Phosphoinositide hydrolysis in mitogen-stimulated human peripheral-blood T lymphocytes

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Both phytohaemagglutinin and antibodies to the CD3 molecule induced proliferation and phosphoinositide hydrolysis in human peripheral-blood T lymphocytes, but the magnitude of the inositol phosphate response was small and the rate of accumulation slow [significant increases in $\text{Ins}(1,4,5)\text{P}_3$ were observed only after 10 min]. Hence this response differs from the well-characterized $\text{Ins}(1,4,5)\text{P}_3$ responses of many other systems. This slow response, its abrogation in Ca^{2+} -depleted medium, the slow and maintained increase in Ca^{2+} as measured by Quin-2, and the ability of the Ca^{2+} ionophore A23187 to stimulate $\text{Ins}(1,4,5)\text{P}_3$ accumulation all suggest that the increase in $\text{Ins}(1,4,5)\text{P}_3$ occurs, at least in part, as a result of receptor-mediated Ca^{2+} influx in mitogen-stimulated T lymphocytes.

INTRODUCTION

The production of secondary-messenger signals by phosphoinositide hydrolysis has been proposed for several cell types (Berridge, 1987). In this model, receptor–ligand binding activates a phospholipase C which hydrolyses $\text{PtdIns}(4,5)\text{P}_2$, liberating $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. The evidence for these secondary messengers as the sole mechanism of transduction of antigenic and mitogenic stimulus in T lymphocytes is less convincing than that for most tissues. This is due in part to a lack of studies on physiologically relevant cells, e.g. resting T lymphocytes isolated from peripheral blood (King, 1988).

We report here that increases in inositol phosphates can be detected by ion-exchange chromatography and h.p.l.c. in mitogen-stimulated human peripheral-blood T lymphocytes. However, we demonstrate that accumulation of $\text{Ins}(1,4,5)\text{P}_3$ is both small and slow and may not occur as a direct consequence of receptor binding, but rather it is secondary to the elevation in cytoplasmic $[\text{Ca}^{2+}]$ observed soon after the addition of mitogen to cells.

EXPERIMENTAL

Cell culture

Mononuclear cells were isolated from buffy cell coats by dextran sedimentation and centrifugation on Ficoll-Paque gradients (Pharmacia; Johnstone & Thorpe, 1987). For most of the experiments, the mononuclear-cell preparation was enriched for T lymphocytes by passing the cells through a nylon wool column (Johnstone & Thorpe, 1987). Cells were stimulated with phytohaemagglutinin (PHA; Pharmacia) at final concentrations of 2.5 and 25 $\mu\text{g}/\text{ml}$. OKT3 (Ortho Diagnostics), a monoclonal antibody which recognizes the CD3 molecule on T lymphocytes, was used at 25 ng/ml. The Ca^{2+} ionophore A23187 (Sigma) was dissolved in dimethyl sulphoxide at a concentration of 20 mM and diluted

in medium, giving final concentrations of 4, 2 and 1 μM .

Cell labelling

Cells were suspended at $10^7/\text{ml}$ in medium 199 containing glutamine (20 mM), 0.1% (w/v) bovine serum albumin and 20 μCi of $[\text{H}]\text{inositol}$ (Amersham; sp. radioactivity 16.6 Ci/mmol)/ml. After overnight incubation at 37 °C in 5% CO_2 in air, the cells were washed (for separation of inositol phosphates on ion-exchange columns) and resuspended at $(4\text{--}8) \times 10^6/\text{ml}$, in medium 199 containing 0.1% bovine serum albumin and 10 mM-LiCl. To determine the Ca^{2+} -dependence of the production of inositol phosphates, identical populations of inositol-labelled cells were resuspended in normal or Ca^{2+} -depleted phosphate-buffered saline (Cockcroft *et al.*, 1987), supplemented with 0.1% glucose and 10 mM-LiCl. After 30 min, mitogens or equal volumes of media were added to samples containing $(1\text{--}2) \times 10^6$ cells, and at various times later (from 1 to 30 min), 1 ml of ice-cold 10% (w/v) HClO_4 was added to stop the reaction. Acidified samples were centrifuged at 500 *g* for 10 min, and the supernatant was collected and diluted with 5 ml of distilled water. The pH was adjusted to 7.0 by addition of 3 M- KH_2PO_4 .

For determination of the early time course of inositol phosphate formation by h.p.l.c., labelled cells $[(1\text{--}2) \times 10^6 \text{ cells/sample}]$ were resuspended and stimulated by mitogen in a balanced salt solution (137 mM-NaCl, 2.7 mM-KCl, 1 mM- MgCl_2 , 1 mM- CaCl_2 , 20 mM-Hepes, 5.6 mM-glucose and 1 mg of bovine serum albumin/ml). The reaction was terminated at various times by addition of 1 ml of ice-cold 10% (w/v) trichloroacetic acid. Acidified samples were centrifuged at 500 *g* for 10 min, the supernatant was collected and its pH adjusted to 7.0 by addition of NaOH.

Chromatographic separation of inositol phosphates

Separation by ion-exchange resin was based on the

Abbreviations used: GroPIs, glycerophosphoinositol; PHA, phytohaemagglutinin.

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method described by Berridge *et al.* (1982). The sample was loaded on to 1 ml Dowex-1 (Sigma; formate form) columns and washed with 10 ml of distilled water to remove inositol. Glycerophosphoinositol (GroPIns) was eluted with 6 ml of 5 mM-sodium tetraborate plus 60 mM-sodium formate. InsP , InsP_2 and InsP_3 were eluted sequentially by stepwise addition of 10 ml of 0.1 M-formic acid containing 0.2 M-, 0.4 M- and 1.0 M-ammonium formate. A 3 ml portion of each fraction was counted for radioactivity after addition of scintillant.

H.p.l.c. was performed by using gradients developed from the methods introduced by Irvine *et al.* (1985) and Batty *et al.* (1985), as detailed by Salmon & Bolton (1988). Internal ^{32}P -labelled standards were added to each sample (Salmon & Bolton, 1988). The system was verified by comparison of an h.p.l.c. trace of resting [^3H]inositol-labelled nylon-wool-adhered T lymphocytes with a trace of labelled standards. Samples were injected in a volume of 2 ml on to a 25 cm $\times$ 0.4 cm column of

Fig. 1. Separation of inositol phosphates from PHA-stimulated T lymphocytes by ion-exchange chromatography

Nylon-wool-enriched T lymphocytes were incubated with PHA (2.5 $\mu\text{g}/\text{ml}$; $\square$) or PHA (25 $\mu\text{g}/\text{ml}$; $\square$) for various times, after which GroPIns and inositol phosphates were separated on 1 ml columns of ion-exchange resin; $\square$, unstimulated cells. Results (c.p.m.) are expressed as means $\pm$ S.E.M. for three separate experiments, in which each time plot was performed in triplicate. Results were analysed by the Wilcoxon Sign Rank Test (* $P \leq 0.05$).

Partisil SAX 10 equipped with a 5 cm $\times$ 0.4 cm guard column, packed with the same material. At 5 min after injection, a linear gradient up to 0.2 M-ammonium formate was initiated, followed by a hyperbolic gradient (Waters curve 4) up to 0.4 M-ammonium formate, then a gradient (Waters curve 4) up to 1 M-ammonium formate and completed by a linear increase to 2 M-ammonium

Fig. 2. Separation of inositol phosphates from OKT3-stimulated T lymphocytes by ion-exchange chromatography

Unseparated mononuclear cells were incubated with OKT3 (25 ng/ml; $\square$) for various times, after which GroPIns and inositol phosphates were separated on 1 ml columns of ion-exchange resin. The response is compared after 30 min activation with the increases in inositol phosphates stimulated by PHA (25 $\mu\text{g}/\text{ml}$) in untreated T lymphocytes ($\square$) and those stimulated by OKT3 in nylon-wool-enriched T lymphocytes ($\square$). Unstimulated values are represented by $\square$ for untreated lymphocytes and by $\square$ for nylon-wool-enriched T lymphocytes. Results (c.p.m.) are expressed as means $\pm$ S.E.M. for three separate experiments, in which each time point was performed in triplicate. Results were analysed by the Wilcoxon Sign Rank Test (* $P \leq 0.05$).

formate. Each gradient increased over 10 min. The flow rate was 1.25 ml/min, and the eluate was monitored for A_{245} and with an on-line detector (Beckman model 170) for ^{32}P β -emission. Fractions were collected at 0.4 min intervals.

Proliferation assays

Cells ($1 \times 10^6/\text{ml}$) were stimulated with mitogens in RPMI 1640 containing 5% (v/v) foetal-calf serum. After 30 min, the cells were washed three times, resuspended at $1 \times 10^6/\text{ml}$ in RPMI 1640/5% foetal-calf serum and cultured for 72 h in flat-bottomed micro-titre plates, at 0.2 ml/well. [^3H]Thymidine was added to a final concentration of 2 $\mu\text{Ci}/\text{ml}$, 4 h before the cells were harvested on to GF/C paper (Whatman) by using a cell harvester. The radioactivity incorporated into DNA was assessed by liquid-scintillation counting.

Measurement of intracellular Ca^{2+}

Cells were loaded with the fluorescent quinoline Ca^{2+} indicator Quin-2 by incubation with Quin-2 acetoxymethyl ester (50 μM) in RPMI 1640/10% foetal-calf serum. After 30 min, 10^7 cells were washed and resuspended in 4 ml of a 10 mM-Hepes-buffered salt solution as described by Tsien *et al.* (1982). This was added to a 10 mm cuvette and was stirred continuously throughout the experiment. Then 20 μl of PHA was added, giving a final concentration of 2.5 $\mu\text{g}/\text{ml}$. Fluorescence was measured at the normal excitation and emission wavelengths for Quin-2 of 340 and 492 nm respectively and calibrated by the addition of digitonin and then Tris/EDTA, pH 8.4, to determine maximum and minimum fluorescence (Tsien *et al.*, 1982).

RESULTS

In the presence of 10 mM-LiCl, levels of inositol phosphates, separated on ion-exchange resin, increased in PHA- and OKT3-stimulated T cells in a dose- and time-dependent manner (Figs. 1 and 2) which correlates with mitogenic stimulation (Table 1). The same concentration of PHA (25 $\mu\text{g}/\text{ml}$) was responsible for both maximal phosphoinositide hydrolysis and uptake of [^3H]thymidine; OKT3 produced neither response in the absence of monocytes (Fig. 2).

The appearance of InsP_3 after stimulation was slow

Table 2. Production of inositol phosphates in response to A23187

Nylon-wool-enriched T lymphocytes were stimulated with PHA (25 $\mu\text{g}/\text{ml}$) or A23187 (4, 2 and 1 μM). After 30 min, inositol phosphates from these and an unstimulated control sample were separated on 1 ml columns of ion-exchange resin. Results (c.p.m.) are expressed as means $\pm$ S.E.M. for three separate experiments, in which each measurement was performed in triplicate.

	Production (c.p.m.)				
	A23187				PHA (25 $\mu\text{g}/\text{ml}$)
	Control	(1 μM)	(2 μM)	(4 μM)	
InsP	140 $\pm$ 13	268 $\pm$ 27	364 $\pm$ 33	429 $\pm$ 37	3364 $\pm$ 551
InsP ₂	112 $\pm$ 18	494 $\pm$ 75	807 $\pm$ 62	1067 $\pm$ 112	379 $\pm$ 46
InsP ₃	137 $\pm$ 26	391 $\pm$ 86	551 $\pm$ 54	642 $\pm$ 53	270 $\pm$ 11

[significant levels were only detected after 10 (25 $\mu\text{g}/\text{ml}$) to 30 (2.5 $\mu\text{g}/\text{ml}$) min exposure to PHA]; the degree of stimulation was small (20%). However, significant levels of both InsP and InsP_2 were detected after 5 min. In contrast, considerable levels of InsP_3 , InsP_2 and InsP were detected 30 min after stimulation with A23187 (Table 2).

The production of inositol phosphates was further studied by using h.p.l.c. (Fig. 3), including an investigation of very early times. For this study LiCl was omitted, resulting in a decrease in the overall increase in the formation of $\text{Ins}(1)\text{P}$. $\text{Ins}(1,3,4,5)\text{P}_4$ was undetectable, as was an increase in $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$; only $\text{Ins}(1,4)\text{P}_2$ was increased after 5 min stimulation with PHA.

The slow rise in the production of inositol phosphates observed on stimulation with PHA was preceded by an increase in intracellular Ca^{2+} , which reached a plateau after 5 min as determined by Quin-2 (Fig. 4). In the absence of added Ca^{2+} in the extracellular medium, the formation of InsP in response to 25 μg of PHA/ml fell by approx. 60%, as did the production of InsP_2 (Fig. 5). The generation of InsP_3 was also decreased in the absence of Ca^{2+} , but the poor stimulation in the presence of Ca^{2+} , even after 30 min, meant that it was difficult to quantify the inhibition. Cells loaded with Quin-2 and then stimu-

Table 1. Mitogenic stimulation of T cells

The uptake of [^3H]thymidine by nylon-wool-enriched T lymphocytes or unseparated mononuclear cells in response to PHA and OKT3 was measured. Results (c.p.m.) are expressed as means $\pm$ S.E.M. of three separate experiments, in which each measurement was performed in triplicate; n.d., not determined.

	Uptake (c.p.m.)			
	Unstimulated	PHA		OKT3 (25 ng/ml)
		(2.5 $\mu\text{g}/\text{ml}$)	(25 $\mu\text{g}/\text{ml}$)	
T lymphocytes	2100 $\pm$ 230	3540 $\pm$ 4400	12800 $\pm$ 3500	4900 $\pm$ 420
Unseparated mononuclear cells	1700 $\pm$ 340	n.d.	n.d.	21800 $\pm$ 250

Fig. 3. Determination of GroPIs and inositol phosphates by h.p.l.c. in mitogen-stimulated T lymphocytes

GroPIs and inositol phosphates were separated by h.p.l.c. from nylon-wool-enriched T lymphocytes stimulated for various times with PHA (25 µg/ml). The total radioactivities (d.p.m.) for each peak of GroPIs (■), Ins(1)P (●), Ins(1,4)P₂ (▲) and Ins(1,4,5)P₃ (▼) are shown from one experiment of three performed. Levels of GroPIs (□), Ins(1)P (○), Ins(1,4)P₂ (△) and Ins(1,4,5)P₃ (▽) after stimulation with A23187 (4 µM) for 30 min are also shown.

lated with 25 µg of PHA/ml in the presence of Ca²⁺ showed a similar degree of inhibition (Table 3).

When ion-exchange resin was used, levels of 'GroPIs' eluted with sodium tetraborate/sodium formate increased after PHA stimulation (Fig. 1). This observation could not be repeated when h.p.l.c. was used for analysis. Subsequent h.p.l.c. analysis of the GroPIs fraction from the Dowex columns revealed that most of the label was associated with Ins(1)P which had been eluted before the addition of 0.2 M-ammonium formate (results not shown).

Fig. 4. Ca²⁺ response to PHA

PHA (2.5 µg/ml) was added to Quin-2-labelled nylon-wool-enriched T lymphocytes, and the response was monitored for 5 min. After addition of PHA, in three separate experiments, the concentration of intracellular Ca²⁺ rose slowly from a basal value of 157 ± 20 nM until it reached a plateau at 305 ± 33 nM 5 min later.

Fig. 5. Ca²⁺-dependency of inositol phosphate formation in response to PHA

The production of inositol phosphates by nylon-wool-enriched T lymphocytes was determined by using 1 ml columns of ion-exchange resin, after activation with PHA (25 µg/ml) for 30 min in (a) medium containing normal concentrations of Ca²⁺ and (b) Ca²⁺-depleted medium; □, unstimulated values. Results (c.p.m.) are expressed as means ± S.E.M. of three separate experiments, in which each measurement was performed in triplicate. Results were analysed by the Wilcoxon Sign Rank Test (*P ≤ 0.05).

Table 3. PHA-stimulated InsP₃ formation in Quin-2-loaded cells

Cells were labelled with [³H]inositol and incubated for 30 min in the absence or presence of Quin-2. After this the cells were stimulated for a further 30 min with 25 µg of PHA/ml before analysis of InsP₃ content by ion-exchange chromatography. Results (d.p.m.) are expressed as means ± S.E.M. of triplicate determinations.

	Quin 2	InsP ₃ formation (c.p.m.)		Inhibition (%)
		Control	+ PHA	
Expt. 1	—	82.8 ± 13.6	185 ± 7.2	
	+	73.2 ± 7.6	124 ± 12.4	50.3
Expt. 2	—	141 ± 1.3	263 ± 20	
	+	141 ± 2.2	236 ± 2.4	22.1

DISCUSSION

InsP₃ has been implicated in the activation of a number of cell types in response to stimulation by a variety of agonists. A rapid increase in Ca²⁺ within 5 s has been shown to precede a secondary slow increase in intracellular Ca²⁺ in many systems, by using both Quin-2 and Fura-2. The initial rapid rise is believed to be from non-mitochondrial internal stores, whereas the sustained rise is from Ca²⁺ influx. The present observations on the PHA stimulation of human T lymphocytes are very different from inositol phosphate responses of other cells. Thus we can only find a small increase in InsP₃, and only after 30 min stimulation, i.e. after the rise in intracellular Ca²⁺. Similar results were obtained with OKT3, the anti-CD3 antibody which binds to the T-cell receptor complex. This indicates that a similar mech-

anism might be involved in response to this more physiological stimulus.

The inability to detect rapid changes in $\text{Ins}(1,4,5)\text{P}_3$ is contrary to studies with T-cell lines (Imboden & Stobo, 1985; Pantaleo *et al.*, 1986, 1987), clones (Imboden *et al.*, 1987), hybridomas (Cockcroft *et al.*, 1987), thymocytes (Taylor *et al.*, 1984; Gelfand *et al.*, 1987) and activated T cells (Mills *et al.*, 1986). This may reflect the fact that, unlike peripheral-blood T cells, many T-cell lines are pre-activated, as indicated by a response to interleukin 2. Individual T-cell clones differ in their stimulated inositol hydrolysis, and thus may not be representative of physiologically occurring populations (Goronzy *et al.*, 1987; Hasegawa-Sasaki & Sasaki, 1983).

Direct studies on the role of Ca^{2+} are often difficult to interpret (Michell *et al.*, 1981). The total depletion of Ca^{2+} by using chelators will affect not only processes that depend on increases in Ca^{2+} but also those that require basal cytosolic Ca^{2+} concentrations. Therefore we studied the Ca^{2+} -dependence of the responses to stimulation by PHA by examining the effect of medium without added Ca^{2+} , but we chose not to investigate the effect of Ca^{2+} chelators because of difficulties in interpretation of the results.

Under conditions of low Ca^{2+} the formation of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1)\text{P}$ was significantly decreased, indicating a dependence on extracellular Ca^{2+} . The remaining response observed may be the result of residual Ca^{2+} present in the medium. The basal formation of InsP_3 was unaffected by the Ca^{2+} depletion, and again there was little or no response to stimulation by PHA.

A23187 significantly enhanced the production of inositol phosphates by human T lymphocytes. Similar results have been demonstrated in basophilic cells and T and B lymphoma cell lines (Abraham *et al.*, 1987; Fahey & DeFranco, 1987; Lo *et al.*, 1987). Several research groups have used A23187 to activate lymphocytes, at concentrations which induce phosphoinositide hydrolysis in our system (Koretzky *et al.*, 1983; Croll *et al.*, 1987). Hence the effect of ionophore cannot be solely ascribed to increased cytoplasmic $[\text{Ca}^{2+}]$, as other secondary messengers, such as diacylglycerol, will almost certainly be generated, as shown in the liver (Bocckino *et al.*, 1985).

The results presented here suggest that, although $\text{Ins}(1,4,5)\text{P}_3$ may be involved in the proliferative response of human T lymphocytes to PHA, it is not responsible for the slow rise in intracellular Ca^{2+} , as it is unlikely that such a response could be sustained by the limited intracellular Ca^{2+} concentration. The experiments using low Ca^{2+} suggest a role for extracellular Ca^{2+} in the stimulation of the InsP_3 response. An alternative mechanism may be that T-cell activation results in a sequence of events leading separately to an increase in Ca^{2+} influx and the stimulation of inositol phosphate production. Mire-Sluis *et al.* (1987) put forward GroPIns as a possible candidate for an alternative secondary messenger. Those authors used Dowex ion-exchange chromatography; however, in our hands a similar 'GroPIns' fraction subsequently run on h.p.l.c. was

found to be predominantly InsP . Thus claims of the involvement of GroPIns based solely on Dowex chromatography should be viewed with caution.

In conclusion, the increase in InsP_3 may play a role in the activation of T lymphocytes, but it would appear not to be the primary event.

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