Metabolism of inositol 1,4,5-trisphosphate in permeabilized rat aortic smooth-muscle cells

Dependence on calcium concentration

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The metabolism of [^3H]inositol 1,4,5-trisphosphate {[^3H]Ins(1,4,5) P_3 } was studied in permeabilized rat aortic smooth-muscle cells. Addition of [^3H]Ins(1,4,5) P_3 to the leaky cells led to formation of several labelled metabolites. Amounts of [^3H]inositol bisphosphate and [^3H]inositol 1,3,4,5-tetrakisphosphate ([^3H]Ins P_4) reached a maximum within 2 min of incubation, whereas production of [^3H]inositol monophosphate and [^3H]inositol 1,3,4-trisphosphate {[^3H]Ins(1,3,4) P_3 } was delayed. Formation of Ins P_4 and Ins(1,3,4) P_3 was Ca^{2+} -sensitive in the physiological intracellular range (0.06–5 μM), showing a maximum at 1 μM - Ca^{2+} . A correlation between the formation of Ins P_4 and that of Ins(1,3,4) P_3 was observed, suggesting that the former is the precursor of the latter. These results suggest that, in vascular smooth-muscle cells, Ins(1,4,5) P_3 is metabolized via two distinct pathways: (1) a dephosphorylation pathway, leading to formation of inositol bis- and mono-phosphate; and (2) a Ca^{2+} -sensitive phosphorylation/dephosphorylation pathway, involving formation of Ins P_4 and leading to formation of Ins(1,3,4) P_3 .

INTRODUCTION

It is now well established that Ins(1,4,5) P_3 plays a key role in cellular responses to stimuli involving Ca^{2+} mobilization from intracellular Ca^{2+} pools (Berridge & Irvine, 1984). As a second messenger, Ins(1,4,5) P_3 is expected to be rapidly metabolized after its generation. Indeed, the specific phosphatases involved in the stepwise degradation of Ins(1,4,5) P_3 to inositol have been characterized, and their subcellular localization has been determined in the liver (Storey *et al.*, 1984; Joseph & Williamson, 1985). In addition, a soluble enzyme, specifically hydrolysing the 5-phosphate of Ins(1,4,5) P_3 , has been isolated from platelets and found to be inhibited by high Ca^{2+} concentrations ($K_i = 70 \mu\text{M}$) (Connolly *et al.*, 1985). In contrast, the degradation of Ins(1,4,5) P_3 in cytosolic fractions from coronary smooth muscle was enhanced by physiological concentrations of free Ca^{2+} (0.1–1 μM) and by Mg^{2+} (Sasaguri *et al.*, 1985). It has been proposed that protein kinase C could be involved in the activation of the 5-phosphatase (Molina y Vedia & Lapetina, 1986; Connolly *et al.*, 1986).

This metabolic scheme has become even more complex since the discovery of the presence of a second inositol trisphosphate isomer in stimulated parotid glands, Ins(1,3,4) P_3 (Irvine *et al.*, 1984). The generation of this isomer was delayed (Irvine *et al.*, 1985), but Ins(1,3,4) P_3 appeared to be the predominant form in various cells types after prolonged exposure to agonists (Turk *et al.*, 1986; Downes *et al.*, 1986; Lew *et al.*, 1986). Since the theoretical phospholipid precursor for Ins(1,3,4) P_3 , i.e. phosphatidylinositol 3,4-bisphosphate, has not been found in membranes (Downes *et al.*, 1986), it was proposed that Ins(1,3,4) P_3 was generated from a higher-phosphorylated form of inositol, Ins(1,3,4,5) P_4 , which formed early, e.g. after muscarinic stimulation of

brain slices (Batty *et al.*, 1985). An Ins(1,4,5) P_3 3-kinase activity has been observed and characterized after addition of [^3H]Ins(1,4,5) P_3 to various cell homogenates (Irvine *et al.*, 1986; Hansen *et al.*, 1986; Hawkins *et al.*, 1986), and has been shown to be Ca^{2+} -sensitive in intact HL-60 cells (Lew *et al.*, 1986), in permeabilized adrenal glomerulosa cells (Rossier *et al.*, 1986) and in subcellular fractions from insulin-secreting RINm5F cells (Biden & Wollheim, 1986). In the present work, the existence of this metabolic pathway and its Ca^{2+} -sensitivity were tested in vascular smooth-muscle cells.

MATERIALS AND METHODS

Materials

Hepes, bovine serum albumin, MgATP, phosphocreatine and creatine kinase were obtained from Sigma. Trypsin was purchased from Gibco, and EGTA from Fluka. [^3H]Ins(1,4,5) P_3 was purchased from New England Nuclear, and Ins(1,4,5) P_3 was generously given by Dr. R. F. Irvine, Cambridge, U.K.

Preparation of cultured rat aortic smooth-muscle cells

Rat aortic smooth-muscle cells were prepared and cultured as described elsewhere (Capponi *et al.*, 1985). Upon confluence after five passages, the cells were detached by treatment with 2 ml of 0.25% trypsin for 15 min at 37 °C and then resuspended in a medium containing 20 mM-Hepes, 137 mM-NaCl, 3 mM-KCl, 5.6 mM-D-glucose and 0.05% albumin, pH 7.2. The cells were washed twice in the same buffer and once in 250 mM-sucrose/5 mM-Hepes, pH 7.2.

Permeabilization of the cells

The cells were resuspended in the above non-ionic buffer at a concentration of 3×10^6 cells (1.5 mg of

Abbreviations used: Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; Ins(1,3,4) P_3 , inositol 1,3,4-trisphosphate; Ins(1,3,4,5) P_4 , inositol 1,3,4,5-tetrakisphosphate; Ins P_2 , inositol bisphosphate; Ins P , inositol monophosphate.

Fig. 1. Kinetics of formation of the various phosphoinositols

The time course of appearance of the peaks corresponding to the various phosphoinositols was determined after addition of [³H]Ins(1,4,5)P₃ to permeabilized cells, as described in the Materials and methods section. (a) Appearance of InsP₂ and InsP; the disappearance of [³H]Ins(1,4,5)P₃ is also shown. (b) Appearance of Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃. [Ca²⁺] in the medium was 5 μM. Each point represents the mean value from three separate experiments.

protein)/50 μl and then permeabilized by exposure to a high-voltage electric field (2 kV/cm), by the method of Knight & Baker (1982), and as previously described for adrenal glomerulosa cells (Rossier *et al.*, 1986).

Incubation of the cells

A portion (50 μl) of this cell preparation was mixed with 350 μl of various buffers containing an 'intracellular cationic background' (5 mM-NaCl, 115 mM-KCl, 5 mM-NaHCO₃, 1 mM-KH₂PO₄, 1 mM-MgCl₂), an ATP-regenerating system (2 mM-MgATP, 10 mM-phosphocreatine and 8 units of creatine kinase/ml), 0.05% albumin and 20 mM-Hepes, pH 7.2. The free Ca²⁺ concentration was buffered in these media with 1 mM-EGTA and various amounts of CaCl₂, and was measured with a Ca²⁺-selective mini-electrode as previously described (Rossier *et al.*, 1986).

After 5 min of preincubation at 37 °C, 0.5 μM-Ins(1,4,5)P₃, containing 0.05 μCi of [³H]Ins(1,4,5)P₃/ml, was added to the incubation media; samples (50 μl) were removed at the indicated times and mixed with 1 ml of ice-cold 10% (v/v) trichloroacetic acid.

Fig. 2. Ca²⁺-dependence of the formation of Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃

(a) The amount of Ins(1,3,4,5)P₄ produced after 2 min was determined as a function of [Ca²⁺]. Statistical significance was assessed by Student's *t* test. (b) The amount of Ins(1,3,4)P₃ generated after 10 min as a function of [Ca²⁺]. Each point is the mean ± S.E.M. from three to six separate experiments.

Separation of phosphoinositols

The samples were then centrifuged for 10 min at 2500 *g*, and the supernatant was extracted with 3 × 5 vol. of diethyl ether. Finally, phosphoinositols were separated by anion-exchange h.p.l.c. with an ammonium formate gradient, by the method described by Irvine *et al.* (1986). The peaks of inositol and of Ins(1,4,5)P₃ were identified by comparison with the elution profile of pure standards. The nature of the other peaks was presumed on the basis of their previously described chromatographic behaviour [for example, ATP always co-eluted with Ins(1,3,4)P₃].

RESULTS

Kinetics of Ins(1,4,5)P₃ metabolism

Addition of [³H]Ins(1,4,5)P₃ to permeabilized aortic smooth-muscle cells led to the rapid formation of several ³H-labelled phosphoinositols. After 2 min, 70% of the initial radioactivity of [³H]Ins(1,4,5)P₃ was recovered as InsP₂, InsP and inositol, whereas only 8% was eluted as Ins(1,3,4)P₃ and Ins(1,3,4,5)P₄. The kinetics of Ins(1,4,5)P₃ degradation and of the formation of other phosphoinositols were studied (Fig. 1). Ins(1,4,5)P₃ decreased rapidly (*t*_{1/2} = 1.5 min), and less than 2% of the initial amount remained after 10 min of incubation. InsP₂ and Ins(1,3,4,5)P₄ increased immediately, to reach a maximum at 2 min, whereas the appearance of InsP and Ins(1,3,4)P₃ was delayed. Ins(1,3,4)P₃ remained in elevated amounts after 10 min, while InsP kept increasing. The maximal amounts of Ins(1,3,4,5)P₄ were always reached within 2 min, independently of the Ca²⁺ concentration (results not shown).

Ca²⁺-dependence of Ins(1,4,5)P₃ metabolism

Fig. 2 shows the Ca²⁺-dependence of the formation of Ins(1,3,4,5)P₄ and of Ins(1,3,4)P₃. Increasing [Ca²⁺] from 0.1 to 1 μM led to a 3-fold increase in the amount of Ins(1,3,4,5)P₄ present at 2 min and in that of Ins(1,3,4)P₃ at 10 min. This effect was reversed by [Ca²⁺] > 1 μM. The degradation of Ins(1,4,5)P₃ did not depend on [Ca²⁺] (results not shown).

Fig. 3. Correlation between the amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ present at 2 min and of $\text{Ins}(1,3,4)\text{P}_3$ present at 10 min in the same incubation medium

The linear-regression analysis was performed on the data of Fig. 2, obtained at various $[\text{Ca}^{2+}]$.

A significant correlation was found between the amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ measured after 2 min and those of $\text{Ins}(1,3,4)\text{P}_3$ detected after 10 min (Fig. 3). This relationship was not affected by the $[\text{Ca}^{2+}]$ in the medium.

DISCUSSION

The recovery of the radioactivity from $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ in various phosphoinositols clearly demonstrates not only the presence of a degradative pathway involving stepwise dephosphorylations, but also the presence of an alternative pathway, leading to the formation of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$. There is considerable experimental evidence to suggest that the conversion of $\text{Ins}(1,4,5)\text{P}_3$ into $\text{Ins}(1,3,4)\text{P}_3$ occurs through the formation of $\text{Ins}(1,3,4,5)\text{P}_4$. In the present work with vascular smooth-muscle cells, firstly, $\text{Ins}(1,3,4,5)\text{P}_4$ appears early and only transiently, whereas the formation of $\text{Ins}(1,3,4)\text{P}_3$ is delayed (Fig. 1), and secondly, a significant correlation exists between the maximal amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ (reached at 2 min) and those of $\text{Ins}(1,3,4)\text{P}_3$ (at 10 min) (Fig. 3). In addition, it is known that removal of ATP from the medium prevents the appearance of $\text{Ins}(1,3,4)\text{P}_3$ in permeabilized adrenal glomerulosa cells (Rossier *et al.*, 1986). Finally, incubation of parotid homogenates with $\text{Ins}(1,3,4,5)\text{P}_4$ leads to the formation of $\text{Ins}(1,3,4)\text{P}_3$ (Hawkins *et al.*, 1986). These results are in agreement with the model proposed by Irvine *et al.* (1986).

The pathway by which $\text{Ins}(1,3,4)\text{P}_3$ is metabolized is not yet established, but such a pathway seems to be

slower than the dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$: the amounts of $\text{Ins}(1,3,4)\text{P}_3$ remain elevated, even when $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ have almost completely disappeared (Fig. 1). Most of the InsP and InsP_2 present at 10 min seem therefore to result from $\text{Ins}(1,4,5)\text{P}_3$ and not from $\text{Ins}(1,3,4)\text{P}_3$.

An interesting feature of this alternative pathway is its sensitivity to Ca^{2+} concentrations in the physiological intracellular range. These results are similar to those obtained in other systems (Lew *et al.*, 1986; Rossier *et al.*, 1986; Biden & Wollheim, 1986). It is known that $\text{Ins}(1,4,5)\text{P}_3$ is able to mobilize Ca^{2+} from intracellular pools, whereas $\text{Ins}(1,3,4,5)\text{P}_4$ is inactive in this regard. Thus $\text{Ins}(1,4,5)\text{P}_3$ can control its own metabolism. The presence of this second pathway, which can be modulated by Ca^{2+} , suggests a second-messenger role for $\text{Ins}(1,3,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$.

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