Antigen-stimulated metabolism of inositol phospholipids in the cloned murine mast-cell line MC9

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Cells of the murine mast-cell clone MC9 grown in suspension culture were sensitized with an anti-DNP (dinitrophenol) IgE and subsequently prelabelled by incubating with [32 P] P_i . Stimulation of these cells with DNP-BSA (bovine serum albumin) caused marked decreases in [32 P]polyphosphoinositides (but not [32 P]phosphatidylinositol) with concomitant appearance of [32 P]phosphatidic acid. Whereas phosphatidylinositol monophosphate levels returned to baseline values after prolonged stimulation, phosphatidylinositol bisphosphate levels remained depressed. Stimulation of sensitized MC9 cells with DNP-BSA increased rates of incorporation of [32 P] P_i into other phospholipids in the order: phosphatidylcholine > phosphatidylinositol > phosphatidylethanolamine. In sensitized cells prelabelled with [3 H]inositol, release of inositol monophosphate, inositol bisphosphate and inositol trisphosphate, was observed after stimulation with DNP-BSA. When Li^+ was added to inhibit the phosphatase activity that hydrolysed the phosphomonoester bonds in the sugar phosphates, greater increases were observed in all three inositol phosphates, particularly in inositol trisphosphate. The IgE-stimulated release of inositol trisphosphate was independent of the presence of extracellular Ca^{2+} . In addition, the Ca^{2+} ionophore A23187 caused neither the decrease in [32 P]polyphosphoinositides nor the stimulation of the release of inositol phosphates. These results demonstrate that stimulation of the MC9 cell via its receptor for IgE causes increased phospholipid turnover, with effects on polyphosphoinositides predominating. These data support the hypothesis that hapten cross-bridging of IgE receptors stimulates phospholipase C activity, which may be an early event in stimulus–secretion coupling of mast cells. The results with the Ca^{2+} ionophore A23187 indicate that an increase in intracellular Ca^{2+} alone is not sufficient for activation of this enzyme.

INTRODUCTION

In the mast cell, receptors for IgE on the plasma membrane designated 'Fc $_{\epsilon}$ ' are bridged by a variety of multivalent stimuli such as anti-(Fc $_{\epsilon}$ receptor) antibody (Ishizaka *et al.*, 1980), the lectin concanavalin A (Hirata *et al.*, 1979), or the hapten to which the IgE is directed (Liu *et al.*, 1978). The antigenic stimulation of mast cells requires the bridging and subsequent clustering of the Fc $_{\epsilon}$ receptors (Ishizaka & Ishizaka, 1978). Bridging of the Fc $_{\epsilon}$ receptors stimulates a number of biochemical events, including phospholipid methylation (Hirata *et al.*, 1979; Chiang *et al.*, 1978; in refutation, see Moore *et al.*, 1984), increases in cellular cyclic AMP (Ishizaka *et al.*, 1980), increased calcium influx (Ishizuka & Nozawa, 1983; Ishizaka *et al.*, 1980), hydrolysis of membrane inositol phospholipids (Moore *et al.*, 1984; Beavan *et al.*, 1984); Ishizuka *et al.*, 1984), phospholipase activation (Ishizuka *et al.*, 1984; Musch & Siegel, 1985) and leukotriene synthesis (Musch & Siegel, 1985). Many of these enzymes stimulated during activation are Ca^{2+} -dependent; however, certain mast-cell stimuli, such as compound 48/80, or the peptide somatostatin, activate mast-cell secretion without extracellular Ca^{2+} (Baxter & Adamik, 1978), indicating a second, or additional, pathway of activation.

Phospholipase C activation and hydrolysis of membrane phosphoinositides was initially proposed by Michell (1975) to mediate the cellular effects of a number of hormones and neurotransmitters. It includes a diverse number of agonists, as recently reviewed by Berridge (1984). Fc $_{\epsilon}$ receptor stimulation of PtdIns turnover has been demonstrated in 2H3 cells (Moore *et al.*, 1984; Beaven *et al.*, 1984), mast cells (Ishizuka *et al.*, 1984) and lymphocytes (Allan & Michell, 1977; Schellenberg & Gillespie, 1977). The polyphosphoinositides are minor (less than 10% of the total phosphoinositides) membrane phospholipids, and their rapid disappearance after specific stimulation has implicated their hydrolysis as the first event in stimulus–secretion coupling (Creba *et al.*, 1983). Stimulation of the phosphodiesteratic hydrolysis of PtdIns(4,5) P_2 results in the formation of Ins P_3 (Berridge *et al.*, 1983; Agranoff *et al.*, 1983; Downes & Wustemann, 1983) and diacylglycerol, both of which may perform critical roles in cellular activation. Ins P_3 has been shown to release Ca^{2+} from non-mitochondrial intracellular stores (Streb *et al.*, 1983; Burgess *et al.*, 1984; Joseph *et al.*, 1984), providing Ca^{2+} essential for activation. Additionally, the diacylglycerol formed may activate protein kinase C (Nishizuka, 1984), which has been implicated in stimulus–secretion coupling in platelets

Abbreviations used: Ptd, phosphatidyl; Ins, inositol; P_1 , P_2 and P_3 , mono-, bis- and tris-phosphate; DNP, dinitrophenol; BSA, bovine serum albumin; PtdSer, phosphatidylserine; PtdA, phosphatidic acid; PtdEtn, phosphatidylethanolamine; MEM, minimal essential medium; PtdCho, phosphatidylcholine.

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(Nishizuka, 1984), neutrophils (White *et al.*, 1984) and many other cell types. The nomenclature utilized in the present paper is PtdIns P_2 rather than PtdIns(4,5) P_2 and, to maintain consistency, PtdIns P . As first described in rat parotid (Irvine *et al.*, 1984) and then verified by h.p.l.c. and structural analysis (Irvine *et al.*, 1985), Ins P_2 produced may be of two isomers, Ins(1,3,4) P_3 and Ins(1,4,5) P_3 . The source of the 1,3,4-isomer is unknown, but it may be released from PtdIns(3,4) P_2 . Presumably this would co-migrate with PtdIns(4,5) P_2 on t.l.c.

The murine mast-cell clone MC9 (derived from foetal liver) structurally resembles mast cells isolated from a variety of sources, notably those of the lung and intestinal mucosa (Galli *et al.*, 1982). These mast-cell types are characterized by granules that contain chondroitin sulphates rather than heparin. Biochemically, MC9 cells can be stimulated by calcium ionophore A23187, concanavalin A, and IgE+hapten, plus, presumably, a variety of other agents. MC9 cells release arachidonic acid from membrane phospholipids and synthesize large amounts of leukotriene C_4 (Musch *et al.*, 1985; Musch & Siegel, 1985). Additionally, they release their granule contents, e.g. histamine, β -hexosaminidase and neutral proteinases, when stimulated. They can easily be grown in large numbers in suspension cultures, providing the opportunity to perform accurate biochemical analysis. The following studies were designed to evaluate the effect of IgE receptor cross-bridging on phospholipid metabolism and to determine if a PtdIns cycle might be involved in Fc ϵ receptor stimulation in these cells.

MATERIALS AND METHODS

Cell culture

MC9 cells were grown and sensitized as described previously (Musch *et al.*, 1985; Musch & Siegel, 1985). Briefly, MC9 cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 2 mM-glutamine, penicillin (50 μ g/ml), streptomycin (50 i.u./ml) (all GIBCO), 10% (v/v) heat-inactivated (56 °C for 60 min foetal-calf serum (HyClone, Logan, UT, U.S.A.) plus 5% (v/v) conditioned medium from a murine T-lymphocyte clone D9 stimulated with concanavalin A (10 μ g/ml) to produce interleukin 3, a necessary factor for MC9 cell growth and viability (obtained from Dr. D. Rennick, DNAX Institute for Molecular Biology, Palo Alto, CA, U.S.A.). Cells were passed every 2 days to maintain cell density between 2×10^5 and 2×10^6 cells/ml. For experiments, cells which had been passed the day before were always used. To sensitize cells, 0.1 μ g of anti-DNP IgE (obtained from Dr. Rennick) was added for every 8×10^5 cells in culture when passing cells on the day before the experiments. Before experimentation, cells were washed first with RPMI 1640 medium and then with Ca^{2+} -free assay buffer consisting of (concns. in mM): NaCl (125), KCl (2.5), $MgCl_2$ (0.7), Hepes (25), EGTA (0.5) and glucose (10), pH 7.4. $CaCl_2$ was added to the final incubations to achieve a final $[Ca^{2+}]$ of 1.0 mM.

[^{32}P]P $_i$ labelling and phospholipid analysis

MC9 cells, grown as described and sensitized when appropriate, were centrifuged (150 g for 10 min at 4 °C) and resuspended in Ca^{2+} -free assay buffer with 0.1% (w/v) fatty-acid-free BSA (Sigma Chemical Co., St.

Louis, MO, U.S.A.) at a density of 25×10^6 cells/ml. Carrier-free [^{32}P]P $_i$ (ICN, Irvine, CA, U.S.A.) was added to a final concentration of 100 μ Ci/ml. Cells were initially labelled for periods up to 180 min. Subsequently, a 90 min labelling period with [^{32}P]P $_i$ was used. After labelling, cells were centrifuged and resuspended in assay buffer without BSA equilibrated to 37 °C. In experiments when Dnp-BSA was used as the stimulus, PtdSer (50 μ g/ml) (Avanti Polar Lipids, Birmingham, AL, U.S.A.) was included in the incubation buffer. After 2 min at 37 °C, Ca^{2+} was added along with the stimulus, Dnp-BSA (Calbiochem, La Jolla, CA, U.S.A.) at 100 ng/10 6 cells or 1 μ M Ca^{2+} ionophore A23187 (Calbiochem, La Jolla, CA, U.S.A.). Six 200 μ l aliquots were removed at designated intervals. The reactions were stopped by the addition of cells directly to 17 mm $\times$ 100 mm polypropylene tubes containing 2.4 ml of chloroform/methanol/conc. HCl (25:50:1, by vol.). After all incubations were concluded, 600 μ l of chloroform and 600 μ l of 2 M-KCl were added to each tube. Samples were vortex-mixed vigorously and then centrifuged to separate phases (2000 g for 10 min at 4 °C). The lower (organic) phase was then washed with 1.9 ml of methanol/water/conc. HCl (100:90:1, by vol.) and phases were separated by centrifugation (1000 g for 10 min at 4 °C) and the organic phase removed. The organic phase was then dried under N_2 at 50 °C and resuspended in 100 μ l of chloroform/methanol (2:1, v/v) immediately before spotting on silica-gel G t.l.c. plates. The t.l.c. plates used to resolve the polyphosphoinositides had been dipped in a solution of 2 mM- Na_2EDTA plus 1% (w/v) sodium oxalate (both Sigma Chemical Co.) and allowed to air-dry overnight. These plates were developed in a solvent system of chloroform/methanol/conc. NH_3 /water (450:350:26:74, by vol.). Another set of plates was spotted and desiccated for 90 min before development in a solvent system of chloroform/methanol/acetic acid/water (25:15:4:1, by vol.). After air-drying for 5 min, the plates were again desiccated for 90 min and then redeveloped in a solvent system consisting of the organic phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10, by vol.). This second solvent system assured that PtdA was well resolved from PtdEtn. The regions corresponding to the various phospholipids were localized by autoradiography with Kodak Blue Brand Film (Eastman-Kodak, Rochester, NY, U.S.A.) and the corresponding areas of the t.l.c. plates were scraped into 5 ml glass scintillation vials. A 4 ml portion of scintillation fluid (Scintiverse II; Fisher Chemical Co., Springfield, NJ, U.S.A.) were added and radioactivity quantified by liquid-scintillation spectroscopy.

[3H]Ins labelling, release, and analysis of Ins phosphates

Preliminary experiments attempting to label MC9 cells with *myo*-[3H]Ins (specific radioactivity 14.5 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) at 2 μ Ci/ml in RPMI 1640 medium resulted in very small amounts of label being incorporated into the cells (less than 2%), with less than 50% of this amount incorporated into cellular phospholipids. Therefore MEM with Earle's salts and Medium 199 (both from M.A. Bioproducts, Walkersville MD, U.S.A.), both supplemented, as was RPMI 1640, were tested. It is noteworthy that RPMI 1640 has 35 mg of Ins/litre, whereas MEM has only 2 mg and medium 199 only 0.05 mg. [3H]Ins incorporation varied, but

averaged approx. 30000 d.p.m./ 10^6 cells. Subsequently, 24 h labelling in Medium 199 was used, during which incubation IgE was added when appropriate. Before each experiment, labelled cells were washed three times, once with Medium 199, and twice with assay buffer to remove unincorporated [^3H]Ins. Accumulation of the water-soluble hydrolysis products InsP, InsP₂ and InsP₃ were determined as described by Berridge *et al.* (1983), with minor modifications. Briefly, cells were resuspended in thermally equilibrated assay buffer. After 2 min, stimuli were added along with Ca²⁺ and, at various times, samples were removed and stopped by addition of 200 μl aliquots of cells to 800 μl of 3.125% (w/v) trichloroacetic acid. The samples were then kept on ice for 30 min and protein separated by centrifugation (2000 *g* for 10 min at 4 °C). The trichloroacetic acid supernatant was extracted eight times with water-saturated diethyl ether and then 1 ml of 10 mM-Na₂B₄O₇ adjusted to pH 11 with conc. NaOH was added. The samples were then applied to 2 ml Dowex AG1 (X8; formate form) columns (generated from AG1 X8 chloride-form resin; Sigma Chemical Co.). Samples were eluted as described by Berridge *et al.* (1983). A 3 ml portion of scintillation fluid was added to each fraction and radioactivity quantified by liquid-scintillation spectroscopy.

The performance of the Dowex AG1 (X8) was monitored by using the trichloroacetic acid extract from [^{32}P]P_i-labelled erythrocyte ghosts stimulated with 1 mM-Ca²⁺ to release labelled Ins phosphates as described by Downes & Michell (1981). Dowex resin generated from its chloride form proved to provide reproducible elution profiles, whereas the commercially available formate form of Dowex resin did not.

Histamine-release analysis

MC9 cells were grown, labelled and prepared for assay as described in the above subsection. To determine histamine release, cells were allowed to equilibrate for 2 min at 37 °C and then 'zero-time' samples were taken. The 200 μl sample was added to 200 μl of Ca²⁺-free Hepes assay buffer plus 4 mM-EDTA to stop the release

reaction. Samples were then processed as previously described (Musch *et al.*, 1985). Briefly, samples were spun for 30 s in a Microfuge and the supernatants removed. Histamine content was measured fluorimetrically in both supernatant (S) and cell pellet remaining after release (C). Percentage release was calculated as 100 S/(S + C) and corrected for blanks.

Materials

All solvents used were of analytical grade and obtained from Mallinkrodt, St. Louis, MO, U.S.A. All salts used were of reagent grade.

RESULTS

Kinetics of [^{32}P]labelling of phospholipids

Initially, the time course of [^{32}P]P_i labelling of membrane phospholipids in MC9 cells was investigated to determine the time at which PtdInsP and PtdInsP₂ reach constant specific radioactivities. As shown in Fig. 1, the monophosphate ester groups exchange rapidly and their labelling possibly lags only slightly behind the labelling of cellular ATP with [^{32}P]P_i. As demonstrated by [^{32}P]P_i incorporation into PtdCho, PtdIns and PtdEtn, labelling of the diester phosphate groups linking the glycerol backbone to the head groups does not reach isotopic equilibrium even after 180 min. Since PtdInsP and PtdInsP₂ achieve constant specific radioactivity before the 90 min used in subsequent studies, any decrease seen in the radioactivity of the polyphosphoinositides should parallel changes in mass. If, however, an increase in PtdIns kinase occurs upon stimulation, then breakdown of ^{32}P -labelled polyphosphoinositides may not be representative of mass. We have attempted to measure mass of PtdInsP and PtdInsP₂ by PO₄ determination; however, MC9 cells contain less than 0.5 nmol of either phospholipid per 10^7 cells, making determination difficult. However, loss of [^{32}P]polyphosphoinositides is evidence for hydrolysis by some enzyme activity, presumably either a phospholipase

Fig. 1. Time courses for the incorporation of [^{32}P]P_i into membrane phospholipids

Cells were resuspended in Ca²⁺-free assay buffer plus 0.1% (w/v) BSA at a density of 25×10^6 cells/ml. At indicated times, 200 μl aliquots (5×10^6 cells) in triplicate were removed and analysed either for PtdInsP₂ (▲) and PtdInsP (●) or for PtdA (○), PtdCho (△), PtdEtn (■) and PtdIns (□). Data represent values obtained in one experiment repeated with similar results on three occasions. Standard errors between triplicates in all experiments were always less than 5% of the mean.

Fig. 2. Time courses for the changes in membrane phospholipids after addition of DNP-BSA to sensitized MC9 cells

Cells were labelled with [^{32}P]P_i for 90 min before the experiment. After they had been washed, cells were resuspended at a density of 20×10^6 cells/ml in Ca^{2+} -free assay buffer. Zero-time samples were taken (all samples taken in triplicate), and DNP-BSA was added (100 ng/ 10^6 cells) along with 1.5 mM- CaCl_2 . At 5, 15 and 30 s and 1, 2, 5, 10 and 30 min later, triplicates were taken both for the analysis of polyphosphoinositols [PtdInsP₂ (▲) and PtdInsP (●)] and other phospholipids [PtdA (○), PtdC (△), PtdIns (□) and PtdE (■)]. Certain early data points where no change occurred are omitted from the graphs for clarity. Data represent values obtained in one experiment repeated with similar results on five occasions. Standard errors between triplicates in all experiments was always less than 5% of the mean. Zero-time values in the experiment presented were: PtdInsP₂, 56721 ± 1201 c.p.m.; PtdInsP, 39787 ± 689 c.p.m.; PtdA, 7089 ± 236 c.p.m.; PtdCho, 561870 ± 21981 c.p.m.; PtdIns 175661 ± 6239 c.p.m.; and PtdEtn, 387635 ± 16810 c.p.m. In each experiment, activation of the cells was checked by stimulation of leukotriene C₄ production at 10 min.

A₂, D, or C. As discussed by Michell & Kirk (1981), this breakdown is important to observe, since it may be the initiating reaction.

Changes in phospholipid metabolism during antigenic stimulation

When sensitized MC9 cells are labelled with [^{32}P]P_i and stimulated with DNP-BSA, marked decreases in the polyphosphoinositides are observed as early as 1 min after stimulation (Fig. 2). [^{32}P]PtdInsP₂ decreases maximally by 5 min, and the levels do not recover even at 30 min. Decreases in [^{32}P]PtdInsP parallel those in [^{32}P]PtdInsP₂, but subsequently the levels return to baseline values. PtdA increases in a nearly coincident time course, except that increases can be noted as early as 30 s after stimulation with DNP-BSA. No decrease in PtdIns is observed at any time point after stimulation.

Stimulation with DNP-BSA also increases the rate of incorporation of ^{32}P into other membrane phospholipids (Fig. 2). In non-sensitized cells exposed to DNP-BSA or sensitized cells not stimulated with DNP-BSA, only small increases in [^{32}P]PtdCho, [^{32}P]PtdIns and [^{32}P]PtdEtn occur (results not shown). In sensitized cells stimulated with DNP-BSA, significant increases in PtdIns and PtdCho can be measured at 5 min after stimulation (129 and 116% initial values) and by 30 min the labelling is 438% and 316% of zero-time values respectively.

Formation of inositol phosphates during antigen stimulation

The loss of the polyphosphoinositides may be the result of phospholipase A₂ activity, phospholipase D activity, or by a hydrolysis by phospholipase C yielding InsP, InsP₂ and InsP₃. To determine whether phospholipase C activity is involved, experiments were performed with

[^3H]inositol-labelled cells to measure the water-soluble hydrolysis products, namely InsP, InsP₂ and InsP₃. Stimulation with DNP-BSA causes significant increases in the amounts of all three Ins phosphates (Fig. 3a).

The amount of [^3H]inositol also increases after stimulation with DNP-BSA (results not shown), suggesting rapid metabolism of the Ins phosphates by cellular phosphatase activity. Li⁺ has been shown to inhibit inositol-1-phosphatase activity (Hallcher & Sherman, 1980), resulting in greater accumulations of InsP in whole cells and tissues (Berridge *et al.*, 1982). When LiCl (5 mM) is added at the same time as stimulation, much larger accumulations of all the Ins phosphates occur (Fig. 3b). Particularly important is the release of InsP₃ detectable 5 s after stimulation, which precedes release of InsP₂ and InsP.

Kinetics of histamine release in the presence and absence of Li⁺

If Fc_ε-receptor-stimulated polyphosphoinositide breakdown is responsible for mediating stimulus-secretion coupling, then [^3H]Ins phosphate release should precede histamine release. In Fig. 4 the kinetics of histamine release in sensitized MC9 cells stimulated with DNP-BSA in the absence and presence of Li⁺ are shown. As has been demonstrated previously (Musch & Siegel, 1985), this process requires extracellular Ca^{2+} . When the kinetics of InsP₃ and histamine release are compared, it is apparent, particularly when Li⁺ is present, that InsP₃ formation precedes the functional response of the cell.

Effects of Ca^{2+} on phosphoinositide metabolism

To determine whether hydrolysis of PtdInsP₂ required Ca^{2+} , MC9 cells labelled with [^3H]inositol were washed in the usual way, except only 100 μM-EGTA was used in the buffer to prevent extensive leaching of Ca^{2+} from the

Fig. 3. Time courses for release of $[^3\text{H}]$ Ins phosphates [InsP ($\square$), InsP_2 ($\triangle$), and InsP_3 ($\circ$)] stimulated by DNP-BSA in the absence (a) and presence (b) of 5 mM-LiCl

MC9 cells prelabelled with $\text{myo}-[^3\text{H}]$ inositol were washed extensively and then resuspended in Ca^{2+} -free assay buffer at a density of 40×10^6 cells/ml. Zero-time samples [$200 \mu\text{l}$ aliquots (8×10^6 cells) in duplicate] were taken and then DNP-BSA added along with 1.5 mM- CaCl_2 and 4 mM-LiCl when appropriate. At 5, 15 and 30 s and 1, 2, 5, 10 and 30 min later, samples were taken and stopped by addition of trichloroacetic acid and processed as described in the Materials and methods section. Data represent values obtained in one experiment repeated with similar results on six occasions. Standard errors between triplicates in all experiments were generally less than 10% of the mean. Zero-time values for the experiments presented were (a) without Li^+ : InsP , 1417 ± 109 d.p.m.; InsP_2 , 524 ± 39 d.p.m.; and InsP_3 , 367 ± 19 d.p.m.; (b) with Li^+ , respectively: 2817 ± 274 d.p.m., 491 ± 51 d.p.m. and 387 ± 43 d.p.m.

Fig. 4. Time course for the release of histamine in the absence ($\triangle$) and presence ($\circ$) of 5 mM-LiCl

MC9 cells prelabelled with $\text{myo}-[^3\text{H}]$ Ins were washed extensively and then resuspended in two equal aliquots of Ca^{2+} -free assay buffer. Zero-time samples ($200 \mu\text{l}$) in duplicate were taken and then DNP-BSA was added along with 1.5 mM- Ca^{2+} and 5 mM-LiCl when appropriate. Samples were taken at 15 and 30 s and 1, 2, 5, 10 and 30 min later and stopped by addition to ice-cold Ca^{2+} -free buffer plus 4 mM-EDTA. Data represent those obtained in one experiment repeated with similar results on three occasions. Standard errors between duplicates in all experiments were always less than 3% of the mean.

Fig. 5. Time course for release of $[^3\text{H}]$ Ins phosphates InsP ($\square$), InsP_2 ($\triangle$) and InsP_3 ($\circ$) stimulated by DNP-BSA in the absence of extracellular calcium in the presence of 5 mM-LiCl

MC9 cells prelabelled with $\text{myo}-[^3\text{H}]$ inositol were washed twice in Ca^{2+} -free assay buffer with 0.1 mM-EGTA and resuspended at a density of 40×10^6 cells/ml. Zero-time samples ($200 \mu\text{l}$ aliquots; 8×10^6 cells) were taken and then DNP-BSA added along with 5 mM-LiCl. At 5, 15 and 30 s and 1, 2, 5, 10 and 30 min later, samples were taken and stopped by addition to trichloroacetic acid and processed as detailed in the Materials and methods section. Data represent values obtained in one experiment repeated with similar results on four occasions. Standard errors between triplicates in all experiments was always less than 10% of the mean. Zero-time values for the experiment presented were: InsP , 4631 ± 217 d.p.m.; InsP_2 , 295 ± 15 d.p.m.; and InsP_3 , 317 ± 34 d.p.m.

Fig. 6. Time courses for the changes in membrane phospholipids after the addition of the Ca^{2+} ionophore A23187 to non-sensitized MC9 cells

Cells were labelled with $[^{32}\text{P}]\text{P}_i$ for 90 min before the experiment. After they had been washed, cells were resuspended at a density of 20×10^6 cells/ml in Ca^{2+} -free assay buffer. Zero-time samples were taken and $1 \mu\text{M}$ -A23187 added along with 1.5 mM - CaCl_2 . At 5, 15 and 30 s and 1, 2, 5, 10 and 30 min later, triplicates were taken both for polyphosphoinositol [PtdInsP_2 ($\blacktriangle$) and PtdInsP ($\bullet$)] and PtdA ($\circ$), PtdC ($\triangle$), PtdIns ($\square$) and PtdE ($\blacksquare$) analysis. Certain early data points where no change occurred are omitted from the graphs for clarity. Data represent values obtained in one experiment repeated with similar results on four occasions. In each experiment, activation of the cells was checked by stimulation of leukotriene C_4 production at 10 min. Standard errors between triplicates in all experiments were always less than 5% of the mean. Zero-time values for the experiment presented were: PtdInsP_2 , 63060 ± 3117 c.p.m.; PtdInsP , 32987 ± 1181 c.p.m.; PtdA , 3083 ± 129 c.p.m.; PtdCho , 531907 ± 22521 c.p.m.; PtdIns , 121776 ± 5431 c.p.m.; and PtdEtn , 423073 ± 12122 c.p.m.

Fig. 7. Time courses for release of $[^3\text{H}]\text{Ins}$ phosphates InsP ($\square$), InsP_2 ($\triangle$) and InsP_3 ($\circ$), stimulated by Ca^{2+} ionophore A23187 in the absence (a) and the presence (b) of 5 mM - LiCl

MC9 cells prelabelled with $\text{myo}-[^3\text{H}]\text{inositol}$ were washed extensively and then resuspended in Ca^{2+} -free assay buffer at a density of 40×10^6 cells/ml. Zero-time samples [$200 \mu\text{l}$ aliquots (8×10^6 cells) in duplicate] were taken and then ionophore added along with 1.5 mM - CaCl_2 and 5 mM - LiCl when appropriate. At 5, 15 and 30 s and 1, 2, 5, 10 and 30 min later, samples were taken and stopped by addition to trichloroacetic acid and processed as detailed in the Materials and methods section. Data represent values obtained in one experiment repeated with similar results on four occasions. Standard errors between triplicates and all experiments were always less than 10% of the mean. Zero-time values for the experiment presented were: InsP , 2219 ± 140 d.p.m.; InsP_2 , 231 ± 17 d.p.m.; and InsP_3 , 175 ± 13 d.p.m. (without Li^+) and, respectively, 2891 ± 174 , 306 ± 27 and 371 ± 29 d.p.m. with Li^+ .

cells. The cells were then stimulated with DNP-BSA alone in the presence of Li^+ . No Ca^{2+} was added. As shown in Fig. 5, the formation of InsP_3 is smaller when no extracellular Ca^{2+} is present, yet it is only reduced 20% (in other experiments, the reduction varied up to 35%). There is no leukotriene C_4 production and no stimulation

above spontaneous release of histamine when the cells are activated in such a fashion (Musch & Siegel, 1985).

Additionally, experiments using the calcium ionophore A23187 were performed. In ^{32}P -labelled cells, ionophore does not stimulate changes in the polyphosphoinositides (Fig. 6). Although the ionophore stimulates the

functional response of the cell as well as DNP-BSA, it appears to bypass this particular step. Ionophore does, however, affect phospholipid metabolism, as evidenced by the increase in PtdA (Fig. 6). This result may simply be due to a rise in intracellular Ca^{2+} stimulating some event in PtdA formation unrelated to polyphosphoinositide breakdown.

As further evidence that ionophore does not stimulate polyphosphoinositide breakdown, release of [^3H]inositol phosphates was measured during ionophore stimulation, in both the presence and absence of Li^+ (Figs. 7a and 7b). In neither case are changes in the Ins phosphates observed to be significantly larger than changes seen in unstimulated cells, except at the last time point taken (30 min), where only modest elevations occurred.

DISCUSSION

The rapid hydrolysis of PtdIns P_2 and PtdIns P and the appearance of their water-soluble products indicate that polyphosphoinositide breakdown mediated by phospholipase C may play a central role in the stimulation of the MC9 cell by cross-bridging of its IgE receptors by hapten. These results resemble those of the 2H3 variant of the RBL-1 cell (Beavan *et al.*, 1984) and those in the peritoneal mast cell (Ishizuka & Nozawa, 1983; Ishizuka *et al.*, 1984) which document similar changes in [^3H]Ins phosphate release and changes in membrane Ins phospholipids. It should be noted that, in all three cell types investigated, the time course of these biochemical events correlates well with the functional response (histamine release) of the cell. Whereas the peritoneal mast cell responds rapidly to antigenic stimulation (less than 30 s), both the cloned MC9 cell and the 2H3 variant of the RBL-1 cell respond appreciably slower, as measured by histamine release (maximal release at 2–6 min). This correlation does not necessarily imply a cause-and-effect relationship, but it is supportive of the hypothesis that activation of phospholipase C hydrolysis of polyphosphoinositides is intimately linked to cellular activation.

Hydrolysis of PtdIns P and PtdIns P_2 in the MC9 cells appears to be mediated by phospholipase(s) C activity. The release of the water-soluble Ins phosphates is an anticipated result of phospholipase C action. This release, however, was observed before significant changes in phospholipid breakdown in the presence of Li^+ . This may indicate that rapid cycling of the membrane phospholipids occurs and that changes in ^{32}P labelling are only observed when large amounts of PtdIns P and PtdIns P_2 are hydrolysed. In addition, the observation that [^{32}P]PtdA increases coincidentally with decreases in the membrane polyphosphoinositides supports the contention that phospholipase C is activated by antigen stimulation. The logical source of the diacylglycerol made available to diacylglycerol kinase is PtdIns P and PtdIns P_2 .

As observed in hepatocytes (Creba *et al.*, 1983; Thomas *et al.*, 1984), pancreatic acini (Rubin *et al.*, 1984) and cultured GH $_3$ cells (Martin, 1983), as examples, the event that occurs first after receptor cross-bridging is the release of Ins P_3 . In the MC9 cell this event is not dependent on addition of extracellular Ca^{2+} . As reviewed by Michell *et al.* (1981), in most systems studied the initial breakdown of polyphosphoinositides is not dependent on Ca^{2+} . The initial step in mobilization of Ca^{2+} and stimulus–secretion coupling must require levels of cytoplasmic Ca^{2+} present

in the resting cell and must precede a measurable change in cytoplasmic Ca^{2+} . The Ins P_3 released may have the same effect in the mast cell as it has been shown to have in pancreatic acinar cells (Streb *et al.*, 1983) and in hepatocytes (Burgess *et al.*, 1984). In these systems Ins P_3 stimulates the release of Ca^{2+} from ATP-dependent non-mitochondrial intracellular pools. How the Ins P_3 releases the Ca^{2+} is unknown, but this event most probably occurs at the endoplasmic reticulum (Prentiki *et al.*, 1984). In addition to IP $_3$ -stimulated Ca^{2+} release, the diacylglycerol formed by phospholipase action in mast cells (Kennerly *et al.*, 1979) may activate protein kinase C (Nishizuka, 1984). Since protein kinase C requires PtdSer, this may explain why IgE-receptor-mediated stimulation of mast cells of certain types is potentiated by addition of this phospholipid. In studies where phorbol esters (protein kinase C activators) have been used as rat serosal mast-cell stimuli (Heiman & Crews, 1985; Katakami *et al.*, 1984), phorbol myristate acetate causes phosphorylation of mast-cell proteins without addition of PtdSer. As Heiman & Crews' (1985) data show, however, this is not correlated well with histamine release, which is PtdSer-dependent. In other systems where stimulus–secretion occurs via protein kinase C, as in platelets (Nishizuka, 1984) or neutrophils (White *et al.*, 1984), no exogenous PtdSer is required. Thus the requirement for PtdSer in rat serosal mast cells is unlikely to be related to the phospholipid-dependence of the kinase. Others (Hirata *et al.*, 1979; Ishizuka *et al.*, 1980) have proposed that the PtdSer might be decarboxylated and serve as a source of PtdEtn for methylation. Results of Moore *et al.* (1984) and a limited number of experiments in our laboratory (results not shown) fail to demonstrate increased methylation concomitantly with stimulation of the Fc $_c$ receptor. This makes a hypothesis for PtdSer-dependence as a source of PtdEtn tenuous.

Release of Ca^{2+} from an intracellular store may be the trigger to activate the MC9 cell, but cannot explain the entire activation cascade, since extracellular Ca^{2+} is absolutely required for stimulation of granule release in this cell system (Musch & Siegel, 1985). Similarly, fMet-Leu-Phe stimulates polyphosphoinositide turnover and Ins phosphate production in HL-60 cells (Dougherty *et al.*, 1984), but activation of the 5-lipoxygenase by fMet-Leu-Phe requires addition of extracellular Ca^{2+} (J. Anthes, personal communication) as does lysosomal-enzyme release (Dougherty *et al.*, 1984). Thus, whereas Ins P_3 may play a critical role in initial activation, either it must also be able to cause influx of extracellular Ca^{2+} or additional pathways of Ca^{2+} mobilization must be involved. One possibility is that the PtdA formed may act as a Ca^{2+} ionophore (Serhan *et al.*, 1981). Other possibilities include opening of a Ca^{2+} channel or stimulation of a Ca^{2+} -transport protein in the plasma membrane.

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