Metabolism of inositol phosphates in ATP-stimulated vascular endothelial cells

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The accumulation of InsP_1 , InsP_2 , InsP_3 and InsP_4 isomers was investigated in bovine aortic endothelial cells labelled with [^3H]inositol and stimulated with ATP. The separation of these isomers was performed by ion-pairing reverse-phase h.p.l.c. on a $\mu\text{Bondapak C}_{18}$ column for the InsP_3 and InsP_4 isomers and by ion-exchange h.p.l.c. on a Partisil SAX column for the InsP_1 and InsP_2 isomers. In unstimulated endothelial cells, a large amount of material was co-eluted with InsP_5 and InsP_6 , whereas amounts of InsP_3 and InsP_4 were small. The addition of ATP (100 μM) induced a striking (35-fold stimulation) and transient increase of $\text{Ins}(1,4,5)\text{P}_3$ that was maximal around 15 s. This peak was followed by a more sustained accumulation of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$, but the amounts of these two metabolites accumulated in response to ATP were much smaller than that of $\text{Ins}(1,4,5)\text{P}_3$. The increase in InsP_2 isomers in response to ATP had similar characteristics: a rapid and transient accumulation of $\text{Ins}(1,4)\text{P}_2$, followed by an increase of $\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}(1,3)\text{P}_2$, which was more sustained but had a smaller magnitude. ATP also induced the accumulation of both $\text{Ins}1\text{P}$ and $\text{Ins}4\text{P}$, but with different time courses: the level of $\text{Ins}4\text{P}$ was maximal at 1 min (60 times the control value) and returned to baseline after 5 min, whereas the increase in $\text{Ins}1\text{P}$ was undetectable at 1 min and reached a maximum after 5 min, which represented 240 % of the basal level. These data indicate that $\text{Ins}(1,4,5)\text{P}_3$, which is rapidly formed in aortic endothelial cells as a result of activation of P_{2Y} receptors, is preferentially metabolized at early times (< 1 min) by a 5-phosphatase, with the sequential formation of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}4\text{P}$. Afterwards, a small but sustained increase in the content of $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}1\text{P}$ was observed, reflecting the activation of the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase.

INTRODUCTION

Stimulation of InsP_3 formation in response to agonists is now well established. The first event is the hydrolysis of PtdInsP_2 by a phospholipase C (PLC), leading to the accumulation of two second messengers: $\text{Ins}(1,4,5)\text{P}_3$ (mobilizing Ca^{2+} from intracellular stores) and diacylglycerol, activating protein kinase C (for review, see Berridge & Irvine, 1989). $\text{Ins}(1,4,5)\text{P}_3$ can be metabolized by two different enzymes (for reviews see Berridge & Irvine, 1989; Shears, 1989): a 5-phosphatase removes the 5-monoester phosphate, producing $\text{Ins}(1,4)\text{P}_2$ (Downes *et al.*, 1982; Storey *et al.*, 1984; Erneux *et al.*, 1986), which is then degraded to inositol via $\text{Ins}4\text{P}$ (Delvaux *et al.*, 1987a; Inhorn *et al.*, 1987; Dean & Moyer, 1987). $\text{Ins}(1,4,5)\text{P}_3$ can also be phosphorylated by a specific $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase with the generation of $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine *et al.*, 1986), which is dephosphorylated by the same 5-phosphatase to $\text{Ins}(1,3,4)\text{P}_3$ (Batty *et al.*, 1985; Shears *et al.*, 1987b; Erneux *et al.*, 1987). In several systems, $\text{Ins}(1,3,4,5)\text{P}_4$ has been shown to act in synergy with $\text{Ins}(1,4,5)\text{P}_3$ in controlling intracellular Ca^{2+} mobilization (see, e.g., Irvine, 1990). Various pathways have been described for the metabolism of $\text{Ins}(1,3,4)\text{P}_3$. It can be phosphorylated to $\text{Ins}(1,3,4,6)\text{P}_4$ (Shears *et al.*, 1987b; Balla *et al.*, 1987) or degraded to InsP_2 . Two isomers, $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$, have been identified as dephosphorylation products of $\text{Ins}(1,3,4)\text{P}_3$ (Dean & Moyer, 1987; Shears *et al.*, 1987a; Balla *et al.*, 1987; Bansal *et al.*, 1987). Other inositol phosphates, such as InsP_5 and InsP_6 (phytic acid), have been detected in pituitary gonadotrophs (Morgan *et al.*, 1987) and in bovine adrenal glomerulosa cells (Balla *et al.*, 1989). It has been proposed that $\text{Ins}(1,3,4,6)\text{P}_4$ (Shears *et al.*, 1987b) and $\text{Ins}(3,4,5,6)\text{P}_4$ (Stephens *et al.*, 1988a) are precursors for InsP_5 .

P_{2Y} -purinergic receptors mediate the responses of vascular endothelial cells to extracellular ATP: mainly, the release of prostacyclin (PGI_2) and endothelium-derived relaxing factor ('EDRF') (Pearson *et al.*, 1983; Van Coevorden & Boeynaems, 1984; Houston *et al.*, 1986). These responses are likely to play an important role in the interaction between platelets (a rich source of adenine nucleotides) and the vessel wall. We have previously shown that, in endothelial cells from bovine aorta (BAEC), the P_{2Y} receptors are coupled to the hydrolysis of PtdInsP_2 by a PLC, resulting in a rapid and transient accumulation of $\text{Ins}(1,4,5)\text{P}_3$, followed by a rise in cytosolic Ca^{2+} content (Piroton *et al.*, 1987).

We have now further characterized the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in BAEC. At early times after the addition of ATP, $\text{Ins}(1,4,5)\text{P}_3$ is rapidly dephosphorylated by a 5-phosphatase to $\text{Ins}(1,4)\text{P}_2$ and ultimately to $\text{Ins}4\text{P}$. Later, a significant proportion of $\text{Ins}(1,4,5)\text{P}_3$ is converted into $\text{Ins}(1,3,4,5)\text{P}_4$, which is sequentially degraded to $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}1\text{P}$.

MATERIALS AND METHODS

Materials

Collagenase type IA was from Cooper (Worthington Biochemical Corp.), trypsin was from Flow Laboratories, and all the other culture media and reagents were purchased from GIBCO. LiCl and tributylamine were from Merck. Proteinase inhibitors (leupeptin, phenylmethanesulphonyl fluoride) and ATP were from Sigma. Dowex AG1X8 (formate form) was from Bio-Rad. The $\mu\text{Bondapak C}_{18}$ column (3.9 mm $\times$ 300 mm) and the Partisil 10 SAX column (4.6 mm $\times$ 250 mm) were purchased from Waters Millipore and Whatman respectively. The solvents for h.p.l.c.

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's Modified Eagle's Medium; RP-h.p.l.c.: reverse-phase h.p.l.c.; PGI_2 , prostacyclin; PLC, phospholipase C; MEM, Minimum Essential Medium.

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were obtained from Romil Chemicals. The radioactive products *myo*-D-[2-³H]inositol (10–20 Ci/mmol), D-*myo*-[U-¹⁴C]Ins3P (50–60 mCi/mmol) and D-*myo*-[5-³²P]Ins(1,4,5)P₃ (> 5000 Ci/mmol) were from Amersham; D-*myo*-[2-³H]Ins(1,4)P₂ (15 Ci/mmol) and D-*myo*-[1-³H]Ins(1,3,4)P₃ (17 Ci/mmol) were purchased from DuPont de Nemours (NEN).

Cell culture

BAEC were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow (Booyse *et al.*, 1975) and cultured in a medium composed of Minimum Essential Medium (MEM) D-valine (80%, v/v), fetal-calf serum (20%, v/v), 2 mM-glutamine, 100 units of penicillin/ml, 100 µg of streptomycin/ml and 2.5 µg of amphotericin B/ml. MEM D-valine was used to prevent survival of contaminating smooth-muscle cells (Marcum *et al.*, 1986). The cells were incubated at 37 °C in a humidified air/CO₂ (19:1) incubator. When the primary culture formed a confluent monolayer, the cells were harvested with trypsin (0.1%, w/v) in a Ca²⁺- and Mg²⁺-free Hanks buffer and subcultured in 35 mm-diam. Petri dishes. For the second and the following passages, the medium was replaced by Dulbecco's Modified Eagle's Medium (DMEM) (60%, v/v), Ham's F₁₂ medium (20%, v/v), fetal-calf serum (20%, v/v), antibiotics, amphotericin B and glutamine at the same concentrations. The cells were used between the second and the fifth passages.

Incubation of BAEC

Subconfluent cells (approx. 8 × 10⁵ cells/dish) were incubated for 48 h in a medium containing Ins-free DMEM and Ham's F₁₂, fetal-calf serum (5%, v/v), antibiotics and amphotericin B as described above, supplemented with 10 µCi of D-*myo*-[³H]Ins (10–20 Ci/mmol)/ml. The cells were washed twice with Ins-free DMEM and incubated in this medium for 30 min before addition of ATP. When tested, Li⁺ was added during this preincubation at 10 mM.

Separation of inositol phosphates on Dowex columns

The inositol phosphates were extracted as described previously (Piroton *et al.*, 1987). Briefly, the medium was rapidly replaced by 1 ml of HClO₄ (3%, w/v). The cells were scraped, the dishes rinsed with 1 ml of HClO₄ (1%) and the suspension was centrifuged (1000 g, 10 min). The supernatant was neutralized with KOH (0.765 M) and Hepes (0.375 M), and the inositol phosphates were separated on Dowex AG1X8 columns (0.8 ml wet bed volume) with increasing concentrations of ammonium formate: Ins was eluted with water (20 ml), glycerophosphoinositol with 0.06 M-ammonium formate/5 mM-sodium tetraborate (8 ml), InsP₁ with 0.15 M-ammonium formate/5 mM-sodium tetraborate (20 ml), InsP₂ with 0.4 M-ammonium formate/0.1 M-formic acid (18 ml), InsP₃ with 0.8 M-ammonium formate/0.1 M-formic acid (12 ml), and the mixture of InsP₄, InsP₅ and InsP₆ with 1.5 M-ammonium formate/0.1 M-formic acid (20 ml). The inositol phosphates were quantified by liquid-scintillation counting of a 4 ml portion of each fraction.

Separation of InsP₁ isomers

After incubation with ATP, the inositol phosphates were extracted and separated on Dowex columns, as described above. The fraction containing InsP₁ (16 ml) was re-applied to a small Dowex column (1 ml) and directly eluted with 1 ml of 0.4 M-ammonium formate/0.1 M-formic acid to concentrate the material. After a 2.6-fold dilution, 2 ml of each sample was injected on to a Partisil 10 SAX column (Van Lookeren Campagne *et al.*, 1988). The isomers of InsP₁ were separated by using ammonium acetate (0.15 M) at pH 4. The column was eluted isocratically at a 1 ml/min flow rate, and 1 min fractions were collected. The

radioactivity in the fractions was quantified by liquid-scintillation counting. Standards of [¹⁴C]Ins3P and [³H]Ins4P were used to calibrate the column. [³H]Ins4P was prepared by dephosphorylation of [³H]Ins(1,4)P₂ by a purified Ins(1,4)P₂ 1-phosphatase (Delvaux *et al.*, 1987b). The mean recovery of [³H]InsP₁ isomers was 89.9 ± 9.6% (mean ± S.D. from 45 injections).

Separation of InsP₂, InsP₃ and InsP₄ isomers

The incubation with ATP was stopped by replacing the medium by 0.5 ml of 18% (v/v) trichloroacetic acid, 10 mM-EDTA and 0.2 mg of phytic acid hydrolysate/ml (Wreggett *et al.*, 1987). The cells were scraped and the dishes were rinsed with an equal volume of water. The proteins were eliminated by centrifugation (1000 g, 10 min), and the trichloroacetic acid in the supernatant was removed after extraction with 5 × 3 ml of diethyl ether. The samples were adjusted to pH 4 with 0.1 M-KOH and concentrated by Speed-Vac. They were resuspended in 50 mM-tributylammonium, pH 4 (0.4 ml), for the separation of InsP₃ + InsP₄ isomers, 1 ml for the InsP₂ isomers. The InsP₂ were first isolated in one peak by using ion-pairing reverse-phase (RP-) h.p.l.c. on a µBondapack C₁₈ column (Van Haastert *et al.*, 1989). The column was eluted isocratically with 5 mM-tributylammonium/10% methanol/100 mM-KH₂PO₄ (pH 4) at a 1.2 ml/min flow rate. The InsP₂ isomers were recovered in four or five 0.5 min fractions, and were then resolved on an ion-exchange Partisil 10 SAX column, by the protocol described by Wreggett & Irvine (1989) with some modifications. The column was eluted with Na₂HPO₄ (200 mM, pH 3.8) at a 1 ml/min flow rate; 0.25 min fractions were collected. The isomers were identified on the basis of their co-elution with radioactive standards. The Ins(1,3)P₂ standard was prepared from [³H]Ins(1,3,4)P₃ with a soluble extract of bovine brain (Bansal *et al.*, 1987). [³H]Ins(3,4)P₂ was obtained from [³H]Ins(1,3,4)P₃ by incubation with a purified Ins(1,3,4)P₃ 1-phosphatase (Delvaux *et al.*, 1989).

InsP₃ and InsP₄ isomers were directly separated on the C₁₈ µBondapack column, eluted as described above; 0.25 and 0.5 min fractions were collected for InsP₃ and InsP₄ respectively. The samples were co-injected with about 300 c.p.m. of [³²P]Ins(1,4,5)P₃ and 300 c.p.m. of [³²P]Ins(1,3,4,5)P₄. [³²P]Ins(1,3,4,5)P₄ was prepared from [³²P]Ins(1,4,5)P₃ (approx. 10⁵ c.p.m.) with a purified Ins(1,4,5)P₃ 3-kinase (Takazawa *et al.*, 1989). A standard of [³H]Ins(1,3,4,6)P₄ was synthesized from [³H]Ins(1,3,4)P₃ with a purified 6-kinase (Verjans *et al.*, 1991). These standards were then isolated by h.p.l.c. as described above.

RESULTS

H.p.l.c. separation of InsP₃ and InsP₄ isomers

We have previously shown that the only InsP₃ isomer which accumulates in [³H]Ins-labelled BAEC, 15 s after stimulation by ATP, is Ins(1,4,5)P₃ (Piroton *et al.*, 1987). In the present study we have used ion-pairing reverse-phase h.p.l.c. on a µBondapack C₁₈ column (Van Haastert *et al.*, 1989) to separate the InsP₃ as well as the InsP₄ isomers present in these cells, during the 10 min after stimulation by ATP. These isomers were identified on the basis of their co-elution with standards of [³²P]Ins(1,4,5)P₃, [³²P]Ins(1,3,4,5)P₄, [³H]Ins(1,3,4)P₃ and [³H]Ins(1,3,4,6)P₄. Fig. 1 illustrates characteristic h.p.l.c. profiles corresponding to extracts of BAEC incubated with or without ATP (100 µM). Fig. 1(a) shows that in unstimulated cells the amounts of InsP₃ and InsP₄ isomers were very low. An interesting finding is that most of the radioactivity was recovered in one peak, which was more polar than any of the InsP₄ isomers. We have calculated that this peak represented about 56% of the radioactivity associated with the inositol phosphates. Ion-exchange h.p.l.c. of this material on a Zorbax SAX column resolved two peaks, co-eluted respectively

Fig. 1. H.p.l.c. elution profiles of InsP_3 , InsP_4 and InsP_5 from BAEC

$[\text{^3H}]\text{Ins}$ -labelled BAEC were incubated in the absence (a) or in the presence of ATP ($100\ \mu\text{M}$) for 15 s (b) or 10 min (c). The inositol phosphates contained in the trichloroacetic acid extracts of BAEC were separated on a $\mu\text{Bondapak C}_{18}$ column as described in the Materials and methods section. The retention times of the different standards are indicated. Results are representative of three distinct experiments.

with standards of InsP_5 and InsP_6 . The ratio of $\text{InsP}_5/\text{InsP}_6$ peaks was approx. 8:3 (results not shown). After 15 s of stimulation (Fig. 1b), ATP induced a large increase in $\text{Ins}(1,4,5)\text{P}_3$ and a smaller one in $\text{Ins}(1,3,4,5)\text{P}_4$. No significant change in $\text{Ins}(1,3,4)\text{P}_3$ was observed. At 10 min (Fig. 1c), the level of $\text{Ins}(1,4,5)\text{P}_3$ had virtually returned to the baseline, whereas $\text{Ins}(1,3,4)\text{P}_3$ was increased. Two minor peaks appeared in the InsP_4 region of the profile. The first one was eluted a few minutes before $\text{Ins}(1,3,4,5)\text{P}_4$ and had the same retention time as a standard of $[\text{^3H}]\text{Ins}(1,3,4,6)\text{P}_4$. The other compound was slightly more polar than $\text{Ins}(1,3,4,5)\text{P}_4$, and might correspond to $\text{Ins}(3,4,5,6)\text{P}_4$ or its enantiomer $\text{Ins}(1,4,5,6)\text{P}_4$, according to Stephens *et al.* (1988b) and Balla *et al.* (1989). In comparison with its action on InsP_3 isomers and $\text{Ins}(1,3,4,5)\text{P}_4$, ATP had only a small effect on these two compounds.

Fig. 2. Time course of the accumulation of (a) $\text{Ins}(1,4,5)\text{P}_3$, (b) $\text{Ins}(1,3,4)\text{P}_3$ and (c) $\text{Ins}(1,3,4,5)\text{P}_4$ in ATP-stimulated BAEC

BAEC were labelled with $[\text{^3H}]\text{Ins}$ and incubated with (●) or without (○) ATP ($100\ \mu\text{M}$) for different times between 15 s and 10 min. The inositol phosphates were extracted and separated on a $\mu\text{Bondapak C}_{18}$ column as described in the Materials and methods section. The results are expressed as c.p.m. of $[\text{^3H}]\text{inositol}$ phosphates isomers/dish from one representative experiment out of three. Each point represents the mean of duplicate determinations with a range of less than 10%.

Time courses of InsP_3 isomers and $\text{Ins}(1,3,4,5)\text{P}_4$ formation are presented in Fig. 2. $\text{Ins}(1,4,5)\text{P}_3$ rapidly increased in ATP-stimulated cells (Fig. 2a). It reached a maximum around 15 s, which represented 3500% of the control value (mean of 3 experiments; range 1400–5600%), and then decreased to a new steady state slightly above baseline. The time course of $\text{Ins}(1,3,4)\text{P}_3$ was markedly different (Fig. 2b); there was no significant increase $\text{Ins}(1,3,4)\text{P}_3$ until 1 min after ATP addition. At this time, the content of $\text{Ins}(1,3,4)\text{P}_3$ in ATP-stimulated cells was 18-fold greater than in unstimulated cells (mean of 3 experiments; range of stimulation 12–23-fold), and this level was maintained for at least 10 min. The amount of $\text{Ins}(1,3,4,5)\text{P}_4$ was also rapidly enhanced in response to ATP (Fig. 2c): a major rise was observed after 15 s of incubation, and the level of $\text{Ins}(1,3,4,5)\text{P}_4$ was maximally elevated within 1 min (23-fold increase, range 16–29; mean of 3 experiments). After 1 or 5 min, the level of $\text{Ins}(1,3,4,5)\text{P}_4$ began to fall more or less rapidly, depending on the cell culture. As shown in Fig. 1(c), ATP also stimulated the production of two other InsP_4 isomers, $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$. After a time lag of 15 s, these

Fig. 3. Kinetics of Ins P_3 accumulation in BAEC: comparison between ATP and ATP[S]

BAEC were labelled with ^{3}H Ins and incubated with either ATP (100 μM) (●) or ATP[S] (100 μM) (◆) for different times between 15 s and 10 min. The inositol phosphates were extracted and separated on Dowex columns as described in the Materials and methods section. Results represent the radioactivity in the Ins P_3 fraction (mean of triplicate determinations in one representative experiment out of two).

Fig. 4. H.p.l.c. analysis of Ins P_2 from BAEC

^{3}H Ins-labelled BAEC were incubated in the absence (○) or presence (●) of ATP (100 μM) for 5 min. The various Ins P_2 isomers were collectively isolated by ion-pairing RP-h.p.l.c. They were then separated by ion-exchange h.p.l.c. on a Partisil SAX column as described in the Materials and methods section. The retention times of ^{3}H -labelled Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(3,4) P_2 are indicated. Results are representative of two different experiments.

two isomers accumulated continuously during the 10 min period of the experiment and at the end of the incubation their level in ATP-stimulated BAEC represented respectively 560 % (range 460–650) and 610 % (range 290–840) of their basal level (mean of 3 different experiments; results not shown). On the contrary, ATP did not significantly change the size of the late-eluted peak containing Ins P_5 and Ins P_6 (results not shown).

As shown in Fig. 3, the time course of Ins P_3 accumulation was similar whether the endothelial cells were stimulated by ATP or by its phosphorothioate analogue adenosine 5'-[γ -thio]triphosphate (ATP[S]), which is not hydrolysed by the ecto-ATPase present on these cells (Cusack *et al.*, 1983).

Analysis of Ins P_2 isomers

The h.p.l.c. profile of Ins P_2 isomers isolated from control and ATP-stimulated BAEC is shown in Fig. 4. Three peaks, co-eluted with standards of Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(3,4) P_2 respectively, were resolved. The kinetics of Ins P_2 isomers accumulation in response to ATP are shown in Fig. 5. The accumulation of Ins(1,4) P_2 was rapid in onset and transient: a maximum,

Fig. 5. Time course of the accumulation of Ins P_2 isomers in ATP-stimulated BAEC

^{3}H Ins-labelled endothelial cells were incubated with (●) or without (○) ATP (100 μM) for various times (15 s to 10 min). The Ins P_2 isomers were isolated by RP-h.p.l.c. and then separated by ion-exchange h.p.l.c. on a Partisil SAX column, as described in the Materials and methods section. Results are expressed as means $\pm$ range of duplicate determinations in one representative experiment out of two.

representing 1075 % of the control value (mean of 2 experiments; range 960–1190 %), was reached 15 s after ATP addition. The levels of the other two isomers increased with a completely different time course: no detectable change before 1 min and a plateau between 5 and 10 min. At 10 min, the levels of Ins(1,3) P_2 and Ins(3,4) P_2 were respectively 8-fold (range 5.2–11.4) and 21-fold (range 21–22) greater in ATP-stimulated than in control cells.

Separation of Ins P_1 isomers

Ins4 P is derived from a stepwise dephosphorylation of Ins(1,4,5) P_3 via Ins(1,4) P_2 by an Ins(1,4) P_2 1-phosphatase (Downes *et al.*, 1982; Delvaux *et al.*, 1987a; Dean & Moyer, 1987). This activity has been detected in the soluble fraction of a BAEC homogenate (results not shown). Ins4 P thus represents a specific marker of the dephosphorylation pathway of Ins(1,4,5) P_3 . On the contrary, Ins1 P may be generated in different ways. Ins1 P and its enantiomer, Ins3 P , are dephosphorylation products of Ins(1,3) P_2 and Ins(3,4) P_2 respectively (Bansal *et al.*, 1987; Shears, 1989). At least in rat brain, Ins1 P can also be derived directly from the breakdown of PtdIns (Ackermann *et al.*, 1987). Since Ins1 P

Fig. 6. H.p.l.c. analysis of InsP_1 from BAEC

[^3H]Ins-labelled BAEC were incubated in the absence (○) or in the presence (●) of $100\ \mu\text{M}$ -ATP for 1 min (a) or 10 min (b). The inositol phosphates were extracted and separated on Dowex columns. The fraction containing the InsP_1 isomers was then analysed by h.p.l.c. on a Partisil SAX column as described in the Materials and methods section. The retention time of the standards, D-[^{14}C]Ins1P and [^3H]Ins4P, are indicated. Results are representative of three different experiments.

Fig. 7. Time course of the accumulation of InsP_1 isomers in ATP-stimulated BAEC

[^3H]Ins-labelled endothelial cells were incubated in the absence (○) or in the presence (●) of $100\ \mu\text{M}$ -ATP for various times (15 s–10 min). The inositol phosphates were extracted, separated on Dowex columns, and the InsP_1 isomers were analysed on a Partisil SAX column as described in the Materials and methods section. Results are expressed as c.p.m. of ^3H -labelled InsP_1 isomers/dish (mean $\pm$ range of duplicate determinations from one representative experiment out of three).

and Ins3P are not separated by ion-exchange chromatography, we called Ins1P the material which was co-eluted with the [^{14}C]Ins3P standard, regardless of the precise 1 or 3 position of the phosphate group. We have studied the accumulation of InsP_1 isomers in [^3H]Ins-labelled BAEC before and after stimulation by ATP. As shown in Fig. 6, two major isomers were detected in resting BAEC: they were co-eluted with standards of [^{14}C]Ins1P and [^3H]Ins4P. A third peak appeared in the profile just after Ins1P elution. According to Hughes & Drummond (1987), it might represent Ins2P . In unstimulated BAEC, Ins1P was

predominant: Ins4P represented only $4.5 \pm 2.1\%$ of the total InsP_1 fraction (mean $\pm$ s.d. from 3 different experiments). ATP ($100\ \mu\text{M}$) enhanced the accumulation of Ins1P (Fig. 7), but with different time courses. Ins4P increased immediately in response to ATP and reached a maximum after 1 min of incubation: a 56–61-fold increase was observed in two experiments. Within 5 min, the level of Ins4P had returned to a steady state slightly above the baseline. On the contrary, the effect of ATP on Ins1P became significant only after 5 min and was maintained until

Table 1. Effect of Li^+ on the ATP-induced accumulation of InsP_3 , InsP_2 and InsP_1 isomers

[^3H]Ins-labelled BAEC were incubated for 30 min with or without LiCl ($10\ \text{mM}$), before addition of ATP ($100\ \mu\text{M}$) for the indicated times. The inositol phosphates were analysed by h.p.l.c. as described in the Materials and methods section. Results are expressed as c.p.m. of ^3H -labelled inositol phosphate isomers (mean $\pm$ range of duplicate determinations in one representative experiment of three for InsP_3 and InsP_4 , or one of two for InsP_1 and InsP_2). The three panels correspond to distinct experiments.

Conditions	$\text{Ins}(1,4,5)\text{P}_3$	$\text{Ins}(1,3,4)\text{P}_3$	$\text{Ins}(1,3,4,5)\text{P}_4$	$\text{Ins}(1,4)\text{P}_2$	$\text{Ins}(1,3)\text{P}_2$	$\text{Ins}(3,4)\text{P}_2$	Ins4P	Ins1P
(a) Without Li^+								
Control 10 min	129 $\pm$ 6	80 $\pm$ 55	71 $\pm$ 2	470 $\pm$ 21	49 $\pm$ 32	94 $\pm$ 18	573 $\pm$ 23	50 $\pm$ 11
ATP 15 s	5022	190	375	5174 $\pm$ 76	40 $\pm$ 10	153 $\pm$ 57	781 $\pm$ 83	830 $\pm$ 123
ATP 1 min	1218 $\pm$ 30	1083 $\pm$ 203	720 $\pm$ 70	1447	192	1176	1087 $\pm$ 97	2810 $\pm$ 23
ATP 5 min	673 $\pm$ 2	1557 $\pm$ 63	644 $\pm$ 21	868 $\pm$ 54	246 $\pm$ 16	1751 $\pm$ 141	1780 $\pm$ 153	316 $\pm$ 100
ATP 10 min	559 $\pm$ 46	1559 $\pm$ 76	541 $\pm$ 6	859 $\pm$ 56	257 $\pm$ 18	1984 $\pm$ 57	1454 $\pm$ 37	347 $\pm$ 37
(b) With Li^+								
Control 10 min	102	248	62	503 $\pm$ 206	59 $\pm$ 1	101 $\pm$ 17	642 $\pm$ 30	55 $\pm$ 9
ATP 15 s	4913 $\pm$ 244	145 $\pm$ 6	334 $\pm$ 41	5598 $\pm$ 22	46 $\pm$ 4	104 $\pm$ 13	617 $\pm$ 65	436 $\pm$ 46
ATP 1 min	1495 $\pm$ 146	1217 $\pm$ 26	816 $\pm$ 27	3731 $\pm$ 91	153 $\pm$ 16	604 $\pm$ 18	972 $\pm$ 40	2453 $\pm$ 27
ATP 5 min	537 $\pm$ 27	1364 $\pm$ 122	524 $\pm$ 36	1262 $\pm$ 21	708 $\pm$ 25	1619 $\pm$ 8	2704 $\pm$ 219	3561 $\pm$ 401
ATP 10 min	579 $\pm$ 90	3000 $\pm$ 288	741 $\pm$ 27	1920 $\pm$ 102	671 $\pm$ 9	1779 $\pm$ 54	4604 $\pm$ 216	3683 $\pm$ 203

10 min. It was also less marked than the effect on Ins4P production: at the end of the 10 min incubation, the amount of Ins1P in ATP-treated cells represented $240 \pm 80\%$ of the control value.

Effect of Li⁺ on accumulation of inositol phosphates

In order to characterize further the metabolism of inositol phosphates in BAEC, we incubated the cells in the presence and absence of LiCl. Li⁺ is an experimental tool often used for measurement of the production of inositol phosphates because of its well-known inhibitory effect on the Ins(1,4)P₂ 1-phosphatase, Ins(1,3,4)P₃ 1-phosphatase and InsP₁ phosphatase (Delvaux *et al.*, 1987a,b; Erneux *et al.*, 1987; Bansal *et al.*, 1987; Gee *et al.*, 1988; Ragan *et al.*, 1988). The effect of Li⁺ on the ATP-induced accumulation of InsP₁, InsP₂, InsP₃ and InsP₄ isomers is illustrated in Table 1. The pretreatment of endothelial cells with Li⁺ (10 mM) had little effect on the basal content of these compounds. Li⁺ enhanced the accumulation of Ins(1,3,4)P₃, Ins(1,4)P₂, Ins4P and Ins1P induced by ATP, but had no significant or reproducible effect on the production of the other inositol phosphates presented in Table 1. The effect of Li⁺ was detected after 5 min of incubation. In Li⁺-treated cells, the maximal production of Ins(1,3,4)P₃ and of Ins(1,3)P₂ in response to ATP was increased, Ins1P was continuously accumulated over the 10 min period investigated, and the maximal level of Ins4P was maintained until the end of this incubation. The levels of Ins(1,3,4,6)P₄ and of Ins(3,4,5,6)P₄ were not affected by pretreatment with Li⁺ (results not shown).

DISCUSSION

The original proposal by Berridge (1983), that Ins(1,4,5)P₃ is a second messenger linking receptor-activated phosphoinositide turnover to intracellular Ca²⁺ mobilization, is now well documented. In particular, Ins(1,4,5)P₃ appears to be the major second messenger of adenine nucleotides in their action on vascular endothelial cells (Piroton *et al.*, 1987; Boeynaems & Pearson, 1990). The present study shows that, shortly after stimulation of endothelial cells by ATP, Ins(1,4,5)P₃ is metabolized mainly by dephosphorylation to Ins(1,4)P₂, with little conversion into Ins(1,3,4,5)P₄. Indeed, 15 s after ATP addition, the levels of Ins(1,4,5)P₃ and Ins(1,4)P₂ were maximally increased (35-fold and 11-fold respectively) and a significant rise in Ins4P was already observed. At that time, Ins(1,3,4,5)P₄ content was also significantly enhanced, but to a much lesser extent than Ins(1,4,5)P₃ (about 10-fold less), with no change in Ins(1,3,4)P₃, Ins(1,3)P₂, Ins(3,4)P₂ and Ins1P. The time course and the amount of Ins(1,4)P₂ and Ins4P produced in ATP-treated cells are consistent with a main pathway of Ins(1,4,5)P₃ metabolism through a stepwise dephosphorylation to Ins(1,4)P₂ and Ins4P. This indeed constitutes the classical route of dephosphorylation of Ins(1,4,5)P₃, which has now been established in most mammalian tissues. However, we cannot exclude that a fraction of Ins(1,4)P₂ and Ins4P accumulated in response to ATP is generated by a direct attack of PLC on PtdIns4P rather than by dephosphorylation of Ins(1,4,5)P₃. A different profile of metabolites appeared after 1 min of incubation with ATP: the level of Ins(1,4,5)P₃ declined to a value slightly above the baseline, whereas the level of Ins(1,3,4,5)P₄ was maximal and that of Ins(1,3,4)P₃ reached a plateau. The InsP₂ and InsP₁ isomers were modified in a similar way: at 5 min, Ins(1,4)P₂ and Ins4P decreased to near their control value, but, in contrast, Ins(1,3)P₂, Ins(3,4)P₂ and Ins1P reached a maximum, which was maintained up to 10 min. This change in metabolic profile might be explained by the activation of the 3-kinase by the Ca²⁺-calmodulin complex (Biden *et al.*, 1987b; Morris *et al.*,

1987; Takazawa *et al.*, 1988). Indeed, ATP increases the cytosolic concentration of free Ca²⁺ in vascular endothelial cells (Luckhoff & Busse, 1986; Hallam & Pearson, 1986), resulting in the activation of Ca²⁺-calmodulin-dependent kinases, detectable within 1 min after ATP addition (Demolle *et al.*, 1988).

The accumulation of InsP₃ in ATP-stimulated BAEC was a transient phenomenon (Piroton *et al.*, 1987; Fig. 3). This is unlikely to result simply from the sequential degradation of ATP to ADP, AMP and adenosine by ectoenzymes located on the surface of endothelial cells. The kinetics were indeed identical for ATP and its analogue ATP[S], which is not a substrate for the endothelial ecto-ATPase (Cusack *et al.*, 1983). The agonist-induced desensitization of a P_{2y}-purinergic-receptor-regulated PLC has been demonstrated in turkey erythrocytes (Martin & Harden, 1989).

The balance of 5-phosphatase/3-kinase pathways of Ins(1,4,5)P₃ metabolism is clearly variable from one cell type to the other. In dog thyroid cells, for example, the Ins(1,4,5)P₃ formed in response to carbachol is rapidly converted into Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃, which is the major InsP₃ isomer in these cells (Laurent *et al.*, 1989; Verjans *et al.*, 1991). Similar observations were reported in pancreatic islets (Biden *et al.*, 1987a) and in cerebral-cortex slices (Batty *et al.*, 1989) stimulated with carbachol, as well as in ATP-stimulated turkey erythrocytes (Berrie *et al.*, 1989). On the contrary, Ins(1,3,4,5)P₄ formation was undetectable in AR42J pancreatoma cells stimulated with substance P (Horstman *et al.*, 1988) or in *Dictyostelium discoideum* incubated with chemoattractant or guanosine 5'-[γ-thio]triphosphate (Van Haastert *et al.*, 1989). Our results with endothelial cells are rather similar to those obtained in vasopressin-stimulated rat hepatocytes (Hansen *et al.*, 1986), angiotensin-stimulated adrenal glomerulosa cells (Balla *et al.*, 1988) and in cardiomyocytes under α₁-adrenergic stimulation (Guse *et al.*, 1989): both 5-phosphatase and 3-kinase contribute to Ins(1,4,5)P₃ metabolism, the 5-phosphatase appearing as the most active pathway at early times. A previous study of Ins(1,4,5)P₃ metabolism in BAEC and human umbilical-vein endothelial cells ('HUVEC') stimulated with bradykinin and thrombin (Bartha *et al.*, 1989) provided results which were less clear than those in the present report: bradykinin and thrombin were much less effective to stimulate accumulation of inositol phosphates in BAEC and, in particular, the increase in Ins(1,3,4,5)P₄ was barely detectable. This can be due to the use of a different agonist or to the higher basal level of inositol phosphates that they observed, possibly in relation to differences in labelling conditions.

Several studies show that the PtdInsP₂-specific PLC is not the only phospholipase coupled to the P_{2y}-receptors in endothelial cells. Indeed, ATP, via a rise in intracytoplasmic Ca²⁺, stimulates the activation of a phospholipase A₂, leading to the mobilization of arachidonic acid, which is then converted into PGI₂ (Carter *et al.*, 1988). An ATP-induced activation of phospholipase D, hydrolysing phosphatidylcholine to phosphatidic acid and choline, has been observed in bovine pulmonary-artery (Martin & Michaelis, 1989) and in bovine aortic (Piroton *et al.*, 1990) endothelial cells. In the present paper we show that, although InsP₃ isomers and Ins4P were present at very low levels in unstimulated BAEC, large amounts of Ins1P were detected. This observation could be explained by a high PtdIns-specific PLC activity. Bartha *et al.* (1989), who measured the accumulation of InsP₁ isomers in BAEC in response to bradykinin and thrombin, postulated that the delayed increase in Ins1P resulted from the breakdown of PtdIns. Although the time course and the content of Ins1P detected in ATP-treated BAEC are consistent with its direct formation by dephosphorylation of Ins(1,3,4)P₃, we cannot exclude the activation of a PtdIns-PLC by ATP.

Other InsP_4 isomers [$\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$] as well as InsP_5 and InsP_6 were detected in BAEC. $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$ levels were very low in resting cells, and slowly increased after the addition of ATP. They have been proposed to be the precursors of InsP_5 (Shears *et al.*, 1987b; Stephens *et al.*, 1988a). However, unstimulated BAEC contained large amounts of material that was co-eluted with InsP_5 and InsP_6 standards, and the level of these compounds was not affected by the exposure to ATP, at least during the 10 min incubation investigated. High levels of InsP_5 and InsP_6 have also been observed in HL60 cells, where their production could be stimulated by the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine within 1 min (Pittet *et al.*, 1989). In adrenal glomerulosa cells treated with angiotensin II, InsP_5 slightly decreased and was then enhanced after 10 h of incubation, although $\text{Ins}(1,3,4,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$ were increased in the first 1 h of this incubation (Balla *et al.*, 1989).

The effect of Li^+ on inositol polyphosphate 1-phosphatase and InsP_1 phosphatase and the insensitivity of other phosphatases is well documented. Most of our results obtained with Li^+ -treated BAEC are in agreement with the data reported in other systems. However, the fact that the accumulation of $\text{Ins}(3,4)\text{P}_2$ in ATP-stimulated cells was not affected by Li^+ was somehow unexpected. Hughes & Drummond (1987) observed a similar phenomenon in GH3 pituitary cells incubated with thyrotropin-releasing hormone. The lack of decrease in $\text{Ins}(3,4)\text{P}_2$ might result from the simultaneous inhibition of $\text{Ins}(1,3,4)\text{P}_3$ 1-phosphatase by Li^+ and of $\text{Ins}(3,4)\text{P}_2$ 4-phosphatase by InsP_3 ('product inhibition'), as observed for the $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase (Delvaux *et al.*, 1989).

In conclusion, both 5-phosphatase and 3-kinase are involved in the metabolism of $\text{Ins}(1,4,5)\text{P}_3$, formed in ATP-stimulated endothelial cells. At early times (< 1 min), the dephosphorylation pathway clearly predominates, but later a significant flux through the 3-kinase pathway is observed.

During the revision of this manuscript, Allsup & Boarder (1990) published their work on the production of inositol phosphates in ATP-stimulated endothelial cells from aorta and adrenal medulla. The results that they obtained with the AG4762 cell line, derived from BAEC, are quite similar to ours and are in agreement with our conclusions.

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