Inositol 1,4,5-trisphosphate concentrations increase after adherence in the macrophage-like cell line J774.1

Vivian ZABRENETZKY and Elaine K. GALLIN*

Department of Physiology, Armed Forces Radiobiology Research Institute, Bethesda, MD 20814-5145, U.S.A.

Several properties of macrophages change when suspended cells become adherent. To determine the intracellular signals involved in these changes, concentrations of the second messenger inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] were monitored during adherence of J774.1 cells, a macrophage-like cell line. When cells grown in suspension were allowed to adhere to a glass surface, there was a transient increase in InsP_3 that reached a peak between 100 and 120 s after plating. Inositol mono- and bis-phosphate concentrations were also elevated 100 and 120 s after plating. Analysis of isomer distribution showed significant 3-fold increases in $\text{Ins}(1,4,5)\text{P}_3$ and inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] at 100 s after plating. These values were maintained at 120 s, with the additional appearance of a 4-fold increase in inositol 1,3,4-trisphosphate. The adherence-induced generation of $\text{Ins}(1,4,5)\text{P}_3$ was decreased, and $\text{Ins}(1,3,4,5)\text{P}_4$ formation was blocked, in Ca^{2+} -free medium. However, doubling intracellular $[\text{Ca}^{2+}]$ by addition of the Ca^{2+} ionophore ionomycin ($1\text{ }\mu\text{M}$) did not increase $\text{Ins}(1,4,5)\text{P}_3$ in suspended cells. Adherence of J774.1 cells to fibronectin-coated glass also induced an increase in InsP_3 .

INTRODUCTION

The migration of macrophages to areas of chronic inflammation and infection involves adherence to a variety of surfaces. Studies *in vitro* have shown that the process of adherence triggers a number of functional changes in macrophages. These changes include an increase in peroxidase staining in the rough endoplasmic reticulum in rabbit blood monocytes 2 h after adherence (Bodel *et al.*, 1977), an increased expression of the FMC 17 antigen in human monocytes 12 h after plating of cells that were suspended for 16 h (Triglia *et al.*, 1985), and increases in the transport of lysine and adenosine 30 min after monolayer formation in rabbit lung macrophages (Pofit & Strauss, 1977). In the murine-derived macrophage-like cell line J774.1, membrane-potential measurements using a lipophilic radiolabelled cation indicate that adherence is associated with a hyperpolarization from -15 mV to -80 mV (Sung *et al.*, 1985). Patch-clamp studies in J774.1 cells have demonstrated the differential expression of two voltage-dependent K^+ conductances with time after adherence (Gallin & Sheehy, 1985). One of these conductances is responsible for maintaining the -80 mV membrane potential in adherent cells (Gallin & Sheehy, 1985). In all of these studies, the nature of the signal(s) that mediates the adherence-induced changes in macrophages is unknown.

Signal transduction in many cells, including white blood cells, occurs via receptor-mediated stimulation of phospholipase C that cleaves the plasma-membrane phospholipid phosphatidylinositol 4,5-bisphosphate. Two products which possess second-messenger function are formed: inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], which releases Ca^{2+} from intracellular stores, and *sn*-1,2-diacylglycerol, which activates protein kinase C (Berridge & Irvine, 1984; Nishizuka, 1984; Downes & Michell,

1985). In macrophages, platelet-activating factor, *N*-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) (Downes & Michell, 1985; Whetton *et al.*, 1986) and bacterial lipopolysaccharide activate this pathway (Prpic *et al.*, 1987). The present paper addresses the question of whether the process of adherence of J774.1 cells is associated with the generation of the second messenger $\text{Ins}(1,4,5)\text{P}_3$.

MATERIALS AND METHODS

Culture of J774.1 cells

J774.1 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and were stored at $-70\text{ }^\circ\text{C}$ until needed. Cells were maintained in tissue-culture medium containing RPMI 1640 supplemented with penicillin (10 units/ml), streptomycin ($10\text{ }\mu\text{g/ml}$), 0.03% (w/v) glutamine and 10% (v/v) heat-inactivated fetal bovine serum. Stock cultures were maintained as a non-adherent population in spinner flasks treated with Sigmacote. Cultures were kept at $37\text{ }^\circ\text{C}$ in a 5%- CO_2 incubator and fed every 2 days. Only early-passage cells (< 20) were used in these studies. In all cases freshly thawed cells were able to generate the InsP_3 signal when measured at 100 s after plating.

Labelling of cells with *myo*-[2- ^3H]inositol

Approx. 7×10^5 cells/ml in a final volume of 45 ml of supplemented RPMI 1640 medium were incubated with $100\text{ }\mu\text{Ci}$ of *myo*-[^3H]inositol (13 Ci/mmol) for 18 h. Each portion of [^3H]inositol was purified by centrifugation through Dowex AG 1-X8 resin before use. Longer periods of labelling cells, from 20 to 42 h, did not yield a substantially higher specific radioactivity of radioisotope incorporation.

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,3,4,5)\text{P}_4$, inositol 1,3,4,5-tetrakisphosphate; InsP_1 , inositol 1-phosphate; InsP_2 , inositol 1,4-bisphosphate; Ca^{2+}_o , extracellular Ca^{2+} ; Ca^{2+}_i , intracellular Ca^{2+} .

* To whom reprint requests should be sent.

Adherence protocol

Labelled cells were pelleted (850 g for 5 min) and then resuspended in serum-free RPMI 1640 for 10 min at room temperature. The cells were pelleted again and resuspended in 15 ml of NaCl Hanks (145 mM-NaCl, 4.6 mM-KCl, 1.3 mM-MgCl₂, 1.6 mM-CaCl₂ and 10 mM-Hepes, pH 7.3). This suspension was immediately pelleted and the cells were resuspended in NaCl Hanks at approx. 14×10^6 cells/ml. After equilibration for 10 min, 1.5 ml of cell suspension was applied to a 50 mm-diameter glass dish and allowed to adhere for various times. At the end of the adherence period, the non-adherent cells were removed by flooding the surface with 5 ml of NaCl Hanks and immediately aspirating the supernatant. The reaction was stopped by addition of 4.5 ml of cold 4.5 % HClO₄/NaCl Hanks (2:1, v/v) to the glass dish. The dishes were chilled and then scraped. Suspended cells served as the control group; 3 ml of 4.5 % HClO₄ was added to 1.5 ml of the suspended cell sample. Portions (100 μ l) of suspended and adherent samples were set aside for protein determination.

In preliminary experiments incubation with 10 mM-LiCl for 10 min did not affect InsP₃ generation. Therefore LiCl was not used in subsequent studies.

Separation of [³H]inositol metabolites

The HClO₄ extracts from suspended and adherent cells were chilled at 0 °C and then centrifuged to pellet the acid-extractable material. Lipid-soluble material was extracted from these pellets. The lipids from the acid-extracted pellet were solubilized by addition of chloroform/methanol (1:1, v/v) (containing 1 M-KCl and 10 mM-inositol) and centrifuged (6000 g, 3 min). Portions (100 μ l) of the chloroform extract were evaporated before radioactivity counting in 10 ml of Aquasol (New England Nuclear) to determine d.p.m. in the lipid extract for the normalization procedure (see below).

The supernatants were prepared for separation of the [³H]inositol metabolites by adjusting the pH to 8.0 with a solution of 0.5 M-KOH/9.0 mM-Na₂(BO₄)₂/1.9 mM-EDTA (Burgess *et al.*, 1985). This separation procedure was compared with one using trichloroacetic acid precipitation followed by diethyl ether extraction (Berridge *et al.*, 1983), and similar recoveries were obtained. The samples were stored at -20 °C. After thawing, the KClO₄ salts had precipitated and were pelleted (850 g, 5 min). The supernatant was applied to 1 ml of a 50 % (w/v) suspension of Dowex AG 1-X8 resin (formate form; 100–200 mesh). The [³H]inositol phosphates were eluted by the method of Berridge *et al.* (1983). Inositol, glycerophosphoinositol, inositol 1-phosphate (InsP₁), inositol 1,4-bisphosphate (InsP₂) and inositol trisphosphates (InsP₃) were sequentially eluted with water (24 ml), 5 mM-disodium tetraborate/60 mM-sodium formate (24 ml), 100 mM-formic acid/200 mM-ammonium formate (36 ml), 100 mM-formic acid/400 mM-ammonium formate (24 ml), and 100 mM-formic acid/1.0 M-ammonium formate (38 ml). Each eluate was collected in at least two fractions, and 1 ml of each fraction was counted for radioactivity in 10 ml of Aquasol. *myo*-[³H]inositol, [¹⁴C]inositol 1-phosphate and [³H]-Ins(1,4,5)P₃ were run as standards with and without the water extract from unlabelled cells to verify the elution sequence. There was a 96 % recovery of standards.

Protein determination

Protein was determined in the acid extracts of suspended and adherent cells. Portions (100 μ l) from each sample were frozen and thawed, and the acid-extracted protein was then pelleted (6000 g, 3 min). NaOH (50 μ l; 1 M) was added to dissolve the protein, and 10 μ l samples were used in the Bradford micro-assay (Bio-Rad, Richmond, CA, U.S.A.).

Normalization procedure

The adherent cell population represented only a fraction of the suspended cell population at each of the time points surveyed. Therefore, to determine if concentrations of inositol phosphates changed after adherence, it was necessary to normalize the adherent-cell data to the suspended-cell data. Because it was not clear which normalization procedure was best, data were normalized in two different ways: normalizing to cell protein and normalizing to d.p.m. in the lipid-soluble extract.

Normalized InsP₃ was calculated by the following equation: $[(I_a \times N)/I_s] \times 100 = \text{adherent InsP}_3$ (expressed as % of suspended cells), where $L_s/L_a = N$ (normalization factor), L_s = d.p.m. in the suspended-cell lipid-soluble extract, L_a = d.p.m. in the adherent-cell lipid-soluble extract, I_s = d.p.m. in the suspended-cell InsP₃ fraction, and I_a = d.p.m. in the adherent-cell InsP₃ fraction. For normalization to protein, the term 'lipid-soluble extract' can be replaced by 'cell protein' in these expressions.

Separation of InsP₃ isomers by h.p.l.c.

The InsP₃ fractions from the Dowex columns were acidified and salt-extracted by passing them over Dowex 50 resin (H⁺ form); they were frozen, freeze-dried, and stored at -70 °C until further analysis. The freeze-dried [³H]InsP₃ fractions were solubilized in 2 ml of water and filtered through 0.22 μ m-pore-size filter before manual injection on to a Whatman Partisil 10 SAX column (H₂PO₄⁻ form) and chromatographed in accordance with modifications of the procedure of Batty *et al.* (1985). The sample was eluted at 1.2 ml/min by a non-linear gradient, consisting first of water for 4.9 min, followed by a linear gradient of 0.8 M-ammonium formate (adjusted to pH 3.7 with H₃PO₄) to 1.7 M-ammonium formate (adjusted to pH 3.7 with H₃PO₄) at 5–30 min. The 1.7 M-ammonium formate buffer (100 %) was run for a further 5 min, and then the gradient was returned linearly to water over 5 min. Throughout, 1 min fractions were collected, and 0.5 ml of each fraction was counted for radioactivity in 1.0 ml of water and 10 ml of Aquasol. Standard retention times were determined by running a mixture of 0.025 μ Ci of D-[4,5-³²P]Ins(1,4,5)P₃, 0.025 μ Ci of D-[inositol]-2-³H(n)inositol 1,3,4,5-tetrakisphosphate-{[³H]Ins(1,3,4,5)P₄} and 150 μ M-ATP in water and monitoring each fraction for both radioactivity and ATP (by the A₂₄₅). Another run, consisting of the ³H-labelled cell fraction eluted from the Dowex column with 1.0 M-ammonium formate (InsP₃ fraction) mixed with 0.025 μ Ci of [³²P]Ins(1,4,5)P₃ fraction, was done. This run gave the same retention time for Ins(1,4,5)P₃ as the standard run. Approx. 96 % of each standard was recovered in both standard runs, and approx. 90 % of each cell-derived sample was recovered in the experimental runs.

Statistical analysis

The significance of the differences between means was assessed by Student's two-tailed *t* test for paired samples.

Materials

myo-[2-³H(n)]Inositol, D-[inositol-2-³H(n)]Ins(1,4,5)*P*₃, D-[4,5-³²P]Ins(1,4,5)*P*₃ and D-[inositol-2-³H(n)]Ins(1,3,4,5)*P*₄ were obtained from New England Nuclear (Boston, MA, U.S.A.). L-*myo*-[U-¹⁴C]Inositol-1-phosphate was purchased from Amersham (Arlington Heights, IL, U.S.A.). Ammonium formate and sodium formate were obtained from Sigma (St. Louis, MO, U.S.A.). Whatman Partisil 10 SAX columns were obtained from American Scientific Products (Columbia, MD, U.S.A.). AG 1-X8 resin (formate form) and Dowex 50 W-X8 resin were obtained from Bio-Rad.

RESULTS

Time-dependence of adherence

Fig. 1(a) shows data from three different experiments in which the adherent-cell protein was measured at various times after plating on a glass surface. There was a time-dependent increase in adherence, with approx. 40% of all cells adhering 100 s after plating, and 50% of the cells adhering 160 s after plating. Experiments carried out for longer time periods (190 and 310 s) did not result in a significant increase in the number of adherent cells, indicating that by 160 s cell adherence had plateaued. A visual examination of the dishes indicated that the cells were approaching confluence. A study was performed to determine whether the cells that did not adhere after the first plating were able to adhere if plated a second time. Non-adherent cells from a dish plated for 300 s were harvested and replated for another 300 s; 83% of these cells adhered during the second plating, indicating that approx. 90% of the total population of J774.1 cells was adherent. Results (not shown) from one batch of J774.1 cells (probably plated at a lower density) showed 90% adherence 160 s after the first plating. These cells did not differ in their ability to generate InsP₃ after adherence.

InsP₃ generation as a consequence of adherence

Fig. 1(b) shows the time-dependent increase in InsP₃ produced after adherence in J774.1 cells. The [³H]inositol phosphate content of adherent cells was normalized to the radioactivity (d.p.m.) in the lipid extracts of suspended cells and was expressed as a percentage of the [³H]inositol content of the suspended cells. A peak value of approx. 145% of control was reached at 100 s after

Fig. 1. (a) Percentage of cell protein adhering to glass, and (b) time course of adherence-induced InsP₃ generation

(a) Cells were allowed to adhere for the indicated times, and the adherent protein was measured. Each time point represents the mean $\pm$ S.E.M. for three experiments (done in duplicate), from different passages of J774.1 cells. The data are presented as percentages of total protein for suspended cells. (b) InsP₃ content was analysed by Dowex chromatography of cells plated for the indicated times. Data, normalized to lipid, are expressed as percentages of suspended-cell values. Each point represents the mean $\pm$ S.E.M.: *P < 0.001, **P < 0.05.

plating and remained significantly elevated at 120 s. InsP₃ contents decreased to control values by 190 s and remained at 100% of control until 310 s. Though the number of cells adhering between 100 and 160 s was increasing (from ~40% to ~50%), the observed InsP₃ signal had reached a peak and was decreasing. If the process of adherence simply selected cells with higher basal contents of InsP₃, a time-dependent increase followed by a decrease in InsP₃ would not have been expected. Therefore these data indicate that adherence can generate an InsP₃ signal in J774.1 cells.

Table 1. Time course of formation of inositol phosphates

Data (means $\pm$ S.E.M.), expressed as percentages of suspended-cell inositol phosphate contents, were normalized either to d.p.m. in the lipid-soluble extract of suspended cells or to suspended cell protein: ^a*P* < 0.001, ^b*P* < 0.01, ^c*P* < 0.02, ^d*P* < 0.05.

Time of adherence (s)	No. of expts.	InsP ₁		InsP ₂		InsP ₃	
		Lipid	Protein	Lipid	Protein	Lipid	Protein
100	8	170 $\pm$ 11 ^a	189 $\pm$ 12 ^a	113 $\pm$ 3.2 ^b	122 $\pm$ 3.5 ^a	145 $\pm$ 8.1 ^a	160 $\pm$ 14 ^b
120	3	133 $\pm$ 6.5 ^d	150 $\pm$ 7 ^c	126 $\pm$ 6.9 ^d	153 $\pm$ 9.2 ^d	140 $\pm$ 6.4 ^c	180 $\pm$ 14 ^c
190	3	97 $\pm$ 16	106 $\pm$ 5.2	93 $\pm$ 16	118 $\pm$ 9.8	101 $\pm$ 2.0	115 $\pm$ 12

Comparison of normalization procedures

Table 1 compares data from the same studies that were normalized to either protein or lipid. The average value of InsP_3 at 100 s after plating was slightly higher when normalized to protein than when normalized to lipid. At 120 s the InsP_3 signal was still significantly elevated when either normalization procedure was used. At 190 s after plating, both normalization procedures indicated that the InsP_3 signal had returned to control.

There were also adherence-associated changes in the contents of InsP_1 and InsP_2 , when either normalization procedure was used (Table 1). At 100 s there was a significant increase in InsP_1 and a smaller but significant increase in InsP_2 . At 120 s InsP_1 and InsP_2 were still significantly increased. By 190 s InsP_1 , InsP_2 and InsP_3 had returned to basal values. These data show that normalizing to lipid or protein gave equivalent results; therefore data presented below have been normalized to d.p.m. in the lipid-soluble fraction.

Distribution of InsP_3 isomers

At 100 and 120 s after plating, InsP_3 contents were elevated to $\sim 145\%$ of control. To identify the isomers that were increased at these times, the InsP_3 fractions were separated into their component isomers by h.p.l.c. In five separate studies, at 100 s after plating the $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ contents were 3.11 ± 0.22 ($P < 0.01$) and 3.14 ± 0.35 ($P < 0.01$) times suspended-cell values respectively. A representative experiment is shown in Fig. 2(a). An additional peak was eluted at a later retention time than $\text{Ins}(1,3,4,5)\text{P}_4$ (which was eluted at 32 min), suggesting that another inositol phosphate, possibly $\text{Ins}(1,3,4,5,6)\text{P}_5$, was present (Stephens *et al.*, 1988). Further studies will have to be done to determine its identity.

At 120 s after plating (Fig. 2b) the increases in both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were sustained. In addition, there was a 4-fold increase in $\text{Ins}(1,3,4)\text{P}_3$, which is consistent with other studies indicating that $\text{Ins}(1,3,4)\text{P}_3$ is a metabolic product of the action of 5'-phosphomonoesterase on $\text{Ins}(1,3,4,5)\text{P}_4$ (Hawkins *et al.*, 1986). The control (suspended-cell) inositol phosphate profiles were somewhat variable, as depicted by the difference between the control runs in Figs. 2(a) and 2(b).

Role of Ca^{2+} in the generation of the InsP_3 signal

In other cells the stimulation of InsP_3 production sometimes requires extracellular Ca^{2+} (Ca^{2+}_o) (Lew *et al.*, 1986; Beaven *et al.*, 1984). In order to examine the role of Ca^{2+}_o in adherence-induced InsP_3 formation, cells that had equilibrated for 5 min in Ca^{2+} -free Hanks medium containing 1 mM-EGTA were compared with cells from the same batch plated in Ca^{2+} -containing media. The percentage of cells adhering in Ca^{2+} -free medium + 1 mM-EGTA at 100 s after plating was equivalent to the percentage of cells adhering in normal Hanks medium (Fig. 3a). In contrast, the increase in InsP_3 , InsP_1 and InsP_2 was inhibited by 77%, 60% and 58% respectively (Table 2). In these studies the cells were incubated for 5 min in Ca^{2+} -free media; therefore it was possible that intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was lowered at the time of adherence, and the decrease in the contents of the inositol phosphates was due to this decrease rather than to the removal of Ca^{2+}_o . To obviate this possibility, experiments were performed in which a bolus of 5 mM-

Fig. 2. H.p.l.c. profiles of InsP_3 fractions from Dowex columns

(a) Fractions from adherent cells 100 s after plating (●) and from suspended cells (□). Data are from a single experiment, with each point derived from duplicates. Adherent-cell data were normalized to the d.p.m. in the lipid-soluble fraction. The means $\pm$ S.E.M. for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ peaks for the suspended cells in five experiments were 53 ± 27 and 45 ± 23 d.p.m. respectively. (b) Fractions from adherent cells 120 s after plating (●) and from suspended cells (□). Derivation of data is as presented in Fig. 2(a). The mean values for $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ peaks for the suspended cells in two experiments were 20, 15 and 13 d.p.m. respectively.

EGTA was added to cells in normal Hanks medium (containing 1.6 mM- CaCl_2) immediately before plating. The generation of InsP_3 in these cells was compared with

Fig. 3. Effect of Ca²⁺-free media on (a) time course adherence and (b) h.p.l.c. profile of InsP₃ fraction

Each point represents the mean $\pm$ S.E.M. for three experiments, each done in duplicate. The data are expressed as percentages of suspended-cell protein in either normal Hanks medium (●) or Ca²⁺-free Hanks medium + 1 mM-EGTA (□) as appropriate. (b) H.p.l.c. profile of InsP₃ fraction from cells plated for 100 s in Ca²⁺-free medium + 1 mM-EGTA (●) and from cells suspended in Ca²⁺-free medium + 1 mM-EGTA (□). Data are from a representative experiment, with each point derived from duplicates. The adherent-cell data were normalized to the d.p.m. in the lipid-soluble fraction. The means $\pm$ S.E.M. for the Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ peaks for suspended cells from three separate studies were 52 ± 12 and 62 ± 19 d.p.m. respectively.

that in cells plated in Ca²⁺-containing media. These conditions (results not shown) also inhibited adherence-induced InsP₃ formation at 100 s, suggesting that generation of the signal requires Ca²⁺.

Analysis of the isomer distribution of the InsP₃ fraction was examined for cells suspended in Ca²⁺-free medium + 1 mM-EGTA for 5 min before adherence (two experiments), and for cells in which a bolus of 5 mM-EGTA was added to Ca²⁺-containing medium immediately before plating (one experiment). All three experiments showed that there was no change in the Ins(1,3,4,5)P₄ peak. Summing fractions 19–21, the Ins(1,4,5)P₃ fraction, showed an average increase of $42 \pm 31\%$, compared with an average increase of $251 \pm 91\%$ observed in the presence of Ca²⁺. Fig. 3(b)

Table 2. Distribution of inositol phosphates at 100 s after plating

Cells were plated in normal Hanks (+Ca₀) or Ca²⁺-free Hanks + 1 mM-EGTA (−Ca₀). The data (means $\pm$ S.E.M. of three experiments in duplicate), expressed as percentages of total suspended-cell inositol phosphate content, were normalized to d.p.m. in the lipid-soluble extract of suspended cells. *P < 0.05.

	InsP ₁	InsP ₂	InsP ₃
+Ca ₀	200 $\pm$ 20*	137.5 $\pm$ 3.4	162 $\pm$ 7*
−Ca ₀	135 $\pm$ 20	115 $\pm$ 26	113 $\pm$ 15

Fig. 4. Effect of fibronectin-coated glass on (a) time course of adherence and (b) amounts of inositol phosphates

(a) The data, presented as percentages of suspended-cell protein, represent means $\pm$ S.E.M. of three separate experiments done in duplicate. Protein corresponding to fibronectin was subtracted from the total protein to determine cell protein. (b) Distribution of inositol phosphates 120 s after adherence to fibronectin. Data, presented as percentages of suspended-cell values, normalized to d.p.m. in lipid-soluble fraction, represent means $\pm$ S.E.M. of three experiments done in duplicate: *P < 0.05.

shows one of these studies. Suspended cells in Ca²⁺-free medium had Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ contents that did not differ from values obtained for suspended cells in Ca²⁺-containing medium. These data show that the generation of InsP₃ and its isomers during adherence is inhibited in Ca²⁺-free media.

Since the preceding studies demonstrated that Ca²⁺ is

required for the generation of the InsP_3 signal, experiments were done to determine whether raising $[\text{Ca}^{2+}]_i$ with the Ca^{2+} ionophore ionomycin could induce formation of InsP_3 in suspended cells. Studies in these cells using the Ca^{2+} indicator fura-2 indicated that ionomycin ($1\ \mu\text{M}$), in the presence of $1.6\ \text{mM}\text{-Ca}^{2+}_o$, doubled $[\text{Ca}^{2+}]_i$ within 30 s after its addition and that this increase was maintained for the 2 min assay period (results not shown). However, incubation in $1\ \mu\text{M}$ -ionomycin for 30 s, 1 min or 3 min did not affect the contents of InsP_3 , InsP_2 or InsP_1 in suspended cells, indicating that doubling $[\text{Ca}^{2+}]_i$ is not a sufficient stimulus to increase InsP_3 .

Adherence to fibronectin-coated surfaces and InsP_3 generation

J774.1 cells were placed on fibronectin-coated dishes to determine whether adherence to other surfaces resulted in similar increases in InsP_3 . As shown in Fig. 4(a), there was a slight decrease (10%) in the number of cells adhering to fibronectin-coated dishes compared with adherence to glass. We chose the 120 s time point to measure InsP_3 contents, since more cells were adherent at this time than at 100 s. Fig. 4(b) shows that InsP_3 was significantly increased above control, which is similar to the results obtained after adherence to glass. However, InsP_1 and InsP_2 were not significantly elevated.

DISCUSSION

This report demonstrates that there is a transient increase in the second messenger $\text{Ins}(1,4,5)\text{P}_3$ in J774.1 cells 100–120 s after adherence to glass and that this increase is dependent on Ca^{2+}_o . An increase in InsP_3 was also noted 120 s after adherence to fibronectin-coated glass.

In these studies adherence was not synchronized, since cells settle at different rates. The peak of an adherence-induced transient signal (measured in a population of cells) will be diminished by differences in adherence times. Plating the cells in a volume sufficient just to cover the surface of the dish minimized this problem. Despite an unavoidable (but small) variation in adherence times, the total InsP_3 values 100 s after plating were significantly higher than suspended-cell values, and the isomers $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were increased 3-fold above values for suspended cells.

The increases in InsP_3 contents described in this paper could result from the selection of a subpopulation of adherent cells that have endogenously higher contents of InsP_3 . Three findings argue strongly against this possibility. First, whereas only 50% of the J774.1 cells plated adhered to the glass surfaces (presumably because of physical constraints caused by the high cell density), ~90% of the cells were capable of adhering. Second, there is a time-dependence for the increase in InsP_3 , with the contents returning to suspended-cell values 3 min after plating. Third, the InsP_3 signal was decreased in cells plated in Ca^{2+} -free medium, even though there was no decrease in the percentage of cells adhering or any change in the time course of adherence.

The generation of the InsP_3 signal has been shown to be dependent on $[\text{Ca}^{2+}]_o$ in some studies on different cells. For example, the peak and rate of rise of the substance-P-induced InsP_3 and InsP_4 signals in parotid cells is dependent on $[\text{Ca}^{2+}]_o$ (Merritt & Rink, 1987), and

Beaven *et al.* (1984) have shown that antigen-stimulated production of inositol phosphates is dependent on $[\text{Ca}^{2+}]_o$, with a concentration-dependence similar to that of histamine release. Pribluda & Metzger (1987) have shown that antigen-stimulated production of inositol phosphates is decreased to 25% of control in the 2H3-HR⁺ subline of rat basophilic leukaemia cells in Ca^{2+} -free media. These reports are consistent with our data that demonstrate a decrease in InsP_3 (77%), InsP_2 (58%), and InsP_1 (60%) in cells plated in Ca^{2+} -free media compared with cells in Ca^{2+} -containing media. Isomer determination of the InsP_3 fraction revealed an inhibition of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ production in Ca^{2+} -free media. These findings indicate that Ca^{2+} -free medium may inhibit phospholipase C, which is consistent with previous reports of a Ca^{2+} requirement for the activity of purified phospholipase C (Ryu *et al.*, 1987). Since the adhesion process is not impaired by the removal of extracellular Ca^{2+} , the data suggest that the production of inositol phosphates is not required for adherence, but plays a role in the functional changes that follow adherence. Attenuation of the inositol phosphate signal consequent to adherence in Ca^{2+} -free media may effect these changes.

Lew *et al.* (1986) have reported that dimethyl sulphoxide-differentiated HL-60 cells (neutrophil-like cells) respond to fMet-Leu-Phe with an increase in $\text{Ins}(1,4,5)\text{P}_3$ that is followed by a slower and more sustained increase in $\text{Ins}(1,3,4)\text{P}_3$. Increasing $[\text{Ca}^{2+}]_i$ to micromolar with ionomycin enhanced the contents of inositol phosphates and the $\text{Ins}(1,3,4)\text{P}_3$ isomer in the absence of fMet-Leu-Phe. These findings are in contrast with our data indicating a lack of effect of a 30 s, 1 min or 3 min incubation with ionomycin on InsP_3 contents in suspended J774.1 cells. The reason for this difference is not clear. Our measurements of $[\text{Ca}^{2+}]_i$ after ionomycin treatment indicated that $[\text{Ca}^{2+}]_i$ doubled. It is possible that greater increases in $[\text{Ca}^{2+}]_i$ are necessary to generate inositol phosphates, or, conversely, that changes in $[\text{Ca}^{2+}]_i$ in the absence of other changes have no effect on the generation of inositol phosphates in J774.1 cells.

In HL-60 cells $\text{Ins}(1,3,4)\text{P}_3$ generation was not directly linked to receptor activation, but resulted from an increased $[\text{Ca}^{2+}]_i$ and $\text{Ins}(1,4,5)\text{P}_3$ (Lew *et al.*, 1986). A comparison of the h.p.l.c. data in the present paper obtained 100 s after plating (Fig. 2a) and 120 s after plating (Fig. 2b) indicates that $\text{Ins}(1,3,4)\text{P}_3$ increases after the increase in $\text{Ins}(1,4,5)\text{P}_3$, which is similar to the sequence found in HL-60 cells.

It will be of interest, in future studies, to determine whether $[\text{Ca}^{2+}]_i$ in J774.1 cells is increased after adherence, and whether this is related to the increases in $\text{Ins}(1,4,5)\text{P}_3$ described in this paper. A paper by Kruskal & Maxfield (1987) demonstrated that adherence of mouse thioglycollate-induced peritoneal macrophages to either opsonized or unopsonized tissue-culture plates induces transient increases in $[\text{Ca}^{2+}]_i$. In a large percentage of the cells studied, $[\text{Ca}^{2+}]_i$ oscillated after adherence. In addition to changes in $[\text{Ca}^{2+}]_i$, the adherence and spreading of macrophages are associated with a redistribution of actin (Amato *et al.*, 1983). Interestingly, Janmey *et al.* (1987) have shown that gelsolin, the Ca^{2+} -activated actin-binding protein, binds to phosphatidylinositol bisphosphate. This binding inhibits the filament-severing activity of gelsolin and dissociates gelsolin-actin complexes. The changes in actin that occur

after adherence may be mediated by both a rise in $[Ca^{2+}]_i$ and hydrolysis of phosphatidylinositol bisphosphate, resulting in a subsequent increase in $InsP_3$.

Adherence may be acting via a non-receptor-mediated pathway to generate $Ins(1,4,5)P_3$. Conversely, the membrane perturbations occurring during adherence may cause the rearrangement and thereby the activation of receptors linked to phospholipase C, or adherence may be acting to induce the activation of phospholipase A_2 . In macrophages the generation of arachidonic acid through activation of phospholipase A_2 requires Ca^{2+} (Wightman *et al.*, 1981), an thromboxane, an arachidonic acid metabolite, has been shown to generate $Ins(1,4,5)P_3$ via receptor-mediated activation of phospholipase C (Rittenhouse, 1985).

The relationship of the adherence-induced changes in inositol metabolism to macrophage function is unknown. A variety of changes occur in macrophages after adherence. Rabbit and human blood monocytes with no cytochemically observable peroxidase activity in rough endoplasmic reticulum will develop such activity within 2 h post-adherence (Bodel *et al.*, 1977). Adherent rabbit lung macrophages transport lysine and adenosine at faster rates than do suspended cells (Pofit & Strauss, 1977). Human blood monocytes lose FMC-17-antigen expression after 4 h in suspension and regain expression after adherence (Triglia *et al.*, 1985). In J774.1 macrophages, both the resting membrane potential (Sung *et al.*, 1985) and the K^+ conductances (Gallin & Sheehy, 1985) change after adherence. Future studies are needed to determine if the adherence-induced alterations in secretory capacity, antigen expression, enzyme appearance, nutrient uptake and membrane electrophysiology that have been described in macrophages are mediated by an increase in $InsP_3$ contents.

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