiated arachidonic acid was used. Cell killing was assessed by failure to exclude 0.023% trypan blue dye for 2 min at room temperature.

RESULTS: Divalent cation ionophore A23187 stimulates phospholipid hydrolysis in 3T3 Swiss mouse fibroblasts biosynthetically labeled with [^{3}H]arachidonate as previously observed in platelets (5,6) and other cultured fibroblast lines (7). Optimal hydrolysis of labeled phospholipids in 3T3 cells occurs at near physiological extracellular Ca^{2+} concentrations (1 to 5 mM, Fig. 1A) and at 5 to 10 μM A23187. Examination of the phospholipid composition of incorporated [^{3}H]arachidonate in parallel cultures of 3T3 cells before and after 20 min treatment with 5 μM A23187 in 1.8 mM $CaCl_2$ indicated that [^{3}H]arachidonate was released predominantly from phosphatidylcholine (39.4% of total incorporated label reduced

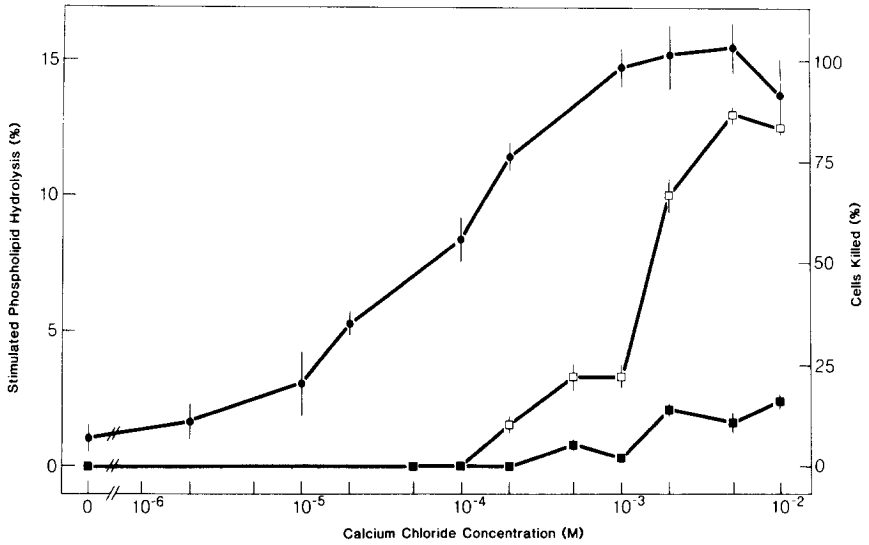

Fig. 1: Effect of Ca^{2+} and Mn^{2+} concentration on activation of phospholipid hydrolysis and on cell killing by 5 μM A23187. The phospholipid hydrolysis (●) stimulated in cultured 3T3 fibroblasts for 20 min was assayed as described in Materials and Methods in the presence of 5 μM A23187 and the indicated concentrations of $CaCl_2$, in medium prepared without $CaCl_2$ or calcium pantothenate. Cell killing was assessed in parallel cultures of 3T3 fibroblasts exposed for 60 min to 5 μM A23187 in the same medium supplemented with a range of concentrations of $CaCl_2$ alone (□) or in the presence of 100 μM $MnCl_2$ (■). Cell killing was assessed by failure to exclude trypan blue dye as described in Materials and Methods.

to 19.4%) and to a lesser extent from phosphatidylethanolamine (21.8% reduced to 16.2%) whereas no significant release was observed from phosphatidylinositol (15.0% to 15.1%) or phosphatidylserine (2.3% to 2.3%). During the 20 min incubation 21.6% of incorporated label was released from the cells as free arachidonate plus metabolites and an additional 4.0% free arachidonate generated in the process was retained in the cells. Daniel, et al. (18) have also observed release of [^{3}H]arachidonic acid from multiple phospholipids on stimulation of phospholipid hydrolysis in cultured cells.

Hydrolysis of arachidonate-labeled phospholipids stimulated in 3T3 cells by 5 μM A23187 is inhibited by manganese chloride ($MnCl_2$) (Fig. 1 and 2). Ten-fold increases in the extracellular Ca^{2+} concentration necessitate aproximately ten-fold higher concentrations of $MnCl_2$ for half-maximal inhibition (Fig. 2), consistent with Mn^{2+} acting as a competitive inhibitor of Ca^{2+} at a binding site in the cell. The nature of the binding site has not been identified, but it has been shown that inhibition by $MnCl_2$ is not due to competition for A23187 carrier sites; similar degrees of inhibition of phospholipid hydrolysis in 1.8 mM $CaCl_2$ by 100 μM $MnCl_2$ were observed at 1, 5, and 25 μM A23187 (data not shown). Mn^{2+} has been shown to compete for Ca^{2+} binding sites on some proteins (19), and it inhibits a Ca^{2+}-dependent step in programming for T-lymphocyte-mediated cytolysis (20).

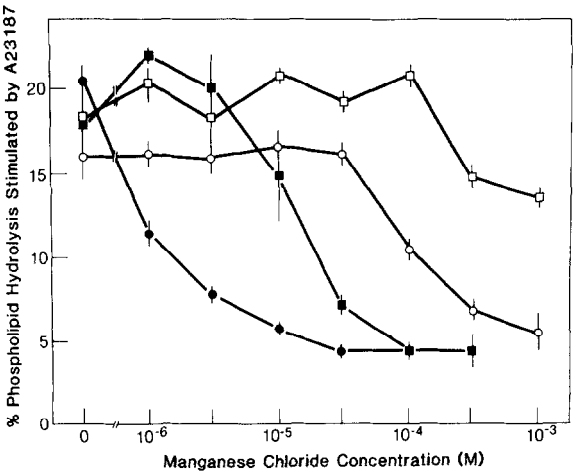

Fig. 2: Inhibition of phospholipid hydrolyzing activity by manganese chloride. Phospholipid hydrolysis was measured in 3T3 fibroblasts as described in Materials and Methods for 20 min in the presence of 5 μM A23187, the indicated concentrations of $MnCl_2$, and $CaCl_2$ at one of the following concentrations: 0.02 mM (●), 0.2 mM (■), 2 mM (◕) or 20 mM (□).

Treating 3T3 cells with 5 μM A23187 in medium (1.8 mM $CaCl_2$) results in extensive cell death within 60 min (Fig. 3). 3T3 cells are killed at Ca^{2+} concentrations which stimulate hydrolysis of greater than 10% of labeled phospholipids (Fig. 1). 3T3 cells can be protected from killing by low extracellular concentrations of Ca^{2+} (2,4) or by 100 μM $MnCl_2$ (Fig. 1 and 3). However, prolonged treatment with 5 μM A23187 results in modest levels of cell killing in the absence of extracellular Ca^{2+} or in the presence of 1.8 mM Ca^{2+} and 100 μM Mn^{2+} (Fig. 3).

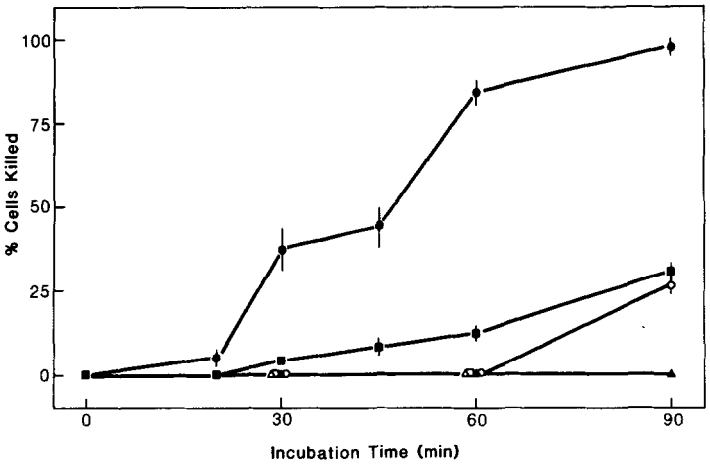

Fig. 3. Effect of Ca^{2+} and Mn^{2+} on the rate of cell killing by ionophore A23187. Parallel cultures of 3T3 fibroblasts were exposed for the indicated times to medium containing no $CaCl_2$ or calcium pantothenate (△), or the same medium supplemented with 1.8 mM $CaCl_2$ (▲), 1.8 mM $CaCl_2$ plus 100 μM $MnCl_2$ (□), 5 μM ionophore A23187 (○), 5 μM A23187 plus 1.8 mM $CaCl_2$ (●), or 5 μM A23187 plus 1.8 mM $CaCl_2$ and 100 μM $MnCl_2$ (■). Cell killing was assessed by failure to exclude trypan blue dye as described in Materials and Methods.

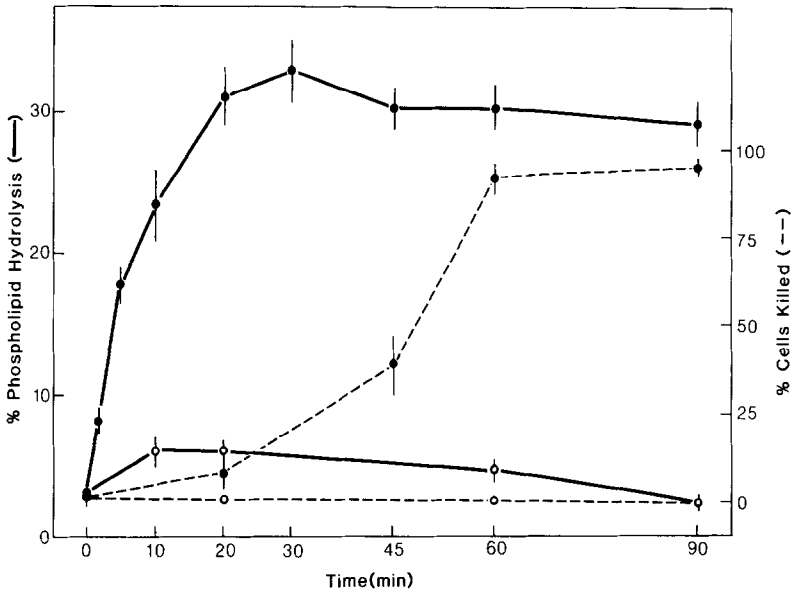

Fig. 4. The rates of phospholipid hydrolysis and cell killing stimulated by ionophore A23187. Parallel cultures of 3T3 fibroblasts were treated with medium containing 1.8 mM $CaCl_2$ with 5 μM A23187 (●) or without A23187 (○). Cell killing (---) and phospholipid hydrolysis (——) were determined at the indicated times as described in Materials and Methods.

A comparison of the rates of phospholipid hydrolysis and of cell killing stimulated in parallel cultures by 5 μM A23187 (Fig. 4) indicates that phospholipid hydrolysis begins within 2 min and is essentially complete in 20 min whereas cell killing begins at about 20 min and is not complete until after 60 min treatment with A23187. This observation indicates that the degradation of arachidonate-containing phospholipids monitored in these studies is not the step in the cytotoxic mechanism of A23187 which causes the membrane permeabilization used to define cell death in the trypan blue dye exclusion assay. Additional studies have shown that cell killing by A23187 is not mediated by known metabolites of arachidonic acid. Neither indomethacin (1 μM; an effective inhibitor of prostaglandin synthesis in 3T3 cells (12)) nor phenidone (50 μM, an effective inhibitor of lipoxygenase (12,15)) inhibited killing of 3T3 cells by 5 μM A23187 in medium containing 1.8mM CaCl (data not shown).

DISCUSSION: The use of $MnCl_2$ has made it possible to confirm in an easily-studied experimental system the proposal of Chien, et al. (8,9) that phospholipid hydrolysis is involved in cell death associated with Ca^{2+} influx (1,2). A major source of support for this proposal was provided by studies in animals with chlorpromazine to inhibit phospholipid hydrolysis, Ca^{2+} influx and cell death in liver (8) and myocardial cells (9). Chlorpromazine is not an ideal experimental tool for these types of studies because it is surface active and it

exerts a wide variety of effects on membranes at the concentrations used. In the experimental system used in these studies chlorpromazine inhibited phospholipid hydrolysis only at concentrations which caused cell loss from the substratum. The observations reported here with Mn^{2+} as an inhibitor complement the earlier studies with chlorpromazine. Mn^{2+} probably interacts with numerous processes in the cells, but its general spectrum of effects can be expected to be very different from those caused by chlorpromazine.

The more readily manipulated experimental system used in these studies has allowed the demonstration (Fig. 4), that the phospholipid hydrolysis stimulated by A23187 plus Ca^{2+} is largely complete before cell death begins. This observation is in contrast to results with a series of toxins which act by stimulating phospholipid hydrolysis (10,11). These toxins stimulate prolonged hydrolysis of phospholipids (up to 3 hr at low toxin concentrations) with cell death occurringconcomitant with hydrolysis after a level of approximately 10% hydrolysis of labeled lipids has been achieved. With some of these toxins (21,22) it has been demonstrated that the phospholipid hydrolyzing activity that is stimulated is a phospholipase A_2 with little or no specificity for arachidonic acid containing phospholipids. However, we have confirmed the observation of Hong and Deykin (7) that phospholipid hydrolysis stimulated by A23187 exhibits specificity for phospholipids containing arachidonic acid and other polyunsaturated fatty acids. Because arachidonic acid represents a modest percentage of the total fatty acids in mammalian phospholipids (21), hydrolysis of 20 to 30% of the arachidonate-containing lipids may be achieved without hydrolyzing enough of the total phospholipid to cause membrane destabilization. The different relationship of phospholipid hydrolysis to cell killing suggests that these toxins kill cells by a different mechanism than does A23187 plus Ca^{2+}. Additional evidence is provided by the observation (data not shown) that 100 μM $MnCl_2$ neither inhibits stimulation of phospholipid hydrolysis or protects against cell killing by any of the following toxins: mellitin (21), staphylococcal delta toxin (24), prymnesin (10), lysolecithin (14), the direct lytic factor from Hemachatus hemachatus venom (21) or phallolysin (22).

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