Extracellular ATP Induces Rapid Cell Rounding in Cultured Human Chang Liver Cells

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Summary Extracellular ATP ($7\ \mu\text{M}$ – $14\ \text{mM}$) induced monolayer Chang liver cells (ATCC CCL 13) to retract and round up in dose-dependent and time-dependent manners. ATP-induced rounding was concomitant with raised intracellular pH (7.3 to 7.8) and suppressed (a) in sodium-free medium (250 mM sucrose), (b) with the addition of 1 mM quinidine, an inhibitor of Na^+/H^+ exchanges, and (c) with the addition of 10 nM staurosporine, a potent inhibitor of protein kinase C that is known to activate Na^+/H^+ exchanges. These findings suggest that ATP-induced cell rounding is due to activation of Na^+/H^+ antiporters.

Key words: ATP, cell rounding, intracellular pH, sodium/proton antiporter.

Activation of the Na^+/H^+ antiport by (a) motivation of downgradient exchanges [1, 2], (b) phosphoinositide (PI) signal transduction with sulphate [3], or (c) a combination of both mechanisms [4, 5], produces a cell retraction and rounding phenomenon concomitantly with a distinctive endocytosis [6]. Apparently, tumorigenic transformation of normal fibroblasts [7] and shape change in malignant melanoma cells with phorbol ester treatment, are associated with alkalization [8] as well.

We now show that extracellular ATP, an established first messenger in phosphoinositide (PI) signal transduction [9, 10] induces monolayer cells to retract from anchorage and round up with concomitant intracellular alkalization.

Monolayer cultures of human Chang liver cells (ATCC CCL 13) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal calf serum (Cytosystems). Parallel cultures were seeded in $25\ \text{cm}^2$ flasks (Costar) at 3×10^5 cells/flask from stirred stock suspension.

Parallel monolayer cultures were washed with $2 \times 10\ \text{ml}$ (a) Na^+ -HEPES buffer (NaCl 120 mM and 10 mM n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid (HEPES, Sigma), adjusted to pH 7.2 with NaOH) or (b) 250 mM sucrose

(saccharose, Extra Pure, Merck) and induced to round up by incubation at 37°C (non-gassed) with another 1 ml of respective buffer containing varying concentrations (7 μ M to 14 mM) of ATP (adenosine 5'-triphosphate, free acid, Sigma) for 15 min or varying times. In some experiments, 10 nM staurosporine (AM-2282, mol. wt = 466.5, Sigma, stock solution is 100 μ M prepared in 1:1 H₂O:DMSO) a potent inhibitor of phospholipid/Ca²⁺-dependent protein kinase (protein kinase C) [11], or 1 mM quinidine hydrochloride monohydrate (mol. wt = 378.9, Sigma, prepared in 100 mM stock solution in DMSO), an inhibitor of Na⁺/H⁺ exchanges [12], were added with ATP. To evaluate whether raised cytosolic Ca²⁺ *per se* mediates cell retraction and rounding, monolayer cultures were incubated in the presence of various concentrations of CaCl₂ in Na⁺-HEPES buffer containing 1 μ M of rapid-acting 4-bromo-calcium ionophore A23187, B-7272 (Sigma) prepared as 1 mM stock in 1:1 H₂O:DMSO. Cell profile area and perimeter were quantitated using a Reichert-Jung Univar microspectrophotometer as previously described [1]. Roundness form factor was estimated by the following equation: $4\pi \times (\text{profile area}) / (\text{profile perimeter})^2$. To evaluate cell viability, cells were rinsed in Na⁺-HEPES buffer and incubated for 7 min at 37°C with 0.25% trypsin (Gibco) and cell suspensions were stained with 0.1% trypan blue in PBS (Gurr) for 10 min. The stained cell proportion was counted in an Improved Neubauer chamber. For intracellular pH (pH_i) measurements, cells were first loaded with BCECF-AM (2'-7'-bis-(2-carboxyethyl)-(and-6) carboxyfluorescein, acetoxymethyl ester, mol. wt = 688, Molecular Probes, Oregon) by incubating at 37°C in DMEM with 10 μ M of the dye for 30 min. The fluorescence change on incubation with 7 mM ATP in Na⁺-HEPES buffer was recorded in the Shimadzu RF5000 recording spectrophotometer which allowed overlay tracing. Protocol was as previously described [1].

Figure 1 shows effects of ATP on (a) cell profile area, (b) perimeter, and (c) roundness. ATP-induced cell retraction and rounding in (A) dose-dependent and (B) time-dependent manners in the presence of Na⁺. The minimum effective dose and half-maximal dose were 7 and 700 μ M, respectively. These effects were markedly suppressed by removal of extracellular Na⁺ as demonstrated in Fig. 1A. Even at minimal-effective ATP dosages (7 to 70 μ M range), incubation in Na⁺-free medium shows significant suppression of the retraction and rounding responses (Fig. 2).

The addition of quinidine, a Na⁺/H⁺ antiporter inhibitor [12], inhibited the cell retraction and rounding responses due to added ATP (Fig. 1B). Cytotoxicity is not the cause since the dead cell indices (trypan blue dye inclusion) with and without added ATP in the incubation medium are similar at 3.2–3.3%. Inhibiting Na⁺/H⁺ exchanges therefore seems correlated with the inhibition of cell retraction responses due to added ATP. In fact, in the presence of Na⁺ (with Na⁺-HEPES), ATP causes marked pH_i elevation (a) from 7.32 (± 0.02) to 7.84 (± 0.07) in 3 separate measurements, at maximal level of stimulation using 7 mM ATP, and (b) from 7.32 (± 0.02) to 7.60 (± 0.05) in 3 separate measurements, at half-maximal level of stimulation using 0.7 mM ATP (Fig. 3). ATP cannot induce pH_i elevation

Fig. 1. Cell retraction and rounding in ATP dose (A) and time (B) responses. In 1A-(a), sodium-free sucrose medium (dotted line, filled-triangle symbols), retraction is practically abolished. In 1B, the addition of 1 mM quinidine, a Na^+/H^+ exchange inhibitor (dashed line, square symbols), and 10 mM staurosporine, a potent inhibitor of protein kinase C (dotted line, triangle symbols) cause obvious suppression of the cell retraction and rounding phenomena. The lines in 1B-(a) and 1B-(b) are bi-exponential regressions. Error bar = 2 SE (95% confidence limits). As shown by the uninhibited, solid lines in 1A-(c) and 1B-(c), the cell approaches complete roundness with ATP stimulation in both dose and time responses. The inhibited, i.e., dotted and dashed, lines (in sodium-free medium, and with either quinidine or staurosporine) are at a much lower level.

Fig. 2. Cell retraction and rounding with 0.007 and 0.07 mM ATP concentrations in sodium-free medium (250 mM sucrose) and in Na⁺-HEPES buffer. While there are marked differential changes in cell area (a) and perimeter (b) (retraction phenomena) between sodium-free and Na⁺-HEPES buffered reactions, the rounding responses (c) are not dramatic. These reactions represent the minimal effective level of ATP-induced rounding.

in Chang cells when incubated in sodium-free medium whether it were isotonic 250 mM sucrose or hypertonic 600 mM sucrose (traces not shown).

In many cell types, the functional effects elicited by exposure to extracellular ATP can be correlated with intracellular mobilization of Ca²⁺ from stores and phosphoinositide (PI) hydrolysis that should cause protein kinase C activation, notwithstanding the possibility of feedback control [9, 10, 13–17]. Therefore, there is a possibility that intracellular Ca²⁺ mobilization is a causal factor for morphological changes in response to ATP. However, replacing the added ATP with 1 μM rapid-acting brominated A23187 calcium ionophore in the Na⁺-HEPES incubation medium that contains respectively 1, 5, 10, 50, and 100 mM CaCl₂ produced absolutely no hint of any retraction response or rounding response even after 30 min incubation. Apart from intracellular mobilization, extracellular Ca²⁺ can enter the cytosol via 3 mechanisms in the plasma membrane, viz., the Ca²⁺ channel, the Ca²⁺-ATPase pump and the Ca⁺/Na⁺ exchanger [18]. The ATP-induced rounding (7 μM–14 mM) response was rather abolished by increasing the extracellular Ca²⁺ level to 5 mM (data not shown). This observation corroborates our previous findings about the anti-rounding effect of added Ca²⁺ on antiporter-mediated cell retraction and rounding motivated via high [Na⁺]_o/[Na⁺]_i and [H⁺]_i/[H⁺]_o gradients imposed across the plasma membrane [1]. However, despite the observation that raised cytosolic Ca²⁺ *per se* did not appear to be the causation of the rounding response, an activation of the phospholipid / Ca²⁺-dependent protein kinase (protein kinase C) [13] seems vital. This is seen from the fact that significant blocking of the rounding response was achieved with just 10 nM staurosporine, a protein kinase C inhibitor [11, 19] (Fig. 1B). Cytotoxicity was not the cause of the inhibition because the dead cell index from trypan blue dye inclusion in cultures exposed to even 100 nM staurosporine (10 times more than what we used

Fig. 3A

Fig. 3. Intracellular pH in ATP-induced rounding. 3A shows overlay tracings of consecutive experiments. ATP is applied at 0 time (arrow). The "calibration" plot is the average of individual calibrations. From the calibration equation pH values from individual RFU (relative fluorescence unit) values of points in each trace are obtained and plotted in 3B (see overleaf).

Fig. 3B

in our experiments) was similar ($\approx 4.5\%$) to that without added staurosporine. Definite suppression of cell retraction and rounding was observed at just 2 min exposure to 10 nM staurosporine. At 95% confidence limits, the twice standard error bars between the solid line (ATP without staurosporine) and the dotted line (ATP with 10 nM staurosporine) do not overlap at all incubation times greater than zero in Fig. 1B.

Thus the ATP-induced rounding response is blocked (a) in the presence of antiporter blocker quinidine, (b) when Na^+/H^+ exchanges are inhibited without available Na^+ in sodium-free incubations, and (c) when the allosteric activator of Na^+/H^+ exchanges, protein kinase C, is blocked by staurosporine. Taken together with the production of raised pH_i in the rounding incubations, a correlation with raised antiporter activity seems suggested.

Cell detachment is a necessary prerequisite in developmental and metastatic cell movements [20]. While there appears various means of producing rounding and cell detachment, viz., (a) competitive inhibition using RGD (Arg-Gly-Asp) peptides [21] in RGD-directed integrin-fibronectin interactions, (b) inducing the expression of transformation-associated proteases that attack fibronectin-related contact sites [22], (c) adhesion downregulatory signals postulated in EGF-induced rounding of A431 human epidermoid carcinoma cells [23, 24], (d) activation of protein kinase C by phorbol ester in Cloudman melanoma cells [8], (e) forskolin and cyclic AMP induction of cell rounding [25, 26], and (f) expression of a yeast proton pump that also causes cell transformation [7], nevertheless, given the documented presence of the antiporter in virtually all vertebrate cells [27, 28] and its involvement in cell rounding from several corroborative lines of evidence [1–6, 29, 30], pH_i regulation [31, 32] seems to have a cell detachment function or at least

a permissive one.

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