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Role of the Purinergic P2Z Receptor in Spontaneous Cell Death in J774 Macrophage Cultures[1]

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J774 mouse macrophages express an ionotropic receptor gated by extracellular ATP. Activation of this receptor, currently named purinergic P2Z, causes transmembrane ion fluxes, plasma membrane depolarization, cell swelling and eventual cell death. The physiological role of this receptor is as yet unknown. In the present report we show that macrophage cell clones that hypo-express the P2Z receptor showed a very low degree of spontaneous cell death in culture, while hyper-expressing clones were exceedingly susceptible to cell death. To further support a role for ATP receptors in spontaneous cell death, addition to the macrophage cell cultures of oxidized ATP, a selective inhibitor of ionotropic purinergic receptors, or the ATP-hydrolysing enzyme apyrase, also reduced spontaneous death.

Macrophage cells are known to express at least two P2 purinergic receptor subtypes: metabotropic P2Y and ionotropic P2Z (1, 2). Metabotropic P2Y receptors are activated by UTP and ADP besides ATP, and are coupled to G-protein, inositol 1, 4, 5-trisphosphate generation and mobilization of intracellular Ca^{2+}. On the contrary, the P2Z receptor is selectively activated by ATP, while other naturally occurring nucleotides are inactive. This receptor is thought to consist of a channel/pore that allows transmembrane fluxes of ions and small hydrophylic molecules of MW up to 900 Da (3).

The possible physiological role of the P2Z receptor is unknown. We and others have shown that sustained stimulation of this receptor by extracellular ATP (ATP_c) or P2Z-selective ATP analogues causes cell death by necrosis or apoptosis, depending on the cell type and the experimental conditions (4–7). However, a role for this receptor in naturally-occurring cell death has never been demonstrated.

In the present paper we checked whether the P2Z receptor might be involved in spontaneous cell death in macrophage cultures. Two approaches were followed: on one hand, we selected macrophage cell clones that either hypo or hyper-expressed the P2Z receptor; on the other, we tested the effect of the specific P2Z inhibitor oxidized ATP (oATP) (8), or the ATP-hydrolysing enzyme apyrase.

MATERIALS AND METHODS

Cells

The J774 mouse macrophage cell line and P2Zhyper or hypo variants were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) (complete

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Abbreviations: Extracellular ATP, ATP_c; oxidized ATP, oATP; J774 macrophages hypo-expressing the P2Z receptor, P2Zhypo; J774 macrophages hyper-expressing the P2Z receptor, P2Zhyper; wild-type J774 macrophages, P2Zwt.

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DMEM medium). P2Zhypo variants were selected by repeated rounds of incubation in the presence of 5 mM ATP_c followed by cloning by limiting dilution. P2Zhyper variants were obtained by cloning by limiting dilution and selection of the clones that showed a higher ATP_c-dependent uptake of lucifer yellow. Unless otherwise indicated, experiments were performed in saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5.5 mM glucose, 5 mM $NaHCO_3$ and 20 mM Hepes. We will refer to this medium as standard saline. When indicated, 1 mM $CaCl_2$ was also present.

Microscopy and Measurement of Changes in Plasma Membrane Permeability

Phase contrast and fluorescence pictures were taken with an inverted microscope (Olympus IMT-2, Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a 40X objective. ATP_c-dependent increases in plasma membrane permeability were measured with the extracellular fluorescent tracer lucifer yellow (Molecular Probes, Inc., Eugene, OR, USA). Cell monolayers were incubated in standard saline for 15 min at 37°C in the presence of 250 μM sulfinpyrazone (9) and 1 mg/ml lucifer yellow. After rinsing with complete DMEM medium to remove extracellular dye, cells were analysed with the fluorescence microscope.

Fluorimetric Measurement of Plasma Membrane Potential

Changes in plasma membrane potential were measured with the fluorescent dye bis[1, 3-diethylthio barbiturate]trimethineoxonal (bisoxonol) (Molecular Probes) at ethe wavelength 540/580 nm, as previously described (10). Experiments were performed in a spectrofluorimeter (model LS50, Perkin- Elmer Ltd., Beaconsfield, UK) equipped with a thermostat-controlled (37°C) cuvette holder and magnetic stirrer.

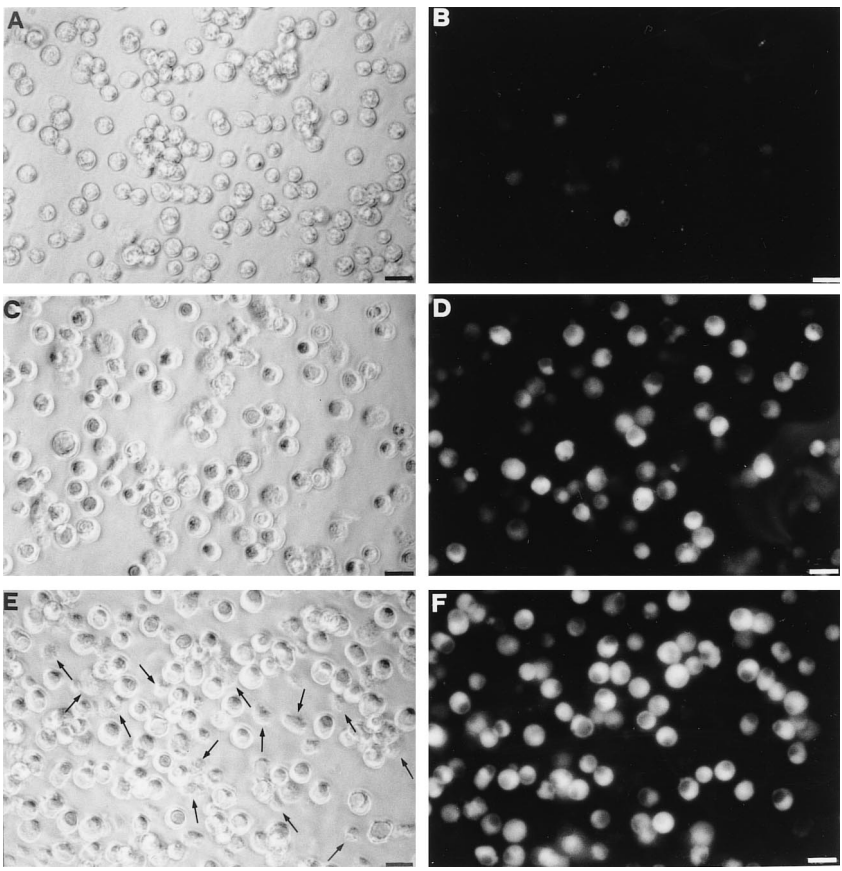

FIG. 1. ATP_c-stimulated lucifer yellow uptake in wild type J774 macrophages and clones selected for hypo or hyper expression of the P2Z receptor. Cells were stimulated with 5 mM ATP, for 15 min in the presence of 1 mg/ml of lucifer yellow, as described in Materials and Methods. After this incubation time, they were rinsed several times and photographed with a fluorescence microscope (40× objective). Panels A and B, P2Zhypo; panels C and D, P2Zwt; panels E and F, P2Zhyper. Arrows in panel E, indicate dead cells. Bar = 25 μm.

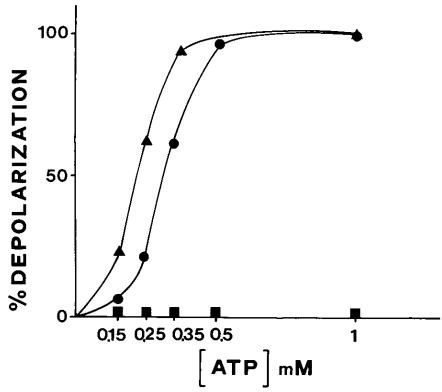

FIG. 2. Dose-dependency for ATP-dependent plasma membrane potential changes. Cells were incubated at 37°C in Ca^{2+}-containing standard saline solution at a concentration of 5×10^5/ml in the presence of 100 nM bisoxonol and stimulated with increasing ATP_e concentrations. Membrane potential changes are expressed as percent of maximal depolarization induced by 1 mM ATP_e. ▲–▲, P2Zhyper; ●–●, P2Zwt; ■–■ P2Zhypo.

Measurement of Enzymatic Activity

Lactate dehydrogenase activity was measured according to standard methods (11).

RESULTS AND DISCUSSION

Fig. 1 reports the pattern of ATP_c-stimulated lucifer yellow uptake by J774 cells that were selected for low (P2Zhypo, panels A and B), or high (P2Zhyper, panels E and F) expression of the P2Z receptor. Panels C and D show the pattern of lucifer yellow uptake by wild-type (P2Zwt) J774 cells. Frankly ATP_c-sensitive cells (bright fluorescence) were less than 1% in panel B, about 25% in panel D and over 50% in panel F. Cell positive for lucifer yellow uptake are clearly underes-

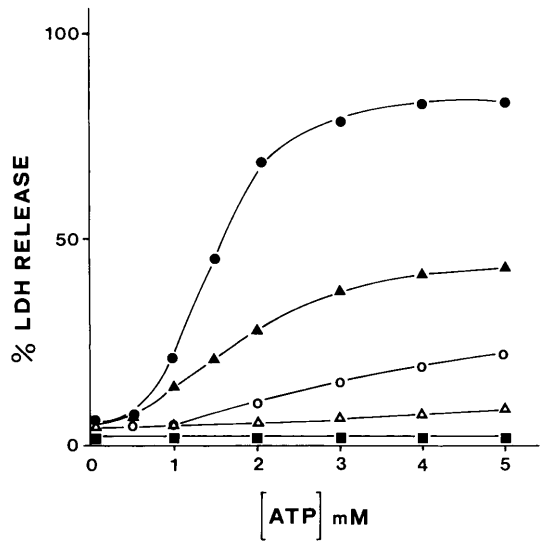

FIG. 3. Dose-dependency for ATP_c-dependent release of lactic dehydrogenase. Cells were incubated at 37°C in DMEM medium for 5 h in the presence of increasing ATP_e concentrations. At the end of this incubation, the supernatants were collected and the content of lactic dehydrogenase was measured. ●–●, P2Zhyper; ○–○, P2Zhyper plus oATP; ▲–▲, P2Zwt; △–△, P2Zwt plus oATP; ■–■, P2Zhypo.

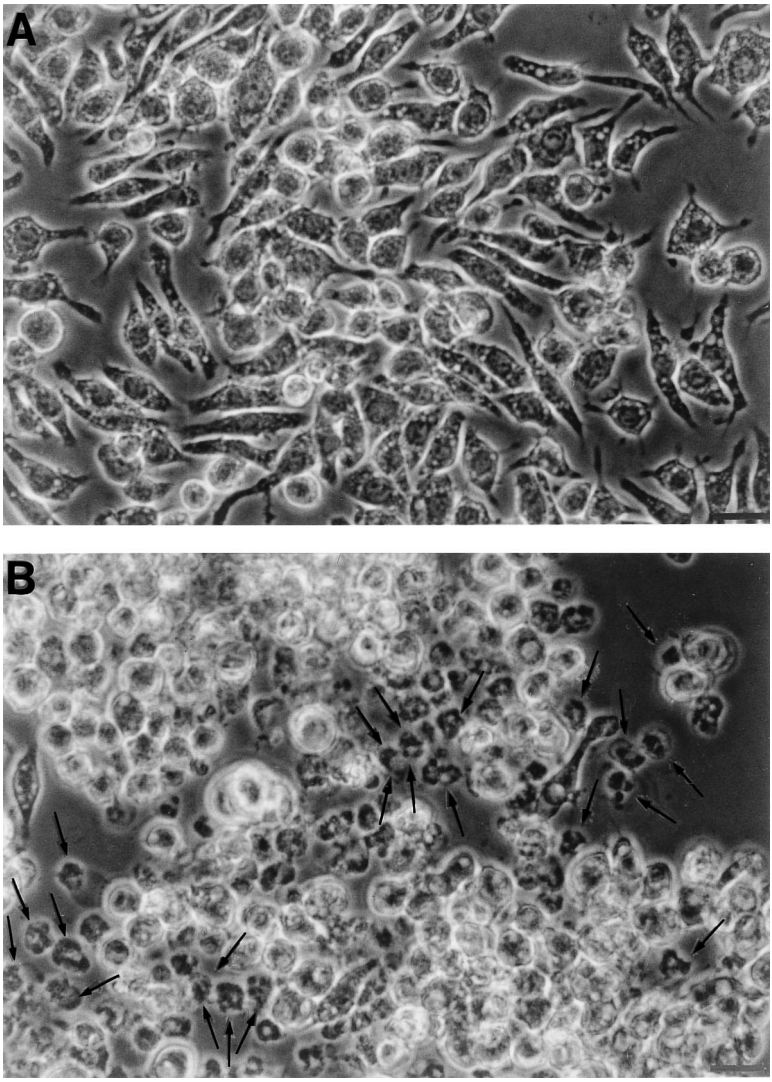

FIG. 4. Morphology of wild-type macrophages and clones selected for hyper expression of the P2Z receptor. Cells were grown to confluence and photographed with a 40× objective. Panels A and B, P2Zwt and P2Zhyper, respectively. Arrows in panel B, indicate dead cells. Bar = 25 μm.

timated in panel F due to the high percentage of dead cells (see arrows in panel E) in the P2Zhyper population. P2Zhyper cells were depolarized by lower ATP_c concentrations with respect to P2Zwt (Fig. 2), but the EC50 was only slightly shifted to the left (225 μM compared to 325 μM for P2Zhyper and P2Zwt, respectively), suggesting that hypersensitivity to ATP_c was not due to a different receptor subtype, but rather to higher expression of the same P2Z receptor. A different susceptibility to ATP_c of the three cell populations was also confirmed by measuring release of lactic dehydrogenase (Fig. 3). Again, ATP_c threshold was shifted to the left, but EC_{50} was unchanged in P2Zhyper cells compared to P2Zwt. ATP_c-dependent cytotoxicity was inhibited by pre-incubation with the selective P2Z blocker oATP.

During the process of isolation of P2Zhyper macrophage clones, we observed that it was very difficult to propagate these cells in culture as they underwent a high rate of spontaneous death that was exacerbated upon reaching confluence. Fig. 4, compares the morphology of P2Zwt and

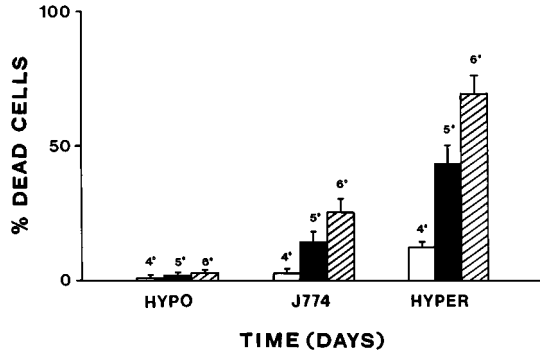

FIG. 5. Spontaneous cell death in macrophage cell monolayers. Cell monolayers were supplemented with fresh culture medium and incubated overnight in 24 well culture dishes (12 h). The following morning, the percentage of dead cells in the monolayers was evaluated by phase microscopy. Open bar, P2Zhypo; closed bar, P2Zwt; hatched bar, P2Zhyper. Numbers over the bars indicate the day after plating at which cell viability was evaluated.

P2Zhyper macrophage monolayers, clearly documenting the high incidence of dead cells in this latter culture (panel B, arrows). Quantitative evaluation of cell death (Fig. 5) confirmed this finding.

These observations suggested the possibility that the P2Z receptor was somehow involved in causing the high incidence of death in P2Zhyper macrophages. To test this hypothesis, we investigated the ability of oATP to block spontaneous death.

Fig. 6 shows that this nucleotide greatly reduced the number of dead cells in P2Zhyper cultures. As a further proof for the involvement of ATP_c receptors, the ATP-hydrolysing enzyme apyrase was also able to prevent spontaneous cell death.

The P2Z receptor, which alone among P2 receptors has not yet been cloned, mediates a reversible permeabilization of the plasma membrane that causes large ion fluxes and leakage of small metabolites such as nucleotides (3, 9). Cell death then occurs by apoptosis (5, 7), or, in case of more prolonged exposures to ATP_c (and therefore sustained permeabilization of the plasma membrane), by colloido-osmotic lysis (necrosis) (5, 12). A possible interpretation of the physiological role of the P2Z receptor is that this molecule might be involved in the elimination of unwanted cells during physiological or pathological cell and tissue turnover, or maybe in the amplification of tissue damage during immune and inflammatory reactions (13). This "auto-cytotoxic" role of the macrophage P2Z receptor could also be maintained during *in vitro* culture, thus causing a small but significant rate of cell death.

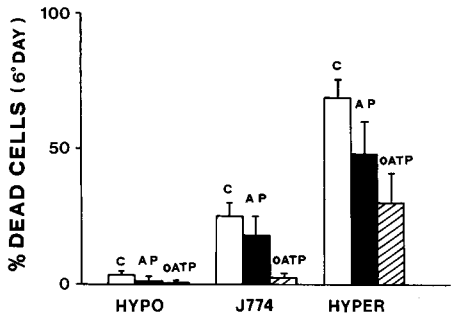

FIG. 6. Inhibition of spontaneous cell death by oATP. Cell monolayers were incubated in the presence of 0.2 U/ml of apyrase (closed bar), 300 μM oATP (hatched bar), or in the absence of these reagents (open bar) as described in Fig. 5.

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