Biochem. J. (1985) **227**, 971–979
Printed in Great Britain

Breakdown of phosphatidylinositol 4,5-bisphosphate in a T-cell leukaemia line stimulated by phytohaemagglutinin is not dependent on Ca^{2+} mobilization

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(*Received 31 December 1984; accepted 10 January 1985*)

Addition of phytohaemagglutinin (PHA) to the [^{32}P]P_i-prelabelled JURKAT cells, a human T-cell leukaemia line, resulted in a decrease of [^{32}P]phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to about 35% of the control value. The decrease was almost complete within 30s after the PHA addition. This decrease was followed by an increase in the ^{32}P-labelling of phosphatidic acid (maximally 2.8-fold at 2min). The stimulation of *myo*-[2-^{3}H]inositol-prelabelled JURKAT cells by PHA induced an accumulation of [2-^{3}H]inositol trisphosphate in the presence of 5mM-LiCl. The result indicates hydrolysis of PtdIns (4,5)P_2 by a phospholipase C. The PHA stimulation of JURKAT cells induced about 6-fold increase in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, which was reported by Quin-2, a fluorescent Ca^{2+} indicator. Studies with partially Ca^{2+}-depleted JURKAT cells, with the Ca^{2+} ionophore A23187, and with 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate indicate that the breakdown of PtdIns(4,5)P_2 is not mediated through changes of $[Ca^{2+}]_i$. These results therefore indicate that the PHA-induced breakdown of PtdIns(4,5)P_2 in JURKAT cells is not dependent on the Ca^{2+} mobilization.

Activation of one family of cell-surface receptors for intercellular messages, including hormones, neurotransmitters and antigens, brings about a rise in $[Ca^{2+}]_i$ in stimulated cells (Ca^{2+} mobilization). Stimulus-induced Ca^{2+} mobilization in a wide variety of cells is associated with enhanced breakdown of inositol lipids; and this breakdown is usually followed by a compensatory resynthesis, with phosphatidic acid as a key intermediate in this process. It has been proposed that the receptor-stimulated breakdown of inositol lipids functions in the translation of intercellular messages into a rise in the $[Ca^{2+}]_i$ (for reviews, see Michell, 1975, 1979; Michell & Kirk, 1981; Berridge, 1984).

Lymphocytes stimulated by polyclonal T-cell mitogens, e.g. ConA and PHA, are known to exhibit the receptor-stimulated inositol lipid metabolism (Fisher & Mueller, 1968; Masuzawa *et al.*, 1973; Maino *et al.*, 1975; Hasegawa-Sasaki & Sasaki, 1981, 1982). The T-cell mitogens initiate blast transformation and mitosis in small resting lymphocytes. The T-cell proliferative response results from the interaction of three or more distinct types of cells, such as interleukin-2 producer precursor and interleukin-2 responder precursor. The plant lectins presumably have an effect on each of these cells either in the induction of the synthesis and secretion of lymphokines or in the expression of lymphokine receptors (Larsson & Coutinho, 1979; Robb *et al.*, 1981; Smith & Ruscetti, 1981; Palacios, 1982). The lymphocytes prepared from lymph nodes and peripheral blood consist of various cell types, and it is possible that only a small fraction of the lymphocyte population participates in the stimulated inositol lipid metabolism previously studied. In order to understand the early processes in T-lymphocytes which are set in motion by the lectins, we studied the effect of PHA on the inositol lipid metabolism of human T-lymphoid cell lines. A human T-lymphoblastoid cell line designated CCRF-CEM was found to respond to PHA with rapid hydrolysis of inositol lipids, whereas the other four human T-lymphoid cell lines screened, PEER, MOLT-4f, RPMI-8402 and SKW-3, were negative in this response to PHA (Hasegawa-Sasaki & Sasaki, 1983). The cell-biological effect of PHA on CCRF-CEM cells is not known. In the present paper, a human T-cell

Abbreviations used: ConA, concanavalin A; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PHA, phytohaemagglutinin; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; TMB-8, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate.

leukaemia line designated JURKAT-FHCRC was used to study the early processes in T-lymphocytes induced by T-cell mitogens. JURKAT cells produce relatively high quantities of interleukin-2 after a 6–24h stimulation with either PHA or ConA (Gillis & Watson, 1980). The stimulus-induced Ca^{2+}-mobilization and the receptor-stimulated breakdown of PtdIns(4,5)P_2 were measured in parallel in order to learn the interrelation of these two cellular events.

Materials and methods

Cell culture

JURKAT-FHCRC cells were kindly supplied by Dr. James Watson (University of Auckland). The cells were cultured in RPMI 1640 medium supplemented with 6.7% (v/v) heat-inactivated (56°C for 30min) foetal-calf serum, 50 units of pencillin/ml, 50 μg of streptomycin/ml, and 300 μg of L-glutamine/ml.

Phospholipid analysis of [^{32}P]P_i-prelabelled JURKAT cells stimulated by PHA

Cultured JURKAT cells were suspended at 10^7 cells/ml in a medium containing 50 μCi of carrier-free [^{32}P]P_i (Japan Atomic Energy Research Institute)/ml. The medium was a mixture (84.6:15.4, v/v) of P_i-free Tris-buffered saline (Hasegawa-Sasaki & Sasaki, 1981) and an iso-osmotic solution of substrates (Krebs, 1950). After labelling at 37°C for 1h, cells were washed twice with the medium and suspended at 10^7 cells/ml in P_i-free Tris-buffered saline. Portions (0.25ml) of the cell suspension were transferred to warmed test tubes containing either 5 μl (12.5 μg of protein) of PHA (leucoagglutinin; Sigma) or 5 μl of 0.9% NaCl and then incubated at 37°C for the indicated period of time. The incubation was stopped by addition of 1ml of chloroform/methanol/conc. HCl (100:200:1, by vol.) The mixture was partitioned into two phases by addition of 0.33ml each of chloroform and 0.1M-HCl. Insoluble residues were re-extracted with 2ml of chloroform/methanol/conc. HCl (200:100:1, by vol.). The second extract was partitioned into two phases. The first and second lower phases were combined and washed three times with new upper phase. PtdIns(4,5)P_2, PtdIns4P, PtdIns, phosphatidic acid, and other phospholipids in the extracts were separated by two-dimensional t.l.c. on silica gel H plates impregnated with 1% potassium oxalate/2mM-EDTA. Chloroform/methanol/aq. 4M-NH_3 (9:7:2, by vol.) and butanol/acetic acid/water (6:1:1, by vol.) were used as developing solvents in the first and second dimensions respectively. Phospholipids separated on t.l.c. plates were located by autoradiography. Each located area was scraped into a vial, the sample was mixed with 5ml of ACS II (Amersham) and 0.3ml of water, and the radioactivity was determined with a Beckman LS-9000 liquid-scintillation spectrometer.

Analyses of breakdown of Ptd[^{3}H]Ins(4,5)P_2 and accumulation of [^{3}H]inositol phosphates in PHA-stimulated JURKAT cells prelabelled with myo-[2-^{3}H]inositol

JURKAT cells were collected from cultures by centrifugation (30*g* for 10min). The cells were suspended at 3×10^5 cells/ml in a modified RPMI 1640 medium, which contained *myo*-inositol at 0.3mg/l (one-117th of the regular concentration), supplemented with 7% dialysed and heat-inactivated foetal-calf serum, 50 units of penicillin/ml and 50 μg of streptomycin/ml. To this cell suspension, *myo*-[2-^{3}H]inositol (15.8Ci/mmol; New England Nuclear) was added at 0.5 μCi/ml. After labelling for 1 day, the cells were washed twice with Dulbecco's phosphate-buffered saline containing 1mg of glucose/ml and 10mM-*myo*-inositol, in which a portion (5mM) of NaCl had been replaced with 5mM-LiCl. The cells were then suspended at 5×10^6 cells/ml in the saline containing glucose and LiCl, but no *myo*-inositol. Portions (0.25ml) of the cell suspension were transferred to warmed test tubes containing either 12.5 μg of PHA in 10 μl of 0.9% NaCl or 10 μl of 0.9% NaCl and incubated at 37°C for the indicated time. The incubation was terminated by adding 0.5ml of ice-cold 10% (w/v) trichloroacetic acid. The precipitate was collected by centrifugation (500*g* for 15min). Trichloroacetic acid was removed from the supernatant by five extractions with diethyl ether, and the extract was neutralized with 0.5ml of 6.25mM-sodium tetraborate.

The trichloroacetic acid-soluble [^{3}H]inositol phosphates were separated from each other by using 1ml of Dowex-1 (8% cross-linkage; formate form) packed in Pasteur pipettes by the method of Berridge *et al.* (1983) and Berridge (1983). [^{3}H]Inositol phosphates were eluted from the column sequentially by using 10ml each of the eluting solutions for inositol, glycerophosphoinositol, Ins$P$, InsP_2 and InsP_3 described by Berridge *et al.* (1983). A 5ml portion of each fraction was mixed with 10ml of ACS II (Amersham) and counted for radioactivity with a Beckman LS-9000 liquid-scintillation spectrometer. Separation of individual [^{3}H]inositol phosphates by this procedure was confirmed by collecting the column effluent in 1ml fractions.

[^{3}H]Inositol lipids in the trichloroacetic acid precipitate were extracted with chloroform/methanol/conc. HCl (200:100:1, by vol.), and separated after addition of PtdIns, PtdIns4P

and PtdIns(4,5)P_2 as carriers by t.l.c. on silica gel H plates impregnated with 1% potassium oxalate/2mM-EDTA. The plates were developed in chloroform/methanol/aq. 4M-NH_3 (9:7:2, by vol.). Inositol lipids separated on the t.l.c. plates were located by iodine vapour. Each located area was scraped into a vial, and the radioactivity was determined.

Results and discussion

Breakdown of PtdIns(4,5)P_2 and accumulation of phosphatidic acid induced by PHA

JURKAT cells were labelled for 1h with [^{32}P]P$_i$ in P$_i$-free Tris-buffered saline. Under the labelling conditions, ^{32}P radioactivity in PtdIns(4,5)P_2 and PtdIns4P reached steady values after labelling for 1h; ^{32}P radioactivity in phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine continued to increase during the labelling for 90min. The labelled cells were incubated in the presence and absence of PHA for various periods of time. Phospholipids were extracted, and separated by two-dimensional t.l.c. Addition of 50μl of PHA/ml to the prelabelled cells induced a decrease of [^{32}P]PtdIns(4,5)P_2 to about 35% of the control value (Fig. 1*b*). The decrease was evident as early as 10s after the PHA addition and was almost complete within 30s. The radioactivity of [^{32}P]PtdIns(4,5)P_2 stayed at the low value at least for 5min after the PHA addition. A decrease of [^{32}P]PtdIns4P occurred with a similar time course to that of [^{32}P]PtdIns(4,5)P_2 (results not shown); however, the ^{32}P radioactivity of PtdIns4P in the prelabelled cells was 15–35% of that of PtdIns(4,5)P_2. These decreases in [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P in the PHA-treated cells were followed by an increase in the ^{32}P labelling of phosphatidic acid (Fig. 1*c*), which was not detectable until after a 10s lag period. The radioactivity of [^{32}P]phosphatidic acid reached 280% of the control value 2min after the PHA addition. A gradual increase in the ^{32}P labelling of PtdIns was found at 3–5min after the PHA addition (Fig. 1*a*). No significant change was induced by PHA in other ^{32}P-labelled phospholipids. Autoradiograms of [^{32}P]phospholipids separated by t.l.c. are shown in Fig. 2. The PHA-induced changes in [^{32}P]phospholipids described above are obvious in the autoradiograms.

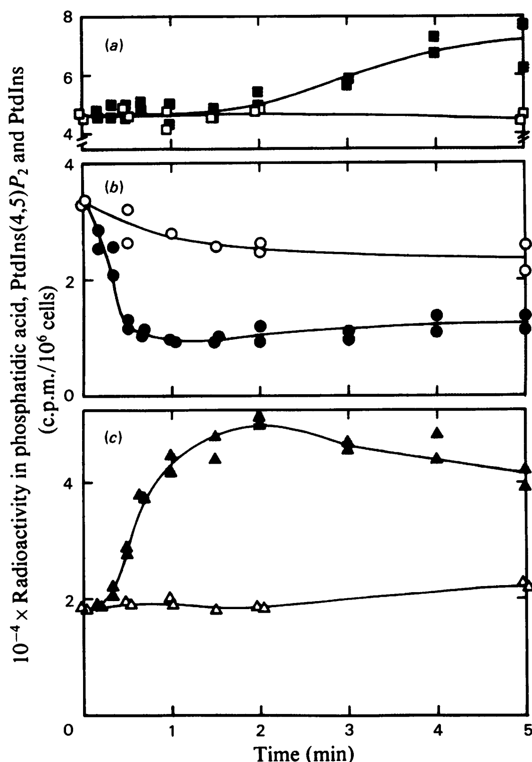

Fig. 1. *Time courses of PHA-induced breakdown of [^{32}P]PtdIns(4,5)P_2 and PHA-induced accumulation of [^{32}P]phosphatidic acid and [^{32}P]PtdIns in JURKAT cells prelabelled with [^{32}P]P$_i$*

JURKAT cells were cultured and labelled with [^{32}P]P$_i$ at 37°C for 1h (see the Materials and methods section). The labelled cells were washed twice and suspended at 10^7 cells/ml in P$_i$-free Tris-buffered saline. Portions (0.25ml) of the cell suspension were incubated at 37°C for the indicated time either in the absence (□, ○, △) or in the presence of 12.5μg of PHA (■, ●, ▲). Phospholipids were extracted and separated by two-dimensional t.l.c. as described in the Materials and methods section. Results obtained in an experiment performed in duplicate are shown. (*a*) ■ and □, PtdIns; (*b*) ● and ○, PtdIns(4,5)P_2; (*c*) ▲ and △, phosphatidic acid.

The breakdown of PtdIns(4,5)P_2 in PHA-stimulated JURKAT cells was studied by the use of cells doubly labelled with [2-^{3}H]glycerol and [^{32}P]P$_i$ in order to find whether the decrease in [^{32}P]PtdInds(4,5)P_2 shown in Fig. 1 is equivalent to the decrease in the chemical quantity of PtdIns(4,5)P_2. For this purpose, growing JURKAT cells were labelled with [2-^{3}H]glycerol for 2 days in the standard culture medium and then the cells were labelled with [^{32}P]P$_i$ for 1h. Stimulation of the doubly labelled cells with PHA for 1 and 2min caused breakdown of PtdIns(4,5)P_2 and PtdIns4P and accumulation of phosphatidic acid (Table 1). The extent of the breakdown and accumulation is expressed in Table 1 by the ratio of the ^{3}H or ^{32}P radioactivity in each lipid found in PHA-stimulated cells to that in control cells. In the breakdown of PtdIns(4,5)P_2 and PtdIns4P of the doubly labelled cells, a good correlation was found between ^{3}H label and ^{32}P label (Table 1). Since growth of JURKAT cells in a culture containing

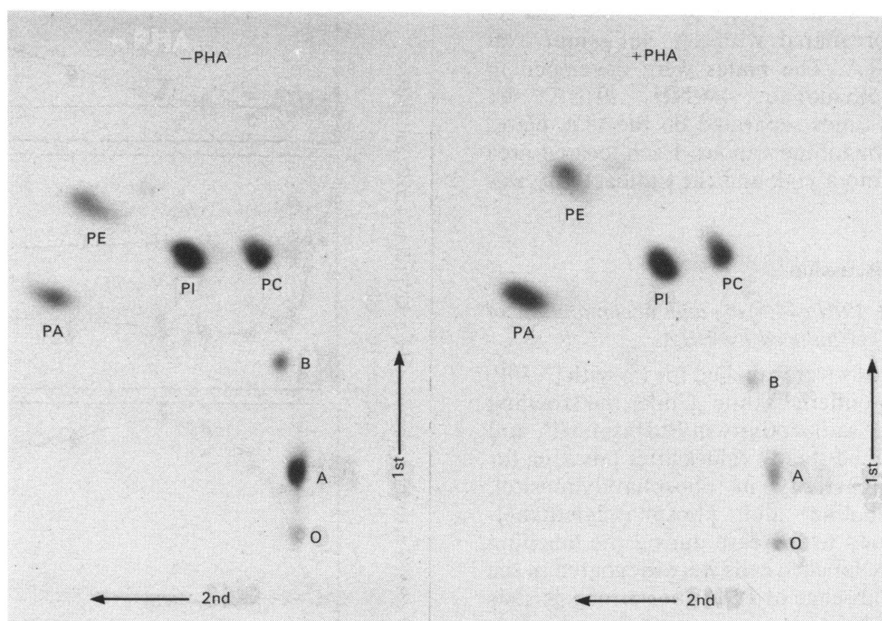

Fig. 2. *Two-dimensional t.l.c separation of [^{32}P]phospholipids of JURKAT cells*
Samples (0.25 ml) of JURKAT cells (2.5×10^6 cells) labelled with ^{32}P were incubated at 37°C for 2 min either in the absence ('−PHA') or in the presence of 12.5 μg of PHA ('+PHA'). The lipids were extracted and separated as described in the Materials and methods section. The [^{32}P]phospholipids were located by autoradiography: A, PtdIns(4,5)P_2; B, PtdIns4P; PI, PtdIns; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; O, origin.

Table 1. *Breakdown of PtdIns (4,5)P_2 and PtdIns4P and accumulation of phosphatidic acid in PHA-stimulated JURKAT cells doubly prelabelled with [2-^{3}H]glycerol and [^{32}P]P_i*
Growing JURKAT cells were labelled with 2.5 μCi of [2-^{3}H]glycerol (10 Ci/mmol; New England Nuclear)/ml in the standard culture medium for 2 days. The ^{3}H-labelled cells were washed once and suspended at 1.2×10^7 cells/ml in the [^{32}P]P_i-labelling medium (see the Materials and methods section). After ^{32}P labelling at 37°C for 1 h, cells were washed once and suspended at 5×10^6 cells/ml in P_i-free Tris-buffered saline containing 1 mg of glucose/ml. Portions (0.5 ml) of the cell suspension were transferred to warmed test tubes containing either 10 μl (25 μg of protein) of PHA or 10 μl of 0.9% NaCl and then incubated at 37°C for 1 or 2 min as indicated. Lipids were extracted and separated by two-dimensional t.l.c. as described in the Materials and methods section. Results are expressed as means ± S.E.M. for two experiments. Abbreviations: PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine.

		Radioactivity in lipids (d.p.m./1.25×10^6 cells)					
		Incubated for 1 min			Incubated for 2 min		
Phospholipid	Label	(*a*) Control	(*b*) PHA added	Ratio *b/a*	(*a*) Control	(*b*) PHA added	Ratio *b/a*
PtdIns(4,5)P_2	^{3}H	1305 ± 154	711 ± 164	0.54	1428 ± 17	950 ± 31	0.67
	^{32}P	26453 ± 3527	12827 ± 1689	0.48	27797 ± 323	15585 ± 121	0.56
PtdIns4P	^{3}H	638 ± 84	447 ± 47	0.70	653 ± 29	583 ± 37	0.89
	^{32}P	9998 ± 948	6365 ± 79	0.64	9377 ± 35	7905 ± 327	0.84
PtdOH	^{3}H	795 ± 58	1876 ± 5	2.36	793 ± 31	2343 ± 67	2.95
	^{32}P	17860 ± 524	31441 ± 260	1.76	17450 ± 456	37509 ± 264	2.15
PtdIns	^{3}H	58425 ± 788	59676 ± 58	1.02	60036 ± 665	58396 ± 664	0.97
	^{32}P	51854 ± 2152	52849 ± 83	1.02	53351 ± 302	52167 ± 457	0.98
PtdCho	^{3}H	181904 ± 1599	192072 ± 1132	1.06	194769 ± 191	195177 ± 1524	1.00
	^{32}P	9913 ± 46	10175 ± 89	1.03	10758 ± 308	10509 ± 129	0.98

[2-^{3}H]glycerol for 2 days evenly labels cellular lipids with [2-^{3}H]glycerol, the breakdown observed in ^{3}H-labelled lipids can be taken as changes in chemical quantity. The results shown in Table 1 therefore indicate that the breakdown of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P shown in Fig. 1 are closely related with the changes in chemical quantities of these lipids. The accumulation of phosphatidic acid induced by PHA appears much greater when measured with [2-^{3}H]glycerol than with [^{32}P]P_i (Table 1). The breakdown of PtdIns(4,5)P_2 is catalysed by a phospholipase C, as described below. Since the accumulated phosphatidic acid is most probably formed by the phosphorylation of diacylglycerol produced by this hydrolysis (Sasaki & Hasegawa-Sasaki, 1985), the ^{32}P label of the phosphatidic acid is not derived from [^{32}P]PtdIns(4,5)P_2. Therefore one cannot expect a correlation between ^{32}P label and ^{3}H label in phosphatidic acid.

The breakdown of PtdIns(4,5)P_2 described above may possibly be the earliest event found so far in the phytomitogen-stimulated T-lymphocytes, and therefore may represent the receptor-linked event. Stimulation of [^{32}P]P_i incorporation into PtdIns (Fisher & Mueller, 1968; Masuzawa *et al.*, 1973; Maino *et al.*, 1975), decrease of PtdIns (Hasegawa-Sasaki & Sasaki, 1982), and activation of lysolecithin acyltransferase (EC 2.3.1.23) (Ferber *et al.*, 1976) can all be regarded as slower responses to phytomitogens than the breakdown of PtdIns(4,5)P_2. The PHA-stimulated JURKAT cells add one good example showing the receptor-stimulated breakdown of PtdIns(4,5)P_2, which has been found in smooth muscles (Abdel-Latif *et al.*, 1977), hepatocytes (Kirk *et al.*, 1981; Creba *et al.*, 1983; Thomas *et al.*, 1983; Rhodes *et al.*, 1983), platelets (Agranoff *et al.*, 1983; Billah & Lapetina, 1982*b*, 1983; Vickers *et al.*, 1982), parotid acinar cells (Berridge *et al.*, 1983; Downes & Wusteman, 1983; Weiss *et al.*, 1982), exocrine pancreas (Putney *et al.*, 1983), insect salivary glands (Berridge, 1983; Berridge *et al.*, 1983) and GH_3 pituitary cells (Martin, 1983; Rebecchi & Gershengorn, 1983).

Evidence for phospholipase C-catalysed hydrolysis of PtdIns(4,5)P_2

Formation of InsP_3 by the hydrolysis of PtdIns(4,5)P_2 is shown in Fig. 3(*c*) by the use of *myo*-[2-^{3}H]inositol-prelabelled JURKAT cells stimulated by PHA. In this experiment, stimulation with PHA was performed in the presence of 5mM-LiCl to inhibit inositol-1-phosphatase (Hallcher & Sherman, 1980). Addition of 50μg of PHA/ml to the prelabelled cells induced a decrease of Ptd[^{3}H]Ins(4,5)P_2 to about 75% of the control value at 2min after the addition (Fig. 3*d*). This percentage decrease of PtdIns(4,5)P_2 was much smaller than that found in [^{32}P]P_i-prelabelled cells and [2-^{3}H]glycerol-prelabelled cells. The time course of the breakdown in Fig. 3 is also slower than that shown in Fig. 1. It seems possible that the culture of JURKAT cells in a medium with low concentration of *myo*-inositol affected the response of the cells to PHA. In parallel with the decrease in

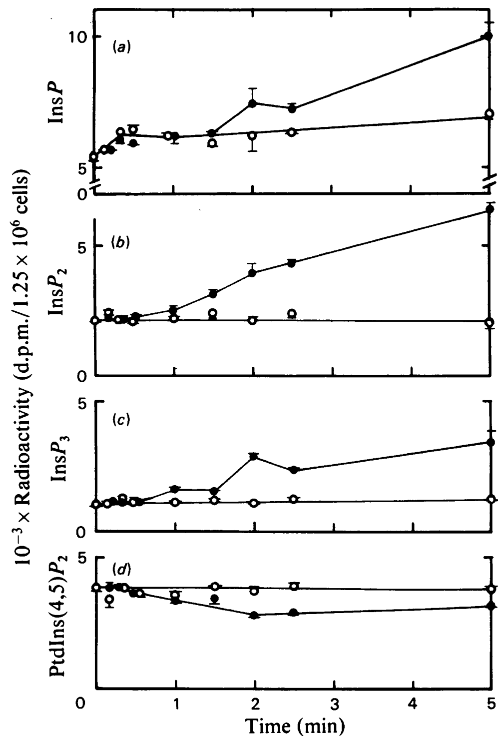

Fig. 3. *Time course of PHA-induced accumulation of [^{3}H]inositol phosphates and PHA-induced breakdown of Ptd[^{3}H]Ins(4,5)P_2 in Li^+-treated JURKAT cells prelabelled with myo-[2-^{3}H]inositol*

JURKAT cells were cultured and labelled with *myo*-[2-^{3}H]inositol for 1 day (see the Materials and methods section). The labelled cells were washed twice and suspended at 5×10^6 cells/ml in Dulbecco's phosphate-buffered saline containing 1mg of glucose/ml and 5mM-LiCl. Portions (0.25ml) of the cell suspension were incubated at 37°C for the indicated time either in the absence (○) or in the presence of 12.5μg of PHA (●). The incubations were stopped by the addition of 0.5ml of cold 10% (w/v) trichloroacetic acid. Acid-soluble metabolites were separated from each other by anion-exchange chromatography (see the Materials and methods section). [^{3}H]Inositol lipids in the acid precipitate were extracted and separated by t.l.c. as described in the Materials and methods section. Results of two experiments performed in duplicate are shown as means ± S.E.M. (*a*), [^{3}H]inositol monophosphate ([^{3}H]Ins$P$); (*b*), [^{3}H]InsP_2; (*c*), [^{3}H]InsP_3; (*d*), Ptd[^{3}H]Ins(4,5)P_2.

Ptd[^{3}H]Ins(4,5)P_2, an increase in [^{3}H]InsP_3 was found (Fig. 3c). The results indicate that PtdIns(4,5)P_2 is hydrolysed by a phospholipase C. At 2min after PHA addition and thereafter, the increase in ^{3}H label in InsP_3 was larger than the decrease in the ^{3}H label in PtdIns(4,5)P_2. These results suggest active resynthesis of Ptd[^{3}H]-Ins(4,5)P_2 accompanying the PHA-induced hydrolysis. An accumulation of [^{3}H]InsP_2, which was larger than that of [^{3}H]InsP_3, was found in the PHA-stimulated JURKAT cells (Fig. 3b).

Effect of PHA on [Ca^{2+}]$_i$ in JURKAT cells loaded with Quin-2

A hypothesis has been advocated by Michell on the role of inositol lipid hydrolysis in receptor-controlled Ca^{2+} mobilization in cells (Michell, 1975, 1979; Michell & Kirk, 1981). The hypothesis predicts that the enhanced breakdown of inositol lipids should not be dependent on the elevation of [Ca^{2+}]$_i$. The interrelation between the receptor-stimulated depletion of PtdIns(4,5)P_2 and the Ca^{2+} mobilization was examined in JURKAT cells. The Ca^{2+}-selective fluorescent indicator Quin-2 (Tsien *et al.*, 1982*a*,*b*) was used to monitor continuously [Ca^{2+}]$_i$ in JURKAT cells. In accordance with the reports by Tsien *et al.* (1982*a*,*b*) and Hesketh *et al.* (1983) on lymphocytes isolated from lymph nodes, spleen and thymus, PHA induced a rise in the [Ca^{2+}]$_i$ in JURKAT cells from 93 to 580nM (Fig. 4*a*). A lag period of about 15s was observed in the PHA-induced increase in the [Ca^{2+}]$_i$. Stimulation by PHA in either Ca^{2+}-free medium or Ca^{2+}-free medium containing 2mM-EGTA markedly decreased the increase in [Ca^{2+}]$_i$ (Figs. 4*b* and 4*c*). Addition of TMB-8 (0.8mM) to the medium almost blocked the PHA-induced increase in [Ca^{2+}]$_i$; in the presence of 0.8mM-TMB-8, PHA induced an increase in [Ca^{2+}]$_i$ from 60 to 70nM in Ca^{2+}-free medium (Fig. 4*d*), and an

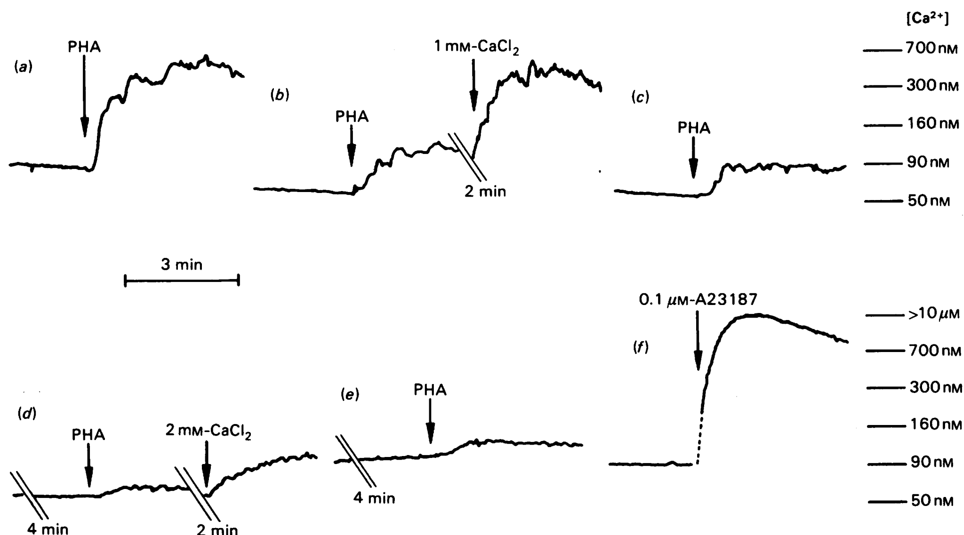

Fig. 4. *Resting [Ca^{2+}]$_i$ of JURKAT cells and responses to A23187 and PHA measured by the use of Quin-2 fluorescence*
Quin-2 tetra-acetoxymethyl ester (Dojin Chemicals, Kumamoto, Japan) was added at 50μM to a JURKAT-cell suspension in RPMI 1640 medium containing 3×10^{7} cells/ml and incubated for 20min at 37°C. The suspension was then diluted 10-fold with RPMI 1640 medium and incubated for a further 60min. The resulting Quin-2 loading was 4.9nmol of dye/5.4×10^{6} cells. After loading, the cells were washed once with Ca^{2+}- and P_i-free Tris-buffered saline (medium A) containing 2mM-EGTA. The cells were resuspended in medium A at 2.7×10^{6} cells/ml and kept at room temperature. For measurement of fluorescence, 2ml of this stock suspension was centrifuged for 10min at 50*g*. The cells were resuspended in 2ml of medium A containing 1mM-$CaCl_2$ (*a*, *f*), medium A (*b*), medium A containing 2mM-EGTA (*c*), medium A containing 0.8mM-TMB-8 (Aldrich Chemical Co.) (*d*), or medium A containing 1mM-$CaCl_2$ and 0.8mM-TMB-8 (*e*). The resuspended cells were transferred to the cuvette. Quin-2 fluorescences were recorded at 37°C with a Hitachi 650-10S fluorescence spectrophotometer fitted with a magnetic stirrer and a thermostatically controlled cell holder. Excitation and emission wavelengths were 339 and 492nm, with 4 and 10nm slits respectively. PHA was added to the cuvette by injection of 40μl of 2.5mg/ml solution through polyethylene tubing. A23187 (Calbiochem-Behring) was added from dimethyl sulphoxide stock solution (100μM-A23187). Ca^{2+} was added from 1M-$CaCl_2$ in medium A. Fluorescence was calibrated in terms of [Ca^{2+}]$_i$ from the fluorescence value at 1mM-Ca^{2+} in the presence of 0.1μM-A23187 and the fluorescence value at 0.5mM-$MnCl_2$ in the presence of 0.1μM-A23187 (Hesketh *et al.*, 1983; Tsien *et al.*, 1982*a*,*b*).

increase from 110 to 130nM in a medium containing 1mM-$CaCl_2$ (Fig. 4*e*). TMB-8 had been shown to interfere with the intracellular mobilization of Ca^{2+} (Malagodi & Chiou, 1974; Chiou & Malagodi, 1975; Rittenhouse-Simmons & Deykin, 1978; Matsumoto *et al.*, 1979). A rise in the $[Ca^{2+}]_i$ is induced by PHA in CCRF-CEM cells, but not in the other three human T-lymphoid cell lines, PEER, MOLT-4f, or SKW-3. It has been suggested that PtdIns(4,5)P_2 and PtdIns4P are the membrane receptors for aminoglycosidic antibiotics (Schacht, 1976). Removal of streptomycin from the culture medium had no appreciable effects on the PHA-induced changes in phospholipids or $[Ca^{2+}]_i$ in JURKAT cells.

Independence of PtdIns(4,5)P_2 breakdown on Ca^{2+} mobilization

Depletion of Ca^{2+} from medium by the use of Ca^{2+}-free medium and by the addition of EGTA did not affect the PHA-induced breakdown of PtdIns(4,5)P_2 (Table 2). Addition of TMB-8 (0.8mM) to the incubation medium did not inhibit the breakdown (Table 2), as has been reported in thrombin-stimulated platelets (Billah & Lapetina, 1982*a*). Moreover, addition of Ca^{2+} ionophore A23187 to the [^{32}P]P_i-prelabelled JURKAT cells did not induce the breakdown of [^{32}P]PtdIns(4,5)P_2 nor the accumulation of [^{32}P]phosphatidic acid (Fig. 5*a*). In cells pretreated with ionophore A23187, PHA induced the breakdown and accumulation (Fig. 5*a*). A procedure which exhaustively depletes cellular Ca^{2+}, i.e. suspension of cells in Ca^{2+}-free medium containing 2mM-EGTA and 5μM-A23187, did not affect the breakdown of [^{32}P]PtdIns(4,5)P_2 nor the accumulation of [^{32}P]phosphatidic acid induced by PHA in the [^{32}P]P_i-prelabelled JURKAT cells (Fig. 5*b*). These results indicate that the breakdown of PtdIns(4,5)P_2 is not mediated through changes in the $[Ca^{2+}]_i$. The results therefore support the view that the PHA-induced breakdown of PtdIns(4,5)P_2 in JURKAT cells is not a consequence of PHA-stimulated Ca^{2+} mobilization, but is instead involved in the processes leading to the Ca^{2+} mobilization. The $[Ca^{2+}]_i$ in JURKAT cells rises at an early time after the stimulation with PHA when the PtdIns(4,5)P_2 breakdown is in progress and phosphatidic acid has just begun to accumulate (Figs. 1 and 4). This may indicate that sufficient intracellular signal(s) for the maximum increase in $[Ca^{2+}]_i$ is generated by the hydrolysis of only a small fraction of cellular PtdIns(4,5)P_2.

The interrelation between the Ca^{2+} mobilization in cells and the receptor-stimulated hydrolysis of inositol lipids has been most extensively studied in isolated rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilizing hormones. For rat hepatocytes, different results have been presented concerning the interrelation (Kirk *et al.*, 1981; Prpić *et al.*, 1982; Charest *et al.*, 1983; Creba *et al.*, 1983; Rhodes *et al.*, 1983; Thomas *et al.*, 1983).

Table 2. *Effects of Ca^{2+}-free medium, EGTA treatment and TMB-8 treatment on PHA-induced breakdown of [^{32}P]PtdIns(4,5)P_2 and [^{32}P]PtdIns4P and accumulation of [^{32}P]phosphatidic acid in JURKAT cells prelabelled with [^{32}P]P_i*

JURKAT cells were cultured and labelled with [^{32}P]P_i at 37°C for 1h (see the Materials and methods section). The labelled cells were washed once with Ca^{2+}- and P_i-free Tris-buffered saline (medium A) containing 2mM-EGTA and then suspended at 6.3×10^6 cells/ml in medium A. Portions (0.2ml) of the cell suspension were incubated in the presence or absence of 12.5μg of PHA (5μl) for 2min in 0.25ml of (*a*) medium A containing 1mM-$CaCl_2$, (*b*) medium A, (*c*) medium A containing 2mM-EGTA, (*d*) medium A containing 1mM-$CaCl_2$ and 0.8mM-TMB-8, or (*e*) medium A containing 0.8mM-TMB-8. In incubations (*d*) and (*e*), the cells were preincubated with TMB-8 for 5min before the PHA addition. The incubations were terminated and the ^{32}P radioactivity of PtdIns(4,5)P_2, PtdIns4P and phosphatidic acid was determined as described in the Materials and methods section. Results are expressed as means ± S.E.M. for three experiments. Abbreviation: PtdOH, phosphatidic acid.

			Gain or loss (−) in ^{32}P radioactivity after 2min incubation in the presence or absence of PHA (c.p.m.)		
Expt.	Condition	PHA	PtdIns(4,5)P_2	PtdIns4P	PtdOH
1	(*a*) 1mM-Ca^{2+}	−	809 ± 1060	−758 ± 14	−1408 ± 274
		+	−18369 ± 2273	−3918 ± 676	17192 ± 183
	(*b*) Ca^{2+}-free	−	−1736 ± 701	−641 ± 438	−998 ± 1197
		+	−17452 ± 886	−2169 ± 378	15364 ± 514
	(*c*) Ca^{2+}-free + 2mM-EGTA	−	−3917 ± 955	−1882 ± 211	−568 ± 821
		+	−17526 ± 555	−2288 ± 175	12931 ± 1204
	(*d*) 1mM-Ca^{2+} + 0.8mM-TMB-8	−	−2770 ± 510	−535 ± 213	4089 ± 2453
		+	−16627 ± 266	−2957 ± 379	20828 ± 1628
2	(*e*) Ca^{2+}-free + 0.8mM-TMB-8	−	−5302 ± 524	−107 ± 0	9563 ± 739
		+	−19379 ± 1219	−3983 ± 294	36867 ± 925

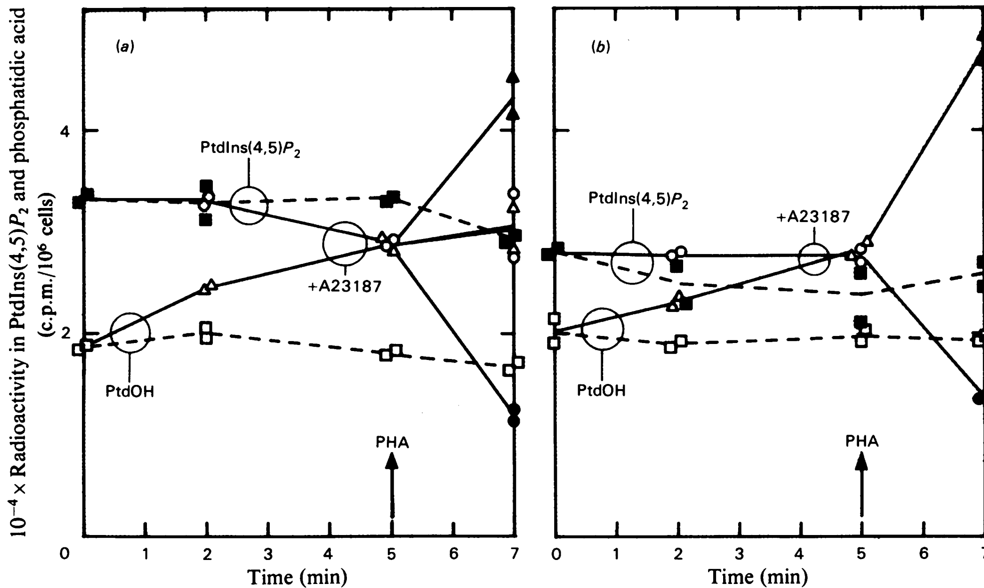

Fig. 5. *Effect of pretreatment with A23187 on PHA-induced [^{32}P]PtdIns(4,5)P_2 breakdown and [^{32}P]phosphatidic acid accumulation in JURKAT cells prelabelled with [^{32}P]P_i*

A suspension of ^{32}P-labelled JURKAT cells was prepared as described in the Materials and methods section. Portions (0.2ml) of the cell suspension were incubated for the indicated period of time in 0.25ml of either (*a*) medium A containing 1mM-$CaCl_2$ or (*b*) medium A containing 2mM-EGTA. Where indicated, A23187 (5μM) was added at zero time. All incubation mixtures contained dimethyl sulphoxide (final concn. 0.05%). Where indicated, 12.5μg of PHA in 5μl was added after a 5min exposure to A23187. ^{32}P radioactivity of PtdIns(4,5)P_2 and phosphatidic acid was determined as described in the Materials and methods section. ■ and □, A23187 not added; ●, ○, ▲ and △, A23187 added. ■, ○ and ●, PtdIns(4,5)P_2; □, △ and ▲, phosphatidic acid (PtdOH). ■, □, ○ and △, PHA not added; ● and ▲, PHA added.

The argument now seems to have been settled in favour of the opinion that PtdIns(4,5)P_2 can be broken down independently of the Ca^{2+} mobilization (Creba *et al.*, 1983; Thomas *et al.*, 1983). The results in the present paper indicate that the receptor-stimulated breakdown of PtdIns(4,5)P_2 is not dependent on the Ca^{2+} mobilization. It was reported that Ins(1,4,5)P_3 induces a rapid release of Ca^{2+} from intracellular stores and therefore functions as a second messenger for the hormonal mobilization of intracellular Ca^{2+} (Joseph *et al.*, 1984; Streb *et al.*, 1983). However, the regulatory mechanism of cell-surface Ca^{2+} channels remains unknown. The rise in $[Ca^{2+}]_i$ in the PHA-stimulated JURKAT cells depends on extracellular Ca^{2+} (Fig. 4*b*), which indicates the importance of Ca^{2+} influx in the Ca^{2+} mobilization in JURKAT cells.

We thank Dr. James Watson for providing us with the JURKAT cell line. We are indebted to Ms R. Ohtani for typing the manuscript.

References

Abdel-Latif, A. A., Akhtar, R. A. & Hawthorne, J. N. (1977) *Biochem. J.* **162**, 61–73

Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **258**, 2076–2078

Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858

Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360

Berridge, M J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482

Billah, M. M. & Lapetina, E. G. (1982*a*) *Biochem. Biophys. Res. Commun.* **109**, 217–222

Billah, M. M. & Lapetina, E. G. (1982*b*) *J. Biol. Chem.* **257**, 12705–12708

Billah, M. M. & Lapetina, E. G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 965–968

Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773

Chiou, C. Y. & Malagodi, M. H. (1975) *Br. J. Pharmacol.* **53**, 279–285

Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. (1983) *Biochem. J.* **212**, 733–747

Downes, C. P. & Wusteman, M. M. (1983) *Biochem. J.* **216**, 633–640

Ferber, E., Reilly, C. E. & Resch, K. (1976) *Biochim. Biophys. Acta* **448**, 143–154

Fisher, D. B. & Mueller, G. C. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1396–1402

Gillis, S. & Watson, J. (1980) *J. Exp. Med.* **152**, 1709–1719

Hallcher, L. M. & Sherman, W. R. (1980) *J. Biol. Chem.* **225**, 10896–10901
Hasegawa-Sasaki, H. & Sasaki, T. (1981) *Biochim. Biophys. Acta* **666**, 252–258
Hasegawa-Sasaki, H. & Sasaki, T. (1982) *J. Biochem.* (*Tokyo*) **91**, 463–468
Hasegawa-Sasaki, H. & Sasaki, T. (1983) *Biochim. Biophys. Acta* **754**, 305–314
Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. & Metcalfe, J. C. (1983) *J. Biol. Chem.* **258**, 4876–4882
Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 3077–3081
Kirk, C. J., Creba, J. A., Downes, C. P. & Michell, R. H. (1981) *Biochem. Soc. Trans.* **9**, 377–379
Krebs, H. A. (1950) *Biochim. Biophys. Acta* **4**, 249–267
Larsson, E. L. & Coutinho, A. (1979) *Nature* (*London*) **280**, 239–241
Maino, V. C., Hayman, M. J. & Crumpton, M. J. (1975) *Biochem. J.* **146**, 247–252
Malagodi, M. H. & Chiou, C. Y. (1974) *Pharmacology* **12**, 20–31
Martin, T. F. (1983) *J. Biol. Chem.* **258**, 14816–14822
Masuzawa, Y., Osawa, T., Inoue, K. & Nojima, S. (1973) *Biochim. Biophys. Acta* **326**, 339–344
Matsumoto, T., Takeshige, K. & Minakami, S. (1979) *Biochem. Biophys. Res. Commun.* **88**, 974–979
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Michell, R. H. (1979) *Trends Biochem. Sci.* **4**, 128–131
Michell, R. H. & Kirk, C. J. (1981) *Trends Pharmacol. Sci.* **2**, 86–89
Palacios, R. (1982) *Immunol. Rev.* **63**, 73–110
Prpić, V., Blackmore, P. F. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 11323–11331
Putney, J. W., Burgess, G. M., Halenda, S. P., McKinney, J. S. & Rubin, R. P. (1983) *Biochem. J.* **212**, 483–488
Rebecchi, M. J. & Gershengorn, M. C. (1983) *Biochem. J.* **216**, 287–294
Rhodes, D., Prpić, V., Exton, J. H. & Blackmore, P. F. (1983) *J. Biol. Chem.* **258**, 2770–2773
Rittenhouse-Simmons, S. & Deykin, D. (1978) *Biochim. Biophys. Acta* **543**, 409–422
Robb, R. J., Munck, A. & Smith, K. A. (1981) *J. Exp. Med.* **154**, 1455–1474
Sasaki, T. & Hasegawa-Sasaki, H. (1985) *Biochim. Biophys. Acta* **833**, in the press
Schacht, J. (1976) *J. Neurochem.* **27**, 1119–1124
Smith, K. A. & Ruscetti, F. W. (1981) *Adv. Immunol.* **31**, 137–175
Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, J. (1983) *Nature* (*London*) **306**, 67–69
Thomas, A. P., Marks, J. S., Coll, K. E. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 5716–5725
Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982*a*) *Nature* (*London*) **295**, 68–71
Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982*b*) *J. Cell Biol.* **94**, 325–334
Vickers, J. D., Kinlough-Rathbone, R. L. & Mustard, J. F. (1982) *Blood* **60**, 1247–1250
Weiss, S. J., McKinney, J. S. & Putney, J. W. (1982) *Biochem. J.* **206**, 555–560