Inositol phosphate production and Ca^{2+} mobilization in human umbilical-vein endothelial cells stimulated by thrombin and histamine

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Human umbilical-vein endothelial cells (HUVECs) were cultured, and their inositol phosphate formation and Ca^{2+} mobilization in response to thrombin and histamine were studied. Evidence from measurement of intracellular Ca^{2+} in the absence of extracellular Ca^{2+} established that the two agonists were both acting on a single cell population, and suggested that a Ca^{2+} -influx component was stimulated which was dependent on receptor-occupancy. After 30 s of stimulation in the presence of 10 mM-LiCl, the effects of 20 μM -histamine and 1 unit of thrombin/ml on formation of inositol phosphates were additive, but at 5 min they were not. HUVECs labelled with *myo*-[^3H]inositol for 72 h synthesized radiolabelled inositol pentakis- and hexakis-phosphate. The predominant isomers of inositol mono-, bis- and tris-phosphates whose formation was stimulated were the 4-phosphate, the 1,4-bisphosphate and the 1,3,4-trisphosphate.

INTRODUCTION

Endothelial cells play a central role in the maintenance of normal vascular homoeostasis, by providing a continuous barrier between the blood circulation and all other body tissues. In response to specific humoral agents, for example histamine, thrombin, bradykinin and ATP, endothelial cells synthesize and secrete prostacyclin [1–4], which is a potent vasodilator and an inhibitor of blood platelet activation [5]. Endothelial cells are also reported to contract in response to histamine and thrombin [6,7], an effect which would cause an increase in vascular permeability *in vivo*. Both synthesis of prostacyclin and endothelial-cell contractility are probably controlled by agonist-dependent elevations in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), the most likely mechanisms being respectively through activation of phospholipase A_2 [8] and myosin light-chain kinase.

It is now established that receptor-mediated generation of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] from phosphatidylinositol 4,5-bisphosphate in the plasma membrane is a means by which some hormones and neurotransmitters can promote the discharge of Ca^{2+} from intracellular stores (for reviews see [9,10]). Recent studies using cultured endothelial cells from human umbilical vein (HUVECs), loaded with Ca^{2+} -indicating fluorescent dyes such as quin-2 or fura-2, have demonstrated that both histamine and thrombin stimulate an elevation in $[\text{Ca}^{2+}]_i$ [6,7,11]. In model systems such Ca^{2+} signals are comprised of an initial transient, presumably representing mobilization of intracellular stores evoked by $\text{Ins}(1,4,5)\text{P}_3$, and an influx component, which recent studies suggest could be controlled, at least in part, by inositol 1,3,4,5-tetrakisphosphate [12,13].

While the present experiments were in progress,

independent studies appeared which showed that both histamine and thrombin were able to stimulate the production of inositol phosphates in HUVECs [14–16]. In the present study we confirm these observations, and extend them to show that a variety of inositol phosphate isomers are generated during receptor stimulation, that these two agonists are acting on the same cell populations, and that at early time points their effects are additive. Hence, in addition to being cells of interest in their own right with respect to vascular homoeostasis, HUVECs are a cell type well suited to studying the interaction of different receptors coupled to the same intracellular signalling system.

MATERIALS AND METHODS

Materials

Human umbilical cords were obtained from the Rosie Maternity Hospital, Cambridge (U.K.). Human thrombin (3000 units/mg), histamine dihydrochloride and collagenase (type II) were from Sigma. Penicillin (5000 units/ml)/streptomycin (5000 $\mu\text{g}/\text{ml}$), foetal-calf serum and trypsin (0.05%)/EDTA (0.02%) were obtained from Flow Laboratories. Medium 199 with Earl's salts, NaHCO_3 and L-glutamine was obtained from Gibco.

The acetoxymethyl ester of fura-2 was from Molecular Probes (Junction City, OR, U.S.A.). Accell QMA SEP-PAKs (lot no. P5270A1) were from Waters Associates (Harrow, Middx., U.K.). Freon (1,1,2-trichlorotrifluoroethane) and tri-n-octylamine were from BDH. *myo*-[2- ^3H]inositol (10–20 Ci/mmol), [^{14}C]Ins(3) P and [^3H]Ins(1,4,5) P_3 were purchased from Amersham International.

Abbreviations used: InsP_1 , InsP_2 , InsP_3 etc., *myo*-inositol mono-, bis- and tris- (etc.) phosphates respectively, with isomeric numbering (all D) as appropriate; GroPIns , glycerophosphoinositol; HUVECs, human umbilical-vein endothelial cells; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concn.

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Preparation of cells

Human umbilical cords (15–20 cm) were collected and stored for up to 12 h at 4 °C in medium 199 containing penicillin (50 units/ml)/streptomycin (50 µg/ml). Venous endothelial cells were harvested by collagenase digestion as originally described by Jaffe *et al.* [17]. Briefly, veins were cannulated and flushed with 20 ml of sterile phosphate-buffered saline (138 mM-NaCl, 2.7 mM-KCl, 0.5 mM-MgCl₂, 1.47 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄, pH 7.5). The cord was then clamped at one end, the vein was filled with 10–15 ml of collagenase (0.5 mg/ml dissolved in medium 199), and then the cord was incubated at 37 °C for 10 min. Detached endothelial cells were flushed out, harvested by centrifugation (500 g for 5 min at 15–20 °C), and the cells were resuspended in 5 ml of medium 199 containing 20% (v/v) foetal-calf serum (FSM). The cells from each cord were plated out in Nunc 25-cm² tissue-culture flasks and incubated at 37 °C in an humidified atmosphere of air/CO₂ (19:1). Non-adherent cells and debris were removed by changing the medium after 4–6 h. Cells were then allowed to grow to confluence, achieving a contact-inhibited monolayer, with a characteristic 'cobblestone' appearance, within 3–5 days. At this stage each flask contained approx. 1.6×10^6 cells, representing about 1 mg of protein. Only cultures essentially free of fibroblast-like cells or other contaminants were used. Cells were subcultured by washing with phosphate-buffered saline, detaching the cells with 2 ml of trypsin/EDTA per flask (2 min at 37 °C), and then adding 0.5 ml of serum. The cells were harvested as above, resuspended in 4 ml of FSM per original flask and plated out in 35 mm-diam. Corning culture dishes (1 ml/dish). Alternatively, the cells were resuspended in 1 ml of FSM per original flask and plated out on 20 mm × 11 mm glass coverslips (0.2 ml/slip placed in a 35 mm-diam. Petri dish). Sub-cultured cells were then left in the incubator for at least 24 h to re-form contact-inhibited monolayers.

Fluorescence studies

For fura-2 fluorescence studies, HUVECs on coverslips were incubated for 45 min at 37 °C in 2 ml of FSM containing 2 µM-fura-2-acetoxymethyl ester, as described by Hallam & Pearson [11]. The medium was then aspirated and replaced with 2 ml of Hepes-buffered saline, consisting of 145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-Hepes and 10 mM-glucose, pH 7.4, and the cells were kept at room temperature until used. Coverslips were placed diagonally inside a cuvette (5 mm × 10 mm optical path length) containing 1.5 ml of Hepes-buffered saline. The cuvette was then placed in cuvette holder maintained at 37 °C in a Perkin-Elmer MPF 44A spectrofluorimeter with the 10 mm face orientated perpendicular to the emission path. Fluorescence was monitored continuously with excitation at 339 nm (6 mm slit width) and emission at 500 nm (10 mm slit width). After additions of receptor agonists, $F_{\max}$ was recorded in the presence of 1 mM external Ca²⁺ by lysing the cells with 50 µM-digitonin; cell auto-fluorescence ($F_{\text{Mn}^{2+}}$) was obtained by quenching the dye with 1 mM-MnCl₂. In the presence of EGTA, a small steady decline in fura-2 fluorescence was observed in HUVEC monolayers. This can be attributed to some leakage of dye from the patch of excited cells, as has been observed in aortic endothelial-cell monolayers [18]. As

this loss of fluorescence was linear with time, $F_{\max}$ was extrapolated back to compensate for any leakage of dye during the recording. $F_{\text{Mn}^{2+}}$ was unaffected, because under these conditions there is no contribution from the dye to the auto-fluorescence signal. Values for [Ca²⁺]_i were then calculated exactly as described previously [19].

Production of radiolabelled inositol phosphates

For studies of inositol phosphate production in HUVECs, the FSM in 35 mm dishes was replaced with 1 ml of medium 199 containing 5 µCi of [³H]inositol, and the cells were incubated at 37 °C for 24 h. Alternatively cells were incubated with 2 µCi of [³H]inositol for 72 h. After labelling, cells were washed with 2 × 2 ml of Hepes-buffered saline supplemented with 10 mM-LiCl and 1 mM-CaCl₂. The cells were preincubated for 5–10 min at 37 °C before the buffer was aspirated, and replaced with buffer containing receptor agonists where indicated. Incubations were terminated by aspirating the buffer and adding 1 ml of ice-cold 6% (v/v) HClO₄ to the dish, which was placed on ice. After 10 min, the lysed cells were scraped into an Eppendorf Microfuge tube and pelleted by centrifugation (12000 g for 2 min) in an MSE Microfuge; the supernatant was transferred to a conical glass centrifuge tube.

The acid extract was neutralized by the method of Sharpes & McCarl [20], as described by Wreggett & Irvine [21], by adding 950 µl of the supernatant to 1.8 ml of freshly prepared freon/octylamine (1:1, v/v). At this stage, phytate hydrolysate (25 µg of P) was added as recommended by Wreggett *et al.* [22]. This mixture was then vortex-mixed for 30 s, and then centrifuged (3000 g for 5 min). The upper phase, pH 6–6.5, containing the water-soluble radioactivity was diluted to 4 ml in distilled water and stored at –20 °C until analysis.

Analysis of radiolabelled inositol phosphates

Inositol phosphates extracted from HUVECs were fractionated by anion-exchange chromatography on Accell QMA SEP-PAKs (Waters Associates) as described by Wreggett & Irvine [21].

Samples were loaded on to a SEP-PAK, previously converted into the formate form, and were batch-processed by sequential elution with 10 ml each of water, 5 mM-Na₂B₄O₇, 0.1 M-ammonium formate/0.01 M-formic acid/5 mM-Na₂B₄O₇, 0.2 M-ammonium formate/0.02 M-formic acid/5 mM-Na₂B₄O₇, and 0.3 M-ammonium formate/0.03 M-formic acid/5 mM-Na₂B₄O₇, followed by 5 ml of 1 M-ammonium formate/0.1 M-formic acid, to elute inositol, GroPIIns, and mono-, bis-, tris- and then higher-phosphorylated forms of inositol respectively. The cartridge was then washed with 10 ml of water and the next sample was loaded. A 3.5 ml portion of each eluted fraction was added to 10 ml of Beckman X12 scintillation fluid, and radioactivity was determined in a Beckman LS2100 liquid-scintillation counter operating at 40% efficiency. The remaining portions of the eluted fractions were stored for further analysis. Before use, the elution profile of each SEP-PAK was verified by using [¹⁴C]InsP monophosphate and [³H]InsP₃ standards. For example, a mixture of approx. 250 d.p.m. of [³H]Ins-(1,4,5)P₃ and 300 d.p.m. of [¹⁴C]Ins(3)P was loaded on a SEP-PAK and eluted as described above. At least 90% of the ³H was recovered in the InsP₃ fraction and 100% of the ¹⁴C was recovered in the InsP₁ fractions. No

Fig. 1. Fluorescence records from fura-2-loaded HUVECs

Fura-2 fluorescence was recorded from HUVEC monolayers as described in the Materials and methods section. Where indicated, 1 mM-EGTA, (E); 20 μM -histamine (H); 1 unit of thrombin/ml (T) and 1 mM- CaCl_2 (C) was added to the cuvette. (a) Histamine in presence of 1 mM- CaCl_2 ; (b) and (c), stimulation in low extracellular Ca^{2+} by histamine followed by thrombin (b) and by thrombin followed by histamine (c). F_{max} was extrapolated back to compensate for dye leakage over the course of the recording. Calculated $[\text{Ca}^{2+}]_i$ values (nM) are indicated. Each trace is one of three similar records from one experiment. Identical results were observed in at least one other experiment.

significant radioactivity was recovered in any other fraction.

Analysis of inositol phosphate isomers

In some experiments, fractions of inositol phosphates eluted from SEP-PAKs were further analysed by isocratic anion-exchange h.p.l.c., with a Partisil SAX column and Na_2HPO_4 as the mobile phase. This enabled us to measure the distribution of inositol phosphate isomers in the SEP-PAK fractions. InsP_1 , InsP_2 and InsP_3 isomers were separated by elution with 0.04 M-, 0.24 M- and 0.55 M- Na_2HPO_4 (pH 3.8) respectively. More detailed study and analysis of these isomers has been performed (K. A. Wregget & R. F. Irvine, unpublished work).

Analysis of higher inositol phosphates

Cells labelled with ^3H inositol for up to 72 h were extracted as above, and InsP_{1-6} were separated by h.p.l.c. exactly as described by Heslop *et al.* [23].

RESULTS AND DISCUSSION

Measurements of $[\text{Ca}^{2+}]_i$ and examination of the possibility of two cell populations

In the presence of external Ca^{2+} , stimulation of HUVECs with 20 μM -histamine resulted in a rapid transient increase in $[\text{Ca}^{2+}]_i$, within 1 min, followed by a sustained phase of $[\text{Ca}^{2+}]_i$ elevated above resting values, which was maintained for at least 5 min in the presence of the receptor agonist (Fig. 1a); similar fluorescence profiles were obtained with 1 unit of thrombin/ml (results not shown).

In the absence of external Ca^{2+} , both histamine (Fig. 1b) and thrombin (Fig. 1c) rapidly increased $[\text{Ca}^{2+}]_i$ in HUVEC monolayers, from a resting value of 130 nM to peak values greater than 2 μM . Thereafter, $[\text{Ca}^{2+}]_i$ returned to near-resting values within 2–3 min. We used these observations to determine whether thrombin and histamine were acting on the same cell population. After the initial receptor-mediated mobilization of Ca^{2+} , addition of the heterologous agonist caused no subsequent increase in $[\text{Ca}^{2+}]_i$ when thrombin followed histamine (Fig. 1b), and only a small increase in $[\text{Ca}^{2+}]_i$, to around 240 nM, where histamine followed thrombin (Fig. 1c). It is worth noting that there is no loss of the phasic response to the first agonist with time after addition of EGTA. For example, 20 μM -histamine could still evoke a $[\text{Ca}^{2+}]_i$ transient to a peak of 2 μM if the agonist was added 5 min after EGTA addition (results not shown). These results indicate that over 95% of the dischargeable intracellular Ca^{2+} stores were released by the first agonist, which argues strongly that both agonists are acting on the same population of cells.

After stimulation of the cells with a receptor agonist for 5 min, restoration of the external $[\text{Ca}^{2+}]$ to 1 mM caused the $[\text{Ca}^{2+}]_i$ to rise rapidly to 1.5–2 μM (Figs. 1b and 1c). This suggests that, provided that the agonist is still present, there is a receptor-regulated Ca^{2+} influx into the cytosol. This stimulated Ca^{2+} entry is unlikely to be regulated by cytosolic Ca^{2+} (because $[\text{Ca}^{2+}]_i$ returned to resting values before the extracellular Ca^{2+} was restored; ref. [24], and see refs. [25–27]).

One significant difference in the fluorescence evoked by histamine and thrombin is that a small secondary transient with a maximum value of around 150 nM was

Table 1. Histamine and thrombin stimulation of inositol phosphates in HUVEC monolayers

HUVEC monolayers were exposed to buffer in control cells, or to 20 μ M-histamine, 1 unit of thrombin/ml or combined agonists, for either 30 s (Expt. *a*) or 5 min (Expt. *b*). Inositol phosphates were extracted and fractionated by using Accell QMA SEP-PAKS as described in the Materials and methods section. The results shown (total d.p.m./sample) are mean values $\pm$ s.e.m. of triplicate determinations. Similar results were observed in two other identical experiments. Statistical analysis was by Student's *t* test: with **P* < 0.05, ***P* < 0.01, ****P* < 0.001 for agonists versus control cells. In addition, *P* < 0.05 for combined agonists versus single agonists are indicated by Σ .

		GroPIIns	InsP ₁	InsP ₂	InsP ₃	InsP ₄₋₆
(a) 30 s	Control	1927 $\pm$ 204	1446 $\pm$ 89	141 $\pm$ 23	347 $\pm$ 54	195 $\pm$ 42
	Histamine	1015 $\pm$ 35	1957 $\pm$ 74 *	815 $\pm$ 99 ***	832 $\pm$ 61 ***	167 $\pm$ 23
	Thrombin	1311 $\pm$ 172	1882 $\pm$ 154	672 $\pm$ 29 ***	801 $\pm$ 115 ***	196 $\pm$ 29
	Histamine + thrombin	1346 $\pm$ 154	2330 $\pm$ 222 ***	1518 $\pm$ 75 *** Σ	459 $\pm$ 28 Σ	509 $\pm$ 27 *** Σ
(b) 300 s	Control	380 $\pm$ 65	463 $\pm$ 45	195 $\pm$ 25	180 $\pm$ 38	178 $\pm$ 2
	Histamine	622 $\pm$ 89	3757 $\pm$ 139 ***	1117 $\pm$ 145 ***	547 $\pm$ 23 ***	242 $\pm$ 22 *
	Thrombin	593 $\pm$ 99	3708 $\pm$ 84 ***	1135 $\pm$ 69 ***	493 $\pm$ 40 ***	240 $\pm$ 36
	Histamine + thrombin	548 $\pm$ 73	3560 $\pm$ 352 ***	1270 $\pm$ 244 ***	497 $\pm$ 95 *	189 $\pm$ 22

observed after stimulation with histamine (Fig. 1*b*). Whether this is a secondary Ca²⁺ signal or else some fluorescence artefact, perhaps as a consequence of cell contraction [6,7], is unclear. Notwithstanding this difference, the data obtained with fura-2 establish that both histamine and thrombin generate a similar maximum rise in [Ca²⁺]_i and that they are both acting predominantly on a single population of cells.

Inositol phosphate metabolism

Having established that histamine and thrombin appeared to evoke Ca²⁺ mobilization/influx on the same cells in our preparation (see above), the inositol phosphate profile in response to these two agonists, acting either alone or in combination, was investigated. Inositol phosphate production by HUVECs was examined at times after stimulation which would coincide with the fluorescence signal either near the peak of the transient (30 s; Fig. 1) or after the initial transient when Ca²⁺ influx could still be observed (5 min; Fig. 1).

Incubation of HUVECs for 30 s with either histamine or thrombin caused similar increases in the accumulation of InsP₁ and InsP₂; in these fractions the combined increases caused by each agonist appeared to be equivalent to the accumulation when the two agonists were added together (Table 1). Although this was most apparent in the InsP₂ fractions, changes in the InsP₁ fractions were not always statistically significant; the apparent increases in InsP₁ and the additivity were, however, consistently observed in other experiments at 30 s. This additivity at 30 s was not seen in the InsP₃ fraction (see also Table 2, below). Only with both agonists in combination was there any significant increase in the accumulation of InsP₄₋₆ over control values.

It would appear that the concentration of histamine used for these experiments (20 μ M) is about 90% of maximal for this agonist [15] and, although higher doses of thrombin (10 unit/ml) caused a higher inositol

phosphate production (results not shown), we were reluctant to increase the concentration of this agonist further to avoid thrombin's proteolytic activity. Also, 1 unit of thrombin/ml was maximal in evoking [³H]arachidonate release from HUVECs (results not shown). Thus we cannot say whether at maximal doses thrombin and histamine are additive (and consequently whether they have access to separate pools of inositol lipid substrate); at the concentrations used in this study, the responses to histamine and thrombin are additive at 30 s, and this provides us with a system for studying possible interactions between the two agonists.

In HUVECs that have been stimulated with either histamine or thrombin for 5 min, much larger increases were seen in the accumulation of InsP₁, InsP₂ and InsP₃, and a small (20–25%) but reproducible increase in InsP₄₋₆ was also measured (Table 1*b*). At this time the effects of the two agonists were not at all additive, perhaps owing to depletion of a common substrate. Alternatively, it may be that some of the phosphatases involved in breaking down the higher inositol phosphates become saturated such that either agonist acting alone generates maximum amounts of InsP₁ and InsP₂.

Isomers of inositol phosphates

Analysis of inositol phosphate isomers by h.p.l.c. (see the Materials and methods section) revealed that Ins(4)P, Ins(1,4)P₂ and Ins(1,3,4)P₃ are the predominant InsP₁, InsP₂ and InsP₃ isomers occurring 30 s after agonist addition (Table 2). The predominant accumulation of Ins(1,4)P₂ and Ins(4)P in response to both histamine and thrombin indicates that the dephosphorylation of Ins(1,4,5)P₃ by Ins(1,4,5)P₃ 5-phosphatase [28,29] is the principal metabolic pathway in these cells, at least initially. The predominance of Ins(4)P over Ins(1)P in the presence of Li⁺ is consistent with previous observations in, for example, human platelets [30] and GH₃ cells [31]. The rapid accumulation of Ins(1,3,4)P₃

Table 2. Inositol phosphate isomers in HUVECs after 30 s stimulation with histamine and thrombin

Data, presented as total d.p.m./sample, are from a single experiment repeated once, and are mean values $\pm$ S.E.M. for duplicate incubations. HUVECs were stimulated for 30 s with 20 μM -histamine, 1 unit of thrombin/ml or a combination of both (HIS/THR) in the presence of 10 mM-LiCl. Ins(1/3) P indicates the enantiomeric pair of Ins(1) P and Ins(3) P , which cannot be resolved by the anion-exchange method applied in this analysis. The percentage of an isomer in its total inositol phosphate fraction [i.e. Ins(1,4,5) P_3 as % of total Ins P_3] is shown in parentheses. Note that the identification of all inositol phosphate isomers is based entirely on their elution relative to internal standards.

	Ins(1/3) P	Ins(4) P	Ins(1,3) P_2	Ins(1,4) P_2	Ins(3,4) P_2	Ins(1,3,4) P_3	Ins(1,4,5) P_3
Control	700 $\pm$ 120	210 $\pm$ 30 (22 %)	80 $\pm$ 24	190 $\pm$ 46 (51 %)	100 $\pm$ 72	30 $\pm$ 32	149 $\pm$ 4 (84 %)
Histamine	500 $\pm$ 68	1015 $\pm$ 10 (68 %)	110 $\pm$ 33	2500 $\pm$ 150 (93 %)	80 $\pm$ 80	900 $\pm$ 270	350 $\pm$ 16 (28 %)
Thrombin	735 $\pm$ 5	800 $\pm$ 160 (52 %)	80 $\pm$ 10	2030 $\pm$ 33 (91 %)	100 $\pm$ 48	610 $\pm$ 37	680 $\pm$ 74 (53 %)
HIS/THR	890 $\pm$ 33	1810 $\pm$ 80 (69 %)	150 $\pm$ 57	4800 $\pm$ 780 (96 %)	40 $\pm$ 44	790 $\pm$ 80	870 $\pm$ 31 (50 %)

relative to Ins(1,4,5) P_3 (as shown in Table 2) suggests that the Ins P_3 / P_4 pathway [32] is also active. In addition to providing information about the metabolic fate of inositol phosphates, further resolution of the SEP-PAK fractions reveals additivity in the Ins(4) P and Ins(1,4) P_2 isomers when histamine and thrombin are combined to stimulate HUVECs.

Accumulation of higher inositol phosphates

It has recently become clear that many cell types will incorporate [^3H]inositol into Ins P_5 and Ins P_6 when labelled for several days (e.g. refs. [23,27,33,34]). A typical elution profile of unstimulated HUVECs labelled for 72 h and analysed by h.p.l.c. as described by Heslop *et al.* [23] is shown in Fig. 2. It is clear that Ins P_5 and Ins P_6 are

Fig. 2. [^3H]Inositol phosphates in 72 h-labelled HUVECs

[^3H]Inositol phosphates were extracted and combined from three 35 mm Petri dishes of 72 h-labelled unstimulated HUVECs as described in the Materials and methods section. Individual [^3H]inositol phosphates were eluted from a Partisil SAX h.p.l.c. column with ammonium formate as described by Heslop *et al.* [23]. The positions of internal standards of [^{32}P]GroPIns P_2 (glycerophosphoinositol 4,5-bisphosphate) and [^{32}P]Ins(1,4,5) P_3 are marked, and also the elution position of [^3H]Ins(1,3,4,5) P_4 in a preceding run.

synthesized in these cells, although not to the same very high degree (compared with InsP_{1-4}) as is seen in some established cell lines (see, e.g., [27,33]) and in brain [34]. We have also observed in another experiment that the peak of InsP_4 activity did not exactly co-elute with an $\text{Ins}(1,3,4,5)\text{P}_4$ standard, a result similar to that obtained by Stephens *et al.* [35], and, in view of their observations, this peak is likely to be $\text{L-Ins}(1,4,5,6)\text{P}_4$ [= $\text{Ins}(3,4,5,6)\text{P}_4$], a precursor in the pathway to InsP_5 . In summary, HUVECs produce a complex pattern of inositol phosphate isomers, which includes InsP_5 and InsP_6 .

Conclusions

These results confirm and extend the observations by other groups (e.g. refs. [15–18]) which demonstrate that both histamine and thrombin can stimulate inositol phosphate formation and Ca^{2+} mobilization in HUVECs. This study also shows, however, that these agonists are acting on the same cell population, and that their effects on inositol phosphate formation can be additive.

Our results demonstrate that quantitative analysis of InsP isomers is possible in HUVECs; how histamine and thrombin may differ in the pattern of isomers that they produce, and how these agonists interact (if at all), requires further investigation.

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REFERENCES

1. Weksler, B. B., Ley, C. W. & Jaffe, E. A. (1978) *J. Clin. Invest.* **62**, 923–930
2. Hong, S. L. (1980) *Thromb. Res.* **18**, 787–795
3. MacIntyre, T. M., Zimmerman, G. A., Satoh, K. & Prescott, S. M. (1985) *J. Clin. Invest.* **76**, 271–280
4. Pearson, J. D., Slakey, L. L. & Gordon, J. L. (1983) *Biochem. J.* **214**, 273–276
5. Moncada, S., Gryglewski, R., Bunting, S. J. & Vane, J. R. (1976) *Nature (London)* **263**, 663–665
6. Rotrosen, D. & Gallin, J. I. (1986) *J. Cell Biol.* **103**, 2379–2387
7. Killakey, J. J. F., Johnston, M. G. & Morat, H. Z. (1986) *Am. J. Pathol.* **122**, 50–61
8. Hong, S. L. & Deykin, D. (1982) *J. Biol. Chem.* **257**, 7151–7154
9. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–317
10. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
11. Hallam, T. J. & Pearson, J. D. (1986) *FEBS Lett.* **207**, 95–99
12. Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
13. Morris, A. P., Gallacher, D. V., Irvine, R. F. & Peterson, O. H. (1987) *Nature (London)* **330**, 653–655
14. Resink, T. J., Grigorian, G. Y., Moldabaeva, A. K., Danilov, S. M. & Bühler, F. R. (1987) *Biochem. Biophys. Res. Commun.* **144**, 438–446
15. Lo, W. W. Y. & Fan, T. P. D. (1987) *Biochem. Biophys. Res. Commun.* **148**, 47–53
16. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) *J. Biol. Chem.* **262**, 8559–8565
17. Jaffe, E. A., Nacham, R. L., Becker, C. G. & Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2756
18. Pirotton, S., Raspe, E., Demolle, D., Erneux, C. & Boeynaems, J.-M. (1987) *J. Biol. Chem.* **262**, 17461–17466
19. Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) *Biochem. J.* **235**, 869–877
20. Sharpes, E. S. & McCarl, R. L. (1982) *Anal. Biochem.* **124**, 421–424
21. Wreggett, K. A. & Irvine, R. F. (1987) *Biochem. J.* **245**, 655–660
22. Wreggett, K. A., Howe, L. R., Moore, J. P. & Irvine, R. F. (1987) *Biochem. J.* **245**, 933–934
23. Heslop, J. P., Irvine, R. F., Tashjian, A. H., Jr. & Berridge, M. J. (1985) *J. Exp. Biol.* **119**, 395–401
24. Von Tscharner, V., Prod'homme, B., Baggiolini, M. & Reuter, H. (1986) *Nature (London)* **324**, 369–372
25. Irvine, R. F. (1987) *Nature (London)* **328**, 386
26. Merritt, J. E. & Rink, T. J. (1987) *J. Biol. Chem.* **262**, 17362–17369
27. Jackson, T. R., Hallam, T. J., Downes, C. P. & Hanley, M. R. (1987) *EMBO J.* **6**, 49–54
28. Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177
29. Connolly, T. M., Bross, T. E. & Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 7868–7874
30. Seiss, W., Stifel, M., Binder, H. & Weber, P. C. (1986) *Biochem. J.* **233**, 83–91
31. Hughes, P. J. & Drummond, A. H. (1987) *Biochem. J.* **248**, 463–470
32. Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
33. Tilly, B. C., Van Paridon, P. A., Verlaan, I., Wirtz, K. W. A., de Laat, S. W. & Moolenaar, W. H. (1987) *Biochem. J.* **244**, 129–135
34. Vallejo, M., Jackson, T. R., Lightman, S. & Hanley, M. R. (1987) *Nature (London)* **330**, 656–658
35. Stephens, L., Hawkins, T. P., Carter, N., Chahwala, S. B., Morris, A. J., Whetton, A. D. & Downes, C. P. (1988) *Biochem. J.* **249**, 271–282

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