Increased Proliferation Rate of Lymphoid Cells Transfected with the P2X$_7$ ATP Receptor*

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Human leukocytes can express the P2X$_7$ purinergic receptor, an ionic channel gated by extracellular ATP, for which the physiological role is only partially understood. Transfection of P2X$_7$ cDNA into lymphoid cells that lack this receptor sustains their proliferation in serum-free medium. Increased proliferation of serum-starved P2X$_7$ transfecants is abolished by the P2X$_7$ receptor blocker oxidized ATP or by the ATP hydrolyase apyrase. Both wild type and P2X$_7$-transfected lymphoid cells release large amounts of ATP into the culture medium. These data suggest the operation of an ATP-based autocrine/paracrine loop that supports lymphoid cell growth in the absence of serum-derived growth factors.

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Human and mouse leukocytes express the purinergic P2X$_7$ receptor (1–4). There is evidence that this plasma membrane receptor/pore participates in various macrophage, microglia, and dendritic cell responses such as plasma membrane permeabilization, cytokine release, multinucleated giant cell formation, and apoptosis (5–10), but its physiological function in lymphocytes is unknown. Several authors have proposed extracellular ATP as a novel mediator of cell proliferation, and evidence for such a role also in lymphoid cells has been presented in the past (11–14). Although the P2 receptor subtype involved has never been clearly defined, it is generally believed that the growth-stimulating effects of ATP are mediated by P2Y receptors. In a previous study we showed that human P2X$_7$ receptor: sense amplimer, 5'-AGATCGTGGAAGAATGGAGTG-3'; antisense amplimer, 5'-AGATCGTGGAAGAATGGAGTG-3'; and probe, 5'-CATCGACTACA-CACCTTCC-3'. Amplification primers for $\beta$-actin used as a reference for the amount of total mRNA loaded were: 5' primer, 5'-TGACGGGTCACCCAC-TTGCGGCATA-3'; 3' primer, 5'-AGCTATGATCGCCGTAGAGCA-TTTGCGGTT-3'.

All oligonucleotides were synthesized by Genenco Life Science Laboratories (Genenco Medical, Firenze, Italy). Blots and labeling of probes were carried out under standard conditions described in digoxigenin labeling and detection protocols from Roche Molecular Biochemicals. RT-PCR amplification (30 cycles) produced a fragment of the expected size, 399 base pairs, for P2X$_7$. RT-PCR products were separated in 1.2% agarose gel and transferred to a positively charged nylon membrane (Roche Molecular Biochemicals) by a vacuum blotter system (Bio-Rad Laboratories, Hercules, CA) for 2 h. After hybridization with the digoxigenin-labeled P2X$_7$-specific internal oligoprobe, P2X$_7$ cDNA was visualized by chemiluminescent detection after incubation with a dilution of anti-digoxigenin Fab fragments conjugated to alkaline phosphatase.

P2X$_7$, Transfectants—The vector used for transfection was pcDNA3 from Invitrogen (amplR) with a Not I-Not I insert for the human P2X$_7$ cDNA. The plasmid was transfected into K562 and LG14 cells by electroporation (Bio-Rad gene pulsar) at 250 V, 960 microfarads. After 24 h, cells were selected with 0.6 mg/ml genetin.

Proliferation—Cells were resuspended in Iscove's medium in the presence or absence of 10% fetal calf serum and seeded in 86-well Falcon 3072 plates (Becton Dickinson, Lincoln Park, NJ) in triplicate at 37 °C. After various times, the cell number was established by counting with a phase-contrast Leitz microscope.

Cytoplasmic Free Ca$^{2+}$ Concentration Measurements—Changes in [Ca$^{2+}$], were measured with the fluorescent indicator Fura-2/AM, as described previously (7). Briefly, cells were loaded for 15 min with 2 μM Fura-2/AM and incubated in a thermostat-controlled (37°C), magnetically stirred fluorometer microcuvette (LS50, Perkin-Elmer) at a concentration of 10 μM in the presence of 250 μM sulfipyrazone. The intracellular Ca$^{2+}$ concentration was determined with the 340/380 ex.
ATP Measurement—Cells (25,000/well) were seeded in microtiter plastic dishes in a total volume of culture medium of 100 μl, then rinsed and supplemented with 100 μl of diluent buffer (FireZyme Ltd., San Diego, CA) to stabilize extracellular ATP, and placed directly in the test chamber of a luminometer (FireZyme). Then, 100 μl of a luciferin-luciferase solution (FireZyme) was added, and light emission was recorded.

Data Presentation—Data shown in the graphs are quadruplicate determinations ± S.D. from a single experiment representative of three similar experiments. In some graphs, error bars are not shown because their width exceeded the dimensions of the symbol.

RESULTS

K562 and LG14 cells have been shown previously to be insensitive to stimulation with extracellular ATP (19). RT-PCR analysis (Fig. 1, inset) showed that these cells do not express the P2X7 receptor mRNA. We therefore used those cells as a model to investigate the effect of P2X7 transfection on human lymphocytes.
leukemic cell responses. P2X<sub>7</sub> transfection rendered both K562 and LG14 cells fully sensitive to activation by ATP, as shown by changes in the cytoplasmic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]i) (Fig. 1), whereas wild type cells were unresponsive. The resting [Ca<sup>2+</sup>]i level of P2X<sub>7</sub>-transfectants was consistently higher than that of wild type cells, but the difference was not statistically significant.

Under standard culture conditions, the growth rates of mock- and P2X<sub>7</sub>-transfected cells did not significantly differ (Fig. 2, A and C). However, in the absence of serum, the proliferation of LG14 and K562 cells transfected with the plasmid vector without the P2X<sub>7</sub> insert progressively declined, but the growth of P2X<sub>7</sub>-transfected cells (both K562 and LG14) was much less affected (Fig. 2, B and D). After 72 h, the number of K562P2X<sub>7</sub> and LG14P2X<sub>7</sub> cells was 2- and 4-fold higher, respectively, than that of control cells. The growth rates of mock-transfected and wild type cells were the same whether in the presence or absence of serum.

There are currently very few pharmacological blockers of the P2X<sub>7</sub> receptor. The most potent, KN-62, an isoquinoline derivative, is however also a calmodulin inhibitor and is thus unsuited for long term studies on cell proliferation. In this respect, a better, albeit less potent, agent is oxidized ATP (oATP), suited for long term studies on cell proliferation. In this regard, is however also a calmodulin inhibitor and is thus unsuited for long term studies on cell proliferation. In this respect, an incidental observation, being followed up in our laboratory, is that LG14P2X<sub>7</sub> cells appear to release significantly more ATP than their wild type counterpart. Many tumor cell lines exhibit high P2X<sub>7</sub> receptor levels, and early studies by Heppel and co-workers (27) suggested that transformation increases expression of this receptor. P2X<sub>7</sub> receptor expression may therefore be a factor that confers a selective advantage to tumor cells and improves their survival in an unfavorable environment.

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thymocytes do not express functional P2Y receptors (15, 26). A few reports on the mitogenic effect of ATP in mouse lymphocytes have appeared in the past (12, 13), and a study on human T lymphocytes was published by our laboratory in 1996 (15). In this study we showed that ATP potentiated the mitogenic effect of phytohemagglutinin and anti-CD3 antibodies, although by itself ATP had no effect on lymphocyte proliferation. We also observed that this activity was mimicked by benzoyl-ATP, a selective P2X<sub>7</sub> agonist, and inhibited by oATP, thus pointing to an involvement of P2X<sub>7</sub> in lymphocyte proliferation. Our present data support the hypothesis that the P2X<sub>7</sub> receptor in lymphoid cells mediates a growth-promoting signal, but only under those conditions in which other, maybe more powerful, growth factors are absent. It appears that under serum starvation P2X<sub>7</sub>-transfected cells are able to take advantage of an autocrine/paracrine loop based on the leak of ATP into the pericellular milieu and the consequent purinergic receptor stimulation. In this respect, an incidental observation, being followed up in our laboratory, is that LG14P2X<sub>7</sub> cells appear to release significantly more ATP than their wild type counter part. Many tumor cell lines exhibit high P2X<sub>7</sub> receptor levels, and early studies by Heppel and co-workers (27) suggested that transformation increases expression of this receptor. P2X<sub>7</sub> receptor expression may therefore be a factor that confers a selective advantage to tumor cells and improves their survival in an unfavorable environment.