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Calcium signals and phospholipid methylation in eukaryotic cells

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Rat basophil leukaemic (2H3) cells, mast cells and mouse thymocytes respond to stimulation by specific ligands with an increase in the free cytosolic Ca^{2+} concentration. The time courses of these Ca signals and the biological responses have been compared with changes in phospholipid metabolism. Increased phosphoinositide metabolism coincides with the Ca signals and the responses in each cell system, whereas any increase in phospholipid methylation during the response is less than one molecule per receptor and at least 5–50-fold less than the increases reported previously. Furthermore, no significant changes were detected in the concentration of *S*-adenosylmethionine, the methyl-group donor in the synthesis of methylated phospholipids. The hypothesis that phospholipid methylation is obligatory for receptor-mediated Ca signals is not supported by these data and requires critical re-evaluation.

The binding of ligands to specific receptors on the surface of eukaryotic cells causes the transmission of biochemical signals to the cell interior. In some instances the identity of the primary intracellular response and the coupling mechanism are well-established (e.g., the stimulation of adenylate cyclase by hormones acting on external receptors), but in many other systems the primary responses are incompletely characterized and the molecular mechanisms of signal transmission are correspondingly obscure. It has recently been proposed that, in a very wide range of cells, an increase in phospholipid methylation in the plasma membrane is directly involved in signal transmission mediated by surface receptors (reviewed by Hirata & Axelrod, 1980; Mato & Alemany, 1983). The metabolic pathway that is stimulated is the sequential transmethylation of phosphatidylethanolamine (PtdEtn) to phosphatidylcholine (PtdCho) via phosphatidylmonomethylethanolamine (PtdMeEtn) and phosphatidyldimethylethanolamine ($PtdMe_2Etn$) with AdoMet as methyl-group donor (Bremer *et al.*, 1960; Hoffman & Conatzer, 1981; Åkesson, 1983). The increase in phospholipid methylation is reported to be transient in all cell systems studied, although the duration of the response varies between cell types. It was suggested that the subsequent decline in the amounts of methylated phospholipids is due to the action of phospholipase A_2 on the newly-methylated PtdCho, and that this degradation also provides arachidonic acid for subsequent metabolism to, for example, prostaglandins (Hirata *et al.*, 1979*a*, 1980; Crews *et al.*, 1980, 1981; Hoffman *et al.*, 1981; Bareis *et al.*, 1983). More specifically, it has been proposed that an increase in phospholipid methylation is involved in the mechanism by which an increase in $[Ca]_i$ is generated in a variety of cells in response to stimulation, including leukaemic basophilic (2H3) cells (Crews *et al.*, 1981), mast cells (Ishizaka *et al.*, 1980), lymphocytes (Hirata *et al.*, 1980), fibroblasts (Bareis *et al.*, 1983) and neutrophils (Bareis *et al.*, 1982). Hirata, Axelrod and colleagues therefore attribute a central role to phospholipid methylation in the transmission of Ca signals. An alternative proposal

Abbreviations used: AdoMet, *S*-adenosylmethionine; $[Ca]_i$, cytoplasmic free Ca^{2+} concentration; h.p.l.c., high-pressure liquid chromatography; Ins1*P*, inositol 1-phosphate; Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; lyso-$PtdHO_2CEtOH$, lyso-phosphatidyl-2-carboxyethanol; PtdCho, phosphatidylcholine; $PtdMe_2Etn$, phosphatidyldimethylethanolamine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdIns-4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdMeEtn, phosphatidylmonomethylethanolamine; PtdSer, phosphatidylserine; Ig, immunoglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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is that a quite distinct pathway of phospholipid metabolism, the degradation of phosphatidylinositol (PtdIns) and its derivatives phosphatidylinositol 4-phosphate (PtdIns-4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], is directly involved in generating receptor-mediated Ca signals in a wide variety of cells (reviewed by Michell, 1975, 1982*a*; Michell *et al.*, 1981; Irvine *et al.*, 1982; Cockroft, 1982; Berridge, 1982). It is well-established by independent studies that metabolism of the phosphoinositides is stimulated in all of the above cell systems, and many others, by ligands acting on specific receptors (Michell, 1975, 1982*b*; Cockroft, 1982; Irvine *et al.*, 1982).

We are interested in the mechanism by which an increase in $[Ca]_i$ is stimulated in lymphocytes (Hesketh *et al.*, 1983*a*) and fibroblasts (Morris *et al.*, 1984) in response to mitogens and growth factors, and more generally in the mechanism by which intracellular Ca signals are generated by the cross-linking of receptors by specific ligands (Beaven *et al.*, 1984*a*). We have therefore compared in detail the time courses of the Ca signal with that of both phosphoinositide metabolism and phospholipid methylation in three of the cell systems examined by Hirata, Axelrod and their colleagues.

Materials and methods

Cell preparations and cultures

2H3 cells were grown in Eagle's Minimal Essential Medium with Earle's Balanced Salts Solution supplemented with 15% (v/v) foetal-calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml and were primed with monoclonal IgE antibody to ovalbumin (MAS 038c; Sera Lab, Crawley, Sussex, U.K.), as described previously (Beaven *et al.*, 1984*a*,*b*). All radiochemicals were obtained from Amersham International. Where indicated, the cells were labelled with [*methyl*-^{3}H]methionine (75 μCi/ml) or *myo*-[2-^{3}H]inositol (2 μCi/ml) by sterile incubation for 16 h in suspension in this medium (methionine concentration 100 μM, inositol concentration 11 μM) at 5×10^5 cells/ml at 37°C in an atmosphere of air/CO_2 (19:1). The cells were washed twice by centrifugation (2 min, 500***g***) and resuspended in a salts solution [150mM-NaCl/3.7mM-KCl/1.0mM-$CaCl_2$/3mM-Na_2HPO_4/3.5mM-KH_2PO_4/0.4mM-$MgSO_4$/5.6mM-glucose/0.1% (w/v) bovine serum albumin, pH 7.2]. This solution was supplemented with 5mM-LiCl for experiments on [^{3}H]inositol-labelled cells (Berridge *et al.*, 1982). Antigen was added after incubation of the cells at 1×10^6/ml for 15 min at 37°C. In some experiments (see Table 1) IgE-primed 2H3 cells were loaded with a range of [*methyl*-^{3}H]methionine concentrations (10–100 μM; 0.1–1 mCi/ml) by incubation in the salts solution for 1–3 h.

Rat peritoneal mast cells were prepared in 150mM-NaCl/3.7mM-KCl/3.0mM-Na_2HPO_4/3.5mM-KH_2PO_4/0.9mM-$CaCl_2$/5.6mM-glucose/0.1% (w/v) gelatin/heparin (10 units/ml)/0.1% (w/v) bovine serum albumin/10mM-Hepes, pH 7.0, as described by Smith *et al.* (1979) and incubated (5×10^5 cells/ml) with [*methyl*-^{3}H]methionine for 0.5–1.5 h at 37°C. The cells were washed three times by centrifugation (5 min, 50***g***), resuspended at the same cell density in fresh medium and incubated for 15 min at 37°C before addition of concanavalin A. The cells were not primed with IgE and did not release histamine in response to anti-IgE antibody.

Thymocytes from Balb/c mice were prepared in Earle's Balanced Salts Solution buffered with 10mM-Hepes, pH 7.3, as described elsewhere (Hesketh *et al.*, 1983*a*; Moore *et al.*, 1983). Where indicated, the cells were labelled with [*methyl*-^{3}H]methionine by incubation at 3×10^7 cells/ml for 0.5–3 h at 37°C. The cells were washed twice by centrifugation (250***g***, 3 min) into fresh medium at the same cell density and incubated at 37°C for 15 min before addition of concanavalin A. For [^{3}H]inositol loading, thymocytes were prepared at 3×10^7/ml in Basal Diploid Eagle's Medium (inositol-free; Flow Laboratories, Irvine, Ayrshire, Scotland), buffered with 20mM-Tris, 24mM-$NaHCO_3$ and supplemented with penicillin G (12 μg/ml) and streptomycin sulphate (100 μg/ml). They were labelled with *myo*-[2-^{3}H]inositol (20 μCi/ml; 15 Ci/mmol) by continuous stirring for 16 h at 37°C in an atmosphere of air/CO_2 (19:1), then washed twice by centrifugation (3 min, 275***g***) into Balanced Salts Solution supplemented with 5mM-LiCl.

Quin 2 loading and determination of $[Ca]_i$

Mouse thymocytes and 2H3 cells were loaded with quin 2 as described by Hesketh *et al.* (1983*a*) and Beaven *et al.* (1984*a*). Fluorescence measurements and calibrations were made using the procedures of Hesketh *et al.* (1983*a*).

Phospholipid preparation

PtdSer and $PtdHO_2CEtOH$ were prepared from dioleoyl-PtdCho (Robles & van den Berg, 1969) by treatment in diethyl ether with phospholipase D in the presence of aqueous solutions of L-serine or 2-carboxyethanol (Smith *et al.*, 1978). The corresponding lyso-lipids were prepared by treating the isolated phospholipids with cobra (*Naja naja*) venom as described by Smith *et al.* (1979).

Analysis of methylated phospholipids by h.p.l.c.

Cells (1 ml), dual-labelled with [*methyl*-^{3}H]methionine and [2-^{14}C]ethanolamine, were disrupted by addition to 3.76ml of chloroform/methanol (1:2). Chloroform (1.24ml) and water (1.24ml) were added and the lower phase was mixed with 3ml of chloroform/methanol (2:1, v/v). After washing three times with 2ml of methanol/1M-KCl + 10mM-methionine (1:1, v/v) the chloroform was evaporated and the lipids processed for h.p.l.c. analysis by using the solvents described by Moore *et al.* (1982). The solvent gradient used to separate the phospholipids on a 10μm R-Sil column (Perkin–Elmer) is represented by the line in Fig. 2(*a*) below. Recovery of [^{14}C]PtdEtn was used to normalize the recovery of methylated phospholipids between samples (Moore *et al.*, 1982). The ^{3}H label was shown to be located in the head-groups of the methylated phospholipids by digestion with phospholipase C (Smith & Stein, 1982).

Determination of intracellular [^{3}H]AdoMet

Cells (0.5ml) labelled with [*methyl*-^{3}H]methionine were mixed with 20nmol (20000 d.p.m.) of *S*-adenosyl[*methyl*-^{14}C]methionine and [*methyl*-^{14}C]methionine and immediately frozen in liquid N_2. After thawing, the samples were centrifuged (approx. 3s at 14000***g***), the supernatant was removed and the pellet extracted with 4ml of water. The combined supernatants were desalted by passage through mixed-bed ion-exchange resin (Amberlite IR-45 and IRC-50), freeze-dried, redissolved in 100μl of 10mM-sodium formate, pH4, filtered through cellulose acetate and analysed by h.p.l.c. using a Partisil-SCX column (Perkin–Elmer). The proportion of 2M-sodium formate, pH4 (solvent A) in a mixture with 10mM-sodium formate, pH4 (solvent B) is indicated by the continuous line in Fig. 2b. Recovery of [^{14}C]AdoMet (30–60%) was used to normalize the recovery of [^{3}H]AdoMet.

Phosphoinositide breakdown

Phosphoinositide breakdown was assayed by measurement of the production of [^{3}H]inositol phosphates as described by Berridge *et al.* (1982, 1983). Briefly, cells (0.5ml) were treated with 1.88ml of chloroform/methanol (1:2, v/v). After addition of chloroform (0.62ml) and water (0.62ml), the aqueous phase was removed and the interfacial material was washed twice with 0.2ml of a solution of cyclohexane-1,2-diaminetetra-acetic acid (prepared as described by Berridge *et al.*, 1983). The combined aqueous phases were applied to a column of Dowex (formate form) resin and [^{3}H]Ins1*P*, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 were eluted and quantified exactly as described in Beaven *et al.* (1984*b*). When either [^{3}H]Ins1*P*, [^{3}H]Ins(1,4)P_2 or [^{3}H]Ins(1,4,5)P_3 (10000 d.p.m.) was added to unlabelled cells, and the cells extracted as described above, recovery of ^{3}H label in the aqueous phase was more than 85%. *myo*-[2-^{3}H]Inositol was treated with Dowex (formate form) resin before use to remove radiolytic decomposition products that otherwise interfere with the determination of [^{3}H]inositol phosphates.

Arachidonic acid release

IgE-primed 2H3 cells (2×10^5) were incubated for 3h as monolayer cultures in microplate wells (Beaven *et al.*, 1984*b*) containing 200μl of Minimal Essential Medium supplemented with 0.2% (w/v) bovine serum albumin and 0.4μCi of [1-^{14}C]arachidonic acid (58mCi/mmol)/ml. The cells were washed twice with 500μl of salts solution containing 0.2% (w/v) bovine serum albumin and incubated in 200μl of the same medium with or without antigen. Aliquots (150μl) of the supernatant solution were removed from the microplate wells, centrifuged (3s at 14000***g***) and the ^{14}C content of a portion (100μl) was determined by scintillation counting.

Mouse thymocytes (6×10^6/ml) were incubated for 1h at 37°C in RPMI 1640 medium (Flow Laboratories) buffered with 20mM-Tris/24mM-$NaHCO_3$ and supplemented with 1% calf serum and 1μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid/ml (18μM). The cells were washed three times by centrifugation (1000***g***, 1 min) with RPMI 1640 supplemented with 0.5% (w/v) defatted bovine serum albumin and incubated with or without concanavalin A. Reactions were terminated by addition of 500μl of ice-cold phosphate buffered saline, pH7.2, to 200μl of cells, which were immediately centrifuged (3s at 14000***g***). The ^{3}H content of the supernatant solution (500μl) was determined by scintillation counting.

Histamine release

Histamine release from 2H3 cells was assayed as described by Beaven *et al.* (1984*a*) using the single-step radioenzymic method of Beaven *et al.* (1983). Release from rat mast cells was determined by the procedure of Smith *et al.* (1979), using the fluorescence assay of Sullivan *et al.* (1975).

Results

Phospholipid methylation in 2H3 cells

2H3 cells (Barsumian *et al.*, 1981) sensitized with IgE release histamine in response to specific antigens (Crews *et al.*, 1980). The $[Ca]_i$ in 2H3 cells can be measured by using the fluorescent Ca^{2+} indicator quin 2 (Tsien, 1980, 1981), and we have

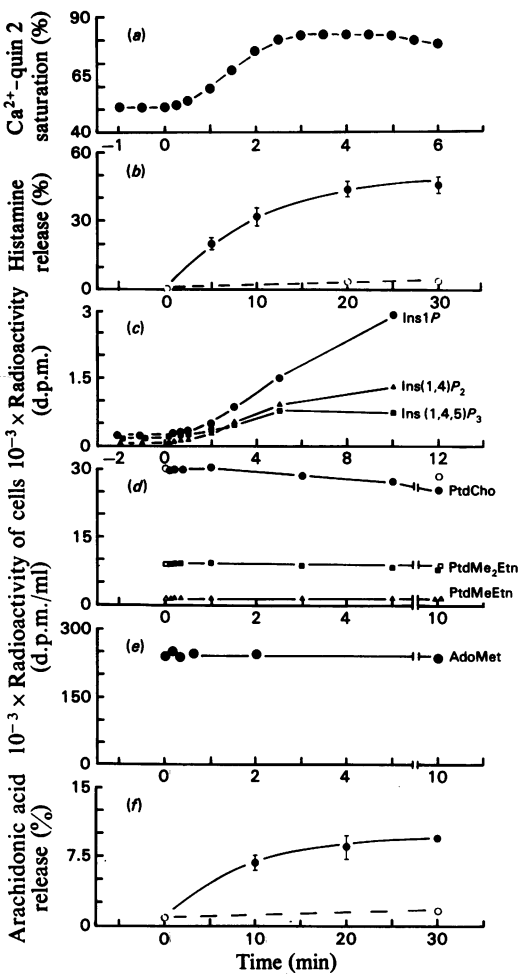

Fig. 1. *Stimulation of 2H3 cells by antigen*
In all experiments, antigen (aggregated ovalbumin, 2.5 μg/ml) was added to IgE-primed cells at zero time. In the experiments described in (*a*), (*d*) and (*e*) the cells were labelled with [*methyl*-^{3}H]methionine (100 μM) as described in the Materials and methods section. Unless otherwise indicated, data points in this and subsequent Figures are single determinations at each time point, but are typical of at least three experiments. (*a*) The effect of antigen on $[Ca]_i$ in quin 2-loaded cells. Saturations of quin 2 by Ca of 50, 60 and 90% are equivalent to 89 nM-, 134 nM- and 800 nM-$[Ca]_i$ respectively. Quin 2 at internal concentrations ≤5 mM did not affect the magnitude or time courses of any of the responses shown in (*b*) to (*f*). (*b*) Histamine release from cells incubated with (●) or without (○) antigen. Data are the means of duplicate determinations. (*c*) Effect of antigen on the amounts of Ins1*P* (●), Ins(1,4)P_2 (▲) and Ins(1,4,5)P_3 (■) in 2H3 cells. (*d*) Amounts of PtdMeEtn (▲, △), PtdMe$_2$Etn (■, □) and PtdCho (●, ○) in cells incubated with (closed symbols) or without (open symbols) antigen. Between 30 and 40% of the chloroform-soluble ^{3}H label was in methylated phospholipids. [2-^{14}C]Ethanolamine (1 μCi/ml; 60 mCi/mmol) was present for the last hour of the incubation with [*methyl*-^{3}H]methionine to provide an internal standard for phospholipid recovery. The recovery of [^{14}C]PtdEtn was 28400 ± 980 d.p.m. (mean ± S.D., $n = 9$). (*e*) Effect of antigen on the amount of [^{3}H]AdoMet in 2H3 cells. (*f*) Release of [1-^{14}C]arachidonic acid from 2H3 cells incubated with (●) or without (○) antigen. Data are means ± S.D. of triplicate determinations and are plotted as a percentage of the total ^{14}C label in the cells at zero time.

shown that histamine release is very closely correlated with the increase in $[Ca]_i$ (Beaven *et al.*, 1984*a*). The increase in $[Ca]_i$ stimulated by antigen varies from 5–10-fold, depending on the cell culture, from a value in unstimulated cells of approx. 100 nM, and we have shown previously (Beaven *et al.*, 1984*b*) that the onset of the Ca signal (Fig. 1*a*) and histamine release (Fig. 1*b*) are closely associated with the degradation of the phosphoinositides (Fig. 1*c*). Crews *et al.* (1980, 1981) reported that antigen stimulated a transient 20–40% increase in phospholipid methylation that was maximal within 3–5 min. The increase was found to be coincident with, and necessary for, $^{45}Ca^{2+}$ influx into the cells (Crews *et al.*, 1981; McGivney *et al.*, 1981*a*). In their experiments, phospholipid methylation was assayed by measuring the incorporation of [^{3}H]methyl groups from [*methyl*-^{3}H]methionine into chloroform-soluble material. We have used (Fig. 2*a*) h.p.l.c. to separate completely the methylated phospholipids from each other and from other ^{3}H-labelled material (Moore *et al.*, 1982). The major proportion (50–70%) of the chloroform-soluble ^{3}H label was not incorporated in methylated phospholipid (Moore *et al.*, 1982); the ^{3}H-labelled material eluted in 2–5 ml in Fig. 2(*a*) is mainly neutral lipid (Zatz *et al.*, 1981; Bougnoux *et al.*, 1983). We could detect no increase in the level of any of the methylated phospholipids within 10 min of antigen addition (Fig. 1*d*) under conditions where there was a large increase in $[Ca]_i$, degradation of the phosphoinositides and release of both histamine and arachidonic acid into the medium (see Fig. 1). Small (5–15%) decreases in PtdCho and PtdMe$_2$Etn were detectable 5–10 min after antigen addition, possibly due to degradation of these phospholipids by phospholipase A_2 (Crews *et al.*, 1980, 1981; McGivney *et al.*, 1981*b*). The recovery of authentic [^{14}C]PtdCho added to each sample was the same (within ± 5%) as the recovery of endogenous [^{14}C]PtdEtn labelled by incubation of the cells with [2-^{14}C]ethanolamine. This implies that PtdEtn was not degraded significantly by phospholipase A_2 and that recovery of this

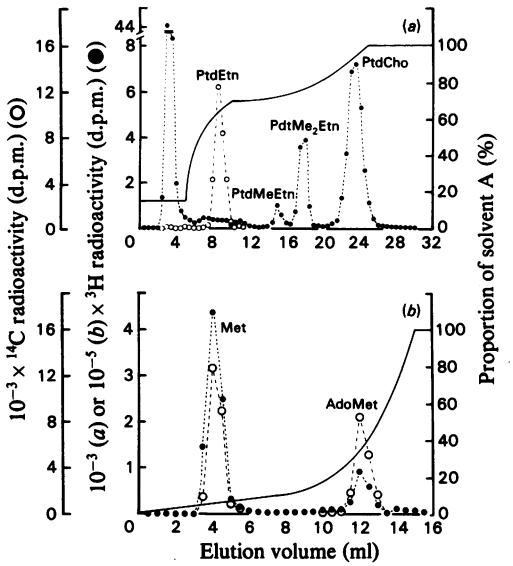

Fig. 2. *H.p.l.c. analysis of [³H]methylated phospholipids and [³H]AdoMet*

(*a*) 2H3 cells were labelled with [*methyl*-³H]methionine (100 μM) and [2-¹⁴C]ethanolamine as described in the legend to Fig. 1(*d*) before determination of their [³H]methylated phospholipid content by the procedures indicated in the Materials and methods section. The elution of ³H (●)- and ¹⁴C (○)-labelled material is shown. (*b*) 2H3 cells were labelled with [*methyl*-³H]methionine (100 μM) before determination of their content of [³H]AdoMet by the procedures indicated in the Materials and methods section. The elution of ³H (●)- and ¹⁴C (○)-labelled material is shown.

phospholipid can be used to normalize the recovery of the methylated phospholipids with respect to the control samples (Moore *et al.*, 1982).

The stimulation of [*methyl*-³H]phospholipid synthesis would only be detected if there was sufficient intracellular [*methyl*-³H]AdoMet as a source of [³H]methyl groups. In several tissues the specific radioactivities of extracellular methionine and intracellular AdoMet have been shown to equilibrate rapidly (Baldessarini & Kopin, 1966; Lombardini & Talalay, 1971; German *et al.*, 1983). We have used cation-exchange h.p.l.c. (Fig. 2*b*) to measure the amount of [³H]AdoMet in 2H3 cells under the same conditions as for the phospholipid-methylation assays. There was at least 6-fold more ³H label in AdoMet than in the methylated phospholipids, but no change in the amount of [³H]AdoMet within 10 min of antigen addition (Fig. 1*e*). It is therefore very unlikely that our failure to detect any increase in [³H]methylated phospholipid was due to insufficient [³H]AdoMet.

Phospholipid methylation in rat mast cells

Mast cells are similar to 2H3 cells in that they release histamine and other mediators of immediate hypersensitivity in response to the cross-linking of receptors on the cell surface by ligands [e.g. concanavalin A (Keller, 1974) or by anti-IgE antibody on cells primed with IgE (Ishizaka & Ishizaka, 1978)]. There is strong indirect evidence that an increase in $[Ca]_i$ is necessary for histamine release (Foreman *et al.*, 1976; Pearce, 1982) and $^{45}Ca^{2+}$ influx into mast cells stimulated by anti-IgE antibody is maximal within 2 min (Ishizaka *et al.*, 1980). However, we have been unable to use quin 2 to measure the time course of the Ca signal directly in these cells because the high intrinsic fluorescence of the granules masks the fluorescence of non-toxic concentrations of the indicator. The prolonged incubation at high cell densities necessary for labelling the phospholipids with [*methyl*-³H]methionine tends to impair the ability of the cells to release histamine in response to concanavalin A. Histamine release from the [*methyl*-³H]methionine-labelled mast cells was complete within 5 min (Fig. 3*a*), but the extent of release was variable (15–45% of the total histamine content of the cells). It was, however, comparable with, or greater than, the 15–20% stimulated release obtained by Hirata *et al.* (1979*b*) and Ishizaka *et al.* (1980) under similar conditions. It has not been possible to incorporate sufficient [³H]inositol into the phosphoinositides for measurements of changes in the degradation of these lipids because of the sensitivity of mast cells to the incubation conditions referred to above. However, increases in PtdIns synthesis within 2–5 min of stimulation of mast cells by agonists are well-documented (Kennerly *et al.*, 1979; Cockcroft & Gomperts, 1979; Schellenberg, 1980; Cockcroft, 1982; Ishizuka *et al.*, 1983).

It has been reported by Hirata *et al.* (1979*b*) that concanavalin A stimulates a 2-fold increase in methylated-phospholipid levels within 2.5 min of addition to rat mast cells, and by Ishizaka *et al.* (1980) that antibodies to the IgE receptor stimulate an 11-fold increase within 10–30s. This increase in [³H]methylated phospholipid was accompanied by an equivalent 12-fold decrease in the [³H]AdoMet content of the cells (Ishizaka *et al.*, 1980). Although we used a range of conditions for loading with [*methyl*-³H]methionine, we could detect no change in the amount of ³H label in chloroform-soluble material, assayed by the procedure of Hirata *et al.* (1979*b*), from 5s–10 min after addition of concanavalin A. Furthermore, there were no changes in the levels of any of the methylated phospholipids or in AdoMet, assayed by h.p.l.c., within 2.5 min of stimulation of the cells with concanavalin A (Figs.

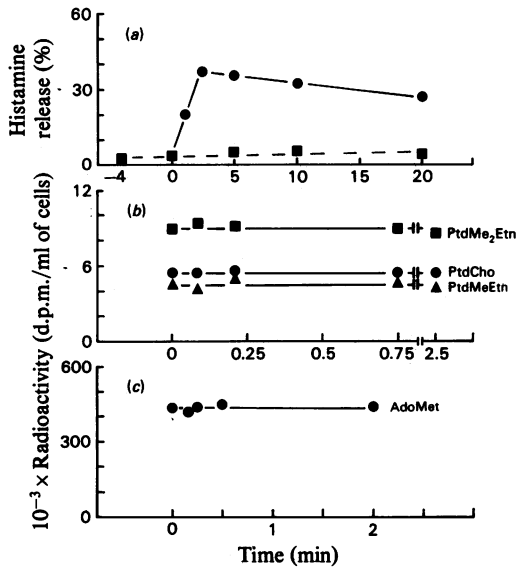

Fig. 3. *Stimulation of rat mast cells by concanavalin A*
Rat mast cells were incubated (5×10^5 cells/ml) with [*methyl*-^{3}H]methionine (1 mCi/ml; 12.5 μM) for 1.5 h at 37°C. After washing, the cells were incubated with 5 μM-lyso-PtdSer for 15 min before addition of concanavalin A (100 μg/ml) at zero time. (*a*) Histamine release from control (■) and concanavalin A-stimulated (●) cells. (*b*) The effect of concanavalin A on the amounts of PtdMeEtn (▲), $PtdMe_2Etn$ (■) and PtdCho (●) in mast cells. [2-^{14}C]Ethanolamine (2.5 μCi/ml) was included with [*methyl*-^{3}H]methionine in the incubation. Between 25 and 30% of the total chloroform-soluble ^{3}H label was in the methylated phospholipids. (*c*) The effect of concanavalin A on the amount of [^{3}H]AdoMet in mast cells.

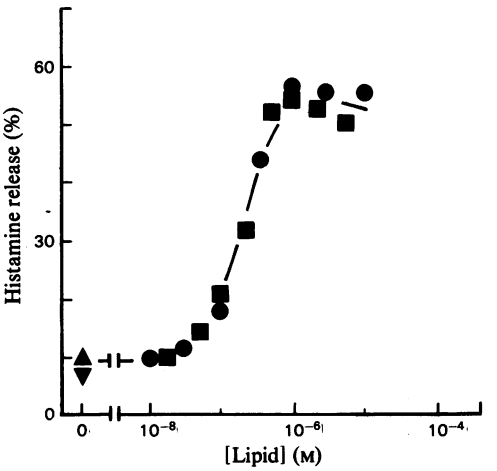

Fig. 4. *Histamine release from rat mast cells stimulated with concanavalin A and lyso-phospholipids*
Mast cells (5×10^4 in 0.5 ml) were incubated as described by Smith *et al.* (1979) for 15 min at 25°C with concanavalin (50 μg/ml) and lyso-PtdSer (●) or lyso-$PtdHO_2CEtOH$ (■) at the concentrations indicated before assay of histamine release. Release from cells incubated with (▲) or without (▼) concanavalin A, but without lyso-lipid is also shown. In the absence of concanavalin A, histamine release was less than 10% at lyso-lipid concentrations of less than 10 μM. When lyso-$PtdHO_2CEtOH$ was used in place of lyso-PtdSer in the experiment described in Fig. 3(*b*), there was no change in the amount of methylated phospholipids after concanavalin A addition.

3*b* and 3*c*). We note that, under the loading conditions for [*methyl*-^{3}H]methionine in this experiment, an 11-fold increase in the incorporation of [^{3}H]methyl groups into the phospholipids would cause a decrease of less than 5% in the [^{3}H]AdoMet content of the cell (see the discussion of assay sensitivity below).

In rat peritoneal mast cells unprimed by IgE the stimulation of secretion by concanavalin A is strongly and specifically potentiated by either exogenous PtdSer or lyso-PtdSer (Sullivan *et al.*, 1975; Martin & Lagunoff, 1979; Smith *et al.*, 1979). Hirata *et al.* (1979*b*) report that the exogenous PtdSer is incorporated into the cell and enzymically decarboxylated to PtdEtn before methylation of the PtdEtn amino group to PtdCho via the transmethylation pathway, and they suggest that this is the molecular mechanism of the action of PtdSer as a co-stimulant with concanavalin A. Lyso-PtdSer is 10^3-fold more potent than PtdSer as a co-stimulant with concanavalin A (Smith *et al.*, 1979), and in Fig. 4 we demonstrate that lyso-$PtdHO_2CEtOH$ is as effective as lyso-PtdSer as a co-stimulant. Lyso-$PtdHO_2CEtOH$ has no amino group and could not therefore be methylated. The conversion of exogenous PtdSer into methylated phospholipids is not therefore necessary for the response.

Phospholipid methylation in mouse thymocytes

Concanavalin A acts as a mitogen on mouse thymocytes (Hesketh *et al.*, 1983*a*; Moore *et al.*, 1983) and stimulates a 1.5–2-fold increase in $[Ca]_i$ that is maximal within 2–3 min (Tsien *et al.*, 1982; Hesketh *et al.*, 1983*a*; Smith *et al.*, 1983) and is sustained for many hours (Hesketh *et al.*, 1983*b*). The time courses of the increases in $[Ca]_i$ and phosphoinositide degradation in response to concanavalin A are shown in Fig. 5(*a*) and 5(*b*). Concanavalin A did not, however, cause changes in the amounts of any of the [^{3}H]methylated phospholipids or in [^{3}H]AdoMet within 5–120 s (Fig. 5*c* and 5*d*). We

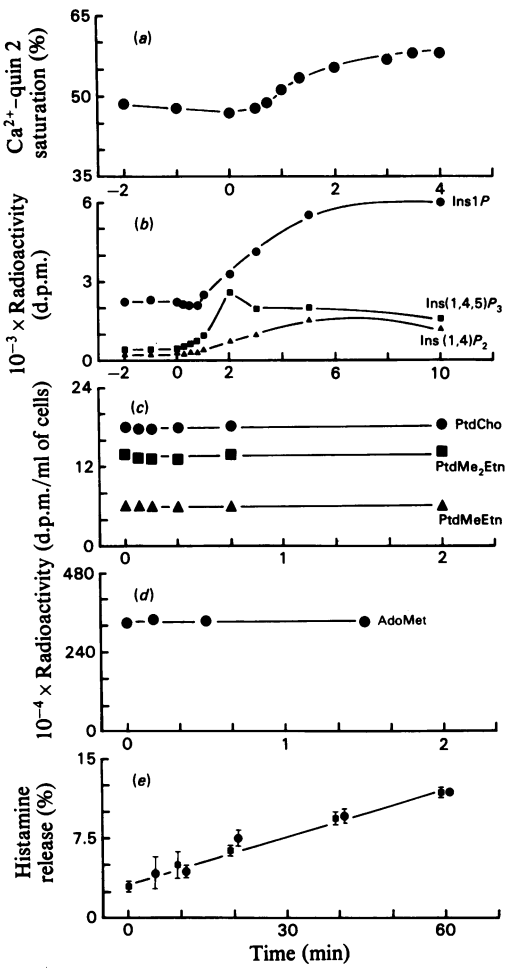

Fig. 5. *Stimulation of mouse thymocytes by concanavalin A*
Except where otherwise indicated, concanavalin A (1 μg/ml) was added to the cells at zero time. In the experiments described in (*a*), (*c*) and (*d*), thymocytes were labelled with [*methyl*-^{3}H]methionine (200 μCi/ml; 2.5 μM) by incubation at 3×10^7 cells/ml for 3 h at 37°C. (*a*) Effect of concanavalin A on $[Ca]_i$ in quin 2-loaded cells. Quin 2 at internal concentrations <0.7 mM did not affect the magnitude or time courses of any of the responses shown in (*b*) (*c*) and (*d*). (*b*) Effect of concanavalin A on the amounts of Ins1*P* (●), Ins(1,4)P_2 (▲) and Ins(1,4,5)P_3 (■) in thymocytes. (*c*) Effect of concanavalin A on the amounts of PtdMeEtn (▲), PtdMe$_2$Etn (■) and PtdCho (●) in thymocytes. Between 40 and 50% of the chloroform-soluble ^{3}H label was in methylated phospholipids. [2-^{14}C]Ethanolamine (0.5 μCi/ml) was present during the incubation to provide an internal standard for phospholipid recovery. The recovery of [^{14}C]PtdEtn was 5937 ± 400 d.p.m. (mean ± S.D., $n = 6$). (*d*) Effect of concanavalin A on the amount of [^{3}H]AdoMet in thymocytes. (*e*) [^{3}H]Arachidonic acid release from thymocytes incubated with (●) or without (■) concanavalin A (2 μg/ml). Data are means ± S.D. of triplicate determinations and are plotted as a percentage of the total ^{3}H label in the cells at zero time.

have previously shown that there were no changes in the [^{3}H]methylated phospholipids of mouse thymocytes or splenocytes or in pig lymph-node lymphocytes within 10 min of concanavalin A addition (Moore *et al.*, 1982), and this has been confirmed independently for the action of phytohaemagglutinin on human lymphocytes (Chien & Ashman, 1983). These observations are inconsistent with those of Hirata *et al.* (1980) who reported that concanavalin A caused a 2-fold increase in the [^{3}H]methylated phospholipid content of mouse splenocytes that was maximal within 10 min of addition and that larger effects were observed in thymocytes. It was also reported that concentrations of concanavalin A that were supramitogenic for the cells did not stimulate increases in phospholipid methylation. However, all of the other early metabolic responses to concanavalin A, including the increase in $[Ca]_i$ (Hesketh *et al.*, 1983*a*), in PtdIns metabolism (Maino *et al.*, 1975; Hesketh *et al.*, 1983*a*) and in the rates of glycolysis (Hesketh *et al.*, 1983*a*) and uridine uptake (A. N. Corps & J. C. Metcalfe, unpublished work) also occur at concanavalin A concentrations supraoptimal for mitogenic stimulation [see McClain & Edelman (1976) and Pozzan *et al.* (1981)].

We could detect no effect of concanavalin A on the rate of arachidonic acid release from mouse thymocytes (Fig. 5*e*), mouse splenocytes or pig lymph-node lymphocytes, although we can confirm the observation of Parker *et al.* (1979) that concanavalin A increases the rate of arachidonic acid release from human blood lymphocytes by 1.2–1.5-fold (results not shown). In contrast, Hirata *et al.* (1980) reported that concanavalin A stimulated the rate of arachidonic acid release from mouse splenocytes by 2–3-fold within 10–60 min and suggested that this was a consequence of enhanced degradation of methylated phospholipids.

Discussion

For each cell system, experiments similar to those described have been performed under a wide range of conditions for loading with [*methyl*-^{3}H]methionine (2–100 μM for 1–16 h) before stimulation, as exemplified by the various protocols in the legends to Figs. 2,3 and 5. No significant changes (< 10%) in phospholipid methylation or in the amount of [^{3}H]AdoMet have been detected in

response to stimulation in any of these experiments, for which the data in Figs. 2,3 and 5 are typical.

[*methyl*-^{3}H]Methionine is taken up into cells from the medium (Moore *et al.*, 1982; German *et al.*, 1983) and is rapidly converted into [^{3}H]AdoMet, the half-time for equilibration being typically 10–15 min (Lombardini & Talalay, 1981; German *et al.*, 1983), which is consistent with the data in Figs. 1(*e*), 3(*c*) and 5(*d*). By loading with [*methyl*-^{3}H]methionine for relatively short periods (approx. 1 h) the incorporation of ^{3}H label into AdoMet is almost maximal, whereas the incorporation of ^{3}H label into the methylated phospholipids, which occurs at an approximately constant rate for several hours in unstimulated cells, is minimal. These conditions are consequently the most sensitive for assaying any changes in [^{3}H]methylated phospholipids that might occur on stimulation of the cells. The data in Table 1 show the amounts of [^{3}H]methylated phospholipids in unstimulated cells loaded for 1 h with [*methyl*-^{3}H]methionine concentrations from 2–100 μM. The amounts are calculated by using the rates of equilibration of [*methyl*-^{3}H]methionine with the AdoMet pool [similar to those reported by Lombardini & Talalay (1971) and by German *et al.* (1983)] and assuming that the specific radioactivity of [^{3}H]-AdoMet is determined mainly by the specific radioactivity of the extracellular [*methyl*-^{3}H]methionine (Lombardini & Talalay, 1971). Consistent with this assumption, the calculated amounts of [^{3}H]methylated phospholipids varied by only 2–3-fold in different experiments when the [*methyl*-^{3}H]methionine concentration was varied by 50-fold (2-100 μM). The highest calculated amounts of [^{3}H]methylated phospholipids were obtained at [*methyl*-^{3}H]methionine concentrations of 10–100 μM, and these values were used to set the limit to any change in methylated phospholipids after stimulation shown in Table 1. The limit is estimated as less than 10% of the corresponding amount of [^{3}H]methylated phospholipid in the cells immediately before stimulation and is based on the consistency of recovery of phospholipid in the samples using [^{14}C]PtdEtn as an internal standard (Moore *et al.*, 1982), which varied by less than $\pm$5% (see the legend to Fig. 1*d*). From the

Table 1. *Limits to the increase in [^{3}H]methylated phospholipids in response to stimulation*

Cells were incubated with concentrations of [*methyl*-^{3}H]methionine from 10 to 100 μM for 1 h at 37°C (see the Materials and methods section) before determination of [^{3}H]methylated phospholipids ([a]) as described in Fig. 2(*a*). The amounts of [^{3}H]methylated phospholipids were calculated as described in the text and represent the highest values obtained to avoid underestimating the limit to any change in [^{3}H]methylated phospholipid. Any effect of successive ^{3}H-labelling of $PtdMe_2Etn$ and PtdCho from the [^{3}H]AdoMet pool on the calculated amounts of these lipids has been ignored for the same reason. The average number of receptors per 2H3 cell ([b]) was taken from Barsumian *et al.* (1981). A range of values from 1.4 to 11 $\times 10^5$ was obtained over a culture period of 6 months. For mast cells, the number of receptors ([c]) is taken from Coutts *et al.* (1980), although a higher value of 3×10^5 has been reported (Conrad *et al.*, 1975). The number of concanavalin A molecules bound per thymocyte ([d]) was measured as 2.4×10^5 using ^{125}I-concanavalin A at the optimal mitogenic dose of 1 μg/ml. Values between 1.5 and 2.5×10^5 have been reported elsewhere (Betel & van den Berg, 1972; Resch *et al.*, 1978). The limit to any increase in [^{3}H]methylated phospholipids ([e]) is estimated as 10% of the [^{3}H]methylated phospholipids before stimulation as described in the text. Data calculated from reported increases in the incorporation of [^{3}H]methyl group into chloroform-soluble material ([f]) were approx. 6 pmol/2×10^6 2H3 cells (Fig. 1*a* of Crews *et al.*, 1981), 0.4 pmol/3×10^5 mast cells (Fig. 1 of Hirata *et al.*, 1979*b*) and 0.6 pmol per 1×10^6 lymphocytes (Fig. 1*a* of Hirata *et al.*, 1980). The value marked ([g]) was calculated from the data given for mouse spleen lymphocytes (Fig. 1*a* of Hirata *et al.*, 1980); larger (but unquantified) increases in phospholipid methylation were reported for thymocytes.

Cell type	Phospholipid	[a]Calculated [^{3}H]methylated phospholipids (amol/cell)	Receptors (no./cell)	[e]Limit to change in number of [^{3}H]methylated phospholipids incorporated per receptor	[f]Reported increase in number of [^{3}H]methyl groups incorporated per receptor
2H3	PtdMeEtn	0.5	5×10^5[b]	0.06	—
	$PtdMe_2Etn$	1.5		0.18	—
	PtdCho	2.2		0.26	—
	Total	4.2		0.50	3.6
Mast	PtdMeEtn	0.14	0.7×10^5[c]	0.12	—
	$PtdMe_2Etn$	0.30		0.26	—
	PtdCho	0.26		0.22	—
	Total	0.70		0.60	11
Thymocytes	PtdMeEtn	0.025	2.4×10^5[d]	0.006	—
	$PtdMe_2Etn$	0.059		0.015	—
	PtdCho	0.046		0.012	—
	Total	0.130		0.033	1.5[g]

data in Table 1, the limit to any increase in total [^{3}H]methylated phospholipid is calculated as less than one molecule per receptor in each cell system.

In the data given previously for the same cell systems it was assumed that all of the increase in the ^{3}H label in chloroform-soluble material extracted from the cells after stimulation was [^{3}H]methylated phospholipid [see Vance & de Kruijff (1980) and Axelrod & Hirata (1980)]. On that assumption, the total number of phospholipid molecules methylated per receptor after stimulation has been calculated directly from the data reported, using the same values for the number of receptors per cell as for our data in Table 1. The stimulated changes are greater by 5–50-fold than the limits set in the present experiments. The comparisons summarized in Table 1 therefore indicate a major discrepancy between the present data and those reported previously. We cannot determine the cause of the increase in the amount of ^{3}H label in chloroform-soluble material in previous studies, since we do not observe the response, measured either as authentic methylated phospholipid or as chloroform-soluble material. We conclude that changes in phospholipid methylation are not necessary for the transduction of Ca signals in these cell systems and that the hypothesis proposed by Hirata, Axelrod and colleagues for the generation of calcium signals requires critical re-evaluation.

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