The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus

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When Swiss 3T3 cells are treated with Insulin-like Growth Factor I, a rapid decrease in the mass of polyphosphoinositol lipids (phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate) occurs within the nuclei, with a concomitant increase in nuclear diacylglycerol and translocation of protein kinase C to the nuclear region. This is in contrast to the effects of the regulatory peptide, bombesin, which causes similar inositol lipid changes in the plasma membrane, has no effect on nuclear inositide levels and causes a translocation of protein kinase C to post-nuclear membranes. These results suggest the existence of a discrete nuclear polyphosphoinositide signalling system entirely distinct from the well-known plasma membrane-located system, which is under regulatory control by cell surface-located receptors.

Key words: insulin-like growth factor/nucleus/polyphosphoinositide cycle/protein translocation/receptors

Introduction

There exists in the plasma membrane of most eukaryotic cells a signal transduction system that utilizes the inositol lipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], as a precursor of three second messengers, diacylglycerol, a physiological stimulator of protein kinase C (PKC) (Nishizuka, 1988), and the inositol polyphosphates Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ which regulate intracellular Ca²⁺ (Berridge and Irvine, 1989). The primary location of the pool of diacylglycerol involved in the activation of PKC is believed to be the plasma membrane (Nishizuka, 1988). However, a number of reports have shown that if cells are treated with tumour-promoting phorbol esters (which stimulate PKC), there is a translocation of PKC to the nucleus (e.g. Kiss *et al.*, 1988; Leach *et al.*, 1989), and this has led to the suggestion that there are intranuclear targets for PKC (Rozengurt and Sinnett-Smith, 1987; Kiss *et al.*, 1988; Fields *et al.*, 1989; Leach *et al.*, 1989), perhaps involved in the control of mitogenesis. It has been generally assumed that the activation of PKC would occur first in the plasma membrane or cytoplasm, and that this would then result in translocation to the nucleus.

However, as PKC translocation is generally considered to be a phenomenon of binding to diacylglycerol (DAG), an alternative mechanism for initiating nuclear translocation could be the generation of a nuclear pool of DAG. The presence of an entirely separate nuclear phosphoinositide

signalling system has been hinted at by the circumstantial evidence that purified nuclei have the ability to synthesize polyphosphoinositide lipids *in vitro* (Cocco *et al.*, 1987), and that some aspects of this metabolism might change when Insulin-like Growth Factor I (IGF-I), a tyrosine kinase-stimulating growth factor, is applied to intact Swiss 3T3 cells but not to the isolated nuclei (Cocco *et al.*, 1989).

Here we have set up picomole sensitivity mass assays for PtdIns, PtdIns(4)P, PtdIns(4,5)P₂ and DAG, and have used these to document the lipid changes that take place in the nuclei of Swiss 3T3 cells when the cells are treated with IGF-I. We demonstrate, as far as is possible, that these changes are indeed nuclear, and also show that co-incident with the increase in nuclear DAG there is a specific translocation of PKC to the nuclear region. As these effects are near maximal within a few minutes they are amongst the earliest intranuclear events to be documented in response to tyrosine kinase(s) activation, and so they may represent a part of the mechanisms whereby information is transmitted from the plasma membrane to the nucleus to initiate cell division.

Results

Assays for inositides and diacylglycerol

Because the radiolabelling of inositides by [³H]inositol in the nucleus occurs at a very low level (Cocco *et al.*, 1989), and also because all radiolabelling experiments are subject to difficulties in interpretation unless equilibrium labelling can be demonstrated unequivocally, we have circumvented these difficulties by setting up mass assays of picomole sensitivity. The PtdIns(4)P and DAG assays are based on incorporation of radioactivity from [³²P]ATP using PtdIns(4)P-5-kinase and DAG kinase activities, partially purified from bovine and rat brain respectively (see Materials and methods and Figure 1). The DAG assay is based on the same principles as the assay of Preiss *et al.* (1986) but is more sensitive.

For PtdIns (Figure 1) we have not used PtdIns kinase as this is primarily a membrane-bound enzyme, and soluble enzymes are easier to deal with in this sort of assay. Instead we have used a coupled assay employing a phospholipase C and the DAG kinase (measuring incorporation of radioactivity from [³²P]ATP into PtdOH). This assay is sensitive to ~10 pmol and is, under these conditions, entirely specific for PtdIns [as opposed to PtdIns(4)P, PtdIns(4,5)P₂, or other glycerophospholipids, see Materials and methods]. For PtdIns(4,5)P₂ we have miniaturized the chemical procedures whereby the fatty acids and the glycerol are removed sequentially from this lipid (Irvine *et al.*, 1985), and then assayed the resulting Ins(1,4,5)P₃ by a well-established radioreceptor assay (Palmer and Wakelam, 1990); the entire procedure is conducted in one tube to avoid losses. We have not investigated the degree to which any of the lipids studied here change during preparation of the

Fig. 1. Standard curve of DAG, PtdInsP₂, PtdInsP and PtdIns mass measurements DAG. For assay details see Materials and methods. In the PtdInsP₂ panel, (●); PtdInsP₂ standards; (○) Ins(1,4,5)P₃ processed (i.e. taken through deacylation and deglyceration in an identical manner) as the PtdInsP₂.

nuclei; it may be that all our measured levels are underestimates of those *in vivo*, although the reproducibility of the overall results argue against extensive changes during nuclear isolation. Finally, we should note that the possible presence of nuclei in other polyphosphoinositides, phosphorylated in the 3 position (Whitman and Cantley, 1988) has not been addressed here. The assays for PtdIns(4)P and PtdIns(4,5)P₂ are isomer-specific by their nature, and no evidence for PtdIns(3,4)P₂ (Cocco *et al.*, 1987), nor for PtdIns(3)P (R.F.Irvine and D.J.Lander, unpublished observations) was found in nuclei of Friend cells labelled *in vitro* with [γ -³²P]ATP.

IGF-I-induced changes in nuclear lipid metabolism

Table I records the characteristics, in the cells used here, of the induction of cell growth by IGF-I or bombesin, or the two together, and their effects on nuclear polyphosphoinositide metabolism. IGF-I leads to a small increase in thymidine incorporation, as does bombesin, whereas together they lead to a synergistic increase as has been found previously (see Rozengurt and Sinnett-Smith, 1987 for further references); this is not maximal however, as fetal calf serum (FCS) was the most effective stimulator of both thymidine incorporation and nuclear polyphosphoinositide metabolism (Table I). However, because the central point of this paper is to dissect out the differential control and function of nuclear, as opposed to cytoplasmic, inositide metabolism, we have not used FCS, but have employed IGF-I or bombesin, mostly alone, and only rarely the two together, in these experiments.

Table I shows that within 5 min of treatment of cells with IGF-I, a change in DAG mass occurs in the nuclei. To investigate this in more detail we undertook an extensive series of experiments which are recorded in Figure 2. It is apparent that within 1–2 min of treatment of the cells,

a decrease occurs in PtdIns(4)P and PtdIns(4,5)P₂ and a concurrent rise is seen in DAG. Note that the nuclear isolation procedure takes 4–5 min before the nuclei could be described as ‘quenched’ (see Materials and methods) so we cannot be precise about the actual minimum time for an effect to be detectable; times of stimulation discussed here are from addition of the agonists to addition of cold isotonic medium (as the first stage in nuclear isolation). The mass increase in DAG is greater than the combined loss in the former two lipids, and we assume that, as occurs in the plasma membrane in other tissues (Downes *et al.*, 1989), some re-synthesis of PtdIns(4)P and PtdIns(4,5)P₂ has taken place; however, this would not produce an experimentally detectable decrease in PtdIns. We should also note that we cannot rule out a contribution to DAG from other lipids such as occurs from phosphatidylcholine in whole Swiss 3T3 cells in response to bombesin (Cook and Wakelam, 1989). The effect on nuclear polyphosphoinositides and DAG lasts for 30–45 min, and has disappeared by 60 min (Figure 2). This is, to our knowledge one of the most rapid nuclear metabolic effects detected in response to a tyrosine kinase-linked cell surface receptor and is entirely consistent with the activation of a phosphoinositide C within the nucleus which may be part of the sequence of events which lead ultimately to cell activation.

Is this a true nuclear event?

The nuclei prepared in this way, using Triton X-100 and deoxycholate (DOC), are apparently almost entirely clear of any cytoplasmic contamination (see below)—as were nuclei from Swiss 3T3 cells which incorporated radioactivity from [γ -³²P]ATP into polyphosphoinositides *in vitro* (Cocco *et al.*, 1988). Those nuclei (and also the ones used here, see below, and Figure 4) are stripped of their outer nuclear envelope; furthermore, nuclei from Friend cells and

Table I. Incorporation of [^3H]thymidine (16 h) into Swiss 3T3 cells, and concentrations of DAG (5 min) and PtdIns (5 min) in cell nuclei

	Control	IGF-I (20 ng/ml)	Bombesin	IGF-I (20 ng/ml) + bombesin (1 nM)	10% FCS
[^3H]Thymidine incorporation (c.p.m./well)	193 $\pm$ 15	546 $\pm$ 46*	211 $\pm$ 37 (1 nM) 572 $\pm$ 37* (5 nM)	1294 $\pm$ 242*	7015 $\pm$ 380*
DAG in nuclei (pmol/mg protein)	111 $\pm$ 6	173 $\pm$ 14*	112 $\pm$ 8 (1 nM) 117 $\pm$ 13 (5 nM)	212 $\pm$ 12*	257 $\pm$ 31*
PtdIns in nuclei (nmol/mg protein)	2.14 $\pm$ 0.1	1.98 $\pm$ 0.07	2.02 $\pm$ 0.09 (1 nM) 2.14 $\pm$ 0.12 (5 nM)	2.06 $\pm$ 0.14	2.27 $\pm$ 0.08

For details see Materials and methods. Asterisks indicate significantly different ($P < 0.05$, Student's t -test) from controls.

Fig. 2. Time course of changes in DAG, PtdInsP₂, PtdInsP and PtdIns concentration in nuclei obtained from Swiss 3T3 cells; control (●—●) or IGF-I (○—○) (20 ng/ml). Each point represents the mean $\pm$ SE from three different experiments in duplicate for DAG and PtdIns, and single measurements for PtdInsP₂ and PtdInsP. Bars of half length indicate that at these times the difference between the corresponding experimental and control values is not statistically specific. All other details are described in Materials and methods.

hepatocytes retain the ability to incorporate radioactivity into polyphosphoinositides *in vitro* even when their nuclear envelopes are removed (Cocco *et al.*, 1987). Thus we can interpret these results to mean that the changes in Figure 2 may be occurring in the laminar layer/inner envelope or further inside the nucleus, and are probably not a result of cytoplasmic contamination of the nucleus (caused by, for example, the known continuity between the endoplasmic reticulum and the outer nuclear envelope). However, given the novelty and potential importance of the effects documented in Figure 2, which resemble so closely at a superficial level the 'classic' inositide response in the cytoplasm/plasma membrane of so many cells (Nishizuka, 1988; Berridge and Irvine, 1989), we considered it essential to establish unequivocally that these phenomena are not the result of cytoplasmic contamination of the nuclei which may be undetectable by more conventional methods (e.g. enzyme markers). We have done this in two ways.

First (Figure 3), we have demonstrated that in Swiss 3T3 cells IGF-I does not cause any detectable increase in whole

cell DAG (or a decrease in PtdInsP₂), whereas bombesin does (yet it causes no change in nuclear lipids, Table I). Note that as judged by the DNA:protein ratios given in Table II, the purified, detergent-treated nuclei contain only ~2.5% of the original whole cell protein (the latter no doubt containing also serum proteins adsorbed onto cells and substratum); thus the changes in nuclear DAG and PtdInsP₂ seen in Figure 2 would only represent 2.5% of the totals in Figure 3, and so any changes induced in nuclear lipids by IGF-I would be experimentally undetectable in the whole cells. In our cells, even when primed in the way that Kojima *et al.* (1988) primed their cells, we could not detect a rise in intracellular Ca^{2+} or DAG in response to IGF-I alone (results not shown). The data in Figure 2 effectively eliminate a straightforward contamination artefact, but there still exist the very real possibilities either that treatment of the cells with IGF has in some way changed the contamination of nuclei with plasma membranes (by an alteration in cytoskeleton for example), or alternatively that the effect is taking place in the cytoplasm, near the nucleus.

Table II. DNA, PtdIns, PtdInsP and PtdInsP₂ concentrations in nuclei prepared either with NP-40 or Triton and deoxycholate (T+D)

		Control	IGF-I (20 ng/ml for 2 min)	
Whole cells		10 ± 1	12 ± 2	μg DNA/mg protein
Nuclear preparations	NP-40	127 ± 12	134 ± 17	μg DNA/mg protein
	T+D	380 ± 16	413 ± 21	μg DNA/mg protein
	NP-40	2.09 ± 0.17 [16.4]	2.22 ± 0.4 [16.6]	nmol PtdIns/mg protein [/mg DNA]
	T+D	2.17 ± 0.22 [5.7]	1.91 ± 0.19 [4.6]	nmol PtdIns/mg protein [/mg DNA]
	NP-40	88 [693]	83 [619]	pmol PtdInsP/mg protein [mg DNA]
	T+D	54 [142]	28 [68]	pmol PtdInsP/mg protein [/mg DNA]
	NP-40	15.1 ± 1.7 [119]	14.1 ± 1.1 [105]	pmol PtdInsP ₂ /mg protein [/mg DNA]
	T+D	7.2 ± 0.6 [19]	3.8 ± 0.5 [9]	pmol PtdInsP ₂ /mg protein [/mg DNA]

Nuclei were prepared and lipids measured as described in Materials and methods. DNA was measured as described by Burton (1955). For DNA and PtdIns, each data point represents mean values ± SE from duplicate determinations from three different experiments. For PtdInsP, data are mean values from single measurements in two experiments, while for PtdInsP₂ data are mean values ± SE from single measurements in three experiments. Also given in square brackets are the lipid data calculated on a DNA basis (see text); these are calculated from the numbers in the table (because lipids and DNA were not necessarily always measured in the same samples).

Fig. 3. A time course of changes of DAG and PtdInsP₂ concentration in (Δ···Δ), control ●—● IGF-I (20 ng/ml); and ○—○, IGF-I + bombesin (20 ng/ml, 1 nM)-stimulated Swiss 3T3 cells. Each point represents the mean ± SE from a single measurement in three different experiments. The significance of error bars is as in the legend to Figure 2.

We have tried to eliminate these possibilities by using an alternative method of preparing nuclei, a method which we abandoned at an early stage because it produces nuclei that are demonstrably impure and contaminated by cytoplasm. Figure 4 shows that if nuclei are prepared in NP-40 [a method which in other Swiss 3T3 cells produced very clean nuclei (Cocco *et al.*, 1988)—this is a problem of variability

between cell batches], they have some 'cytoplasm' associated with them and some also retain their nuclear envelopes. The simplest way to assess this contamination is on a basis of DNA; whereas the DNA:protein of NP-40 nuclei is greatly enriched over whole cells (Table II), this ratio is lower than in Triton/DOC nuclei. Also, it is clear that much of the PtdInsP and PtdInsP₂ in NP-40-prepared nuclei (>80%

Fig. 4. Phase contrast and electron microscopy of nuclear preparations from Swiss 3T3 cells. (A) Nuclei prepared as described in Materials and methods. (B) Nuclei prepared as described in Materials and methods but with 1% Nonidet P-40 instead of 1% Triton X-100 and 1% Na-DIC. (A') Electron micrograph of nuclei prepared as in (A). This extraction method produces a uniform preparation of nuclear remnants (N) with no traces of nuclear membranes and little cellular debris. (B') Electron micrograph of a nuclei prepared as in (B). NP-40 extraction results in a more varied nuclear remnant morphology (N) with occasional areas of recognizable nuclear envelope and a considerable contribution from cell fragments (asterisks). Bar = 1 μ M for both A' and B'.

calculated relative to DNA) is not there in Triton/DOC nuclei (Table II), and we interpret these data as showing that whereas pure nuclei contain levels of PtdIns per mg protein similar to the cytoplasm and whole cells, they have less PtdInsP and (particularly) PtdInsP₂. The data in Table II also demonstrate that the NP-40 nuclei do not show the lipid changes if the cells are stimulated with IGF-I, but that these changes are only detectable in the more purified nuclei; in the NP-40 nuclei the changes would be diluted 6-fold by the polyphosphoinositides which are removed by DOC and Triton (see above and Table II), so a 40–50% decrease (Figure 2 and Table II) would be experimentally undetectable in NP-40 nuclei if those latter polyphosphoinositides do not change. Taken together, these results suggest strongly that

we are observing effects occurring in the nuclei, and not in non-nuclear membranes contaminating them.

Translocation of protein kinase C to the nucleus

The simplest interpretation of the data described above is that IGF-I is inducing an intranuclear phosphoinositidase-C-catalysed hydrolysis of polyphosphoinositides. The subsequent processes that occur when a similar event is induced in the plasma membrane are increases in cytoplasmic Ca²⁺ induced by inositol phosphates (Berridge and Irvine, 1989) combined with the activation of PKC (Nishizuka, 1988). The latter is probably mediated by translocation of PKC from the cytosol to membranes, a process caused primarily by the generation of DAG, but probably also

Fig. 5. Translocation of PKC to nuclear and non-nuclear fractions. 3T3 cells grown for 7 days were quiesced overnight in DMEM + 0.1% BSA and stimulated with either IGF (20 ng/ml) for 2 min or 1 h, or with bombesin (10 nM) for 2 min. Cells were then lysed using a non-detergent hypotonic lysis buffer, and the nuclear and post-nuclear fractions were isolated (see Materials and methods). 50 μ g of protein was subjected to SDS-PAGE, transferred to nitrocellulose and probed with an anti-PKC antibody. Lanes 10 and 9 contain lysates from 3T3 whole cells before (lane 10) and after (lane 9) long-term treatment with TPA, demonstrating the specificity of the antibody. Lanes 1–4 are nuclear fractions while lanes 5–8 are post-nuclear membranes. Lanes 1 and 5, control; lanes 2 and 6, IGF 2 min; lanes 3 and 7, bombesin 2 min; lanes 4 and 8, IGF 1 h.

requiring the increased Ca^{2+} (Regazzi *et al.*, 1989; Trilivas and Brown, 1989). Both these branches of the inositide signalling system could potentially occur in nuclei: a recent report suggests that $\text{Ins}(1,4,5)\text{P}_3$ could regulate nuclear Ca^{2+} (Nicotera *et al.*, 1990), and, as discussed in the Introduction, the possibility of translocation of PKC to the nucleus has been considered by several groups (Rozengurt and Sinnett-Smith, 1987; Fields *et al.*, 1989; Kiss *et al.*, 1989; Leach *et al.*, 1989). Clearly, it is an intriguing possibility that some of the early events of IGF-I-mediated cell stimulation could involve a translocation of PKC to the nucleus, in turn mediated by the nuclear phosphoinositide metabolism and increase in nuclear DAG described here.

We have investigated this using a PKC antibody (one recognizing a consensus sequence common to α , γ and δ PKCs). For Western blotting experiments of this sort it was not possible to use nuclei prepared with Triton and DOC because these detergents removed the PKC, so instead nuclei had to be prepared without detergents—in fact the purity of the preparations does not differ obviously from NP-40 nuclei (as in Figure 4A—results not shown), but nevertheless these experiments can only address the possibility of PKC translocation to the nuclear region. Figure 5 shows that after 2 min treatment of Swiss 3T3 cells with IGF-I, there is a clear translocation of PKC, with a molecular weight of $\sim 80\text{K}$, to the nuclear region. This effect is no longer evident after 1 h (when nuclear DAG levels have returned to control levels, Figure 2), and is not mimicked by bombesin. Furthermore, when in the same experiment the post-nuclear membrane pellet is examined, bombesin does cause a translocation, and IGF does not—consistent with the

whole-cell data in Figure 3, and with the 'classic' cellular phosphoinositide responses induced by bombesin. Thus the translocation to the nucleus (or nuclear region) of Swiss 3T3 cells induced by IGF-I is clearly distinct from that induced by bombesin, and it seems very likely that it is a result of the stimulated nuclear polyphosphoinositide metabolism documented above.

Discussion

It is possible that the changes in nuclear inositide metabolism which are apparently caused by IGF-I are an integral part of the mechanism by which IGF-I stimulates cell division. However, we do not yet know whether this is a universal phenomenon caused by all tyrosine kinases; in parallel experiments with epidermal growth factor we detected no increase in nuclear DAG (results not shown). In these experiments IGF-I has served merely as an agonist that affects nuclear, but not cytoplasmic, inositide metabolism to a measurable degree, and given the differences that are manifest between different growth factors whose receptors contain tyrosine kinases (Klein *et al.*, 1985; Ullrich and Schlessinger, 1990), it remains to be explored whether nuclear inositide metabolism is necessarily a result of tyrosine kinase stimulation in general, and what role it plays in cell division. We assume for the purposes of discussion that it is tyrosine kinase activity of the IGF-I receptor which is the initial signal for those nuclear events, an assumption supported by preliminary data using tyrosine kinase inhibitors which attenuate nuclear DAG formation in response to IGF-I, but not the whole-cell response to bombesin (manuscript in preparation).

The nature of the next (downstream) stage of the signal transduction induced by nuclear phosphoinositide metabolism is an intriguing question. A recent report documenting a change in nuclear Ca^{2+} induced by $\text{Ins}(1,4,5)\text{P}_3$ (Nicotera *et al.*, 1989) implies that if this is formed (as is inevitable under our present interpretation), there may be Ca^{2+} changes to aid the DAG stimulation of PKC (or to do something else). However, in those experiments the $\text{Ins}(1,4,5)\text{P}_3$ was applied to the nuclei from outside, and caused a small decrease in intranuclear Ca^{2+} , so just how that response fits into the current context is unclear. We cannot exclude the possibility that $\text{PtdIns}(4)\text{P}$ is also hydrolysed by a phosphoinositidase C, and it is noteworthy that Sylvia *et al.* (1988) have suggested an intranuclear function for $\text{Ins}(1,4)\text{P}_2$.

As to targets for the putative nuclear PKC activation, there are reports of a rapid phosphorylation of lamins (Friedman and Ken, 1988) induced by stimulation of cells with tyrosine kinase-linked growth factors and by phorbol esters (Fields *et al.*, 1989, 1990), and it is certainly possible that it is PKC activated within the nucleus that is responsible for this (Hornbeck *et al.*, 1990). It has been suggested that PKC has a regulatory role in mRNA translocation from the nucleus (Schröder *et al.*, 1988), and this is another possible end-point for the metabolic events that we have observed. In Swiss 3T3 cells, *c-fos* induction can be stimulated by bombesin, or TPA, but not by insulin (presumed to be acting through IGF-I receptors, Bravo *et al.*, 1987) and this has interesting implications for the present results in so far as if that bombesin effect is truly due to PKC stimulation, it would be more likely to be indirect, or to involve a different

isozyme of PKC (otherwise IGF-I would be expected to have the same effect).

We are also not certain whether or not the translocation of PKC is truly within the nucleus (as has been suggested by others using TPA as a stimulus, e.g. Kiss *et al.*, 1989; Leach *et al.*, 1989). As discussed above, the DAG generated presently seems most likely to be in the laminar layer (or further inside the nucleus) rather than in the nuclear envelope, and it may be that DAG could transfer into the envelope to induce a translocation of PKC from the cytoplasm; if the PKC actually enters the nucleus it must presumably pass through the nuclear pores, and how intranuclear DAG could control such a process is unclear. Also, the concentration of DAG in the nuclei will, assuming uniform distribution, be lower than that in the plasma membrane (when the latter is induced by bombesin—see Figure 3 and Table II), so a localization of DAG to specific sites in the nucleus might be necessary to explain the PKC translocation shown in Figure 5. Clearly, there is much to learn about the details of the link which we infer here (on what we regard as strong circumstantial evidence) between nuclear DAG generation and the translocation of PKC; localization by immunocytochemistry of both DAG and PKC will be very enlightening.

Finally, the mechanism of the activation of a nuclear phosphoinositidase C (PIC) is obscure. Some tyrosine kinases can phosphorylate cytoplasmic PIC and activate it (Nishibe *et al.*, 1990; Ullrich and Schlessinger, 1990), but if that is the mechanism here, is it a cytosolic enzyme which is phosphorylated and then translocated to the nucleus, or is the enzyme already there? The clear changes that we have observed in nuclear lipids could be used as simple markers when developing *in vitro* assays to identify the nature of the signal which traverses the cytoplasm. Whatever the mechanism of activation, and of its consequences, we believe that the data here point to a discrete role for nuclear polyphosphoinositide turnover in the regulation of nuclear function.

Materials and methods

Culture of Swiss 3T3 cells

Swiss 3T3 fibroblasts were grown in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% FCS and containing 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Treatment of cells and extraction of lipids

Confluent cells were incubated overnight with DMEM containing 0.1% BSA. Cells were then incubated for 30 min in a water bath in Hanks' physiological salt solution, in which stimulation with growth factors was induced. The Hanks' solution was removed by suction, and the cells were quenched with 1.5 ml chloroform/methanol (1:2) for DAG measurements, or 1.5 ml chloroform/methanol/HCl (180:160:1) for PtdInsP₂ measurements. DAG and PtdInsP₂ were extracted and measured as described below.

Thymidine incorporation following growth factor treatment

Thymidine incorporation was based on that of Corps *et al.* (1985). Briefly, cells were plated at 20 000 cells per 3 cm dish, grown for 7 days then kept quiescent overnight using DMEM, 0.1% BSA. Growth factors were incubated with cells in the above medium together with 1 μ Ci [³H]thymidine and 1 μ M cold thymidine for 16 h. Cells were then washed three times with 2 ml ice-cold phosphate-buffered saline followed by precipitation with 10% TCA on ice for 15 min. Cells were then washed three times in 100% ethanol and finally solubilized in 2 ml of 0.1 M NaOH. 1 ml of this was mixed with 1 ml of 0.2 M HCl and counted after the addition of 18 ml of scintillation fluid (Ready Value, Beckman).

Isolation of 3T3 fibroblast nuclei and extraction of lipids

The following preparation of nuclei is based on a modification of that described by Thomas *et al.* (1977). Swiss 3T3 cells were washed with ice-cold hypotonic cell-wash buffer (1.5 mM KCl, 2.5 mM MgCl₂, 5 mM Tris-HCl, pH 7.4) and all subsequent procedures were carried out at 4°C. Cells were swelled for 4 min in the same buffer, and after removal of the buffer, 15 ml of cell lysis buffer [hypotonic cell-wash buffer containing 1% Na-DOC, 1% Triton X-100, 0.1 mM PMSF, 1 mM EGTA and 1:10 000 leupeptin (25 mg/ml)] was added. Cells were broken by gentle pipetting. The dish was washed with an additional 5 ml of buffer and 6.66 ml of 1.2 M sucrose and 50 μ l of 1 M MgCl₂ were added. Nuclei were then sedimented at 500 g for 10 min. The pellet was resuspended in 4 ml of nuclei-wash buffer (1.5 mM KCl, 5 mM MgCl₂, 5 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.32 M sucrose, 1 mM EGTA and PMSF + leupeptin, as in cell lysis buffer) and layered over 20 ml of the same buffer containing 0.5 M sucrose. Nuclei were then sedimented at 500 g for 10 min. The pellet was resuspended in 20 ml of the final buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.32 M sucrose, 5 mM β -mercaptoethanol, 1 mM EGTA and PMSF + leupeptin as in cell lysis buffer), and nuclei were sedimented at 500 g for 10 min.

Mass assay of lipid extraction and quantification

DAG from the nuclei was extracted using 0.75 ml chloroform/methanol (1:2), while if inositides were to be measured the nuclei were extracted using 0.75 ml chloroform/methanol/1 M HCl (80:160:1). Further extraction was performed as described by Folch *et al.* (1957). After the lipids were dried, PtdIns mass measurement was performed as described below, while DAG was dissolved in 0.5 ml of chloroform and loaded on a silicic acid column (0.5 ml made in a Pasteur pipette), eluted with 1 ml of chloroform, dried once again and the mass measurement for DAG was then performed as described below. Protein concentration was measured as described by Bradford (1976) using a Bio-Rad protein assay kit. Each data point represents mean value $\pm$ SE of duplicate determinations from three different experiments (PtdInsP values are means of duplicate experiments). For statistical analyses, the Student's *t*-test for unpaired samples at the level of significance of 0.05 was used; *significantly different from control.

DAG. DAG kinase purification was achieved in a single step from rat brain by using a DEAE-Sephacel column as described by Divecha and Irvine (1990). The mass measurement for DAG was performed in the following manner. The dried lipid was dissolved by the addition of 20 μ l of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 s. After the addition of 80 μ l of buffer (50 mM Tris-acetate, 80 mM KCl, 10 mM magnesium acetate, 2 mM EGTA, pH 7.4), the reaction was started by the addition of 20 μ l of DAG kinase enzyme followed by 80 μ l of buffer containing 5 μ M ATP and 1 μ Ci [³²P]ATP. After 1 h at room temperature the reaction was stopped by the addition of 750 μ l of chloroform/methanol/HCl (80:160:1). PtdOH was extracted as described by Folch *et al.* (1957) and chromatographed on oxalate (1%) sprayed TLC plates using the following solvent system: chloroform/methanol/concentrated ammonia/water (45:35:2:8, by vol) (Irvine, 1986). After autoradiography, the spots corresponding to PtdOH were scraped off and their ³²P content was determined by scintillation counting. Each point represents a single measurement in typical experiments.

PtdIns(4,5)P₂. To the dried PtdIns(4,5)P₂, 150 μ l monomethylamine reagent (prepared as described by Clarke and Dawson, 1981) was added and the sample was incubated at 53°C for 60 min. The sample was dried and 100 μ l of 50 mM sodium periodate was added. After 30 min at room temperature, 20 μ l of ethylene glycol (10% v/v) was added to stop the reaction, and after 15 min, 50 μ l of 1% (v/v) 1,1-dimethylhydrazine/formic acid (pH 7), and the mixture was allowed to stand for a further 3 h. The pH of the sample was adjusted to 7 with 1 M Tris-HCl and the sample was evaporated to dryness in a Vac-Fuge. Preparation of binding protein and procedure for the binding assay for Ins(1,4,5)P₃ was exactly as described by Palmer and Wakelam (1990). Each point represents a mean value $\pm$ SE for four measurements performed in duplicate.

PtdIns(4)P. This was performed using a highly purified bovine brain phosphatidylinositol 4-phosphate kinase. Briefly, bovine brain homogenates were chromatographed on DEAE-Sephacel and PtdInsP kinase activity eluted using a linear gradient of 0–500 mM NaCl. The activity eluted at ~250 mM and was then applied to a column of blue Sepharose and eluted with 2.5 M NaCl. After dialysis the pooled activity was applied to a heparin agarose column. Elution of the PtdInsP kinase was achieved using a linear gradient of 300 mM–1 M NaCl. This preparation was stored at +4°C

in buffer containing 0.01% Triton and 50 μ M ATP, and was stable for 3 months.

Mass measurement was performed as follows. Standard quantities of PtdInsP or lipid extractions from isolated nuclei were dried by vacuum centrifugation and resuspended in 100 μ l of PIPkin buffer (50 mM Tris, 10 mM MgSO₄, 80 mM KCl, 2 mM EGTA) followed by bath sonication. The reaction was started by the addition of 100 μ l PIPkin buffer containing 20 μ l of enzyme, 10 μ M ATP and 1 μ Ci [³²P]ATP (3000 Ci/mmol). After 1.5 h at room temperature the reaction was terminated by the addition of 750 μ l chloroform/methanol/concentrated HCl (80:160:1) and lipids extracted as in Folch *et al.* (1957). After separation of lipids by TLC (as for DAG assay) PIP₂ spots were scraped after identification by autoradiography and quantified by scintillation counting.

PtdIns. PtdIns mass measurement was performed using a coupled assay employing a mixture of PtdIns-specific phospholipase C and the DAG kinase. One rat brain was homogenized in 5 ml of the same buffer used for DAG kinase purification, and centrifuged at 50 000 g for 45 min. The resulting supernatant (5 ml) was loaded onto a DEAE-Sephacrose column. The enzyme mixture was found to elute in the flow-through of the column. The mass measurement for PtdIns was performed as follows. The dried lipid was dissolved by the addition of 50 μ l of buffer (80 mM Tris-acetate, 10 mM magnesium acetate, 2 mM EGTA, 10 mM KCl, 70 mM NaCl, pH 7.4) and sonicated for 15 s. Fifty nmol of PtdEtn in 80 μ l of the above buffer was then added. The reaction was started by the addition of 20 μ l of enzyme mixture followed by 50 μ l of buffer containing 5 μ M ATP and 1 μ Ci [³²P]ATP. The reaction was continued for 1 h at room temperature and the PtdOH was extracted, separated and quantified as for the DAG assay (above). Under the above experimental conditions, the phospholipase C in the enzyme mixture will hydrolyse only PtdIns, since no incorporation of ³²P into PtdOH was observed when 0.1–10 nmol of either PtdInsP₂, PtdInsP₃, PtdCho, PtdEtn or PtdSer were used as substrate; we have no idea what the substrate of this enzyme is *in vivo*, but only that it shows the stated specificity in these assays. Note also that PtdIns radiolabelled in the inositol ring released water-soluble radioactivity in this assay, and PtdIns radiolabelled with ¹⁴C in the fatty acyl chains was converted to [¹⁴C]PtdOH in the expected quantity, confirming the validity of this coupled assay. Each point represents a single measurement in a typical experiment.

Western blotting with anti-PKC antibody

Nuclei were isolated as described in Table I except that none of the buffers contained detergent. After the first spin to remove nuclei, the post-nuclear supernatant was removed and centrifuged at 25 000 r.p.m. (SW27 Beckman L10 ultracentrifuge) to give post-nuclear membranes. The pellet was resuspended in 300 μ l of final buffer (10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.32 M sucrose, 5 mM β -mercaptoethanol and 1 mM EGTA).

50 μ g of nuclear and post-nuclear fractions were subjected to electrophoresis on 10% SDS gels. Transfer to nitrocellulose was achieved using a 10 mM sodium carbonate, 3 mM sodium bicarbonate buffer pH 9.9 for 1.5 h. The blot was blocked in 20 mM Tris pH 7.4, 0.5 M NaCl, 5% BSA, 0.2% Tween 20, followed by incubation of primary antibody (1:1000) overnight at 4°C in the above buffer. After incubation with an anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, 1:2000 at 4°C), visualization was performed using 3,3'-diaminobenzidine as a substrate.

Electron microscopy

Nuclei were pelleted at 100 000 g, resuspended and fixed in 1% glutaraldehyde, 3% (*para*)formaldehyde in 0.1 M cacodylate buffer (pH 7.2) plus 5% sucrose for 30 min at room temperature. They were then pelleted on a microfuge and stored in cacodylate buffer at 4°C. The pellets were then treated with 1% osmium tetroxide in veronal acetate buffer, pH 7.0, for 1 h, dehydrated in an alcohol series and embedded in Araldite epoxy resin. Sections were stained with uranyl acetate and lead citrate.

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