RESEARCH COMMUNICATION

Simultaneous oscillations of cytoplasmic free Ca^{2+} concentration and Ins(1,4,5)P_3 concentration in mouse pancreatic β-cells

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Changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in stimulated cells are often oscillatory, but the mechanisms that drive these oscillations are still a matter of controversy: different models of the generation of these $[Ca^{2+}]_i$ oscillations make different assumptions as to whether oscillations in Ins(1,4,5)P_3 concentration are necessary for this process. We have looked for changes in inositol polyphosphate levels that might occur in suspensions of murine pancreatic β-cells when these cells are induced to display synchronized oscillations in $[Ca^{2+}]_i$ by the sequential addition of glucose, an α_2-adrenergic stimulus and extracellular Ca^{2+}. The intracellular level of Ins(1,4,5)P_3 oscillated in a manner approximately in synchrony with changes in $[Ca^{2+}]_i$. Oscillations in the levels of Ins(1,4,5)P_3 metabolites [Ins(1,3,4)P_3 and inositol bisphosphates] were slightly delayed relative to the Ins(1,4,5)P_3 oscillations, and the concentration of Ins(1,3,4,5,6)P_5 remained approximately constant during the $[Ca^{2+}]_i$ oscillations. These results demonstrate that [Ins(1,4,5)P_3] and $[Ca^{2+}]_i$ oscillate in synchrony in at least one type of cell. Whether such oscillations in intracellular [Ins(1,4,5)P_3] provide a primary driving force for $[Ca^{2+}]_i$ oscillations either in β-cells or in other stimulated cells remains to be determined. Even if they do not, the [Ins(1,4,5)P_3] oscillations will at least provide an amplifying influence on the $[Ca^{2+}]_i$ changes.

INTRODUCTION

An important recent insight into intracellular regulation has been recognition that changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in stimulated cells are often oscillatory, and that increased stimulus intensity often increases the frequency, rather than the amplitude, of these repetitive $[Ca^{2+}]_i$ changes [1]. A major factor controlling the $[Ca^{2+}]_i$ in eukaryotic cells is the intracellular concentration of Ins(1,4,5)P_3 [2]. However, the mechanisms that drive these $[Ca^{2+}]_i$ oscillations are still a matter of controversy. Available models of the generation of these oscillations make different assumptions as to whether oscillations in Ins(1,4,5)P_3 concentration are necessary for this process [3–7]. In particular, only one report [8] has directly asked whether oscillatory changes in the intracellular concentration of the second messenger Ins(1,4,5)P_3 are involved in driving oscillations in $[Ca^{2+}]_i$.

Exposure to glucose at concentrations capable of stimulating insulin secretion can initiate synchronized $[Ca^{2+}]_i$ oscillations in the β-cells of murine pancreatic islets [9]. Moreover, patch-clamped β-cells that are stimulated with glucose display an Ins(1,4,5)P_3-dependent periodic activation of a Ca^{2+}-dependent K^+ conductance [10], and there are conditions under which synchronized $[Ca^{2+}]_i$ oscillations can consistently be provoked in suspensions of murine β-cells [11]. Insulin secretion from β-cells in response to glucose is dependent on the voltage-gated entry of extracellular Ca^{2+}. Although this is a process in which Ins(1,4,5)P_3 has no essential role, it is accompanied by formation of Ins(1,4,5)P_3, at least a part of which may be a response to the rise in $[Ca^{2+}]_i$ [12]. Many neural and endocrine agents which modulate this central mechanism for the control of insulin secretion do so by signalling through Ins(1,4,5)P_3 generation, and Ins(1,4,5)P_3-mediated Ca^{2+} mobilization can elicit modest levels of insulin secretion [13–15]. Suspensions of murine β-cells thus offer a rare opportunity to measure both $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 concentration during a single oscillation.

Using these cell suspensions, we have explored the temporal relationships between oscillatory changes in $[Ca^{2+}]_i$ and the intracellular levels of Ins(1,4,5)P_3 and other inositol polyphosphates. We find that oscillatory changes in Ins(1,4,5)P_3 levels parallel the first $[Ca^{2+}]_i$ oscillation, whilst Ins(1,3,4,5,6)P_5, a major inositol polyphosphate of these cells, shows no consistent change. Although it is known that $[Ca^{2+}]_i$ oscillations can be provoked in some cells without accompanying oscillations in [Ins(1,4,5)P_3], these results add experimental support to the idea that at least one mechanism for generating $[Ca^{2+}]_i$ oscillations involves simultaneous oscillatory changes in intracellular [Ins(1,4,5)P_3].

MATERIALS AND METHODS

Isolation and incubation of β-cells

For each experiment, pancreatic β-cells were isolated from two *ob/ob* mice [16]. The basic medium used for cell preparation and experiments was a Hepes buffer, pH 7.4, with Cl^- as the sole anion and containing 1.28 mM Ca^{2+} and 1 mg/ml BSA [17]. The isolated cells were cultured for 24 h in inositol-free RPMI 1640 medium containing 40 μCi/ml [2-^{3}H]inositol (Amersham International), 10% (v/v) foetal calf serum, 100 i.u./ml penicillin, 100 mg/ml streptomycin and 60 mg/ml gentamycin. The labelled cells were loaded with Fura-2 by incubation with 1 mM Fura-2/AM for 45 min, washed twice in buffer, suspended in 3.5 ml of buffer and transferred to a stirred fluorimeter cuvette held at 37 °C. As shown in Figure 1(a), the subsequent additions were 20 mM glucose, 10 nM clonidine and 5 mM Ca^{2+}. $[Ca^{2+}]_i$ was monitored with a SPEX Fluorolog-2 CM1T11I fluorimeter; the

Abbreviation used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration.

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excitation wavelengths (340 nm and 380 nm) were generated by two monochromators, and emitted light was collected through a 510 nm filter [18]. $[Ca^{2+}]_i$ is expressed as the ratio of fluorescence at 340 nm and at 380 nm.

The methods used to follow the $[Ca^{2+}]_i$ and $[Ins(1,4,5)P_3]$ oscillations in the present experiments did not permit us directly to calibrate the intracellular Ca^{2+} levels and, in any case, such calibrations on suspensions of cells are at best gross estimates. However, our previous experience with this cell system enables us to be confident that at no time does the raised $[Ca^{2+}]_i$ reach a level at which the $Ins(1,4,5)P_3$ receptors will be down-regulated [19,20].

Analysis of β-cell inositol phosphates

Samples were withdrawn at appropriate times (see Figure 1a), quenched in 10% (w/v) trichloroacetic acid and phytate hydrolysate was added. Trichloroacetic acid was removed by three washes with diethyl ether, and the samples were brought to pH 6–7 with 30 μl of 0.1 M EDTA, pH 7.0, and stored at −20 °C. H.p.l.c. on a 25 cm Whatman Partisphere-WAX column (Laserchrom U.K. Ltd.) was optimized to achieve separation of labelled $Ins(1,4,5)P_3$ from a second ^{3}H-labelled $InsP_3$, possibly D/L-$Ins(1,4,6)P_3$, that eluted slightly earlier and was present in mouse β-cells. The column was equilibrated with water, the sample loaded and elution, at a flow rate of 1 ml/min, was achieved with the following gradient, generated from water (Pump A) and 0.5 M $(NH_4)_2PO_4$ that had been adjusted to pH 3.2 with H_3PO_4 (Pump B): 0 min, 0% B; 5 min, 0% B; 30 min, 20% B; 75 min, 22% B; 80 min, 35% B; 100 min, 43% B; 105 min, 80% B; 115 min, 100% B; 125 min, 100% B; 126 min, 0% B. Samples were collected (every 15 s in the $InsP_3$ region) and radioactivity was determined using Uniscint BD (National Diagnostics) and a Packard Tricarb liquid scintillation counter. As illustrated in Figure 1(b), which shows the $InsP_3$ region of such a chromatogram in the shallow gradient between 30 and 75 min, endogenously formed $[^3H]Ins(1,4,5)P_3$ {identified by its co-chromatography with an internal standard of $[^{14}C]Ins(1,4,5)P_3$} was resolved from the putative $Ins(1,4,6)P_3$ peak.

RESULTS

Definition of oscillatory behaviour and of appropriate sampling and analysis techniques for inositol phosphates

Isolated β-cells and small β-cell aggregates from the mouse show 2–5 min oscillations in $[Ca^{2+}]_i$ [18]. Moreover, β-cells of a single murine islet of Langerhans display synchronized $[Ca^{2+}]_i$ oscillations when exposed to 5.5–22 mM glucose [9]. Using suspensions of murine β-cells, synchronized $[Ca^{2+}]_i$ oscillations of fairly long period can be initiated by the protocol that is illustrated in Figure 1 [11]. This involves sequential addition of: (1) 20 mM glucose, which depolarizes the cells and leads to Ca^{2+} entry through potential-regulated Ca^{2+} channels; (2) somatostatin, galanin or an α_2-adrenergic agonist such as clonidine, any of which cause β-cell repolarization as a result of pertussis toxin-sensitive activation of a low-conductance K^+ channel [21]; and, finally, (3) elevation of extracellular $[Ca^{2+}]$ from 1.28 mM to 6.28 mM. We have used the experimental system illustrated in Figure 1 to explore the relationship between the induced $[Ca^{2+}]_i$ oscillations and changes in the intracellular concentration of $Ins(1,4,5)P_3$.

In the absence of a continuous technique for following temporal changes in intracellular $[Ins(1,4,5)P_3]$ in single cells, we elected to use multiple sampling from synchronized β-cell suspensions to determine whether changes in $[Ins(1,4,5)P_3]$ might accompany, and possibly drive, the $[Ca^{2+}]_i$ oscillations. Murine β-cells that had been labelled with [^{3}H]inositol were loaded with Fura-2 and induced into $[Ca^{2+}]_i$ oscillations. Changes in $[Ca^{2+}]_i$ were followed spectrofluorimetrically and used to dictate at what key times, such as the trough, midpoints and peak of the first and best defined oscillation, samples should be withdrawn for analysis of inositol phosphates.

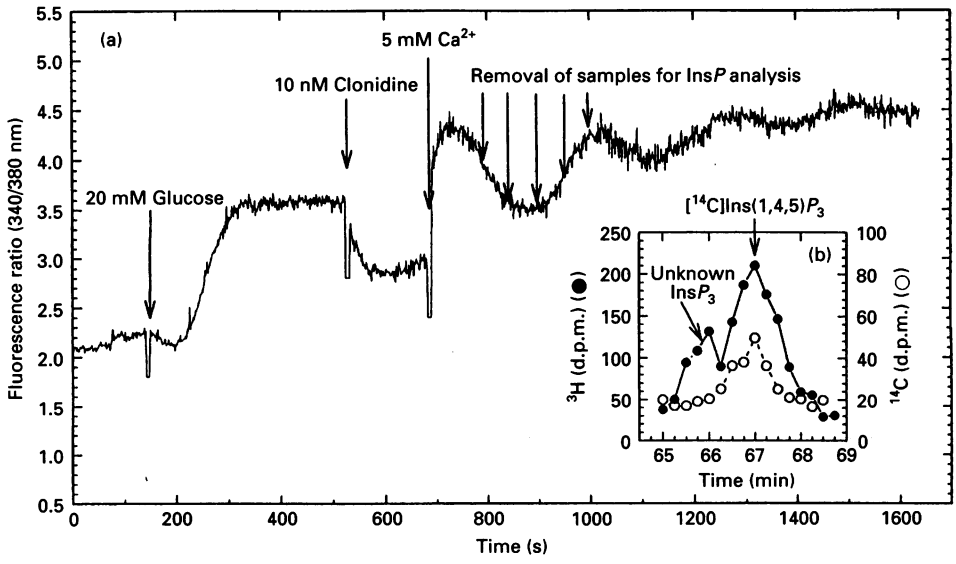

Figure 1 (a) Stimulus and sampling procedures used to initiate $[Ca^{2+}]_i$ oscillations in suspensions of β-cells and to sample for inositol phosphate analysis during such oscillations; (b) h.p.l.c. resolution of $Ins(1,4,5)P_3$ from a second unidentified $InsP_3$ isomer in these extracts

The experimental procedures were exactly as described in the Materials and methods section.

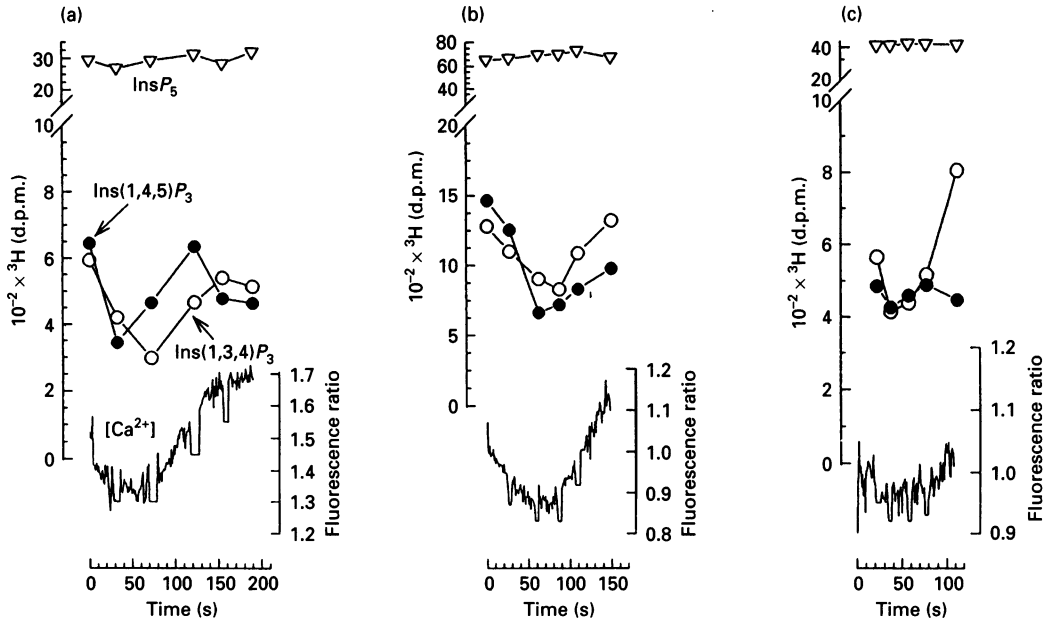

Figure 2 Temporally linked oscillations of intracellular $[Ca^{2+}]_i$ and of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ levels in stimulated murine pancreatic β-cells

Cells were isolated, stimulated and their inositol phosphates extracted and analysed as described in the Materials and methods section. The data shown are the results of continuous $[Ca^{2+}]_i$ traces and of single h.p.l.c. analyses of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ in samples withdrawn during Ca^{2+} recordings on three cell suspensions, representative of five experiments that gave similar results. The major deflections of the $[Ca^{2+}]_i$ trace, which have been truncated for clarity, are artefacts caused by the sampling of the cell suspension for inositol phosphate analysis. The levels of $Ins(1,4)P_2$ and of $Ins(3,4)P_2/Ins(1,3)P_2$ in these samples showed temporal changes that were similar to those of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$, except that the $InsP_2$ changes were slightly delayed (not shown).

The presence of an unidentified inositol trisphosphate

Initial results suggested that $[Ins(1,4,5)P_3]$ might rise and fall slightly in synchrony with the $[Ca^{2+}]_i$ oscillations, but detailed analysis of these experiments revealed that an unidentified inositol trisphosphate, which showed no change during the oscillations and has the elution time of D- or L-$Ins(1,4,6)P_3$, contributed up to half of the '$Ins(1,4,5)P_3$' peak from our usual h.p.l.c. separation. Once an effective separation of $Ins(1,4,5)P_3$ from this contaminating $InsP_3$ had been established (see Figure 1b), detailed measurements of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ levels during $[Ca^{2+}]_i$ oscillations showed major changes that largely parallelled the $[Ca^{2+}]_i$ changes. The small quantities of $Ins(1,3,4,5)P_4$, the metabolic intermediate that links $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$, that were present were insufficient for reliable analysis.

Synchronized oscillations of inositol phosphates and of $[Ca^{2+}]_i$

Figure 2 presents, from three of five consecutive experiments, the relevant parts of the first $[Ca^{2+}]_i$ oscillation following the triggering addition of Ca^{2+}. Essentially similar and temporally linked changes, of varying magnitude, were seen in $[Ca^{2+}]_i$, $[Ins(1,4,5)P_3]$ and $[Ins(1,3,4)P_3]$ in all experiments.

The limitations imposed by the small number of samples, and thus data-points, that could be obtained from each incubation of a β-cell suspension meant that no single experiment could yield a definitive picture of the relationships between these three key variables. However, a number of consistent features were seen throughout the experiments. (1) The level of labelled $Ins(1,3,4,5,6)P_5$, an abundant inositol polyphosphate in these cells whose metabolism is not closely linked to that of $Ins(1,4,5)P_3$, did not change during the period over which oscillations occurred both in $[Ca^{2+}]_i$ and in the levels of the various inositol phosphates downstream of phosphoinositidase C activation. The stable level of $Ins(1,3,4,5,6)P_5$ thus serves as an internal control: it excludes trivial technical explanations for the oscillations in $Ins(1,4,5)P_3$ (and its metabolites) and indicates that the changes in labelling of other inositol phosphates reflect changes in concentrations rather than artefacts caused by intracellular isotope pooling effects. (2) Large $[Ca^{2+}]_i$ oscillations (as in Figures 2a and 2b) were accompanied by larger excursions in the levels of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ than were smaller $[Ca^{2+}]_i$ oscillations (e.g. Figure 2c). (3) Excursions in $[Ins(1,4,5)P_3]$ generally preceded excursions in $[Ins(1,3,4)P_3]$, as expected if $Ins(1,4,5)P_3$ is the initial product of phosphoinositidase C activation and $Ins(1,3,4)P_3$ lies two metabolic steps downstream of $Ins(1,4,5)P_3$. (4) The minima in the excursions of $[Ca^{2+}]_i$ and of $Ins(1,4,5)P_3$ were clearly in phase, but the resolving power of the experimental design was insufficient to reveal whether there was any time-lag between achievement of minimum $[Ins(1,4,5)P_3]$ and minimum $[Ca^{2+}]_i$. (5) The levels of three early products of $Ins(1,4,5)P_3$ metabolism, namely $Ins(1,4)P_2$ [the product of 5-phosphatase attack upon $Ins(1,4,5)P_3$] and $Ins(3,4)P_2/Ins(1,3)P_2$ [products of $Ins(1,3,4)P_3$ dephosphorylation] also oscillated, but these changes lagged behind those of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ (results not shown).

DISCUSSION

In principle, intracellular oscillations in $[Ins(1,4,5)P_3]$ could be caused either by oscillations in phosphoinositidase C activity or, with the phases reversed, by oscillations in $Ins(1,4,5)P_3$ catabolism. The fact that the oscillations of all of the products of $Ins(1,4,5)P_3$ metabolism lagged behind, rather than preceded, those of $Ins(1,4,5)P_3$ implies that the main controlled event in these β-cell suspensions was phosphoinositidase C activity. How much of this control of phosphoinositidase C activity was due to Ca^{2+} activation and how much to other influences remains undefined. In these murine β-cell suspensions, there is an

immediate increase in [Ins(1,4,5)P_3] following glucose addition (C. J. Barker, T. Nilsson, C. J. Kirk, R. H. Michell and P.-O. Berggren, unpublished work), at least a part of which might be a result of the inhibitory effects of glucose metabolites on Ins(1,4,5)P_3 5-phosphatase activity [22]. Oscillatory regulation of the mobilization of intracellular Ca^{2+} will be an inevitable consequence of the resulting [Ins(1,4,5)P_3] excursions, but the information presented here does not allow any clear conclusion as to what fraction of the observed $[Ca^{2+}]_i$ oscillations is likely to be driven by the oscillations in [Ins(1,4,5)P_3].

Early hypothetical models of the generation of repetitive $[Ca^{2+}]_i$ changes in stimulated cells suggested either: (a) that an initial elevation of $[Ca^{2+}]_i$ [caused by Ins(1,4,5)P_3 or initiated by an alternative mechanism] might initiate oscillations that would subsequently become self-sustaining [3]; or (b) that $[Ca^{2+}]_i$ oscillations might be driven directly by [Ins(1,4,5)P_3] oscillations [4]. As the number of situations in which $[Ca^{2+}]_i$ oscillations can be observed has proliferated, so has the variety of subtly different Ca^{2+}-driven triggering mechanisms become better appreciated, but the question of whether $[Ca^{2+}]_i$ oscillations are ever accompanied by, and possibly caused by, oscillations in intracellular [Ins(1,4,5)P_3] has remained unresolved.

There is one previous report [8] which suggests a phenomenon partially similar to that reported here. In that study, REF52 fibroblasts were provoked into a synchronized elevation of cytosolic $[Ca^{2+}]_i$ by the withdrawal and subsequent re-admission of extracellular Ca^{2+} during a period of continuous vasopressin stimulation, and the $[Ca^{2+}]_i$ rise following Ca^{2+} re-admission was accompanied by an approximately simultaneous rise and fall of [Ins(1,4,5)P_3]. However, the $[Ca^{2+}]_i$ rise in these cells was not followed by clear-cut synchronized $[Ca^{2+}]_i$ oscillations of the type depicted in Figure 1(a) of the present paper, so the results were uninformative about the behaviour of Ins(1,4,5)P_3 during intervals between maxima in a $[Ca^{2+}]_i$ oscillatory system. Recent experiments of quite different design [23] have also come, albeit indirectly, to the conclusion that sinusoidal oscillations in $[Ca^{2+}]_i$ can probably be driven by oscillatory changes in the intracellular concentration of Ins(1,4,5)P_3. In that study, it was observed that cholinergically stimulated $[Ca^{2+}]_i$ oscillations in mouse lacrimal cells were suppressed by activation of protein kinase C, whereas the $[Ca^{2+}]_i$ rise provoked by injection of Ins(2,4,5)P_3 was neither oscillatory nor modified by the activity state of protein kinase C. These results were interpreted in terms of a model based in part on Cobbold's ideas [4]: it was proposed that $[Ca^{2+}]_i$ oscillations are caused directly by oscillations in the level of activation of phosphoinositidase C, and thus of the formation of both Ins(1,4,5)P_3 and 1,2-diacylglycerol, and that this regulation of phosphoinositidase C is achieved through a negative feedback loop involving its inhibition by the 1,2-diacylglycerol which it liberates. In contrast, the results reported here provide a direct demonstration that the downstroke and subsequent upward inflection of $[Ca^{2+}]_i$ during well-defined intracellular $[Ca^{2+}]_i$ oscillations are, at least in murine β-cells, accompanied by simultaneous oscillations in Ins(1,4,5)P_3 formation by phosphoinositidase C. Whether such oscillations in intracellular [Ins(1,4,5)P_3] provide a primary driving force for $[Ca^{2+}]_i$ oscillations either in β-cells or in other stimulated cells remains to be determined. Even if they do not, it is hard to avoid the conclusion that the [Ins(1,4,5)P_3] oscillations will at least provide an amplifying influence on the changes in $[Ca^{2+}]_i$.

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