Inositol phospholipid metabolism and myoblast fusion

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1. The fusion of chick embryonic myoblasts has been studied in tissue culture. Myoblasts are maintained at $0.1\ \mu\text{M}$ - Ca^{2+} for 50 h. During this time they achieve fusion competence. Fusion is initiated by raising the medium Ca^{2+} concentration to $1.4\ \text{mM}$. 2. A rapid breakdown of the polyphosphoinositides was detected within 3 min of Ca^{2+} addition. 3. Rapid synthesis of phosphatidic acid was also detected at this time. 4. Breakdown of phosphatidylinositol and synthesis of 1,2-diacylglycerol were also detected. Other phospholipids were unaffected. 5. Sr^{2+} could replace Ca^{2+} in this process but Mg^{2+} could not and also inhibited the Ca^{2+} effect. The Ca^{2+} -ionophore A23187 stimulated further apparent polyphosphoinositide breakdown in the presence of Ca^{2+} . 6. The results are discussed with respect to myoblast fusion.

The fusion of cell membranes is an extremely important process in many aspects of cellular function. It has (mainly) been studied in model systems, which have provided information on its possible mechanism (for review, see e.g. Papa-hadjopoulos, 1978). The study of model systems can only be of limited use and, although presenting obvious difficulties, the study of naturally occurring fusion processes is desirable; one such process is the fusion of mononucleated myoblasts to produce multinucleated myotubes. The process of myoblast differentiation and fusion can be studied in tissue culture and has the advantage of being made synchronous by culturing at a low Ca^{2+} medium concentration. Like other forms of membrane fusion, fusion of myoblasts is Ca^{2+} -dependent, most of the cells reach fusion competence after approx. 50 h culture at a medium Ca^{2+} concentration of 0.1 – $20\ \mu\text{M}$ and, on raising the ion concentration to $1.4\ \text{mM}$, they fuse rapidly (van der Bosch *et al.*, 1972).

The fusion of membranes involves changes in the lipid structure of the bilayers during the fusion process. This remains the least understood area of fusion. We have recently shown (Wakelam & Pette, 1982) that a rapid breakdown of myoblast phosphatidylinositol occurs upon initiation of fusion by Ca^{2+} . These studies have been extended and show that the minor inositol phospholipids phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate are also broken down at this time and that there is also rapid increase in the production of phosphatidic acid in these fusing cells. Some of these results have been presented in a preliminary form (Wakelam & Pette, 1981).

Methods

Isolation and use of myoblasts

Cells were prepared and cultured as previously described (Wakelam & Pette, 1982) with the following modifications. First trypsin was replaced by 0.1% (w/v) dispase (EC 3.4.24.4) in the muscle digestion and secondly cultures were maintained at a Ca^{2+} concentration of approx. $0.1\ \mu\text{M}$ - Ca^{2+} . EGTA buffers were used when definite Ca^{2+} concentrations were required.

In experiments using *myo*-[2- ^3H]inositol half of the medium was replaced after 24 h with fresh medium containing *myo*-[2- ^3H]inositol (sp. radioactivity $5\ \text{Ci/mmol}$) to give a final radioactive concentration of $2\ \mu\text{Ci/plate}$. A similar procedure was used for experiments involving [U- ^{14}C]glycerol (sp. radioactivity $152\ \text{Ci/mmol}$); in this case the 'half change' was performed after 30 h in culture and resulted in a final radioactive concentration of $1.25\ \mu\text{Ci/plate}$. After 49 h in culture the medium was removed and replaced with a fresh label-free medium; after a further hour this medium was also removed and replaced by media containing Ca^{2+} and/or other test substances as specified in the Results section. At this point some plates were taken for zero-time controls. After incubation for the time period stated in the Results section the medium was rapidly removed and the plates were washed with ice-cold 0.9% NaCl before addition of $1\ \text{ml}$ of 10% (w/v) trichloroacetic acid to stop the reaction.

Preliminary experiments were performed to determine the length of label exposure time to produce equilibrium labelling into the inositol phospholipids. These were found to be 22 h for *myo*-[2- ^3H]inositol

and 17 h for [U- ^{14}C]glycerol. The slightly longer times chosen did not appreciably affect the labelling.

In experiments with [^{32}P]P_i the radioactive compound was added to medium at the plates after 49 h in culture at a concentration of approx. 10 μCi of [^{32}P]P_i (carrier-free)/plate. After 1 h Ca^{2+} was added where stated and the incubations were performed as described above.

Examination of lipids

The trichloroacetic acid/cell mixtures were transferred to glass tubes and lipids were extracted by the method of Shukla *et al.* (1979) in experiments where the polyphosphoinositides were measured and by the method of Lapetina & Michell (1972) where other lipids were measured.

For polyphosphoinositide analysis lipids were deacylated and the glycerol inositol phosphates were separated on 0.75 ml Dowex 1-X 10 (formate form) columns as described by Downes & Michell (1981). The eluents from these columns were diluted 3-fold with water and reloaded on to the same re-activated columns; the glycerol inositol phosphates were then eluted with 2 ml of 2 M-ammonium formate. This was mixed with 15 ml of Aquassure (New England Nuclear) and the mixture was counted for radioactivity with an efficiency of approx. 38% in a Packard liquid-scintillation counter.

In experiments where other lipids were determined the vacuum-dried lipid extracts were dissolved in chloroform/methanol (19:1, v/v) and applied to activated thin-layer silica-gel chromatography plates and separation was achieved using the solvents specified below. After chromatography spots were located using I_2 vapour and after sublimation the lipids were scraped into either 10 ml of Aquassure for scintillation counting or into glass tubes for extraction of the lipids. Elution was achieved by the method of Skipski & Barclay (1969).

The following solvent systems were used for separating the various lipids studied: (a) neutral lipids, successive unidimensional t.l.c. using diethyl ether/benzene/ethanol/acetic acid (200:250:10:1, by vol.) as the first and diethyl ether/hexane (3:47, v/v) as the second solvent (Freeman & West, 1966); (b) phosphatidylcholine, phosphatidylinositol and phosphatidylserine, unidimensional t.l.c. using chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as solvent (Skipski *et al.*, 1964); (c) phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid, successive unidimensional t.l.c. using acetone/light petroleum (b.p. 60–80°C) (1:3, v/v) as the first and chloroform/methanol/acetic acid/water (800:130:80:3, by vol.) as the second solvent (Skipski *et al.*, 1967). When phosphatidic acid alone was determined, samples were separated using chloroform/pyridine/formic

acid (50:3:7, by vol.) as solvent (Farese *et al.*, 1981); (d) polyphosphoinositides were separated using successive unidimensional t.l.c. with chloroform/methanol/aq. 4.3 M- NH_3 (18:13:4, by vol.) as the first and propan-1-ol/aq. 4.3 M- NH_3 (13:7, v/v) as the second solvent (Hauser *et al.*, 1971). Where phosphatidic acid and the polyphosphoinositides were separated by t.l.c., carrier phospholipids were added to ease detection of spots after I_2 staining.

Measurement of protein

All results are related to total cellular protein. This was determined upon portions of the trichloroacetic acid/cell mixtures before lipid extraction by the method of Peterson (1977).

Expression of results

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed using Student's *t*-test.

Materials

Dispace was from Boehringer-Mannheim G.m.b.H. Lipid standards were from Sigma G.m.b.H. Radiochemicals were from Amersham Buchler G.m.b.H. or New England Nuclear G.m.b.H. Culture media and other chemicals were from sources given previously (Wakelam & Pette, 1982).

Results

The addition of 1.4 mM- Ca^{2+} to cells that had been maintained at a Ca^{2+} concentration of 0.1 μM for 50 h produces a rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. This breakdown occurs extremely rapidly, being maximal within 10 min, and accounts for over 40% of the total cellular contents of these lipids (Fig. 1). The results shown in Fig. 1 were obtained using the Dowex column method of Downes & Michell (1981). Similar checks on the validity of this method as utilized by the author were performed and in addition several experiments were performed using a t.l.c. method for separation of the lipids (see the Methods section). These experiments yielded comparable results, e.g. breakdowns of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were $50 \pm 7\%$ and $40 \pm 9\%$ respectively in the presence of 1.4 mM- Ca^{2+} and $3 \pm 6\%$ and $5 \pm 4\%$ respectively in the absence of a raised Ca^{2+} concentration ($n = 5$ in each case). These results gave confidence in the methods used.

The Ca^{2+} dependence of this lipid breakdown was examined. Table 1 shows that raising the medium Ca^{2+} concentration produces polyphosphoinositide breakdown at very low concentrations but this is not significant until the concentration is raised to

Fig. 1. The time course of polyphosphoinositide breakdown

Myoblasts were cultured for 50h at a Ca²⁺ concentration of 0.1μM and labelled with myo-[³H]inositol as described in the Methods section. The Ca²⁺ concentration was raised to 1.4mM and breakdown of phosphatidylinositol 4-phosphate (a) and phosphatidylinositol 4,5-bisphosphate (b) was determined as described in the Methods section. *n* = 10 in each case and the results are expressed as means ± s.e.m. and are pooled from three similar experiments. The broken lines join the two measurements made under low-Ca²⁺ conditions.

0.01 mM-Ca²⁺. Myoblast fusion is a Ca²⁺-dependent event; it has been shown to be replaceable by Sr²⁺ and to be strongly inhibited by Mg²⁺. Table 2 shows that Sr²⁺ can stimulate significant breakdown of phosphatidylinositol 4-phosphate and of phosphatidylinositol 4,5-bisphosphate; this breakdown is less

Table 1. The effect of Ca²⁺ concentration upon the breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate

Myoblasts were cultured for 50h at a Ca²⁺ concentration of 0.1μM. The cells were labelled with myo-[³H]inositol as described in the Methods section. The Ca²⁺ concentration was then adjusted using Ca²⁺/EGTA buffers and the breakdown of the lipids measured after a 15min incubation as described in the Methods section. *n* = 12 in each case and is a pool of two experiments. Significance values are comparisons between the breakdown at a particular Ca²⁺ concentration and the value at 0.1μM-Ca²⁺. Results are significantly different from control values where indicated: **P* < 0.01; ***P* < 0.001.

[Ca ²⁺] (M)	Breakdown (%)	
	Phosphatidylinositol 4-phosphate	Phosphatidylinositol 4,5-bisphosphate
10 ⁻⁷	1 ± 4	2 ± 4
10 ⁻⁶	8 ± 7	3 ± 5
10 ⁻⁵	13 ± 6*	16 ± 6*
10 ⁻⁴	29 ± 5**	22 ± 6**
10 ⁻³	34 ± 5**	24 ± 5**

Table 2. The effect of Ca²⁺, Mg²⁺, Sr²⁺ and the Ca²⁺-ionophore A23187 upon the breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate

Myoblasts were cultured for 50h and labelled with myo-[³H]inositol as described in the legend to Table 1. They were then incubated under various conditions for 15min and the lipid breakdown was determined. Other details are as in Table 1; values for *n* are in parentheses.

Additions to medium	Breakdown (%)	
	Phosphatidylinositol 4-phosphate	Phosphatidylinositol 4,5-bisphosphate
None	1 ± 3 (10)	1 ± 2 (10)
1.4 mM-Ca ²⁺	26 ± 8 (10)	27 ± 5 (10)
1.4 mM-Ca ²⁺ + 5 μg of A23187/ml	42 ± 4 (8)	45 ± 5 (8)
5 μg of A23187/ml	3 ± 4 (6)	2 ± 4 (6)
1.4 mM-Ca ²⁺ ± 0.1% (v/v) ethanol	21 ± 9 (6)	25 ± 9 (6)
0.1% (v/v) ethanol	2 ± 2 (6)	4 ± 8 (6)
1.4 mM-Ca ²⁺ + 20 mM-Mg ²⁺	4 ± 4 (6)	3 ± 5 (6)
2.4 mM-Sr ²⁺	19 ± 6 (6)	18 ± 2 (6)

than that achieved when 1.4 mM-Ca²⁺ is present. The Table also shows that 20 mM-Mg²⁺ inhibits the breakdown stimulated by 1.4 mM-Ca²⁺. Since this process is Ca²⁺-dependent the action of the Ca²⁺-ionophore A23187 upon this process was examined. This result is also shown in Table 2. A23187 in the

Fig. 2. Phosphatidic acid synthesis in myoblasts exposed to 1.4 mM- Ca^{2+}

Myoblasts cultured for 50h were prelabelled for 1h with [^{32}P]P_i and were exposed to Ca^{2+} for the time stated. Plates were 'stopped' by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The incorporation of the label into phosphatidic acid was determined as described in the Methods section. The results are means $\pm$ S.E.M. where $n = 7$ or 8 and are obtained from 2 similar experiments. Other details as in the legend to Fig. 1.

absence of Ca^{2+} has no effect upon the two lipids; nor does 0.1% (v/v) ethanol, the solvent used for the ionophore. However, the presence of the ionophore with 1.4 mM- Ca^{2+} results in a further stimulation of the breakdown of the two lipids.

Similar results to those quoted in Tables 1 and 2 were obtained after a 5 min incubation with the test substances. The results, however, showed much larger S.E.M. values, probably due to the difficulty with timing medium changes and maintaining exact temperatures. Experiments of this sort were therefore not as informative as those where a 15 min incubation time was utilized.

The breakdown of the inositol phospholipids results in the production of 1,2-diacylglycerol, which is then rapidly phosphorylated by a diacylglycerol kinase to produce phosphatidic acid (see Michell, 1975). The production of phosphatidic acid was therefore monitored. Fig. 2 shows that addition of 1.4 mM- Ca^{2+} causes a rapid stimulation of phosphatidic acid synthesis, which has a time course

parallel to that of the polyphosphoinositide breakdown (Fig. 1). Fig. 2 also shows that the non- Ca^{2+} -stimulated myoblasts also synthesize phosphatidic acid but at a much lower rate. In view of this, it is not clear from these data whether the increase in phosphatidic acid synthesis is a result of inositol phospholipid breakdown. Therefore, cells were labelled with [^{14}C]glycerol, treated with or without 1.4 mM- Ca^{2+} , and the changes in radioactive content of an assortment of lipids were examined. Table 3 shows that radioactivity is lost only from phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, and radioactivity is gained only by 1,2-diacylglycerol and phosphatidic acid. These experiments also yielded results on the proportions of the inositol lipids in these cells. A ratio for c.p.m./mg of protein of 20:1:1 ($n = 18$) for phosphatidylinositol/phosphatidylinositol 4-phosphate/phosphatidylinositol 4,5-bisphosphate was found.

Discussion

The results reported in the present paper show that radioactively labelled phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate are broken down (Tables 1 and 3) and 1,2-diacylglycerol and phosphatidic acid are synthesized (Fig. 2 and Table 3) when fusion-competent myoblasts are stimulated to fuse by raising the medium Ca^{2+} concentration to 1.4 mM. Similar results were obtained for phosphatidylinositol (Table 3; Wakelam & Pette, 1982). The other common phospholipids are unaffected (Table 3). Table 3 also shows that the loss in label from the three inositol phospholipids is roughly equal to the gain in label in phosphatidic acid and 1,2-diacylglycerol. Thus the synthesis of phosphatidic acid and 1,2-diacylglycerol can reasonably be assumed to be a result of the breakdown of the inositol phospholipids; 1,2-diacylglycerol is one of the initial products of inositol phospholipid breakdown and this is then phosphorylated by a diacylglycerol kinase to phosphatidic acid (see Michell, 1975). Sr^{2+} , which can replace Ca^{2+} in stimulating myoblast fusion (Schudt *et al.*, 1973), also stimulates the breakdown of all three inositol phospholipids, whereas Mg^{2+} inhibits both the Ca^{2+} -stimulated fusion and the breakdown of the three lipids (Table 2; Wakelam & Pette, 1982). There appears therefore to be a link between the stimulation of myoblast fusion and the breakdown of the inositol phospholipids.

Grove *et al.* (1981) also examined phosphatidylinositol 4,5-bisphosphate metabolism in fusing myoblasts. These authors found no effects and concluded that the inositol phospholipids had no role in the fusion process. However, several important differences between their work and that presented

Table 3. *The effect of raising the Ca^{2+} concentration upon myoblast neutral lipids and phospholipids*

The lipids of 50h-cultured myoblasts, labelled with $[\text{U-}^{14}\text{C}]\text{glycerol}$ as described in the Methods section, were extracted, separated and their radioactive content measured as described in the Methods section, before and after exposure (or without exposure) of the cells to 1.4 mM-Ca^{2+} for 8 min. $n = 4$ in each case and the results are means $\pm$ S.E.M. of one of two experiments that gave similar results. Significance values refer to differences between radioactivity (c.p.m./mg) at zero time and after the incubations. Results were significantly different at: * $P < 0.01$; ** $P < 0.001$.

Lipid	Radioactivity (c.p.m./mg of cellular protein)		
	Zero time	+ Ca^{2+}	- Ca^{2+}
Phosphatidylinositol 4,5-bisphosphate	147 $\pm$ 25	84 $\pm$ 20*	129 $\pm$ 35
Phosphatidylinositol 4-phosphate	120 $\pm$ 18	65 $\pm$ 20**	108 $\pm$ 21
Phosphatidylinositol	3041 $\pm$ 307	1963 $\pm$ 255**	2989 $\pm$ 513
Phosphatidic acid	885 $\pm$ 129	1856 $\pm$ 195**	1256 $\pm$ 281
Phosphatidylcholine	4890 $\pm$ 267	4920 $\pm$ 721	4450 $\pm$ 821
Phosphatidylethanolamine	11460 $\pm$ 1408	11231 $\pm$ 989	10689 $\pm$ 1064
Phosphatidylserine	2928 $\pm$ 847	3016 $\pm$ 641	3104 $\pm$ 1011
Phosphatidylglycerol	6260 $\pm$ 772	5947 $\pm$ 958	6385 $\pm$ 1095
1,3-Diacylglycerol	5597 $\pm$ 997	5263 $\pm$ 590	5442 $\pm$ 694
1,2-Diacylglycerol	1597 $\pm$ 76	2220 $\pm$ 238**	1698 $\pm$ 195

here stand out. First, they studied the lipid under labelling conditions, that is they studied synthesis and not breakdown; therefore their conclusions on breakdown cannot be valid (for a discussion of this point see Michell & Kirk, 1981a). Secondly, they used myoblasts cultured at 0.1 mM-Ca^{2+} , compared with a concentration of $0.1\text{ }\mu\text{M}$ used in the present work. Whereas at 0.1 mM-Ca^{2+} myoblasts do not fuse (though up to 10% fusion is observed), it is a Ca^{2+} concentration at which the lipids can be broken down (Table 1). This probably explains why these authors did not observe the breakdown.

Inositol phospholipid breakdown has been mainly studied as a receptor-occupation-stimulated event where Ca^{2+} is the second messenger for such agonists. Michell *et al.* (1981) have recently extended their studies on phosphatidylinositol breakdown (see Michell & Kirk, 1981b) and now are of the view that it is the breakdown of phosphatidylinositol 4,5-bisphosphate that is the primary event in a possible Ca^{2+} -gating process. David *et al.* (1981) have shown that Ca^{2+} entry into myoblasts occurs just before fusion. One of the roles of the lipid breakdown observed in this work could therefore be in Ca^{2+} -gating. It is the author's view that such a process is involved here, since Ca^{2+} is unable to initiate fusion or inositol lipid breakdown in the absence of chick embryo extract (M. J. O. Wakelam, unpublished work). However, one of the strongest arguments for inositol phospholipid involvement in Ca^{2+} -gating is that lipid breakdown is not observed when cells are treated with the Ca^{2+} -ionophore A23187 (Michell *et al.*, 1981), though physiological responses are still observed.

A23187 has no effect upon myoblast fusion at 50h (Wakelam & Pette, 1982), but it has been shown in cells cultured at 1.4 mM-Ca^{2+} to induce precocious fusion in the 9h period before normal fusion (David *et al.*, 1981). Table 2 shows that addition of the ionophore to the cells in the presence of 1.4 mM-Ca^{2+} causes an approximate doubling of the breakdown of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate. Wakelam & Pette (1982) showed that under these conditions only half the normal amount of phosphatidylinositol breakdown was observed. From these data the existence of two functionally differing pools was postulated. The erythrocyte, the synaptosome and probably other cell membranes (Allan & Michell, 1977; Griffin & Hawthorne, 1978; Michell *et al.*, 1981) contain a Ca^{2+} -activated polyphosphoinositide phosphodiesterase as well as a non- Ca^{2+} -controlled enzyme and it is the former enzyme that has possibly produced this increase in lipid breakdown in response to A23187 and Ca^{2+} in myoblasts. An alternative explanation for such results is that the high intracellular Ca^{2+} concentration produced in response to the ionophore treatment has inhibited compensatory inositol phospholipid synthesis. Such an effect has been observed in the rabbit vas deferens (Egawa *et al.*, 1981) and the blowfly salivary gland (Berridge & Fain, 1979). This seems to be an effect upon CDP-diacylglycerol:inositol phosphatidate transferase (see Berridge, 1982).

It is, however, possible from the above discussion to postulate the existence of two functionally different pools of inositol phospholipids in myoblasts, one maybe involved in Ca^{2+} -gating and the

other having some role in the fusion process. In this context it is noteworthy that myoblasts contain more polyphosphoinositides than do other cells, e.g. hepatocytes (Michell *et al.*, 1981), i.e. a ratio of 20:1:1 for phosphatidylinositol/phosphatidylinositol 4-phosphate/phosphatidylinositol 4,5-bisphosphate compared with 100:1:1 (see the Results section). If the above conclusions are valid it is necessary to consider what role the inositol phospholipids could play in fusion.

A problem exists in assigning a role to phosphatidylinositol in that it is found in all cellular membranes. The polyphosphoinositides, however, appear to be plasma-membrane-specific (see Michell *et al.*, 1981), which would make them stronger candidates for involvement in the fusion process.

The increased synthesis of 1,2-diacylglycerol and phosphatidic acid could have a role in fusion. 1,2-Diacylglycerol has been shown to have activity as an exogenously added erythrocyte fusogen, albeit a weak activity (Ahkong *et al.*, 1973). Allan *et al.* (1975) showed accumulation of this lipid during erythrocyte vesiculation. Sundler & Papahadjopoulos (1981) showed that vesicles composed of phosphatidic acid fused in the presence of millimolar concentrations of Ca^{2+} or Mg^{2+} , but vesicles made up of phosphatidylinositol only aggregated. Sundler *et al.* (1981) have proposed that head-group hydration is critical to fusion since phosphatidic acid and phosphatidylethanolamine, which have low head-group hydration, fuse in artificial systems, whereas non-fusogens, such as phosphatidylinositol and phosphatidylcholine, are more heavily hydrated. It is thus possible that removal of the even more highly hydrated head groups of the polyphosphoinositides and the production of phosphatidic acid stimulates fusion.

Thus the role of polyphosphoinositide breakdown in stimulated myoblast fusion could be 2-fold. First, it could be part of a Ca^{2+} -entry process (cf. Michell *et al.*, 1981) and secondly it could be involved with fusion itself. The polyphosphoinositides could constitute a fusion block, as proposed by Allan & Thomas (1981) for erythrocyte vesiculation, to permit full myoblast differentiation. Breakdown could remove this block and the production of 1,2-diacylglycerol and phosphatidic acid stimulate fusion.

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