SIGNALING THROUGH P2X7 RECEPTOR IN HUMAN T CELLS

INVOLVES p56<sup>LCK</sup>, MAP KINASES, AND TRANSCRIPTION FACTORS AP-1 AND NF-κB

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Abbreviations: anti-pTyr, anti-phosphotyrosine; CaM kinase, Ca$^{2+}$/calmodulin-dependent protein kinase; MAP kinase, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; EMSA, electromobility shift assay; JNK, Jun N-terminal kinase; Bz-ATP, 3-O-(4'-benzoyl)-benzoyl-ATP; NF-κB, nuclear factor-κB; oATP, oxidized ATP; PIP$_2$, phosphatidylinositol; PI-3-K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase.
SUMMARY

ATP-gated ion channel P2X receptors are expressed on the surface of most immune cells and can trigger multiple cellular responses, such as membrane permeabilization, cytokine production, and cell proliferation or apoptosis. Despite broad distribution and pleiotropic activities, signaling pathways downstream of these ionotropic receptors are still poorly understood. Here, we describe intracellular signaling events in Jurkat cells treated with millimolar concentration of extracellular ATP. Within minutes, ATP treatment resulted in the phosphorylation and activation of p56\textsuperscript{lck} kinase, extracellular-signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), but not p38 kinase. These effects were wholly dependent upon the presence of extracellular Ca\textsuperscript{2+} ions in the culture medium. Nevertheless, a calmodulin antagonist calmidazolium and CaM kinase inhibitor KN-93 both had no effect on the activation of p56\textsuperscript{lck} and ERK, whereas a pretreatment of Jurkat cells with MAPK kinase (MEK) inhibitor P098059 was able to abrogate phosphorylation of ERK. Further, expression of c-Jun and c-Fos proteins and activator protein (AP-1) DNA-binding activity were enhanced in a time-dependent manner. In contrast, DNA-binding activity of nuclear factor-κB (NF-κB) was reduced. ATP failed to stimulate the phosphorylation of ERK and JNK and activation of AP-1 in p56\textsuperscript{lck}-deficient isogeneic T cell line, JCaM1, suggesting the critical role for p56\textsuperscript{lck} kinase for the downstream signaling. Regarding biological significance of the ATP-induced signaling events we show that, while extracellular ATP was able to stimulate proliferation of both Jurkat and JCaM1 cells, an increase in IL-2 transcription was observed only in Jurkat cells. The nucleotide selectivity and pharmacological profile data supported the evidence that the ATP-induced effects in Jurkat cells were mediated through the P2X7 receptor. Taken together, these results demonstrate the ability of extracellular ATP to activate multiple downstream signaling events in human T-lymphoblastoid cell line.
INTRODUCTION

Extracellular ATP and other nucleotides act through specific cell surface receptors and can regulate a variety of cellular responses in many cell types and tissues (1-7). Among them are such different phenomena as platelet aggregation, smooth muscle contractility, excitatory transmitter function, mitogenic stimulation or induction of cell death (reviewed in Refs. 1, 8). The biological effects of extracellular nucleotides are mediated via stimulation of two primary classes of purinergic receptors, P1 and P2. The P1 receptors are responsive to adenosine, whereas the P2 receptors respond to a variety of nucleotides, including ATP (7, 9). The P2 receptors are subdivided in two mechanistically distinct subclasses, the metabotropic G protein-coupled P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-13), and the ionotropic ligand-gated channel P2X receptors (P2X1-7) (9-12). Activation of the P2Y receptors generally induces downstream signaling through the G-protein coupled activation of phospholipase C, followed by Ca$^{2+}$ mobilization from intracellular stores (13, 14). The P2Y11 also activates adenylyl cyclase, whereas the P2Y12 inhibits it (15). The seven subunits of the ionotrophic ATP-gated P2X receptor family comprise a different subclass, ranging from 379 to 595 amino acids in length and regulating intracellular level of Ca$^{2+}$ by ligand-stimulated increase in cell membrane permeability for extracellular Ca$^{2+}$ ions (7, 16, 17).

The P2X7 receptor is a 595-amino acid polypeptide and shares similar for other P2X receptors structure with two membrane-spanning domains, a large extracellular loop and intracellular N- and C-terminal domains (18, 19). In contrast to other P2X receptors, the P2X7 COOH-terminal intracellular chain is about 200 amino acids longer (20, 21). The P2X7 has a pharmacological profile similar of the receptor previously designated as
P2Z, with prominent expression in many immune cells (lymphocytes, monocytes/macrophages, dendritic, mesangial and microglial cells) (7, 20), and requires millimolar levels of ATP in the presence of divalent cations to achieve activation (22, 23). While each of the P2X receptors is capable of forming heteromers with other family members in a specific pattern (24), the P2X7 cannot heteropolymerize with any other P2X subunit (25). Activation of the P2X7 receptor results in the formation of a nonselective cationic channel with low affinity for ATP and increased permeability to Ca\(^{2+}\), intracellular depolarization, and equilibration of sodium and potassium gradients (18, 26). In addition, the P2X7 receptor may also induce a nonselective pore with uncharacterized structure able to pass molecules up to 900 Da, sharing this ability, albeit to a lesser degree, with other P2X family members (7, 27). Continuous activation of the receptor and the formation of a large transmembrane pore can cause perturbations in ion homeostasis and finally result in cell death (20, 28). Depending on the cell background, stimulation of the P2X7 receptor by ATP triggers diverse biological responses, such as posttranslational processing of precursors of IL-1\(\beta\) and IL-18 in human monocytes (29, 30), altered cytokine production in mice lacking this receptor (31), stimulation of JNK activity and induction of apoptosis in murine macrophages (26), inhibition of osteoclastic resorption (32), and activation of transcription factor NF-\(\kappa\)B (33) and NFAT (34) in microglial cell lines. Despite the accumulating evidence for an important function of the P2X7 receptor in many cellular systems, intracellular signaling events underlying the biological processes upon the P2X7 stimulation in immune cells are still obscure.

The activation of protein tyrosine kinases (PTKs) constitutes one of the initial steps for the induction of signaling cascades, which ultimately results in the activation of T cell effector function (35). p56\(^{lk}\) kinase is a lymphoid-specific cytoplasmic PTK with
molecular size of about 56 kDa that mediates initial events in CD3/TCR signaling, such as phosphorylation of the TCR complex within amino acid sequences known as immunoreceptor-based tyrosine activation motifs (ITAMs) (35, 36), which serve as docking sites for Src homology domain 2 (SH2)-containing molecules, predominantly Zap-70 and Syk (35, 37), and activation of mitogen-activated protein (MAP) kinases (38). The MAP kinase cascade represents another key signaling pathway, critical for the linking membrane receptors to cytoplasmic and nuclear effectors. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 are serine/threonine kinases, which constitute major components of this inducible signaling pathway and regulate many intracellular events, including cell proliferation and differentiation (39, 40). Upon activation, the MAP kinases phosphorylate various cytoplasmic effector proteins and are translocated to the nucleus, where they participate in the regulation of the gene expression by acting on transcription factors (39, 41).

The present study focuses on intracellular signaling events in Jurkat cells treated with millimolar concentrations of extracellular ATP. We show here that ATP induces phosphorylation and activation of p56\textsuperscript{ck} kinase, ERK and JNK. These events are prerequisite for the subsequent increase in the expression of c-Jun and c-Fos proteins and activation of transcription factor AP-1. On the contrary, DNA-binding activity of p50 and p65 (RelA) subunits of NF-\kappa B transcription factor was reduced. The observed effects were wholly dependent on the extracellular Ca\textsuperscript{2+} influx and, in regard to the pharmacological profile and expression pattern, are likely to be exclusively mediated by the P2X7 receptor. Finally, the experiments aimed to characterize the biological significance of the signaling pathway involved showed that ATP was able to stimulate proliferation of Jurkat cells and to induce an increase in transcription of IL-2.
data presented in this study demonstrates the ability of extracellular ATP to elicit diverse cellular responses in human T-lymphoblastoid cell line.
MATERIALS AND METHODS

Reagents and antibodies – All reagents used were of analytical grade. ATP was obtained from Roche (Mannheim, Germany). PD098059, inhibitor of ERK (42); suramine, antagonist of the P2 receptors (43); calmidazolium, an antagonist of calmodulin (44); KN-93, inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase, 44); and KN-92, an inactive analog of KN-93, were purchased from Calbiochem-Novabiochem (La Jolla, CA). \(1[N,O\text{-bis}(5\text{-isoquinolinesulphonyl})\text{-N-methyl-L-tyrosyl}]-4\text{-phenylpiperazine}\) (KN-62), oxidized ATP (oATP), both antagonists of the P2X7 receptor (7, 45, 46); 3-\(O\)-(4\text{'-benzoyl})-benzoyl-benzoyl-ATP (Bz-ATP), agonist of the P2X7 receptor (7); UTP, GTP, adenosine and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO). Anti-phosphotyrosine (anti-pTyr) antibodies (horseradish peroxidase conjugated (RC20H) and biotinylated (RC20B)) were obtained from Transduction Laboratories (Lexington, KY). Antibodies against ERK (C-16), pERK (E-4), pJNK (G-7), pp38 (D-8), Lck (3A5), NF-κB p50 (C-19), p65 (A), c-Rel (N-466), cyclin D (R-124), Fos (4), Jun (N), Myc (C-19), P2X7 (L-20) were purchased from Santa Cruz (Santa Cruz, CA); anti-pLck (pY505) and anti-pSrc (pY416) were purchased from BioSource Int. (Camarillo, CA).

Cell culture and stimulation conditions – Human T-lymphoblastoid cell line Jurkat, its p56\(^{\text{lck}}\)-deficient derivative variant JCaM1 as well as stable transfectants of JCaM1 expressing p56\(^{\text{lck}}\) cDNA in pBP1 vector or mock pBP1 vector (last three lines were kindly provided by Dr. D. Straus, University of Chicago) (36) were maintained in RPMI-1640, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100
mg/ml streptomycin. Transfectants were maintained in the presence of 0.5 mg/ml of G418 and 0.5 mg/ml hygromycin. Before treatment cells were washed twice with Dulbecco’s PBS and incubated in RPMI-1640 without FCS at 37°C for 3 h.

The cells (5 x 10^6) were activated by ATP (final concentration 3 mM) for different time intervals at 37°C. Activation was interrupted by adding ice-cold PBS with 10 mM EDTA and 100 mM sodium vanadate. Then, the cells were pelleted and stored at -80°C before electrophoresis.

**Immunoprecipitation, Western blotting and kinase assay** – Cell pellets were lysed in 1% NP-40 cell extraction buffer (20 mM Tris-HCl buffer, pH 8.0 with 15 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 10 mM phenylmethylsulphonylfluoride (PMSF) and 100 mM sodium vanadate). The detergent insoluble material was removed by centrifugation for 15 min at 13.000 and 4°C. Protein concentrations were determined using BSA protein assay kit (Bio-Rad, Munich, Germany), and 50 µg of proteins were analyzed by electrophoresis in 10% SDS-PAGE. For immunoprecipitation, 500 µg of proteins were incubated overnight at 4°C with 5 µg/ml of RC20B or anti-Lck antibodies. Immunocomplexes were captured on protein A-agarose (Bio-Rad) or streptavidin-agarose (Pierce, Rockford, IL), respectively, with gentle mixing for 1 h at 4°C and analyzed in 10% SDS-PAGE. Visualization of specific proteins was carried out by an enhanced chemiluminescence (ECL) method using ECL Western blotting detection reagents (Amersham Life Science, Buckinghamshire, England) according to the manufacturer’s recommendations.

For kinase assay, immunocomplexes were washed with 25 mM HEPES (pH 7.4), 2 mM MnCl₂, 10 mM MgCl₂, 1 mM Na₃VO₄ and incubated in 60 µl of 5 mM HEPES, 2
mM MnCl₂, 10 mM MgCl₂, 1 mM Na₃VO₄, 10 µCi of [γ³²P]ATP (3000 Ci/mmol; Amersham), 10 µM ATP and 1 µg GST-ζTCR fusion protein (p56lek substrate, generously provided by Dr. D. Straus) for 15 min at 37°C. The reaction was stopped by adding 20 µl of 4x sample buffer. Samples were boiled for 5 min and proteins were resolved in 10% SDS-PAGE. Phosphotyrosine containing proteins were detected by autoradiography.

**RNA extraction and RT-PCR** – Cellular RNA was extracted from the cells using TRIZOL reagent (Invitrogen, Groningen, Netherlands) according to the manufacturer’s instruction. A 5 µg aliquot of total cellular RNA was reverse transcribed using random hexanucleotides as primers and SUPERSCRIPT II preamplification kit (Invitrogen). cDNA was amplified in 50 µl PCR reaction mixture, containing 250 µM of each dNTPs, 200 nM primers, 5 µl of 10-fold PCR buffer with 1.5 mM MgCl₂ and 1 U Taq DNA polymerase (“Amplitaq”, Applied Biosciences, Warrington, UK). The primers used were: human P2X7 receptor (GenBank accession N Y09561): sense, 5’-TCCGAGAAACAGGCGATAA-3’, anti-sense, 5’-ACTCGCACTTCTTCTCTGT A-3’; human IL-2 (GenBank accession N NM 000586): sense, 5’-TACAACTGGAGCATTTACTGC-3’; anti-sense, 5’-TTGACAAAAGGTAATCCATCT-3’; human β-actin (GenBank accession N NM001101): sense, 5’-GTGGGGCGCCCCAGGCACCA-3’; anti-sense, 5’-CTCCTTAATGTCACGCAGCATTTACTGC-3’. All primers were purchased from TIB Molbiol (Berlin, Germany). Samples were amplified in a DNA Thermocycler (Eppendorf, Hamburg, Germany) for 35 cycles. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 1 min. Aliquots of
PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. To evaluate mRNA expression semi-quantitatively, in addition to the PCR product from 35 cycles, 15 µl of the PCR product from the 25 cycles and the 30 cycles was run simultaneously. β-actin message was used to normalize the cDNA amount to be used. A mock PCR (without cDNA) was included to exclude contamination in all experiments.

Electromobility shift assay (EMSA) – Nuclear extracts from 2 x 10⁶ cells were prepared according to the method of Schreiber et al. (47). EMSA was performed by incubating 4 mg of nuclear extract with 16 fmol of ³²P end-labeled double-stranded NF-κB oligonucleotides from the HIV long terminal repeat, 5’-TTGTAACAAGGACTTTCCGCTGGGGACTTTCCAGGGAGGCCTGG-3’ or with AP-1 oligonucleotides, 5’-CGCTTGATGACTCAAGCCGGAA-3’ and 3’-GCGAACTACTGAGTGCGGCCTT-5’ (underlined regions are consensus NF-κB or AP-1 sites). Binding reaction was performed in 20 µl of reaction buffer (20 mM Tris-Cl, pH 7.9, 20% glycerol, 50 mM KCl, 1 mM DTT and 2.5 mM MgCl₂) containing 4 µg of nuclear proteins, 2 µg poly(dI-dC) and 1 ng end-labeled DNA (10,000-30,000) at room temperature, loaded on 4% polyacrylamide gel and run in 0.5x TBE buffer (89 mM Tris, 89 mM Boric acid, pH 8.3). Gels were dried in a gel dryer and exposed to an X-ray film. A double-stranded mutated oligos were used to prove the specificity of binding of NF-κB or AP-1 to the DNA. The specificity was also proven by competition with the unlabelled (“cold”) oligos. To characterize NF-κB/DNA and AP-1/DNA complexes, supershifting
assay was performed using anti-p65, anti-p50, anti-c-Rel antibodies, and anti-c-Fos/c-Jun antibodies, respectively.

**Proliferation assay, cell survival assay and flow cytometric analysis** –

Proliferation of Jurkat cells was assessed by (³H)thymidine incorporation. 1 x 10⁵/well cells were cultured in triplicates in 96-well flat-bottom plates, in a final volume of 200 µl for 24 h and then incubated with (³H)thymidine (1 µCi/well) for additional 12 h. Cells were harvested onto glass filters, and incorporation of (³H)thymidine was determined by liquid scintillation counting.

For cell survival assays, Jurkat cells were cultured at 1 x 10⁵ cells/ml in triplicate at 37°C for 1 to 3 days in RPMI medium in the presence of 3 mM ATP. Cells were harvested and cell viability was determined by FACS analysis with 5 µg/ml of propidium iodide (PI). Percentage of apoptotic cells was evaluated by ApoTarget Annexin-V-FITC Apoptosis kit (BioSourse Int.) according to the manufacturer’s protocol and analyzed on a FACScalibur (Becton Dickinson, San Jose, CA) using CELLQuest software.

**Intracellular Ca²⁺ measurement** – Cells were pre-incubated with 2 µM of Fura-2 (Molecular Probes, Eugene, OR) for 30 min at 37°C and Ca²⁺ influx was measured in Hitachi F-2500 spectrophotometer (Hitachi, Tokio, Japan) using 350/385 excitation filters. After recording of background for 20 sec, cells were stimulated with 3 mM ATP and concentration of Ca²⁺ was calculated using equation of Grinkievich (48).
Changes in plasma membrane permeability – ATP-dependent increase in plasma membrane permeability were measured with the extracellular fluorescent tracers Lucifer yellow (Molecular Probes) and ethidium bromide (Sigma), as described earlier (25). For Lucifer yellow uptake, cells were incubated for 15 min at 37°C in buffer (125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM Na2HPO4, 5.5 mM glucose, 5 mM NaHCO3, 20 mM HEPES, pH 7.4) containing 250 µM sulfinpyrazone and 1 mg/ml Lucifer yellow, and stimulated with 3 mM of ATP. After several washing to remove the extracellular dye, cells were analyzed with an inverted fluorescence microscope (Nikon Diaphot 300, Tokyo, Japan) using a 40X objective and a fluorescein filter. For ethidium bromide uptake, cells were incubated in a fluorometer cuvette (37°C) at a concentration of 10^6 cells/ml in the presence of 20 µM ethidium bromide, and challenged with various ATP concentrations. Fluorescence changes were monitored at the wavelength 360-580 nm.

Statistical analysis – All experiments were performed in at least three independent assays, which yielded highly comparable results. Data are presented as mean values ±SD as indicated in the figure legends. The Mann-Whitney-U test was used to determine the level of statistical significance.
RESULTS

*Extracellular ATP induces activation of p56^{lck} kinase* – Protein tyrosine kinases (PTKs) are critically involved in signaling pathways that regulate cell growth, differentiation and activation. The activation of downstream signaling events, including the Ras/Raf/MAP kinase pathway, the phosphatidylinositol (PIP$_2$) pathway, and the phosphatidylinositol 3-kinase (PI-3-K) pathway was shown to be dependent upon protein tyrosine kinase functions (39, 49). Thus, we studied first the ability of purinoreceptors to mediate intracellular signaling in response to extracellular ATP by incubating Jurkat cells with various concentrations of ATP for different time intervals and evaluating the phosphorylation of PTKs. In the experiments illustrated in Fig. 1, Jurkat cells were treated with 3 mM ATP. Immunoprecipitation and probing with anti-pTyr antibodies at the indicated time points showed that a number of proteins were phosphorylated upon the exposure of the cells to extracellular ATP. These included 40-42, 56, 70 and 80-85 kDa proteins (Fig. 1A). Such phosphorylation pattern was already detectable within first 5 min of ATP administration, reaching its maximum at 15 min and returning to near basal level within 30 min. Reportedly, T-cell activation often involves the recruitment of p56$^{lck}$ kinase, which is essential for T-cell development and function (36). Re-probing of the blots with anti-p56$^{lck}$-specific antibodies allowed us to identify among the phosphorylated proteins p56$^{lck}$ kinase (Fig. 1B). Other kinases of the Src family, such as c-Src, Lyn and Fyn, as well as Syk and ZAP-70, which are often recruited for signal transduction in T-cell (50), were not involved (data not shown). To confirm the phosphorylation of p56$^{lck}$ kinase upon ATP administration, immunoblotting of anti-p56$^{lck}$ precipitates with anti-pTyr antibodies was carried out (Fig. 1C).
ATP induced transient phosphorylation of p56\textsuperscript{Lck} within 5-15 min of treatment, and this effect significantly declined within 15-30 min. For loading control, stripped membranes were re-probed with anti-p56\textsuperscript{Lck} antibodies (Fig. 1D). Thus, the kinetic experiments revealed that ATP stimulated the rapid and transient phosphorylation of p56\textsuperscript{Lck} kinase.

According to the several reports, activity of p56\textsuperscript{Lck} is regulated by phosphorylation at two tyrosine residues, Y394 and Y505 (51-53). Phosphorylation at Y505 leads to stabilization of p56\textsuperscript{Lck} in a biologically inactive conformation (51, 52). On the contrary, phosphorylation at Y394 stimulates the catalytic activity of p56\textsuperscript{Lck} (52, 53). We immunoprecipitated p56\textsuperscript{Lck} and probed the membrane with phosphospecific antibodies to Y416 of human c-Src (corresponds to Y394 in p56\textsuperscript{Lck} (53)) and with antibodies to Y505-Lck, in order to detect which tyrosine residues are phosphorylated in p56\textsuperscript{Lck} kinase after exposure to extracellular ATP. As shown in Fig. 2A, ATP induced the phosphorylation of Y394 (upper panel), but did not change the phosphorylation of Y505 (middle panel).

Since phosphorylation of Y394 usually leads to an increase in the kinase activity of p56\textsuperscript{Lck} (53), a kinase assay was performed to determine whether ATP also affects p56\textsuperscript{Lck} kinase catalytic activity. We incubated p56\textsuperscript{Lck}-precipitates with \[^{32}\text{P}]\text{ATP} and an endogenous substrate consisting of the cytosolic domain of the ζ-chain of the TCR fused to GST (38). As it is shown in Fig. 2B, ATP activates the kinase activity of p56\textsuperscript{Lck} within 5-15 min of treatment. To investigate whether Ca\textsuperscript{2+} was essential for ATP-induced p56\textsuperscript{Lck} phosphorylation, Jurkat cells were stimulated with ATP in the presence of the Ca\textsuperscript{2+} chelator EGTA. As shown in Fig. 2C, pretreatment of cells with 2 mM EGTA completely prevented p56\textsuperscript{Lck} phosphorylation, indicating that the activation of p56\textsuperscript{Lck} required extracellular Ca\textsuperscript{2+}. Thus, extracellular ATP mediates Ca\textsuperscript{2+}-dependent activation of p56\textsuperscript{Lck}.
in Jurkat cells by inducing phosphorylation at Y394 and increases the activity of the kinase.

**Extracellular ATP activates the MAP kinases ERK1, ERK2 and JNK1/2, but not p38** – It was shown that ATP can induce phosphorylation of ERK in PC12 cells and fetal astrocytes (54-56), and of JNK in BAC1 murine macrophages (26). Several studies reported an important role for p56\(^{\text{Lck}}\) kinase in the activation of the MAP kinase pathway (38, 57). Based on these findings, our next goal was to determine whether ATP is able to induce the activation of ERK and JNK MAP kinases in Jurkat cells. The cells were incubated with ATP and the activation of the MAP kinases ERK and JNK was evaluated by employment of phosphospecific antibodies directed against C-termini of ERK1 and ERK2 (both phosphorylated at Y204) (55) and JNK1 and JNK2 (phosphorylated at T183 and Y185, respectively) (40). Kinetic experiments revealed that ATP induced rapid and transient phosphorylation and activation of ERK1 (p44) and ERK2 (p42), as well as JNK1 (p54) and JNK2 (p52) in a time-dependent manner (Fig. 3). Phosphorylation of both kinases was already detectable within 5 min of ATP administration, reaching its maximum after 15 min and returning to near basal levels within 3 h. On the other hand, the phosphorylation state of p38 kinase was not affected, remaining at the same level throughout the time-course of the experiment (Fig. 3). For loading control, membranes were stripped and re-probed with anti-ERK, anti-JNK or anti-p38 antibodies, respectively. The activation of MAP kinases by ATP required a sustained increase in the intracellular Ca\(^{2+}\) concentration and Ca\(^{2+}\) influx across the plasma membrane, since it was inhibited by the depletion of extracellular C\(^{2+}\) ions in extracellular medium (data not shown).
To investigate the mechanism of activation of ERK, we examined the effects of a specific inhibitor of MEK, PD098059 (42). The ERK activation cascade is believed to proceed through sequential activation of three protein kinases, MAP kinase kinase kinase, MAP kinase kinase (MEK) and MAP kinase (ERK). The MEK1/2 activates ERK by phosphorylation of the conserved Thr and Tyr residues. PD098059 inhibits activation of both MEK1 (IC$_{50}$ = 5-10 µM) and MEK2 (IC$_{50}$ = 50 µM) (42). Thus, we used PD098059 in order to study whether it can block phosphorylation of ERK in response to the ATP treatment. The cells were incubated with 50 µM of PD098058 for 90 min, and then stimulated with ATP. Non-treated cells were used as a control. As shown in Fig. 4A, PD098059 was able to inhibit the phosphorylation of ERK (upper panel). The second panel shows the control of loading. At the same time, the phosphorylation pattern of p56$^{lck}$ was not altered. The lysates from PD098059-treated and ATP-activated cells were precipitated with anti-p56$^{lck}$ antibodies and phosphorylation of p56$^{lck}$ at Y394 was evaluated by SDS-PAGE using anti-pY416(c-Src) specific antibodies. As shown in Fig. 4A (third panel), PD098059 did not affect ATP-mediated phosphorylation of p56$^{lck}$ kinase. For control of loading, these membranes after stripping were probed with anti-p56$^{lck}$ antibodies (Fig. 4A, lower panel).

Recently, Franklin and coauthors (44) demonstrated that calcium ionophore-induced Ca$^{2+}$ influx is able to trigger a rapid activation of ERK in the similar cell model through CaM-kinase-dependent stimulation of p56$^{lck}$. To test the involvement of calmodulin and CaM kinase in ATP-mediated activation of p56$^{lck}$ and ERK1/2, Jurkat cells were pretreated with antagonist of calmodulin, calmidazolium or an inhibitor of CaM kinase, KN-93 (44) for 30 min at 37°C, stimulated with 3 mM ATP for different time intervals (5-15 min), and analyzed for the activation of ERK1/2 and p56$^{lck}$. Calcium
ionophore A23187 and an inactive analog of KN-93, KN-92, were used as controls. As shown in Fig. 4B, left panel, ATP alone induced a rapid activation of ERK1/2 after 5 min of treatment, with a peak within 15 min. In agreement with the previous report (44), A23187 also was able to trigger activation of ERK. However, calmidazolium in concentration of 5 µM was able to abrogate A23187-induced but not ATP-induced activation of ERK1/2 (Fig 4B, upper left panels). Lower panel shows control of loading. Then, we immunoprecipitated p56^\text{ck}\text{kinase} and probed the membrane with phosphospecific antibodies to detect the activation of p56^\text{ck}. As shown in Fig. 4B, ATP induced the phosphorylation and activation of the kinase in a time-dependent manner both in the absence and presence of calmidazolium, clearly indicating that ATP-mediated activation of p56^\text{ck} is not dependent on calmodulin. Lower membrane was probed with anti-p56^\text{ck} antibodies to prove the control of loading. The experiments with CaM kinase inhibitor KN-93 revealed that, although ATP preserved the ability to activate both p56^\text{ck} and ERK1/2 (Fig. 4B, right panels), this effect was slightly diminished when compared with its inactive analog, KN-92. Lower membranes show the control of loading. Notably, KN-93 was able to completely inhibit the activation of ERK and p56^\text{ck} in response to calcium ionophore A23187, while KN-92 had no effect. The difference between action of KN-93 and KN-92 on Jurkat cells after stimulation with ATP deserves additional exploration and may reflect the partial involvement of CaM kinase to the observed activation of p56^\text{ck} and ERK1/2 in response to ATP, although this speculation requires further proof. Nevertheless, the ability of ATP to induce the activation of ERK1/2 and p56^\text{ck} kinase even in the presence of CaM kinase inhibitor KN-93 strongly supports the idea that such an effect is elicited mainly via CaM kinase-independent mechanism.
Taken together, these results confirm the ability of extracellular ATP to trigger the phosphorylation and activation of ERK1/2 and JNK1/2, but not p38 kinase, and show the contribution of MEK to the activation of ERK. In addition, such activation of p56^\text{Lck} kinase and ERK1/2 appears not to be dependent on calmodulin, and only partially, if at all, dependent on CaM kinase.

Activation of p56^\text{Lck} and MAP kinases by ATP is mediated through the P2X7 receptors – To identify both the specificity of the response and the identity of the receptors involved in ATP-induced activation of p56^\text{Lck} and MAP kinases in Jurkat cells, a panel of purinoreceptors was tested for their ability to activate these kinases. Each purinoreceptor subtype is defined by its relative response to different purinergic ligands (7, 9). In our preliminary experiments, we did not observe the activation of p56^\text{Lck} and MAP kinases in response to the treatment with adenosine, ADP, AMP, GTP or UTP (data not shown). Adenosine has been demonstrated to be a selective agonist of P1 receptors (13). UTP serves as a high potency agonist for human P2Y2 and P2Y4 receptors, while at P2Y1 and P2Y11 it is inactive (7). ADP activates P2Y12 and P2Y13 and was reported to be equipotent or even more potent as ATP for P2Y1, while for P2Y11 ATP is more potent than ADP (7, 12, 13). UDP selectively activates P2Y6 (7, 58). The unique, naturally occurring agonist of P2X receptors is ATP (7, 13, 14).

Adenosine and UTP showed no ability to induce phosphorylation of ERK and p56^\text{Lck} kinase in Jurkat cells at concentration 100 \mu M (Fig. 5A) or higher (data not shown). These results indicated that the P1 receptor as well as the P2Y2 and P2Y4 were not involved in the observed ATP-mediated effects. Next, RT-PCR was performed to test mRNA expression in Jurkat cells of five mammalian P2Y receptors (P2Y1, P2Y2, P2Y4,
P2Y6, P2Y11). We were not able to detect a message codifying for these receptors (data not shown). More controversial results were obtained when primers designed to amplify the seven receptors of the P2X family were utilized. The expression of three receptors of the P2X family, P2X1, P2X5 (data not shown) and P2X7 (Fig. 5B, left panel), was identified at mRNA level. Since the fact that only rather high concentrations of ATP (1 mM and above) were required to induce the typical phosphorylation pattern supports the involvement of the P2X7 receptor (22, 23, 26), our major interest was focused on the P2X7 subunit. Therefore, we performed Western blotting experiments with antibodies to the P2X7 receptor to confirm the presence of the P2X7 protein in the cell lysates (Fig. 5B, right panel).

As a next step, a number of various agonists and antagonists were used to assess the involvement of each P2X receptor in the ATP-mediated signaling. Reportedly, suramine antagonizes the effect of ATP on the P2X purinoreceptors, and was shown at concentration of 30 µM to block ATP-induced calcium influx (7, 59). Pretreatment of Jurkat cells with suramine at concentration 30 µM for 30 min prior to stimulation with ATP abolished the phosphorylation of MAP and p56\textsuperscript{ck} kinases (Fig. 5A, first and third panels). Treatment of Jurkat cells with 100 µM Bz-ATP, which is believed to be a more potent agonist for the P2X7 receptors than ATP (7, 26), resulted in the increase of phosphorylation of ERK as well as p56\textsuperscript{ck} kinase at levels similar to those induced by ATP (Fig. 5A). Further, we employed KN-62, an isoquinoline derivative and inhibitor of CaM kinase II, which is widely used as a most potent and selective antagonist of the P2X7 receptor (45). In addition, another effective inhibitor of the P2X7 receptor, oATP, was used (46), although data are available indicating multiple P2X receptor targets for this agent (26). Pretreatment of Jurkat cells with KN-62 (1 µM) for 5 min or with oATP
(300 µM) for 30 min prior to stimulation with ATP was able to abrogate the activation of the MAP and p56\textsuperscript{\textit{lk}} kinases (Fig. 5A, first and third panels). For loading control, we detected ERK or p56\textsuperscript{\textit{lk}} kinase on the same membranes after stripping (Fig. 5A, second and forth panels).

It was repeatedly reported that signal transduction through the P2X7 receptor is associated with Ca\textsuperscript{2+} influx across the cellular membrane (5, 32), and may also open a non-selective pore capable of allowing uptake of low molecular weight hydrophilic solutes (up to 900 Da), such as Lucifer yellow and ethidium bromide (7, 27). Therefore, we investigated whether these effects take place in stimulated with ATP Jurkat cells. ATP at various concentrations (1-3 mM) was not able to induce uptake of Lucifer yellow (Fig. 5C) or ethidium bromide (data not shown) by Jurkat cells. Human macrophages were used as a positive control. Notwithstanding, as shown in Fig. 5D, both ATP and Bz-ATP were able to induce transmembrane Ca\textsuperscript{2+} influx and this effect was completely abrogated by KN-62, thus providing a further support for the identification of the P2X7 receptor as responsible for the ATP-induced effects in Jurkat cells. In addition, as mentioned above, chelation of extracellular Ca\textsuperscript{2+} in the culture medium by EGTA was able to abrogate the ATP-mediated activation of p56\textsuperscript{\textit{lk}} and MAP kinases. Taken together, RT-PCR and pharmacological selectivity data indicate the P2X7 receptor as a most probable candidate responsible for the induction of the downstream signaling events after stimulation of Jurkat cells with ATP.

Extracellular ATP stimulates the expression of c-Jun and c-Fos proteins and activation of AP-1 — Numerous studies have focused on the regulation of transcription factors by members of MAP kinase family. AP-1 proteins have been identified as...
substrates of the MAP kinases (39, 60, 61). The MAP kinase cascade plays a role in the stimulation of fos gene products, which heterodimerize with Jun proteins to form more stable AP-1 dimers (60). A major role in jun induction is played by JNK, which phosphorylates and enhances the transcriptional activity of two major factors, c-Jun and ATF2 (60). The observed activation of p56^{lck} kinase and MAPK pathway in Jurkat cells after ATP treatment strongly invites one to explore further the changes in the activity of transcription factors. AP-1 transcription factor usually consists of Jun/Fos heterodimers (62, 63). To understand, whether AP-1 activity is regulated by the activated MAP kinases upon ATP treatment, we studied first the changes in the expression of AP-1 components c-Jun and c-Fos, followed by the evaluation of AP-1 DNA binding activity. Jurkat cells were stimulated with ATP for different time intervals and assayed for the expression of c-Jun and c-Fos proteins by SDS-PAGE. As shown in Fig. 6A (upper and middle panels), non-activated cells express both proteins at relatively high level. However, stimulation of Jurkat cells by ATP resulted in a further increase in the expression of both c-Jun and c-Fos and, likely, also in an enhanced phosphorylation of c-Jun, as shown by the appearance of shifted up bands of slightly higher molecular weight (Fig. 6A), although this speculation requires further proof. The expression of another transcription factor, c-Myc, which served as a control, remained at the same level (Fig. 6A, lower panel).

Finally, nuclear extracts from activated cells were analyzed for AP-1/DNA binding activity using oligonucleotides carrying a consensus for an AP-1 binding site. ATP induced an enhancement in the AP-1/DNA-binding activity, which was already detectable within the first 30 min and continued to increase, reaching the peak to 3rd h of activation (Fig. 6B). To determine the composition of ATP-induced AP-1 complex, nuclear extracts from ATP-treated Jurkat cells were incubated with different antibodies,
and then assayed for AP-1 by EMSA. The ability of antibodies against c-Fos and c-Jun to supershift the band to the higher molecular weight position, as shown in Fig. 6C, suggested that these two proteins are the part of activated AP-1 complex. Irrelevant antibodies (anti-cyclin D) had no effect on the mobility of AP-1. Specificity of the AP-1-DNA complexes was demonstrated by competition assays where the addition of “cold” AP-1 oligonucleotides inhibited the formation of AP-1/DNA complex, while mutated AP-1-consensus motif oligonucleotides failed to show any binding. Taken together, these results indicate that ATP induces the increase in the expression of c-Jun/c-Fos proteins and enhances AP-1/DNA-binding activity in Jurkat cells.

**Extracellular ATP down-regulates NF-κB transcription factor** – The NF-κB family of transcription factors controls the expression of numerous genes involved in T cell function and can be activated in response to a broad variety of stimuli. Many agents that activate NF-κB also activate AP-1 transcription factor (57, 64). Therefore, as a next step, we wanted to study the effect of ATP on NF-κB activity. Jurkat cell were treated for different time intervals with ATP and nuclear extracts were examined for NF-κB DNA binding activity by EMSA. Fig. 7A shows, that ATP transiently inhibits NF-κB binding activity within 1-2 h of treatment. To prove that the retarded band visualized by EMSA is NF-κB, we incubated nuclear extracts with antibodies against p50 (NF-κB1), p65 (Rel A) or c-Rel, and then performed EMSA. Antibodies against p50 and p65 of NF-κB supershifted the band to the higher molecular weight position (Fig. 7B), suggesting that ATP-activated complex consists of these two subunits. Either anti-c-Rel or irrelevant antibodies (anti-cyclin D) had no effect on the mobility of NF-κB. Specificity of binding
was proved using EMSA with “cold” NF-κB oligonucleotides. Incubation of nuclear extracts with unlabelled oligonucleotides completely blocked NF-κB/DNA complex formation (Fig. 7B) and mutated NF-κB consensus motif oligonucleotides failed to bind NF-κB. Therefore, extracellular ATP is able to down-regulate NF-κB transcription factor in Jurkat cells.

Effects of extracellular ATP on p56\textsuperscript{lek}-deficient JCaM1 cells - To study the possible role of p56\textsuperscript{lek} in ATP-mediated activation of MAP kinases and up-regulation of AP-1, we used JCaM1 cell line, a derivative of Jurkat cells deficient in p56\textsuperscript{lek} expression, JCaM1 cells reconstituted and stably expressing p56\textsuperscript{lek} (JCaM1/Lck+) or transfected with a pBP1 mock plasmid (JCaM/vector+). As shown in Fig. 8A, JCaM1 cells as well as JCaM/vector+, do not express p56\textsuperscript{lek} kinase. JCaM1/Lck+ cells have restored expression of p56\textsuperscript{lek} at the level similar to that of Jurkat cells. These three cell lines exhibit the same pattern of the P2X7 receptor expression at the mRNA and protein level as Jurkat cells, as evaluated by RT-PCR and Western blotting, respectively (Fig. 8B and C). Absence of p56\textsuperscript{lek} in JCaM1 and JCaM/vector+ cells did not affect ATP-mediated Ca\textsuperscript{2+} influx as shown in Fig. 8D, while KN-62, an antagonist of the P2X7 receptor, was able to completely abrogate this effect in all cell lines used (data not shown). In addition, ATP was not able to trigger uptake of Lucifer yellow or ethidium bromide by JCaM1, JCaM1/Lck+ or JCaM/vector+ cells (data not shown).

Further, JCaM1 cell lines described above were utilized to study the importance of p56\textsuperscript{lek} for the ATP-induced intracellular events. In striking contrast, stimulation with ATP of JCaM1 and JCaM/vector+ cells did not result in the phosphorylation of ERK and JNK, increased expression of c-Jun and c-Fos proteins (Fig. 9), and AP-1 activation, but
induced \( \text{Ca}^{2+} \) influx across the plasma membrane (data not shown). However, JCaM1/Lck+ cells displayed a phosphorylation pattern, which resembles that of Jurkat cells, namely the activation of MAP kinases (Fig. 9) and increased expression of c-Jun and c-Fos (data not shown). Thus, these results strongly suggest that ATP-mediated effects in JCaM1 cells, such as ERK and JNK phosphorylation, increased expression of c-Jun and c-Fos proteins and activation of AP-1, but not \( \text{Ca}^{2+} \) influx, are dependent on the proper expression and function of p56\(^{\text{lk}}\) kinase.

Extracellular ATP stimulates proliferation of Jurkat cells and increases transcription of IL-2 – To understand the biological significance of the described intracellular events in response to ATP, we evaluated the consequences of activation of the P2X7 by ATP on Jurkat cell survival and growth. Given the well-documented ability of ATP to induce cell death via both apoptotic or necrotic mechanisms in many cell types (28, 31-34), we wanted to test whether such effect could also be observed in Jurkat and JCaM1 cells. The cells were cultured in presence of 3 mM ATP for 24 or 48 h, and the cell viability was assessed on 24, 48 and 72 h by exclusion of propidium iodide. Additionally, number of the apoptotic cells was evaluated by flow cytometry analysis using FITC-conjugated anti-Annexin V antibody staining. As shown in Fig. 10A and B, ATP had no influence on cell survival and the number of apoptotic cells. About 96% of the cells remained alive after 24, 48 and 72 h. Next, we evaluated the proliferation activity of Jurkat cells in response to ATP. As shown on Fig. 10C, ATP was able to stimulate the proliferation of Jurkat cells in dose-dependent manner and ATP antagonist, oATP, was able to block the observed proliferation response. This effect, however, was detectable not only in Jurkat cells but also, although to a slightly lesser extent, in p56\(^{\text{lk}}\)-
deficient JCaM1 cells, which do not exhibit the characteristic phosphorylation pattern in response to extracellular ATP, indicating the proliferation of the cells in response to the ATP treatment as a more general phenomenon. This finding strongly invited to explore further the biological relevance of the activation of p56\(^{ck}\) and MAP kinases in Jurkat cells. Therefore, as a next step, we tested by semi-quantitative RT-PCR a panel of various cytokines (e.g. IL-1\(\beta\), IL-2, IL-4, IL-7, IL-15 and IL-18) for possible changes in the level of transcription. Among these, only transcript codifying for IL-2 was clearly up-regulated (Fig. 10D, upper panel). Noteworthy, the MAP kinase pathway and activation of AP-1 transcription factor was reported by several groups to be required for IL-2 production (65, 66). Unstimulated Jurkat and JCaM1 cells showed a weak transcription of IL-2 cytokine. However, stimulation with 3 mM ATP induced an increase in amount of IL-2 transcript in Jurkat cells already after 3 h of ATP action, reaching its peak within 24 h of stimulation, while in JCaM1 cells IL-2 transcription remained at the same level throughout the time-course of the experiment. Taken together, these results indicate that extracellular ATP has ability to induce proliferation of both Jurkat and JCaM1 cell and, presumably through the activation of p56\(^{ck}\), MAP kinases and AP-1, to selectively up-regulate IL-2 at transcriptional level in Jurkat cells alone.
DISCUSSION

Despite the obvious significance and broad distribution of cell surface receptors for extracellular nucleotides in many cellular systems, including immune, intracellular signaling pathways induced by extracellular ATP in lymphocytes are not understood well. This study provides evidence that extracellular ATP is able to induce activation of p56\textsuperscript{ck} and MAP kinases in Jurkat cells, followed by up-regulation of AP-1 and down-regulation of NF-κB transcription factors. By using p56\textsuperscript{ck}-deficient cells, we demonstrate that p56\textsuperscript{ck} is required for the ATP-mediated downstream signaling, while the transfection with p56\textsuperscript{ck} gene reconstituted the ATP-induced cellular responses. To our knowledge, this is the first report to indicate that p56\textsuperscript{ck} kinase is required for ATP-mediated activation of AP-1, ERK and JNK, stimulation of IL-2, and down-regulation of NF-κB in Jurkat cells.

The ability of Jurkat cells to proliferate in response to extracellular ATP, as shown by increased \[^{3}H\]thymidine incorporation, confirms the existence of functional purinoreceptors on the cell surface. Stimulation of DNA synthesis by extracellular nucleotides in similar cell model was already documented (67). The apparent lack of a P2X7-dependent cell death response in this T cell line is intriguing and deserves further exploration in follow-up experiments. Interestingly, transfection of P2X7 DNA into lymphoid cells lacking endogenous P2X7 receptors, K562 and LG14, was able to enhance cell proliferation in the absence of exogenous growth factors (31). Furthermore, tumor transformation may lead to an increase in expression of the P2X7 receptor as well as to a release of considerably higher amount of extracellular ATP (68). The possible increase in proliferation rate, which expression of the P2X7 receptor appears to bestow
upon the cells may serve as a factor that confers a selective advantage and enhances their survival. In our experiments, however, both Jurkat and JCaM1 cells were able to proliferate after ATP stimulation. Therefore, we made an attempt to identify the biological significance of the observed signaling pathway in Jurkat cells more precisely. Reportedly, ATP stimulation may trigger a number of biologically relevant processes, such as cytokine production, cell proliferation or apoptosis (26, 29-34). Semi-quantitative RT-PCR showed that the up-regulation of IL-2 on transcriptional level was already detectable after first three hours of ATP treatment, continuing to increase with the time course and reaching its peak after 24 h of stimulation. In striking contrast, JCaM1 cells exhibited no changes in expression pattern of IL-2. Based on these findings, one might conclude that the up-regulated IL-2 transcription in Jurkat, but not JCaM1 cells results as a direct consequence of the activation of the p56\textsuperscript{lck}, MAP kinases and AP-1, thus indicating the biological significance of the particular signaling pathway involved.

The nucleotide and pharmacological selectivity data strongly suggest that the ATP-induced downstream intracellular signaling events are the result of activation of the P2X7 receptor. The involvement of P1 receptors was ruled out on the basis of the absence of an effect of adenosine, which can act as agonist at these receptors. Further, only millimolar concentrations of extracellular ATP had capacity to induce the activation of signaling molecules, while ADP, AMP, UTP and GTP were not able to trigger such changes in the phosphorylation pattern (data not shown). Next, the fact that the P2X7 receptor has capacity to trigger a long-lasting transmembrane \( \text{Ca}^{2+} \) influx complies with our observation that ATP-induced intracellular signaling events in Jurkat cells were wholly dependent on cytoplasmic influx of extracellular \( \text{Ca}^{2+} \) ions, as shown by the ability of EGTA, a chelator of divalent cations, to inhibit the protein phosphorylation via
depletion of Ca^{2+} ions in the culture medium prior to the ATP treatment. Noteworthy, the presence of mRNA for the P2X1, P2X4 and, particularly, for the P2X7 receptors was reported to be prominent in many immune cells (7, 20, 21). Thus, we tested by RT-PCR expression of messages corresponding to the mammalian metabotropic P2Y receptors and to the ionotropic P2X receptors, with a particular interest to expression of the P2X7 receptor. RT-PCR confirmed the presence of mRNA for the P2X7, P2X1 and P2X5 receptors (data not shown) in Jurkat and JCaM1 cells, but failed to detect messages corresponding to the other receptors of P2X family (P2X2-4 and P2X6) as well as to the P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptors. The expression of the P2X7 protein in the cells was also confirmed by Western blotting. As a next step, we evaluated the pharmacological selectivity characteristics, to resolve the P2X receptor subtype underlying the intracellular consequences of the ATP action. First, oATP has been extensively utilized as an effective ATP antagonist, which covalently binds to and inhibits the P2X7 receptor (46), although recent data indicates that this agent may not differentiate between multiple P2X receptor targets (26). Therefore, we also used KN-62, an isoquinoline derivative and a CaM kinase inhibitor, which exhibits a strong species-dependent sensitivity for human P2X7 and is widely used as a most potent and selective antagonist of the P2X7 receptor (45). The action of KN-62 upon the P2X7 does not involve its inhibitory properties on calmodulin kinase in short-term studies; however, prolonged exposures to KN-62 require cautious interpretation, since it may also result in the inhibition of the CaM kinase II, and is thus unsuited for long-term studies on cell proliferation. Second, we used a well-known agonist of the P2X7 receptor, Bz-ATP, which is believed to be more potent than ATP and can mimic its action at lower concentrations (below 1mM) (7). Pretreatment of the cells with oATP, KN-62, or
suramin abrogated ATP-induced phosphorylation of p56lk and MAP kinases, whereas treatment with Bz-ATP mimicked ATP effects, resulting in the similar phosphorylation pattern. The ability of Bz-ATP to induce Ca^{2+} influx in the Jurkat cell line used in this study appears to be in a controversy with a recent report of Vigne et al (69), where Bz-ATP (up to 100 µM) had no effect by itself to increase Ca^{2+} in Jurkat cells and served as an antagonist of human and rat P2Y1 receptors. It should, however, be mentioned that expression of the purinoreceptors may vary considerably among different clonal variants of the same cell line maintained under different culture conditions, thus favoring different selection pressures on the cells that may account for the loss of the original morphological phenotype due to phenotypic instability, as it was already shown for PC12 cells (54). Therefore, the diverse pattern of purinoreceptor sensitivity among cell line batches utilized in different laboratories might contribute to a distinct pattern of response to extracellular nucleotides (7). The requirement of ATP in millimolar range and the ability of submillimolar amounts of Bz-ATP to mimic its effects, taken together with inhibitory properties of KN-62 and αATP, are typical for the pharmacological profile of the P2X7 receptor, strongly suggesting a role for it as the sole mediator of the following signaling events in Jurkat cells.

p56lk kinase activity is essential for the initiation of downstream signaling pathways in T cells (51, 52). The phosphorylation state of Y394 stimulates the catalytic activity of p56lk (52, 53), whereas phosphorylation of Y505 helps to stabilize it in a relatively inactive, “closed” biological conformation, resulting in binding of pY505 to the SH2 domain of p56lk (51, 52). Nevertheless, positive regulation of p56lk by phosphorylation at Y394 was shown to be dominant over any inhibition induced by Y505 phosphorylation (53). ATP at high concentrations, and its analog Bz-ATP, both were able
to induce a rapid and transient tyrosine phosphorylation of several proteins in Jurkat cells, including p56\textsuperscript{ck} kinase (Fig. 1 and 5), and this effect was dependent on the presence of calcium in the extracellular medium. Through the use of phosphorylation-state-specific antibodies, we have shown that p56\textsuperscript{ck} was phosphorylated at Y394, but not at Y505, resulting presumably in the kinase activation. To prove this further, we performed a kinase assay which confirmed catalytic activation of p56\textsuperscript{ck} kinase by specific phosphorylation of TCR\(\zeta\)-chain /GST fusion protein as an endogenous substrate for the p56\textsuperscript{ck}.

The p56\textsuperscript{ck} SH3 domain was shown to play an important role in the activation of MEK and ERK following TCR stimulation (38). In addition, p56\textsuperscript{ck} kinase was recently shown to be required for the ceramide-induced and HIV-tat protein-induced activation of MEK and JNK, AP-1 and NF-\(\kappa\)B transcription factors in Jurkat cells (59, 70). Importantly, calcium ionophores can trigger activation of p56\textsuperscript{ck} and ERK1/2 in Jurkat cells via calmodulin and CaM kinase-dependent mechanism (44). Furthermore, one signaling pathway that is required for IL-2 production is MAP kinase cascade through the action of the transcription factor AP-1 (65, 66). The involvement of p56\textsuperscript{ck} in TCR signaling appears to be complex, indicating its selective requirement for the activation of downstream signaling pathways, such as MAPK pathway, despite the activation of ZAP-70 and PIP\textsubscript{2} in p56\textsuperscript{ck} deficient JCaM1 cell line (38). To examine the role of p56\textsuperscript{ck} in the ATP-induced downstream signaling more precisely, we also employed JCaM1 cells, a genetic variant of Jurkat cell line, deficient in p56\textsuperscript{ck} protein expression due to the deletion of exon 7 (36, 38). ATP failed to stimulate activation of MAP kinases ERK1/2 and JNK1/2, and to induce an increase in the DNA binding activity of AP-1 in these cells, whereas the transfection with the gene coding for p56\textsuperscript{ck} reconstituted the ATP-
induced cellular responses. Thus, the selective disruption of the MAP kinase pathway in p56\textsuperscript{Lck}-deficient cells demonstrates that exists an additional requirement of this kinase for the activation of downstream signaling events, and suggesting an upstream location for p56\textsuperscript{Lck} in the ATP signaling pathway. Noteworthy, the activation of p56\textsuperscript{Lck} kinase upon the ATP action does not seem to depend on, or be preceded by, the activation of other mediator of Ca\textsuperscript{2+} signaling, Pyk2 (data not shown), nor does it require the involvement of calmodulin and CaM kinase, as shown by inability of both calmidazolium, a calmodulin antagonist, and CaM kinase inhibitor KN-93, to abrogate the activation of p56\textsuperscript{Lck} and ERK, although CaM kinase appears to participate, at least partially, in the observed activation of ERK. Furthermore, a specific inhibitor of MEK, PD098059, had capacity to abrogate phosphorylation of ERK in response to ATP, while not affecting activation of p56\textsuperscript{Lck} kinase. The fact that extracellular ATP, acting over the P2X7 receptor, is able to stimulate p56\textsuperscript{Lck}-dependent phosphorylation and activation of ERK and JNK, but not p38 kinase, demonstrates a novel pathway by which this agent can modulate the intracellular responses on various levels. The precise molecular mechanisms underlying the ability of ATP to trigger the activation of p56\textsuperscript{Lck} and MAP kinases through the P2X7 receptor remain unknown. Albeit an increase in the intracellular level of Ca\textsuperscript{2+} appears to be critical for the subsequent signaling events, the inability of the P2X7 to mediate activation of the MAP kinases in the absence of p56\textsuperscript{Lck} in JCaM1 cells unambiguously indicates that simple rise in the level of intracellular Ca\textsuperscript{2+} ions is not enough. Though MEK seems to contribute to the observed activation of ERK, it is not clear yet how p56\textsuperscript{Lck} kinase might couple to MEK and, in general, mediate its effects on activation of the MAP kinases and AP-1 in response to ATP, and future studies are required to address this question in more detail.
A plethora of physiological and pathological stimuli is able to induce and activate a group of DNA binding proteins that form AP-1 complex, which binds to AP-1 regulatory elements in the promoter and enhancer regions of numerous mammalian genes, including IL-2 (65, 66). In unstimulated T-cells, AP-1 expression is low or undetectable, with a rapid induction of AP-1 activity after T-cell stimulation, such as TCR/CD3 binding and action of cytokines or hormones (71-73). The ATP-mediated induction of c-Jun and c-Fos expression, followed by the up-regulation of DNA binding activity of AP-1 may mirror, at least in part, the initial steps in T cell activation, which are usually orchestrated through the recruitment of several transcriptional factors, such as AP-1, NFAT and NF-κB (73). AP-1 probe and nuclear proteins were able to form complexes within 30 min from the action of ATP and were still present after 3 h. The supershifting experiments showed that c-Jun and c-Fos proteins are the constituents of the activated AP-1 transcription complex. As already mentioned above, the MAP kinase cascade is crucial for AP-1 induction and IL-2 gene expression in T cells (65, 66), since it regulates tightly at the transcriptional and post-translation levels AP-1 activity, increasing its stability and binding activity through phosphorylation (73, 74).

Another important inducible transcription factor that plays a pivotal role in many cellular responses to environmental changes is NF-κB. The factor is ubiquitously found as an inactive complex in the cytoplasm bound to its inhibitory subunit IκB, and plays an important role in cell growth, differentiation, development, apoptosis, inflammation and immune responses (75, 76). NF-κB is also able to function in concert with other transcription factors, such as AP-1 (57). In contrast to the activation of AP-1, we detected simultaneous, although slightly accelerated decrease in DNA-binding activity of NF-κB p65 (Rel A) and p50 proteins, as evidenced by the supershift experiments, within the first
hour of the ATP action. The level of NF-κB proteins slowly returned nearly to basal after 3 h. It should be mentioned that we did not observe an increase in the expression of IκBα upon ATP treatment (E. Bulanova and V. Budagian, unpublished observation). The biological relevance of such opposite responses as the activation of AP-1 and down-regulation of NF-κB is not clear and future experiments must be aimed to shed light on this issue.

Taken together, our results demonstrate that ATP induces Ca\(^{2+}\)-dependent activation of p56\(^{\text{lck}}\) tyrosine kinase, which is prerequisite for the activation of ERK and JNK MAP kinases, up-regulates transcriptional factor AP-1 and stimulates IL-2 transcription, while down-regulating DNA binding activity of NF-κB. Thus, the intracellular events occurring in Jurkat cells in response to extracellular ATP may represent a heretofore-unappreciated mechanism of its ability to modulate cellular processes within the immune system.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. **ATP-induced tyrosine phosphorylation of intracellular proteins.** Jurkat cells were serum starved for 3 h and incubated at 37°C in the absence (control) or presence of 3 mM ATP for different time intervals, as indicated. Proteins were precipitated using biotinylated anti-pTyr antibodies (RC20B). A, phosphorylated proteins were detected in immunoblotting using RC20H antibodies. B, the same blot was stripped with 62.5 mM Tris-HCl buffer, pH 6.7, containing 2% SDS and 100 mM β-mercaptoethanol overnight at 4°C and re-probed with anti-Lck antibodies. C, lysates from ATP-activated Jurkat cells were precipitated with anti-Lck antibodies and immunoblotting with anti-pTyr antibodies was performed. D, detection of p56^lck after immunoprecipitation as a control of loading. Positions of phosphorylated proteins are indicated on the left and phosphorylated p56^lck is indicated on the right. The picture presents one of three independent experiments with same results.

Fig. 2. **ATP stimulates phosphorylation of Y394, but not Y505 of p56^lck and increases its kinase activity.** Serum-starved Jurkat cells were stimulated with 3 mM ATP for the indicated time periods at 37°C. A, p56^lck kinase was precipitated from lysates and phosphorylation of Y394 (upper panel) and Y505 (middle panel) was detected using anti-Src (pY416) and anti-Lck (pY505) antibodies, respectively. Lower panel shows the loading control. B, in vitro kinase assay of p56^lck immunoprecipitates from ATP-activated Jurkat cells. Immunocomplexes were incubated with [γ^32P]ATP and GST-ζTCR fusion protein as an exogenous substrate (upper panel). A fraction of each immunoprecipitate was immunoblotted with anti-Lck antibodies to prove the equal loading (lower panel). C,
Jurkat cells were treated with 3 mM ATP for 5 min and the activation of p56^{lck} was assessed in absence or presence of calcium chelator EGTA. The chelation of Ca^{2+} ions in extracellular medium was performed by adding to the medium of 2 mM EGTA. The results are representative of three independent experiments.

Fig. 3. **ATP induces phosphorylation and activation of ERK and JNK, but not p38 kinase.** Serum-starved Jurkat cells were stimulated at 37°C with 3 mM ATP for the indicated times and lysed. Then, equal amounts of the proteins were loaded onto 10% gel and SDS-PAGE was performed. Phosphorylation of ERK 1/2, JNK 1/2 and p38 kinases at specific sites was detected using phosphospecific antibodies. Positions of phosphorylated kinases are indicated on the right. The equal amounts of ERK, JNK and p38 are shown as controls for loading. The data shown are representative of three separate experiments with similar results.

Fig. 4. **Effect of PD098059, calmidazolium and KN-93 on phosphorylation of ERK and p56^{lck} kinase.** A. Serum-starved Jurkat cells were incubated at 37°C with 50 µM of MEK inhibitor, PD98059, for 90 min prior to the ATP treatment and stimulated thereafter for different time intervals with 3 mM ATP. Cells were analyzed for ERK phosphorylation using specific anti-pERK antibodies (*upper panel*). Phosphorylation of p56^{lck} was evaluated by immunoprecipitaion with anti-Lck antibodies and Western blotting with anti-Src(pY416) antibodies (*third panel*). Equal loading of ERK and precipitation of p56^{lck} are shown at *second and forth panels*, respectively. B. Jurkat cells were serum-starved and treated at 37°C with 50 mM calmidazolium or 1 µM KN-93, for 30 min prior to the activation with 3 mM ATP (5 and 15 min). Then, the membranes
were probed with activation state-specific anti-pERK antibodies to determine the phosphorylation of ERK, whereas the activation of \( p56^{\text{lck}} \) was detected by immunoprecipitation with anti-Lck antibodies and Western blotting with anti-Src(pY416) antibodies (third panel). Pretreatment of cells with DMSO (1:100) or KN-92 (1 \( \mu \text{M} \)), and activation with 500 nM of calcium ionophore A 23187 were used as controls. Equal loading of ERK and precipitation of \( p56^{\text{lck}} \) are shown at second and forth panels.

Fig. 5. ATP induces the phosphorylation of ERK, \( p56^{\text{lck}} \) kinase and \( \text{Ca}^{2+} \) influx in Jurkat cells through the P2X7 receptors but does not influence cell membrane permeability. A, Jurkat cells were serum-starved for 3 h and stimulated for 15 min at 37\(^\circ\)C with 3 mM ATP or with appropriate purinoreceptor agonists (Bz-ATP, adenosine or UTP) at concentration of 100 \( \mu \text{M} \). Additionally, the cells were incubated for 30 min with 30 \( \mu \text{M} \) suramine, for 15 min with 300 \( \mu \text{M} \) oATP or for 5 min with 1 \( \mu \text{M} \) KN-62 prior to stimulation with 3 mM of ATP for the next 15 min. Then, the cell lysates were analyzed for the phosphorylation of ERK and \( p56^{\text{lck}} \) kinase. B, total RNA extracted from Jurkat cells was reverse transcribed into cDNA and PCR using primers to the P2X7 receptor was performed (left panel). The amplified products from this reaction were analyzed by 1.5% agarose gel electrophoresis. A mock PCR (no cDNA) was included as a negative control. Expression of the P2X7 receptor was analyzed in Jurkat cell lysates using specific anti-P2X7 antibodies (right panel). Position of the P2X7 receptor is indicated on the right. C. Membrane permeabilization was analyzed in Jurkat cells by fluorescence microscopy (right panels). Cells were incubated in the absence (control) or presence of 3 mM ATP for 15 min in a buffer containing 1 mg/ml Lucifer yellow, washed and examined under phase contrast (left panels) or fluorescent light using a
fluorescent filter (*right panels*). Membrane permeability induced by ATP in human macrophages is shown as a control. Cells were observed with 40X objective. *D*, ATP- and Bz-ATP-mediated Ca\(^{2+}\) influx is inhibited by KN-62 in Jurkat cells. Ca\(^{2+}\) influx in Jurkat cells was measured as described in *Materials and Methods* after stimulation with 3 mM ATP or 100 µM Bz-ATP in control cells (*left graphs*) or after treatment with 1 µM KN-62 for 5 min (*right graphs*). The data shown are representative of three independent experiments with similar results.

Fig. 6. **ATP treatment stimulates an increase in the expression of c-Jun and c-Fos and enhances AP-1 DNA-binding activity.** *A*, serum-starved Jurkat cells were stimulated with 3 mM ATP, lysed, and proteins were analyzed by Western blotting. Then, the membranes were probed with anti-c-Jun (p39) and anti-c-Fos (p60) antibodies. Expression of c-Myc (p65) was assayed at the same time for comparison and loading control. *B*, nuclear extracts from the stimulated cells were incubated with double-stranded oligonucleotides with an AP-1 consensus site, followed by electrophoretic mobility shift assay (EMSA) as described in *Materials and Methods*. Position of AP-1/DNA binding complex is indicated on the *right*. *C*, nuclear extracts were prepared from treated for 3 h with ATP (3 mM) Jurkat cells and incubated with c-Jun, c-Fos and irrelevant anti-cyclin D1 antibodies for 15 min and then assayed for AP-1 as described in *Materials and Methods*. Competition with unlabelled intact or mutated AP-1 probes shows specificity of binding.

Fig. 7. **Effect of ATP on DNA-binding activity of NF-κB.** *A*, Jurkat cells were treated with ATP for different time intervals as shown. Then, nuclear extracts were incubated for
1 h with $^{32}$P-labeled double-stranded oligonucleotides with consensus sequence for NF-κB and evaluated by EMSA as described in Materials and Methods. Position of NF-κB/DNA complex is indicated on the right. B, the nuclear extracts from non-activated Jurkat cells were incubated for 15 min with indicated antibodies and then for additional 1 h with NF-κB consensus oligonucleotides and complexes were analyzed by EMSA. Controls with competing unlabelled intact or mutated probes for NF-κB confirm specificity of binding. The data shown are representative of three independent experiments with similar results.

Fig. 8. Expression of p56$^{lck}$ kinase and the P2X7 receptor, and Ca$^{2+}$ influx in response to ATP in Jurkat and JCaM1 cells. A, expression of Lck protein was detected by Western blotting using anti-Lck antibodies in Jurkat cells, JCaM1 cells deficient for p56$^{lck}$ expression, JCaM1 cells reconstituted for p56$^{lck}$ expression (JCaM1/Lck+), and JCaM1 cells transfected with a mock plasmid (JCaM/vector+). B, expression of the P2X7 receptor was detected by RT-PCR in all cell lines used. C, expression of the P2X7 protein was analyzed in cell lysates using anti-P2X7 antibodies. D, cells were stimulated with 3 mM of ATP and Ca$^{2+}$ influx was measured as described in Materials and Methods. A representative of three independent experiments is shown.

Fig. 9. ATP failed to stimulate phosphorylation of ERK and JNK and expression of c-Jun and c-Fos in p56$^{lck}$-deficient JCaM1 cells. A, Jurkat, JCaM1, JCaM1/Lck+ and JCaM/vector+ cells were serum starved for 3 h and activated with 3 mM ATP for 5 and 15 min at 37°C. Protein lysates were analyzed for ERK and JNK phosphorylation. Lower panel represents control of loading. B, Jurkat and JCaM1 cells were activated for
different time intervals and expression of c-Jun and c-Fos was detected by SDS-PAGE. Expression of c-Myc is shown for loading control (lower panel). Position of specific proteins is indicated on the right. The data shown represent three separate experiments, all of which yielded highly comparable results.

Fig. 10. ATP stimulates proliferation of Jurkat and JCaM1 cells and increases transcription of IL-2 in Jurkat cells. Jurkat and JCaM1 cells were incubated at concentration of 1 x 10^5 cells/ml in absence or presence of 3 mM ATP for indicated time intervals. Cell viability (A) was analyzed by propidium iodide exclusion and FACS analysis after 24, 48 and 72h, and percentage of apoptotic cells (B) was determined by Annexin-FITC staining after 24 and 48 h. C, Jurkat and JCaM1 cells were seeded in triplicates (10^5 cells/well) and incubated for 24 h with 3 mM ATP. Samples treated with oATP (300 µm) were cultured in the presence of this inhibitor throughout the experiment. Then, (³H)thymidine was added for additional 12 h. *- P<0.05 versus medium and ATP + oATP. B, Jurkat and JCaM1 cells were cultured for 3 or 24 h in the presence of 3 mM of extracellular ATP. Then, mRNA was extracted, reverse-transcribed to cDNA and amplified with respective primers (human IL-2, upper panel) for 35 cycles. KN-62 (1 µM) was used to inhibit the action of ATP. Beta-actin message was used to equalize the amount of cDNA used (lower panel). The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis. For semi-quantitative analysis, in addition to 35 cycles, 15 ml aliquots of the PCR product from 25 and 30 cycles were also evaluated. Picture shows the amplified bands after 35 cycles. A mock PCR (no cDNA) was included as a negative control. The data represent three separate experiments with comparable results.
A

B

C

D

Jurkat ATP

JCaM/Lck+

ATP

JCaM/vector+

ATP

[Ca^{2+}] \text{[M]}$

2 \text{ min}$
Mechanisms of Signal Transduction: Signaling through P2X7 receptor in human T cells involves p56LCK, MAP kinases, and transcription factors AP-1 and NF-kB

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