Supporting Online Material for

ATP Release Guides Neutrophil Chemotaxis via P2Y2 and A3 Receptors

Yu Chen, Ross Corriden, Yoshiaki Inoue, Linda Yip, Naoyuki Hashiguchi, Annelies Zinkernagel, Victor Nizet, Paul A. Insel, Wolfgang G. Junger*

*To whom correspondence should be addressed. E-mail: wjunger@ucsd.edu

Published 15 December 2006, *Science* **314**, 1792 (2006)

DOI: 10.1126/science.1132559

This PDF file includes:

Other Supporting Online Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/314/5806/1792/DC1)

Materials and Methods

Materials

Adenosine receptor analogs, nucleotides, ATPγS, fMLP, the A1 receptor agonist CPA (N6-cyclopentyladenosine), A2a & A2b receptor agonist CGS-21680 (2-p-[2-carboxyethyl] phenethylamino-5'-N-ethylcarboxamidoadenosine hydrobromide-hemicarbonate salt), A3 receptor agonist IB-MECA (N6-[3-Iodobenzyl]adenosine-5'-N-methyluronamide) and DMSO were obtained from Sigma-Aldrich Co. (St. Louis, MO). W-peptide (WKYMVM) was obtained from Phoenix Pharmaceuticals (Belmont, CA). Platelet activating factor-16 (PAF) and Interleukin 8 (IL-8) were from EMD Calbiochem (San Diego, CA). Microscope studies were performed using a Leica DMIRB microscope (Bensheim, Germany) connected to a Hamamatsu ORCA camera (Hamamatsu, Japan) and images were obtained using Openlab software (Improvision, Lexington, MA).

Cell culture and neutrophil isolation

Human neutrophils were isolated from peripheral blood of healthy adult volunteers as described previously (*23*). Homozygous $A3^{-/-}$ mice were provided by Merck Research Laboratories (West Point, PA), $P2Y2^{-/-}$ mice were obtained from Dr. Beverly H. Koller (University of North Carolina, Chapel Hill, North Carolina), and C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) served as wild-type controls. Bone marrow cells of 6-10 week old mice were obtained as described previously (*24*). Murine neutrophils were isolated by Percoll gradient separation (55/68% interface) and washed twice with HBSS before overnight incubation in RPMI (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (FCS). HL60 cells were cultured in Iscove's medium (ATCC, Manassas, VA) supplemented with 20% FCS. Differentiation of HL60 cells was accomplished by incubating cells with 1.3% DMSO seven days prior to experimentation.

HPLC analysis of extracellular nucleotides

Nucleotides released from neutrophils (10^7 cells in 250 µl) were analyzed by HPLC analysis as described previously (*9*), except that the HPLC gradient was slightly modified: 0-10 min, 5-100% elution buffer B; 10-13 min, 100% buffer B; 13-13.5 min, 100-5% buffer B; 13.5-15 min, 5% buffer B. Nucleotides were identified by their retention times and concentrations estimated using known standards.

Assay for visualization of ATP release

Neutrophils were incubated at 37°C in HBSS containing a mixture of 8 U/ml each of hexokinase and glucose 6-phosphate dehydrogenase and 5 mM NADP. In the presence of ATP, these enzymes catalyze the formation of NADPH, a fluorescent molecule that was visualized using fluorescence microscopy with an excitation wavelength of 340 nm and an emission at 460 nm. The fluorescence intensity of regions outside the cells was measured using Openlab software and ATP concentrations were estimated using ATP standard solutions of known concentrations.

Oxidative burst assays

Oxidative burst was measured by assessing superoxide formation using the cytochrome C reduction assay previously described (*9, 23*).

Transwell chemotaxis assays

Transwell assays were performed with 96-well MultiScreen MIC Plate containing a filter plate with a pore size of 3.0 μm (Millipore, Bedford, MA). As chemoattractant we used 1 nM fMLP, 100 nM PAF, 3% autologous zymosan-activated serum (C5a), 2 ng/ml IL-8 (for human neutrophils), or 100 nM W-peptide (for murine neutrophils) in each well (150 μl) of the lower receiver plate. A suspension of 100 μl human or murine neutrophils (1×10^7/ml) was added into each well of the upper filter plate. After 50 min incubation at 37°C, the upper filter plate was removed. For human neutrophil assays, the cells in the lower wells were collected and sedimented by centrifugation at 16,000 x g for 10 s at room temperature. Elastase activity of the lysed cell suspension in the lower well was used as an indicator of the number of migrated cells as previously described (*18*). Briefly, after removal of the remaining supernatants, we mixed cells with 160 μl of a buffer consisting of 50 mM Tris/HCl and 100 mM NaCl, pH 7.4 containing 0.05% (v/v) Triton X-100. Enzymatic reactions were started by the addition of the elastase-specific chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma) at a final concentration of 1 mM. After 30 min at room temperature, the change in optical density was measured at a wavelength of 405 nm. For murine cells, neutrophils migrated into the lower wells were counted by microscopy. As a control, each set of experiments included wells containing HBSS with 10% FCS without chemoattractant in the lower well. Each condition was tested in triplicate.

Chemotaxis assays by microscopy

Human neutrophils, HL60 cells, or murine bone marrow neutrophils (2.5×10^6/ml) were plated on 25 mm glass coverslips (Fisher Scientific, Pittsburgh, PA) coated with 50 μg/ml fibronectin and placed into a temperature-controlled stage incubator (Harvard Apparatus, Holliston, MA) at 37°C. Cells were pre-treated with reagents as described and exposed to a chemoattractant gradient field generated by slowly releasing 100 nM fMLP (for human neutrophils) or 100 nM W-peptide (for mouse bone marrow neutrophils) from a micropipette tip placed in proximity of the cells. Cell migration was tracked by obtaining 20 serial images at 25-s intervals. From these images, the migration paths of individual cells were plotted and the total distance traveled toward the point source of fMLP was measured for each cell. From these data we obtained migration speed and path deviation for each cell.

Cell labeling for fluorescence microscopy

Antibody staining of receptors was performed by plating neutrophils or differentiated HL60 cells (2.5×10^6/ml) on glass coverslips and the cells were stimulated with 100 nM fMLP for 10 min at 37°C. The cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS; Irvine Scientific) for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min, and labeled with antibody as described previously (*9*).

For actin staining, differentiated HL60 cells were plated on glass coverslips, stimulated with 100 nM fMLP for 10 min, fixed by treatment with 3.7% paraformaldehyde for 10 min, washed with PBS, and permeabilized by the addition of a 0.1% solution Triton X-100 of for 10 min at room temperature. Coverslips were blocked for 1 h using a blocking solution containing 4% BSA in PBS, and then stained with rhodamine phalloidin according to the supplier's instructions (Invitrogen, Carlsbad, CA).

Real-Time PCR

The relative level of expression of mRNA of all known mammalian P1 and P2 receptors in human neutrophils and HL60 cells were determined with Real-Time RT-PCR using neutrophils isolated from 3 individual human donors. Primers were designed based on human P1 and P2 receptor sequences obtained from Genbank using the Primer 3 program (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) and published data (*25*).

Table S1: Real-Time RT-PCR Primers

Receptor	Primer	Sequence (5' to 3')	Position	Accession Number	Size (bp)
A_1	S	GCGAGTTCGAGAAGGTCATC	916-1041	L22214	126
	AS	GCTGCTTGCGGATTAGGTAG			
A_{2A}	S	CTTGGGTTCTGAGGAAGCAG	1699-1840	NM_000675	142
	AS	TGCTCTGTGGAGACAAGGTG			
A_{2B}	S	ATGGAACCACGAATGAAAGC	808-866	NM_000676	59
	AS	GGGGACCACATTCTCAAAGA			
A_3	S	TGTTTGGCTGGAACATGAAA	728-793	L20463	66
	AS	ATTGGCATGAAAGGAAGGTG			
$P2X_1$	S	GCGTAATAAGAAGGTGGGCGTTA	513-535	NM_002558	111
	AS	GCCGCTCGAGGTCTGGTA	604-622		
$P2X_2$	S	CAGGTTTGCCAAATACTACAAGATCA	903-928	NM_170682	105
	AS	AACTTCCCGGCCTGTCCAT	1007-989		
$P2X_3$	S	TGTAGGGTGGGTTTTCTTGC	279-298	Y07683	73
	AS	GGTTACCACCGAGGACTCAA	332-351		
$P2X_4$	S	CATCATCCCCACTATGATCAACA	714-736	U87270	52
	AS	AGCACGGTCGCCATGC	761-765		
$P2X_5$	S	CGCTGGGGAAGCGGTTA	444-460	AF016709	100
	AS	GCACCAGGCAAAGATCTCACA	543-523		
$P2X_6$	S	CACTGCCGCTATGAACCACAA	693-713	AF065385	85
	AS	CGAAGGTCCCTCCAGCCTT	777-759		
$P2X_7$	S	ATCGGCTCAACCCTCTCCTAC	1981-2001	Y12854	155
	AS	CTGGAGTAAGTGTCGATGAGGAAG	2112-2135		
$P2Y_1$	S	CGTGCTGGTGTGGCTCATT	516-534	AY136752	68
	AS	GGACCCCGGTACCTGAGTAGA	563-583		
$P2Y_2$	S	CGGTGGACTTAGCTCTGAGG	2101-2120	NM_176072	80
	AS	GCCTCCAGATGGGTCTATGA	2161-2180		
$P2Y_4$	S	TGTCCTTTTCCTCACCTGCAT	546-566	X91852	64
	AS	TGCCCGAAGTGGGTGG	594-609		
$P2Y_6$	S	CCTGCCCACAGCCATCTT	753-770	X97058	68
	AS	GGCTGAGGTCATAGCAGACAGTG	798-820		
$P2Y_{11}$	S	GTTGGTGGCCAGTGGTGTG	1272-1290	BC009877	69

	AS	TTGAGCACCCGCATGATGT	1322-1340		
$P2Y_{12}$	S	AACTGGGAACAGGACCACTG	501-520	NM_022788	135
	AS	TAAATGGCCTGGTGGTCTTC	616-635		
$P2Y_{13}$	S	AGGGCTCATAGCCTTTGACA	512-531	NM_023914	139
	AS	GATCGTATTTGGCAGGGAGA	631-650		
$P2Y_{14}$	S	TCTTTTACGTGCCCAGCTCT	352-371	NM_014879	75
	AS	GGCTCATCACAAAGTCAGCA	407-426		
β-actin	S	CTCTTCCAGCCTTCCTTCCT	1266-1285	BC014861	100
	AS	AGCACTGTGTTGGCGTACAG	1346-1365		

"S" and "AS" refer to sense or anti-sense primers based on sequences obtained from GenBank.

Total RNA was extracted from $4x10^8$ neutrophils using Trizol (Invitrogen), treated with 1 U/μg total RNA DNase I (Invitrogen), and first strand cDNA was synthesized using Superscript II RNase H^- reverse transcriptase (Invitrogen), according to the manufacturer's instructions. A negative control sample, lacking reverse transcriptase, was prepared simultaneously. The amplification efficiency of each primer set was determined to ensure equal efficiency. Templates were generated by PCR using Platinum PCR Supermix (Invitrogen), containing 100 nM sense and anti-sense primers and 1 μl cDNA. PCR products were serially diluted 10^5 to 10^{11} fold and used as the template for Real-Time PCR with the Qiagen QuantiTect SYBR Green PCR kit. Reactions were amplified and subjected to melting curve analysis using DNA Engine Opticon 2 Real-Time PCR detection system (BioRad, Hercules, CA) and the following cycling parameters: 1 cycle at 95°C (10 min) and 45 cycles at 94°C (30 s), 60°C (30 s), 72°C (30 s). A standard curve was generated by plotting the threshold cycle (C_t) against the Log (initial starting quantity). The efficiency of each primer set was calculated using the slope of this curve and the following equation: Efficiency = $10^{(-1/slope)}$-1. P1 and P2 receptor gene expression levels were measured in samples using Real-Time PCR as described above (n = 3) and the comparative C_t method for relative quantification of gene expression, which was normalized against β-actin mRNA levels.

Determination of cell surface expression of A3 receptors by flow cytometry

Neutrophils (1×10^6) were stimulated with 100 nM fMLP at 37°C for different times. Samples were then placed on ice and stained with a rabbit anti-human A3 adenosine receptor antibody (Alpha Diagnostic, San Antonio, TX; 10 ng/ml) followed by a goat anti-rabbit IgG-FITC antibody (Sigma; at 1:100 dilution). The cells were washed with HBSS, fixed with sheath fluid containing 0.5% paraformaldehyde, and analyzed by flow cytometry using a BD FACSCalibur System (BD Biosciences, Rockville, MD) equipped with a 488 nm air-cooled argon-ion laser and interfaced to a computer equipped with Cellquest Pro software (BD Biosciences). Neutrophils were gated to exclude from analysis other cell types, debris, and aggregates. A total of 10^4 neutrophils per sample were analyzed.

Live cell imaging of P1 and P2 receptor localization

Using the mammalian expression vector pEGFP-N1 (Clontech Laboratories, Mountain View, CA), we generated plasmids encoding EGFP-fusion proteins of the human A3 and P2Y2 receptors using genes obtained from UMR cDNA Resource Center (Rolla, MO).

The constructs were transfected into HL60 cells by electroporation (5 μg/ml plasmid) using a pre-set protocol on the GenePulser II electroporation system (BioRad, Hercules, CA). After 48 h in complete Iscove's medium supplemented with 20% FCS, stably transfected clones were selected by the addition of 2% G418 (Invitrogen) and incubated under the same conditions for 4 weeks. Fluorescent imaging was carried out using the microscope assays described above.

Endothelial Transcytosis Assay

Human brain microvascular endothelial cells (hBMEC) immortalized by transfection with SV40 large T antigen were obtained from Kwang Sik Kim (Johns Hopkins University, Baltimore, Maryland, USA); these cells show the morphological and functional characteristics of primary brain endothelium. hBMEC were propagated in RPMI 1640 medium (Life Technologies and Co, Bedford, Massachusetts, USA) supplemented with nonessential amino acids +10% FBS + 10% Nu-Serum^{TM} IV (BD Biosciences, San Jose, CA) at 37°C in 5% CO_2. Polar hBMEC monolayers were established on collagen-coated Transwell^{TM} plates 6.5 mm in diameter and with 3 μm pores (BioCoat, BD Biosciences, San Jose, CA). Human neutrophils (10^6 cells in 100 μl HBSS) were added to the upper well of the transwell chamber and treated with either 10 U/ml ADA or 10 μM MRS 1191. As a chemotactic stimulus, either 2 ng/ml IL-8 or 6 x 10^5 CFU of *Staphylococcus epidermidis* (ATCC 12228) in 600 μl HBSS were added to the bottom of the transwell chamber. The plates were incubated for 160 min at 37°C in 5% CO_2 and numbers of neutrophils that crossed the monolayers were enumerated using microscopy. Integrity of the hBMEC monolayers was confirmed by impermeability to 4% Evans blue-labeled bovine serum albumin (BSA). Each assay condition was performed in triplicate and repeated three times with similar results.

Assessment of neutrophils migration in mouse peritonitis models

Male WT, P2Y2$^{-/-}$, and A3$^{-/-}$ mice (8-12 weeks old; 6 animals per group) were used. To determine the role of P2Y2 and A3 receptors in neutrophils migration *in vivo*, either 1 ml of W-peptide (1 nM), *Staphylococcus aureus* (10^8/ml), or vehicle (saline) was injected intraperitoneally. After 2 h (in the W-peptide groups) or 4 h (in the *S. aureus* groups), animals were euthanized. The peritoneal cavities were lavaged with 3 ml ice cold HBSS containing 1% mouse serum, and neutrophil numbers were counted.

Figure Legends

Supplemental Fig 1: *ATPase and alkaline phosphatase hydrolyze ATP and impair neutrophil chemotaxis.* (A) Effect of ATPase (porcine cerebral cortex) on the chemotaxis of human neutrophils towards fMLP assessed with the transwell assay. (B) Cell traces of human neutrophils migrating towards a micropipette tip containing 100 nM fMLP in the absence (left) or presence of 10 U/ml phosphatase (calf intestine; CIP). (C) Effect of the P2X specific P2 receptor antagonists 1-[N,O-bis(5-isoquinolinesulphonyl)-N -methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) and oxidized ATP (o-ATP) on neutrophil

chemotaxis using transwell assays with antagonists loaded in either the upper or lower wells.

Supplemental Fig 2: *P1 and P2 receptor localization in polarized human neutrophils and HL60 cells*. (A) Immunostaining of A2a, A3, and P2Y2 receptors in neutrophils using fixed purified human neutrophils (A) or HL60 cells (B) uniformly stimulated with 100 nM fMLP prior to fixation. Images were arranged to show cells with their leading edges oriented upwards.
(C) Images of HL60 cells expressing A3-EGFP (left) or P2Y2-EGFP fusion protein (right) migrating towards a micropipette tip filled with 100 nM fMLP (at right of the images). Insets show density slice of cross sections along the cell axes.

Supplemental Fig 3: *Roles of adenosine and A3 receptors in chemotaxis.* (A) Effect of 10 μM adenosine on traces of human neutrophils migrating towards fMLP analyzed with the microscope assay. (B-D) Effect of ADA or the A3 receptor antagonist MRS 1191 on chemotaxis of human neutrophils towards 100 nM platelet activating factor (PAF), C5a (3% zymosan-activated autologous serum), 2 ng/ml IL-8, or 10^6/ml living *Staphylococcus epidermidis* (ADA, 10 U/ml; MRS 1191, 10 μM) added together with drugs to the lower wells. (E) Effect of inosine added to the lower well of the transwell system on chemotaxis of human neutrophils toward 100 nM fMLP.

Movie Captions

Video 1 and 2: ATP release from human neutrophils in an fMLP gradient
Video 3: Negative control for ATP release assay (without glucose 6-phosphate dehydrogenase or NADP)
Video 4 and 5: Neutrophils chemotaxis in the absence or presence of apyrase
Video 6: Neutrophils chemotaxis in the presence of 100 μM ATPγS
Video 7 and 8: Neutrophils chemotaxis in the absence or presence of 10 μM MRS 1191
Video 9: Chemotaxis of HL60 cells expressing A3-EGFP fusion protein
Video 10: Confocal image sequence of HL60 cell expressing A3-EGFP fusion protein

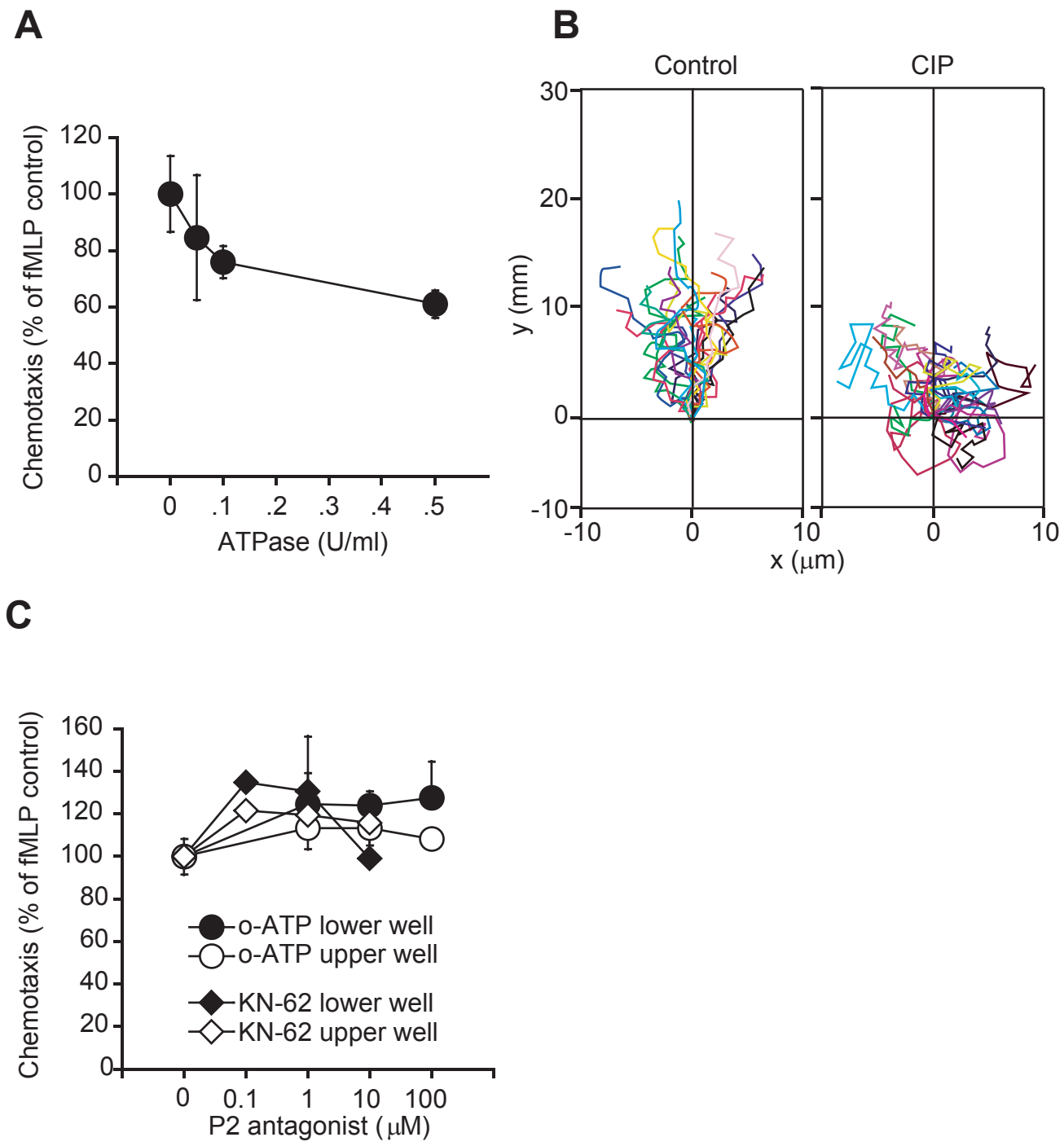
References

23. W. G. Junger *et al*., *J. Clin. Invest.* **101**, 2768 (1998).

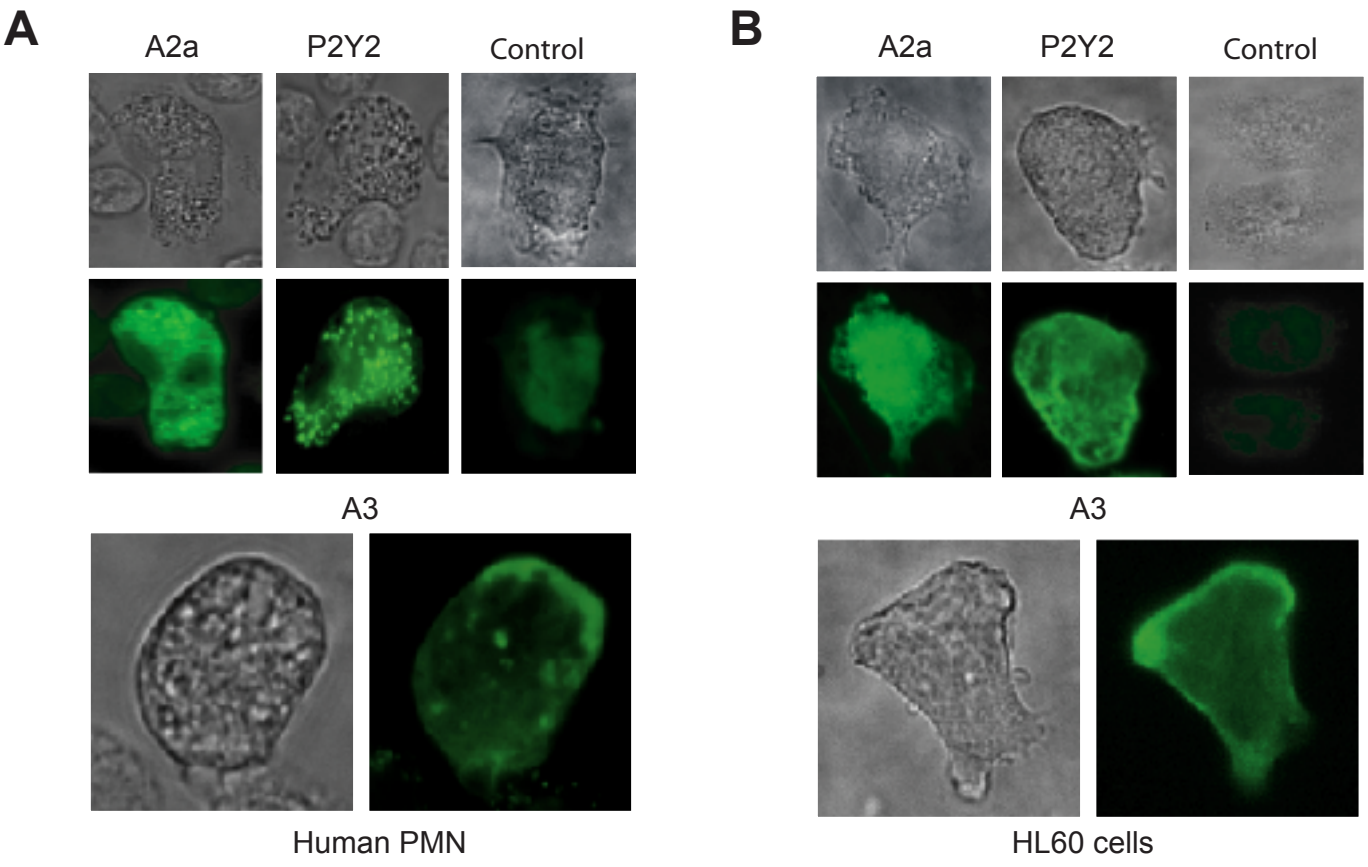
24. K. Hestdal *et al*., *J. Immunol.* **147**, 22 (1991).

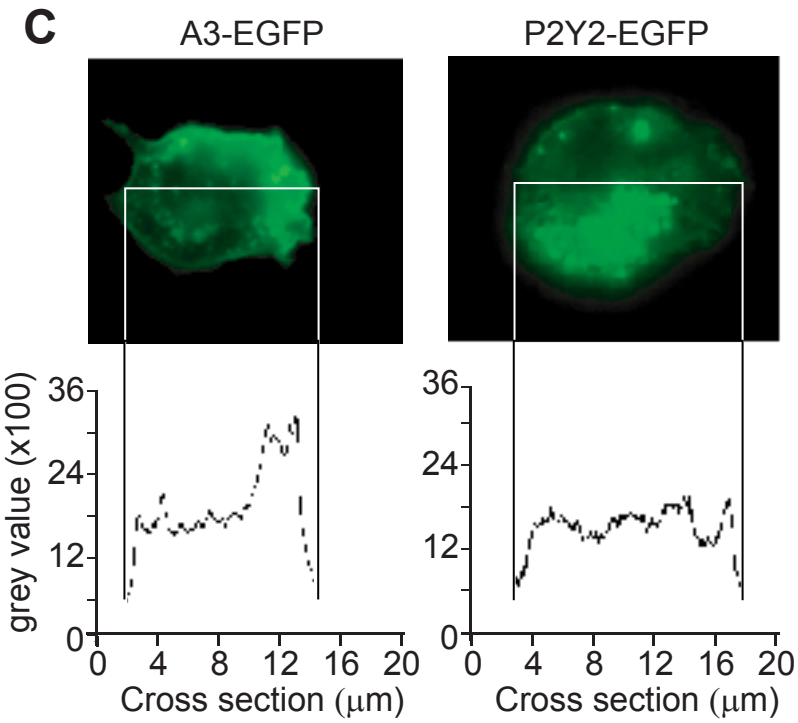
25. L. Wang *et al*., *J. Cardiovasc. Pharmacol.* **40**, 841 (2002).

supplemental Fig. 1



supplemental Fig. 2





supplemental Fig. 3