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Mitochondrial P2Y-Like Receptors Link Cytosolic Adenosine Nucleotides to Mitochondrial Calcium Uptake

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Abstract ATP is a known extracellular ligand for cell membrane purinergic receptors. Intracellular ATP can work also as a regulatory ligand via binding sites on functional proteins. We report herein the existence of $P2Y_1$-like and $P2Y_2$-like receptors in hepatocyte mitochondria ($mP2Y_1$ and $mP2Y_2$), which regulate mCa^{2+} uptake though the uniporter. Mitochondrial $P2Y_1$ activation stimulates mCa^{2+} uptake; whereas, $mP2Y_2$ activation inhibits mCa^{2+} uptake. ATP acts preferentially on $mP2Y_2$ receptors, while ADP and AMP-PNP stimulate both the $mP2Y_1$ and $mP2Y_2$. PPADS inhibits ADP stimulated $mP2Y_1$-mediated mCa^{2+} uptake. In addition, UTP, a selective $P2Y_2$ agonist, strongly inhibits mCa^{2+} uptake. The newly discovered presence and function of these receptors is significant because it explains increased mCa^{2+} uptake in the setting of low cytosolic [ATP] and, therefore, establishes a mechanism for direct feedback in which cytosolic [ATP] governs mitochondrial ATP production through regulation of mCa^{2+} uptake. J. Cell. Biochem. 92: 1062–1073, 2004.

Key words: mitochondria; purinergic receptors; P2Y; calcium uniporter

ATP and its metabolites can function as ligands [Chari et al., 1996]. By binding to specific purine receptors, ATP can function as a signaling moiety. When first proposed, it was difficult to conceive that a ubiquitous and intracellular compound such as ATP acted outside of cells in a controlled and selective way. Initially, it was believed that the effects of extracellular ATP were due to its final breakdown product, adenosine. Now, 30 years later, the actions of extracellular ATP are widely accepted, and the presence of specific cell surface receptors (P2 to distinguish them from adenosine-selective P1 receptors) is clearly established [Unwin et al., 2003]. P2 receptors are structurally and functionally divided into gated-channel ionotropic P2X receptors ($P2X_{1-7}$) or metabotropic G-protein coupled P2Y receptors ($P2Y_{1,\ 2,\ 4,\ 6,\ 11-13}$) [Chen et al., 1995; Ralevic and Burnstock, 1998]. The only common feature of these two classes of receptors is their ability to bind ATP. More recent reports have extended the concept of purine signaling by establishing a direct effect of intracellular ATP on cellular functions as a ligand and not as an energy source [Szewczyk and Pikula, 1998]. To date, the only known targets for intracellular ATP signaling are $mitoK_{ATP}$ channels [Inoue et al., 1991], the sarcoplasmic reticulum ryanodine receptor/Ca^{2+} channel receptor [Ehrlich et al., 1994], ATP-regulated cardiac Ca^{2+} channel [Belles et al., 1988; McDonald

Abbreviations used: ADP, adenosine 5′-diphosphate; AMP-PNP, 5′-adenylyl-b,g-imidodiphosphate; ATP, adenosine 5′-triphosphate; EM, electron microscopy; mCa^{2+}, mitochondrial calcium; $mP2Y_1$, mitochondrial $P2Y_1$-like receptor; $mP2Y_2$, mitochondrial $P2Y_2$-like receptor; PPADS, Pyridoxalphosphate-6-azophenyl-2′,4′,-disulfonic acid; PSU, physiological state uptake; RR, ruthenium red; UTP, uridine 5′-triphosphate; F_0F_1, mitochondrial F_0F_1 ATP synthase; PLC, phospholipase C; WC, whole cells; WM, whole mitochondria; MM, mitochondrial membranes; PM, plasma membrane.

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et al., 1994], and the ATP-dependent anion channel [Haussinger and Lang, 1992; Petersen, 1992]. None of these intracellular targets have been characterized as a purinergic receptor.

Mitochondria are active participants in cellular Ca^{2+} signaling and can rapidly accumulate and release large quantities of Ca^{2+}. Mitochondrial Ca^{2+} accumulation occurs via the RR-sensitive Ca^{2+} uniporter [Gunter et al., 1994] whose activation is dependent on elevated cytosolic Ca^{2+} concentrations [Kroner, 1986; Gunter and Pfeiffer, 1990; Rizzuto et al., 1993; Bernardi, 1999]. The uniporter transfers Ca^{2+} into the mitochondrial matrix across the inner MM in charge uncompensated movement, and down the electrochemical gradient ($\Delta\psi$); $\Delta\psi$ is created by the work of the respiratory chain in the presence of oxygen and normally is used by F_0F_1-ATP synthase for ATP production. Under physiological conditions, when cytosolic [ATP] is normal, mCa^{2+} uptake is limited. When cellular activity increases, there is increased ATP utilization, which is associated with a reduction of cytosolic [ATP] and a rise of cytosolic [Ca^{2+}] and mitochondrial [Ca^{2+}]. Increased mitochondrial [Ca^{2+}] activates respiratory enzymes [Hansford, 1994] and increases mitochondrial ATP production [Territo et al., 2001]. In effect, mitochondrial ATP production is regulated by mCa^{2+} uptake, and mCa^{2+} uptake, in turn, is regulated by cytosolic [ATP]. While this relationship is recognized, the basis of the feedback mechanism between cytosolic [ATP] and mCa^{2+} uptake has not been established.

In this article, we report the presence of $P2Y_1$-like and $P2Y_2$-like receptors in mitochondria ($mP2Y_1$ and $mP2Y_2$). Our data implicate these receptors in the regulation of mCa^{2+} uptake. Activation of $mP2Y_1$ stimulates mCa^{2+} uptake; $mP2Y_2$ activation inhibits mCa^{2+} uptake. The function of these receptors explains increased mCa^{2+} uptake in the setting of low cytosolic [ATP]. Based on our findings, we identify the basis of the feedback mechanism in which cytosolic [ATP] regulates mCa^{2+} uptake, which in turn governs mitochondrial ATP production.

METHODS

Reagents

Unless otherwise noted, all reagents were obtained from Sigma chemicals (St. Louis, MO).

Animals

All animal studies were performed in accordance with NIH animal care procedures, and they were approved by and carried out under the guidelines of the Vanderbilt University Institutional Animal Care and Use Committee. Male Sprague–Dawley rats, body weight ~300 g, were used for these experiments. Animals were housed in cages, kept on a 12:12h-light/dark cycle, fed daily with rat chow, and given water ad libitum. The rats were acclimatized in the animal facility for 2–3 days prior to any experimentation.

Liver Perfusion

Animals were anesthetized with isoflurane and sacrificed by cervical dislocation. A 20 G cannula was inserted in the proximal portal vein, and the liver was perfused with 50 ml of cold (4°C) liver homogenization medium [LHM: 0.2 M mannitol, 50 mM sucrose, 10 mM KCl, 1 mM Na_2EDTA (pH = 7.4, T° = 4°C)].

Preparation of Mitochondria and Mitochondrial Membranes

Mitochondria were separated from rat liver as described previously [Vieira et al., 2000; Belous et al., 2003]. Briefly, LHM perfused liver was minced with scissors and homogenized using Potter–Elvehjem homogenizer (0.09 mm clearance; 25 ml working volume) in LHM. The homogenate was filtered through cotton mesh and centrifuged for 10 min at 1,000*g* at 4°C in a swinging-bucket rotor. The supernatant was collected and centrifuged for 10 min at 3,000*g* at 4°C in a fixed-angle rotor. The pellets were resuspended in LHM without EDTA and centrifuged twice for 10 min at 3,000*g* at 4°C. LHM used for the final steps of mitochondrial separation was made Ca^{2+}-free by filtration through an ion exchange resin, Chelex-100. The final pellets were resuspended in 4 ml of LHM without EDTA. Protein concentration was determined using Bradford assay [Bradford, 1976] and normalized to 2 mg/ml by dilution. Rat brain and heart mitochondria were isolated using modifications of this method as previously described [Evans, 1992]. To obtain a mixed MM fraction, isolated mitochondria were sonicated (VirSonic 100 sonicator, VirTis, Gardiner, NY) with 15 s pulses for a total time of 2 min per 0.5 ml sample and then centrifuged at 10,000*g*

to pellet unbroken mitochondria. The supernatant was collected and centrifuged at 144,000*g* for 60 min. The resulting pellet was resuspended in RIPA [Kim et al., 2000] buffer and stored at −70°C as MMs. All steps were performed at 4°C.

Western Analysis

Purity of the mitochondrial preparation was confirmed by Western analysis as previously reported [Belous et al., 2003]. Sixty microgram samples by protein weight of liver WC homogenate, PM, isolated WM, and MM were electrophoresed on 12% acrylamide gel and transferred to PVDF membranes. Rabbit anti-alkaline phosphatase (RDI, Flanders, NJ) and rabbit anti-Na/K ATPase antibodies (Novus Biologicals, Littleton, CO) were used to detect heavy and light PM fraction contamination. Anti-calreticulin antibodies (Novus Biologicals) determined ER contamination. Anti-prohibitin antibodies were used to confirm the presence of mitochondria. Densitometry was performed to determine relative levels of calreticulin, Na/K ATPase, and alkaline phosphatase in MM fractions relative to positive controls using a Versa Doc 3000 (Bio-Rad Laboratories, Inc., Hercules, CA). Quantitation was performed using Quality One Quantitation software v4.4.1 (Bio-Rad Laboratories, Inc.).

Purinergic Receptor Antibodies

Two antibodies from independent vendors were used to determine presence of the $P2Y_1$ and $P2Y_2$ receptors in mitochondria. Anti-$P2Y_1$ antibody, from Alomone Labs (Jerusalem, Israel) [Fumagalli et al., 2003], is a rabbit polyclonal recognizing the $P2Y_1$ peptide residues 242–258 (accession P49651), which correspond to the 3rd intracellular loop between TM5 and TM6 domains. A second $P2Y_1$ antibody from US Biological (Swampscott, MA) was used for comparison and confirmation. Anti-$P2Y_2$ antibody from Alomone Labs is a rabbit polyclonal antibody raised against $P2Y_2$ receptor peptide residues 227–244, which correspond to the 3rd intracellular loop between TM5 and TM6 domains [Lustig et al., 1993; Fumagalli et al., 2003]. Again, a second $P2Y_2$ antibody obtained from US Biological was used for comparison and confirmation. Specificity of the anti-$P2Y_1$ (US Biological) and anti $P2Y_2$ (Alomone Labs) ($n = 2$ for each $P2Y_1$ and $P2Y_2$) was verified with control antigen supplied by the manufacturer (per manufacturers' protocol) prior to Western analysis. $P2X_{1,\ 2,\ 4,\ \text{and}\ 7}$ and $P2Y_{4\ \text{and}\ 6}$ antibodies were obtained from Alomone Labs.

Electron Microscopy Studies

Tissue embedding and transmission electron microscopy (TEM) studies were performed using the facilities of the Vanderbilt Research Electron Microscopy Resource Lab. Post-embedding TEM immunocytochemistry was performed on thin sections of rat liver using anti-$P2Y_1$ and $P2Y_2$ antibodies obtained from Alomone Labs, and anti-rabbit secondary antibody conjugated with 25-nm gold beads (Electron Microscopy Sciences, Fort Washington, PA) as described in Shostak et al. [2003] with modifications. Rat liver sections were fixed with 0.5–2% glutaraldehyde and 4% paraformaldehyde and embedded in EPON resine (Electron Microscopy Sciences).

Measurement of Mitochondrial Calcium Uptake

All buffers used for mCa^{2+} uptake studies were void of Na^+ in order to exclude any effects of mitochondrial Ca^{2+}/Na^+ exchanger [Bernardi, 1999]. Mitochondrial Ca^{2+} uptake was measured in incubation buffer (100 mM KCl, 1 mM malic acid, 1 mM pyruvic acid, 20 mM HEPES, pH = 7.4, KOH). Stock solutions for each component were filtered through an ion-exchange resin (Chelex-100) in order to remove Ca^{2+}. Only plastic hardware was used during these experiments in order to prevent possible Ca^{2+} diffusion from glass. Mitochondria were resuspended in Ca^{2+}-free LHM at a protein concentration of 2 mg/ml and added to the incubation buffer (final concentration of 0.2 mg/ml). Various compounds were added alone or in combinations to the mitochondrial suspension to establish their effect on mitochondrial Ca^{2+} uptake and are as follows: ATP (0.1–3 mM), ADP (0.1–3 mM), UTP (0.1–3 mM), AMP-PNP (0.1–3 mM), MRS2179 30 μM, PPADS 30 μM, oligomycin (5 mg/ml), and RR (5–10 μM).

The only Ca^{2+} present in the incubation buffer was radioactive $^{45}Ca^{2+}$ as $CaCl_2$ (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA); therefore, radioactivity of the mitochondria was proportional to mCa^{2+} uptake during the period of incubation. The 0.5 ml aliquots of mitochondrial suspension were removed after 30 min of incubation at 37°C and vacuum-filtered through 25 mm cellulose nitrate filters (0.45 μm pore size, PALL Corporation, Ann

Arbor, MI) that had been pre-wet in deionized Ca^{2+}-free water and pre-washed with 2 ml of washing solution (100 mM KCl and 20 mM HEPES; pH = 7.4, KOH). $^{45}Ca^{2+}$-loaded mitochondria were trapped on the filters. The filters were washed with 10 ml of ice-cold washing solution. After overnight drying, the filters were placed in vials containing scintillation counting cocktail (BioSafe II, Research Products International Corp. Mount Prospect, IL), and the $^{45}Ca^{2+}$ activity was assessed in a Beckman LS6000IC beta counter (Beckman Coulter, Inc., Fullerton, CA). The time of incubation and specific activity of the $^{45}Ca^{2+}$ enabled calculation of mCa^{2+} uptake/min/mg of mitochondrial protein. The amount of total $^{45}Ca^{2+}$ in the incubation buffer was also monitored by scintillation counting. Blank results were obtained by incubating mitochondria in the buffer without respiratory substrates and with 10 μM RR. Results of mCa^{2+} uptake were expressed as pmol $^{45}Ca^{2+}$ accumulated/mg of mitochondrial protein/minute of incubation time.

Mitochondrial Viability Studies

To assess mitochondrial viability and structural intactness during experimental conditions, two separate techniques were used. To determine whether mitochondria are able to respire and generate transmembrane potential, we used MitoTracker Red CMXRos, which is a red-fluorescent dye that stains mitochondria and its accumulation is dependent upon membrane potential (MTR, Molecular Probes, Eugene, OR). Mitochondria were incubated in 0.5 μM solution of MTR for 10 min at room temperature. Twenty microliters of mitochondrial suspension was placed on microscope slides. After 5 min, excess mitochondrial suspension was washed away with incubation buffer, and the attached mitochondria were fixed with 4% formaldehyde. Fixed mitochondria were covered with a cover slip and studied by fluorescent microscopy. The effect of ATP, ADP, UTP, AMP-PNP, MRS2179, PPADS, oligomycin, and RR on mitochondrial MTR uptake was assessed. Mitochondria are considered viable if they were able to maintain their transmembrane potential and uptake MTR.

Mitochondrial protein release measurement studies after incubation, with and without ATP, ADP UTP, AMP-PNP, MRS2179, PPADS, oligomycin, and RR, was also performed to ensure that the altered mCa^{2+} uptake was not due to lysis/loss of mitochondria. Mitochondria were incubated in incubation buffer with or without experimental compounds for 30 min at 37°C. Following incubation, mitochondrial suspensions were centrifuged at 5,000*g*, and the supernatant was collected for total protein measurements using Bradford assay per manufacturer's instructions.

Statistics

Each experimental condition was tested on three to five animals. All measurements for each condition and for each calcium concentration were performed in triplicate. Data are presented as mean ± SD. Statistical significance of differences was assessed using a two-tailed-homoscedastic Student's *t*-test. Statistical significance was inferred if $P < 0.05$.

RESULTS

Mitochondrial Membrane Preparations are Free of Contamination

The high purity of the mitochondrial preparations was confirmed as previously reported by Belous et al. [2003]. Densitometric analysis indicated that there was no significant activity above background at the band size for the alkaline phosphatase, Na/K ATPase or calreticulin in the MM preparations (n = 3, data not shown).

P2Y Receptors are Present in Mitochondria by Western Analysis

Rat brain is known to express the $P2Y_1$ receptor and served as an internal positive control [von Kugelgen and Wetter, 2000]. Western analysis with both commercially available anti-$P2Y_1$ receptor antibodies revealed a band at 42 kDa in brain, heart, and liver WC lysates. Within the corresponding MM fractions of each of these tissues, there was enrichment of the band (Fig. 1a). The Western analysis with the anti-$P2Y_2$ receptor antibody demonstrated the two bands known to correlate with band sizes of the $P2Y_2$ receptor antibodies, which are 55 and 38 kDa. Lung tissue is a known positive control for $P2Y_2$ and demonstrated the expected two-band pattern on our Western blot with Alomone Labs antibodies. The lower $P2Y_2$ band in heart and liver mitochondria membranes is larger than the 38 kDa band found in heart and liver WC. The upper band is absent in heart and

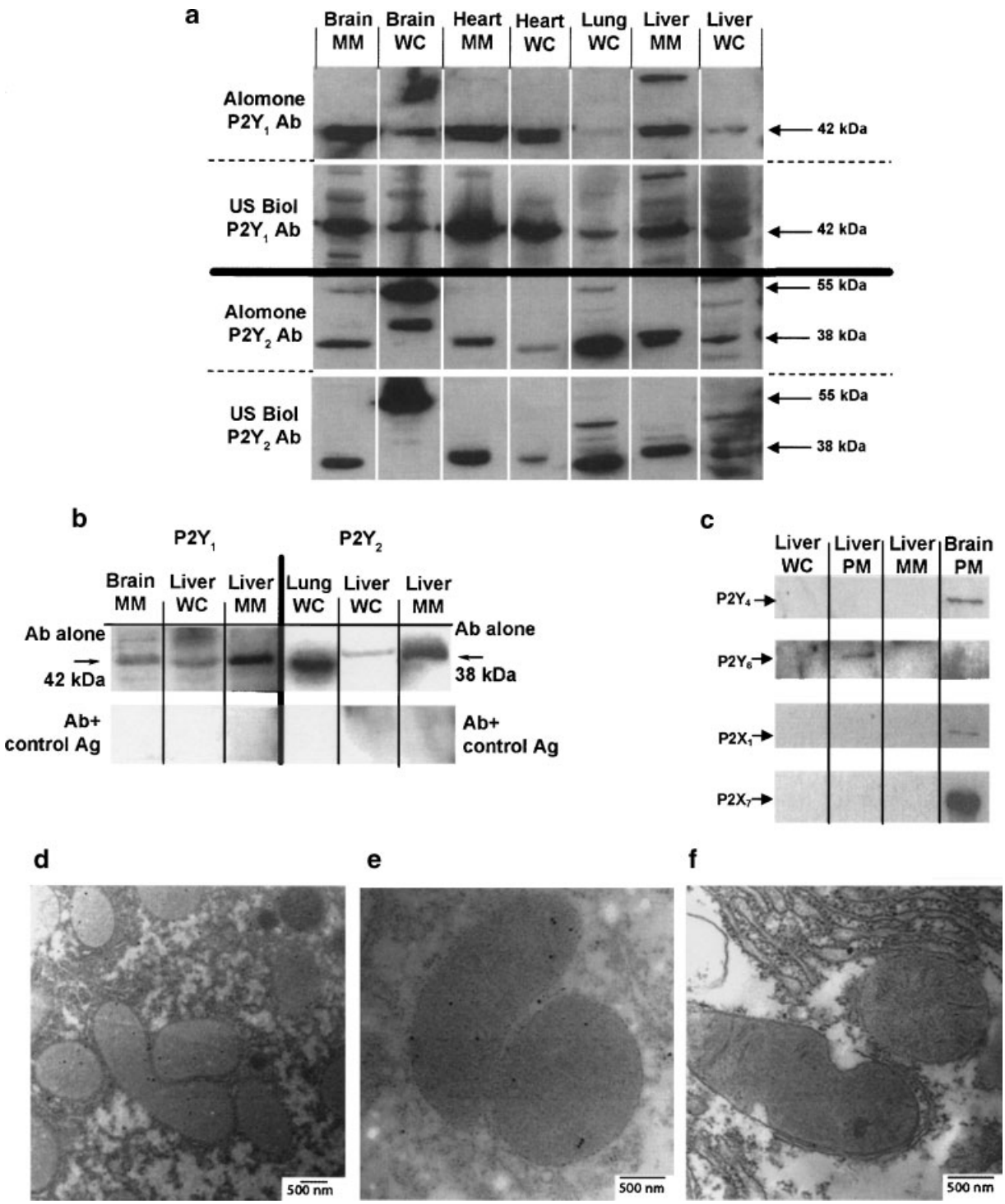

Fig. 1. Localization of mitochondrial purinergic receptors and purity of MMs. **a**: $P2Y_1$ and $P2Y_2$ tissue and mitochondrial distributions. Representative gels are presented with the Alomone Labs and US Biological antibodies. In liver, there was increased band intensity for both of the receptors in the mitochondrial fraction compared to the WC fraction. **b**: Competitive antigen studies with US Biological anti-$P2Y_1$ in rat brain and Alomone Labs anti-$P2Y_2$ in rat lung demonstrates loss of band in control lanes in the presence of peptide antigen. Similarly, there is a loss of bands in liver WC and liver MM when the antibodies are preincubated with control peptide. **c**: Western analysis of rat tissues for other P2 receptors demonstrated no other rat mitochondrial P2 receptor antibody hybridization. Post-embedding TEM immunocytochemistry was performed on thin sections of rat liver using anti-$P2Y_1$ and $P2Y_2$ antibodies obtained from Alomone Labs, and anti-rabbit secondary antibody conjugated with 25-nm gold. **d**: 1:20 anti-$P2Y_1$ with gold labeled secondary demonstrates localization of $P2Y_1$ both on the edges and on mitochondrial surface. **e**: 1:20 anti-$P2Y_2$ with gold labeled secondary antibodies with localization to the MMs and within the mitochondria. **f**: No signal was observed when using control preparation of gold labeled goat anti-rabbit (same control for both $P2Y_1$ and $P2Y_2$ as both of the primaries were rabbit antibodies). Abbrev: MM, mixed mitochondrial membranes; PM, plasma membrane; WC, whole cell lysate; Ab, antibody; Ag, antigen.

liver MMs and in heart WCs homogenate. In brain MMs, only antibodies from Alomone Labs were able to detect the 55 kDa band. For each antibody, western analysis was performed on three separate blots for each anti-$P2Y_1$ and anti-$P2Y_2$ antibody (representative gels shown for each antibody).

The specificity of the US Biological anti-$P2Y_1$ and Alomone Labs anti-$P2Y_2$ was determined with competitive peptide antigen studies (Fig. 1b). In these experiments ($n = 2$ for each $P2Y_1$ and $P2Y_2$), the antibody was preincubated with the control antigen supplied with the antibody (lower panel, Fig. 1b) and then incubated with PVDF membranes with 60 μg samples of liver WC homogenate, isolated liver MMs and brain or lung for positive controls for the $P2Y_1$ and $P2Y_2$, respectively. No bands were seen on the gels when the antibody was preincubated with the control antigen. To assess the presence of other mitochondrial P2 receptors, Western analysis with commercially available $P2X_{1,\ 2,\ 4,\ and\ 7}$ and $P2Y_{4\ and\ 6}$ antibodies was also performed (Fig. 1c); these experiments failed to demonstrate any additional P2 bands in MMs.

Electron Microscopy Localizes $mP2Y_1$ and $mP2Y_2$ Receptors to Mitochondria

Post-embedding TEM immunocytochemistry was performed on thin sections of rat liver using anti-$P2Y_1$ and $P2Y_2$ antibodies obtained from Alomone Labs, and anti-rabbit secondary antibody conjugated with 25-nm gold. EM studies demonstrated mitochondria localization at 1:20 anti-$P2Y_1$ and 1:20 anti-$P2Y_2$ with gold conjugated secondary antibody. The density of gold particle distribution for both $P2Y_1$ and $P2Y_2$ was significantly higher in mitochondria than in cytoplasm, including other organelles such as ER and nucleus. The results are as follows: (1) 0.19 gold particles per unit of mitochondrial area vs. 0.033 per unit of cytoplasmic area for $P2Y_2$ and (2) 0.16 vs. 0.038, respectively, for $P2Y_1$. It is known that both $P2Y_1$ and $P2Y_2$ are widely distributed in tissues, including rat hepatocytes [von Kugelgen and Wetter, 2000], and localization of the signal to liver cell membrane for both $P2Y_1$ and $P2Y_2$ served as internal positive controls (data not shown). The negative control demonstrated insignificant background activity using the anti-rabbit secondary antibody conjugated with 25-nm gold beads (Fig. 1f).

Mitochondrial P2Y Receptors Respond to Agonist and Antagonist

The functional activity of mitochondrial purinergic receptors was confirmed by studying the effects of the purinergic ligands, ATP, ADP, UTP, and AMP-PNP, and the inhibitors, PPADS and MRS2179, on mCa^{2+} uptake in isolated rat liver mitochondria.

First, to establish accurate experimental conditions, we investigated the possibility that mitochondrial F_0F_1-ATP synthase was hydrolyzing ATP and using this energy to produce transmembrane potential across the inner MM [St Pierre et al., 2000] and drive mCa^{2+} uptake. To exclude this, an inhibitor of F_0F_1, oligomycin A, was added. The addition of oligomycin A altered mCa^{2+} uptake only when Mg^{2+} was present in the buffer (data not shown), confirming that only ATP-Mg^{2+} can be hydrolyzed by F_0F_1 [Syroeshkin et al., 1999]. Based on these results, all Mg^{2+} was eliminated from our experimental setup.

Next, we established two reference states: "baseline" and "physiological state uptake" (PSU). The baseline mCa^{2+} uptake was defined as the uptake obtained when pyruvate, malate, and 0.1 mM of ADP were present in the incubation buffer. We chose to add a very low background level of ADP for the following reasons: (i) absence of ATP and ADP in the cytosol is not found in normal physiological conditions, and (ii) addition of ADP prevented the variable release of cytochrome c and stabilized mCa^{2+} uptake (data not shown) when mitochondria were incubated with respiratory substrates and physiological extra-mitochondrial Ca^{2+} concentration (0.2 μM) [Orrenius et al., 2003]. Therefore, 0.1 mM ADP was added to the incubation buffer for all conditions studied to rule out any variations in mCa^{2+} uptake. The baseline value of mCa^{2+} uptake was 5.02 ± 0.51 pmol/min/mg protein. PSU was defined as mCa^{2+} uptake measured in the presence of a physiological concentration of ATP and ADP in the buffer (3 and 0.1 mM, respectively) [Brdiczka and Wallimann, 1994; Folbergrova et al., 1995; Piantadosi et al., 2002]. The mCa^{2+} uptake measured at PSU was 1.3 ± 0.41 pmol/min/mg protein.

Effect of ATP and ADP on mCa^{2+} Uptake

Isolated mitochondria were incubated in incubation buffer in the presence of 0.2 μM

$^{45}Ca^{2+}$ with differing concentrations of ADP and ATP added to the incubation buffer. With increasing ATP in the incubation buffer, mCa^{2+} uptake decreased from 5.10 ± 0.62 pmol/min/mg protein at 0.1 mM ATP to 1.20 ± 0.21 pmol/min/mg protein at 3 mM ATP (76.4% decrease, $P < 0.05$ compared to baseline). Increase of ADP suppressed mCa^{2+} uptake from 4.93 ± 0.91 pmol/min/mg protein to 2.41 ± 0.31 pmol/min/mg protein (51.2% decrease, $P < 0.05$ compared to baseline). When the incubation buffer contained 3 mM ATP, mCa^{2+} uptake was the same as PSU; however, when the incubation buffer contained 3 mM ADP (no ATP), it was significantly greater than PSU and was 2-times greater than uptake stimulated by 3 mM ATP alone (Fig. 2a).

The effect of different ATP:ADP ratios on mCa^{2+} uptake was also studied. When [ATP] is higher than [ADP], it resembles a normal ATP:ADP ratio in the cell. However, an inverted ratio is more characteristic of ischemic conditions, when a lack of oxygen makes aerobic phosphorylation impossible, causing a shift in the intracellular ATP/ADP ratio. When the ATP concentration was stable at 1 or 3 mM, increasing concentrations of ADP failed to change mCa^{2+} uptake. At 3 mM ATP, mCa^{2+} uptake remained the same as PSU, regardless of ADP concentration (Fig. 2b).

When ADP concentration was fixed at 1 or 3 mM, addition of ATP was able to significantly inhibit mCa^{2+} uptake at 3 mM ATP (Fig. 2c). At high (3:1) ATP/ADP ratio, mCa^{2+} uptake was

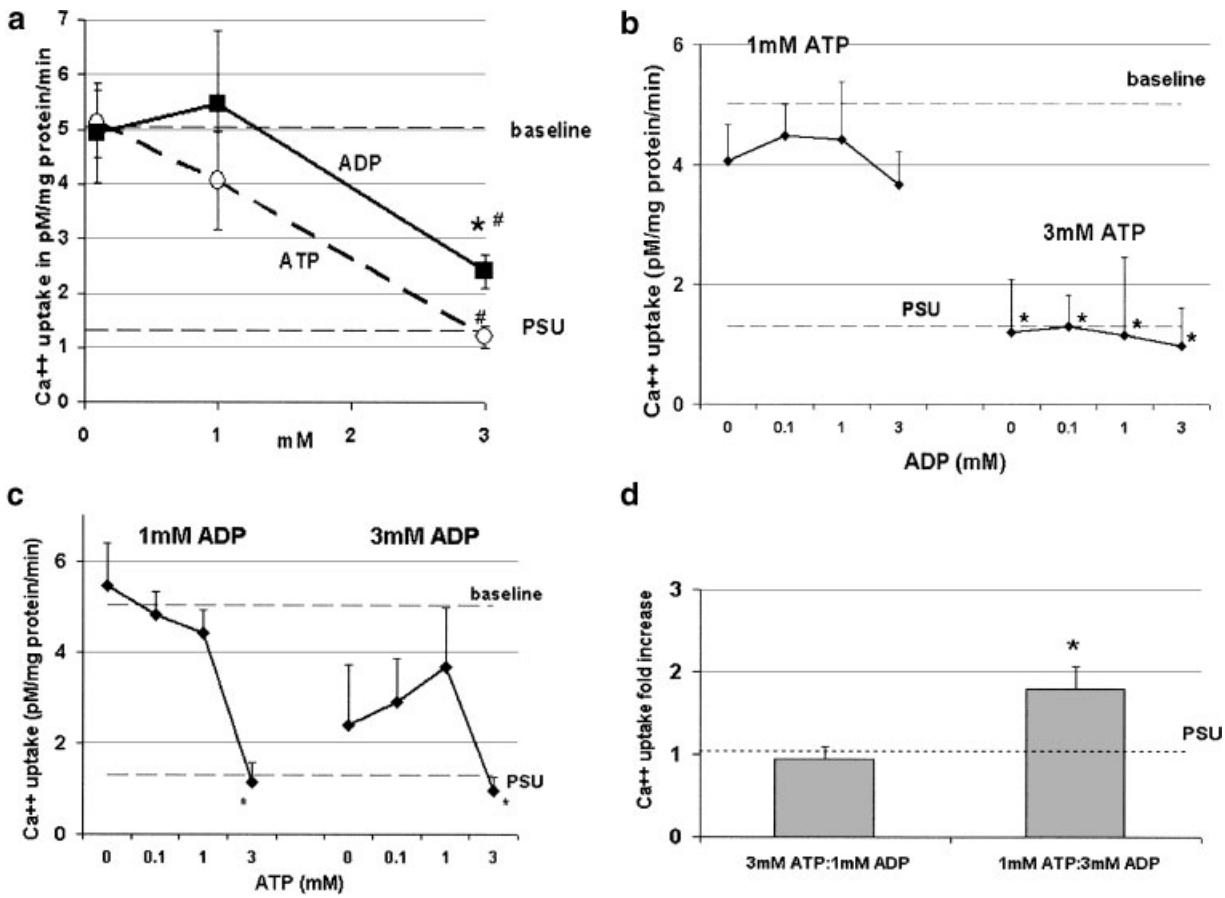

Fig. 2. Effect of ATP and ADP on mitochondrial Ca^{2+} uptake. **a**: Increasing concentration of ATP has an inhibitory effect on mCa^{2+} uptake; at 3 mM, ATP brings mCa^{2+} uptake to PSU. The inhibitory action of ADP is weaker; at 3 mM, mCa^{2+} uptake is still higher than PSU; $^{*}P < 0.05$ vs. ''PSU''; $^{\#}P < 0.05$ vs. ''baseline.'' ■, ADP; ○, ATP. **b**: Mitochondrial Ca^{2+} uptake in the presence of different ATP:ADP ratios. ATP concentration is fixed at 1 or 3 mM, with ADP concentration varying from 0 to 3 mM. $^{*}P < 0.05$ vs. the same ADP concentration on ''0.1 ATP'' curve. At 3 mM ATP, regardless of ADP concentration, mCa^{2+} uptake was decreased to PSU level. **c**: ADP concentration is fixed at 1 or 3 mM, with ATP concentration varying from 0 to 3 mM. Addition of 3 mM ATP causes significant depression of mCa^{2+} uptake in comparison with other conditions. $^{*}P < 0.05$ vs. ''ATP 1 mM''. **d**: ''ischemic'' (1 mM ATP:3 mM ADP) mCa^{2+} uptake is nearly two-fold increased compared to PSU (3 mM ATP:1 mM ADP). $^{*}P < 0.05$ vs. ''3mM ATP:1mM ADP.''

equivalent to that of PSU, but as the ATP/ADP ratio decreased, the mCa^{2+} uptake increased (Fig. 2d).

Effect of Purinergic Receptor Inhibitors

We used the non-selective P2 inhibitor PPADS and the $P2Y_1$-selective inhibitor MRS2179 to study the effect of receptor inhibition on mCa^{2+} uptake in the presence of ATP and ADP. PPADS failed to affect mCa^{2+} uptake in the presence of various concentrations of ATP (Fig. 3a), but it changed the response of mCa^{2+} uptake to ADP significantly. Addition of PPADS decreased mCa^{2+} uptake by 44.38% at 1 mM and by 62.23% at 3 mM ADP, bringing it to PSU level (Fig. 3b). MRS2179, a selective human $P2Y_1$ receptor antagonist, did not alter the ATP or ADP effect on mCa^{2+} uptake in our experiments with rat liver mitochondria (data not shown).

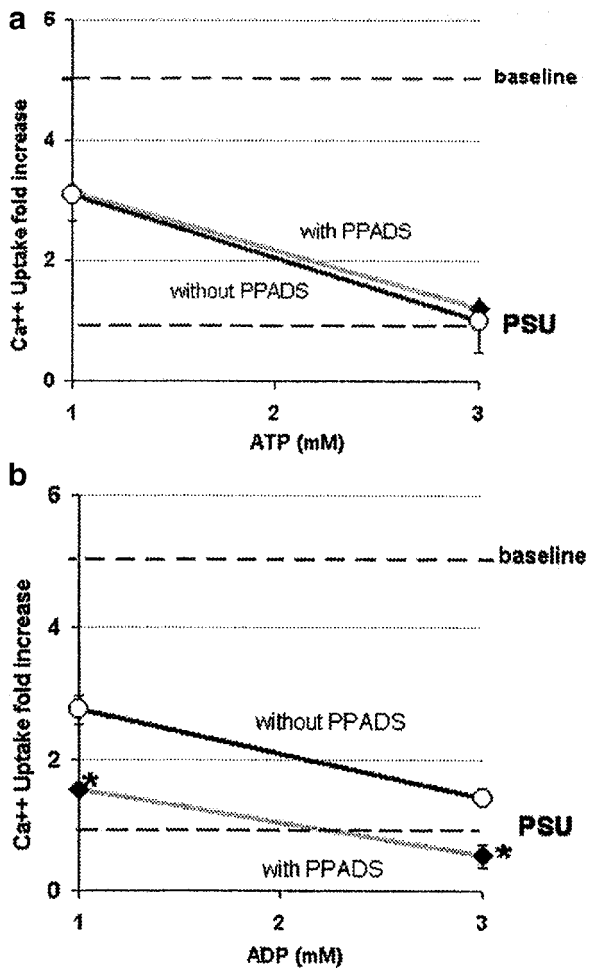

Fig. 3. Effect of PPADS on mCa^{2+} uptake affected by ATP or ADP. **a**: $P2Y_1$ inhibition by PPADS does not alter ATP effect on mCa^{2+} uptake (no ADP added); (**b**) in the presence of PPADS, ADP provided strong inhibition of mCa^{2+} uptake (no ATP added). ○, mCa^{2+} uptake in presence of ATP or ADP without PPADS; ◆, mCa^{2+} uptake in presence of ATP or ADP with PPADS. $^*P < 0.05$ vs. compared to ADP alone without PPADS.

Effect of Non-Hydrolyzable Purine Compounds

A series of experiments were performed with a non-hydrolyzable purine analog, AMP-PNP, at the same concentration ranges as ATP (Fig. 4a). The effect of AMP-PNP on mCa^{2+} uptake was similar to that of ATP at 1 and 3 mM. In Figure 4b, PPADS decreased mCa^{2+} uptake at 3 mM AMP-PNP while there was no change in mCa^{2+} uptake when mitochondria were incubated with AMP-PNP and MRS2179.

UTP is the strongest agonist of $P2Y_2$, and it is not known to have any effect on $P2Y_1$ [Ralevic and Burnstock, 1998]. At 0.1 mM UTP, mCa^{2+} uptake is not different from baseline. However, UTP had a strong inhibitory effect on mCa^{2+} uptake at 3 mM UTP, resulting in a 90.29% ($P < 0.05$) decrease in mCa^{2+} uptake in comparison with the baseline, and a 62.77% ($P < 0.05$) decrease compared to PSU. PPADS failed to affect the UTP mediated mCa^{2+} uptake response (Fig. 4c).

Purine Regulated Changes in mCa^{2+} Uptake Occur Through Uniporter

To determine the relationship between altered mCa^{2+} uptake induced by adenine nucleotides and the mitochondrial Ca^{2+} uniporter, all above described conditions were also tested in the presence of RR. Addition of RR to the experiments reduced mCa^{2+} uptake to "0" at any condition, indicating that all mCa^{2+} uptake observed during the experiments occurred via the mitochondrial Ca^{2+} uniporter (data not shown).

Mitochondrial Viability is not Affected by Addition of Experimental Compounds

MitoTracker Red uptake and mitochondrial protein release studies confirmed that mitochondria were viable and structurally intact under all conditions tested. We incubated with alomethacin (a strong inducer of mitochondrial permeability transition) or 100 μM $CaCl_2$ as positive controls for mitochondrial damage. As a negative control, we incubated mitochondria in the presence of RR. Alomethacin and 100 μM $^{45}CaCl_2$ were able to prevent mitochondria from taking up MitoTracker and yielded a significant

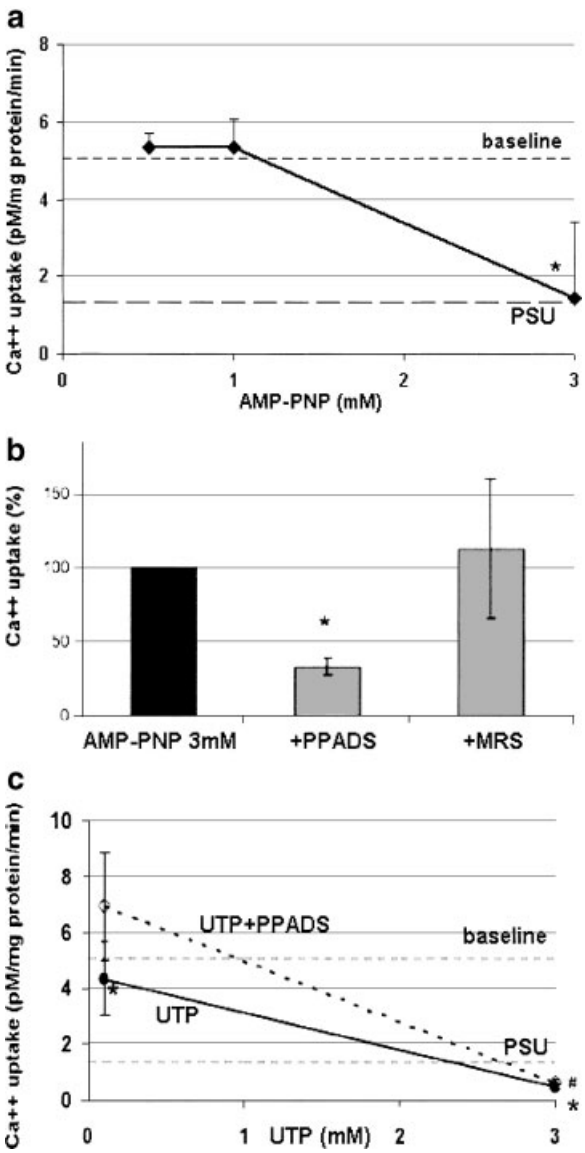

Fig. 4. Effect of non-hydrolyzable analog of ATP–AMP-PNP and UTP on mCa^{2+} uptake. **a**: In the presence of low concentrations of AMP-PNP, mCa^{2+} was at baseline level, and a higher concentration (3 mM) brought mCa^{2+} uptake to the PSU level. $^{*}P = 0.05$ vs. ''1mM''. **b**: Inhibition of P2 receptors with PPADS significantly inhibited mCa^{2+} uptake in the presence of 3 mM AMP-PNP (+PPADS = AMP-PNP 3 mM + PPADS) $^{*}P < 0.05$ vs. ''AMP-PNP'' 3 mM alone. MRS2179 did not have any effect (+MRS = AMP-PNP 3 mM + MRS 2179). **c**: UTP has a strong inhibitory effect on mCa^{2+} uptake, and this effect was not changed by PPADS. $^{*}P < 0.05$ vs. PSU; $^{\#}P < 0.05$ vs. baseline. ●, mCa^{2+} uptake in presence of AMP-PNP without PPADS; ◇, mCa^{2+} uptake in presence of AMP-PNP with PPADS.

amount of protein to be detected in the incubation buffer. Under all experimental conditions MitoTracker Red fluorescence and protein release were not different from those in the presence of RR.

DISCUSSION

ATP and its metabolites have been recognized to function as indirect and direct modulators of mitochondrial Ca^{2+} uptake. Indirectly, ATP-Mg^{2+} can be used by F_0F_1-ATP synthase to increase $\Delta\gamma$ [Belous et al., 2003], which in turn increases mCa^{2+} uptake. Direct regulation of the mitochondrial Ca^{2+} uniporter by ATP has been suggested by Litsky and Pfeiffer [1997]. They have demonstrated that the uniporter behaves like a gated channel that is governed not only by divalent cations, but also purine nucleotides, suggesting that ATP acts at a site located on the outer surface of the inner membrane through a mechanism that does not require its hydrolysis. Our findings concur that the mitochondrial Ca^{2+} uniporter is regulated by adenine nucleotides and indicate that this action is mediated through purinergic-like receptors on MMs.

Increasing the [ATP] in the incubation buffer produced significant inhibition of mCa^{2+} uptake compared to baseline and equalled PSU at 3 mM, while increasing [ADP] had a less marked inhibitory effect on mCa^{2+} uptake. A stimulatory effect of ADP on mCa^{2+} uptake has been described previously [Rottenberg and Marbach, 1990] and has been attributed to the changing conformation of adenine nucleotide translocator (ANT) to M state (the matrix side of the inner MM). This effect of ADP is not completely understood. It is possible that locking ANT in M state can increase the negative charge on the inner surface of the inner MM; however, addition of ADP or AMP does not cause changes in membrane potential [Laris, 1977]. Our data indicate a relative stimulatory effect of ADP on mCa^{2+} uptake and suggest that it is mediated through the $mP2Y_1$ receptor.

P2Y receptors were first characterized as PLC-linked G-protein coupled receptors. There is some diversity in the signaling mechanisms of $P2Y_1$ and $P2Y_2$ receptors. $P2Y_1$ is linked to PLC via G_q and $P2Y_2$ via $G_{i/o}$ [Boarder and Hourani, 1998]. Two different types of $P2Y_1$ receptors have been described. One is coupled with PLC and can be inhibited by PPADS, and another one is negatively coupled to adenylate cyclase [Webb et al., 1996]. We have previously identified and characterized the presence of PLC in mitochondria [Knox et al., 2004], but we have not been able to demonstrate any evidence for the existence of mitochondrial G_{q11} or $G_{i/o}$. The

mechanism by which the mP2Y receptors signal is not clear at this point.

The known $P2Y_1$ receptor is sensitive to ADP, while its sensitivity to ATP is variable; it is not activated by UDP and UTP. Many $P2Y_1$ and $P2Y_1$-like receptors are relatively insensitive to ATP (see [Ralevic and Burnstock, 1998]). MRS2179, a selective antagonist for the $P2Y_1$ receptor, which blocks responses mediated via human and turkey $P2Y_1$ receptors [O'Grady et al., 1996; Boyer et al., 1998], had no consequence on the effect of ATP or ADP on mCa^{2+} in our experiments with rat liver mitochondria. An effect with MRS 2179 would have unequivocally confirmed the presence of $mP2Y_1$ receptors; however, due to the high specificity of MRS2179 to human platelet $P2Y_1$ receptors and the fact that receptors under investigation are mitochondrial, MRS 2179 may not have activity against rat $mP2Y_1$. Alternatively, the mechanism of MRS2179 may be dependent upon a $P2Y_1$–G_{q11} interaction and a lack of mitochondrial G_{q11} also would render this inhibitor ineffective.

Although the P2Y inhibitor PPADS is not selective between P2Y and P2X receptors, its inhibitory action is able to distinguish between $P2Y_1$ and $P2Y_2$ receptors. PPADS is a potent antagonist of $P2Y_1$ receptors but is relatively ineffective as a $P2Y_2$ inhibitor [Charlton et al., 1996; Ralevic and Burnstock, 1998], and it has been shown to completely block the rat $P2Y_1$ but not $P2Y_2$ receptors [Schachter et al., 1997]. The findings of our experiments with PPADS on ADP stimulated mCa^{2+} uptake indicate that ADP loses the stimulatory component of its effect on mCa^{2+} uptake when the $P2Y_1$ receptor is blocked. In the presence of PPADS, ADP inhibits mCa^{2+} uptake. This suggests that ADP had an effect on both $mP2Y_1$ and $mP2Y_2$ and that stimulation of $mP2Y_1$ causes an increase of mCa^{2+} uptake. There are no selective $P2Y_2$ receptor antagonists to verify the 'mCa^{2+} uptake stimulation' effect of $mP2Y_1$ activation [Ralevic and Burnstock, 1998]. The known $P2Y_2$ receptor is activated by ATP and UTP with approximately equal potency and is insensitive to ADP [Nicholas et al., 1996; Hoebertz et al., 2002]. PPADS was not able to change the ATP-induced mCa^{2+} uptake inhibition. This finding suggests that ATP-induced inhibition of mCa^{2+} uptake in mitochondria is mediated by activation of $mP2Y_2$ receptors.

The rate of mCa^{2+} uptake in response to an increasing AMP-PNP concentration in incubation buffer was very close to that of ATP, confirming that ATP can affect mCa^{2+} uptake without being hydrolyzed. Addition of PPADS reduced AMP-PNP mCa^{2+} uptake to an even lower level. As discussed above, activation of $mP2Y_1$ has a stimulatory effect on mCa^{2+} uptake; thus, application of PPADS will remove $mP2Y_1$ effect without affecting $mP2Y_2$ inhibitory signal, resulting in $mP2Y_2$ mediated inhibition of mCa^{2+} uptake. UTP is known to be a pure $P2Y_2$ agonist, and it indeed had a strong inhibitory effect on mCa^{2+} uptake when used at a concentration of 3 mM. As expected, PPADS failed to affect the UTP-mediated mCa^{2+} uptake response.

Under normal physiological conditions, mitochondria are exposed to both ATP and ADP. The ratio of the nucleotides has direct relevance to many pathological conditions, such as ischemia and reperfusion. Interplay of $mP2Y_1$ and $mP2Y_2$ receptor signaling establishes the basis of a logical feedback mechanism. In energy-replete cells, physiological levels of ATP in the

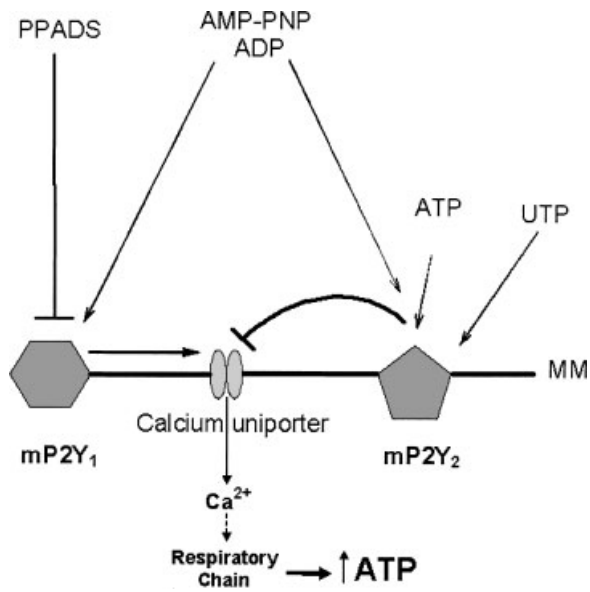

Fig. 5. Schema of proposed mechanism of action of mitochondrial purinergic receptors. Purinergic signal which inhibits mCa^{2+} uptake is mediated by stimulation of $P2Y_2$ receptors in mitochondria while $mP2Y_1$ signal is permissive of mCa^{2+} uptake. AMP-PNP and ADP have an agonistic effect on $mP2Y_2$ and $mP2Y_1$ receptors. The net effect of ADP is less inhibitory than ATP. Mitochondrial $P2Y_1$ signaling caused by ADP has a stimulatory effect on mCa^{2+} uptake, and this effect can be canceled by $mP2Y_1$ inhibition (PPADS). UTP can stimulate the $mP2Y_2$ only. Increase in mCa^{2+} uptake stimulates respiratory chain activity and increases ATP production. Abbrev: MM, mitochondrial membrane.

cytosol act on the $mP2Y_2$ to prevent mCa^{2+} uptake; whereas in low energy states, low [ATP] and increased [ADP] in the cytosol acting on the $mP2Y_1$ result in increased mCa^{2+} uptake, which enhances mitochondrial ATP production (Fig. 5). Prolonged low energy state with a low ATP:ADP ratio favors mCa^{2+} uptake. Increase in intramitochondrial Ca^{2+} level activates four mitochondrial enzymes that are involved in substrate dehydrogenation and production of NADH [Hansford, 1994]. Such conditions as ischemia will cause significant decrease of the ATP:ADP ratio [Stoica et al., 2003], inducing mCa^{2+} uniporter opening and entry of Ca^{2+} into the mitochondrial matrix in an attempt to increase ATP production. Thus, the presence of MM P2Y receptors that regulate mCa^{2+} uptake in response to extramitochondrial [ATP] and [ADP] changes represents the mechanism of an intracellular feedback loop which allows a cell to alter mitochondrial ATP production based on physiological needs.

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