Journal of Cellular Biochemistry 99:1165–1174 (2006)

Mitochondrial Calcium Transport is Regulated by $P2Y_1$- and $P2Y_2$-Like Mitochondrial Receptors

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Abstract Ischemia-reperfusion injury remains a major clinical problem in liver transplantation. One contributing factor is mitochondrial calcium (mCa^{2+}) overload, which triggers apoptosis; calcium also regulates mitochondrial respiration and adenosine 5′-triphosphate (ATP) production. Recently, we reported the presence of purinergic $P2Y_1$- and $P2Y_2$-like receptor proteins in mitochondrial membranes. Herein, we present an evaluation of the functional characteristics of these receptors. In experiments with isolated mitochondria, specific $P2Y_1$ and $P2Y_2$ receptors ligands: 2-methylthio-adenosine 5′-diphosphate (2meSADP) and uridine 5′-triphosphate (UTP), respectively, were used, and mitochondrial calcium uptake was measured. 2meSADP and UTP had a maximum effect at concentrations in the range of the known $P2Y_1$ and $P2Y_2$ receptors. The P2Y inhibitor phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) blocked the effects of both ligands. The phospholipase C (PLC) antagonist U73122 inhibited the effect of both ligands while its inactive analog U73343 had no effect. These data strongly support the hypothesis that mitochondrial Ca^{2+} uptake is regulated in part by adenine nucleotides via a P2Y-like receptor mechanism that involves mitochondrial PLC activation. J. Cell. Biochem. 99: 1165–1174, 2006.

Key words: ischemia; ATP; apoptosis; transplantation; calcium; mitochondria

Hypoxic and ischemic states are common in a variety of clinical conditions. In liver transplantation the importance of ischemia and reperfusion (I/R) injury to the donor organ cannot be overstated, especially as more suboptimal donors are being used in an effort to treat patients on an ever-increasing waiting list. Organ harvest, cold storage and subsequent reperfusion initiate processes that cause cellular injury, leading to organ dysfunction or graft failure. Lack of oxygen during the ischemic period arrests oxidative phosphorylation, leading to a significant decrease of ATP levels in the cytoplasm.

In mammalian cells, energy is produced primarily in the form of ATP via substrate oxidation and oxidative phosphorylation in mitochondria. Regulation of cellular respiration by ADP has been described in the classical work of Chance and Williams [1956]; however, this model works only in vitro. It has been shown that adenosine 5′-diphosphate (ADP) and ATP levels in the cytoplasm do not change during workload under normal conditions and, therefore, cannot serve as regulatory stimuli and that the rate of respiration is regulated by other mechanisms [for review see Balaban, 2002]. Calcium ion (Ca^{2+}) is considered the main

Grant sponsor: National Institute of Health; Grant numbers: DK59390, DK064669; Grant sponsor: AOA Ruth Kuckein fellowship; Grant sponsor: Association of Academic Surgery research fellowship.

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Received 2 March 2006; Accepted 11 April 2006
DOI 10.1002/jcb.20985

signaling agent in respiratory regulation, which couples ATP production to cellular energy demand via its ability to activate respiratory chain enzymes in mitochondria.

Mitochondria also play a key role in cellular apoptosis. They respond to harmful stimuli by sequestering large amounts of Ca^{2+}, undergoing permeability transition, releasing cytochrome c and other pro-apoptotic factors, and triggering apoptotic cell death [Gunter et al., 2004]. Under certain conditions, such as hypoxia, cellular levels of ATP and ADP change significantly. These changes provide an additional regulation of mitochondrial function. In the millimolar range ATP inhibits the mitochondrial Ca^{2+} uniporter [Litsky and Pfeiffer, 1997], preventing Ca^{2+} entry in mitochondria and ATP overproduction. Depletion of ATP in the cytoplasm under ischemic conditions may de-inhibit the mitochondrial Ca^{2+} uniporter, allowing the entry of large amounts of Ca^{2+} into the mitochondria, which triggers permeability transition and the release of apoptotic mediators. It is possible that at low ATP concentrations, mitochondrial Ca^{2+} uptake increases not only because of de-inhibition but also because low doses of ATP can actually stimulate mitochondrial Ca^{2+} accumulation. For instance, in the absence of ATP, Ca^{2+} uptake is approximately equal to that in the presence of normal cellular concentrations of ATP (3–5 mM); however, when ATP is decreased to 1 mM, mitochondrial Ca^{2+} uptake is greater than in the absence of ATP, suggesting a stimulatory action of ATP at lower than normal concentrations [Belous et al., 2004]. If it was just an effect of de-inhibition then mitochondrial Ca^{2+} uptake should be maximal when ATP is not present, which is not the case. We, therefore, hypothesized that the action of ATP on mitochondrial Ca^{2+} uptake is mediated by receptors similar to cell surface purinergic receptors. Abbracchio and Williams [2001] suggested a similar hypothesis.

In a recently published article [Belous et al., 2004] we described our findings, which suggested the existence of mitochondrial purinergic-like receptors (mito$P2Y_1$- and $P2Y_2$-like). We detected $P2Y_1$- and $P2Y_2$-like proteins in mitochondrial membranes by Western blot and EM with immunostaining and found that ATP, ADP, and other ligands can affect mitochondrial Ca^{2+} handling. In the current manuscript, we present new findings which detail the functional role of these receptors. Our data indicate that stimulation of mitochondria by $P2Y_1$- or $P2Y_2$-specific ligands affects mitochondrial Ca^{2+} accumulation in different concentration ranges, specific for each receptor. We found that these effects could be blocked by a non-specific inhibitor of P2Y receptors—PPADS. Known cell surface $P2Y_1$ and $P2Y_2$ receptors are G-protein coupled, and their signaling mechanism involves activation of phospholipase C (PLC). And we have previously demonstrated the presence of PLC in mitochondria and its involvement in Ca^{2+} uptake regulation [Knox et al., 2004]. Our current data implicate the involvement of mitochondrial PLC in purinergic regulation of Ca^{2+} transport in mitochondria because a specific PLC inhibitor, U-73122, was able to block the response to $P2Y_1$ and $P2Y_2$ ligands. Collectively, our data strongly support the hypothesis that mitochondrial Ca^{2+} uptake is regulated in part by adenine nucleotides via a P2Y-like receptor mechanism that involves mitochondrial PLC activation.

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 12:12 h-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 Na2EDTA (pH = 7.4)] for several minutes to wash out the blood.

Preparation of Mitochondria

Mitochondria were separated from rat liver as described previously [Vieira et al., 2000; Belous

et al., 2003a]. 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 rendered 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 the Bradford assay [Bradford, 1976] and adjusted to 2 mg/ml by dilution.

Measurement of Mitochondrial Calcium Uptake

All media used for mCa^{2+} uptake studies were devoid of Na^+ in order to exclude any effects of the 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; T = 37°C). 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+} release 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: UTP (0.1–1,000 μM), AMP-PNP (10–2,000 μM), MRS-2179 (10 μM) (Tocris, Ellisville, Missouri), PPADS (30 μM) (Tocris, Ellisville), and ruthenium red (RR) (10 μM).

The only Ca^{2+} present in the incubation medium was radioactive $^{45}Ca^{2+}$ as $CaCl_2$ (GE Healthcare (formerly Amersham Biosciences), Chandler, Arizona); 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 20 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

MitoTracker Red CM-H_2XRos (Molecular Probes, Eugene, OR) was used as an indicator of mitochondrial functional viability. This dye can be accumulated only by respiring mitochondria with normal transmembrane potential ($\Delta\Psi$) potential. The fluorescence of MitoTracker Red loaded mitochondria was measured using fluorescent microscope and appropriate software (Carl Zeiss, Germany). Respiratory control experiments with 5 mM succinate as a substrate were performed in order to assess states III, IV, and uncoupled rates of respiration and confirm that mitochondrial respiratory function and coupling were not affected by preparation, experimental procedures and various inhibitors and ligands (Clark electrode, Hansatech Instruments, Ltd, Norfolk, England).

Statistics. Each experiment was performed on at least three separate animals. All measurements in each condition and at each Ca^{2+} concentration were performed in triplicate. Data are presented as mean ± SEM. Statistical significance was assessed using 2-tailed Student *t*-test with unequal variance, and $P < 0.05$ was considered significant.

RESULTS

The natural ligands for purinergic receptors in the cell are ATP and ADP. In our first report

we used ATP and ADP to establish the functional role of the receptors in mitochondria [Belous et al., 2004]; however, we since have found that the added ATP and ADP are not stable in the presence of isolated mitochondria due to hydrolysis/phosphorylation by the mitochondrial ATP synthase complex. Preventive measures, such as omission of Mg^{2+} ions from the buffer, failed to eliminate this problem. For this reason, in all current experiments, only non-hydrolysable analogs of nucleotides were used.

The intracellular content of ATP can decrease as much as 100 times in the first 5 min after the onset of hypoxia and almost 1,000 times after 20 min. [Dransfield and Aprille, 1994]. The ATP concentration in the non-ischemic cell is 3–5 mM; therefore, hypoxia results in a decrease of cytosolic ATP concentration to micromolar levels. This range is comparable with the EC_{50} of the known $P2Y_1$ and $P2Y_2$ receptors on the cell surface. Thus, we investigated the mitochondrial P2Y-like receptors' response to this range of concentrations of purinergic ligands.

AMP-PNP Stimulated Mitochondrial Ca^{2+} Uptake

We first tested the effect of AMP-PNP, a non-hydrolysable structural analog of ATP, on mitochondrial Ca^{2+} uptake using concentrations ranging from 0.1 μM to 2 mM. AMP-PNP stimulated mitochondrial Ca^{2+} uptake in the concentration range from 100 to 1,000 μM with maximum stimulation at 1,000 μM. When mitochondria were pre-incubated with the non-specific inhibitor of P2Y receptors PPADS, the stimulating effect of AMP-PNP on mitochondrial Ca^{2+} uptake was lost (Fig. 1). These data suggest that AMP-PNP stimulates mitochondrial Ca^{2+} uptake through a mechanism involving purinergic receptors.

$P2Y_1$ and $P2Y_2$ Receptor Specific Ligands Increase Mitochondrial Ca^{2+} Uptake

Our previous findings confirmed the presence of immunosignals specific for $P2Y_1$ and $P2Y_2$ receptors in the mitochondrial membrane [Belous et al., 2004]. In order to determine a receptor specific effect, selective non-hydrolysable analogs for the known $P2Y_1$ and $P2Y_2$ receptors were used in this set of experiments. 2meSADP is known as the most potent and specific ligand for activation of $P2Y_1$ receptors [North and Barnard, 1997]. 2meSADP provided

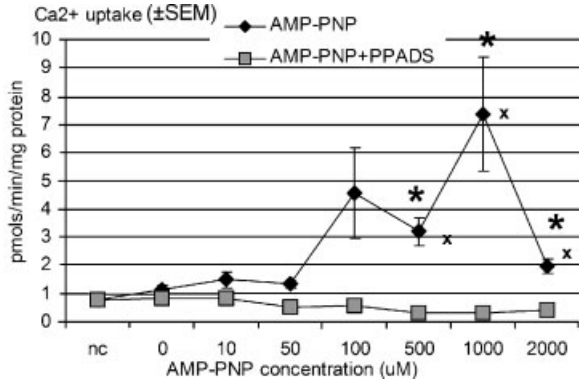

Fig. 1. Effect of AMP-PNP on mitochondrial Ca^{2+} uptake. Maximal stimulatory concentration of AMP-PNP is 1,000 mM. The non-specific P2Y receptor inhibitor PPADS is able to block AMP-PNP stimulated mCa^{2+} uptake. $^{*}P < 0.05$ versus AMP-PNP + PPADS; $^{x}P < 0.05$ versus ''0'' (no AMP-PNP). Error bars represent SEM. nc – negative control, -mitochondrial Ca^{2+} uptake in the absence of respiratory substrates.

maximal stimulation of mitochondrial Ca^{2+} uptake at a concentration range from 0.05 to 0.1 μM; pre-incubation of mitochondria with PPADS prevented this stimulatory response (Fig. 2A). This concentration range is similar to the stimulatory response with $EC_{50} = 0.15$ nM to 2meSADP measured in human $P2Y_1$ cell surface receptor purified after high-level expression from a recombinant baculovirus in Sf9 insect cells [Waldo and Harden, 2004]. Higher concentrations of 2meSADP (over 50 μM) had a strong inhibitory effect on mitochondrial Ca^{2+} uptake. Since PPADS failed to affect this higher-concentration inhibitory action, it is probably mediated by a mechanism described by Litsky and Pfeiffer [1997] and not via the purinergic-like receptors.

For specific activation of $P2Y_2$-like receptors in mitochondria we used UTP, which is reported to be the most potent activator of $P2Y_2$ receptors [Ralevic and Burnstock, 1998]. In UTP-induced [Ca^{2+}] changes in human 1321N1 astrocytoma cells expressing porcine $P2Y_2$ receptors, effective concentrations of UTP were 10–100 μM [Shen et al., 2004]. A similar effective concentration range of UTP stimulation of IP3 production was observed in cultured rat aortic smooth muscle cells [Kumari et al., 2003]. In our experiments UTP effectively stimulated mitochondrial Ca^{2+} uptake with the maximum effective concentration at 100 μM. Higher concentrations produced a decrease in Ca^{2+} uptake. PPADS effectively blocked the stimulatory but not inhibitory effect of UTP (Fig. 2B).

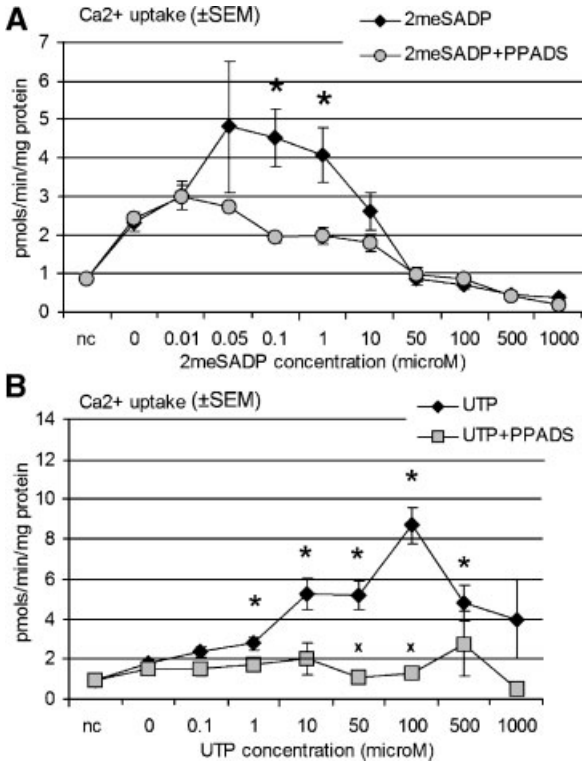

Fig. 2. Mitochondrial Ca^{2+} accumulation in response to variable concentrations of specific $P2Y_1$ and $P2Y_2$ ligands. **A**: 2meSADP stimulated mitochondrial Ca^{2+} uptake with maximum effect at a concentration range of 0.05–0.1 mM. The non-specific P2Y receptor inhibitor PPADS was able to block this effect of 2meSADP. $^{*}P < 0.05$ versus ''2meSADP + PPADS.'' **B**: Maximal stimulatory concentration of UTP was 100 mM. The non-specific P2Y receptor inhibitor PPADS was able to block UTP stimulated mCa^{2+} uptake. $^{*}P < 0.05$ versus ''0'' (no UTP); $^{x}P < 0.05$ versus ''UTP.'' Error bars represent SEM. nc – negative control, -mitochondrial Ca^{2+} uptake in the absence of respiratory substrates.

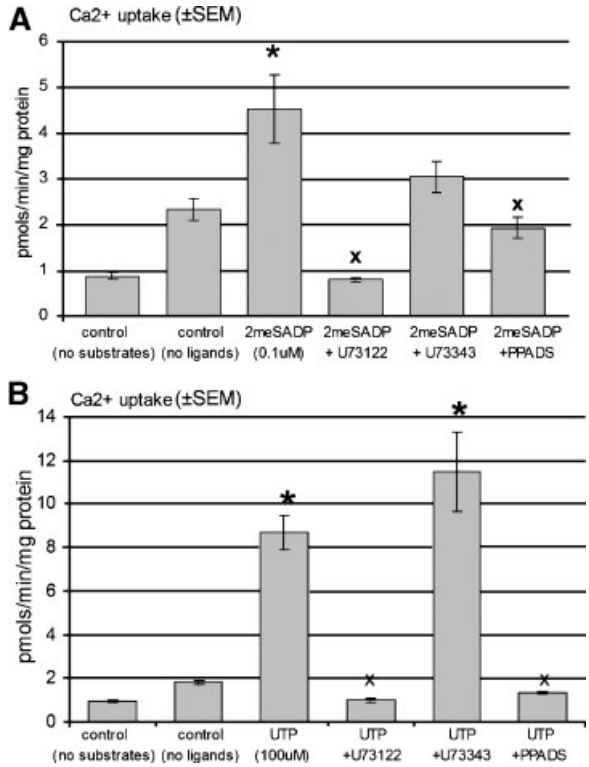

Fig. 3. The role of PLC in mitochondrial Ca^{2+} accumulation. **A**: The maximal stimulatory concentration of 0.1 mM 2meSADP was used to investigate the role of PLC in mitochondrial $P2Y_1$-like signaling. The specific inhibitor of PLC U73122 completely blocks the stimulatory effect of 2meSADP on mitochondrial Ca^{2+} uptake. The inactive analog of U73122, U73343, does not affect the stimulatory action of 2meSADP. PPADS significantly inhibits the effect of 2meSADP. $^{*}P < 0.01$ versus both controls; $^{x}P < 0.05$ versus ''2meSADP (0.1 mM).'' **B**: In the presence of the maximal stimulating concentration of UTP (100 mM), mitochondrial Ca^{2+} uptake is significantly higher than without any ligands. The specific inhibitor of PLC, U73122, completely blocks the stimulatory action of UTP on Ca^{2+}uptake. Inactive U73343 does not change the effect of UTP ($^{*}P < 0.05$ versus both controls). PPADS and U73122 had similar blocking effect on UTP action ($^{x}P < 0.05$ versus ''UTP 100 M'' and ''UTP + U73343''). Error bars represent SEM.

Mitochondrial PLC Inhibition Blocks P2Y Agonist Stimulated Mitochondrial Ca^{2+} Uptake

The known cell surface purinergic $P2Y_1$ and $P2Y_2$ receptors belong to the family of G-protein coupled receptors. Their signaling mechanism involves activation of PLC; thus, an analogous mechanism might be involved in the signaling of mitochondrial purine receptors. The presence of PLC and its involvement in mitochondrial Ca^{2+} transport has been recently reported by our group [Knox et al., 2004]. In the current experiments, we used the specific inhibitor of PLC, U73122, to determine the role of PLC in mitochondrial purinergic signaling. In our current experiments U73122 inhibited mitochondrial Ca^{2+} uptake and blocked its activation by 2meSADP and UTP. The inactive analog of U73122 (U73343) did not affect mitochondrial responses to 2meSADP and UTP (Fig. 3A,B).

MRS-2179 Stimulates Mitochondrial Ca^{2+} Uptake

In order to further characterize the mitochondrial $P2Y_1$-like receptor, we used MRS-2179 as a selective $P2Y_1$ receptor antagonist. MRS-2179 is reported to be a strong inhibitor of ADP-induced platelet aggregation in vitro and ex vivo [Boyer et al., 1998]. In our experiments MRS-2179 (10 μM) failed to inhibit the activation of mitochondrial Ca^{2+} uptake induced by 2meSADP. In Figure 4A, the application of 10 mM MRS-2179 did not cause statistically significant changes in 2meSADP-stimulated mitochondrial Ca^{2+} uptake. In further experi-

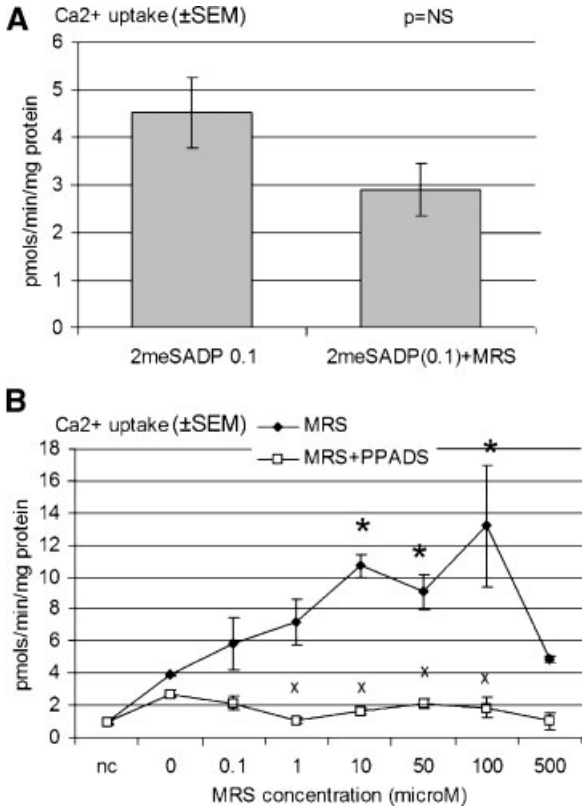

Fig. 4. The role of MRS2179 in mitochondrial Ca^{2+} accumulation. **A**: The specific P2Y1 inhibitor MRS2179 (10 mM) did not block the stimulatory signal of 2meSADP. **B**: MRS2179 increased mitochondrial Ca^{2+} uptake, with a maximum stimulation at 10–100 mM. PPADS was able to block this response. $^{*}P < 0.05$ versus ''0'' (no ligands), $^{x}P < 0.05$ versus ''MRS alone.'' Error bars represent SEM. nc – negative control, -mitochondrial Ca^{2+} uptake in the absence of respiratory substrates.

ments, we discovered that MRS-2179 alone has agonistic properties. It was able to stimulate mitochondrial Ca^{2+} uptake in the concentration range from 10 to 100 μM, and this effect was blocked by PPADS, suggesting agonist properties in mitochondrial purinergic receptor signaling (Fig. 4B).

Mitochondrial Ca^{2+} Uptake Dynamics

In all previously described experiments, we measured Ca^{2+} content in mitochondria after 20 min of incubation. The results reflect a balance between Ca^{2+} uptake and Ca^{2+} release by the mitochondria during the incubation. Previous experiments suggest that application of purinergic ligands resulted in increased or decreased sequestration of Ca^{2+} inside the mitochondria, but it is not clear which mechanism was affected (Ca^{2+} uptake or release) or what the time course of Ca^{2+} accumulation was during the 20 min of incubation. For this reason we determined the dynamics of the Ca^{2+} accumulation process. Most of the Ca^{2+} accumulation in mitochondria occurs during the first minute of incubation. After 2–3 min of incubation with substrates alone, mitochondria start to loose Ca^{2+}. Application of either UTP or 2meSADP in maximal stimulatory concentrations increases Ca^{2+} content of mitochondria. This increase occurs not only during the first minute of incubation but also during the remainder of the incubation period (Fig. 5).

This action of the ligands could be either due to activation of Ca^{2+} uptake or inhibition of Ca^{2+} release. In order to distinguish between these two processes, we used RR, a specific inhibitor of mitochondrial Ca^{2+} uptake via the calcium uniporter. RR was added to the buffer after 2 min of incubation, allowing enough time for initial Ca^{2+} accumulation. When purinergic ligands were absent, application of RR after 2 min of incubation did not change the dynamics of mitochondrial Ca^{2+}, suggesting that after 2

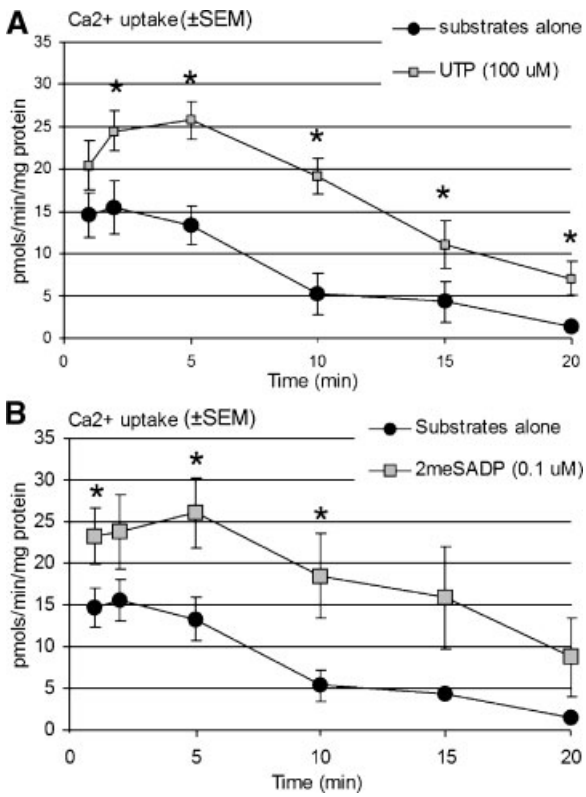

Fig. 5. Mitochondrial Ca^{2+} accumulation during 20 min of incubation in the presence of maximal stimulating concentrations of (**A**) UTP (100 mM) and (**B**) 2meSADP (0.1 mM), versus Ca^{2+} accumulation without any ligands. $^{*}P < 0.05$ versus ''substrates alone.'' Error bars represent SEM.

min of incubation no additional Ca^{2+} accumulation occurs and Ca^{2+} content of mitochondria decreases via slow release mechanisms (Fig. 6A).

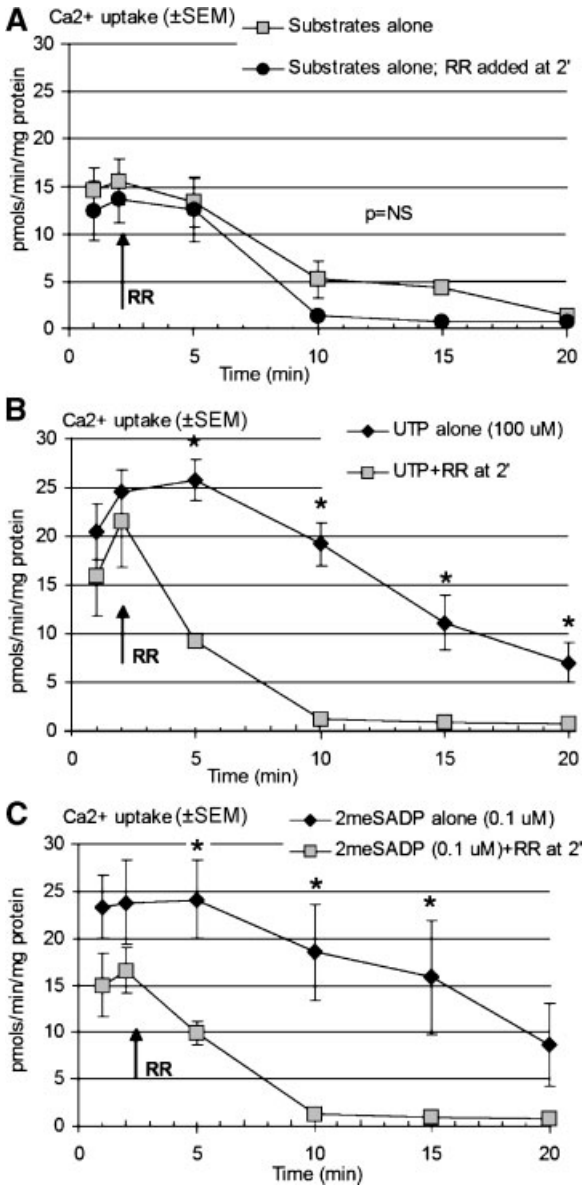

Fig. 6. Mitochondrial Ca^{2+} uptake dynamics. **A**: Blockade of mitochondrial Ca^{2+} uptake with ruthenium red (RR) after 2 min of incubation did not alter the dynamics of mitochondrial Ca^{2+} accumulation when purinergic ligands were not present, indicating that Ca^{2+} uptake occurred only at the beginning of incubation with gradual release thereafter. In the presence of purinergic receptor ligands (**B**) UTP and (**C**) 2meSADP, the addition of RR prevented ligand stimulated mitochondrial Ca^{2+} accumulation, indicating that purinergic stimulation results in higher Ca^{2+} accumulation due to more active and prolonged Ca^{2+} uptake via the uniporter since uniporter inhibition leads to diminished Ca^{2+} accumulation; $^*P < 0.05$. Error bars represent SEM.

On the other hand, when UTP or 2meSADP was present in the buffer at its maximal stimulatory concentration, addition of RR resulted in a significant drop in mitochondrial Ca^{2+} content, suggesting that activated $P2Y_1$ and $P2Y_2$ receptors stimulated Ca^{2+} uptake during the whole period of incubation (Fig. 6B,C). When UTP and 2meSADP were absent, practically 100% of Ca^{2+} accumulation occurred during the first 2 min of incubation only. Addition of UTP or 2meSADP stimulated initial Ca^{2+} uptake and increased duration of Ca^{2+} accumulation beyond the first 2 min without significant effect on Ca^{2+} release.

A final consideration was that all mitochondrial Ca^{2+} uptake studies were performed at atmospheric oxygen partial pressure. However, since we used different types of tubes and different volumes of mitochondrial suspension for the different experiments, we were concerned that oxygen availability might not have been the same. For the experiments with 20 min incubation, we used a total volume of 2 ml in a tube with a diameter of 15 mm; for the experiments where Ca^{2+} uptake was measured in time (mitochondrial Ca^{2+} uptake dynamics), we used larger volumes, with correspondingly increased number of mitochondria (total volume of 20 ml and tube diameter of 30 mm) This raised the possibility that oxygen availability via diffusion was limited in the larger volume experiments. In order to investigate this possibility, in a separate series of experiments atmospheric air was bubbled through the mitochondrial suspension in large tubes during the 20 min of incubation and compared to non-bubbled incubations. We were unable to demonstrate any difference in mitochondrial Ca^{2+} uptake in these experiments compared with regular incubation (data not shown), indicating that oxygen was equally available for mitochondria incubated in larger volumes, and the results were not altered by oxygen availability.

Mitochondrial Viability

MitoTracker Red CM-H_2XRos is only accumulated by respiring mitochondria with normal $\Delta\Psi$ potential [Knox et al., 2004]. The fluorescence of MitoTracker Red loaded mitochondria was measured. Mitochondrial $\Delta\Psi$ potential was not affected by incubation with PPADS, U-73122, U-73353, UTP, AMP-PNP, or 2meSADP (data not shown). Respiratory control experiments with 5 mM succinate as a substrate were

performed in order to assess state III, IV, and uncoupled rates of respiration. These experiments confirmed that mitochondrial respiratory function was not altered by experimental conditions (data not shown).

DISCUSSION

In our previous publication [Belous et al., 2004] we reported the presence of $P2Y_1$- and $P2Y_2$-like receptors on mitochondrial membranes. We found that purified mitochondrial membranes contain proteins similar to known cell-surface $P2Y_1$ and $P2Y_2$ purinergic receptors. We also found that ATP and ADP have a concentration-dependent effect on mitochondrial Ca^{2+} uptake. We attributed these initially observed effects to the action of mitochondrial purinergic receptors. We found that the nonselective P2Y antagonist PPADS was able to inhibit Ca^{2+} uptake in presence of ADP and AMP-PNP, but PPADS failed to affect action of ATP and UTP at the tested concentration range (1–3 mM). In addition, the specific $P2Y_1$ inhibitor MRS-2179 also failed to affect Ca^{2+} uptake response to ATP, ADP, UTP, and AMP-PNP.

Thus, several considerations from that article needed clarification. First, we have subsequently determined that ATP and ADP concentrations over time, when measured by luminometry technique, were not stable in those experiments; probably due to the hydrolysis of ATP and phosphorylation of ADP by mitochondrial complex V (ATP synthase) [Belous et al., 2003b]. Though absence of magnesium is reported by others [Fagian et al., 1986] to inhibit this, it was not the case in our experiments. Secondly, we had questioned the concentrations of the ligands used in our initial experiments since they were significantly higher than the range of sensitivity of known purinergic receptors. Finally, all tested ligands had a biphasic action; at lower concentrations (1 mM) they seemed to stimulate mitochondrial Ca^{2+} uptake, and at high concentrations, inhibitory action prevailed.

To address these issues, we discontinued use of ATP and ADP. The non-hydrolysable analog of ATP, AMP-PNP, was used instead of ATP; 2meSADP was used as a specific ligand of $P2Y_1$, and UTP was selected as a specific ligand of $P2Y_2$. The effect of these compounds on mitochondrial Ca^{2+} uptake was also studied at lower concentrations, compatible with the range of sensitivity of known cell-surface purinergic receptors. The results presented in this publication indicate that in the low concentration range both $P2Y_1$ and $P2Y_2$ ligands have stimulatory effects on mitochondria Ca^{2+} uptake but with different maximal stimulatory concentrations. Based on these observations we now conclude that mitochondrial Ca^{2+} handling is regulated in part by mitochondrial $P2Y_1$- and $P2Y_2$-like receptors, located on mitochondrial membranes: activation of $P2Y_1$ by 2meSADP (0.05-0.1 μM) and $P2Y_2$ by UTP (100 μM) stimulates Ca^{2+} uptake by mitochondria.

The results of our mitochondrial Ca^{2+} uptake dynamic studies indicate that $P2Y_2$ and $P2Y_1$ ligands affect Ca^{2+} uptake, not release. These effects can be cancelled by P2Y inhibition with PPADS or inhibition of PLC with U-73122. The signaling mechanism of these mitochondrial purinergic-like receptors likely involves activation of mitochondrial PLC (previously described by our group [Knox et al., 2004]) via a G-protein coupled process; however, to date, we and others have not been able to confirm the presence of G_{q11} or $G_{i/o}$ subclasses of G-proteins in mitochondria. These are the G-proteins usually associated with cell-surface $P2Y_1$ and $P2Y_2$ receptors, respectively [Boarder and Hourani, 1998]. While the exact signaling mechanism of mitochondrial purinergic receptors is not clear at this time, recent data from our group demonstrate that cold ischemia of the rat liver produced significant increase in mitochondrial PLC-δ1 expression, suggesting involvement of PLC-mediated signaling in mitochondrial Ca^{2+} regulation in ischemic settings [Knox et al., 2006].

In the present data, the maximum stimulatory concentration range for mitochondrial $P2Y_1$- and $P2Y_2$-like receptors is in the nano- and micromolar range. If we assume that natural ligands of these receptors in the cell are ATP ($P2Y_2$) and ADP ($P2Y_1$), it is clear that these receptors would not be functional at normal conditions when cellular ATP and ADP levels are too high to be in the range of sensitivity for these receptors. We propose that these receptors play a role in the low-energy states of the cell when ATP and ADP levels decrease dramatically. In such conditions as hypoxia, levels of ATP and ADP may reach working ranges for the receptors [Dransfield and Aprille, 1994], activating mitochondrial

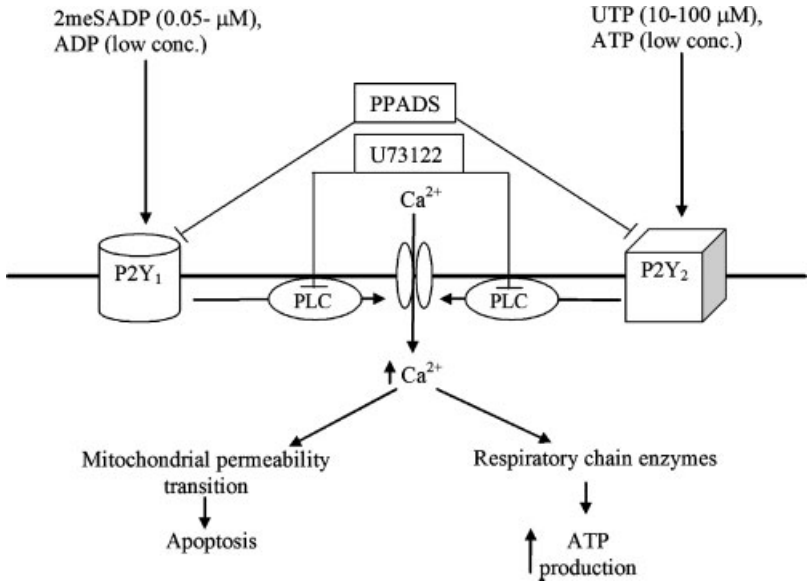

Fig. 7. Schema of the proposed regulation of mitochondrial Ca^{2+} uptake by mitochondrial P2Y-like receptors.

Ca^{2+} uptake in an attempt to stimulate mitochondrial ATP production (via upregulation of respiratory enzymes) as a feedback loop. Alternatively, increased mitochondrial Ca^{2+} uptake may initiate the mitochondrial death pathway, triggering Ca^{2+}-dependent permeability transition of mitochondria. We summarize our experimental results and present a proposed schema in Figure 7.

Surprisingly, the selective inhibitor of cell-surface $P2Y_1$ receptors MRS-2179 had an agonistic action which could be cancelled by PPADS, indicating that it acts via mitochondrial P2Y-like receptors. This suggests functional and structural differences of mitochondrial $P2Y_1$ receptors from known cell membrane counterparts.

The present data strongly support our hypothesis that P2Y-like receptors exist in mitochondria where they regulate mitochondrial calcium uptake in low-energy states, such as hypoxia/ischemia and may be involved in the mitochondrial pathway of cell death. Given the variety of pathological conditions that involve such mechanisms, the understanding of the signaling mechanisms of mitoP2Y receptors and the development of their selective antagonists may have useful clinical applications.

ACKNOWLEDGMENTS

This work was supported by National Institute of Health (DK59390 and DK064669 to RSC), AOA Ruth Kuckein fellowship to CDK, and Association of Academic Surgery research fellowship to CMJ.

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