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Bradykinin and thrombin effects on polyphosphoinositide hydrolysis and prostacyclin production in endothelial cells

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Prostacyclin (PGI_2) production by thrombin- and bradykinin-stimulated bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) was related to the receptor-linked activation of inositide hydrolysis. Bradykinin caused a rapid and transient 3-fold increase in the formation of inositol polyphosphates in BAEC. The increase in InsP_3 reflected changes mainly in the Ins(1,4,5)P_3 isomer. Thrombin was less effective than bradykinin in increasing InsP_3 levels and appeared to only minimally stimulate the production of PGI_2 in BAEC. In HUVEC, thrombin caused a 5-fold elevation of Ins(1,4,5)P_3, closely related to a rise in PGI_2 production. However, bradykinin did not affect inositol phosphates and PGI_2 production in HUVEC. Other inositol phosphates were also assessed to obtain information on putative metabolism of Ins(1,4,5)P_3. The present study supports the notion that formation of Ins(1,4,5)P_3 is linked to an increase in PGI_2 production in endothelial cells and furthermore provides evidence for a large degree of heterogeneity in the responses of BAEC and HUVEC to thrombin and bradykinin.

INTRODUCTION

The inositide cycle as a second messenger system for various stimuli is recognized in many tissues [1–3]. While the precise nature and sequence of post-receptor events are in part yet to be elucidated for the various cell types, different receptors mediate the activation of a common set of biochemical pathways. One of the earliest events detected is the activation of a phosholipase C (PLC) which hydrolyses PtdIns(4,5)P_2 to Ins(1,4,5)P_3 and diacylglycerol (DG). Ins(1,4,5)P_3 mobilizes calcium from the endoplasmic reticulum [1,4]. The increase in cytoplasmic calcium may stimulate phospholipase A_2 (PLA_2) and DG-lipase activity [5,6]. DG alternatively activates protein kinase C by increasing its sensitivity to calcium [4]. PLA_2 may also be activated through protein kinase C, involving phosphorylation of lipocortin [7]. Both PLA_2 and PLC–DG-lipase pathways can be involved in the liberation of arachidonate. The relative contributions of each pathway and the different phospholipid substrates in the liberation of arachidonate are however not yet clear. The protein kinase C and calcium signals might also be involved in mediating mitogenic stimuli [1].

Stimulation of several types of endothelial cells with thrombin, bradykinin, ATP or trypsin (8–10) can result in the release of the arachidonate metabolite prostacyclin (PGI_2). Thrombin and bradykinin exhibit heterogeneity with regard to endothelial cell prostacyclin synthesis [11]. In human umbilical vein endothelial cells, thrombin has been shown to stimulate the release of PGI_2, as well as rapid rise in InsP_3 and calcium levels [8,12]. Stimulation of InsP_3 production and PGI_2 release was also found in bradykinin-stimulated bovine (BAEC) [9] and pig aortic endothelial cells [13]. However, in BAEC [14] and also in human microvascular endothelial cells [15], thrombin was found not to stimulate PGI_2 release. BAEC nevertheless contain binding sites for thrombin [16,17], which mediate the decrease in intracellular plasminogen activator [18], an increase in calcium influx and induction of ornithine decarboxylase [19]. The involvement of inositol phosphates in the differential responsiveness of endothelial cells has so far not been addressed in detail.

In the present study we have investigated and compared inositide metabolism and the production of PGI_2 in response to thrombin and bradykinin stimulation of BAEC and human umbilical vein endothelial cells (HUVEC). Furthermore, we present a first extensive analysis of the individual inositol phosphates in these cells.

MATERIALS AND METHODS

Materials

Bradykinin triacetate, bovine (200 NIH units/mg of protein) and human thrombin (3000 NIH units/mg of protein), antithrombin III (400–600 units/mg of protein), AMP, ADP and ATP were purchased from Sigma. *myo*-[2-^{3}H]Inositol [1 mCi/ml in water/ethanol (9:1, v/v)], D-*myo*-[2-^{3}H]Ins1P D-*myo*-[2-^{3}H]Ins(1,4)P_2 D-*myo*-[2-^{3}H]Ins(1,4,5)P_3 and D-*myo*[2-^{3}H]Ins(1,3,4,5)P_4 were from Amersham. Tissue culture media [Hanks' balanced salt solution (HBSS), medium-199, minimal essential medium] and fetal calf serum (FCS) were purchased from Gibco Laboratories; endothelial cell growth supplement, and human fibronectin were from Sigma. Acetylated-low density lipoprotein labelled with 1,1′-dioactadecyl-1,3,3,3′-tetramethylindocarbocyanine perchlorate (Dil-

Abbreviations used: PGI_2, prostacyclin; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; HUVEC, human umbilical vein endothelial cell; BAEC, bovine aortic endothelial cell; PLC, phospholipase C; PLA_2, phospholipase A_2; DG, 1,2-diacylglycerol; Dil-Ac-LDL, acetylated low-density-lipoprotein labelled with 1,1′-dioactadecyl-1,3,3,3′-tetramethylindocarbocyanine perchlorate; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum.

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Ac-LDL) was purchased from Biomedical Technologies Inc. (Sloughton, U.S.A.). Thrombin–antithrombin III complex was produced by incubation of equimolar amounts of thrombin and antithrombin III for 20 min at 37 °C in the presence of a catalytic amount of heparin.

Cell culture

BAEC, isolated as described [17], were grown in minimal essential medium containing 10% FCS, and used in passages 6–15. HUVEC were isolated as described [20] and used in passages 1–5. After collagenase digestion of the umbilical vein, cells were plated on fibronectin-coated surfaces in Medium-199 containing 10% FCS and 150 μg of endothelial growth supplement/ml. To obtain subcultures, cells were harvested after being grown to confluency using 0.25% trypsin with a split-ratio of 1:5 (BAEC) or 1:2 (HUVEC). For experiments, cells were plated on 6-well plates (Costar) and grown to confluency.

The individual cultures were defined as endothelial cells by their typical morphology and their Dil-Ac-LDL uptake employing a fluorescence-activated cell sorter (kindly made available to us by Dr. Claus Lennartz; University of Essen, Germany). Furthermore, in HUVEC, we have previously shown the release of Factor VIII-related antigen in earlier cultures similarly prepared [20].

Measurement of inositol polyphosphate production

Confluent cell cultures were labelled with 10 μCi of ^{3}[H]inositol/ml in Medium-199 without inositol and containing 1% FCS for 36 h. The monolayers were washed once with HBSS and incubated for 10 min in this medium (1 ml) also containing 10 mM-$LiCl_2$. Bradykinin and thrombin in HBSS (< 20 μl) were then added to the wells, and after appropriate times the medium was removed by suction, followed by immediate addition of trichloroacetic acid (10%) to the monolayers. Denatured cells were then collected by scraping and particulate material was subsequently removed by centrifugation (400 **g**). Trichloroacetic acid was then removed from the supernatant by extraction (four times) with diethyl ether. Samples were diluted in 1 vol. of water, and placed on an AG 1-X8 column (BioRad) and eluted essentially as described [21]. After washing the column with 16 ml of 0.1 M-formic acid, the column was eluted stepwise with increasing concentrations of ammonium formate in 0.1 M-formic acid to obtain InsP_1 (0.2 M, 10 ml), InsP_2 (0.4 M, 8 ml) and InsP_3 (1.0 M, 6 ml). Radioactivity was assessed in 2 ml fractions added to 16 ml of Quickszint 2000 (Zinsser), using a Beckman scintillation counter. Counting efficiency (39%) was determined separately with an internal standard.

For h.p.l.c. analysis, samples, after preparation as described above, were neutralized by addition of Tris base, and EGTA and inositol were added to give final concentrations of 1 mM and 50 μM respectively. Inositol phosphates were then separated by h.p.l.c. anion-exchange chromatography [22], using a Whatman Partisil PXS 10/25 SAX column. Briefly, the column was eluted with a 60 ml discontinuous gradient from 0–3.5 M-ammonium formate/0.5 M-phosphoric acid (pH 3.7), with 1.5 ml/min flow rate. The effluent was continuously monitored by a radioactive flow detector (Berthold), which performed automatic peak integration. The counting efficiency was 39% for Ins1P; 37% for Ins(1,4)P_2; 32% for Ins(1,4,5)P_3 and 21% for Ins(1,3,4,5)P_4. For internal calibration of the column, AMP, ADP and ATP were added to selected samples, and absorption at 254 nm was followed during the elution. The column was further calibrated with the ^{3}H-labelled inositol phosphates Ins1P, Ins(1,4)P_2, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4.

Measurement of 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) production

BAEC and HUVEC were plated on 24-well tissue culture plates and grown to confluency. After a preincubation in 0.2 ml of HBSS for 5 min, bradykinin or thrombin were added in 5–10 μl, and the cells were further incubated for the various times indicated. The amount of PGI_2 in the medium was assessed via its stable metabolite 6-keto-$PGF_{1\alpha}$ by radioimmunoassay [12].

RESULTS

Stimulation of inositol phosphate production by thrombin and bradykinin in BAEC

Cultured BAEC labelled with [^{3}H]inositol were stimulated with bradykinin and bovine thrombin. After extraction, inositol phosphates were separated on h.p.l.c. Elution of the column with a discontinuous gradient of 0–3.5 M-ammonium formate/0.5 M-phosphoric acid, pH 3.7, yielded separation of several ^{3}H-labelled peaks. Under unstimulated conditions, the Ins1P peak represented the majority of radioactivity found in inositol phosphates. This h.p.l.c. method gave a separation of two monophosphate isomers; the first eluted was identified as Ins1P, and the second, eluting just after it, as the putative Ins4P as described also in other tissues [23]. Ins(1,4)P_2 and Ins(1,4,5)P_3 could also be detected and were identified by co-elution with appropriate standards. In addition, another InsP_2 isomer was also eluted following Ins(1,4)P_2. The amount of this InsP_2 isomer was found to be much less than that of Ins(1,4)P_2. Based on its retention time compared with Ins(1,4)P_2, this InsP_2 was putatively identified as Ins(3,4)P_2 [24]. Two forms of InsP_3 were also distinguished, the Ins(1,4,5)P_3 isomer and an immediately preceding peak, which co-eluted with ATP as Ins(1,3,4)P_3 [25]. InsP_4 was hardly detectable under unstimulated conditions. The Partisil-10 SAX column could not really separate the various InsP_4 forms; consequently, the InsP_4 peak at the resting state probably represented, in addition to Ins(1,3,4,5)P_4, both Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4, as described in other tissues [26]. InsP_4 was followed by two additional peaks which presumably represented InsP_5 and InsP_6 as characterized by Heslop *et al.* [27].

Bradykinin evoked a marked, rapid (Fig. 1) and dose-dependent (Fig. 2) increase in the levels of inositol phosphates. The rapid rise in the level of Ins(1,4,5)P_3 was associated with an even larger increase in the amount of Ins(1,4)P_2. A rise in the InsP_4 peak was also observed, suggesting that bradykinin also induced the Ins(1,4,5)P_3 kinase pathway. The rise in InsP_4 was very likely to be due to an increase in the Ins(1,3,4,5)P_4 level, identified by using the labelled standard. The real scale of increase in Ins(1,3,4,5)P_4 on stimulation is probably underestimated by measuring just total InsP_4 levels at resting state. With regard to the inositol monophosphates, the Ins4P level was already increased at 30 s stimulation time (Fig. 2, inset), but no significant change was found in the amount of Ins1P.

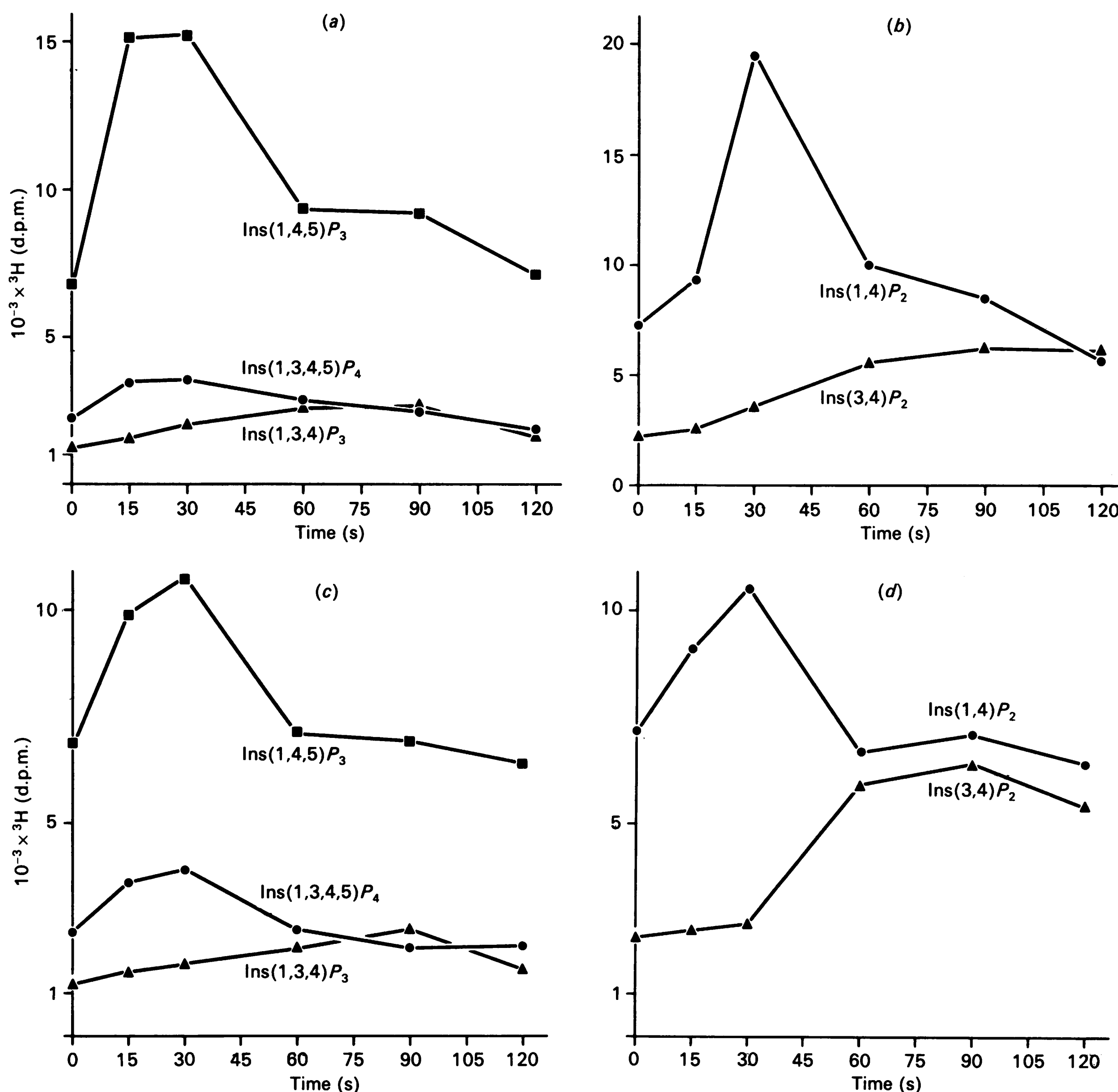

Fig. 1. Kinetics of the production of inositol phosphates in bradykinin and thrombin-stimulated BAEC

Bradykinin (10^{-6} M) or thrombin (2 units/ml) was added (time 0) to the cultures prepared as described under Materials and methods. The reaction was terminated at the times indicated and samples were analysed on h.p.l.c. The eluent was collected in fractions (0.3 min, 370 μl) and the radioactivity in each fraction was determined in 3 ml of Quickscint 2000 using a Packard scintillation counter. (*a*), (*b*), Bradykinin-stimulated BAEC; (*c*), (*d*), thrombin-stimulated BAEC. Each point represents the mean of duplicate determinations with a mean variation of 10%. Data are representative of four similar experiments.

Thrombin (2 units/ml) caused a smaller increase in the level of Ins(1,4,5)P_3 than did bradykinin, and this increase was also associated with a moderate elevation in the amount of Ins(1,4)P_2 (Fig. 1). The amount of Ins4P was hardly affected by thrombin stimulation for 30 s (Table 1), while significant increases in Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 levels were measured (Fig. 1).

Time course of production of inositol phosphates in BAEC

As shown in Fig. 1(*a*) Ins(1,4,5)P_3 increased rapidly within 15 s of exposure to bradykinin, and reached a maximum of 2.5–3 times the basal level. Within 2 min, Ins(1,4,5)P_3 returned to the unstimulated level. The increase in the amount of Ins(1,3,4,5)P_4 followed essentially the same kinetics as with Ins(1,4,5)P_3, while Ins(1,3,4)P_3 peaked at 1 min, consistent with its formation from Ins(1,3,4,5)P_4. A rapid and large rise was observed in the level of Ins(1,4)P_2 (Fig. 1*b*), with a slight delay as compared with changes in Ins(1,4,5)P_3. In contrast, a much more delayed response of the other InsP_2 isomer was found. One of the two monophosphate isomers, Ins4P, had increased considerably after 30 s of bradykinin stimulation, and was elevated 4-fold after 5 min (Table 1). The amount of the other monophosphate isomer, Ins1P, began to increase only after about 1 min. Ins1P was found to predominate over Ins4P, the ratio of the two monophosphates being 4.5:1 in the unstimulated and 2.5:1 in the stimulated state (Table 1).

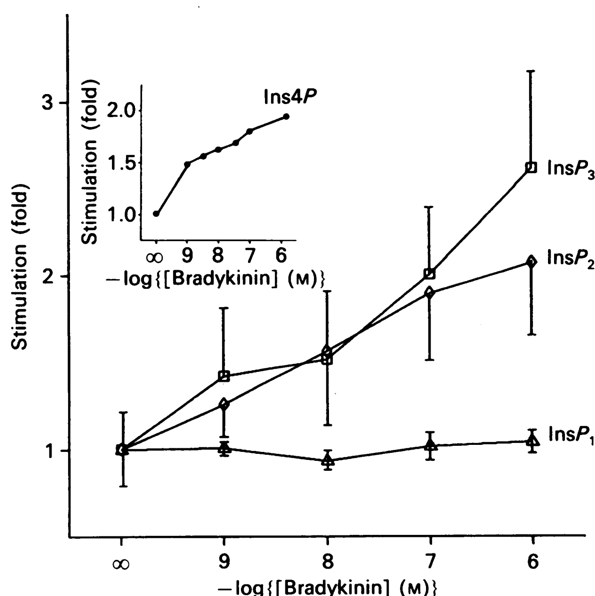

Fig. 2. Concentration-dependence of bradykinin stimulation of inositol phosphate production in BAEC

BAEC were stimulated for 30 s with different concentrations of bradykinin. ^{3}H-labelled inositol phosphates were analysed using AG 1-X8 column separation as described in Materials and methods. Each point represents the mean (±S.E.M.) of the stimulation ratios, measured in three independent experiments, each performed in duplicate. The inset shows Ins4P levels in a different set of experiments, where inositol phosphates were analysed by h.p.l.c. after stimulation with the different concentrations of bradykinin. The eluent was continuously monitored by a radioactive flow detector, and each point represents a mean of duplicate determinations with a mean variation of 10%. Data are representative of two experiments. In these experiments we found similar dose-dependent changes of Ins(1,4,5)P_3 and Ins(1,4)P_2 to those of total InsP_3 and InsP_2 determined with the AG 1-X8 column.

Table 1. Effect of bradykinin and thrombin on inositol monophosphates in BAEC

Bradykinin (10^{-6} M) and thrombin (2 units/ml) was added, and 30 s or 5 min later, the samples were extracted and analysed on h.p.l.c. The data represent integrated radioactivity belonging to Ins1P and Ins4P, measured by a continuous radioactive flow detector and corrected with the counting efficiency described in the Materials and methods section. Data are representative of two similar experiments, each performed in duplicate.

	^{3}H (d.p.m.)			
Time...	30 s		5 min	
Isomer...	Ins1P	Ins4P	Ins1P	Ins4P
Control	25701	5765	26171	5614
Thrombin	25845	6318	27433	12201
Bradykinin	27131	11148	33922	22339

The thrombin-induced changes differed from those of bradykinin in the formation of the relative amounts of individual inositol phosphates and also in the time course of their degradation. Ins(1,4,5)P_3 increased rapidly within 15 s, but reached a maximum of only 1.5 times the basal level (Fig. 1c). Furthermore, after 60 s of thrombin stimulation, no difference as compared with the control was found in the amount of Ins(1,4,5)P_3, whereas this metabolite was still elevated until about 90 s after bradykinin stimulation. Ins(1,4,5)P_3 was converted to Ins(1,3,4,5)P_4, as was seen after bradykinin stimulation. Ins(1,3,4,5)P_4 was dephosphorylated to Ins(1,3,4)P_3, the latter being maximally stimulated by 2-fold at 90 s. Changes in the amount of the Ins(1,4)P_2 isomer (1.5-fold increase over basal level) followed the same kinetics as those of its precursor, Ins(1,4,5)P_3 (Fig. 1d). After a rapid 1.5-fold rise, Ins(1,4)P_2 levels decreased to the basal level. Ins(1,3,4)P_3 was further metabolized to give another InsP_2 isomer, probably Ins(3,4)P_2. The formation of this isomer was much delayed compared with Ins(1,4)P_2 formation.

Ins4P was hardly affected at 30 s after thrombin stimulation, and was elevated only 2-fold after 5 min (Table 1). The amount of Ins1P was unchanged. The two polyphosphorylated compounds eluting after Ins(1,3,4,5)P_4, tentatively identified as InsP_5 and InsP_6, were found to be not affected by either thrombin or bradykinin (results not shown).

Stimulation of inositol phosphate production by thrombin and bradykinin in HUVEC

In contrast to BAEC, thrombin markedly stimulated phosphoinositide hydrolysis in HUVEC. The h.p.l.c. patterns of labelled inositol phosphates in unstimulated HUVEC and BAEC were very similar. In HUVEC, a 5–6-fold stimulation of InsP_3 and InsP_2 production was achieved with 2 units of thrombin/ml (Fig. 3), whereas in BAEC, minimal changes in total inositol phosphates (1.5 times over basal level) had been observed. An example of the elution profile on h.p.l.c. is shown in Fig. 4. A selective increase in Ins4P could also be measured by h.p.l.c. (Fig. 4); InsP_5 and InsP_6 did not change after short incubation times. Pretreatment of thrombin with antithrombin III completely suppressed the ability of thrombin to induce rises in inositol phosphates. In contrast to thrombin, bradykinin (up to 1 μM) did not affect the concentrations of any inositol phosphates in HUVEC (results not shown).

Bradykinin and thrombin stimulation of prostacyclin formation

Bradykinin stimulated the production of PGI_2 (assessed as 6-keto-$PGF_{1\alpha}$) in BAEC (Fig. 5a). The increase in PGI_2 production has ceased by 5 min, and there was no further increase after this time (Fig. 5a, inset). The decline in the amount of 6-keto-$PGF_{1\alpha}$ after 8 min indicates binding or metabolism of PGI_2 or of its stable hydrolysis product 6-keto-$PGF_{1\alpha}$ by the cells. Although some conversion of PGI_2 to 6-keto-$PGF_{1\alpha}$ by cultured endothelial cells has been detected by others [28], whether this or other metabolic events take place under our conditions has not been elucidated. Bradykinin, however, did not affect prostacyclin levels in HUVEC (Fig. 5a). In BAEC, PGI_2 synthesis was only slightly increased by thrombin; in HUVEC on the other hand thrombin caused a concentration-dependent increase in PGI_2 pro-

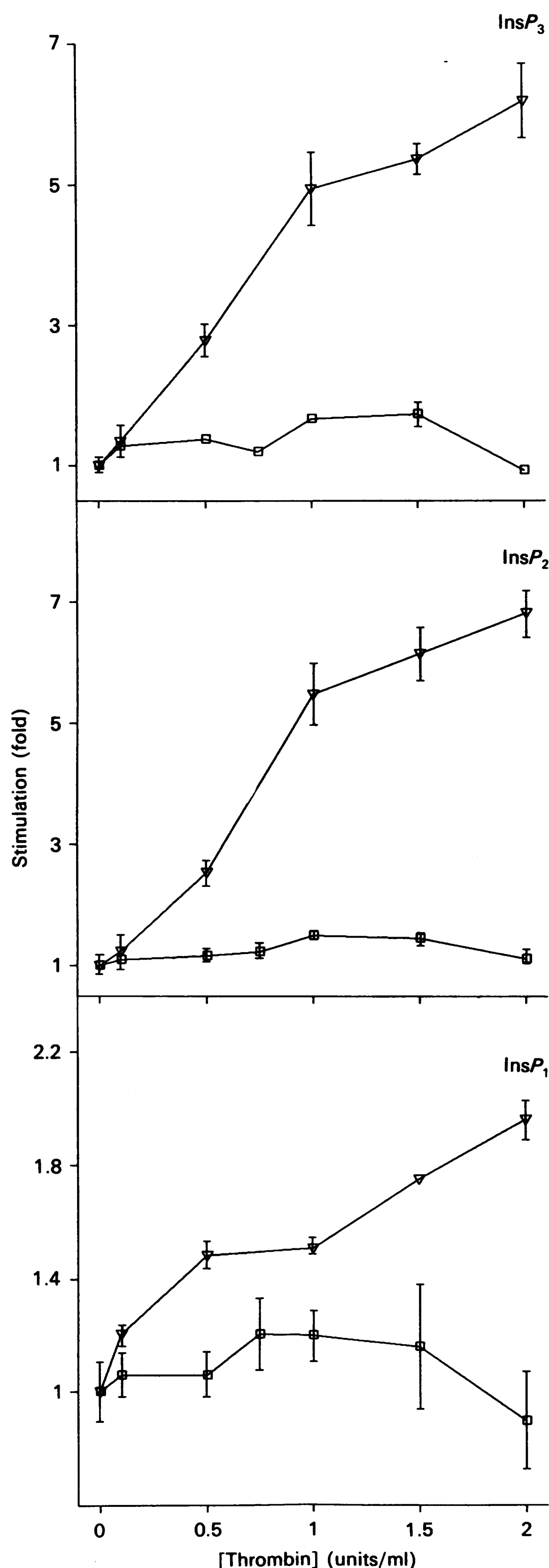

Fig. 3. Concentration-dependence of thrombin stimulation of inositol phosphate production in BAEC and HUVEC

BAEC (□) and HUVEC (▽) were stimulated with different concentrations of bovine and human thrombin respectively for 2 min. ^{3}H-labelled inositol phosphates were analysed using AG 1-X8 column separation as described in Materials and methods. Each point represents the mean (±S.E.M.) of the stimulation ratios in two independent experiments, each performed in duplicate.

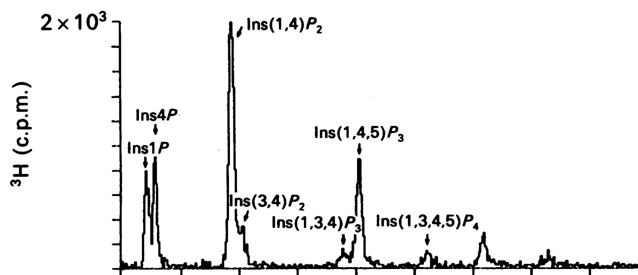

Fig. 4. H.p.l.c. elution profile of inositol phosphates from thrombin stimulated samples

HUVEC were labelled with [^{3}H]inositol for 36 h as described under Materials and methods. Thrombin (2 units/ml) was added for 30 s and the samples were analysed on h.p.l.c.; the eluent was continuously monitored by a radioactive flow detector. Ins1P, Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were identified using ^{3}H-labelled inositol phosphates. Ins4P, Ins(3,4)P_2 and Ins(1,3,4)P_3 were tentatively identified based on their retention times compared with labelled standard.

duction, with 2 units/ml resulting in a 10-fold stimulation of PGI_2 release (Fig. 5*b*). Changes in InsP_3 levels and 6-keto-$PGF_{1\alpha}$ production closely paralleled each other in each case, except in bradykinin-stimulated BAEC. Here bradykinin was more potent in stimulating PGI_2 than InsP_3 production. As we found that most of the InsP_3 after bradykinin stimulation was Ins(1,4,5)P_3 and the fact that Ins(1,4,5)P_3 was metabolized quickly to Ins4P, meant that measuring the increase in Ins4P gave a better estimation of bradykinin-induced inositol polyphosphate turnover. Ins4P was 2-fold stimulated at 10^{-6} M-bradykinin, and about 50% stimulation was reached at 10^{-9} M (Fig. 2, inset). This change in Ins4P concentration correlates well with changes in the prostacyclin release.

DISCUSSION

The present study demonstrates the production and metabolism of Ins(1,4,5)P_3 and other inositol polyphosphates in agonist-stimulated endothelial cells. Recent studies in several other cell types have analysed the reactions by which inositol phosphates are processed, but there is relatively little information about endothelial cells. The inositol phosphates were analysed in two endothelial cell types, BAEC and HUVEC, which differ substantially in their physiological responsiveness. While HUVEC appeared unresponsive to bradykinin, stimulation of BAEC with this ligand resulted in a rapid and transient increase in InsP_3 levels. The protease thrombin was found to act differently in HUVEC and BAEC with regard to production and metabolism of inositol phosphates. In HUVEC, thrombin markedly stimulated the production of InsP_3, mostly reflecting the generation of the Ins(1,4,5)P_3 isomer. In BAEC, however, thrombin caused only minimal changes in inositol polyphosphate metabolism. This finding is not completely in agreement with recent studies by Jaffe *et al.* [8], who could not find any effect of thrombin in elevating inositol phosphates or mobilizing intracellular calcium (determined with quin-2) in BAEC. This however may be due to their use of human rather than bovine thrombin, and also to their use of a cloned cell population.

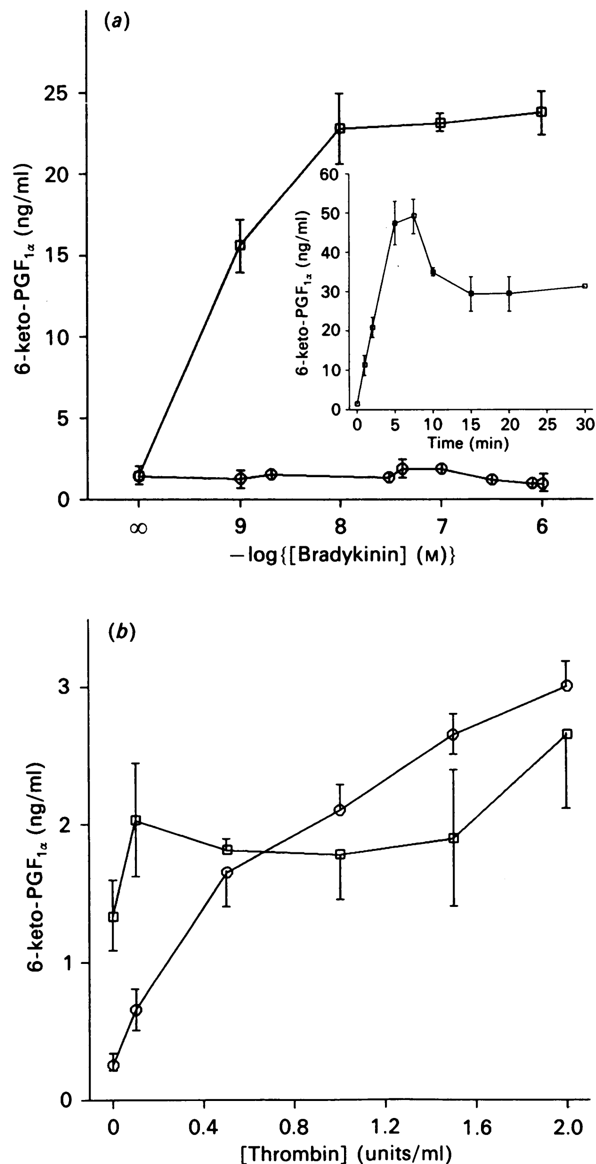

Fig. 5. Bradykinin (*a*) and thrombin (*b*) stimulation of prostacyclin production in BAEC and in HUVEC

BAEC (□) and HUVEC (○) were stimulated with different concentrations of bradykinin (*a*) or thrombin (*b*) for 5 min, and the PGI_2 content in the supernatant was analysed by r.i.a. for 6-keto-$PGI_{1\alpha}$ as described in Materials and methods. Each point represents the mean (±S.E.M.) of the amount of 6-keto-$PGF_{1\alpha}$ of three independent experiments, each performed in duplicate. The time-dependent accumulation of 6-keto-$PGF_{1\alpha}$ after bradykinin stimulation in BAEC is shown in the inset of (*a*).

Recent studies have revealed that the Ins4*P* concentration rises faster than that of Ins1*P* in most agonist-stimulated tissues [23,29]. On the other hand, in endothelial cells, bovine-brain-derived endothelial cell growth factor has been found to induce a rapid breakdown of PtdIns, with a parallel increase in Ins1*P* and DG levels, but no detectable change in the other inositol polyphosphates [30]. In view of these findings, and by analysing the inositol monophosphate isomers with high resolution, we could demonstrate that thrombin and bradykinin induced mainly the formation of Ins4*P* in BAEC and HUVEC, indicating that at the initial phase PLC is possibly acting mainly on PtdIns(4,5)P_2 without hydrolysis of PtdIns. Direct breakdown of PtdIns could be the source of the delayed increase in the Ins1*P* level.

In this study, a substantial increase in Ins(1,4,5)P_3 formation and subsequent inositol polyphosphate breakdown precedes changes in prostacyclin release. Throughout the different studies, we observed a concordance between Ins(1,4,5)P_3 formation and the subsequent production of prostacyclin, suggesting that activation of PLC mediates or regulates arachidonate liberation. The high selectivity with which the two different endothelial cell cultures responded to the two ligands thrombin and bradykinin by generating second messengers also provides a new perspective to study endothelial cell heterogeneity.

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