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Mitogen-stimulated events in nuclei of Swiss 3T3 cells

Evidence for a direct link between changes of inositol lipids, protein kinase C requirement and the onset of DNA synthesis

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Two different clones of Swiss 3T3 cells belonging to the same original cell line have been obtained, one of which was unresponsive to mitogenic stimulation (e.g. insulin-like growth factor-I, bombesin, insulin-like growth factor-I + bombesin), while the other clone showed a very high rate of DNA synthesis under identical conditions as demonstrated by 5-bromodeoxyuridine incorporation. Both types of cells expressed the IGF-I receptor and showed high contact inhibition. When highly purified nuclei from responsive cells, treated for a short time with bombesin and insulin-like growth factor-I or insulin-like growth factor-I alone, were incubated with [γ-^{32}P]adenosine triphosphate, the labelling of phosphatidylinositol-mono- and diphosphate decreased when compared to controls, while this transient effect did not appear in the nuclei from unresponsive cells. Similarly nuclear protein kinase C is activated only in responsive cells. Therefore, it seems that a direct link exists between polyphosphoinositide metabolism, protein kinase C activation and the early events leading to cell division, since the rapid changes in the labelling of both phosphatidylinositol mono- and di-phosphate occur only in nuclei from Swiss 3T3 cells, which respond to the mitogenic stimulus determined by insulin-like growth factor-I on its own, or in combination with bombesin.

Nucleus; Mitogen; Inositol lipid; Swiss 3T3 cell; Protein kinase C; DNA synthesis

1. INTRODUCTION

Cell proliferation consists of a number of highly coordinated events that can be triggered by the binding of a growth factor to its own receptor. This leads to the generation of second messenger molecules among which polyphosphoinositides play an important role [1]. Recently, we have shown that isolated nuclei incorporate ATP into inositol lipids [2] and that changes occurring in their metabolism, when quiescent Swiss 3T3 cells are stimulated to grow with a combination of IGF-I and bombesin at mitogenic concentrations, precede the activation of PKC [3,4]. During routine passages of cells, we selected a clone still maintaining contact inhibition and expressing the IGF-I receptor, but which is unresponsive to the above combination of growth factors. This provided an opportunity to investigate a possible correlation between the onset of DNA synthesis and the early changes in nuclear polyphosphoinositides and the subsequent modulation of PKC by directly comparing the metabolic pattern of polyphosphoinositides and the PKC in vitro activity of the responsive and unresponsive cells. Here we show that this new clone of Swiss 3T3 fibroblasts, when stimulated with IGF-I or IGF-I + bombesin, does not enter the S-phase, as judged by BrdU incorporation, and does not show (in isolated nuclei) the transient decrease in incorporation from [γ-^{32}P]ATP into nuclear PIP and PIP_2, and PKC activation, normally seen in responsive cells.

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Abbreviations: PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-diphosphate; PA, phosphatidic acid; BrdU, 5-bromodeoxyuridine; IGF-I, insulin-like growth factor I; IP_3, inositol 1,4,5-trisphosphate; ATP, adenosine trisphosphate; PKC, protein kinase C; PS, phosphatidyl-serine; DAG, 1,2-dioleyl-rac-glycerol); SPH, sphingosine; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid

2. MATERIALS AND METHODS

2.1. *Cell culture and growth factor stimulation*

Swiss 3T3 fibroblasts (from Dr K. Brown's laboratory, AFRC, Cambridge, UK) were grown in Dulbecco's modified minimum essential medium supplemented with 10% newborn calf serum at 37°C in a humified atmosphere containing 10% CO_2. Aliquots (from American Type Culture Collection no. CCL 92, freeze 6602, passage no. 116) were thawed and seeded in the same medium at high dilution (1:1000) and then, before reaching confluence, reseeded at 3×10^3 per cm^2 for 5 passages. After 6 days cells reached saturation density.

Quiescent and confluent cells were washed with serum-free medium containing 1% bovine serum albumin for growth factor stimulation.

Growth factors were added as follows: (i) IGF-I, 20 ng/ml; (ii) bombesin, 1 nM; (iii) bombesin, 1 nM + IGF-I, 20 ng/ml, for the times indicated in the table and figure legends.

2.2. *BrdU incorporation and immunofluorescence microscopy*
Twenty hours after the stimulation cells were labelled for 60 min with 10 μM BrdU and 1 μM fluorodeoxyuridine. They were then processed for immunofluorescence microscopy as previously described [5].

2.3. *[^{125}I]IGF-I binding assay*
The [^{125}I]IGF-I binding assay was carried out exactly as described by Corps and Brown [6].

2.4. *Isolation of nuclei, phosphorylation of nuclear polyphosphoinositides and thin-layer chromatography of lipid extracts*
All these steps were carried out exactly as described by Cocco et al. [4].

2.5. *PKC assay and quantitation of autoradiograms*
PKC assay and quantitation of autoradiograms were exactly as described by Martelli et al. [7].

2.6. *Protein assay*
This was performed according to Lowry et al. [8].

3. RESULTS AND DISCUSSION

There is a considerable body of evidence suggesting the involvement of a lipid component in some aspects of nuclear structure and function (for a review see [9]). Among phospholipids, polyphosphoinositides seem to be particularly interesting for their hypothesized signalling capability at the nuclear level [10–14]. Here we point out the fact that, when isolated nuclei are studied in vitro, the polyphosphoinositide metabolism undergoes changes, namely a transient decrease in the degree of PIP and PIP_2 phosphorylation, only in mitogen-responsive cells after IGF-I treatment.

The data in Fig. 1 and Table I show that only responsive cells after mitogenic stimulation incorporate BrdU as demonstrated by immunofluorescence microscopy. The number of responsive cells which replicate their DNA is directly related to the stimulus which has been employed, since the combination of IGF-I and bombesin is the more effective condition, while IGF-I and bombesin on their own give rise to a lower response. The unresponsive cells do not replicate either in the presence of growth factors on their own or in the

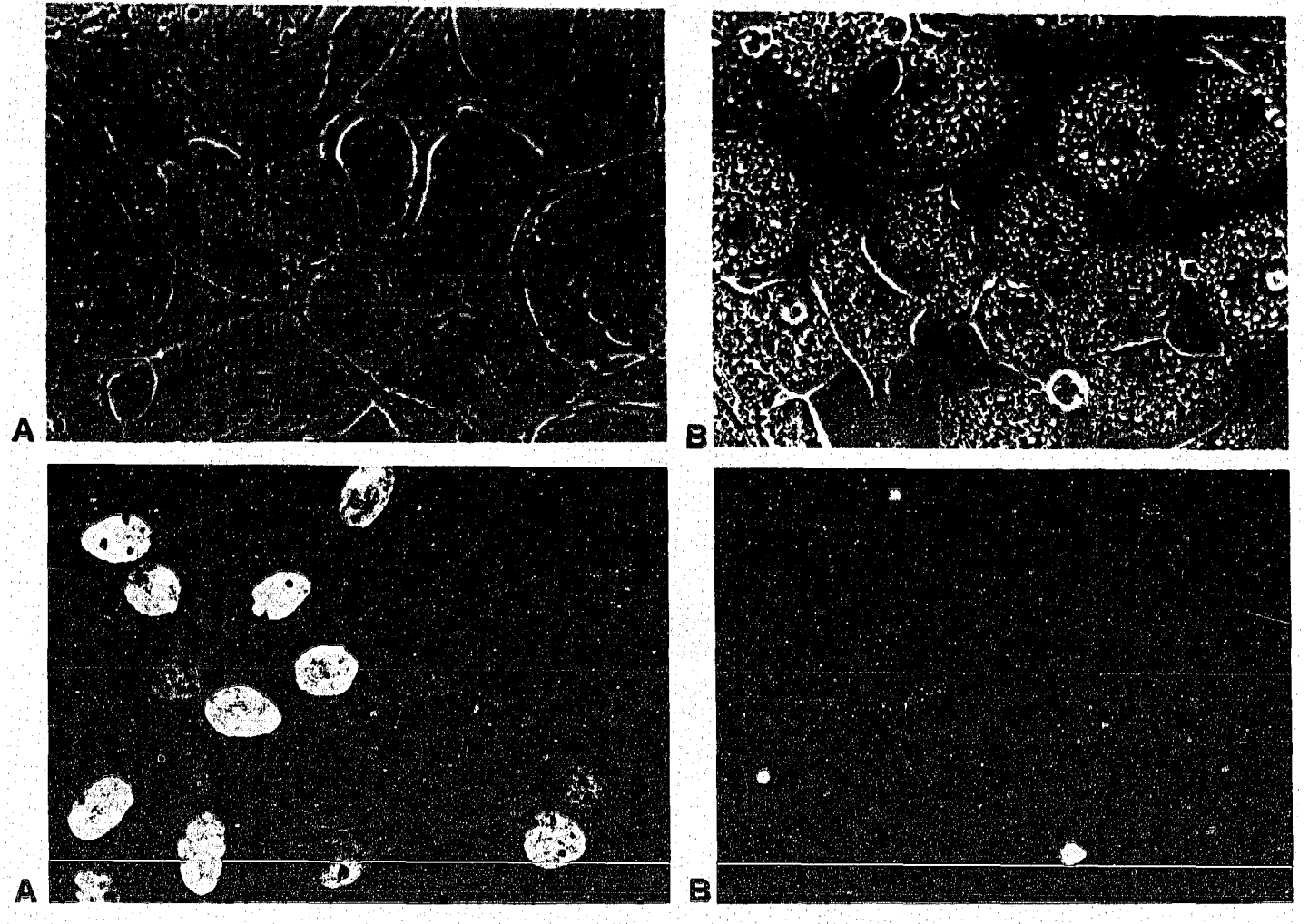

Fig. 1. The upper panel shows the phase-contrast micrograph of responsive (a) and unresponsive (b) Swiss 3T3 cells stimulated with IGF-I and bombesin. The lower panel shows the immunofluorescent staining of anti BrdU monoclonal antibody in the same microscopy field of the same specimen. The conditions were those reported in Table I.

Table I

Effect of IGF-I and/or bombesin on stimulation of DNA synthesis in Swiss 3T3 cells

Addition	% labelled nuclei	
	Responsive cells	Unresponsive cells
None	3	1
IGF-I	25	2
Bombesin	41	2
IGF-I + bombesin	80	3

DNA synthesis was assessed by BrdU incorporation as described in section 2 after growth stimulation of quiescent cells with the above growth factors for 20 h.

presence of the mitogenic combination (Table I). The availability of an unresponsive clone of Swiss 3T3 cells was extremely important, once it was established that these cells express the type I IGF receptor, which, in responsive cells, mediate mitogenic stimulation [6]. The data in Table II show that unresponsive cells express the type I IGF receptor to a higher extent when compared to responsive Swiss 3T3 fibroblasts. It seems that unresponsive cells attempt to override the impaired machinery of signal transduction inside cells by overexpressing the receptor. The binding characteristics indicate that, in both cell populations, at low mitogenic concentration of [^{125}I]IGF-I, the only significant binding is to type I IGF receptors, as previously demonstrated for responsive 3T3 fibroblasts [6]. The data reported here, dealing with polyphosphoinositide metabolism, have been obtained with highly purified nuclei. Indeed the activity of glucose-6-phosphatase, recovered in our isolated nuclei, is less than 0.1% of the activity present in whole cell homogenates, and the routine electron microscope observations show nuclei completely stripped of the nuclear envelope, in agreement with previously published data [2,4]. The phosphorylation pattern of polyphosphoinositides from isolated nuclei (Fig. 2 and Table III) is characterized by a transient decrease of PIP and PIP_2 levels in responsive cells, confirming previous observations [10],

Table II

[^{125}I]IGF-I binding assay in responsive and unresponsive Swiss 3T3 fibroblasts. Cells were incubated at 4°C with labelled IGF-I (1 ng/ml), and the indicated concentrations of unlabelled IGF-I and insulin. Control is the complete binding assay with [^{125}I]IGF-I alone. This is repeated with excess of unlabelled IGF-I and insulin to test the specificity of binding and the receptor type. Results are means ± SD of 3 separate experiments.

Conditions	cpm [^{125}I]IGF-I bound / 10^6 cells	
	Responsive cells	Unresponsive cells
Control	12,925 ± 153	34,159 ± 381
+ unlabelled IGF-I (100 ng/ml)	8,007 ± 213	18,413 ± 197
+ unlabelled insulin (500 μg/ml)	9,602 ± 349	21,834 ± 183
Specific binding cpm / 10^6 cells	4918	12,325

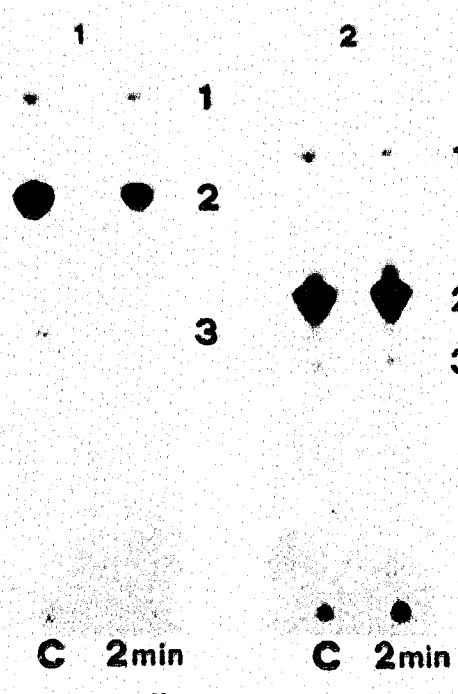

Fig. 2. Incorporation of ^{32}P into lipids in Swiss 3T3 cell nuclei incubated in vitro. Panels A and B show the autoradiograms of TLC separation of lipid extracts from mitogen-responsive and unresponsive cells after 2 min of treatment with IGF-I and bombesin, respectively. Radiolabelled compounds were identified as 1, PA; 2, PIP; 3, PIP_2.

whilst no such changes are seen in unresponsive cells. The decrease is visible in nuclei from IGF-I as well as IGF-I/bombesin treated cells, suggesting that IGF-I but not bombesin elicits the response via polyphosphoinositides at the nuclear level. It is worth noting that the rapid decrease in polyphosphoinositide phosphorylation levels ascribed to changes in lipid mass by in vivo labelling experiments [3], precedes the activation of nuclear PKC [7].

Table III

Phosphorylation of polyphosphoinositides in nuclei from responsive and unresponsive Swiss 3T3 cells after mitogenic stimulation (bombesin 1 nM and IGF-I 20 ng/ml). Results, expressed as dpm/mg protein × 10^{-1}, are the mean of 3 different experiments.

	PA	PIP	PIP_2
Control (responsive cells)	189	1665	140
+ Bombesin, 2 min	196	1621	147
+ Bombesin, 1 h	172	1630	138
+ IGF-I, 2 min	167	705	97
+ IGF-I, 1 h	185	1640	132
+ Bombesin + IGF-I, 2 min	68	532	81
+ Bombesin + IGF-I, 1 h	218	1751	153
Control (unresponsive cells)	209	1821	133
+ Bombesin, 2 min	204	1930	141
+ Bombesin, 1 h	200	1918	135
+ IGF-I, 2 min	197	1803	137
+ IGF-I, 1 h	200	1900	142
+ Bombesin + IGF-I, 2 min	180	1801	140
+ Bombesin + IGF-I, 1 h	207	1921	134

Table IV

Nuclear PKC activity in vitro using exogenous histone H1 as substrate after treatment of responsive and unresponsive cells with IGF-I and bombesin. Values are averages of 5 separate experiments and refer to the percentage increase over the control (100). The relative amount of radioactivity of histone H1 band was determined by densitometric scanning of autoradiograms.

Additions	Responsive cells	Unresponsive cells
PS/DAG	310	125
PS	138	118
PS/DAG/SPH	119	109
PS/DAG/EGTA	115	106

For that reason we checked the nuclear PKC activity after 45 min of mitogenic stimulation in both responsive and unresponsive cells, e.g. the time of treatment at which PKC activity is increased in responsive 3T3 cells [7,14]. Table IV shows that only responsive 3T3 cells exhibit a marked increase of nuclear PKC activity when compared to unresponsive cells.

In responsive cells the transient changes of nuclear inositol lipids are essentially due to IGF-I whilst bombesin on its own does not at all affect both PIP and PIP_2 phosphorylation (Table III). The expression of type I IGF receptors in both cell populations used, seems to indicate that, once the growth factor binds to its receptor at the plasma membrane, something inside the unresponsive cells is changed or imbalanced and the nuclear trigger for initiating DNA synthesis is absent. Thus it is significant that, when the early changes in nuclear polyphosphoinositide metabolism, distinct from those at the plasma membrane [2,3,10], do not occur, and in the absence of nuclear PKC activation, the onset of DNA synthesis cannot take place. In conclusion, these results, which are in good agreement with previously reported data on nuclear inositol lipids, further suggest a direct link inside the nucleus between the transient changes of inositol lipid metabolism, the subsequent activation of PKC and the triggering of the synthetic machinery capable of carrying out DNA synthesis. Moreover, these second messenger functions of nuclear inositol lipids seem to be confirmed by recent observations showing the presence of IP_3-sensitive pools at the nuclear level [15,16].

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