Changes in the surface membrane during myoblast fusion

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1. During fusion of chick-embryo myoblasts in culture, the surface membrane is affected as follows. Uptake of 2-aminoisobutyrate and 2-deoxyglucose, each of which is concentrated 20-fold relative to its concentration in the medium, is unaltered; uptake of α -methyl glucoside and choline (15 mM), each of which equilibrates relative to its concentration in the medium, approximately doubles. An approximate doubling also occurs in iodinated surface protein (and in total protein) and in cell surface area as judged by light-microscopy. Adenylate cyclase (in the absence or the presence of fluoride) increases by more than 2-fold. 2. It is concluded that, during myoblast fusion cells increase in size, and this is reflected in an increased rate of simple diffusion; the rate of facilitated processes such as the uptake of amino acids and sugars, on the other hand, remains unaltered, though the activity of certain enzymes is increased. These results indicate that specific changes in the function of surface membranes occur during myoblast fusion *in vitro*.

Grove & Stockdale (1978) have reported that during myoblast fusion there is a decrease in the specific uptake mechanism for amino acids such as 2-aminoisobutyrate, when expressed as $V_{\max}$ values (nmol of 2-aminoisobutyrate transported/mg of total cell protein). Since total cell protein increases during myoblast fusion, it is possible that 2-aminoisobutyrate uptake/cell actually remains constant; another possibility is that the change in 2-aminoisobutyrate uptake reflects an altered N^+ gradient, to which 2-aminoisobutyrate uptake is linked (see, e.g., Christensen, 1975). We have investigated these possibilities by measuring the uptake of 2-aminoisobutyrate, of 2-deoxyglucose (which is taken up by a facilitated mechanism known not to involve Na^+ linkage) and of two compounds taken up predominantly by simple diffusion, namely α -methyl glucoside and choline (the latter at a concentration in excess of that at which facilitated uptake is saturated). Changes in the amount of ^{125}I bound to the cell surface by the lactoperoxidase technique (Hubbard & Cohn, 1972), and in a membrane function known to change during fusion, namely adenylate cyclase (Zalin & Montague, 1974), were assessed at the same time. To minimize changes that might result from cell proliferation rather than from cell differentiation, and to decrease the number of contaminating fibroblasts, myoblast cells were exposed to cytosine arabinoside during, and beyond, the fusion period (Fischbach, 1972).

Methods and materials

Chick-embryo skeletal-muscle myoblasts were dissociated from the hind-limb musculature of 11- or 12-day Leghorn-chick embryos by vortex agitation (Tepperman *et al.*, 1975). The cells were cultured in 50 mm-diam. collagen-coated Petri dishes (Sterilin, Teddington, Middx., U.K.) in medium 199 (Gibco-Biocult, Hounslow, Middx., U.K.) supplemented with 10% (v/v) horse serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), 2% (v/v) chick-embryo extract, antibiotics (penicillin, 10^4 units/ml; streptomycin, 100 μ g/ml) and 0.125% $NaHCO_3$. The cultures were maintained at 37°C under a humidified atmosphere of air/ CO_2 (19:1) and were fed with fresh growth medium 24 and 72 h after being plated at an initial density of 1.2×10^6 cells/dish. Cytosine arabinoside (1 or 5 μ M) was added in 0.1 ml of Earle's salts (Flow Laboratories) to the cultures at 52 h and replaced after re-feeding at 72 h. Under these conditions the main period of fusion took place between 42 and 70 h of culture.

Radioactive compounds (The Radiochemical Centre, Amersham, Bucks., U.K.) were diluted in Hepes-buffered saline [145 mM- $NaCl$ /5 mM- KCl /5 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid/ $NaOH$ buffer, pH 7.2] and used at the following concentrations (pulse medium): 2-amino- $[^{14}C]$ isobutyrate, 1 μ M (60 μ Ci/mol); 2-deoxy $[^3H]$ -glucose, 2.4 μ M (500 μ Ci/ml); $[U-^{14}C]$ α -methyl

glucoside, 3.3 μM (60 $\mu\text{Ci/mol}$); [^3H]choline, 15 mM (130 $\mu\text{Ci/mol}$); ^3H - and ^{14}C -labelled compounds were used in pairs.

Uptake was measured by washing cells on plates twice with Hepes-buffered saline, and exposing them to pulse medium (1 ml) for various periods of time; uptake was terminated by washing four times with 2 ml of Hepes-buffered saline. All operations were carried out at 37°C. Cells were chilled and extracted with 0.6 M-HClO₄. The acid-soluble fraction was transferred to scintillation vials for assay of radioactivity; the pellet was dissolved in 0.33 M-NaOH and assayed for protein (Lowry *et al.*, 1951), with bovine serum albumin as standard, and DNA [Kissane & Robins (1958) as modified by Hinegardner (1971)]. Uptake was measured at three or more time intervals, and the mean rate was calculated; by 30 min there was little further incorporation of radioactivity.

Cells on plates were iodinated with ^{125}I by the procedure of Hubbard & Cohn (1972). After iodination the monolayers were washed in 5% (v/v) glycerol/0.1 M-Tris/HCl buffer, pH 6.8, and dissolved in a solution containing 5% (v/v) glycerol, 1% (w/v) sodium dodecyl sulphate, 50 mM-dithiothreitol and 0.05% Bromophenol Blue. The ^{125}I content was assayed, and the samples were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Gels were stained with Coomassie Blue and dried on to filter paper *in vacuo*, and photographs, and radioautographs were prepared. These were scanned on a Joyce densitometer. Individual bands were cut out and assayed for ^{125}I in a LKB γ -radiation counter.

Adenylate cyclase activity was measured, by the method of Salomon *et al.* (1974), in homogenates that were prepared as follows. The monolayers were rinsed twice with Earle's salts in 20 mM-Hepes/NaOH buffer, pH 7.0, followed by ice-cold homogenizing buffer (0.96 mM-MgCl₂/2.4 mM-dithiothreitol/25 mM-Tris/HCl, pH 7.4) and removed by gentle scraping. After cell disruption in a hand-operated homogenizer, the homogenates were diluted with buffer to 20–60 μg of DNA/ml or 0.48–1.44 mg of protein/ml, and assayed within 1 h. Incubations were initiated with 50 μl of homogenate and were performed at 30°C in a final volume of 120 μl containing the following reagents in 25 mM-Tris/HCl buffer, pH 7.6: [α - ^{32}P]ATP, 1 mM (3.8–11.7 Ci/mol); MgCl₂, 5 mM; phosphocreatine, 10 mM; creatine kinase (rabbit muscle), 75 units ($\mu\text{mol/min}$)/ml; 3-isobutyl-1-methylxanthine, 0.5 mM; dithiothreitol, 1 mM; cyclic AMP, 1 mM (plus cyclic [^3H]AMP, 10 000 c.p.m.); where appropriate, 10 mM-NaF was included. After 10 min the reaction was terminated by adding 0.1 ml of 'stopping solution' [2% (w/v) sodium dodecyl

sulphate with 40 mM-ATP and 1.4 mM-cyclic AMP; pH adjusted to 7.5]. The cyclic [^{32}P]AMP product was isolated by sequential chromatography on Dowex 50 X4 neutral alumina columns as described by Salomon *et al.* (1974). Determinations of cyclic [^{32}P]AMP were corrected for losses during the incubation and isolation procedures, as determined from the final recovery of cyclic [^3H]AMP. Under the conditions employed the reaction was linear with respect to both time (up to 20 min) and protein concentration (up to 60 $\mu\text{g/tube}$).

Myoblast fusion was estimated by direct microscopic examination (magnification $\times 200$) of nuclei within 16 evenly spaced fields per culture dish as described by Zalin & Montague (1974). Fusion was expressed as an index, which was calculated as follows:

$$\frac{\text{no. of nuclei within myotubes}}{\text{total no. of nuclei (myotubes + single cells)}} \times 100$$

Results and discussion

Cytosine arabinoside halts DNA synthesis and is toxic to replicating cells (Graham & Whitmore, 1970; Cozzarelli, 1977). When the drug is added to fusing myoblast cultures in which the rate of proliferation has substantially fallen, it has no effect on the ability of myoblasts to fuse and differentiate (Curtis, 1979). Plate 1 shows the morphology of myoblasts during the fusion period. Use of cytosine arabinoside therefore maintains the cell number constant during the fusion period, and avoids problems such as that of dealing with a mixed population of cells, some of which (e.g. fibroblasts) are proliferating and some of which are fusing. In fact this proved not to be a major problem in some of the present experiments, in which DNA was approximately the same in the presence and in the absence of cytosine arabinoside. Fig. 1 shows that DNA content and cell number increase in concert before the addition of cytosine arabinoside, and remain constant thereafter. Expression on a 'per protein' basis (Grove & Stockdale, 1978) was considered undesirable, as protein content increases during fusion.

The surface-membrane functions that were measured were found to fall into three categories: (i) those that remain constant during fusion, (ii) those that increase in concert with total protein and (iii) those that increase faster than total protein.

In the first category is the uptake of 2-aminoisobutyrate and 2-deoxyglucose (Fig. 2), and in the second category the uptake of α -methyl glucoside and choline (at high concentration) (Fig. 3). The difference between them is clear. The uptake of α -methyl glucoside and choline increases approximately 2-fold during the fusion period (50–100 h),

EXPLANATION OF PLATE 1

Phase-contrast micrographs of myoblast cultures

Unfixed cultures, grown as described in the Methods and materials section, were photographed on the stage of a Nikon microscope. Exposures were set automatically with a Nikon AF camera attachment. (a) Replicating myoblasts (27 h of culture); (b) myoblasts at start of fusion (40 h); (c) fused cultures grown without cytosine arabinoside (96 h); (d) fused cultures grown in the presence of cytosine arabinoside ($5\text{ }\mu\text{M}$ added at 50 h) (96 h). The length of the bar is $10\text{ }\mu\text{m}$.

Fig. 1. *Effect of cytosine arabinoside on proliferation and fusion*

Cells were plated, half the cultures exposed to cytosine arabinoside (5 μ M) as described in the Methods and materials section, and cell number (Δ and $\blacktriangle$), DNA content ($\square$ and $\blacksquare$) and fusion index ($\circ$ and $\bullet$) were determined as described in the Methods and materials section. Δ , $\square$ and $\circ$, Cytosine arabinoside present; $\blacktriangle$, $\blacksquare$ and $\bullet$, cytosine arabinoside absent.

Fig. 2. *Uptake of labelled 2-aminoisobutyrate and 2-deoxyglucose during myoblast fusion*

Cells were exposed to 14 C-labelled 2-aminoisobutyrate ($\bullet$) or 2-deoxyglucose ($\blacksquare$), and uptake was measured as described in the Methods and materials section.

Fig. 3. *Uptake of labelled α -methyl glucoside and choline during myoblast fusion*

Cells were exposed to 14 C-labelled α -methyl glucoside ($\bullet$) or choline ($\blacksquare$) and uptake was measured as described in the Methods and materials section. Total cellular protein ($\blacktriangle$) is shown for comparison.

and this is true of total protein content also (Fig. 3). Assuming that the rate of uptake is a measure of simple diffusion, and that this is related to the total amount of surface membrane, it would appear that surface area approximately doubles during fusion. That this is probably so is documented below (Table 2).

The amounts of substrate taken up by 30 min (by which time the rate of uptake of each of the substrates has fallen considerably) is shown in Table 1. The amount of choline taken up is approximately the same as that of α -methyl glucoside, and one may assume that, at the high concentration of choline used (15 mM), none is accumulated. That is, α -methyl glucoside and choline equilibrate between medium and cells. In that case, one may infer that the doubling in amount of uptake at 100h compared with that at 50h reflects a doubling in cell volume; certainly fused cells appear larger than the sum of their unfused precursors (Plate 1), and the total surface area, as judged by total iodinated surface proteins (Table 2), doubles.

Between 40 and 120h 2-aminoisobutyrate and 2-deoxyglucose become some 20–50-fold more

concentrated in cells than α -methyl glucoside or choline (Table 1). The mechanism by which this is achieved is presumably by linkage to the Na^+ gradient for 2-aminoisobutyrate, and by phosphorylation for 2-deoxyglucose (cf. Impraime *et al.*, 1980). When choline is present at 1 μM instead of 15 mM, it is concentrated to approximately the same degree as 2-deoxyglucose (results not shown). It may be concluded from Fig. 2 and Table 1 that, during fusion, the capacity of cells to accumulate Na^+ -linked amino acids or phosphorylated intermediates remains constant, despite a doubling in volume and surface area. Whether the rate-limiting step is Na^+ -linked transport or the activity of the Na^+ pump for 2-aminoisobutyrate, and whether it is 2-deoxyglucose transport or the intracellular kinase that phosphorylates 2-deoxyglucose for 2-deoxyglucose, are not known at present. What is clear is that the results are in agreement with those of Grove & Stockdale (1978) insofar as 2-aminoisobutyrate is concerned, and confirm their speculation that total protein and the surface membrane (see Table 2) increase during myoblast fusion. On the other hand a report has appeared (Farfel *et al.*, 1979) showing a

Table 1. Accumulation of labelled substrates

Cells were exposed to radioactive substrates and uptake was measured as described in the Methods and materials section. The total amount of isotope taken up by cells over 30 min is expressed as a percentage of that present in the medium. Note that cellular water (of 2×10^6 cells) is approx. 0.01–0.05% of medium water.

Substrate	Concn. in medium (volume 1 ml)	% uptake by cells	
		At 40h	At 96h
2-Amino[^{14}C]isobutyrate	1 μM	1.4	1.4
Deoxy[^3H]glucose	2.4 μM	1.2	1.1
[U- ^{14}C] α -Methyl glucoside	3.3 μM	0.027	0.042
[^3H]Choline	15 mM	0.01	0.016

Table 2. Amount of [^{125}I]iodinated proteins

Cells at 40 and 96h of culture were labelled with ^{125}I , extracted, subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and scanned, and individual bands (corresponding to those shown in Fig. 4) were extracted and assayed for ^{125}I radioactivity. The radioactivity of plates to which no cells had been added is shown for comparison. The total amount of protein subjected to electrophoresis was 50 μg (840 c.p.m. of $^{125}\text{I}/\mu\text{g}$ of protein) for the 40h sample and 130 μg (960 c.p.m. of $^{125}\text{I}/\mu\text{g}$ of protein) for the 96h sample. The values in parentheses show the percentage distribution of radioactivity among the different bands.

	^{125}I radioactivity (c.p.m.)		
	Cells at 40h	Cells at 96h	No cells
Band 1	4350 (10%)	13 280 (11%)	<20
Band 2	1870 (4%)	5920 (5%)	260
Band 3	1120 (3%)	1560 (1%)	30
Band 4	9800 (23%)	25 010 (20%)	20
Band 5	5200 (12%)	18 750 (15%)	<20
Band 6	980 (2%)	1310 (1%)	<20
Band 7	1260 (3%)	2690 (2%)	<20
Dye front	9030 (21%)	27 470 (22%)	<20

transient increase in 2-aminoisobutyrate transport (expressed on a 'per plate' basis) during myoblast fusion. It is possible that differences in culture conditions explain the discrepancy between the results obtained by Farfel *et al.* (1979) and those obtained by Grove & Stockdale (1978) and ourselves: times of re-feeding, which stimulates uptake, for example, are not stated by Farfel *et al.* (1979); alternatively it is conceivable that the cells used by Farfel *et al.* (1979) were more highly synchronized.

Total surface protein was assessed by measuring the amount of ^{125}I that can be bound to cells by the lactoperoxidase technique. The collagen used to coat

the Petri dishes does not become appreciably labelled, as shown in Table 2. Approximately the same amount of ^{125}I becomes bound (per μg of total cellular protein) at 96 h as at 40 h (Table 2); since the total amount of cellular protein approximately doubles between 40 and 96 h (Fig. 3), this is clearly true of total surface protein also. This conclusion assumes that the accessibility of bulk surface protein does not alter during fusion. Certainly the pattern of ^{125}I -labelled surface proteins (in terms of molecular size, as assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) is the same at 40 and 96 h (Fig. 4 and Table 2); note that the pattern

Fig. 4. Patterns of total and ^{125}I iodinatable proteins

Cells at 40 and 96 h of culture were labelled with ^{125}I , extracted, subjected to polyacrylamide-gel electrophoresis and scanned as described in the Methods and materials section. The numbering of the bands corresponds to that shown in Table 2. (a) Total protein, stained with Coomassie Blue. (b) ^{125}I iodinated protein. The migrations of β -galactosidase (mol.wt. 130000) (G), bovine serum albumin (mol.wt. 70000) (A) and pepsin (mol.wt. 30000) (P) are shown for comparison.

Fig. 5. Activity of adenylate cyclase during myoblast fusion

The activity of adenylate cyclase in the presence (■) and absence (●) of fluoride was measured as described in the Methods and materials section.

of cellular proteins (of which surface proteins are but a fraction) does not alter either (Fig. 4). On the other hand the amount (Hartzell & Fambrough, 1973) and distribution of specific proteins such as acetylcholine receptor (Prives *et al.*, 1976) or large external transformation-sensitive (LETS) protein [fibronectin; Vaheri *et al.*, (1978)] (Chen, 1977) on the cell surface (probably in band 1 of Table 2 and Fig. 4) does change considerably; adenylate cyclase, as documented below, is another example.

The activity of adenylate cyclase, measured in the absence or presence of fluoride, shows a 5–10-fold increase during the fusion period (Fig. 5). The same is true of the activity in the presence of prostaglandin or isoproterenol (D. H. Curtis & R. Zalin, unpublished work; Curtis, 1979).

Note added in proof (received 19 June 1980)

A report confirming the overall constancy in the pattern of ^{125}I -labelled surface proteins during myoblast fusion has recently appeared (Cates & Holland, 1980).

Another aspect of the present work is confirmed by the report by Wyke *et al.* (1980), which shows that a transient change in permeability to low-molecular-weight compounds (such as α -methyl glucoside) and ions (such as Ca^{2+}) is not a prerequisite for membrane fusion; this is to be contrasted with the postulates of Volsky & Loyter (1978) and Blow *et al.* (1979). Only when

osmotically induced swelling follows the fusion event, as in virally mediated or chemically mediated giant-cell formation (Knutton & Pasternak, 1979), does a permeability change appear to be necessary.

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References

- Blow, A. J. J., Botham, G. M. & Lucy, J. A. (1979) *Biochem. J.* **182**, 555–563
- Cates, G. A. & Holland, P. C. (1980) *Biochem. J.* **186**, 211–216
- Chen, L. B. (1977) *Cell* **10**, 393–400
- Christensen, H. N. (1975) *Curr. Top. Membr. Transp.* **6**, 227–258
- Cozzarelli, N. R. (1977) *Annu. Rev. Biochem.* **46**, 641–668
- Curtis, D. H. (1979) D.Phil. Thesis, University of Sussex
- Farfel, Z., Karlisch, S. & Prives, J. (1979) *J. Cell. Physiol.* **98**, 279–282
- Fischbach, G. D. (1972) *Dev. Biol.* **28**, 407–429
- Graham, F. L. & Whitmore, G. F. (1970) *Cancer Res.* **30**, 2627–2635
- Grove, B. K. & Stockdale, F. E. (1978) *Dev. Biol.* **66**, 142–150
- Hartzell, H. C. & Fambrough, D. M. (1973) *Dev. Biol.* **30**, 153–165
- Hinegardner, R. T. (1971) *Anal. Biochem.* **39**, 197–201
- Hubbard, A. L. & Cohn, Z. A. (1972) *J. Cell Biol.* **55**, 390–405
- Impraim, C. C., Foster, K. A., Micklem, K. J. & Pasternak, C. A. (1980) *Biochem. J.* **186**, 847–860
- Kissane, N. M. & Robins, E. (1958) *J. Biol. Chem.* **233**, 184–188
- Knutton, S. & Pasternak, C. A. (1979) *Trends Biochem. Sci.* **4**, 220–223
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Prives, J., Silman, I. & Amsterdam, A. (1976) *Cell* **7**, 543–550
- Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- Tepperman, K., Morris, G., Essien, F. & Heywood, S. M. (1975) *J. Cell. Physiol.* **86**, 561–566
- Vaheri, A., Ruoslahti, E. & Mosher, D. F. (eds.) (1978) *Ann. N.Y. Acad. Sci.* **312**, 1–456
- Volsky, D. J. & Loyter, A. (1978) *J. Cell Biol.* **78**, 456–477
- Wyke, A. M., Impraim, C. C., Knutton, S. & Pasternak, C. A. (1980) *Biochem. J.* **190**, 625–638
- Zalin, R. J. & Montague, W. (1974) *Cell* **2**, 103–108