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TITLE: STIMULATION OF THE $P2Y_1$ RECEPTOR UPREGULATES NTPDase1 IN HUMAN RETINAL PIGMENT EPITHELIAL CELLS

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Running Title: NTPDase1 upregulation by $P2Y_1$ receptor

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Non-standard abbreviations: CT, cycle threshold; DMEM, Dulbecco's modified Eagles medium; eNPP, ectonucleotide pyrophosphatase/phosphodiesterase; FBS, fetal bovine serum; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MRS2179, N6-methyl-2'-deoxyadenosine-3',5'-bisphosphate; MRS2365, (N)-methanocarba-2MeSADP; MRS2500, 2-iodo-N^6-methyl-(*N*)-methanocarba-2'-deoxyadenosine 3',5'-bisphosphate; NMDA, *N*-methyl-D-aspartic acid; NTPDase, nucleoside triphosphate diphosphohydrolase; oATP, oxidized ATP; PVDF, polyvinylidine difluoride; RB2, Reactive Blue 2; RPE, retinal pigment epithelium.

Section: Neuropharmacology

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Abstract

Stimulation of receptors for either ATP or adenosine leads to physiologic changes in retinal pigment epithelial (RPE) cells that may influence their relationship to the adjacent photoreceptors. The ectoenzyme nucleoside triphosphate diphosphohydrolase-1 (NTPDase1) catalyzes the dual dephosphorylation of ATP and ADP to AMP. While NTPDase1 can consequently control the balance between ATP and adenosine, it is unclear how its expression and activity are regulated. Classic negative feedback theory predicts an increase in enzyme activity in response to enhanced exposure to substrate. This study asked whether exposure to ATP increases NTPDase1 activity in RPE cells. While levels of NTPDase1 mRNA and protein in cultured human ARPE-19 cells were generally low under control conditions, exposure to slowly-hydrolyzable ATPγS led to a time-dependent increase in NTPDase1 mRNA level that was accompanied by a rise in levels of the functional 78 kD protein. Neither NTPDase2 nor NTPDase3 message was elevated by ATPγS. The ATPase activity of cells increased in parallel, indicating the upregulation of NTPDase1 was functionally relevant. The upregulation of NTPDase1 protein was partially blocked by $P2Y_1$ receptor inhibitors MRS2179 and MRS2500, and increased by $P2Y_1$ receptor agonist MRS2365. In conclusion, prolonged exposure to extracellular ATPγS increased NTPDase1 message and protein levels and increased ectoATPase activity. This upregulation reflects a feedback circuit, mediated at least in part by the $P2Y_1$ receptor, to regulate levels of extracellular purines in subretinal space. NTPDase1 levels may thus serve as an index for increased extracellular ATP levels under certain pathologic conditions, although other mechanisms could also contribute.

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Introduction

Extracellular ATP and adenosine act at distinct receptors to mediate discrete actions in many tissues (Bucheimer and Linden, 2004). The two transmitters can act as a balanced pair, with diverse effects resulting from their activation of different receptors (Zhang et al., 2006b). The corresponding effects on cell physiology are influenced by the relative availability of each purine. P2 receptors for ATP are typically stimulated by ATP released from cells via physiologic mechanisms (Schwiebert, 2001; Lazarowski et al., 2003), while the adenosine capable of stimulating P1 receptors is frequently produced from the consecutive dephosphorylation of ATP (Dubyak and el-Moatassim, 1993). The extracellular enzymes that mediate this dephosphorylation can dramatically alter responsiveness by simultaneously removing ATP and producing adenosine. It follows that regulation of these enzymes offers great potential to alter the balance of purines and coordinate their effects.

The retinal pigment epithelium (RPE) lies adjacent to the photoreceptors in the posterior eye. Short and long-term communication between the two cell types is necessary for optimal visual function (Strauss, 2005). Adenosine and ATP can both modulate this interaction by stimulating P1 and P2 receptors, respectively (Mitchell and Reigada, In press). For example, activation of the $P2Y_2$ receptor for ATP on the RPE apical membrane elevates Ca^{2+} and enhances fluid movement across the tissue, thus helping to keep photoreceptors in place (Peterson et al., 1997; Maminishkis et al., 2002). Stimulation of an A_2 receptor for adenosine decreases the phagocytosis of photoreceptor outer segments by the RPE (Gregory et al., 1994).

Level of purine agonists in the subretinal space between the RPE and photoreceptors are regulated by various mechanisms. The RPE itself releases ATP into the subretinal space in response to NMDA receptor activation, chemical ischemia, cell swelling and other stimuli (Mitchell, 2001; Reigada and Mitchell, 2005; Reigada et al., 2006a; Mitchell and Reigada, In press). Multiple nucleotidases capable of converting the released ATP into adenosine have been identified. Ecto-5'-nucleotidase (CD73), which converts AMP to adenosine, was recently localized to the apical membrane of RPE cells, with enzyme activity decreased following stimulation of the α1 epinephrine receptor (Reigada et al., 2006b). RPE cells

are also capable of degrading extracellular ATP, with the cells expressing multiple ATPase enzymes. Message for eNPP1, eNPP2 and eNPP3, as well as NTPDase2 and NTPDase3 were clearly detected in cultured human ARPE-19 cells (Reigada et al., 2005). However, message for NTPDase1 was detected only occasionally, with most trials failing to pick up any PCR product.

The variable presence of NTPDase1 suggested that expression of the enzyme in RPE cells was regulated. Exposure to ATP has been shown to increase ectonucleotidase activity (Wiendl et al., 1998), although the specific identity of the contributing enzyme(s) has not been determined. As NTPDase1 uses ATP as a primary substrate, and as the levels of ATP bathing cells can be modified by a variety of environmental and experimental conditions (Grygorczyk and Hanrahan, 1997), we asked whether the presence of ATP in the bath was responsible for the appearance of the enzyme. As the dephosphorylation of ATP by ectonucleotidases would severely limit its half-life, ATPγS was used to prolonging availability of the nucleotide, as the replacement of oxygen with sulfur on the terminal phosphate greatly slows the hydrolysis of ATPγS with respect to ATP. Changes in NTPDase1 levels were examined on molecular, protein and functional levels and demonstrate physiological regulation of the enzyme.

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Methods

Cell Culture and Treatment Regime – The human ARPE-19 cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown grown in a 1:1 mixture of DMEM and Ham's F12 medium with 3 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 mg/ml Fungizone (all Invitrogen Corp, Carlsbad, CA) and 10% FBS (HyClone Laboratories, Inc., Logan, UT) as described (Reigada et al., 2005). Cells were grown for a total of 14-16 days, with confluence reached after 5-8 days. Cells grown for less time gave less consistent results. Cells were maintained for indicated times in growth medium containing 100 µM ATPγS or $P2Y_1$ agonists. For the 48 hrs preincubation time, the medium was replaced with fresh ATPγS solution after 24 hrs. Solution was replaced with growth medium alone at the same time points in controls. P2 inhibitors were added 10 min before ATPγS.

RT-PCR – Total RNA was extracted from ARPE-19 cells using the Trizol reagent (Invitrogen, Corp.). Oligo dTs were used to reverse transcribe 2µg of total RNA at 42°C for 50 min using SuperScript II RT (Invitrogen Corp). Increasing concentrations of cDNA were used for semi-quantitative RT-PCR. β-actin was selected as an endogenous internal standard gene. The primers used were designed to human NTPDase1 (sense: 5'-CTACCCCTTTGACTTCCAGG -3', anti-sense: 5'-GCACACTGGGAGTAAGGGC-3'; Macvector Program, Oxford Molecular Group/Accelrys, Burlington, MA; Reigada et al., 2005); NTPDase2 (sense: 5'- TGGAGGCAGCCGCAGTGAATGT -3', anti-sense: GGAGGCGAAGAGCAGCAGGAGGAC; Wood et al., 2002) NTPDase3 sense:5'-AGCCTGGTCTCTTGGCTACA-3', antisense: 5'-ACCCCAGGCTGACTCTAAGCA -3'; Primer3 software, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and β-actin (sense: 5'-GGACTTCGAGCAAGAGATGG-3' and antisense: 5'- AGCACTGTGTTGGCGTACAG-3'; Primer3). The predicted amplification products were 558, 300, 271 and 244 bp for the NTPDase1, NTPDase2, NTPDase3 and actin primer pairs, respectively. The amplification conditions were 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. This cycle was repeated 33-35 times for the NTPDases and 26 times for β-actin as initial trials determined that the PCR products for the later increased linearly between 23 and 33 cycles. In both cases, final extension was carried out at 72°C for 10 min. To control for genomic

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contamination, parallel PCR reactions were performed using cDNA that had not been reverse transcribed. The reaction products were separated on a 1% agarose gel including 10 ng/mL ethidium bromide and photographed using an ultraviolet transilluminator (Fisher Scientific, Suwanee, GA.). Bands were scanned (Hewlett Packard Scanjet 3570c; Hewlett Packard Corp., Palo Alto, CA) and quantified with ImagePro Plus software (Media Cybernetics, Silver Spring, MD). The lack of increase in message for NTPDase2 and NTPDase3 was seen in two independent samples.

Real-time PCR – Real-time experiments were carried out in a Stratagene Mx3000P system (Stratagene Corp., Cedar Creek, TX). Total RNA was isolated as described above. Each PCR reaction contained 1 µl of RT product (cDNA samples were first diluted 1:10), 0.15 µM of each primer, 10 µl of 2× Brilliant SYBR Green QPCR Master Mix, 300 nM of reference dye (all Stratagene Corp.). Following a 95°C denaturation for 10 min, the reactions underwent 40 cycles of : 95°C for 30 sec, 58°C for 1 min, and 72°C for 1 min. New primers were chosen to give a smaller product appropriate for real-time PCR; primers for human NTPDase1 amplification were 5’ - CAGGGACCCATGCTTTCATCC - 3' and 5’ - GCTGGAATGGAAGAGTCATCTCA - 3' (Primer bank, http://pga.mgh.harvard.edu/cgi-bin/primerbank/search.cgi; 104 bp) and for β-actin: 5’ - CTCCTCCTGAGCGCAAGTACTC - 3’ and 5’ - TCGTCATACTCCTGCTTGCTGAT - 3’; (101 bp). Again, specificity for the NTPDase1 primers was confirmed using BLAST analysis. Three replicates of each reaction were performed and each run included a no-template control. The CT was determined when the level of fluorescence from accumulating amplicons crossed a threshold assigned during the period of linear growth. Relative starting mRNA template concentrations of each sample were calculated using the comparative delta-delta CT method for relative quantification as done previously (Zhang et al., 2006a). Briefly, CT values for the β-actin gene were subtracted from that of the NTPDase1 gene for samples treated with control (ΔCTcont) or ATPγS (ΔCTATPγS) solutions. The mean corrected difference of CT between control and ATPγS-treated cells was defined as ΔCTATPγS-ΔCTcont (mean ΔΔCT). Additional replicates were performed using the ABI 7500 (Applied Biosystems, Foster City, CA) shared by the Vision Research Core of the University of Pennsylvania using the same primers and analogous protocols.

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Western blots – ARPE-19 cells were washed twice with cold Dulbecco's Phosphate-Buffered Saline (PBS; Invitrogen Corp.), and lysed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, protease inhibitor cocktail (Complete; Roche Diagnostics, Germany), 1% Triton X-100, 0.1% SDS, and 10% glycerol. The samples were sonicated and cleared by centrifugation (10,000*g*) for 30 min at 4°C. The protein concentrates were determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Homogenate containing 60 µg of protein was separated using conventional SDS-PAGE under non-reducing conditions, and transferred to a PVDF membrane. Non-specific binding was blocked with 5% nonfat dried milk for 1 hr at 25°C. Blots were then incubated with a mouse monoclonal antibody against human NTPDase1, BU61 (1µg/ml; Ancell Corp., Bayport, MN) overnight at 4°C. This was followed by incubation with anti-mouse IgG conjugated with horseradish peroxidase (1:5000 dilution; Amersham Biosciences Corp., Arlington Heights, IL) at 25°C for 1 hr and developed by chemiluminescence detection (ECL detection system; Amersham Biosciences Corp.). ImagePro Plus software was used to quantify the intensity of the specific bands as above.

For membrane purified extracts, cells were homogenized in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2mM EDTA and protease inhibitor cocktail. Nuclei and cell debris were removed from the homogenate by centrifugation at 10,000 × g for 15 min at 4 °C. The resulting supernatant was centrifuged at 40,000 × g for 35 min at 4 °C. The membrane pellet was solubilized in buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail) for a minimum of 1 hr at 4 °C.

Measurement of ATP hydrolysis – ARPE-19 cells were cultured in 96-well white plates with clear bottoms (Corning Inc., Corning, NY) to confluence. Cells were washed and 90 µl isotonic buffer containing 1 µM ATP was added to the wells immediately before recording began. Isotonic buffer contained (in mM) 105 NaCl; 5 KCl; 6 HEPES Acid; 4 NaHEPES; 5 $NaHCO_3$; 60 mannitol; 5 glucose; 0.5 $MgCl_2$; 1.3 $CaCl_2$, at pH 7.4. The plate was placed into a luminometer (Luminoskan Ascent, Labsystems; Franklin, MA) and 10 µl of luciferin-luciferase solution was injected into each well using the internal injector as described previously (Reigada and Mitchell, 2005). Luminescence levels were measured every 60 sec for 3 hrs, with an integration time of 100 msec for each well.

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Materials and analysis – MRS2500 and MRS2365 were obtained from Tocris Bioscience, (Ellisville, MO). Information concerning the structure and synthesis of these compounds is available at MRS2500 (Kim et al, 2003) and MRS2365 (Chhatriwala et al., 2004). All other materials were from the Sigma Chemical Corp. (St Louis, MO) unless otherwise noted. Statistical analysis was performed using unpaired Students t-test or an ANOVA test with appropriate post-hoc test. Time constants (τ) of ATP hydrolysis were calculated by fitting each individual record to an exponential decay curve $y=ae^{(-bx)}$ using Sigmaplot Software (Systat Software Inc., Richmond, CA), with $\tau=1/b$.

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Results

Upregulation of NTPDase1 message

Exposure of ARPE-19 cells to 100 μM ATPγS for 48 hrs enhanced expression of NTPDase1 message in three separate trials using standard PCR, as defined by the presence of a bright band at the expected 558 bp in cells treated with ATPγS and its absence in control cells. To strengthen the analysis, the amount of PCR-amplified product was analyzed as a function of starting cDNA. The densitometric values of the NTPDase1 amplification products increased linearly with the amount of reverse transcribed cDNA added (Fig. 1A, B). In contrast, no bands were detected using any concentration of cDNA obtained from cells grown in control medium. Similar results were found using message extracted from three independent sets of cells in the presence and absence to 100 μM ATPγS. To ensure that this increase in message after exposure to ATPγS was specific for NTPDase1, the analysis was repeated using primers for β-actin as an internal control. The amount of β-actin product was the same for ATPγS treated and control cells regardless of the amount of starting cDNA (Fig. 1C, D). No increase in message for either NTPDase2 or NTPDase3 was found in cells exposed to 100 μM ATPγS for 48 hrs, even though NTPDase1 was substantially elevated in the same samples (not shown). In all cases, no product was detected when reverse transcriptase was omitted from the reaction.

The time-course of upregulation was examined by collecting RNA from cells exposed to ATPγS for 0, 1, 3, 6, 12, 24 and 48 hrs. PCR product was first detected in material obtained after 12 hrs exposure (Fig 1E, F). In contrast, levels of β-actin did not vary much with the duration of exposure. Similar results were obtained in three independent sets of cells exposed to ATPγS for the indicated durations. In the set shown, levels of message were highest at 12 hrs and declined slightly afterwards, but still remained >100 fold greater than control after 48 hrs of treatment. In one trial, levels at 12 hrs were 80% of the maximum found at 24 hrs, while in the third set levels at 12 hrs were 20% of those found at 24 hrs. While message was first detected in the 12 hr sample in all cases, this variation in relative amount suggests that the greatest increase in message occurs near the 12 hr point.

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The semi-quantitative PCR above suggested that exposure of RPE cells to ATPγS led to an increase in mRNA for NTPDase1. More quantitative evidence for this transcriptional regulation was sought using real-time PCR. Real-time PCR was performed with cDNA isolated from cells exposed to 100 μM ATPγS and control solution for 48 hrs. Message for NTPDase1 was detected at a much lower number of amplification cycles in cells treated with ATPγS than with control solution, indicating a larger amount of starting message (Fig. 2). Treatment with ATPγS had no effect on detection of β-actin message. When adjusted for the amount of β-actin, message from ATPγS-treated cells crossed the threshold after 8.42 cycles while that from control cells crossed at 18.17 cycles, giving a mean ΔΔCT of 9.75. There was no observable amplification without template. A similar increase was seen using cells from three independent trials.

Upregulation of NTPDase1 protein

The relative expression of NTPDase1 was examined using Western blotting techniques to determine if the increase in transcription led to a corresponding increase in protein. Protein from whole cell extracts was run and probed with the NTPDase1 monoclonal anti-human antibody BU61. While nothing was detected from cells under control conditions, protein from cells exposed to ATPγS for 48 hrs showed a clear, single band of 78 kD (Fig 3A). This size band corresponds to the functional monomeric glycosylated form of NTPDase1 (Sevigny et al., 1997). Similar increases were found in three independent trials following 48 hrs treatment with 100 μM ATPγS. The 78kDa band was also detected in a fraction specifically enriched in membrane proteins (Fig. 3B), suggesting the increase could have functional implications.

Further analysis was performed to determine the time course of the increase in protein. Exposure to ATPγS for 12, 24 and 48 hrs led to a corresponding increase in band intensity (Fig. 3C). Densitometric quantification of the blot supported this observation, and indicated the biggest jump in protein occurred between 12 and 24 hrs after exposure to ATPγS began (Fig. 3D). As levels of protein were similar for 24 and 48 hrs exposure, subsequent experiments were performed at 24 hrs. In total, band intensity increased

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in 14 preparations after 24 hrs exposure to 100 μM ATPγS. Band intensity was usually below the limits of detection in material from cells not exposed to ATPγS. Faint bands were found in protein from untreated control cells in 3 of the 14 preparations. The presence of NTPDase1 protein in some controls but not others is consistent with the occasional presence of NTPDase1 found previously (Reigada et al., 2005).

Upregulation of ATP hydrolysis

Functional analysis of extracellular ATP hydrolysis was performed to determine whether the increases in NTPDase1 mRNA and protein levels were accompanied by an increase in biochemical activity. The time-course of ATP dephosphorylation was monitored after injection of 1 μM hydrolyzable ATP into the bath, as this concentration is below that previously demonstrated to trigger an ATP-mediated ATP release in these cells (Reigada et al., 2005). While extracellular ATP was hydrolyzed by control cells, this enzymatic activity was substantially enhanced in cells treated with 100 μM ATPγS (Fig. 4A). Levels of ATP bathing control cells dropped to 23.9±2.9% of the initial values after 3 hrs of enzymatic reaction, but this fell to 2.1±0.5% in cells exposed to ATPγS for 48 hrs. Exposure to ATPγS for intermediate times had intermediate effects on hydrolysis, with 6.9±0.9% and 4.3±0.4% of ATP remaining after 15 hrs and 24 hrs respectively. The speed of decay was also enhanced, with the time constant for hydrolysis falling with increased exposure to ATPγS (Fig. 4B). The majority of the increase in ATPase activity occurred in the first 24 hrs, in agreement with the increase in protein and message (Fig. 3B). In total, the mean time constant fell from 5387 ± 435 s^{-1} to 3515 ± 250 s^{-1} after 48 h preincubation with ATPγS (n=45-50 wells from 3 independent trials, $p<0.0002$). The closeness of the initial luminescence values indicated that residual ATP from the ATPγS pretreatment was not affecting the functional assay. In one set of experiments, ATPγS treatment failed to enhance activity, although mRNA for NTPDase1 was already elevated in untreated controls and did not increase further with ATPγS treatment.

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$P2Y_1$ receptor and upregulation of NTPDase1

We hypothesized that stimulation of P2 receptors present on these cells might initiate the changes that led to increased transcription of NTPDase1 message and elevated levels of protein. The ability of P2 antagonists to inhibit this upregulation was quantified from the 78 kDa band on Western blots, as this provided post-translational information specifically about the functional form of NTPDase1. Two relatively non-specific antagonists were initially tested. Antagonists oATP (100 μM) and RB2 (50 μM) were added to cells 10 min before ATPγS, and proteins extracted 24 hrs later. Levels of NTPDase1 were decreased in all 4 trials with oATP, with a mean decrease of 25 ± 11 % (Fig. 5A). The effect of RB2 was more variable, with levels decreasing by over 90%, in one trial, but increasing by 250% in another, giving a non-significant change overall (Fig. 5B).

The effect of oATP, and perhaps RB2, suggested some type of P2 receptor involvement. The contribution from the $P2Y_1$ receptor in particular was pursued for several reasons. Although oATP has traditionally been used as a $P2X_7$ receptor inhibitor and is now recognized to have actions as a general P2X blocker, it also has distinct effects on the $P2Y_1$ receptor (Beigi et al., 2003). Previous analysis has indicated the presence of the $P2Y_1$ receptor in these cells on a molecular and functional level (Fries et al., 2004; Reigada et al., 2005), and the recent availability of specific pharmacologic tools for the $P2Y_1$ receptor has enabled a more definitive analysis. Addition of the $P2Y_1$ antagonist MRS2179 to cells 10 min before ATPγS inhibited the response in 7 out of 7 trials. In total, the expression of NTPDase1 in cells treated with MRS2179 (100 μM) was reduced by 58% as compared to cells treated with ATPγS alone (Fig. 5C). While MRS2179 is 3 orders of magnitude more selectivite for the $P2Y_1$ receptor than the $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors (Boyer et al., 1998; von Kugelgen, 2006), the compound is not particularly stable (Baurand and Gachet, 2003; Hechler et al., 2006) and requires relatively high levels for effective block over extended periods. In contrast, the recently available inhibitor MRS2500 is substantially more stable and effective in the low nanomolar range, with no binding or action at $P2Y_2$, $P2Y_{12}$ or avian P2Y receptors (Kim et al., 2003; Hechler et al., 2006; Houston et al., 2006). Treatment of cells with 10 nM MRS2500 reduced the amount of NTPDase1 by over 30% (Fig. 5D).

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The block by both MRS2179 and MRS2500 implicated the $P2Y_1$ receptor, but relative ineffectiveness of ATP at the $P2Y_1$ receptor, combined with the potential for contamination of commercially obtained ATPγS with other nucleotide products, led us to seek additional confirmation with a more specific agonist (von Kugelgen, 2006). The agonist MRS2365 is highly specific for the $P2Y_1$ receptor; it was reported to have no activity at the $P2Y_{12}$ receptor and very little at the $P2Y_{13}$ receptor, while stimulating the $P2Y_1$ receptor with an EC_{50} in the low nanomolar range (Chhatriwala et al., 2004). At 10 nM, MRS2365 significantly increased NTPDase1 levels 14 fold over control (n=5). Increasing MRS2365 to 100 nM raised NTPDase1 levels 25-fold over control (Fig. 5E), although the upregulation by MRS2365 was not additive with that of ATPγS. The increase by 2MeSATP (100 μM) was not significant, perhaps due to the ability of NTPDase1 to hydrolyze this agonist (Picher et al., 1996).

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Discussion

This study shows that prolonged exposure to ATPγS increased transcription of NTPDase1 in human ARPE-19 cells. This increase in mRNA was accompanied by an increase in NTPDase1 protein and in the rate of extracellular ATP hydrolysis. This response was mediated, at least in part, by the $P2Y_1$ receptor, and may reflect the need for cells to maintain low levels of extracellular nucleotides.

The E-NTPDase family is composed of eight members and four of them, NTPDase1, 2, 3, and 8 are dominant ectonucleotidases that dephosphorylate extracellular nucleotides (Bigonnesse et al., 2004). NTPDase1 catalyzes the dual dephosphorylation of ATP and ADP to AMP plus inorganic phosphorus (Kaczmarek et al., 1996; Robson et al., 2006). NTPDase1 is an acidic glycoprotein with a molecular mass of 78 kDa that contains two transmembrane regions and several potential glycosylation sites (Sevigny et al., 1997). A truncated 54kDa band is occasionally observed, corresponding to a C-terminal portion created by proteolytic digestion of the larger 78 kDa form (Sevigny et al., 1995; Schulte am Esch et al., 1999; Lemmens et al., 2000). The detection of a 78 kDa band with the monoclonal antibody BU61 corresponds to the active monomeric form of the enzyme and is consistent with an increase in the ATPase activity of RPE cells after treatment with ATPγS.

The ability of MRS2179 and MRS2500 to inhibit the upregulation of NTPDase1 by ATPγS, combined with the increase induced by MRS2365, strongly implicates the $P2Y_1$ receptor in the control of enzyme levels. The shared utilization of both tri- and diphosphate adenines makes the $P2Y_1$ receptor well matched to regulate NTPDase1. ADP is considerable more effective at the $P2Y_1$ receptor than is ATP (von Kugelgen, 2006), while ADP is hydrolyzed more effectively by NTPDase1 than other members of the E-NTPDase family (Kaczmarek et al., 1996). Stimulation of the $P2Y_1$ receptor is also affected by the expression of NTPDase1 (Alvarado-Castillo et al., 2005). A contribution from other P2 receptors in the upreguation of NTPDase1 is possible as relatively high levels of MRS2179 and MRS2500 inhibited only half the response to ATPγS, while a maximally effective concentration of MRS2365 the increased NTPDase1 levels to only half that of ATPγS (Chhatriwala et al., 2004). However, instability of MRS2365 over the course of 24 hours may have led to a sub-maximal response. The second-messenger pathways

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linking receptor stimulation with transcriptional control are presently unknown, but stimulation of the $P2Y_1$ receptor in myotubes can upregulate expression of acetylcholine esterase through a pathway involving intracellular Ca^{2+}, protein kinase C and the transcription factor Elk-1 (Choi et al., 2003), while activation of the $P2Y_1$ receptor in ARPE-19 cells increases intracellular Ca^{2+} (Reigada et al., 2005). Whether this rise in Ca^{2+} is necessary for the upregulation of NTPDase1 remains to be determined.

It is possible that other members of the E-NTPDase family, or those of the eNPP family, are also upregulated after treatment with ATPγS. Ogilvie and colleagues found previous exposure to ATP altered the degradation of numerous nucleotides (Wiendl et al., 1998). However, the consistently high expression of some of these other enzymes in ARPE-19 cells under unstimulated conditions predicts a smaller relative change, (Reigada et al., 2005), perhaps explaining why neither NTPDase2 not NTPDase3 were upregulated by ATPγS in our hands. As ATPγS is hydrolyzed slowly, it is also possible that some of the effects are initiated by ADP. We feel these results may open up an exciting area of investigation into how different nucleotide species stimulate purine receptors to alter expression of nucleotidase enzymes.

Previous examination of the ecto-nucleotidases in RPE cells found levels of NTPDase1 were variable, with mRNA for the enzyme detected only occasionally (Reigada et al., 2005). While it is not possible to examine the reasons for this variability in retrospect, changes in the levels of extracellular ATP may have contributed, and release of ATP is exquisitely sensitive to handling (Grygorczyk and Hanrahan, 1997). Conditions of cell growth may also have modified expression, as initial investigations indicate that enzyme expression was more reliably regulated when cells were grown for two weeks as opposed to the one week used in earlier studies. Even with prolonged growth, however, NTPDase1 was occasionally present in control cells, indicating additional yet unknown parameters may influence expression. The dynamic regulation of NTPDase1 levels does emphasize caution when interpreting evidence for the presence or absence of the enzyme in either cultured or fresh cells.

Cells would not be exposed to ATPγS *in vivo*, of course, but to a sustained supply of fresh, excess ATP released into the extracellular space. As increased protein and ATP hydrolysis was observed after 12 hrs, this feedback system may have implications for the physiological processes regulated by the

light/dark cycle in subretinal space. Glutamate is released by photoreceptors in the dark, and stimulation of an NMDA receptor for glutamate on the apical surface of the RPE leads to a rapid increase in the levels of extracellular ATP (Mitchell, 2001; Reigada and Mitchell, 2005; Reigada et al., 2006a). The present findings suggest this increased ATP in subretinal space would upregulate NTPDase1 levels 12 hrs later, thus altering the dynamics of purinergic signaling in the light.

The causal link between ATPγS and NTPDase1 upregulation above supports the role of excess ATP in several pathologic conditions. For example, changes in nucleotidase activity have been observed after epileptic seizures (Bonan et al., 2000) and global cerebral ischemia (Schetinger et al., 1994), and NTPDase1 regulation at the transcriptional level has been observed in response to transient forebrain ischemia (Braun et al., 1998) and excess noise in the cochlea (Vlajkovic et al., 2004). The rise in the enzyme was often assumed to occur in response to elevated extracellular ATP, but as far as we are aware this has not been directly shown until now. Although identification of the specific enzyme involved in these cases is needed, these observations, and those in epidermoid carcinoma cells (Wiendl et al., 1998), make it unlikely that upregulation of NTPDase1 is restricted to RPE cells.

In addition to confirming a trigger for upregulation of NTPDase1, the demonstration that ATPγS enhanced NTPDase1 levels suggests that the enzyme could provide diagnostic information. Measurements of extracellular ATP are frequently difficult to make in vivo, complicated by high intracellular levels, the release of ATP following physiologic manipulation (Grygorczyk and Hanrahan, 1997), and the restrictive extracellular microenvironment. Levels of NTPDase1 might serve as an index for prolonged increases in extracellular ATP concentrations, although contribution of other mechanisms to enzyme levels is of course, always possible.

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Footnotes

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Legends for Figures

Fig 1. RT-PCR for NTPDase1 from human ARPE-19 cells. (A) PCR with a primer pair specific for NTPDase1 generated a 558-bp band in cDNA from cells treated with ATPγS for 48 hrs, but not in untreated control cells. The band intensity increased with the starting volume of cDNA. (B) The densitometric values of the RT-PCR amplified NTPDase1 products increased linearly with an increasing amount of cDNA in cells exposed to 100 μM ATPγS (black circles). No product was detected from cells in control medium (white triangles). A first order linear regression is fit to the data. Similar increases in NTPDase1 message were found in three independent sets of cells exposed to ATPγS for 48 hrs, and in all cases densitometetric values increased with the amount of cDNA. (C) PCR with β-actin primer pair generated single 244-bp bands in both control and ATPγS treated cells. (D) The densitometric values of β-actin products increased linearly in both ATPγS-treated (black circles) and control cells (white triangles). Data are fit with a single order regression. (E, F) Time-course of upregulation of NTPDase1 mRNA. Cells were exposed to ATPγS continuously for the time indicated. Message for NTPDase1 was first detected after 12 hrs exposure to ATPγS, while levels of β-actin remained constant throughout. NTPDase1 data points are connected with a line. A similar time course was found in 3 independent trials.

Fig 2. Quantitative PCR analysis of NTPDase1 expression in RPE cells. Amplification of NTPDase1 in RPE cells exposed to control or 100 μM ATPγS medium for 48 hrs was performed with SYBR Green real-time PCR. cDNA samples were diluted 1/10 and all reactions performed in triplicate. The CT of housekeeping gene β-actin was similar for both control and ATPγS treated cells (lines 1 and 2 respectively), whereas CT for the NTPDase1 message was attained after considerably fewer amplification cycles in ATPγS treated cells (line 3) as compared with untreated cells (line 4). No signal was detected from the no-template control (line 5). Similar increases were found using message from three independent

trials, each performed in triplicate. The figure indicates the mean of such triplicate reactions from one particular trial.

Fig. 3. Western blot analysis for NTPDase1 protein levels. (A) Immunobloting with antibody BU61 demonstrates that incubation of cells with 100 μM ATPγS for 48 hrs led to detectable 78 kDa bands in protein from whole cell lysates. (B). 78 kDa bands were also detected in protein purified from cell membranes after incubation with 100 μM ATPγS for 48 hrs. (C). Incubation of cells with 100 μM ATPγS for 12, 24 and 48 hrs led to detectable 78 kDa bands on immunoblots of increasing intensity from cell lysate material. (D) Quantification of staining intensity indicates that the largest increase in NTPDase1 protein occurred after 12 to 24 hrs of exposure to ATPγS. A similar increase in NTPDase1 protein levels after 24 hrs in ATPγS was found in 14 trials.

Fig 4. Incubation with ATPγS increased ecto-ATPase activity. (A) Cells exposed to 100 μM ATPγS for 48 hrs (gray triangles) hydrolyzed 1 μM ATP more rapidly than untreated control cells (black circles). Intermediate preincubations of 15 and 24 hrs led to intermediate increases in hydrolysis but are not shown here for reasons of clarity. Results show the mean of 5-8 wells from a trial representative of 3 independent experiments. Light levels represent the photons given off with the luciferin/luciferase reaction, an index of the level of ATP present in the bath (in arbitrary units, A.U.). (B) The time constant of decay decreased exponentially with preincubation time. * $p<0.05$ vs. no preincubation, n=5-8. In (A), error bars are smaller than symbols.

Fig 5. Involvement of $P2Y_1$ receptors in upregulation of NTPDase1. P2 antagonists were given to cells 10 min before 100 μM ATPγS and the 78 kDa band detected with antibody BU61 from protein extracted from whole cell lysates 24 hrs later. (A) Non-specific P2 antagonist oATP (100 μM) decreased the net upregulation of NTPDase1 by 25%. The upper trace is representative of 4 independent trials, with the

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mean ± SE given in the bars below. (B) The effect of RB2 (50 μM) was variable with some trials like that illustrated above indicating a drastic reduction, although the mean decrease was not significant (n=8). (C) The $P2Y_1$ receptor antagonist MRS2179 (100 μM) led to a clear decrease in band intensity (n=7). (D) A second $P2Y_1$ antagonist, MRS2500 (10 nM), also decreased band intensity (n=4). (E) $P2Y_1$ agonists MRS2365 (100 nM) significantly increased NTPDase1 levels compared to control after 24 hrs exposure, while the rise with 100 μM 2MeSATP was not significant (n=2-3). * $p<0.05$ vs. ATPγS alone (A-D) or vs. control (E).

Figure 1

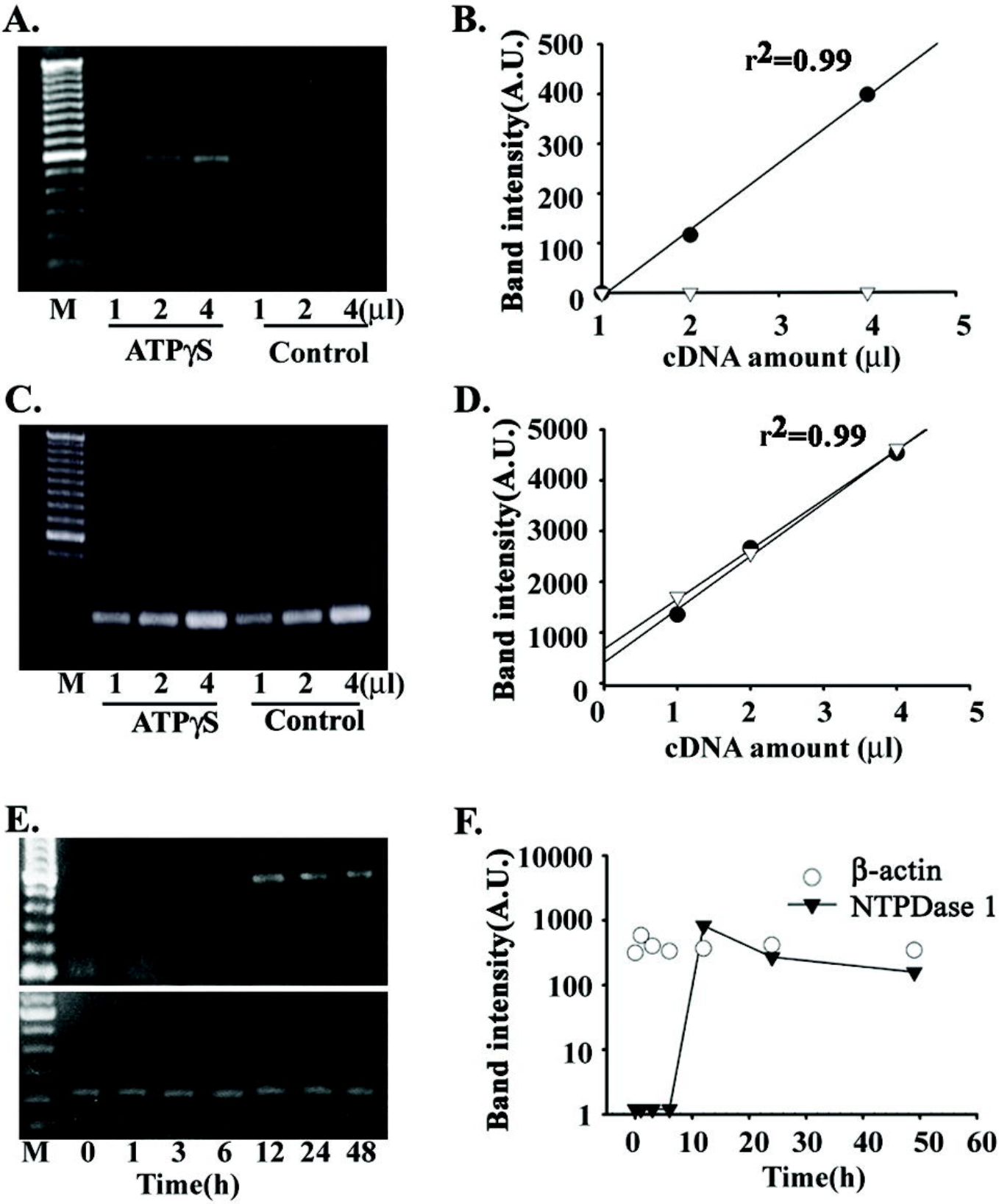

A.
M 1 2 4 1 2 4(μl)
ATPγS
Control
B.
r2=0.99
Band intensity(A.U.)
500
400
300
200
100
0
1 2 3 4 5
cDNA amount (μl)
C.
M 1 2 4 1 2 4(μl)
ATPγS
Control
D.
r2=0.99
Band intensity(A.U.)
5000
4000
3000
2000
1000
0
0 1 2 3 4 5
cDNA amount (μl)
E.
M 0 1 3 6 12 24 48
Time(h)
F.
β-actin
NTPDase 1
Band intensity(A.U.)
10000
1000
100
10
1
0 10 20 30 40 50 60
Time(h)

Figure 2

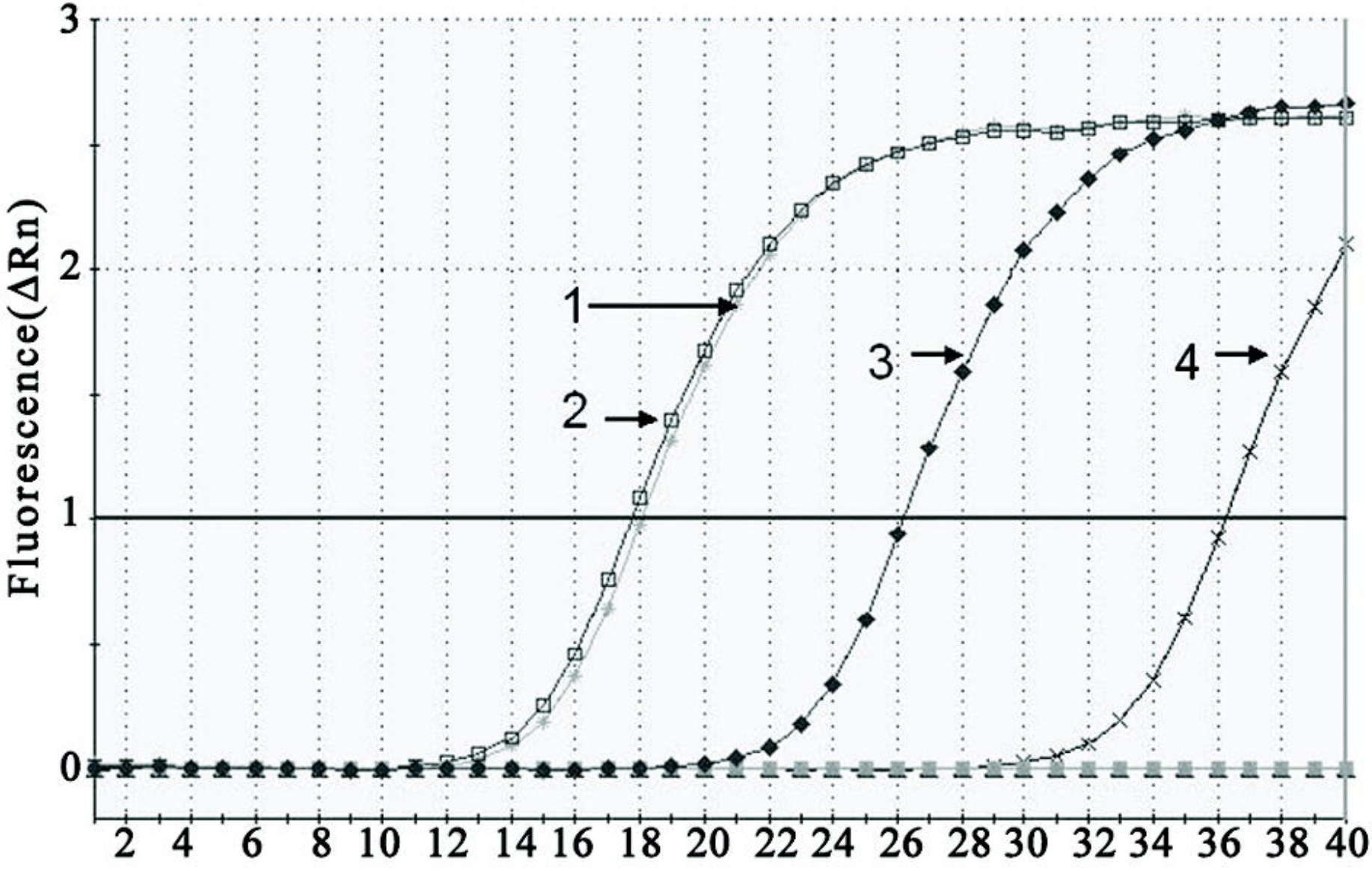

Fluorescence(ΔRn)
3
2
1
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
1
2
3
4

Figure 3

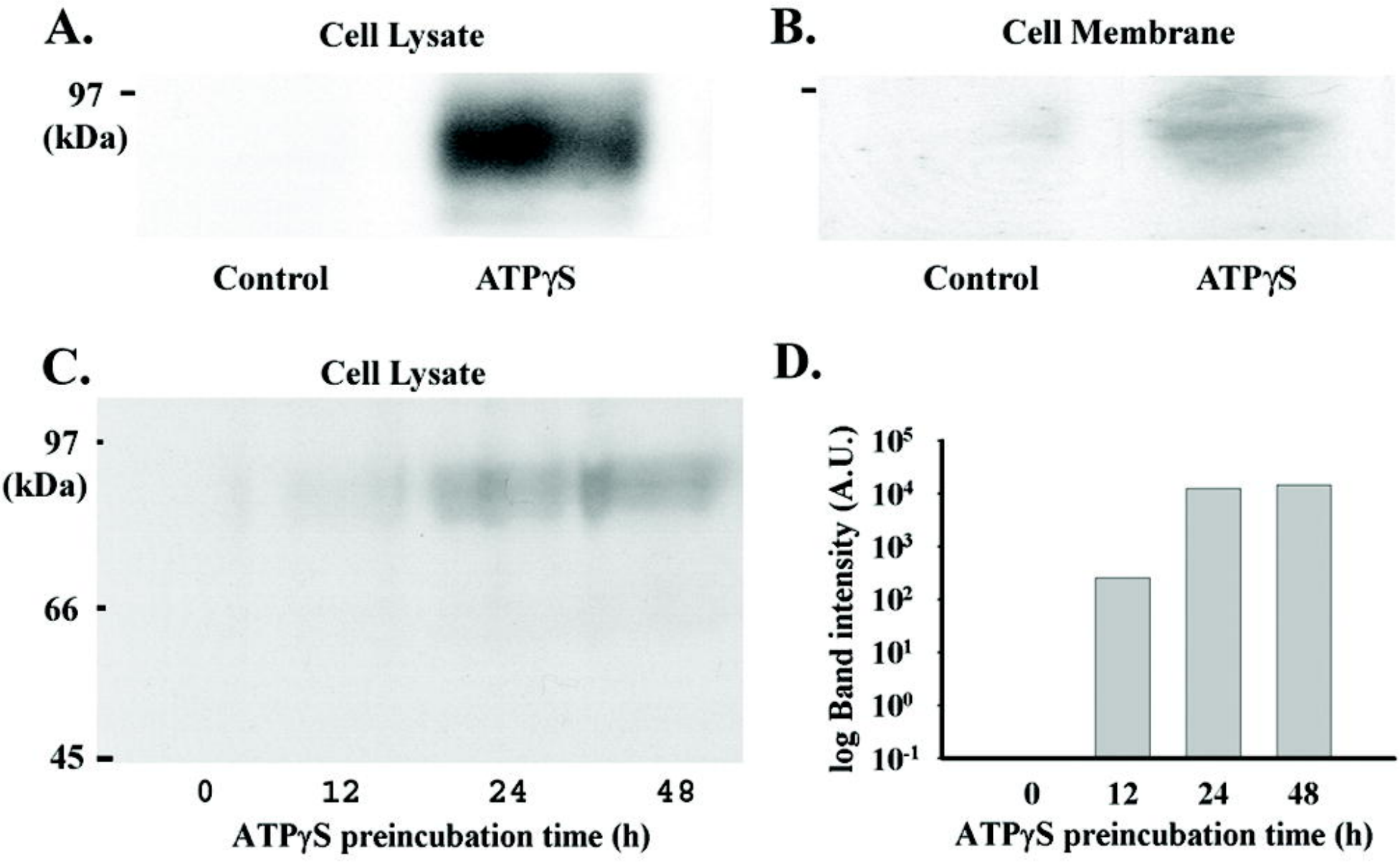

A.
Cell Lysate
97 -
(kDa)
Control
ATPγS
B.
Cell Membrane
-
Control
ATPγS
C.
Cell Lysate
97 -
(kDa)
66 -
45 -
0
12
24
48
ATPγS preincubation time (h)
D.
log Band intensity (A.U.)
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
0
12
24
48
ATPγS preincubation time (h)

Figure 4

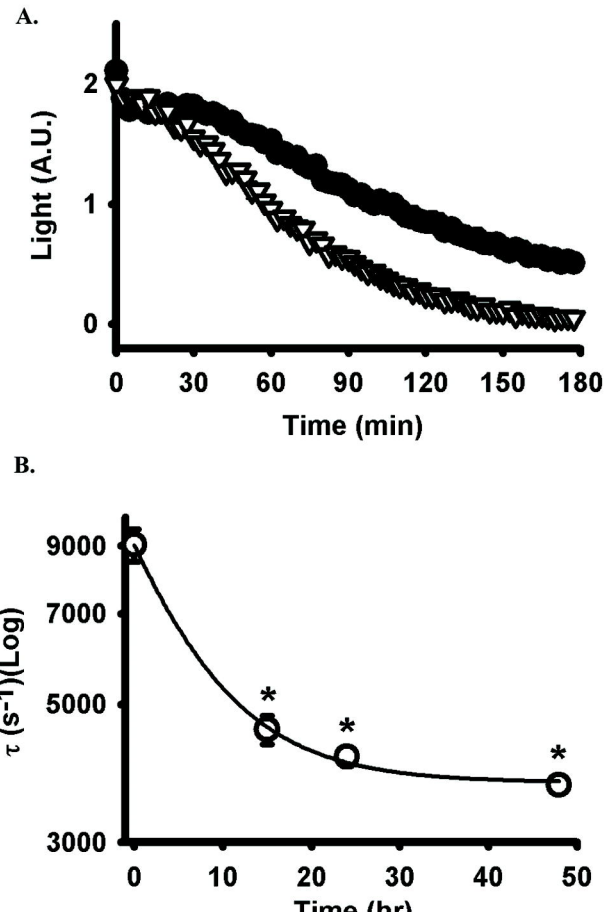
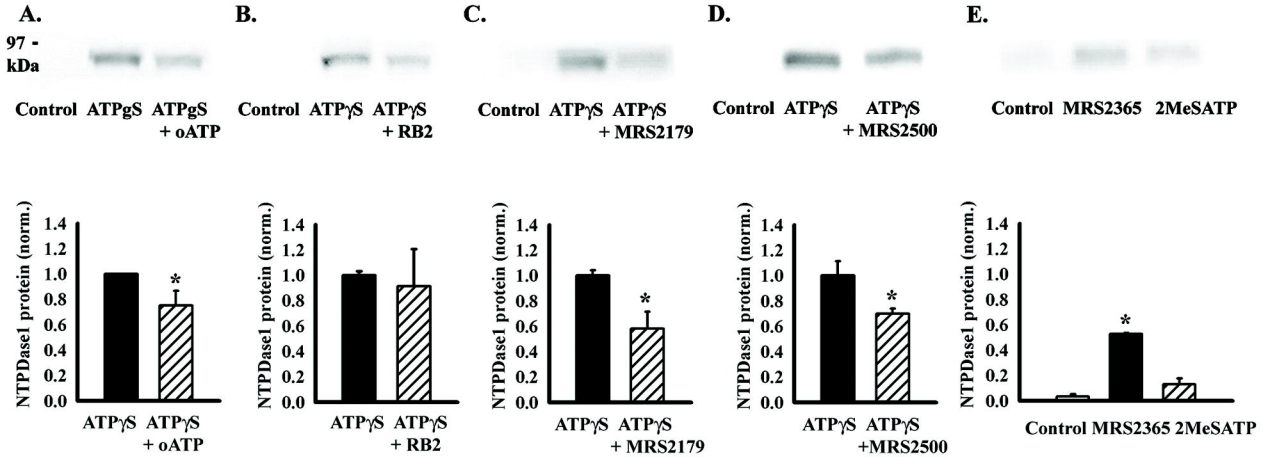

A.
Light (A.U.)
2
1
0
0
30
60
90
120
150
180
Time (min)
B.
τ (s-1)(Log)
9000
7000
5000
3000
*
*
*
0
10
20
30
40
50
Time (hr)

Figure 5


A.
B.
C.
D.
E.
97 -
kDa
Control ATPgS ATPgS + oATP
Control ATPγS ATPγS + RB2
Control ATPγS ATPγS + MRS2179
Control ATPγS ATPγS + MRS2500
Control MRS2365 2MeSATP
NTPDase1 protein (norm.)
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
*
ATPγS ATPγS + oATP
ATPγS ATPγS + RB2
ATPγS ATPγS + MRS2179
ATPγS ATPγS +MRS2500
Control MRS2365 2MeSATP