Effects of Temperature, Metabolic Inhibitors and Some Other Factors on Fluid-Phase and Adsorptive Pinocytosis by Rat Peritoneal Macrophages

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Low temperature, NaF and 2,4-dinitrophenol could each abolish the pinocytic uptake of ^{125}I -labelled poly(vinylpyrrolidone) or colloidal ^{198}Au gold by rat peritoneal macrophages cultured *in vitro*. Cytochalasin B caused only partial inhibition, even at $10\text{ }\mu\text{g/ml}$, and colchicine (10 or $25\text{ }\mu\text{g/ml}$) inhibited uptake of colloidal ^{198}Au gold much more than that of ^{125}I -labelled poly(vinylpyrrolidone). Dibutyl cyclic AMP and ouabain were without effect on uptake of ^{125}I -labelled poly(vinylpyrrolidone), and slight stimulation was seen with ATP and theophylline. Uptake of ^{125}I -labelled poly(vinylpyrrolidone) was abolished by EGTA (5 mM), but restored by adding CaCl_2 (5 mM). The results appear not to support the conventional criteria for the division of pinocytic phenomena into macropinocytosis, requiring a metabolic energy supply and cytoskeletal components, and micropinocytosis, requiring neither.

In a previous paper (Pratten *et al.*, 1977) we described a system for quantification of pinocytosis in rat peritoneal macrophages cultured *in vitro* which allowed those substrates entering in the fluid phase to be distinguished from those entering by adsorption to the membrane. In a further study (Pratten *et al.*, 1978) we attempted to modify the rate of capture of substrates by using polycations and polyanions, but found that it was impossible to alter greatly the basal pinocytic rate with such compounds, although it was possible to enhance the rate of capture of substrates entering by adsorptive pinocytosis. In the work reported here the metabolic and other characteristics of pinocytosis in macrophages were examined and compared by using substrates entering by the two different mechanisms.

Materials and Methods

Chemicals

ATP, colchicine, cytochalasin B, dibutyl cyclic AMP, 2,4-dinitrophenol, EGTA, NaF, ouabain and theophylline were from Sigma (London) Chemical Co., Poole, Dorset, U.K. These substances were dissolved in medium 199 before use and when necessary the pH was re-adjusted to 7.4.

Culture of peritoneal macrophages *in vitro* with radioactive substrates

The rate of uptake of colloidal ^{198}Au gold or ^{125}I -labelled poly(vinylpyrrolidone) was measured by the method of Pratten *et al.* (1977), except that in some cases the culture period was extended to 50 h.

When uptake proceeds at a constant rate throughout the incubation, this rate can be expressed as an Endocytic Index, defined as the volume of culture medium (μl) whose contained substrate has been captured per 10^6 cells per h. In all experiments involving the incubation of macrophages at temperatures other than 37°C , the cells were allowed to equilibrate at the new temperature for 1 h before addition of substrate. In experiments with chemical modifiers, modifier and substrate, dissolved in culture medium, were added simultaneously. Cytochalasin B was dissolved in dimethyl sulphoxide and then diluted with culture medium. Every experiment included a concurrent incubation with substrate at 37°C and with no added modifier, macrophages from the same cell population being used.

Results

The uptake of ^{125}I -labelled poly(vinylpyrrolidone) and colloidal ^{198}Au gold was linear with time in all experiments, permitting the calculation of Endocytic Indices and the expression of each result as a percentage of a matched control value obtained on the same day by using macrophages from the same population.

Effect of temperature and metabolic inhibitors

The effects of temperature on uptake of ^{125}I -labelled poly(vinylpyrrolidone) (Fig. 1*a*) and colloidal ^{198}Au gold (Fig. 1*b*) are very similar in that substrate capture was almost completely abolished below 20°C .

NaF at 10 or $25\text{ }\mu\text{g/ml}$ had little effect on uptake of

¹²⁵I-labelled poly(vinylpyrrolidone) or colloidal [¹⁹⁸Au]gold, but at 50 μ g/ml was very inhibitory (Table 1). At 500 μ g/ml NaF was cytotoxic.

2,4-Dinitrophenol decreased uptake of both substrates, and at a concentration of 100 μ g/ml decreased uptake of ¹²⁵I-labelled poly(vinylpyrrolidone) to 26% of the control and of colloidal [¹⁹⁸Au]gold to 18% of the control (Table 1). At 500 or 1000 μ g/ml this inhibitor proved to be cytotoxic, causing cell death and detachment from the coverslip.

When moniodoacetate was used as an inhibitor it was found to have a cytotoxic effect and cause the detachment of cells from the coverslip, even at a concentration as low as 10 μ g/ml.

Effects of cytoskeletal inhibitors

Cytochalasin B at 1 μ g/ml had no effect on uptake of either substrate, whereas at 10 μ g/ml it caused approx. 60% inhibition of ¹²⁵I-poly(vinylpyrrolidone) and colloidal [¹⁹⁸Au]gold uptake (Table 2). In a single experiment the concentration of cytochalasin B was increased still further with no significant increase in inhibition. Dimethyl sulphoxide (0.1%, v/v), which was present in cultures containing cytochalasin B, had itself no effect on pinocytosis of either substrate.

Fig. 1. Effect of temperature on rate of uptake of (a) ¹²⁵I-labelled poly(vinylpyrrolidone) and (b) colloidal [¹⁹⁸Au]gold by rat peritoneal macrophages

Each experiment was performed concurrently with a matched control incubated at 37°C. The graphs show, for each temperature, the ratio ($\pm$ S.E.M. of at least three experiments) of the Endocytic Index at the temperature indicated to that at 37°C.

Table 1. Effect of 2,4-dinitrophenol and NaF on uptake of colloidal [¹⁹⁸Au]gold and ¹²⁵I-labelled poly(vinylpyrrolidone) by rat peritoneal macrophages

Each experiment with modifier was performed concurrently with a matched control, and the ratio of the Endocytic Index in the presence of modifier to that in its absence was calculated. The Table shows this ratio (mean $\pm$ S.E.M. of three separate experiments) for each concentration of each modifier, except where only one experiment was performed.

Modifier	Concentration (μ g/ml)	Effect on uptake of	
		colloidal [¹⁹⁸ Au]gold	¹²⁵ I-labelled poly(vinyl- pyrrolidone)
NaF	10	0.93 $\pm$ 0.16	1.69 $\pm$ 0.96
	25	0.87 $\pm$ 0.24	0.54 $\pm$ 0.36
	50	0.07 $\pm$ 0.03	0.11 $\pm$ 0.05
	500	cytotoxic	
Dinitrophenol	5	1.28	—
	10	1.59	—
	25	0.55 $\pm$ 0.06	—
	100	0.18 $\pm$ 0.11	0.26 $\pm$ 0.03
	250	0.10 $\pm$ 0.04	—
	500	cytotoxic	
	1000	cytotoxic	

Table 2. *Effect of cytochalasin B and colchicine on uptake of colloidal [^{198}Au]gold and ^{125}I -labelled poly(vinylpyrrolidone) by rat peritoneal macrophages*

Each experiment with modifier was performed concurrently with a matched control, and the ratio of the Endocytic Index in the presence of modifier to that in its absence was calculated. The Table shows this ratio (mean $\pm$ S.E.M. of three experiments) for each concentration of each modifier, except where only one experiment was performed.

Modifier	Concentration ($\mu\text{g/ml}$)	Effect on uptake of	
		colloidal [^{198}Au]gold	^{125}I -labelled poly(vinyl- pyrrolidone)
Cytochalasin B	1	0.90 ± 0.05	1.00 ± 0.16
	10	0.67 ± 0.15	0.64 ± 0.42
	20	0.50	—
Colchicine	10	0.20 ± 0.03	0.92 ± 0.09
	25	0.18 ± 0.07	0.87 ± 0.34
	100	0.15 ± 0.07	0.12 ± 0.07

Table 3. *Effect of ATP, cyclic AMP, theophylline, EGTA, CaCl_2 and ouabain on uptake of ^{125}I -labelled poly(vinylpyrrolidone) by rat peritoneal macrophages*

Each experiment with modifier was performed concurrently with a matched control, and the ratio of the Endocytic Index in the presence of modifier to that in its absence was calculated. This ratio (mean $\pm$ S.E.M. of three separate experiments) is shown in the Table for each concentration of each modifier.

Modifier	Concentration	Effect on uptake of ^{125}I -labelled poly(vinylpyrrolidone)
ATP	0.25 mM	1.28 ± 0.12
	1.0 mM	1.45 ± 0.20
	2.5 mM	0.64 ± 0.10
	5.0 mM	0.47 ± 0.12
Dibutyl cyclic AMP	10 $\mu\text{g/ml}$	1.07 ± 0.15
	100 $\mu\text{g/ml}$	0.66 ± 0.24
Theophylline	1 $\mu\text{g/ml}$	1.57 ± 0.16
	10 $\mu\text{g/ml}$	1.05 ± 0.18
	100 $\mu\text{g/ml}$	0.71 ± 0.24
EGTA	5 mM	0.15 ± 0.04
EGTA + CaCl_2	5 mM	0.86 ± 0.09
CaCl_2	5 mM	1.41 ± 0.25
Ouabain	10 $\mu\text{g/ml}$	1.33 ± 0.23
	100 $\mu\text{g/ml}$	1.18 ± 0.18

Colchicine at 100 $\mu\text{g/ml}$ strongly inhibited uptake of colloidal [^{198}Au]gold or ^{125}I -labelled poly(vinylpyrrolidone) (Table 2). Lower concentrations had a much greater effect on the uptake of colloidal [^{198}Au]gold than on that of ^{125}I -labelled poly(vinylpyrrolidone).

Effects of some other factors on uptake of ^{125}I -labelled poly(vinylpyrrolidone)

The addition of ATP (1 mM) to the culture medium caused a 50% increase in Endocytic Index (Table 3). At a lower concentration the effect was less and at

some higher concentrations there was inhibition of uptake.

Table 3 also shows the effect of dibutyryl cyclic AMP. Some slight inhibition of uptake was seen at 100 $\mu\text{g/ml}$. Theophylline at 1 $\mu\text{g/ml}$ stimulated uptake by about 50%, but higher concentrations were without effect or slightly inhibitory.

The addition of 5 mM-EGTA caused almost complete inhibition of uptake of ^{125}I -labelled poly(vinylpyrrolidone). When equimolar amounts of EGTA and CaCl_2 were added, uptake was at the control value, whereas 5 mM- CaCl_2 alone had a slight stimulating effect (Table 3).

Ouabain at the two concentrations employed had very little effect (Table 3).

Discussion

Pinocytosis of both colloidal [^{198}Au]gold and ^{125}I -labelled poly(vinylpyrrolidone) by rat peritoneal macrophages was temperature-sensitive. The effect of low temperature probably reflects a decrease in the metabolism of the cells and a loss of fluidity of membrane components, especially since the temperature at which pinocytosis effectively ceases is similar to values suggested for the transition temperatures of membrane lipids (Petit & Edidin, 1974). It is possible to calculate a Q_{10} value (the increase in rate of uptake caused by a 10°C rise in temperature) from our data, but the value obtained depends on the temperature range over which it is calculated. Over the range $20\text{--}37^\circ\text{C}$, the steep portion of the graph (Fig. 1), the Q_{10} for uptake of colloidal [^{198}Au]gold is 7.08, whereas the value for the whole temperature range $5\text{--}37^\circ\text{C}$ is 2.14. This latter value, although very similar to values obtained for enzymic reactions, and also to that obtained by Steinman *et al.* (1974) for endocytosis of horseradish peroxidase by fibroblasts over the range $2\text{--}38^\circ\text{C}$, does not best reflect the temperature profile of pinocytosis. Clearly the fall in pinocytic rate over the range $37\text{--}20^\circ\text{C}$ is due to more than metabolic (enzymic) inhibition. The studies using the metabolic inhibitors fluoride and dinitrophenol also indicate that the uptake of both substrates is energy-dependent, requiring both glycolysis and oxidative phosphorylation. These results parallel those of Duncan & Lloyd (1978), who used the rat yolk sac as a model for endocytosis, and those of Munthe-Kaas (1977) using rat Kupffer cells and mouse peritoneal macrophages.

An alternative way of investigating the metabolic requirements for pinocytosis is the addition of energy-providing substrates to the medium. ATP added to the medium has a biphasic effect, with slight stimulation around 1 mM but some inhibition at higher concentrations. The latter effect could be linked with some feedback inhibition of the glycolytic pathway, or high concentrations may be non-

specifically toxic. A far greater stimulating effect was observed morphometrically in mouse peritoneal macrophages by Cohn & Parks (1967), who found a 30-fold increase in the number of vesicles visible. This parameter may not, however, correlate with the amount of substrate being captured by the cells.

It has frequently been suggested that microtubules and microfilaments play an important part in the movement of organelles and vesicles in several cell types, notably in secretory cells (Weissmann *et al.*, 1973) and in endocytic cells (Allison *et al.*, 1971). Cytochalasin B, which is believed to affect polymerization of microfilaments, strongly inhibits phagocytosis of bacteria by mouse peritoneal macrophages (Allison *et al.*, 1971). We found that this inhibitor could not decrease uptake of colloidal [^{198}Au]gold or ^{125}I -labelled poly(vinylpyrrolidone) by more than 40%, which is consistent with the report of Wills *et al.* (1972) that cytochalasin B only slightly inhibited the uptake of colloidal [^{198}Au]gold by mouse peritoneal macrophages, and that of Munthe-Kaas (1977) that uptake of the same substrate by mouse macrophages or rat Kupffer cells was inhibited by about 25%. On the other hand, we found that colchicine, which inhibits the microtubule system, markedly decreased uptake of colloidal [^{198}Au]gold, whereas inhibition of uptake of ^{125}I -labelled poly(vinylpyrrolidone) was only observed at high concentrations, where the specificity of the inhibitor is in doubt (Allison & Davies, 1974).

We have previously shown (Pratten *et al.*, 1977) that, in contrast with ^{125}I -labelled poly(vinylpyrrolidone), colloidal [^{198}Au]gold enters macrophages bound to the plasma membrane, and the simplest explanation of our results is that colchicine interferes with this binding. It should be noted that Munthe-Kaas (1977) found little inhibition by colchicine of colloidal [^{198}Au]gold uptake by Kupffer cells.

These results lend no support to the concept (Allison & Davies, 1974) that fluid-phase uptake by macrophages has two distinct components, macropinocytosis, inhibitable by metabolic inhibitors, cytochalasin B and colchicine, and a non-inhibitable micropinocytosis.

The role of cyclic nucleotides and Ca^{2+} as messengers within cells has been well established (Rasmussen, 1970). Our results indicate that both dibutyryl cyclic AMP and theophylline can slightly elevate uptake of ^{125}I -labelled poly(vinylpyrrolidone) into macrophages, but that at higher concentrations they are inhibitory. Masur *et al.* (1971) have shown an increase in capture of horseradish peroxidase by toad bladder in the presence of either of these elevators of cyclic AMP concentrations, and Atkinson *et al.* (1976) have shown an increase in intracellular cyclic AMP during phagocytosis. However, other groups have reported that cyclic AMP is a potent

inhibitor of endocytosis (Brown & Segal, 1978; Cox & Karnovsky, 1973). It is possible that the anomalous effects observed are a reflection of the concentrations of modifier used and different sensitivities to them in various cell types.

In our system the uptake of ^{125}I -labelled poly-(vinylpyrrolidone) is severely inhibited by removal of exogenous Ca^{2+} but can be restored by adding Ca^{2+} back to the system. This is compatible with results obtained by Rabinovitch (1967) using mouse peritoneal macrophages, by Hemmaplardh & Morgan (1977) using reticulocytes, and by Duncan & Lloyd (1978) using rat yolk sacs. These results all suggest the involvement of Ca^{2+} as a messenger and the release of Ca^{2+} during endocytosis from some site within the cell, as has been observed in many secretory systems (Selinger & Naim, 1970). In amoebae, however, pinocytosis is inhibited by 0.5 mM-Ca^{2+} , although a role has been proposed for Ca^{2+} in the control of pinocytic channel formation which involves its translocation from membrane sites into the cell (Josefsson *et al.*, 1975).

The possible involvement of other changes in distribution of ions in the control of endocytosis was studied with ouabain, but the results are inconclusive, although some stimulation of pinocytosis at low concentrations was observed. This is compatible with the biphasic effect of ouabain on pinocytosis in the rat yolk sac (Duncan & Lloyd, 1978).

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