ATP Induces Large Channel Endocytosis with Concomitant Increase in Cell Density

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Summary: Large channel endocytosis is considered to be the characteristic of specialized endocytic cells like macrophages and phagocytes while small pit endocytosis involving clathrin protein coatings are the membrane recycling macromolecular pathways for most eukaryotic cells. We show here that extracellular ATP induced cells to internalize their plasma membrane by large channel endocytosis. In the process of plasma membrane internalization, flat protracted cells round up and become easily detachable from the substrate. Scanning transmission ion microscopy (STIM) revealed an increase in cell density as the ATP treated cells assumed a rounded morphology. The increase in cell density could be attributed to endocytic internalization of cell membrane and debris.

Materials and Methods

Cell cultures

Human Chang liver cells (American Type Culture Collection, ATCC CCL 13) were cultured in Dulbecco's modified Eagles medium (DMEM, Sigma, USA) supplemented with 10% foetal bovine serum (Cytosystems, Australia) and 10% CO_2 in air. Cells were seeded at 3 \times 10^5 cells per 25 cm² culture flask (Costar, USA) by the Filamatic dispenser (National Instrument, USA) as previously described (Sit et al., 1991a). For transmission electron microscopy, cells were grown on teflon coated coverglass slips. For scanning transmission ion microscopy, cells were seeded in foetal bovine serum on Pioloform (Agar Scientific, UK) substrate and incubated for 1 hour before flooding with DMEM containing 10% foetal bovine serum.

Cell rounding in Chang liver cells with ATP incubation

For ATP induced rounding, parallel 25 cm² cultures were rinsed with 2 \times 10 ml Na⁺-HEPES buffer (140 mM NaCl, 10 mM HEPES (Sigma) adjusted to pH 7.4 with NaOH) and overlaid with 2 ml Na⁺-HEPES buffer containing 7 mM ATP (adenosine 5'-triphosphate, sodium salt, Sigma). Cultures were

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incubated at 37°C for the required duration in time response, otherwise for 15 minutes.

Quantimetr image analysis of profile area and perimeter: ATP time response
Cells were fixed after 0, 4, 8, 15 min 7 mM ATP incubation. Fixation was with NAMETCA (Na+-methanol-TCA; 140 mM NaCl in 70% methanol acidified with 3% trichloroacetic acid) and staining was with Giemsa's stain [Gurr's improved R66 (BDH, UK)] as previously described (Sit & Wong, 1989). Quantitations were done in the Quantimetr 570 image analysis system (Leica, Cambridge, USA). Slides were examined in a Nikon microscope on line with the image analysis system. Cells were detected via 256 grey level discrimination and cursor control in a 262, 144 pixel colour screen interface. The profile area and perimeter were measured using the QUIPS programme.

Transmission electron microscopy
After 1 minute incubation in 7 mM ATP at 37°C, cells were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) followed by 2% osmium

Fig. 1. Chang liver cells incubated for 1 minute in 7 mM ATP. Composite picture. Deep surface invaginations containing extracellular fibrils and ruffles (arrow heads). Fluffy extracellular fibrils in endocytic vacuoles (double arrowheads) are seen in (A). The long endocytic channel in (C) contain a rounded bleb in its depth. Bar = 1 μm.
tetroxide in the same buffer. Details are as previously described (Sit et al., 1991b). Examination was done in a JEOL 1200EX.

**Scanning transmission ion microscopy (STIM)**

The experimental and control cells were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer before rinsing with type I reagent water and dipping in liquid nitrogen. The specimens were then freeze dried before viewing with the Nuclear Microscope Facility at the National University of Singapore which uses a 2 million electron-volt (2 MeV) protons from a small nuclear accelerator (HVEC AN2500 Van de Graaf accelerator) as previously described (Sit et al., 1994b). The density profile and shape of individual cells were detected from transmitted protons.

**Results and Discussion**

It is well known that extracellular ATP exerts a wide variety of effects on cells and tissues. Amongst others, ATP is known to be a signal molecule that triggers intracellular Ca\(^{2+}\) release which in turn modify numerous cellular functions (Dubyak et al., 1988; Sit et al., 1992b; Gosink & Forsberg, 1993). One such effect of extracellular ATP is the induction of rapid cell rounding (Sit et al., 1992a), a process which is important in the cancer metastatic cascade (Juliano, 1987). We have demonstrated that 2,000,000 mol. wt dextran particles as well as 0.92 \(\mu\)m and 2.17 \(\mu\)m fluoresceinated latex beads could be easily internalized with extracellular ATP inductions and postulated that the vacuolar distribution of the internalized material suggested endocytic entry (Sit et al., 1994a).

In this study, we verify that large endocytic channels which contained extracellular ruffles and fibrils, were detected under transmission electron microscopy within a minute of incubation with 7 mM ATP (Fig. 1). The large channels seen in ATP induced cells appeared similar to those reported in antiporter mediated endocytosis due to intracellular alkalinization from downgradient Na\(^+\) and H\(^+\) exchanges in cells incubated with bicarbonate saline buffer (Sit et al., 1990). Concomitant with massive internalization of plasma membrane, there was a drastic reduction of the cell surface area. Fig. 2 and 3 show respectively the profile area (\(p = <1e-6\)) and perimeter (\(p = <1e-6\)) of Chang liver cells decreasing significantly with time when incubated with 7 mM ATP. At the end of 15 minutes the cells were transformed from a flat protracted state (Fig. 4) to globular in shape (Fig. 5). Cell density in ATP treated cells increased simultaneously with cell rounding (Fig. 4 cf 5). STIM outlines the shape of the cells by detection of transmitted protons and measures total cell density as an absolute parameter whereas conventional optical methods measure only relative buoyant density. Increase in cell density observed in ATP treated cells could be ascribed to endocytic internalization of cell membrane and debris. The increased cell density also provides corroborative evidence that such an endocytic phenomenon exists.

For more than a decade, there has been irrefutable evidence that clathrin-coated vesicles mediate the entry of receptors in the plasma membrane and their bound ligands (Goldstein et al., 1985, Watts and Marsh, 1992). However, studies have established that hypertonic medium, potassium depletion or mild cytosolic acidification removes or paralyses the clathrin lattice but only partially inhibits fluid-phase endocytosis (Larkin et al., 1983; Daukas & Zigmond, 1985; Hauser & Anderson, 1989; Heuser, 1989). Furthermore, many studies of membrane protein
turnover have focused on receptors, which comprise only 10% of all membrane plasma proteins (Hubbard, 1989). How then, do the other 90% of non-receptor plasma proteins enter the cell? The search for clathrin independent pathways have yielded three main pathways viz. (a) small (50–100 nm) “non coated” caveolae, (b) surface invaginations (150–300 nm) and (c) membrane ruffles (Orci et al., 1978; Bor-Sagi & Feramisco, 1986; Tran et al., 1987; Sandvig et al., 1987). There now appears to be contradictory evidence as to the role of caveolae in endocytosis (van Deurs et al., 1993). Non-clathrin large endocytic channels which we have demonstrated, appear to be an attractive candidate as an alternate endocytic portal for entry of macromolecules into the cell.

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