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Identification and characterization of a phospholipase C activity in resident mouse peritoneal macrophages

Inhibition of the enzyme by phenothiazines

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Resident mouse peritoneal macrophages contain a phospholipase C of high activity that is specific for phosphatidylinositol. The activity has a neutral pH optimum, is Ca^{2+}-dependent and has a maximum reaction velocity of 525 nmol/h per mg of protein. Certain phenothiazines are potent inhibitors of this activity.

We have reported that mouse peritoneal macrophages synthesize and release large amounts of prostaglandin E_2 and 6-oxoprostaglandin $F_{1\alpha}$ when exposed to inflammatory stimuli (Bonney *et al.*, 1978*a*, 1979*a*, 1980). Hsueh *et al.* (1979) have reported similar findings in rabbit alveolar macrophages responding to zymosan. Hsueh *et al.* (1979) and Scott *et al.* (1980) report that macrophages incubated in the presence of radiolabelled arachidonic acid incorporate this label primarily into phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Bonney *et al.* (1978*a*) and Hsueh *et al.* (1979) report that in such prelabelled macrophages the phosphatidylcholine pool is selectively depleted of its arachidonic acid, the precursor of these prostaglandin products. The phospholipase systems of the macrophage involved in these events are not well characterized.

Although it appears that phosphatidylcholine is the major source of arachidonic acid in the macrophage, several investigators have shown that mononuclear phagocytes responding to various stimuli incorporate ^{32}P specifically into phosphatidylinositol. Karnovsky *et al.* (1966) demonstrated this phenomenon in monocytes undergoing phagocytosis. Graham *et al.* (1967) reported a similar turnover in phosphatidylinositol in endotoxin-treated guinea-pig peritoneal macrophages. Ogmundsdötter & Weir (1979) reported that [^{3}H]-*myo*-inositol incorporation into phosphatidylinositol was stimulated in mouse peritoneal macrophages treated with endotoxin or *Corynebacterium parvum*. In platelets this enhanced turnover of phosphatidylinositol involves in part the specific hydrolysis of phosphatidylinositol by a phospholipase C since a product of this turnover is a phosphatidylinositol-derived diacylglycerol (Rittenhouse-Simmons, 1979). This diacylglycerol is then phosphorylated to give phosphatidic acid and enters the phosphatidylinositol synthetic pathway *de novo*. That there exists a coupling of phosphatidylinositol turnover to a phospholipase A_2-mediated deacylation of phosphatidylcholine is at this time only speculative. However, Lapetina & Cuatrecasas (1979) have suggested that such a coupling may be operative in the platelet. They report that phosphatidic acid is among the earliest detectable products in stimulated platelets and, being a potent Ca^{2+} ionophore (Tyson *et al.*, 1976), can activate Ca^{2+}-dependent phospholipase A_2. This enzyme, well characterized in the platelet (Apitz-Castro *et al.*, 1979), then deacylates phosphatidylcholine and supplies non-esterified arachidonic acid to the prostaglandin synthase system.

We have recently described two phospholipase A_2 activities in the macrophage that may be important in the response of this cell to inflammatory stimuli (Wightman *et al.*, 1981). We now describe for the first time a phosphatidylinositol-specific phospholipase C activity in homogenates of purified resident mouse peritoneal macrophages. We have identified this activity as a phospholipase C by recovering equimolar amounts of its reaction products, phosphoinositol and diacylglycerol. In addition, we further characterized this enzyme by showing that it is inhibited by phenothiazines, known to inhibit the stimulated release of arachidonic acid from macrophages (Humes *et al.*, 1979) and prostaglandins from platelets (Vanderhoek & Feinstein, 1979).

Materials

Male Swiss Webster mice (HLA-SW/ICR SPF) were purchased from Hilltop Lab Animals, Scott-

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dale, PA, U.S.A. M199 medium, components for Higuchi medium (Schroeder *et al.*, 1976) and pig serum were purchased from GIBCO Inc., Grand Island, NY, U.S.A. The pig serum was inactivated by heating at 56°C for 30min. Nunclon tissue culture dishes were from Vangard International, Neptune, NJ, U.S.A. Tissue culture flasks were purchased from Corning Glassworks, Corning, NY, U.S.A. *myo*-[2(n)-^{3}H]Inositol (sp. radioactivity 12.5Ci/mmol) and [1-^{14}C]arachidonic acid (sp. radioactivity 51Ci/mol) were purchased from New England Nuclear, Boston, MA, U.S.A. Silica-gel GF t.l.c. plates were from Analtech, Newark, DE, U.S.A. Lipids used as chromatographic standards were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All solvents (reagent grade) and inorganic salts were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Mouse LM fibroblasts were kindly supplied by Dr. S. Ostrove of these laboratories. Inositol 1-phosphate was supplied by Dr. P. Durette, also of these laboratories.

Methods

Cell culture

Macrophages were collected by peritoneal lavage in 5ml of M199 medium containing 1% heat-inactivated pig serum, 0.1mg of gentamycin and 20 units of heparin/ml. The cells were plated at 5×10^6–6×10^6 cells per 50mm culture dish and incubated for 2h at 37°C in an atmosphere of CO_2/air (1:19). The non-adherent cells were removed by washing the cell sheet with five 5ml volumes of normal saline (0.9% NaCl). The remaining adherent cells have been shown to be greater than 95% macrophages as determined by morphological examination of Giemsa stained cultures.

Mouse LM fibroblasts were routinely maintained in serum-free Higuchi medium.

Preparation of cell homogenates

Adherent macrophages (3×10^6) were scraped into 1ml of normal saline using a rubber policeman, placed in a 15ml conical centrifuge tube and then pulse-sonicated for 30s while suspended in an ice bath. Greater than 95% of the cells were disrupted by this procedure (Bonney *et al.*, 1979*b*). The protein content of this preparation was determined by the method of Lowry *et al.* (1951). Cell sonicates were used immediately for assay of phospholipase C.

Preparation of substrates

Radiolabelled phosphatidylinositol substrates were prepared from mouse LM cells maintained in serum-free Higuchi medium devoid of choline, serine and inositol (Sundler *et al.*, 1978). This medium contained 20μCi of [^{3}H]*myo*-inositol and, for dually-labelled substrates, 20μCi of [^{14}C]-arachidonic acid. Labelled LM cells were scraped from culture flasks in methanol/water (5:2, v/v) and the lipids were extracted by the method of Bligh & Dyer (1959). Radiolabelled phosphatidylinositol was separated to greater than 95% purity on silica-gel GF t.l.c. plates developed in chloroform/methanol/acetic acid/water (25:15:2:1, by vol.). The specific radioactivity of the substrate, averaging (2–4) $\times 10^4$ d.p.m./nmol, was determined by relating lipid phosphorus content to ^{3}H and ^{14}C radioactivity (d.p.m.). Phosphorus determinations were performed using a modification of the method of Kraml (1966). Substrates were stored in chloroform at 0°C under N_2 and were stable for greater than 60 days.

Phospholipase C assay

Enzyme activities were assayed in 80μl of 0.1M-Tris/maleate buffer, pH6.5, with 1mM-Ca^{2+}. This final mixture included 1–2μg of sonicated-macrophage protein and radiolabelled 100μM-phosphatidylinositol added as a liposomal suspension prepared by brief sonication in water. The reactions were performed at 37°C and were terminated after 30min by the addition of 3.75vol. of methanol/chloroform (2:1, v/v). The reactions were extracted by the method of Bligh & Dyer (1959) and released [^{3}H]phosphoinositol was quantified by determining the amount of radioactivity recovered in the aqueous phase. The aqueous product was identified by its co-migration with inositol 1-phosphate on silica-G t.l.c. plates developed in chloroform/methanol/water (6:4:1, by vol.). This system gave R_F values of 0.05 and 0.20 for inositol 1-phosphate and free inositol respectively. [^{14}C]Diacylglycerol was quantified by spotting a portion of the organic phase on silica-gel G t.l.c. plates and developing them in light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (70:30:1, by vol.). Diacylglycerol was detected visually by exposing the plates to I_2 vapour, identified by its co-migration with an authentic standard and quantified by determining the amount of radioactivity associated with the resulting spots. When drugs were included in the assay, all were dissolved in dimethyl sulphoxide. The final dimethyl sulphoxide concentration in the assay was 1% and it was included in the buffer blank and the 'no-drug' controls. The substrate concentration utilized in the phenothiazine experiments was 60μM, the substrate concentration at half-maximum reaction velocity.

All assays were conducted under those conditions wherein the products of phospholipase C activity accumulated in a linearly increasing manner with respect to time of incubation and amount of protein assayed. The assay is linear beyond 1h of reaction time and with greater than 7μg of protein in the reaction mixture.

Results and discussion

To determine if a phospholipase C activity could be detected in the macrophage and to characterize this activity in terms of its Ca^{2+} requirement, substrate specificity and pH optimum, the following experiment was performed. Sonicated macrophages were incubated with 100μM-[^{14}C]arachidonoyl phosphatidyl[^{3}H]inositol in reaction mixtures containing 1mM-Ca^{2+} (its optimal concentration) or 1mM-EDTA and buffers of increasing pH. The data depicted in Fig. 1 show that phosphatidylinositol is hydrolysed to phosphoinositol and diacylglycerol at a neutral pH and is inhibited by EDTA. The equimolar recovery of phospho[^{3}H]inositol and [^{14}C]diacylglycerol throughout the active range of the enzyme establishes this as a phospholipase C activity. When this experiment was performed using dually-labelled phosphatidylcholine or dually-labelled phosphatidylethanolamine as substrates, no products of phospholipase C activity were detected. These results indicate that the macrophage enzyme is specific for phosphatidylinositol. The neutral pH optimum, Ca^{2+} requirement and phosphatidylinositol specificity show that this enzyme resembles the soluble phospholipase C activities reported in the platelet (Mauco *et al.*, 1979; Rittenhouse-Simmons, 1979). It is noteworthy that under these conditions there is neither diacylglycerol lipase nor diacylglycerol kinase activity sufficient to deplete the diacylglycerol produced. Subsequent efforts to identify a diacylglycerol lipase optimally active at pH 7 in the macrophage similar to that reported by Bell *et al.* (1979) in the platelet have not been successful. We have characterized another activity, however, which is very different from the platelet enzyme. This activity is independent of Ca^{2+} concentration, is optimal at pH 4.5, is not augmented by reduced glutathione and has a maximum reaction velocity of 3 nmol/h per mg of protein.

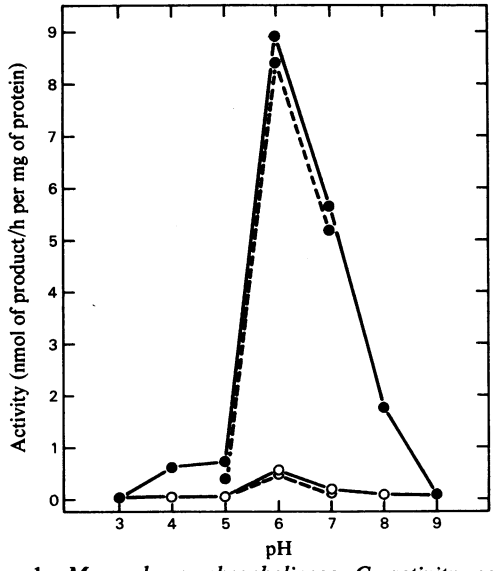

Fig. 1. *Macrophage phospholipase C activity as a function of pH*

Reactions were performed in acetate buffer (pH 3–5), Tris/maleate buffer (pH 6–7) or Tris/HCl buffer (pH 8–9) containing 1mM-Ca^{2+} (●) or 1mM-EDTA (○). [^{14}C]Arachidonoyl phosphatidyl[^{3}H]-inositol was used as substrate. Results are reported as nmol of phosphoinositol (——) or nmol of diacylglycerol (– – – –) recovered/h per mg of protein. Each data point represents the mean of triplicate determinations with less than 10% deviation among determinations.

Sonicated macrophages were assayed for phospholipase C activity with increasing amounts of phosphatidyl[^{3}H]inositol as described in the Methods section. In this experiment phospholipase C activity reached a maximum reaction velocity of 525 nmol of phosphatidylinositol hydrolysed/h per mg of protein and is fully saturated at a substrate concentration of 100μM.

Mouse peritoneal macrophages, when exposed to an inflammatory stimulus such as zymosan or antigen–antibody complexes, release about 1–2 nmol of prostaglandins/h per mg of cell protein (Bonney *et al.*, 1978*a*, 1979*a*). Prelabelled platelets exposed to stimulators such as thrombin and ionophore A23187 release about 20–30% of the incorporated radioactive arachidonic acid (Rittenhouse-Simmons *et al.*, 1976). Vanderhoek & Feinstein (1979) have reported that chlorpromazine, a phenothiazine, inhibits this stimulated release of oxygenated products of arachidonic acid from platelets. Chlorpromazine shows an I_{50} of this release at a concentration of 50μM. Accordingly, we have studied the effects of several phenothiazines on macrophage phospholipase C (Table 1). Although metiazinic acid shows an I_{50} of 300μM, the I_{50} values for each of the other phenothiazines tested against the macrophage phospholipase C are well within the range of activity reported by Vanderhoek & Feinstein (1979) for chlorpromazine inhibition of prostaglandin release. Conceivably, this compound could be exerting this inhibition at the level of the phospholipase C. Promethazine was also titrated against the Ca^{2+}-dependent macrophage phospholipase A_2 activity that we have characterized (Wightman *et al.*, 1981). It failed to show significant inhibition of this activity even at 1mM concentration.

Other investigators have examined the effects of cationic drugs on phospholipase C hydrolysis of phosphotidylinositol. These reports demonstrate that chlorpromazine inhibits both the soluble lymphocyte phospholipase C (Allan & Michell, 1974) and the lysosomal phospholipases C of rat liver and brain (Irvine *et al.*, 1978). Lullmann *et al.* (1980) have reported that this class of drug can displace

Table 1. *Inhibition of macrophage phospholipase C activity by several phenothiazines*

The compounds indicated below were included in the assay mixture described in the Methods section in dimethyl sulphoxide. The concentrations tested were 1 mM, 0.1 mM, 10 μM and 1 μM. The final dimethyl sulphoxide concentration was 1%. I_{50} is the concentration at which the compound inhibits the phospholipase C by 50%. These were calculated from the regression lines $y = a + bx$ relating phenothiazine concentration (x) to percentage inhibition (y). These data are representative of two independent experiments. The correlation coefficient for each regression line was greater than 0.950. The percentage inhibition values were means of three determinations with the coefficients of variation less than 10% of the mean. These data are a representation of two independent experiments.

Compound	I_{50} (μM)
Methdilazine	3.2
Promethazine	5.0
Chlorpromazine	6.3
Prochlorperazine	20.0
Trimeprazine tartrate	25.0
Dimethothiazine mesylate	45.0
Metiazinic acid	300.0

bound Ca^{2+} from phosphatidylinositol monolayers with an I_{50} of about 50 μM. To address the question as to whether this was the mechanism of phenothiazine inhibition of macrophage phospholipase C, promethazine was titrated from 1 μM to 1 mM against the phospholipase C activity in the absence of exogenous Ca^{2+} or increasing amounts of Ca^{2+} added in 2 mM increments up to 10 mM. The mean ($\pm$S.D.) of the I_{50} values at each Ca^{2+} concentration was 5.2 ± 0.01 μM. Hence the inhibition of macrophage phospholipase C is independent of the Ca^{2+} concentration employed. The mechanism of inhibition of macrophage phospholipase C by phenothiazines remains to be resolved. However, it is likely that the active moiety of the phenothiazines may be the ionizable amine. Certain compounds similar in structure to the phenothiazines and bearing an ionizable amine also inhibit macrophage phospholipase C at similar concentrations. These compounds include chloroquine, mepacrine and cyproheptadine tartrate (P. D. Wightman, unpublished work).

In conclusion we have characterized a phospholipase C activity in mouse macrophages that may be of importance in the response of the macrophage to inflammatory stimuli. Furthermore we have shown that this activity can be inhibited by phenothiazines, compounds that have been shown to inhibit the release of prostaglandins from stimulated platelets and arachidonic acid from stimulated macrophages.

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