Regulation of inositol 1,4,5-trisphosphate metabolism in insulin-secreting RINm5F cells

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Factors underlying the transience of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] accumulation following muscarinic stimulation of RINm5F cells were examined. Transience was not due to a protein kinase C-mediated stimulation of $\text{Ins}(1,4,5)\text{P}_3$ dephosphorylation, since pretreatment of cells with tetradecanoylphorbol acetate (TPA) did not alter the rate of this conversion. However, preincubation with TPA did inhibit carbamoylcholine-stimulated $\text{Ins}(1,4,5)\text{P}_3$ formation. In permeabilized cells, the conversion of $\text{Ins}(1,4,5)\text{P}_3$ to inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] was slightly enhanced in the presence of TPA or cyclic AMP, but much more markedly by raising the Ca^{2+} concentration from 10^{-7} M to 10^{-6} or 10^{-5} M. In intact cells the most rapid rate of accumulation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ occurred in the first 2 s following stimulation, whereas the levels of inositol 1,4-bisphosphate were not increased until after 5 s. This suggests that $\text{Ins}(1,4,5)\text{P}_3$ kinase is chiefly responsible for the early disposal of $\text{Ins}(1,4,5)\text{P}_3$ following cellular stimulation. The results are consistent with the proposal that the transient accumulation of $\text{Ins}(1,4,5)\text{P}_3$ is due both to its enhanced metabolism via the Ca^{2+} -calmodulin-sensitive $\text{Ins}(1,4,5)\text{P}_3$ kinase, as well as a down-regulation of phosphatidylinositol 4,5-bisphosphate hydrolysis.

INTRODUCTION

Physiological activation of many cell types following receptor occupation is often associated with enhanced hydrolysis of phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$], a minor phospholipid component of the plasma membrane [1]. This hydrolysis, catalysed by phospholipase C, gives rise to $\text{Ins}(1,4,5)\text{P}_3$, a water-soluble second messenger which releases Ca^{2+} from intracellular stores [2–4], and DAG, which is capable of activating protein kinase C [5]. The peak increase in $\text{Ins}(1,4,5)\text{P}_3$ is often seen at early time points following stimulation: its levels subsequently decline, usually to a new steady state which may or not be elevated above basal [6–15]. The control mechanisms underlying this transient response are of obvious physiological interest, and have been a matter of some debate. One explanation is that the generation of $\text{Ins}(1,4,5)\text{P}_3$ might be shortlived, possibly due to a down-regulation of phospholipase C activity. The reportedly brief duration of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis in tumoural pituitary (GH_3) cells would appear to support this proposal [16]. An alternative explanation is that the further metabolism of $\text{Ins}(1,4,5)\text{P}_3$ is specifically enhanced so as to outstrip the activity of phospholipase C. There are two possible sites at which such a regulation of $\text{Ins}(1,4,5)\text{P}_3$ disposal might occur. The first is at $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase, the enzyme which dephosphorylates $\text{Ins}(1,4,5)\text{P}_3$ to form $\text{Ins}(1,4)\text{P}_2$ [17,18]. In platelets this activity is stimulated in the presence of protein kinase C [19]. The second possible regulatory site is at $\text{Ins}(1,4,5)\text{P}_3$ kinase, by which

$\text{Ins}(1,4,5)\text{P}_3$ is converted to $\text{Ins}(1,3,4,5)\text{P}_4$ [20]. In insulin-secreting RINm5F cells the activity of this enzyme is stimulated by Ca^{2+} [9] in a calmodulin-dependent manner [21]. Two potential mechanisms therefore exist for the regulation of $\text{Ins}(1,4,5)\text{P}_3$ levels by factors attendant upon $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, namely production of DAG and mobilization of Ca^{2+} from intracellular pools. The aim of the present study was to evaluate which of these putative mechanisms might account for the transient accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in stimulated RINm5F cells.

EXPERIMENTAL

Suspensions of RINm5F cells, prelabelled for 48 h with [$2\text{-}^3\text{H}$]inositol, were incubated in a modified Krebs–Ringer bicarbonate medium as previously described [8,9]. In some instances the radiolabelled cells were resuspended in a mannitol-containing medium and permeabilized by exposure ($10 \times 30 \mu\text{s}$) to an electric field of 3 kV/cm. Incubations of the permeabilized cells (5 min) were in a potassium glutamate medium (pH 7.0) containing 10 mM-EGTA and Ca^{2+} to give the required free Ca^{2+} concentration. This procedure and the characterization of the permeabilized cells have been recently reported in detail [22].

For studies on the effect of TPA pretreatment on $\text{Ins}(1,4,5)\text{P}_3$ dephosphorylation, cells were washed and resuspended in the modified Krebs–Ringer bicarbonate medium. Aliquots of the cell suspension (50 μl), con-

Abbreviations used: InsP_1 , inositol monophosphate; InsP_2 , inositol bisphosphate; InsP_3 , inositol trisphosphate; InsP_4 , inositol tetrakisphosphate (with positional determinants of the phosphate groups as indicated); $\text{PtdIns}4\text{P}$, phosphatidylinositol 4-monophosphate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; TPA, tetradecanoylphorbol acetate; $\text{GTP}\gamma\text{S}$, guanosine 5'-[γ -thio]triphosphate; IBMX, isobutylmethylxanthine.

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taining approx. 2×10^6 cells, were added to 50 μ l of the same medium in the presence or absence of TPA (100 nM final). After exactly 10 min of preincubation, 0.4 ml of a buffer (pH 7.0) was added containing 110 mM-KCl, 1 mM-MgSO₄, 10 mM-Hepes, 10 mM-LiCl, 0.3 mM-EGTA, 0.625 μ M-[2-³H]Ins(1,4,5)P₃ (15.9 Ci/mol) and 0.2% Triton X-100. Incubations (0–30 min) were terminated by the addition of 0.5 ml of trichloroacetic acid (20%). Inositol phosphates were extracted and separated by anion-exchange chromatography on 1 ml Dowex 1 $\times$ 8 columns as described [4].

The isomeric forms of various inositol phosphates were resolved by anion-exchange h.p.l.c. using a Partisil SAX 10 column and an ammonium formate (pH 3.7) gradient similar to those previously published [6,23]. After 5 min washing with water the concentration of ammonium formate was increased linearly to 1.12 M at min 32. This concentration was maintained for 3 min, followed by a second linear increase to 1.8 M at min 43. After a further 4 min at this level, the column was washed with water in preparation for the next sample. A flow rate of 1.6 ml/min was used, and fractions were taken every 0.4 min. Using this procedure, seven major peaks of radioactivity were resolved in the extracts of the [2-³H]inositol-labelled cells (Fig. 1). Two of these co-eluted with ³²P-labelled standards of Ins(1,4)P₂ and Ins(1,4,5)P₃. Another (peak V) has been previously shown to have the same retention time as [2-³H]Ins(1,3,4,5)P₄ [9]. Peak IV has been ascribed in earlier studies to Ins(1,3,4)P₃ based on the comparison of its elution position with those of ATP and Ins(1,4,5)P₃ [8]. The existence of a peak (III) running just after Ins(1,4)P₂ has been described in several investigations [10,13,24] and it has been recently identified as Ins(3,4)P₂ [24]. This

is presumably also the case in RINm5F cells. Peaks I and II most probably correspond to Ins1P and Ins4P respectively [13,25].

Radioactive standards of Ins(1,4)P₂ and Ins(1,4,5)P₃ were prepared from ³²P-labelled erythrocyte ghosts as previously described [17,26]. The sources of all other materials were as given [8,9,22], except for TPA and cyclic AMP which were from Sigma, and [³²P]P_i from Amersham International.

RESULTS

It has been previously shown that muscarinic stimulation of RINm5F cells results in a transient increase in Ins(1,4,5)P₃, accompanied by a slightly delayed accumulation of Ins(1,3,4)P₃ [8,9]. There was also a rapid rise in Ins(1,3,4,5)P₄ which could not be resolved from that of its precursor. These results were similar to those seen in many tissues [6,7,10–15,23,26,27] and are confirmed in a further series of experiments (Fig. 2) in which analysis of Ins(1,4)P₂ and (the presumed) Ins(3,4)P₂ was also carried out. Several features of these responses are noteworthy. Firstly, at 2 s after stimulation only Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were significantly elevated. In other words, over the period which sees the steepest rises in these two metabolites, there is no increase at all in Ins(1,4)P₂. The latter starts to accumulate by 5 s, but only reaches significantly elevated levels at the next measured time point (15 s). Secondly, the onset of the rise in Ins(3,4)P₂ (30 s) lags behind that of Ins(1,3,4)P₃ (15 s) which is in turn preceded by the increase in Ins(1,3,4,5)P₄ (2 s). This is consistent with the proposed degradative pathway by which the phosphate groups of Ins(1,3,4,5)P₄ at position 5 [20,23,26] and then at position 1 [24] are sequentially

Fig. 1. Separation of inositol phosphates by anion-exchange h.p.l.c.

RINm5F cells were cultured for 48 h in the presence of 10 μ Ci of [2-³H]inositol/ml. A diethyl ether-washed trichloroacetic acid extract of 10^6 cells stimulated with 100 μ M-carbamoylcholine was injected into a Partisil SAX 10 column, together with ³²P-labelled standards of Ins(1,4)P₂ and Ins(1,4,5)P₃. After washing for 5 min with water a discontinuous gradient of 0–1.8 M-ammonium formate (pH 3.7 with phosphoric acid) was applied (broken line). The flow rate was 1.6 ml/min. Fractions were collected every 0.4 min and counted for both ³H (●) and ³²P (○) by liquid-scintillation spectrometry.

Fig. 2. Early time course of inositol phosphate generation in RINm5F cells following stimulation with 100 μM-carbamoylcholine

Incubations were for 0–30 s in a modified Krebs–Ringer bicarbonate-buffered medium, and followed a 10 min preincubation in the same medium. Inositol phosphates were analysed as described in the legend to Fig. 1. The earliest time points which were significantly different from pre-stimulatory levels were 2 s for Ins(1,4,5)P₃ (*P* < 0.05; ●) and Ins(1,3,4,5)P₄ (*P* < 0.05; ■); 15 s for Ins(1,4)P₂ (*P* < 0.05; ▲) and Ins(1,3,4)P₃ (*P* < 0.005; ○); and 30 s for Ins(3,4)P₂ (*P* < 0.005; △). Each point represents the mean ± S.E.M. of four to six independent observations.

removed. Thirdly, of the measured inositol phosphates only Ins(1,4,5)P₃ displays a transient increase.

The simplest explanation for this transience would be a down-regulation of PtdIns(4,5)P₂ hydrolysis soon after its initial stimulation [16]. Previous studies have established that a potential mechanism by which this might occur is via the activation of protein kinase C [28–34]. To test this hypothesis, RINm5F cells were pretreated for 10 min with 100 nM-TPA (an activator of protein kinase C) prior to agonist addition. The results in Table 1 indicate that carbamoylcholine-stimulated PtdIns(4,5)P₂

hydrolysis was markedly inhibited following this pretreatment.

Alternatively (or in addition), enhanced removal of Ins(1,4,5)P₃ might account for its transient accumulation. Based on several lines of evidence obtained with intact RINm5F cells [9], subcellular fractions [9] and a partially-purified enzyme preparation [21] we have previously proposed that the Ca²⁺-calmodulin-sensitive Ins(1,4,5)P₃ kinase might be a site at which enhanced removal could occur. However, no direct effect of an increase in Ca²⁺ on Ins(1,4,5)P₃ disposal has yet been shown in a preparation of RINm5F cells which retains physiological function. We have therefore used electrically permeabilized cells which undergo exocytosis in response to appropriate stimuli [22] and in which the intracellular Ca²⁺ concentration can be equilibrated with that in the incubation medium. Using RINm5F cells prelabelled with [2-³H]inositol, there was a dose-dependent increase in Ins(1,3,4,5)P₄ levels upon raising the Ca²⁺ concentration of the medium over the range 10⁻⁷–10⁻⁵ M; no significant effect was seen on either isomer of InsP₃ (Fig. 3a). These results are explained by a dual action of Ca²⁺ to stimulate both phospholipase C [22,35] and Ins(1,4,5)P₃ kinase, such that Ins(1,4,5)P₃ produced from PtdIns(4,5)P₂ is rapidly converted to Ins(1,3,4,5)P₄. In previous experiments with intact RINm5F cells [9], we could not rule out the possibility that Ca²⁺ might also stimulate the conversion of Ins(1,3,4,5)P₄ to Ins(1,3,4)P₃. This possibility can now be excluded, since in leaky cells it would have resulted in a decrease, rather than the observed increase, in Ins(1,3,4,5)P₄ as the Ca²⁺ concentration was raised. When GTPγS was used to enhance the coupling of phospholipase C to its GTP-binding protein [22], there was a marked increase in the levels of all three inositol phosphates measured (Fig. 3b). Under these conditions, proportionally more Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃ were formed as the Ca²⁺ concentration was increased, an effect which was most pronounced over the range 10⁻⁷–10⁻⁶ M. Once again, raising the Ca²⁺ concentration in the medium did not significantly alter the levels of Ins(1,4,5)P₃, although the increases in its metabolites are indicative of enhanced phospholipase C activity.

A second mechanism whereby the disposal of Ins(1,4,5)P₃ might be enhanced is via the stimulation of Ins(1,4,5)P₃ phosphomonoesterase, secondary to activation of protein kinase C [19,36]. To test this possibility, further experiments were performed with the permeabilized cells. In this instance exogenous [2-³H]Ins(1,4,5)P₃ (1.5 μM) was added to non-labelled cells and its

Table 1. Effect of pretreatment with TPA on Ins(1,4,5)P₃ formation and metabolism in carbamoylcholine-stimulated RINm5F cells

The experimental procedure was as described in the legend to Fig. 2 except that in some instances TPA was present during the 10 min preincubation and that carbamoylcholine was present for 30 s. Results are expressed as a percentage of the radioactivity seen in the absence of carbamoylcholine, which averaged 5.3, 8.3 and 40.1 for Ins(1,3,4)P₃, Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ respectively. Mean ± S.E.M. of five or six independent observations are given; *P < 0.05, **P < 0.005, ***P < 0.001 versus control.

Carbamoylcholine (100 μM) plus addition	Response (%) of non-stimulated control)		
	Ins(1,4,5)P ₃	Ins(1,3,4,5)P ₄	Ins(1,3,4)P ₃
None	188 ± 30*	128 ± 7*	470 ± 48***
TPA (0.1 μM)	115 ± 36	92 ± 12	273 ± 33**

Fig. 3. Ca^{2+} -dependent metabolism of endogenously formed $\text{Ins}(1,4,5)\text{P}_3$ in permeabilized RINm5F cells

The cells were cultured for 48 h in the presence of $10 \mu\text{Ci}$ of $[2\text{-}^3\text{H}]\text{inositol}/\text{ml}$. They were permeabilized by high-voltage discharge and aliquots (10^6 cells) were resuspended in $300 \mu\text{l}$ of a medium (pH 7.0) containing 140 mM -potassium glutamate, 5 mM - NaCl , 2 mM - MgSO_4 , 5 mM - MgATP , 10 mM -EGTA and CaCl_2 as required. After 10 min preincubation at 4°C , the tubes were warmed to 37°C and incubated for 5 min. Extraction and separation of inositol phosphates was as described in the legend to Fig. 1. Incubations were in the absence (a) or presence (b) of $100 \mu\text{M}$ - $\text{GTP}\gamma\text{S}$. Results are the mean $\pm$ S.E.M. for five independent observations.

conversion to $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,4)\text{P}_2$ monitored (Table 2). However, TPA ($1 \mu\text{M}$) did not stimulate the rate of $\text{Ins}(1,4,5)\text{P}_3$ dephosphorylation; indeed, there was a slight decrease in the ratio of $\text{Ins}(1,4)\text{P}_2$ to

$\text{Ins}(1,3,4,5)\text{P}_4$ formed relative to control. A similar effect was also seen in the presence of cyclic AMP ($100 \mu\text{M}$). It should be stressed that under identical incubation conditions both TPA and cyclic AMP significantly stimulated insulin release (results not shown), thereby indicating that these agents were capable of activating their respective protein kinases to phosphorylate physiologically relevant target proteins. This is also true for the activation of $\text{Ins}(1,4,5)\text{P}_3$ kinase by calmodulin, since a rise in the Ca^{2+} concentration enhanced the production of $\text{Ins}(1,3,4,5)\text{P}_4$, and markedly shifted the metabolic flux away from dephosphorylation. It will be noted, however, that even at 10^{-5} M - Ca^{2+} the contribution of $\text{Ins}(1,4,5)\text{P}_3$ kinase appears greatly underestimated in these experiments as compared with the results obtained with endogenously produced $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (cf. Fig. 3). This may be due to the localization on the inner leaflet of the plasma membrane of $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase [24], which could therefore preferentially metabolize the $\text{Ins}(1,4,5)\text{P}_3$ as it enters the small holes in the electrically permeabilized cells.

Although the above experiments appear to rule out an effect of protein kinase C on $\text{Ins}(1,4,5)\text{P}_3$ phosphomonoesterase activity, we wanted to make absolutely sure that similar results would be obtained following TPA pretreatment of intact cells, under conditions which have been shown to block the muscarinic stimulation of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis. Thus, RINm5F cells were incubated for 10 min in the presence of 100 nM -TPA, then lysed with Triton X-100, and phosphomonoesterase activity assayed (Fig. 4). No effect of the activation of protein kinase C was seen, either on the dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ or on the formation of its metabolites.

DISCUSSION

In the present study we have evaluated three mechanisms by which $\text{Ins}(1,4,5)\text{P}_3$ levels might be regulated following muscarinic stimulation of RINm5F cells. It was shown that $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis could be potentially down-regulated by activation of protein kinase C. This widely observed phenomenon [28–32] might be due either to effects at the receptor itself [32], or at the GTP-binding protein which couples the occupied receptor to phospholipase C [33,34]. However, the physiological significance of this inhibition remains

Table 2. Metabolism of exogenous $\text{Ins}(1,4,5)\text{P}_3$ added to electrically-permeabilized RINm5F cells

Non-labelled cells were permeabilized and incubated as described in the legend to Fig. 3, in the presence of $1.5 \mu\text{M}$ - $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (3200 c.p.m.) and other additions as shown. Inositol phosphates were extracted and separated as indicated in the legend to Fig. 1. Results are means $\pm$ S.E.M. of five independent observations; * $P < 0.05$, ** $P < 0.001$ compared with corresponding value at 10^{-7} M - Ca^{2+} .

Condition	^3H radioactivity (c.p.m./ 10^6 cells)			Ratio [$\text{Ins}(1,4)\text{P}_2/\text{Ins}(1,3,4,5)\text{P}_4$]
	$\text{Ins}(1,4,5)\text{P}_3$	$\text{Ins}(1,4)\text{P}_2$	$\text{Ins}(1,3,4,5)\text{P}_4$	
10^{-7} M - Ca^{2+}	2580 ± 100	362 ± 39	57.9 ± 5.8	6.3 ± 0.5
10^{-7} M - Ca^{2+} plus cyclic AMP ($100 \mu\text{M}$) plus IBMX ($100 \mu\text{M}$)	2580 ± 90	340 ± 32	71.0 ± 8.8	$4.8 \pm 0.3^*$
10^{-7} M - Ca^{2+} plus TPA ($1 \mu\text{M}$)	2590 ± 70	359 ± 37	74.7 ± 8.8	$4.8 \pm 0.2^*$
10^{-5} M - Ca^{2+}	2630 ± 70	274 ± 26	$102 \pm 17^*$	$2.8 \pm 0.3^{**}$

Fig. 4. Dephosphorylation of Ins(1,4,5)P₃ following preincubation of RINm5F cells with TPA

Cells (2×10^6) were preincubated for 10 min in the presence (open symbols) or absence (solid symbols) of TPA (100 nM) followed by addition of Triton X-100 and [^{3} H]Ins(1,4,5)P₃ (0.5 μ M final) in a high-K⁺ containing medium. The InsP (triangles) and InsP₂ (rectangles) formed from Ins(1,4,5)P₃ (circles) were separated by anion-exchange chromatography. For further details see the Experimental section. Each point represents the mean of triplicate observations from a single experiment, representative of three.

uncertain. For example, in human neutrophils leukotriene B₄ promotes an Ins(1,4,5)P₃ response which is shorter-lived than that evoked by the chemotactic peptide fMet-Leu-Phe (despite similar initial peaks), and yet the latter is the much better activator of protein kinase C [37].

The other mechanism whereby protein kinase C has been proposed to regulate Ins(1,4,5)P₃ levels is by stimulation of Ins(1,4,5)P₃ phosphomonoesterase [19,36]. However, pretreatment of intact cells with TPA, or administration of the tumour promoter to permeabilized cells, did not stimulate the rate of Ins(1,4,5)P₃ dephosphorylation. Indeed, to our knowledge there have been no confirmatory reports of such an effect of protein kinase C in any cell other than platelets, suggesting that it may not be widespread. Incidentally the findings reported here and elsewhere [33] that TPA could abolish agonist-induced rises in Ins(1,4,5)P₃ without stimulating phosphomonoesterase activity, argue against a previous proposal that these two phenomena are causally related [19]. The present study does not rule out the possibility that stimulation of Ins(1,4,5)P₃ phosphomonoesterase could conceivably play a role in the transient elevation of Ins(1,4,5)P₃. However, potential mechanisms are not obvious since, apart from the negative findings with TPA, a rise in Ca²⁺ was also without effect on the activity of this enzyme [9].

The third proposed mechanism by which Ins(1,4,5)P₃ levels might be regulated is via the Ca²⁺-calmodulin-sensitive Ins(1,4,5)P₃ kinase [9,21]. This appears widely

applicable, with probable relevance to adrenal glomerulosa [38], brain [39] heart [40], smooth muscle [41], T cells [14,15] and HL60 cells [12]. The original studies with RINm5F cells, as well as showing that the Ins(1,4,5)P₃ kinase was sensitive to Ca²⁺ [9] or Ca²⁺-calmodulin [21], also demonstrated that the decline of Ins(1,4,5)P₃ from its peak level in intact cells coincided with the maximal rise in [Ca²⁺]_i [8], and that the two-step conversion of Ins(1,4,5)P₃ to Ins(1,3,4)P₃ was markedly attenuated if this rise in [Ca²⁺]_i was abolished [9]. These findings have now been extended by the current demonstrations using permeabilized cells, showing that elevations in Ca²⁺ lead both to an enhanced accumulation of Ins(1,3,4,5)P₄, and to a marked decrease in metabolic flux through Ins(1,4)P₂. The latter effect could also be partially mimicked by cyclic AMP and TPA, supporting a recent report showing that Ins(1,4,5)P₃ kinase from a clonal T-cell line was activated by protein kinase C [15], and suggesting multiple control mechanisms for Ins(1,3,4,5)P₄ generation. In addition, the finding that, in stimulated cells, the rise in Ins(1,3,4,5)P₄ preceded that of Ins(1,4)P₂ suggests that the Ins(1,4,5)P₃ kinase predominates in the very early disposal of Ins(1,4,5)P₃. This is consistent with the fact that Ins(1,4,5)P₃ phosphomonoesterase has a relatively low affinity for its substrate [9] and so may come into play at slightly later times, when the low-K_m Ins(1,4,5)P₃ kinase is presumably saturated. According to this explanation, the lag time between the rises in Ins(1,4)P₂ and Ins(1,3,4,5)P₄ would be more pronounced with weak agonists (such as carbamoylcholine in RINm5F cells) or submaximal concentrations of more potent agonists, since the greater the production of Ins(1,4,5)P₃, the more important will be the contribution of the high-capacity phosphomonoesterase.

Of the three mechanisms examined only two appear potentially applicable to the regulation of Ins(1,4,5)P₃ levels in RINm5F cells. Of these the role of the Ca²⁺-calmodulin-activated Ins(1,4,5)P₃ kinase appears physiologically relevant, since manipulations of [Ca²⁺]_i in various cell preparations have been shown to result in reciprocal alterations in the levels of Ins(1,4,5)P₃ versus Ins(1,3,4,5)P₄ or Ins(1,3,4)P₃ [9,12,14,39]. However, this mechanism is unlikely to account solely for the decline in Ins(1,4,5)P₃. In neutrophils, for example, different agonists may give Ins(1,4,5)P₃ transients of varying duration, despite initiating peak increases in [Ca²⁺]_i, which are virtually identical [37]. Therefore, although increased flux through the Ca²⁺-calmodulin-sensitive Ins(1,4,5)P₃ kinase appears to play an important role, some decrease in PtdIns(4,5)P₂ hydrolysis is also likely to contribute to the decline of Ins(1,4,5)P₃ from its initial peak in stimulated RINm5F cells. However, even in GH₃ cells, where PtdIns(4,5)P₂ hydrolysis does appear to decline in the continued presence of agonist [16], the evidence linking this decline to the transient peak of Ins(1,4,5)P₃ remains circumstantial; detailed correlations between the rates of Ins(1,4,5)P₃ generation and accumulation have yet to be performed at the very early time points following stimulation.

We thank Mrs. A.-S. Annen for expert technical assistance in the maintenance of cell cultures. This work was supported by grant no. 3.214.0.85 of the Swiss National Science Foundation. L.V. was the recipient of a long-term fellowship from the European Molecular Biology Organization.

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Received 22 June 1987/19 October 1987; accepted 4 December 1987