Dependence of fluid-phase pinocytosis in arterial smooth-muscle cells on temperature, cellular ATP concentration and the cytoskeletal system

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¹²⁵I-labelled poly(vinylpyrrolidone) was used as a marker of fluid-phase pinocytosis in cultured pig arterial smooth-muscle cells. The rate of pinocytosis was temperature-dependent. A decrease in cellular ATP concentrations as a result of inhibition of either glycolysis or oxidative phosphorylation was associated with a similar decrease in pinocytosis. A microfibrillar-disruptive agent, cytochalasin B, caused a concentration-dependent stimulation of pinocytosis, whereas the microtubular-disruptive agents colchicine and vinblastine decreased pinocytosis to approximately half of control values at all concentrations used. These results indicate that fluid-phase pinocytosis in smooth-muscle cells is dependent on a continuing supply of energy and the integrity of the microtubules. Furthermore, microfilaments appear to exert a certain degree of constraint on pinocytosis, possibly by restricting invagination of the plasma membrane.

Factors regulating fluid-phase pinocytosis in smooth-muscle cells are of considerable interest in view of the possible role of this process in the uptake of lipoproteins during atherogenesis. Smooth-muscle cells take up low-density lipoprotein, the major cholesterol-carrying lipoprotein of the plasma, by two routes involving prior binding to the plasma membrane: a high-affinity receptor-mediated pathway and a low-affinity pathway. The cells also take up low-density lipoprotein by a fluid-phase (receptor-independent) pathway. The high-affinity receptor-mediated pathway is subject to regulation such that low-density lipoprotein uptake causes a decrease in low-density-lipoprotein receptor activity and a suppression of cholesterol biosynthesis. The receptor-independent pathway is not, however, subject to feedback control by low-density-lipoprotein binding. Thus, during atherogenesis, when the cells have accumulated cholesterol and the activity of the low-density-lipoprotein receptor is presumably suppressed (Goldstein & Brown, 1977), fluid-phase pinocytosis probably provides the major route of entry of low-density lipoprotein into the cells. Two key molecular processes on which pinocytosis depends are cell-membrane motility and fusion. Previous studies in other cell types to determine the metabolic requirements of these processes and the cellular elements involved have led to conflicting results. In the present study, therefore, the effects of temperature, inhibition of ATP synthesis,

and disruption of the cytoskeletal system on pinocytosis in pig arterial smooth-muscle cells in culture were investigated. ¹²⁵I-labelled PVP was used as a marker for fluid-phase pinocytosis.

Materials and methods

Sources of materials

Tissue culture medium (Dulbecco's modified Eagle's medium) and foetal-calf serum were from Gibco Europe (Paisley, Scotland, U.K.). Trypsin (1-250 grade) was obtained from Difco (East Molesey, Surrey, U.K.). Falcon tissue-culture dishes and flasks were from Scientific Supplies (London E.C.1, U.K.). Penicillin G (sodium salt), streptomycin sulphate, kanamycin sulphate, colchicine, vinblastine sulphate, cytochalasin B, dimethyl sulphoxide, 2,4-dinitrophenol, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], ATP (sodium salt) and firefly lantern extract were from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). NaF (AnalaR grade) and EDTA (disodium salt) were from BDH Chemicals (Poole, Dorset, U.K.). ¹²⁵I-labelled PVP was from The Radiochemical Centre (Amersham, Bucks., U.K.). Bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.).

Cell culture

Smooth-muscle cells were isolated from the intima and media of pig thoracic aortas by the method of

Abbreviation used: PVP, poly(vinylpyrrolidone).

Ross (1971). The cells were grown to confluence in 75 cm² Falcon tissue-culture flasks containing Dulbecco's modified Eagle's medium with 10% (v/v) foetal-calf serum and a mixture of penicillin G (100 i.u./ml), streptomycin sulphate (100 µg/ml) and kanamycin sulphate (100 µg/ml).

The cells were then detached from the culture vessels with 0.1% (w/v) trypsin in phosphate-buffered saline (Paul, 1973) containing disodium EDTA (54 mM) and seeded into 60 mm-diameter Falcon Petri dishes.

Measurement of pinocytosis

¹²⁵I-labelled PVP was used as the marker for fluid-phase pinocytosis. The use of this substrate as a marker for pinocytosis was first demonstrated in yolk sacs (Williams *et al.*, 1975), and it has since been found to be a suitable marker for the determination of pinocytosis in a number of cell types (Roberts *et al.*, 1977; Ose *et al.*, 1980), including smooth-muscle cells (Leake & Bowyer, 1977).

Pinocytosis was measured by the method of Leake & Bowyer (1981). Dishes of confluent smooth-muscle cells, of the same subculture, were incubated with culture medium of the composition described above, containing ¹²⁵I-labelled PVP. The concentrations of PVP in the medium were 50–100 µg/ml and the cells were subcultures 3–10.

In studies in which the cells were incubated at temperatures other than 37°C, the medium was allowed to equilibrate at the given temperature for 30 min before addition to the cells, which were then incubated for 2 h.

NaF, 2,4-dinitrophenol, 2-deoxyglucose, colchicine and vinblastine were dissolved in culture medium; cytochalasin B was dissolved in dimethyl sulphoxide, and oligomycin in ethanol, and then both were diluted with culture medium to attain the final concentration shown.

In each experiment, the modifier and ¹²⁵I-labelled PVP were added simultaneously. Every experiment included a concurrent control incubation of smooth-muscle cells from the same subculture with ¹²⁵I-labelled PVP, but without modifier. In experiments with cytochalasin B, control cultures contained dimethyl sulphoxide, and in experiments with oligomycin, control cultures contained ethanol. At the end of the incubation period, the medium was removed and a sample taken for assay of radioactivity. Each dish of cells was washed with 6 × 4 ml of Hanks balanced salt solution. The cells were then detached from the dish by trypsin treatment and washed twice more in Hanks balanced salt solution. At the end of this washing procedure, the supernatant contains very little radioactivity in relation to that of the cells (Leake & Bowyer, 1981). Furthermore, the post-trypsin washing procedure removes

any radioactivity released into the cell suspension from desorption from the dish (Leake & Bowyer, 1977), so that only intracellular radioactivity is measured in the cell pellet. Thus the need for a control to determine the effect of various modifiers on adsorption or desorption of PVP from the dish is eliminated.

The cell pellets were solubilized by sonication (MSE Soniprep 150, amplitude 20 µm) in 2.5 ml of glass-distilled water for 15 s at 0°C, and samples were taken for assay of radioactivity and protein. Radioactivity was determined by counting 1.5 ml portions in 3 ml plastic tubes in a gamma spectrometer (Wallac) with a standard counting geometry.

The method of Lowry *et al.* (1951) was used to determine the protein content of the cells, with bovine serum albumin as standard.

Determination of cellular ATP

Smooth-muscle cells were incubated in the presence of metabolic inhibitors for 2 h, detached from the dishes with trypsin, washed once with culture medium, and resuspended in 0.5 ml of culture medium. Then 50 µl of the resulting suspension was taken for assay of ATP. Metabolic inhibitors were present in both the trypsin solutions and the culture medium used to wash and resuspend the cells, to prevent recovery of ATP concentrations, which can occur very rapidly after removal of metabolic inhibitors from the culture medium (Pozzan *et al.*, 1980).

For measurement of ATP, 50 µl samples of cell suspension (containing approx. 10⁶ cells) were treated with 50 µl of HClO₄ (4%, v/v) at 0°C and neutralized with 100 µl of KOH (0.4 M) buffered with Hepes (0.1 M), pH 7.0. Samples of supernatant (20 µl) were injected into a solution of firefly lantern extract (10 mg/ml) in a Du Pont 760 luminescence biometer, calibrated with standard ATP solutions between 1 and 50 µM treated in the same way.

Results

Unless otherwise stated, rates of pinocytosis are expressed as percentages of the rate of accumulation of ¹²⁵I-labelled PVP by control cultures incubated without modifiers. ATP concentrations are also expressed as percentages of those in control cell samples incubated without modifier.

Effects of temperature

Above 20°C the rate of accumulation of PVP was linear with temperature; however, at some temperature between 12°C and 20°C accumulation virtually ceased (Fig. 1).

Effects of metabolic inhibitors

In the presence of NaF, an inhibitor of glycolysis, or 2,4-dinitrophenol, which inhibits oxidative phos-

phorylation, accumulation of ¹²⁵I-labelled PVP by smooth-muscle cells was substantially linear with time, but occurred at a lower rate compared with controls (Fig. 2).

The decrease in pinocytosis was concentration-dependent and was only observed at concentrations of inhibitor that decreased cellular ATP (Table 1). This decrease could not be attributed to cytotoxic effects of higher concentrations of inhibitors, because removal of the inhibitor from the medium

resulted in the return of the pinocytic rate to control values (Table 2).

Other metabolic inhibitors, 2-deoxyglucose and oligomycin, which inhibit glycolysis and oxidative phosphorylation respectively, similarly caused a concentration-dependent decrease in pinocytosis, which could be related to cellular ATP concentrations. The addition of a glycolytic inhibitor simultaneously with an inhibitor of oxidative phosphorylation produced a synergistic effect on

Fig. 1. Effect of temperature on the accumulation of ¹²⁵I-labelled poly(vinylpyrrolidone) by pig smooth-muscle cells

Cells were incubated in culture medium containing ¹²⁵I-labelled PVP (75 µg/ml; sp. radioactivity 1.97 MBq/mg) for 2 h at various temperatures. Each value shown is the mean of five observations.

Fig. 2. Effect of metabolic inhibitors on the accumulation of ¹²⁵I-labelled poly(vinylpyrrolidone) by pig smooth-muscle cells

Cells were incubated in culture medium containing ¹²⁵I-labelled PVP (108 µg/ml; sp. radioactivity 2.05 MBq/mg) and either 2,4-dinitrophenol (5 mM) or NaF (5 mM) for various periods of time. Values shown are means ± S.D. for four observations. —, Control; ■, +NaF; ▲, +2,4-dinitrophenol.

Table 1. Effect of metabolic inhibitors on cellular ATP concentrations and pinocytosis

Smooth-muscle cells were incubated at 37°C for 4 h in the presence of inhibitors and ¹²⁵I-labelled PVP (60 µg/ml; sp. radioactivity 2.0 MBq/mg). The final concentration of ethanol in groups incubated with oligomycin and the matching control group was 0.1% (v/v). Values shown are means ± S.D. for five observations. **Denotes a significant difference compared with controls (*P* < 0.001), and *denotes *P* < 0.01 (analysis of variance).

Inhibitor	Pathway affected	Concn. (mM)	ATP concn. (% of control)	Pinocytosis (% of control)
NaF	Glycolysis	0.1	100 ± 7	100 ± 7
		1	100 ± 9	96 ± 7
		5	43 ± 5	65 ± 5**
2-Deoxyglucose	Glycolysis	1	100 ± 8	100 ± 7
		10	70 ± 9	87 ± 6**
2,4-Dinitrophenol	Oxidative phosphorylation	0.125	80 ± 6	84 ± 5*
		5	43 ± 5	60 ± 4**
Oligomycin	Oxidative phosphorylation	2 (µg/ml)	100 ± 9	100 ± 8
		20 (µg/ml)	72 ± 8	79 ± 5**
NaF +	Glycolysis +	1	47 ± 3	69 ± 5**
2,4-dinitrophenol	Oxidative phosphorylation	0.125		

Table 2. *Effect on pinocytic rate of removal of modifiers from the incubation medium*

Smooth-muscle cells were incubated with NaF (5 mM), 2,4-dinitrophenol (5 mM) cytochalasin B (20 μ M) or colchicine (1 μ M) for 4 h, washed five times in Hanks balanced salt solution, and re-incubated in modifier-free medium for 3 h before a 4 h incubation in modifier-free medium containing ^{125} I-labelled PVP (96 μ g/ml; sp. radioactivity 1.54 MBq/mg). Values shown are means $\pm$ S.D. for five observations. Abbreviation: NS, not significant.

Modifier previously present	Pinocytosis (% of control value in presence of modifier)	Pinocytosis (% of control value after removal of modifier from the culture medium)	P compared with control values
NaF	65 $\pm$ 5	103 $\pm$ 2	NS
2,4-Dinitrophenol	60 $\pm$ 4	98 $\pm$ 2	NS
Cytochalasin B	186 $\pm$ 11	118 $\pm$ 1	< 0.001
Colchicine	54 $\pm$ 3	63 $\pm$ 3	< 0.001

both cellular ATP concentrations and pinocytosis (Table 1).

Effects of microfibrillar- and microtubular-disruptive agents

Concentrations of the microfibrillar-disruptive agent cytochalasin B greater than 5 μ M caused a morphological change in the cells; extensive cytoplasmic retraction occurred, resulting in a ball-like shape of the central cell body, from which cytoplasmic processes projected. The effect of cytochalasin B on pinocytosis is shown in Fig. 3. Accumulation of ^{125} I-labelled PVP in the presence of cytochalasin B (20 μ M) was essentially linear with time but markedly enhanced. This stimulation of pinocytosis was concentration-dependent. At 5 μ M-cytochalasin B no significant effect was observed, but at 20 μ M a significant increase in pinocytosis was observed, which was enhanced at 40 μ M (Table 3). Dimethyl sulphoxide, which was present in cultures containing cytochalasin B, itself had no effect on cell morphology or pinocytosis. At 3 h after removal of cytochalasin B from the incubation medium, the rate of pinocytosis had returned to a value only slightly higher than control values (Table 2), and cell morphology was similar to that of controls.

In contrast with cytochalasin B, in the presence of the microtubular-disruptive agents colchicine and vinblastine a marked decrease in pinocytosis was observed after 2 h incubation (Fig. 4). This effect was concentration-dependent; a decrease in pinocytosis was observed in cells incubated with either modifier at a concentration of 1 μ M, but not at 0.1 μ M. Furthermore, the magnitude of the inhibition was not enhanced by increasing the concentrations of the modifiers (Table 4).

On removal of colchicine from the culture medium, there was only a partial recovery of pinocytic rate (Table 2). This is probably because the binding of colchicine to tubulin is only slowly reversible (Serpinskaya *et al.*, 1981). The decrease in

Fig. 3. *Effect of cytochalasin B on the accumulation of ^{125} I-labelled poly(vinylpyrrolidone) by pig smooth-muscle cells*

Cells were incubated in culture medium containing ^{125} I-labelled PVP (96 μ g/ml; sp. radioactivity 1.54 MBq/mg) and cytochalasin B (20 μ M) for various periods of time. The final concentration of dimethyl sulphoxide in the medium containing cytochalasin B was 0.1% (v/v). Values shown are means $\pm$ S.D. for four observations. —, Control; ■, +cytochalasin B.

pinocytosis produced by colchicine and vinblastine cannot, however, be attributed to a non-specific cytotoxic effect of these agents, since at concentrations sufficient to decrease pinocytosis no leakage of the cytosolic enzyme lactate dehydrogenase could be detected, which would be expected from damaged or dying cells (results not shown).

Table 3. *Effect of cytochalasin B on the accumulation of poly(vinylpyrrolidone) by pig smooth-muscle cells*
Smooth-muscle cells were incubated in the presence of various concentrations of cytochalasin B and ^{125}I -labelled PVP ($54\mu\text{g/ml}$; sp. radioactivity 2.0MBq/ml) for 16 h at 37°C . The final concentration of dimethyl sulphoxide in all groups was 0.16% (v/v). Values shown are means $\pm$ s.d. for four observations. Abbreviation: NS, no significant difference compared with controls.

Concn. of cytochalasin B (μM)	PVP uptake (% of control)	P compared with control values
5	104 ± 6	NS
20	186 ± 10	<0.001
40	287 ± 7	<0.001

Table 4. *Effect of microtubular-disruptive agents on the accumulation of ^{125}I -labelled poly(vinylpyrrolidone) by pig smooth muscle cells*

Smooth-muscle cells were incubated at 37°C for 5 h in the presence of modifier and ^{125}I -labelled PVP ($22\mu\text{g/ml}$; sp. radioactivity 0.67MBq/mg). Each experiment was performed with a matched control. Values shown are means $\pm$ s.d. for four observations. Abbreviation: NS, not significant.

Modifier	Concn. (μM)	PVP uptake (% of control)	P compared with controls
Colchicine	0.1	96 ± 7	NS
	1	54 ± 2	<0.001
	10	53 ± 2	<0.001
	50	60 ± 3	<0.001
	100	59 ± 3	<0.001
Vinblastine	0.1	103 ± 4	NS
	1	56 ± 5	<0.001
	10	50 ± 1	<0.001
	50	60 ± 1	<0.001
	100	46 ± 2	<0.001

Fig. 4. *Effect of microtubular-disruptive agents on the accumulation of ^{125}I -labelled poly(vinylpyrrolidone) by pig smooth-muscle cells*

Cells were incubated in culture medium containing ^{125}I -labelled PVP ($108\mu\text{g/ml}$; sp. radioactivity 2.05MBq/mg) and either colchicine ($1\mu\text{M}$) or vinblastine ($1\mu\text{M}$) for various periods of time. Values shown are means $\pm$ s.d. for four observations. —, Control; O, +colchicine; ●, +vinblastine.

microtubular function at low temperature (Bhattacharyya & Wolff, 1976), since microtubules have been implicated in pinocytosis (Ose *et al.*, 1980; Piasek & Thyberg, 1979). However, the discontinuity in the plot between 20°C and 12°C , the temperature range in which most membrane lipids undergo a phase transition (Petit & Edidin, 1974), suggests that the predominant cause was probably a decrease in membrane fluidity.

The Q_{10} value for pinocytosis calculated over the range 20 – 37°C was 1.85, which is similar to the value obtained in mouse fibroblasts, by using horseradish peroxidase as a marker for pinocytosis (Steinman *et al.*, 1974), and in macrophages, by using vesicle counts to determine pinocytosis (Cohn, 1966).

Effects of metabolic inhibitors

Consistent data on the energy requirements of pinocytosis have not yet emerged; it has even been suggested that pinocytic vesicles can be interiorized in the absence of metabolic energy (Casley-Smith, 1969). Moreover, studies of the effects of metabolic inhibitors on pinocytosis have not usually included concomitant measurements of their effect on cellular ATP concentrations. Experiments in which ATP concentrations have not been measured should be interpreted with caution, as the concentrations of inhibitors required to decrease ATP concentrations may vary between cell types. In addition, there is evidence from studies with mouse fibroblasts that at least some metabolic inhibitors may affect pinocytosis independently of their effects on cellular ATP concentrations (Steinman *et al.*, 1974).

In our experiments the decrease in cellular ATP

Discussion

Effects of temperature

The observed temperature-sensitivity of accumulation of ^{125}I -labelled PVP is in agreement with findings in other cell systems (Williams *et al.*, 1977; Duncan & Lloyd, 1978; Pratten & Lloyd, 1979; Ose *et al.*, 1980).

Contributory factors to the observed temperature-dependence of pinocytosis may include a decrease in the metabolism of the cell, or a loss of

produced by inhibitors of either glycolysis or oxidative phosphorylation was accompanied by a similar decrease in the accumulation of ^{125}I -labelled PVP. Thus smooth-muscle cells derive energy to fuel pinocytosis from both glycolysis and oxidative phosphorylation.

The highest concentrations of inhibitors used were the maximum that were not cytotoxic in the 2 h incubation period. Since it is not possible to decrease ATP below about 40% of control values, data on the threshold concentration of ATP required for pinocytosis could not be obtained.

A similar decrease in pinocytosis in the presence of metabolic inhibitors has been observed in several other cell types (Silverstein *et al.*, 1977; Duncan & Lloyd, 1978; Pratten & Lloyd, 1979; Ose *et al.*, 1980). Also, our findings agree with those of Livesey *et al.* (1980), who showed that there is a correlation between pinocytic activity in yolk sacs and ATP concentrations in the tissue. In contrast, other studies have suggested that pinocytosis is unaffected by metabolic inhibitors (Ryser, 1968; Contractor, 1976). In those studies, however, the cellular ATP concentrations were not measured, and hence the absence of an inhibition of pinocytosis may have been due to the use of insufficient concentrations of inhibitors to lower cellular ATP concentration.

Effects of microfibrillar- and microtubular-disruptive agents

The observed increase in pinocytosis in cells treated with cytochalasin B may be in part due to the shape change induced by this drug, which would increase the surface area of the cells exposed to the culture medium and therefore available for pinocytosis. This effect alone, however, is not sufficient to explain the results, since concentrations of cytochalasin B above those required to produce the shape change continued to increase pinocytosis without causing further morphological changes. One possible explanation for this is that a further increase in drug concentration caused more extensive microfilament disruption. Microfilaments are found in close association with the plasma membrane (Allison & Davies, 1974a) and may thus constrain membrane invagination to a certain extent. Disruption of such microfilaments could thus have resulted in less constraint, facilitating pinocytosis.

A similar stimulation of pinocytosis by cytochalasin B has been observed in hepatocytes (Ose *et al.*, 1980) and human carcinoma cells (Haigler *et al.*, 1979). Furthermore, our findings that the effects of cytochalasin B are readily reversible and occur in the absence of any cytotoxic effects agree with the studies of Wills *et al.* (1972) and Wagner *et al.* (1971).

In contrast with our findings, cytochalasin B at

similar concentrations has been found to decrease the pinocytic uptake of ^{125}I -labelled PVP into yolk sacs (Duncan & Lloyd, 1978) and peritoneal macrophages (Pratten & Lloyd, 1979) and of sucrose into Chang liver cells (Wagner *et al.*, 1971). The discrepancy between these results is unclear, but could possibly be explained by a difference between cell types in the use of glucose as an energy source. Cytochalasin B has been shown to inhibit transport of glucose into fat-cells (Vinten, 1978), and may thus decrease ATP concentrations and hence pinocytosis in cells in which glucose is the prime energy source.

Another possibility is that there are differences in the size of pinosomes formed in the cell types, which may influence their requirements for microfilaments in pinosome formation.

Alternatively the differences may be dependent on the type of actin of which the microfilaments are composed. Actin in smooth-muscle cells is almost entirely composed of the γ -type, whereas most other cell types (except skeletal and cardiac muscle) contain a mixture of β - and γ -actins. Thus differences in microfilament composition may confer different sensitivities to disruption by cytochalasin B. The results could then be explained if partial disruption caused an inhibition of pinocytosis, whereas complete disruption caused a stimulation through its influence on membrane deformability, thus masking the former inhibition.

In our studies colchicine was found to decrease pinocytosis by smooth-muscle cells at concentrations known to disrupt microtubules. Also, the effect of this drug was similar for all concentrations used, suggesting that the effect of colchicine on pinocytosis was due to its action on microtubules rather than an indirect result of its effect on other cellular functions, such as nucleotide transport, which are not affected at the lower concentration of colchicine used (Mizel & Wilson, 1972). In addition, vinblastine, a microtubular disruptive agent which binds to a different site on tubulin from that for colchicine, had a similar effect. These findings thus provide evidence for a role of microtubules in pinocytosis.

Microtubules are involved in vesicle movement within the cell (Bhisey & Freed, 1971), which may be important in the membrane recycling necessary to maintain pinocytosis (Duncan & Pratten, 1977). A similar inhibition of pinocytosis by colchicine treatment has been reported in several other cell types (Ose *et al.*, 1980; Heiniger & Marshall, 1979; Piasek & Thyberg, 1979; Pratten & Lloyd, 1979).

In conclusion, the results obtained in the present study show that pinocytosis in smooth-muscle cells is a temperature-sensitive process, dependent on a source of metabolic energy and on the integrity of the microtubular system. It is also increased when the microfilaments are disrupted.

The results contradict the suggestion (Allison & Davies, 1974*b*) that micropinocytosis is independent of metabolic energy and of the integrity of the cytoskeletal system.

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