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PHAGOCYTIC ACTIVITY AND HYPERPOLARIZING RESPONSES IN L-STRAIN MOUSE FIBROBLASTS

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SUMMARY

1. Fibroblastic L cells not only respond with a slow hyperpolarizing potential change to a mechanical or electrical stimulus but also show spontaneous, repetitive hyperpolarizations (i.e. membrane potential oscillation).

2. Almost all the cells can actively take up latex beads whose surfaces were treated by U.V. irradiation.

3. Non-phagocytic L cells hardly showed hyperpolarizing responses, while hyperpolarizing responses were obtained in all the phagocytic L cells. The exposure of the cell surface to beads, however, did not trigger the generation of hyperpolarizing responses.

4. Metabolic inhibitors, low temperature and cytochalasin B inhibited both the uptake of beads and the hyperpolarizing responses.

5. Increasing the external concentration of Ca^{2+} induced a remarkable stimulation of the phagocytosis of beads. Mg^{2+} and Ba^{2+}, which inhibited hyperpolarizing responses due to competition for Ca^{2+} sites on the outer surface of the membrane, significantly suppressed the uptake of beads.

6. Verapamil, a Ca^{2+} channel blocker, inhibited not only hyperpolarizing membrane responses but also ingestion of beads.

7. It is concluded that the Ca^{2+} inflow on the hyperpolarizing membrane responses is closely associated with the phagocytic activity in L cells, probably through activation of the microfilament assembly.

INTRODUCTION

L-strain fibroblasts in culture have been found to exhibit spontaneous oscillations of membrane potential between about −15 mV and −40 mV under a certain condition (Okada, Doida, Roy, Tsuchiya, Inouye & Inouye, 1977*a*). In addition to these spontaneous repetitive hyperpolarizations, the cells can respond with hyperpolarizing responses to electrical or mechanical stimuli (Nelson, Peacock & Minna, 1972; Okada *et al.* 1977*a*). These spontaneous and evoked hyperpolarizing responses are known to be caused by an increase in the membrane conductance to K^+ (Nelson *et al.* 1972; Okada, Roy, Tsuchiya, Doida & Inouye, 1977*b*; Roy & Okada, 1978). The K^+ conductance increase appears to result from an increase in the intracellular Ca^{2+}

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concentration (Henkart & Nelson, 1979; Okada, Tsuchiya & Inouye, 1979*a*). Recently, we have found that cytochalasin B and local anesthetics inhibited these hyperpolarizing responses and concomitantly suppressed cell motility (Tsuchiya, Okada & Inouye, 1978). Therefore, it is possible that the hyperpolarizing membrane responses in L cells are associated with cell motility, probably through the activation of cytoskeletal systems which are known to be regulated by cytoplasmic Ca^{2+} ions.

Fibroblastic L cells, a 'facultative' phagocyte (Rabinovitch, 1969), show phagocytic activity (Rabinovitch, 1969; Heine & Schnaitman, 1971; Jones & Hirsch, 1971; Hubbard & Cohn, 1975; Vicker, 1977; Byrne & Moulder, 1978). We also observed vigorous ingestion of cell debris or dead cells by normal L cells or non-dividing giant L cells under time-lapse cinematography (Y. Doida, W. Tsuchiya & Y. Okada, *unpublished*). In macrophages, a 'professional' phagocyte (Rabinovitch, 1969), a similar Ca^{2+}-dependent hyperpolarizing response has been demonstrated (Gallin, Wiederhold, Lipsky & Rosenthal, 1975; Gallin & Gallin, 1977; Dos Reis & Oliveira-Castro, 1977; Dos Reis, Persechini, Ribeiro & Oliveira-Castro, 1979) as well as in Kupffer cells (Y. Okada, Y. Doida & W. Tsuchiya, *unpublished*). Thus, the hyperpolarizing response of L cells seems to be closely related to certain steps in the process involved in phagocytosis. For verification, quantitative observations on phagocytosis with latex beads as well as electrophysiological experiments were performed using L cells. Some of these data have appeared in abstract form (Okada, Tsuchiya, Yano, Hongo, Inouye & Sasaki, 1979*b*).

METHODS

Electrophysiology. The cell culture, micro-electrode preparation and electronic instruments employed in the present electrophysiological study were identical with those described in our previous paper (Okada *et al.* 1977*a*). The method of intracellular Ca^{2+} injection has also been described elsewhere (Okada *et al.* 1979*a*). A monolayer of non-dividing large mouse L cells obtained by X-ray irradiation (1·5 kR) was subjected to electrophysiological studies. Data were collected mainly from 'fried-egg'-shaped cells (see Results).

The control medium was Tris-buffered saline (TBS) containing (mM): NaCl, 143·0; KCl, 4·2; $CaCl_2$, 0·9; $MgCl_2$, 0·5; Tris HCl, 10·0; and mannitol, 20·0 (pH 7·3±0·1) or phosphate-buffered saline (PBS) composed of NaCl, 127·0; KCl 2·7; $CaCl_2$, 0·9; $MgCl_2$, 0·5; Na_2HPO_4, 8·0; KH_2PO_4, 1·5; and mannitol, 20·0 (pH 7·3±0·1).

The experiments were performed usually at 35±2 °C, but the temperature of the cells was reduced to desired levels by halting the circulation of warm water or by circulating ice-cold water, if necessary.

Phagocytosis assay. For the phagocytosis experiments, non-irradiated L cells (10^5/ml.) suspended in Fischer culture medium supplemented with 10% bovine serum were dispensed in Leighton-type tissue culture tubes with a cover-glass on the bottom. The cells were grown for 45–48 hr at 37 °C prior to the phagocytosis experiment and were in the log growing stage during the phagocytosis experiment.

Since polystyrene beads cannot be metabolized and are chemically inert, suspension of latex beads (Difco) of 0·8 μm in diameter which had been dispersed in 10 vol. PBS was employed for the present assays of phagocytosis. Surface modification of latex particles was made by U.V. irradiation or by coating with poly-L-lysine (PLL; Sigma); that is, latex beads suspended in PBS were exposed for 20 hr to U.V. irradiation (100 μW/cm^2) with occasional agitation, or were once suspended in PBS containing 0·1% PLL and then twice washed in PBS.

Latex beads were added to the culture medium in Leighton tubes at a final concentration of 0·8, 1·2 or 2·4 × 10^8/ml., and phagocytosis of beads was permitted to proceed at a selected temperature (usually at 37 °C). At definite time intervals the monolayers on the cover-glasses were agitated in PBS for 10–15 sec to remove non-attached particles. The cells were then fixed in 1·25% glutaraldehyde

in PBS, pH 7·0, for 10 min at 4 °C. After washing the cover-glasses in distilled water three times, the number of beads attached to or ingested in a cell in the interphase was counted under an oil-immersion phase-contrast microscope (1500×). Attachment of beads was limited almost exclusively to cells and attachment to the glass was negligible. Whether the beads were attached or ingested could easily be distinguished by the particle position relative to the cell surface and by colour or refractivity of beads. More than about 200 beads ingested in a cell could not accurately be counted because of formidable aggregation of beads in the cytoplasm. Different areas of the monolayers were surveyed in the counts. Triplicate cover-glasses were run for each treatment on the same day, and the data were collected from closer duplicate cover-glasses.

Preliminary experiments showed that non-dividing large L cells obtained by X-ray irradiation also have a similar phagocytic activity to non-irradiated L cells.

Viability assessment. Viability of cells was assessed by exclusion of an impermeable dye. Erythrosin B (20 mg percent w/v) was employed because this dye has been reported to reflect cell viability better than others in L cells (Phillips & Terryberry, 1957). Viability is expressed by percentage of non-stained cells among 800 cells randomly selected.

Microscopy. Cell morphology and phagocytic activity were examined and photographed by employing a Chiyoda photomicroscope (Type T-2) with a 100× phase objective.

For scanning electron microscopy, a sparse monolayer of the cells on a cover-glass was washed three times in PBS at 37 °C, and fixed with 2·5% glutaraldehyde in 0·1 M-cacodylated buffer with 0·25 M-sucrose at 4 °C for 2 hr. After incubating the cells in fresh cacodylated buffer at 4 °C for 2 hr, the cells were post-fixed with 1% osmium-cacodylated buffer at 4 °C for 1 hr. The cells were dehydrated in ten steps, going from 30% to 100% ethanol and washed twice, for 15 min each, in 100% amyl acetate. After drying by the CO_2 critical-point procedure, the specimens were coated with a thin layer of gold with a vacuum evaporator (Shimadzu IC-50) and were examined under the electron microscope (Shimadzu ASM-SX) at about 45 °C tilt operated at 10 kV.

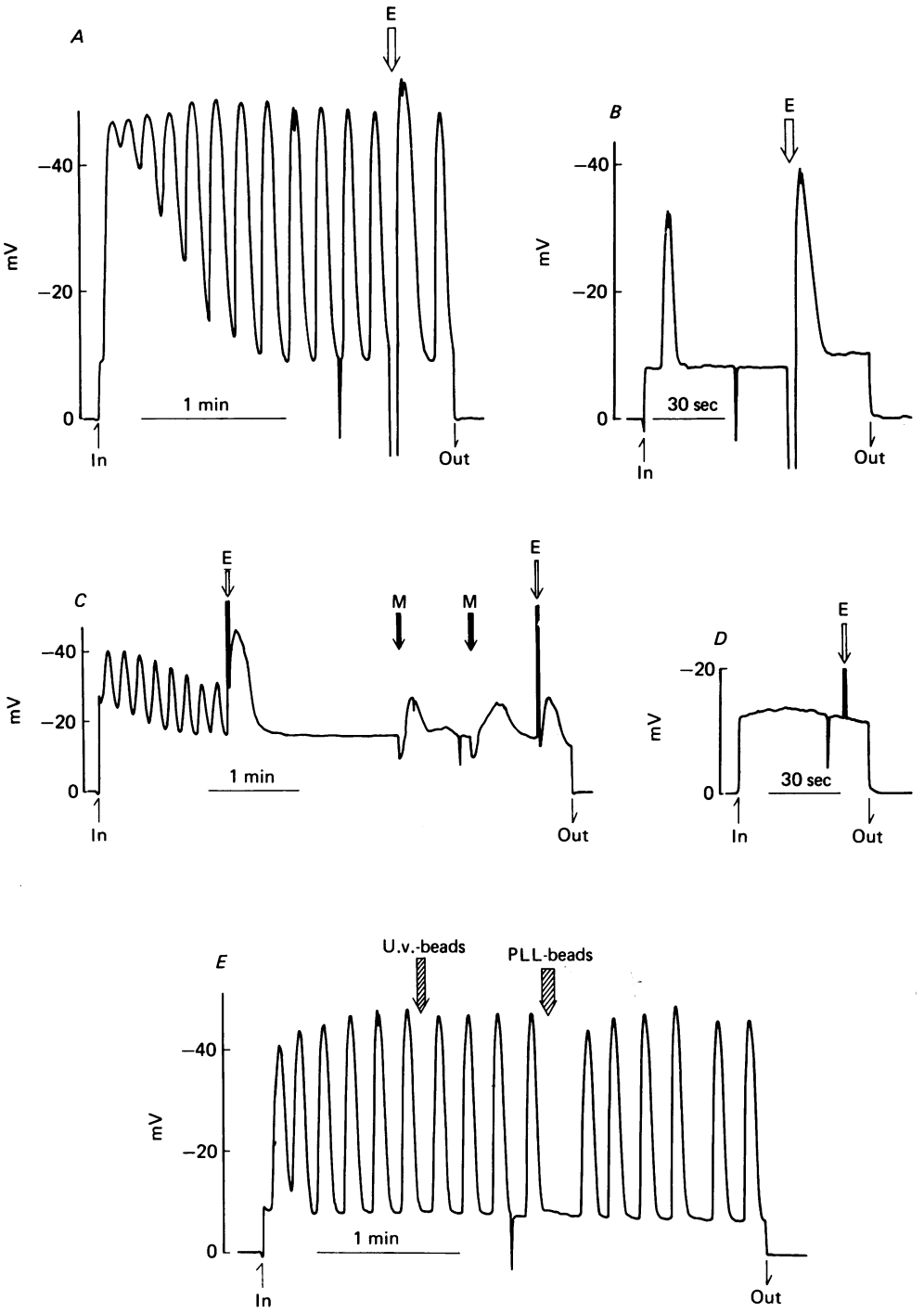
Chemical. EDTA (Na_2 salt) was used as a chelating agent. Ruthenium red was purchased from Chroma-Gesellschaft Schmidt & Co. Verapamil was a gift from Eisai Co. Ethanol was used as a vehicle for the drug. Cytochalasin B was obtained from the Nakarai Chem. Co. The drug was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1 mg/ml. and kept frozen at 4 °C. The addition of ethanol or DMSO alone up to the same dose employed did not affect the electrical and phagocytic properties of L cells. High concentrations of colchicine (Nakarai), vinblastine (Exal®) and metabolic inhibitors (Nakarai) were dissolved in distilled water and kept at −20 °C.

All data are expressed as the mean ± S.E. of mean.

RESULTS

Hyperpolarizing responses. As reported previously (Okada *et al.* 1977*a*), oscillating membrane potentials were obtained in the minority of normal L cells and in most of non-dividing giant L cells under the normal conditions (Fig. 1*A*). In addition to these spontaneous repetitive hyperpolarizations, the cell responded with a single hyperpolarizing response to electrical or mechanical stimuli; that is, a large outward (Fig. 1*A* and *B*) or inward (Fig. 1*C*) current pulse elicited a single hyperpolarizing response, and touching the cell surface with a micropipette also evoked a response (Fig. 1*C*), as reported previously (Nelson *et al.* 1972; Okada *et al.* 1977*a*). Immediately after the micro-electrode impalement, a single hyperpolarizing response thus induced mechanically was sometimes recorded overlapping with intrinsic repetitive hyperpolarizations (Figs. 1*A* and 5*A*).

Typical membrane potential oscillations were most easily recorded from the cells of 'fried-egg' shape, but were rarely recorded from the round or flat cells. The majority of round cells, presumably in mitosis, often responded with a hyperpolarizing membrane response to an electrode penetration or an electrical stimulus without further oscillatory membrane responses, as shown in Fig. 1*B*. Most flat cells showed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an initial hyperpolarizing response immediately after an electrode insertion followed by several abortive responses. Whether the difficulties in obtaining stable oscillations from round or flat cells are due to changes in membrane properties specific to the cell cycle or to the membrane leakage around the micro-electrode has yet to be determined; therefore the relationship between the cell cycle and the hyperpolarizing membrane responses is still under investigation.

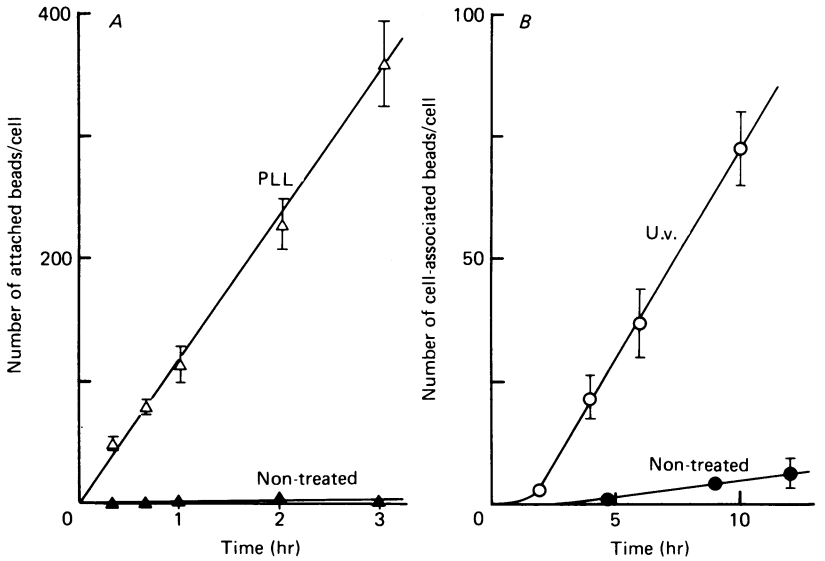


Fig. 2. Time course of phagocytosis of latex beads by monolayer L cells. *A*, comparison between the attachment of PLL-coated beads (△) and that of non-treated beads (▲). The bead concentration in the bulk solution was $2{\cdot}4 \times 10^8$/ml. *B*, comparison between the uptake (attachment plus ingestion) of U.V.-irradiated beads (○) and that of non-treated beads (●). The bead concentration in the bulk solution was $1{\cdot}2 \times 10^8$/ml. Each point represents the mean value of twenty observations with the standard error (vertical bar).

Phagocytosis of latex beads. Although other workers found that L cells cultured in suspension can readily take up latex beads, without surface modification of the beads (Heine & Schnaitman, 1971; Hubbard & Cohn, 1975; Vicker, 1977), few of these non-treated latex beads were taken up by the monolayer L cells cultured on a cover-glass (Fig. 2*A* and *B*).

To stimulate the electrostatic interaction between the beads and the cells, the

Fig. 1. Hyperpolarizing responses in L cells. *A*, stable oscillation of membrane potential composed of spontaneous, repetitive hyperpolarizations recorded in a cell of 'fried-egg' shape. *B*, hyperpolarizing responses obtained after an electrode penetration and after an electrical stimulation (10 nA) at E in a fully round cell. *C*, hyperpolarizing responses occurred spontaneously and were induced electrically (E) as well as mechanically (M). A mechanical stimulation (M) was applied by touching the cell surface with a glass micropipette. *D*, membrane potential recording without hyperpolarizing responses in a non-phagocytic L cell. *E*, effect on the membrane potential (oscillation) of latex beads applied on the cell surface through a glass micropipette. Membrane resistances were monitored by electrotonic potential changes induced by a constant current (0·3 nA) through a recording micro-electrode after compensating for the electrode resistance.

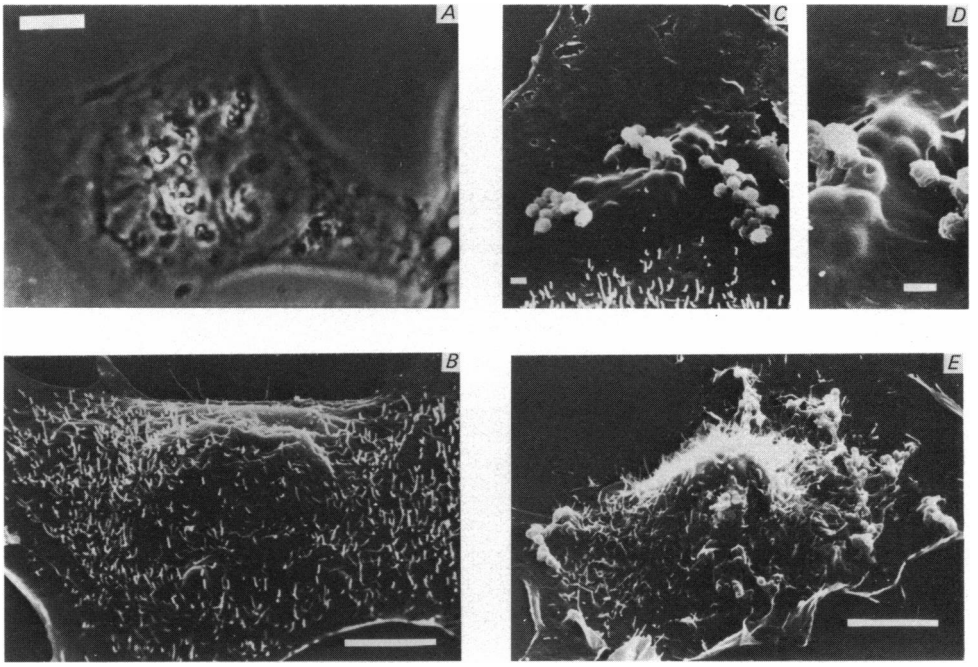
surface of the beads was coated with polycationic PLL, for the cell surface has negative charges. Attachment of these PLL-coated beads to the cell surface occurred immediately after the application, and the number of adsorbed particles increased linearly with time (Fig. 2*A*). Phase microscopy and scanning electron microscopy (Pl. 1*E*) showed that the beads were attached to the cell surface and were not ingested into the cell until 3 hr after the application. After more than 3 hr, the cells exposed to PLL-coated beads were gradually damaged and the viability was rapidly reduced. Therefore, the attachment of phagocytosis was analysed by incubating the cells with PLL-coated beads for less than 2 hr.

The finding that the beads irradiated with U.V. rays can be more vigorously taken up by almost all the L cells was fortuitous. We assume that surface charges are modified by the irradiation, because the effect diminished day by day when the beads were suspended in distilled water in place of PBS. In contrast to PLL-coated beads, a long-term application of U.V.-irradiated beads (even for 20–40 hr) scarcely affected the cell viability. Fig. 2*B* shows that the number of cell-associated (i.e. attached plus ingested) beads was linear between 2 and 10 hr incubation with an initial lag. The presence of such lag in phagocytosis has also been reported by Axline & Reaven (1974) who used macrophages and by Vicker (1977) in L cells. As shown in Pl. 1*A* and *B*, almost all (over 90 %) the cell-associated beads were ingested into the cell in the 10 hr incubation. This incubation time was hence selected for the analyses of phagocytic uptake, using U.V.-irradiated beads. Under the phase microscope, we observed that the application of U.V.-irradiated beads enhanced membrane ruffling (Pl. 1*A*) and the beads were taken up in the peripheral region of the cell, as more clearly seen in a scanning electron micrograph (Pls. 1*C* and *D*), and were then gradually dislocated to the centrosphere region around the nucleus. Incubation of the cells with U.V.-irradiated beads ($1{\cdot}2 \times 10^8$/ml.) for more than 24 hr caused a considerable accumulation of particles inside the cell; for example, approximately 309 beads were taken up by one cell after 28 hr, occupying about 9 % of the entire cell volume.

Phagocytic activity and hyperpolarizing responses. The application of PLL-coated or U.V.-irradiated beads to the cell surface induced no significant changes in the profile

EXPLANATION OF PLATE 1

Micrographs of phagocytic L cells. *A*, phase-microscopic appearance of L cell cultured on a cover-glass 10 hr after incubation with U.V.-irradiated beads ($1{\cdot}2 \times 10^8$/ml.). Ingested beads appear as bright round bodies. Membrane ruffles appear as dark lines. Phase contrast; glutaraldehyde fixation. Bar: 10 μm; *B*, scanning electron microscopic (SEM) appearance of L cell cultured on a cover-glass 10 hr after incubation with U.V.-irradiated beads ($1{\cdot}2 \times 10^8$/ml.). The cell is covered with numerous microvilli. Ingested beads, which had been able to be counted under a phase microscope, are not visible. (A small number of beads loosely attached on the cell surface might be taken away during the preparation procedure for SEM.). Bar: 5 μm; *C*, SEM appearance of L cell in the process of ingesting U.V.-irradiated beads. Bar: 1 μm; *D*, a magnification of *C*. The surface of beads attached on the cell is not smooth because polystyrene is soluble in amyl acetate employed in preparation for SEM. On the other hand, the surface of ingested beads is covered with a smooth and thin enveloping membrane. One bead has a small circular opening at the centre of the enveloping membrane collar. Bar: 1 μm; *E*, SEM appearance of L cell 1 hr after incubation with PLL-coated beads ($2{\cdot}4 \times 10^8$/ml.). Many beads, whose surfaces are, more or less, eroded by amyl acetate, are attached to the cell surface and are linking up. Membrane ruffles are visible near the bottom. Bar: 10 μm.



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of membrane potential in non-dividing large L cells, as shown in Fig. 1*E*, although the attachment of PLL-coated beads started immediately, as was the case in normal L cells (Fig. 2*A*). After 5 hr incubation of non-dividing large L cells with U.V.-irradiated beads, almost all the cells took up a number of beads, as was the case in normal L cells with U.V.-irradiated beads (Fig. 2*B*), but some cells (less than 7%) had neither attached beads on their surfaces nor ingested beads inside. Usually these non-phagocytic large L cells, whose shape was usually very flat, showed no

TABLE 1. Effects of monovalent and divalent cations on phagocytosis

Condition		Viability (%)	Number of beads/cell	Significance of difference (*P*)
Control	—	99·1	44 ± 6·5 (40)	—
+ KCl	30 mM	99·3	44 ± 5·6 (40)	> 0·5
+ NaCl	30 mM	99·0	47 ± 5·0 (40)	> 0·5
+ $CaCl_2$	20 mM	98·0	215* ± 15·7 (40)	< 0·005
+ $SrCl_2$	1 mM	98·5	52 ± 7·6 (40)	> 0·25
+ $MnCl_2$	20 mM	95·1	65 ± 6·4 (40)	< 0·05
+ $MgCl_2$	20 mM	98·5	25 ± 3·9 (40)	< 0·005
+ $BaCl_2$	20 mM	92·3	28 ± 4·4 (40)	< 0·05

The cells were incubated with U.V.-irradiated beads ($1{\cdot}2 \times 10^8$ beads/ml.) for 10 hr. The monovalent and divalent cations were directly added to the culture medium in the Leighton tube. Numbers in parentheses indicate number of cells counted.

* This value could be underestimated because of aggregation of a large number of beads inside the cell.

hyperpolarizing membrane responses (Fig. 1*D*). These results suggest that the hyperpolarizing responses are closely associated with a certain event involved in phagocytosis, but not with the initial attachment.

Effects of cations. The addition of 30 mM-KCl or NaCl to the culture medium had little effect on the phagocytosis of U.V.-irradiated beads by L cells (Table 1), this finding being in good accord with previous observations that monovalent ions appear to have relatively little impact on ingestion (Sbarra, Shirley & Baumstark, 1963; Stossel, Mason, Hartwig & Vaughan, 1972; Stossel, 1973). In contrast to the monovalent cations, divalent cations had a notable influence upon phagocytosis. External high Ca^{2+} markedly enhanced the uptake of U.V.-irradiated beads (Table 1). Such an enhancing effect of Ca^{2+} on ingestion was also found in leukocytes (Greendyke, Brierty & Swisher, 1963; Stossel, Alper & Rosen, 1973) and in macrophages (Rabinovitch, 1967; Mason, Stossel & Vaughan, 1973; Stossel, 1973). Contrary to these 'professional' phagocytes, however, external Mg^{2+} at a high concentration significantly inhibited the uptake of beads in L cells (Table 1). As shown in Fig. 3, both the Ca^{2+} and Mg^{2+} effects were dose-dependent.

In L cells, the external application of an appropriate amount of Ca^{2+}, Sr^{2+} or Mn^{2+} is shown to induce sustained hyperpolarization of the resting potential (Okada *et al.* 1979*a*). However, the addition of Sr^{2+} or Mn^{2+} to the bathing solution did not markedly enhance the phagocytosis, compared with Ca^{2+} (Table 1), although the membrane of phagocytic L cells was significantly hyperpolarized by the application of comparable doses of Sr^{2+} or Mn^{2+}, as seen in Fig. 4. Mg^{2+} and Ba^{2+} suppressed

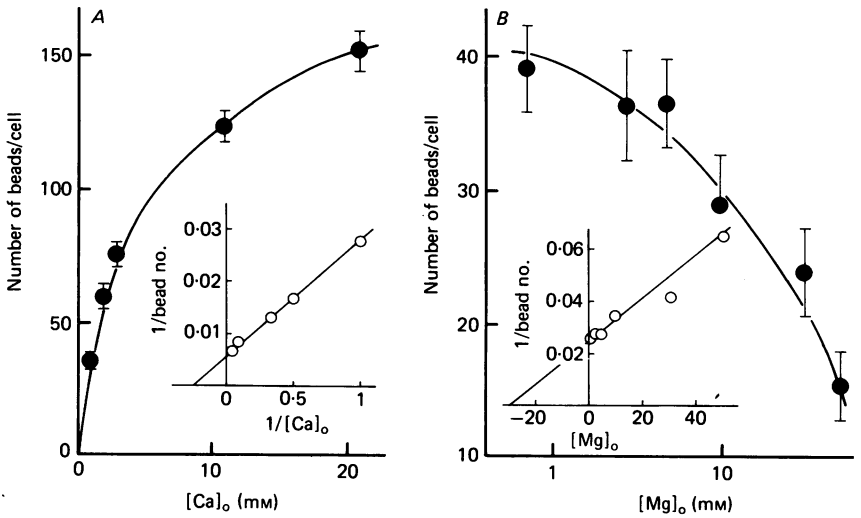

Fig. 3. Effect of Ca^{2+} (*A*) and Mg^{2+} (*B*) added to the external culture medium on the phagocytic uptake of latex beads in L cells. The cells were incubated with U.V.-irradiated beads ($1{\cdot}2 \times 10^8$/ml.) for 10 hr. Each point represents the mean value of twenty (*A*) or forty observations (*B*). The vertical bars represent standard errors on either side of averages. *Insert*. *A*, a double reciprocal plot for the number of cell-associated beads against the extracellular Ca^{2+} concentration ($[Ca]_o$). *B*, a plot for the reciprocal of the number of cell-associated beads against the extracellular Mg^{2+} concentration ($[Mg]_o$).

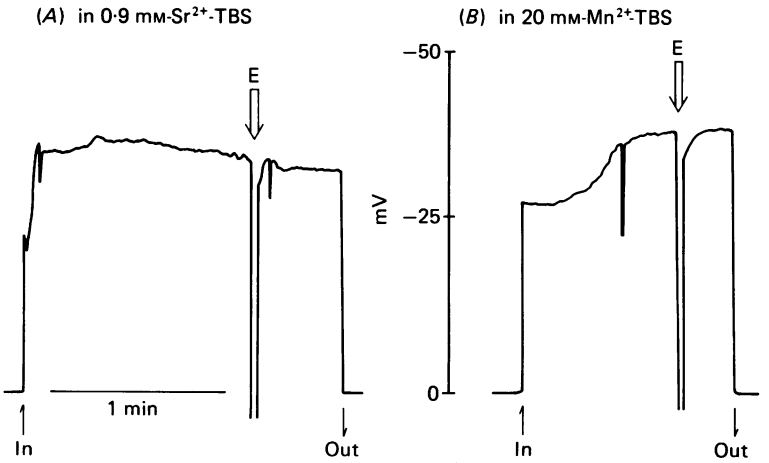

Fig. 4. Effect of 0·9 mM-Sr^{2+} (*A*) and 20 mM-Mn^{2+} (*B*) added to the external medium (TBS) in place of Ca^{2+} on the membrane potential in L cell. Electrotonic potential changes during recording were induced by a current injection (0·3 nA) through a recording micro-electrode after compensating for the electrode resistances to monitor the resistance of the membrane. Electrical stimuli (E) with large currents (10 nA) failed to produce further hyperpolarizations.

significantly the uptake of beads (Table 1). It has been shown (Okada *et al.* 1979*a*) that the external application of Mg^{2+} or Ba^{2+} suppressed the hyperpolarizing membrane responses, probably due to the competitive inhibition for the Ca^{2+} binding site at the outer surface of the cell membrane. Therefore, these effects of divalent cations shown in Fig. 3 and Table 1 could be explained adequately by assuming that the phagocytic activity may be stimulated by an increase in Ca^{2+} influx but not by the membrane hyperpolarization.

Effects of drugs which affect Ca^{2+} transport and cytoskeletal systems. The hyperpolarizing response in L cells appears to be induced by an increase in the concentration of cytoplasmic Ca^{2+} which is mainly derived from the outside of the cell rather than from the internal source (Okada *et al.* 1979*a*). In order to verify this inference, the effect of verapamil, a well known Ca^{2+} channel blocker (Kohlhardt, Bauer, Krause & Fleckenstein, 1972), was examined. As shown in Fig. 5, remarkable inhibition of both spontaneous and induced hyperpolarizing responses occurred within several minutes after the addition of verapamil (0·2–0·8 mM). Such inhibition was reversible if the duration of application did not exceed about 20 min. In the presence of verapamil, the external application of 9 mM-Ca^{2+} failed to produce hyperpolarization of the membrane potential, while intracellular injection of Ca^{2+} was still capable of inducing membrane hyperpolarization (Fig. 5*B*). Thus, it is clear that the drug blocks the Ca^{2+} influx through Ca^{2+} channel without impairing the Ca^{2+}-activated K^+ conductance mechanism. These results also imply that intracellular Ca^{2+} ions responsible for the hyperpolarizing response come mainly from the outside of the cell.

Since the long-term (10 hr) incubation with high doses of verapamil damaged the cells to a certain extent, a relatively low concentration (0·1 mM) of verapamil was employed for phagocytosis experiments. As shown in Table 2, verapamil as well as EDTA and ruthenium red almost completely suppressed the ingestion of beads. Hence, it is obvious that the Ca influx is indispensable not only for the generation of hyperpolarizing responses but also for the ingesting activity seen in the L cells.

In recent investigations (Tsuchiya *et al.* 1978), we found that the hyperpolarizing membrane responses are rapidly inhibited by the external application of cytochalasin B which impairs actin gelation and microfilament function, but not by that of colchicine or vinblastine which depolymerizes cytoplasmic microtubules. A low dose of cytochalasin B (1 μg/ml.) consistently inhibited the uptake of beads by L cells (Table 2), this finding being in good agreement with the observations in macrophages (Klaus, 1973; Axline & Reaven, 1974; Takayama, Katsumato & Takagi, 1975; Munthe-Kaas, 1976) and in polymorphonuclear leukocytes (Allison, Davies & de Petris, 1971; Davis, Estensen & Quie, 1971; Zigmond & Hirsch, 1972; Davies, Fox, Polyzonis, Allison, Phil & Haswell, 1973; Pruzanski & Saito, 1978; Okamura, Ishibashi & Takano, 1979). On the other hand, no significant inhibition was observed with the application of colchicine or vinblastine (Table 2), as in macrophages (Pesanti & Axline, 1975; Takayama *et al.* 1975; Munthe-Kaas, 1976) and in leukocytes (Penny, Galton, Scott & Eisen, 1966; Malawista & Bodel, 1967). These results suggest that the phagocytic process depends upon active movements of the plasma membrane mediated by the actomyosin contractile system which is triggered by an increase in the cytoplasmic Ca level.

Effect of metabolic inhibitors. Phagocytosis is an energy-consuming process (Karnov-

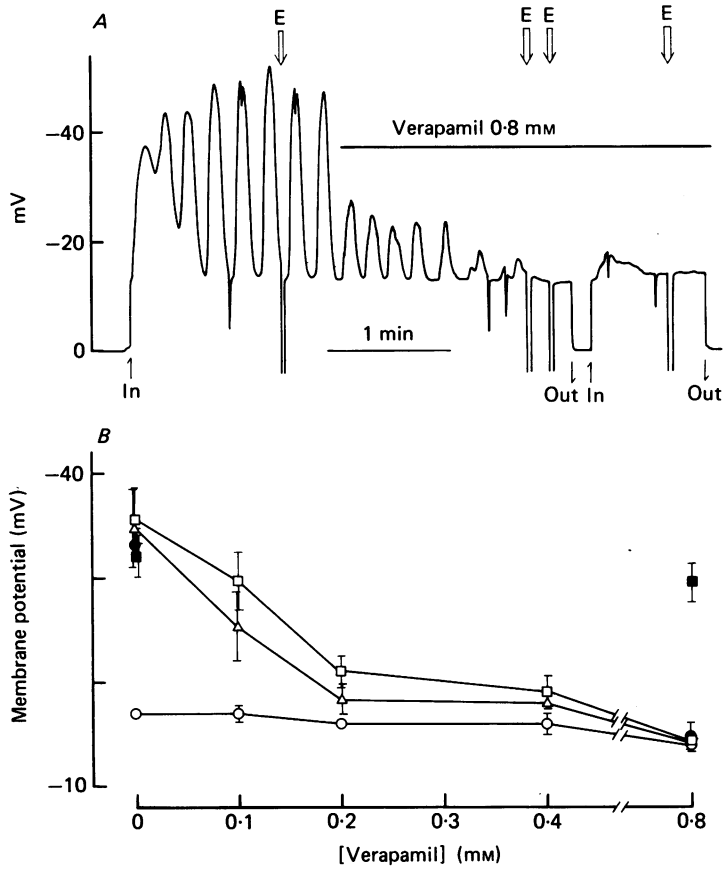

Fig. 5. Effect of verapamil added to the external medium (TBS) on the electrical properties of the cell membrane. *A*, a typical chart of potential recording in the absence and presence of 0·8 mM-verapamil. Membrane resistances were monitored by electrotonic potential changes induced by a constant current (0·3 nA) through a recording micro-electrode after compensating for the electrode resistance. A large current (10 nA) was applied (at E) to examine the response to an electrical stimulation. *B*, effect of verapamil concentrations on the resting potential and hyperpolarizing responses. ○, resting potential in control TBS (0·9 mM-Ca^{2+}); □, peak potential of oscillation in control TBS; △, hyperpolarizing response induced by an electrical stimulus in control TBS; ●, resting potential obtained in high-Ca TBS (10 mM-Ca^{2+}); ■, resting potential obtained in control TBS under the intracellular Ca^{2+} injection. Each point represents the mean value of eight to ninety-one observations with the standard error (vertical bar).

sky, 1962) and dependent upon cellular metabolism, anaerobic glycolysis and/or aerobic oxidation (Stossel, 1973; Silverstein, Steinman & Cohn, 1977). To determine the nature of the main energy source for phagocytosis of L cells, low doses of a variety of metabolic inhibitors were applied to the cells during the exposure to U.V.-treated beads (for 10 hr). The uptake of beads was partially suppressed by cyanide or 2,4-dinitrophenol (DNP), mitochondrial inhibitors, and iodoacetic acid (IAA) or sodium fluoride, glycolysis inhibitors, without affecting cellular viability (Table 3).

A combination of mitochondrial and glycolysis inhibitors causes a greater suppression of the uptake (Table 3). Thus both glycolysis and respiration seem to be involved as the energy source for phagocytosis in L cells. The same conclusion has been reached for phagocytosis in Ehrlich ascites tumour cells (Roberts & Quastel, 1963) and in platelets (Movat, Weiser, Glynn & Mustard, 1965) as well as for pinocytosis in L cells (Steinman, Silver & Cohn, 1974).

TABLE 2. Effects on phagocytosis of drugs which affect the Ca^{2+} transport or the cytoskeletal system

Condition		Viability (%)	Number of beads/cell	*P*
Control	—	99·5	46±4·2 (40)	—
+EDTA	2 mM	99·7	6†±1·6 (40)	< 0·005
+Ruthenium Red	1 mM	n.m.*	2†±0·7 (40)	< 0·005
+Verapamil	0·1 mM	99·2	0·3†±0·2 (40)	< 0·005
+Cytochalasin B	1 μg/ml.	99·0	2†±0·8 (40)	< 0·005
+Colchicine	10 μM	98·7	34±5·6 (40)	> 0·1
+Vinblastine	1 μg/ml.	n.m.*	30±3·3 (40)	> 0·05

The cells were incubated with U.V.-irradiated beads ($1·2 \times 10^8$ beads/ml.) for 10 hr in the absence or presence of the drug. Numbers in parentheses indicate number of cells counted.

* n.m.: cell viability was not measured.

† Almost all the number of beads represents the number of attached beads (but not of ingested).

TABLE 3. Effects of metabolic inhibitors on phagocytosis

Condition		Viability (%)	Number of beads/cell	*P*
Control	—	99·8	48±3·4 (120)	—
DNP	1 μM	n.m.*	27±2·8 (40)	< 0·01
	10 μM	n.m.	26±2·6 (40)	< 0·005
KCN	10 μM	n.m.	22±2·8 (40)	< 0·005
	100 μM	99·7	16±2·7 (40)	< 0·005
IAA	1 μM	99·1	20±2·6 (40)	< 0·005
	10 μM	80·6	12±2·1 (40)	< 0·005
NaF	100 μM	n.m.	26±3·2 (40)	< 0·01
DNP+IAA	1 μM each	99·7	12±3·1 (40)	< 0·005

The cells were incubated with U.V.-irradiated beads ($1·2 \times 10^8$ beads/ml.) for 10 hr in the absence or presence of metabolic inhibitors. Numbers in parentheses indicate number of cells counted.

* n.m.: cell viability was not measured.

Low doses of these metabolic inhibitors did not immediately affect the electrical properties of L cells, whereas high doses rapidly and reversibly suppressed the generation of hyperpolarizing membrane responses (Table 4). Cyanide or IAA applied singly to the bath not only suppressed the amplitude of hyperpolarizing responses but also decreased the number of cells which showed hyperpolarizing responses (i.e. the cell population with hyperpolarizing responses). As previously found (Okada *et al.* 1977*a*), the responses induced by electrical stimuli were more resistant to metabolic inhibitors than those which occurred spontaneously (Table 4). These hyperpolarizing responses were markedly inhibited by a combination of DNP and IAA (Table 4), in a manner similar to that seen in phagocytosis (Table 3).

Effect of temperature. To determine the effects of temperature on the attachment of PLL-coated beads and the uptake (attachment plus ingestion) of U.V.-irradiated beads, the cells were challenged at different temperatures (0–40 °C) during the exposure to beads. Fig. 6*A* shows that the curves for PLL-coated beads and for U.V.-irradiated beads are clearly different; the former was much less sensitive to temperature than the latter, and the latter showed a certain threshold (22 °C) in its

TABLE 4. Effects of metabolic inhibitors on the resting potential and the hyperpolarizing responses

Condition	Number of observation	Resting potential (mV)	Spontaneous repetitive hyperpolarization (mV)	Electrically induced hyperpolarization (mV)
Control	25	−14·9±0·8	−37·8±1·8 (100%)	−39·6±2·1 (100%)
+KCN*	20	−12·1±0·5	−28·0±2·6 (40%)	−25·0±1·9 (80%)
+IAA†	14	−16·6±0·9	−28·2±5·5 (36%)	−29·0±2·7 (93%)
+IAA+DNP‡	9	−12·5±2·0	— (0%)	−21·5±1·0 (33%)

Percentages in parentheses indicate the cell population with hyperpolarizing responses. All data were collected from the cells of 'fried-egg' shape.

* Measured 2–30 min after addition of 1 mM-KCN to the bathing fluid.

† 10–40 min after addition of 0·5 mM-iodoacetic acid (IAA).

‡ 10–30 min after addition of 0·25 mM-iodoacetic acid as well as 0·25 mM-2,4-dinitrophenol.

response to temperature. It is known that particles are not ingested unless the temperature exceeds some critical threshold in LM cells (21 °C; Hoff, Huang, Wisnieski & Fox, 1976) and in macrophages (20 °C; Rabinovitch, 1967). When the logarithms of the number of beads attached or taken up are plotted against the reciprocal of the incubation temperature in degrees Kelvin (Fig. 6*B*), the Arrhenius plots are obtained in which the slopes yield the activation energy (E_a) of attachment or uptake. A distinct inflexion can be seen in the curve for the uptake of U.V.-irradiated beads but not for the attachment of PLL-coated beads. Such an inflexion in the Arrhenius plot has been attributed to lipid phase transitions in the membrane (Wilson & Fox, 1971; Kimelberg & Papahadjopoulos, 1972; Berlin, 1973; Linden, Wright, McConnell & Fox, 1973; Wisnieski, Parkes, Huang & Fox, 1974). Therefore, the results shown in Fig. 6*B* suggest that membrane lipid does play an important role in the ingestion phase (probably on membrane fusion) but not in the attachment phase. Activation energy thus estimated for the uptake of U.V.-irradiated beads was about ten times greater (61 kcal) than that for the attachment of PLL-coated beads (6·3 kcal).

The generation of hyperpolarizing membrane responses was also apparently dependent on the temperature of the incubation medium, as reported previously (Okada *et al.* 1977*a*). The results of a more detailed experiment are depicted in Fig. 7. The cell population with potential oscillations or hyperpolarizing responses induced by electrical stimuli was distinctly decreased by temperatures lower than 15 °C (Fig. 7*A*). At 4–6 °C, a sustained hyperpolarization failed to occur in response to external

high Ca^{2+} (10 mM) as well as to Ca^{2+} injection into the cell (Fig. 7*B*). Therefore, it can be concluded that the temperature dependency of hyperpolarizing responses is due to that of the Ca^{2+}-dependent K^+ conductance mechanism. Moreover, the presence of a distinct inflexion (13–14 °C) in the curve for the cell population with hyperpolarizing responses or for the frequency of potential oscillation (Fig. 7*A*) suggests that such a Ca^{2+}-dependent K^+ conductance mechanism may be K^+ carrier but not a K^+ channel, as previously suggested (Roy & Okada, 1978).

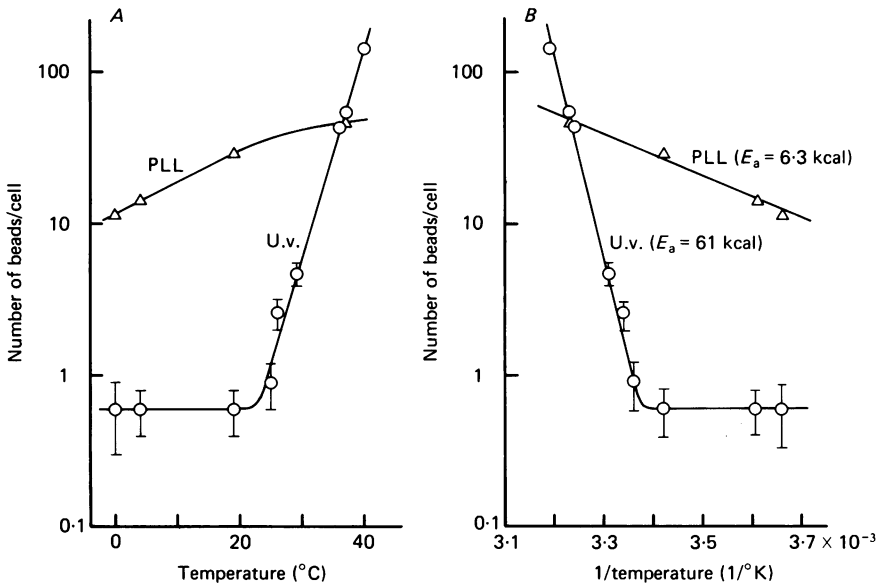

Fig. 6. Effect of temperature on the attachment of PLL-coated beads (△) and on the uptake of U.V.-irradiated beads (○). The cells were incubated with U.V.-irradiated beads ($1{\cdot}2 \times 10^8$/ml.) for 10 hr, or with PLL-coated beads ($1{\cdot}2 \times 10^8$/ml.) for 30 min. It can be seen that there is a distinct inflexion point (about 22 °C) in the curve for U.V.-irradiated beads. E_a: the activation energy. Each point represents the mean value of thirty to sixty observations with the standard error (vertical bar).

Attachment phase and ingestion phase. In an attempt to acquire more information on the different aspects between an attachment phase and an ingestion phase, several additional experiments were performed using PLL-coated and U.V.-irradiated beads. These results are summarized in Table 5. Simultaneous application of DNP and IAA considerably inhibited (by 67 %) the uptake of U.V.-irradiated beads (as shown also in Table 3), while inhibition of the attachment of PLL-coated beads was less marked (by 29 %). This result is in good accord with the temperature effect shown in Fig. 6. Both results provide circumstantial evidence for the view that the ingestion phase of the phagocytic process is more dependent upon temperature and cellular metabolic energy than the attachment phase, although this view cannot, at present, be asserted because no support has been obtained for the assumption that the attachment of PLL-coated beads reflects well the attachment phase for U.V.-irradiated beads. It should be noted that the attachment of PLL-coated beads is also somewhat

dependent on temperature and requires a minute amount of metabolic energy. This finding is consistent with the suggestion by Silverstein *et al.* (1977) that attachment of particles to cell-bearing surface receptors is independent of temperature and metabolic energy, while that of particles for which the cell does not have specific receptors may be slightly temperature dependent.

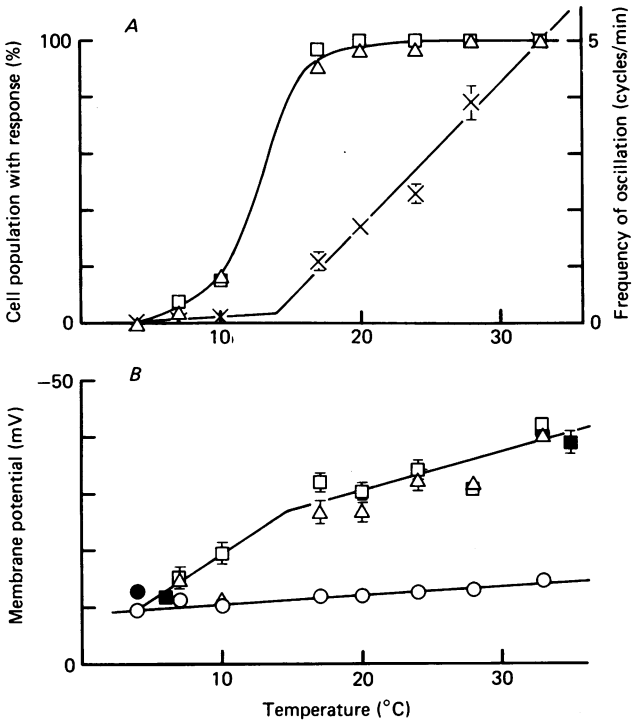

Fig. 7. Effect of temperature on the electrical properties of the cell membrane. *A*, temperature effects on the cell population with oscillations (□) or with hyperpolarizing responses induced by electrical stimuli (△) and on the frequency of oscillations (×). *B*, temperature effects on the magnitudes of resting potentials (○, ●, ■) and hyperpolarising responses (□, △). Symbols are the same as those in Fig. 5. Each point represents the mean value of six to 138 observations with the standard error (vertical bar).

EDTA added to the incubation medium markedly suppressed both the attachment of PLL-coated beads and the uptake of U.V.-irradiated beads (Table 5), so that external divalent cations are essential for both attachment and ingestion phases. High concentrations of external Ca^{2+} markedly enhanced the uptake of U.V.-irradiated beads but significantly suppressed the attachment of PLL-coated beads (Table 5). The suppressing effect of Ca^{2+} on the attachment of particles coated with polycations can be explained by the electrostatic interaction among the divalent cations, the negatively charged surface of the cells and the positively charged surface of the beads, because the same dose of Mg^{2+} also suppressed the attachment (data not shown). The enhancing effect of Ca^{2+} on the uptake of U.V.-irradiated beads might also be explained by the non-specific electrostatic interaction among the divalent cations and

both negatively charged surfaces of the polystyrene beads (Van den Hul & Vanderhoff, 1968) and the cells. The same dose of Mg^{2+} or Ba^{2+}, however, diminished the uptake of the beads (Table 1); therefore such a Ca^{2+} effect on the uptake of U.V.-irradiated beads cannot be accounted for by such a physical role, but rather by physiological roles of Ca^{2+}.

TABLE 5. Comparison between attachment phase and ingestion phase

Condition		Number of beads/cell	
		PLL-coated	U.V.-irradiated
Control		251 ± 34·2 (20)	49 ± 3·7 (40)
+ DNP & IAA	1 μM each	177 ± 18·5† (20)	16 ± 2·2† (40)
+ EDTA	2 mM	30 ± 8·4† (20)	6 ± 1·6† (40)*
+ $CaCl_2$	20 mM	83 ± 12·8† (20)	352‡ ± 14·7† (20)
+ Cytochalasin B	1 μg/ml.	207 ± 28·2 (20)	2 ± 0·8† (40)*
+ Colchicine	10 μM	241 ± 23·8 (20)	34 ± 5·6 (40)*

The cells were incubated with PLL-coated beads ($0·8 \times 10^8$ beads/ml.) for 2 hr or with U.V.-irradiated beads ($1·2 \times 10^8$ beads/ml.) for 10 hr.

* The data from Table 2.

† Significantly different from the control with $P < 0·025$.

‡ This value could be underestimated because of aggregation of a large number of beads inside the cell.

Cholchicine neither significantly affected the attachment of PLL-coated beads nor the uptake of U.V.-irradiated beads (Table 5). Cytochalasin B did not significantly affect the attachment of PLL-coated beads while the drug remarkably suppressed the uptake of U.V.-irradiated beads (Table 5). This result is in good agreement with the observation of dissociated effects of cytochalasin B on the attachment phase and on the ingestion phase in polymorphonuclear leukocytes (Davies *et al.* 1973) and in macrophages (Allison *et al.* 1971; Axline & Reaven, 1974; Munthe-Kaas, 1976), and suggests the essential role of microfilaments in the ingestion phase but not in the attachment phase.

DISCUSSION

Phagocytosis of particles by cells can be, in general, divided into two steps: attachment of the particle to the cell membrane and ingestion of the particle into the cytoplasm (Rabinovitch, 1967; Stossel, 1975; Jones, 1975). The present study using PLL-coated and U.V.-irradiated beads suggests that the ingestion phase is more dependent upon metabolic energy, microfilaments and Ca^{2+} than the attachment phase.

Hyperpolarizing membrane responses in L cells are also highly dependent on metabolic energy (Table 4, Fig. 7) and are inhibited by cytochalasin B (Tsuchiya *et al.* 1978). The generation of the hyperpolarizing responses is mediated by the Ca^{2+}-dependent K^+ conductance in L cells (Henkart & Nelson, 1979; Okada *et al.* 1979*a*) and in macrophages (Gallin *et al.* 1975). Therefore, it seems likely that the hyperpolarizing responses are closely related to the ingestion phase but not to the attachment phase of phagocytosis. This is also supported by the observations that non-phagocytic L cells lack the hyperpolarizing response (Fig. 1*D*) and that the

application of beads on the cell surface did not alter the membrane potential or the pre-existing hyperpolarizing response (Fig. 1*E*).

The addition of 30 mM-K^+, which is known to induce remarkable decreases in the amplitude of hyperpolarizing responses (up to about 20 mV; Okada *et al.* 1977*b*), had little influence on the phagocytosis of beads (Table 1). The addition of 1 mM-Sr^{2+} or 20 mM-Mn^{2+}, which produced significant hyperpolarization of the cell membrane (Fig. 4), did not stimulate the uptake of beads so markedly, in contrast to the notable stimulation by Ca^{2+} (Table 1). Furthermore, the transition temperature for the generation of hyperpolarizing responses (13°–14 °C) was different from that for the ingestion of the beads (22 °C). These observations indicate that the membrane potential level *per se* is not directly related to phagocytic activity, while the latter is associated with some event involved in the generation of the hyperpolarizing response.

Based on the findings that the hyperpolarizing responses are inhibited by the deprivation of external Ca^{2+}, by the addition of appropriate doses of Mg^{2+}, Ba^{2+} or La^{3+} and by the external application of ruthenium red, it has been suggested that hyperpolarizing responses are triggered by the Ca^{2+} entry across the cell membrane (Okada *et al.* 1979*a*; Okada & Tsuchiya, 1980). Henkart & Nelson (1979) have suggested that the primary source of the Ca^{2+} mediating the hyperpolarizing response is intracellular. If this were the case, the application of metabolic inhibitors would be expected to produce membrane hyperpolarization, as a result of the release of Ca^{2+} from the intracellular stores. However, the present investigation showed that the addition of metabolic inhibitors did not hyperpolarize the membrane but rather suppressed the hyperpolarizing responses (Table 4). In addition, our preliminary study with X-ray microprobe analysis failed to show any evidence for intracellular stores of Ca (Okada, Tsuchiya, Yawo, Yada, Sasaki, Nakagaki & Imai, 1980). Furthermore, the present study with verapamil (Fig. 5) demonstrates that Ca^{2+} mediating the hyperpolarizing response is external in origin. Similarly, D-600 and nifedipine, which are known to be Ca^{2+}-channel blockers, inhibit the generation of hyperpolarizing responses (Okada *et al.* 1980).

Hence, it seems feasible that the Ca^{2+} inflow induces the hyperpolarizing response on one hand and on the other hand is closely associated with the ingestion phase of phagocytosis in L cells. If the phagocytic activity is assumed to be linearly dependent upon the concentration of calcium bound to the membrane, the data shown in Fig. 3 can be quantitatively explained by the Ca^{2+} channel model proposed in muscle fibres by Hagiwara (1975). The Ca^{2+} channel may have a site X which is in either the free site (X) or the bound site (CaX) when Ca^{2+} ions penetrate the channel and Mg^{2+} ions are impermeable through this channel competing with Ca^{2+} for the site X. Thus, the CaX concentration ([CaX]) within the membrane can be given by

$$[\mathrm{CaX}] = \frac{[\mathrm{X}]_t}{1 + (1 + [\mathrm{Mg}]_o / k_{\mathrm{Mg}})\, k_{\mathrm{Ca}} / [\mathrm{Ca}]_o},$$

where $[X]_t$ is the total concentration of X within the membrane, and k represents the dissociation constant. The predictions derived from this equation fit the relationships between the number of phagocytized beads and the external Ca^{2+} or Mg^{2+} concentration ($[Ca]_o$ or $[Mg]_o$) as depicted in inserts in Figs. 3*A* and *B*, with $k_{Ca} = 3{\cdot}9$ mM and $k_{Mg} = 23$ mM.

The ingestion process of particle phagocytosis depends upon the integrity of cytoplasmic microfilament (Reaven & Axline, 1973). The present experiment with cytochalasin B supports this notion (Tables 2 and 5). Thus, it may be conceded that the Ca^{2+} entry on the hyperpolarizing membrane response is associated with phagocytic activity through the activation of the microfilament system which is regulated by Ca^{2+} ions.

Similar hyperpolarizing membrane responses or potential oscillations have been found in other cells which have more or less endocytic activity; for example, in macrophages (Gallin *et al.* 1975; Gallin & Gallin, 1977; Dos Reis & Oliveira-Castro, 1977; Dos Reis *et al.* 1979), in rat Kupffer cells (Y. Okada, Y. Doida & W. Tsuchiya, unpublished), in megakaryocytes (Miller, Sheridan & White, 1978), in human syncytiotrophoblasts (J. Yano, Y. Okada & W. Tsuchiya, unpublished), in HeLa cells (K. Izutsu, personal communication; D. Hülser, personal communication) in BICR/M1R-K cells (D. Hülser, personal communication) and in rat thymocytes (Y. Okada, unpublished). Membrane potential responses associated with Ca^{2+}-dependent exocytosis have been also reported in pancreatic aciner cells (Kanno, 1972; Matthews & Petersen, 1973; Petersen & Ueda, 1976). There is, therefore, a possibility that some event associated with such membrane responses with slow potential changes plays an essential role in endocytosis or exocytosis which is dependent on intracellular calcium ions as well as on cytoskeletal microfilaments. Supporting this concept, we have recently found that L cells show sustained hyperpolarization in response to the external application of low density lipoprotein (LDL) followed by receptor-mediated pinocytosis of LDL (Tsuchiya, Okada & Yada, 1979).

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