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Mitochondria play an important role in adenosine-induced ATP release from Madin–Darby canine kidney cells

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ABSTRACT

We previously found that adenosine stimulates ATP release from Madin–Darby canine kidney (MDCK) cells, by activating an Ins(1,4,5)P_3 sensitive-calcium (Ca^{2+}) pathway through the stimulation of A_1 receptors. Thus, we investigated the intracellular pathway of ATP efflux after the rise in intracellular Ca^{2+} in MDCK cells. Adenosine evoked an increase in mitochondrial Ca^{2+} using Rhod-2/AM, a mitochondrial Ca^{2+} indicator. Adenosine-induced ATP release was inhibited by mitochondrial modulators, such as oxidative phosphorylation modulators (carbonyl cyanide 3-chlorophenylhydrazone and oligomycin), mitochondrial ADP/ATP carrier inhibitors (*N*-ethylmaleimide, carboxyatractyloside and bongkrekic acid), a mitochondrial Na^+–Ca^{2+} exchange inhibitor (CGP-37157). In addition, mitochondrial modulators significantly reduced intracellular ATP content. On the other hand, 2-deoxy-glucose (2-DG) induced a greater decrease in intracellular ATP content than mitochondrial modulators. ATP release was still induced by adenosine in the presence of 5 mM 2-DG. These results suggest that mitochondria play an important role in the signaling pathway of adenosine-triggered ATP release in MDCK cells.

1. Introduction

Adenosine triphosphate (ATP), an endogenous ligand of purine receptors, plays an important role in both intracellular and extracellular as a neurotransmitter and an autocrine/paracrine molecule [1]. ATP is released from various cells and tissue types by mechanical stimulation [2], shear stress [3] and hypotonic cell swelling [4]. Our laboratory has previously reported that ATP is released from smooth muscle cells by stimulation with physiologically active substances, such as bradykinin, substance P, histamine, angiotensin II and α,β-methylene ATP [5–7].

In the kidney, it has been shown that P2X and P2Y receptors are expressed in the nephron and renal vasculature [8,9] and that ATP is released from renal tubular epithelial cells [10], endothelial cells [11], smooth muscle cells [11], platelets [11] and nerves [12], suggesting that the purinergic system in the kidney plays an important physiological role by acting in an autocrine/paracrine manner. We recently found that adenosine induced ATP release via activation of A_1 receptors in Madin–Darby canine kidney (MDCK) cells [13]. This release was associated with an increase of intracellular calcium (Ca^{2+}) concentration ($[Ca^{2+}]_i$), which was activated by stimulation of intracellular inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) receptors. However, it is currently unclear how ATP is released from MDCK cells after the increase of $[Ca^{2+}]_i$ in response to activation of the A_1 receptor. With regard to the role of intracellular Ca^{2+}, Rizzuto et al. showed that Ins(1,4,5)P_3-induced Ca^{2+} mobilization from intracellular Ca^{2+} stores caused an increases in mitochondrial Ca^{2+}

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concentration ($[Ca^{2+}]_{mito}$) [14]. As it is well known that mitochondria are important in ATP synthesis and the supply of ATP to the cytosol [15], it is considered that mitochondria are associated with the pathway of ATP efflux from MDCK cells after elevation of $[Ca^{2+}]_i$ in response to adenosine. In the present study, we found that mitochondria play an important role for triggering the release of ATP from MDCK cells.

2. Materials and methods

2.1. Cell culture

MDCK (NBL-2) cells were obtained from the Health Science Research Resources Bank (JCRB9029, HSRRB) and were maintained in D-MEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C, 5% CO_2. Cells were dispersed at 1×10^5 cells in 35 mm plastic plates for each experiment.

2.2. Measurement of ATP release

The release of ATP was quantified by a bioluminescence method using the luciferase-luciferin assay, as previously described [16]. Briefly, MDCK cells were perfused with a HEPES buffer solution (140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose at pH 7.4, 36 °C) at a rate of 0.5 ml/min using a peristaltic pump. Samples were collected by perfusate every 1.5 min. Adenosine was administrated from 0 to 4.5 min. All antagonists were applied for 5–30 min before addition of adenosine. ATP release was measured in each aliquot using a lumitester (C-100, Kikkoman, Japan). The protein content in the cells at the end of the experiment was determined by the Bio-Rad protein assay kit II following cell lysis with 0.1% Triton X-100.

2.3. Measurement of intracellular ATP content

MDCK cells were washed by HEPES buffer solution at 37 °C. After applying mitochondrial modulators or ABC protein modulators to each well, 250 μl of 20% perchloric acid (final concentration, 4%) was added to stop reactions. Cells were then placed on ice for at least 30 min and isolated mechanically. The suspended cells were transferred to a polyester tube and centrifuged at $10{,}000 \times g$ for 8 min at 4 °C. The supernatant was transferred to a new tube and adjusted to pH 7.0 by 1:10,000 dilution of HEPES buffer. Samples were measured by luciferase-luciferin assay using a lumitester (C-100, Kikkoman, Japan). The pellet was used for protein assay. Protein concentrations were determined by the Bio-Rad protein assay kit II following cell lysis with 0.1% Triton X-100.

2.4. Measurement of intracellular Ca^{2+}

Cells were incubated with 10 μM Fluo-4/AM in HEPES buffer solution for 20 min at 37 °C, and then washed with HEPES buffer solution for at least 30 min at room temperature. $[Ca^{2+}]_i$ was observed in a flowing bath positioned on the stage of a fluorescence microscope (Diaphot-TMD, Nikon, Tokyo, Japan). The flow rate was 1 ml/min using a peristaltic pump. Imaging was performed with a 20× objective lens and detected by a cooled CCD camera (CCD300-RC, DAGE-MTI, Michigan City, IN) with an image intensifier unit (c9016-01, Hamamatsu Photonics, Shizuoka, Japan). Fluorescence excitation at 450–490 nm was supplied by a mercury lamp and emission was detected at 520–560 nm. The fluorescence signal intensity was processed using Metamorph software (Universal Imaging Corporation, Downingtown, PA) and stored on a computer. The $[Ca^{2+}]_i$ ratios were calculated using the following formula:

$$\text{ratio of } [Ca^{2+}]_i = \frac{F_2 - F_2}{F_1 - F_1}$$

F_1 is the fluorescence for the first application of adenosine. F_2 is the second application.

2.5. Measurement of mitochondrial Ca^{2+}

MDCK cells were loaded with 5 μM Rhod-2/AM in HEPES buffer solution for 30–60 min at room temperature. After that, cells were loaded with 100 nM MitoTracker green FM (MTG) for 30 min at 37 °C. Fluorescence measurements were performed with a 50× oil-immersion objective (Nikon). MTG and Rhod-2/AM were excited by a mercury lamp at 560 and 450–490 nm, respectively, and emission was detected at more than 590 and 520–560 nm, respectively. Images were collected and recorded using the same system as described in the $[Ca^{2+}]_i$ experiment.

2.6. Data analysis

Values are expressed as the mean ± standard error of the mean value (S.E.M.) for the number of cells. The significance of differences between multiple means was determined by one-way analysis of variance (ANOVA) followed by the Dunnett test. A value of $P < 0.05$ was considered significant.

2.7. Chemicals

D-MEM, fatal bovine serum, penicillin and streptomycin were obtained from Gibco Laboratories (Burlington, Canada). Oligomycin and *N*-ethylmaleimide (NEM) were obtained from Nacalai tesgue (Kyoto, Japan). CGP-37157 was purchased from Tocris (Bristol, UK). Rhod-2/AM was obtained from Dojindo (Kumamoto, Japan). MitoTracker Green FM was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were purchased from Sigma–RBI (St. Louis, MO, USA).

3. Results

3.1. Increase of intracellular Ca^{2+} induced by adenosine from Ca^{2+} store in MDCK cells

Sipma et al. showed that the activation of A_1 receptors was capable of causing a phospholipase C (PLC)-dependent rise in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells [17]. We also confirmed whether adenosine induced a change of $[Ca^{2+}]_i$ in MDCK cells. As shown in Fig. 1A and B, 10 μM adenosine caused a transient increase in the fluorescence intensity of Fluo-4/AM (ratio of $[Ca^{2+}]_i = 0.75 \pm 0.06$, $n = 7$). This increase was completely blocked by 50 μM 2-APB, an $Ins(1,4,5)P_3$ receptor inhibitor

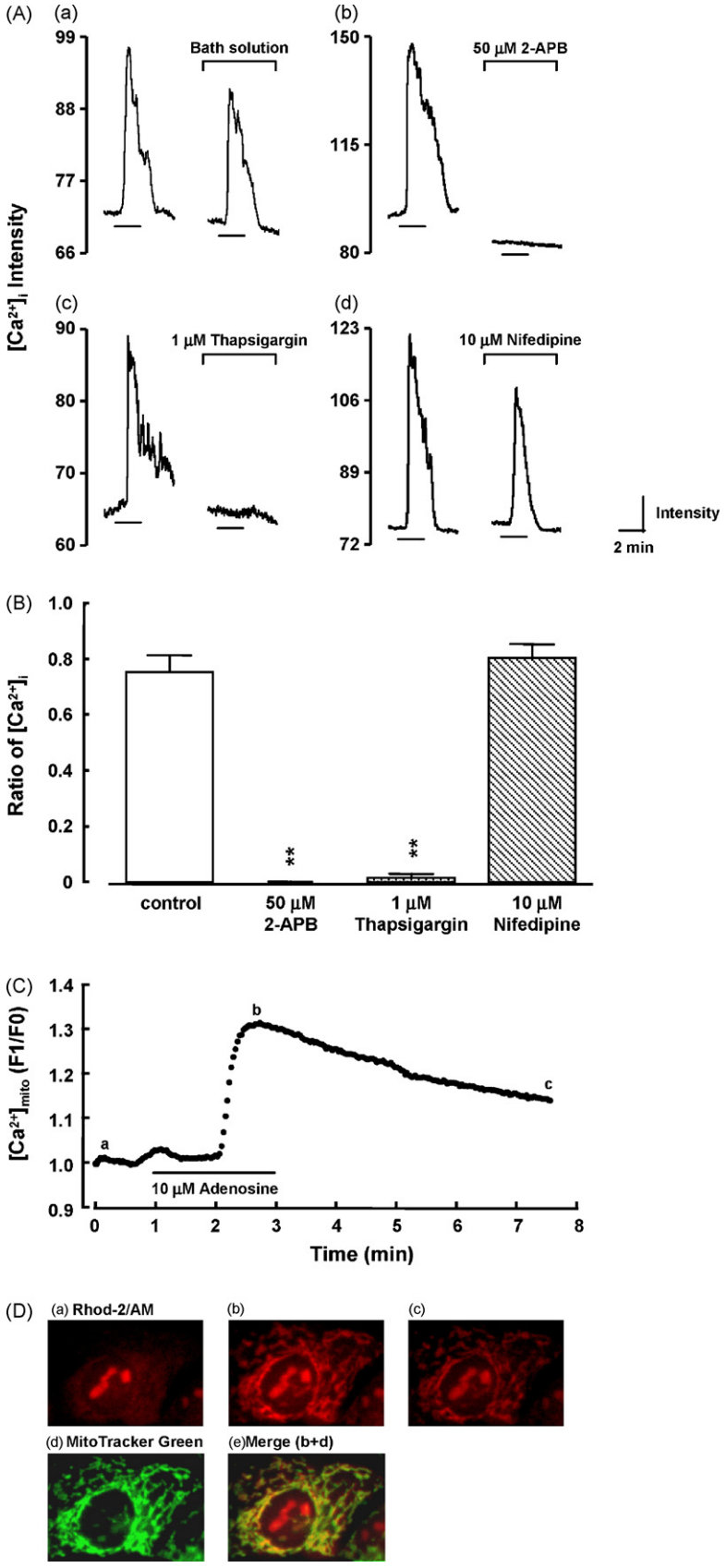

Fig. 1 – Adenosine-induced changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mito}$ in MDCK cells. (A) MDCK cells were loaded with Fluo-4/AM for Ca^{2+} imaging. Fluorescence intensity transiently increased when adenosine was applied to the cells. MDCK cells were incubated with 50 μM 2-APB, 1 μM thapsigargin and 10 μM nifedipine for 5–15 min before the application of 10 μM adenosine. (B) Effects of antagonists of intracellular signals on adenosine-induced increases of $[Ca^{2+}]_i$ in MDCK cells (see

(ratio of $[Ca^{2+}]_i = 0.01 \pm 0.02$, $n = 12$), and 1 μM thapsigargin, a Ca^{2+}-ATPase inhibitor (ratio of $[Ca^{2+}]_i = 0.02 \pm 0.02$, $n = 7$). However, 10 μM nifedipine, a L-type Ca^{2+} channel inhibitor, did not block the increase in $[Ca^{2+}]_i$ (ratio of $[Ca^{2+}]_i = 0.80 \pm 0.05$, $n = 8$). These results indicate that the rise in $[Ca^{2+}]_i$, that was induced by adenosine was derived from Ins(1,4,5)P_3 receptor-sensitive intracellular Ca^{2+} stores in MDCK cells.

3.2. Change of mitochondrial Ca^{2+} induced by adenosine in MDCK cells

Szabadkai et al. reported that mitochondrial Ca^{2+} uptake depends on the preservation of high Ca^{2+} microdomains at the mouth of endoplasmic reticulum Ca^{2+} release sites close to mitochondria [18]. We thus measured whether adenosine caused a change in $[Ca^{2+}]_{mito}$ in MDCK cells using the Ca^{2+} indicator, Rhod-2/AM. The intensity of Rhod-2/AM fluorescence was elevated by 10 μM adenosine (ratio of $[Ca^{2+}]_{mito} = 1.27 \pm 0.02$, $n = 4$) and the fluorescence almost overlapped with MTG fluorescence (Fig. 1C and D). MTG staining sites were observed not only around nuclei but also near the plasma membrane. These results show that Ca^{2+} released from Ins(1,4,5)P_3 receptor-sensitive intracellular Ca^{2+} stores in response to adenosine is rapidly sequestered by the mitochondria.

3.3. Effect of mitochondrial regulators on adenosine-induced ATP release from MDCK cells

Our previous report showed that adenosine stimulates ATP release from MDCK cells, by activating an Ins(1,4,5)P_3 sensitive-Ca^{2+} pathway through the stimulation of A_1 receptors [13]. We also confirmed that CPA, a selective A_1 receptor agonist, stimulates ATP release (data not shown). Next, to examine the involvement of mitochondria in ATP release, we tested the effect of mitochondrial regulators on adenosine-induced ATP release. The application of 10 μM adenosine for 4.5 min enhanced the basal release of ATP in MDCK cells by approximately three-fold (from 1.08 ± 0.15 up to 2.78 ± 0.44 pmol/mg protein), and the peak appeared at 4.5 min (Fig. 2A and B). Pretreatment with modulators of oxidative phosphorylation, carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μM) and oligomycin (10 μM), markedly reduced adenosine-induced ATP release (0.53 ± 0.05 and 0.70 ± 0.35 pmol/mg protein at 4.5 min, respectively). Mitochondrial ADP/ATP carrier inhibitors, *N*-ethylmaleimide (NEM, 50 μM), carboxyatractyloside (CAT, 10 μM) and bongkrekic acid (BKA, 10 μM), also strongly abolished adenosine-induced ATP release (0.38 ± 0.05, 0.72 ± 0.25 and 1.16 ± 0.09 pmol/mg protein at 4.5 min, respectively). Furthermore, ATP release was completely blocked by preincubation with a mitochondrial Na^+–Ca^{2+} exchange inhibitor, CGP-37157 (20 μM) (0.82 ± 0.18 pmol/mg protein at 4.5 min). These results suggest that mitochondria are involved in the pathway of adenosine-induced ATP release.

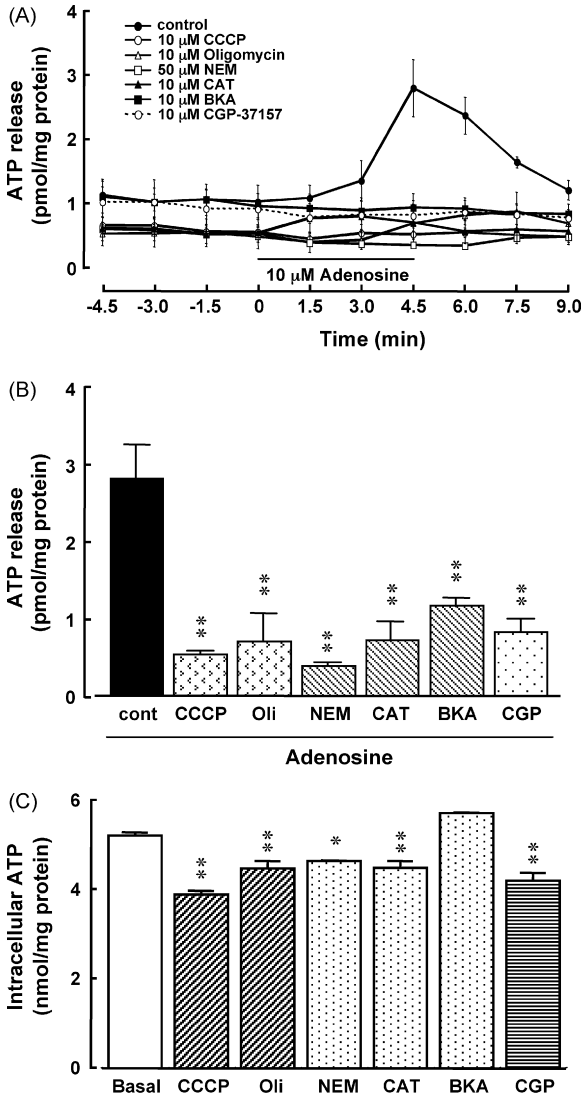

Fig. 2 – Effect of mitochondrial modulators on adenosine-induced ATP release from MDCK cells. (A) The cells were exposed to the mitochondrial modulators (10 μM CCCP, 10 μM oligomycin, 50 μM NEM, 10 μM CAT, 10 μM BKA and 20 μM CGP-37157) for 30–60 min before the application of adenosine. (B) Control (cont), CCCP, oligomycin (Oli), NEM, CAT, BKA and CGP-37157 (CGP) show the release of ATP at 4.5 min after the administration of adenosine. Values are expressed as mean ± S.E.M. of pmol/mg protein of ATP ($n = 3$–20). $^{**}P < 0.01$ vs. control. (C) Effects of mitochondrial modulators on intracellular ATP content in MDCK cells. The cells were exposed to the mitochondrial modulators (10 μM CCCP, 10 μM oligomycin, 50 μM NEM, 10 μM CAT, 10 μM BKA and 20 μM CGP-37157) for 30–60 min before the application of adenosine. Values are expressed as mean ± S.E.M. of pmol/mg protein of ATP ($n = 3$–9). $^{**}P < 0.01$ vs. basal.

Section 2). Values are expressed as mean ± S.E.M. of the $[Ca^{2+}]_i$ ratio ($n = 8$–31). $^{**}P < 0.01$ vs. control. (C) MDCK cells were loaded with Rhod-2/AM. The changes in fluorescence are presented as $[Ca^{2+}]_{mito}$ (F_1/F_0), where F_1 is the measured fluorescence and F_0 is the initial fluorescence. (D) Images of Rhod-2/AM and MTG in MDCK cells. (a–c) Images of Rhod2/AM before (a), at peak (b) and after washing out (c) the application of adenosine; (d) an image of MTG staining; (e) merge of (b) and (d). Yellow colors indicate overlapping sites of Rhod-2/AM fluorescence and MTG staining.

3.4. *Influence of mitochondrial modulators on intracellular ATP content in MDCK cells*

One of the basic mitochondrial functions is ATP synthesis. We thus measured the effect of mitochondrial modulators on intracellular ATP content in MDCK cells. Intracellular ATP content was 5.17 ± 0.07 nmol/mg protein in MDCK cells (Fig. 2C). In separate experiments, exposure of the cells to 10 μM CCCP, 10 μM oligomycin, 50 μM NEM, 100 μM CAT and 20 μM CGP-37157 significantly decreased intracellular ATP content (3.86 ± 0.08, 4.44 ± 0.16, 4.61 ± 0.01, 4.45 ± 0.16 and 4.16 ± 0.19 nmol/mg protein, respectively). However, incubation with 10 μM BKA did not significantly reduce intracellular ATP content (5.67 ± 0.02 nmol/mg protein). These results suggest that a part of intracellular ATP content is regulated by mitochondrial regulators.

3.5. *Effect of 2-deoxy-glucose (2-DG) on adenosine-induced ATP release from MDCK cells*

To characterize the relationship between intracellular ATP content and ATP release, we compared adenosine-triggered ATP release in the presence and absence of the glycolysis inhibitor, 2-DG. Pretreatment of the cells with 5 mM 2-DG significantly decreased intracellular ATP content (3.20 ± 0.14 pmol/mg protein) compared with basal intracellular ATP content (5.51 ± 0.30 pmol/mg protein; Fig. 3A). This decrease was greater than that caused by mitochondrial modulators. In addition, 10 mM 2-DG strongly decreased intracellular ATP content (1.27 ± 0.05 pmol/mg protein; Fig. 3A). On the other hand, adenosine-induced ATP release was not inhibited by treatment with 5 mM 2-DG, although basal ATP release was reduced (Fig. 3B). This release was perfectly inhibited by CCCP. However, adenosine failed to induce ATP release in treatment with 10 mM 2-DG. From these results, adenosine-triggered ATP release does not parallel to the change of intracellular ATP content, although the extreme reduction of intracellular ATP content cannot be negligible for the influence on adenosine-induced ATP release.

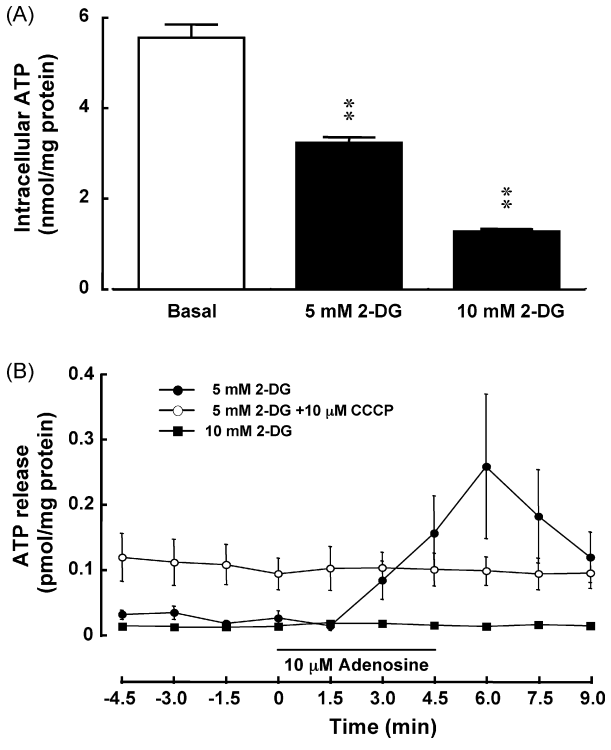

Fig. 3 – (A) Effects of 2-DG on intracellular ATP content in MDCK cells. The cells were exposed to 5 mM 2-DG for 1 h or 10 mM 2-DG for 30 min, respectively. Values are expressed as mean ± S.E.M. of nmol/mg protein of ATP content (n = 4–9). $^{**}P < 0.01$ vs. basal. (B) Effect of 2-DG on adenosine-induced ATP release from MDCK cells. The cells were exposed to 5 mM 2-DG for 1 h and 10 mM 2-DG for 30 min, respectively, before the application of adenosine. The cells were exposed to 10 μM CCCP for 30 min before the application of adenosine in the presence of 5 mM 2-DG.

4. Discussion

MDCK cells, a renal epithelial cell line derived from the distal/tubule collecting duct, respond to extracellular nucleotides, such as ATP and UTP, with hyperpolarization and alterations in short-circuit [19,20] and ion flux [21–23]. We previously showed that ATP was released by stimulation of the A_1 receptor with adenosine, and that the mechanism included an $Ins(1,4,5)P_3$ elevation and subsequent increase in $[Ca^{2+}]_i$ in MDCK cells [13]. The A_1 receptor interacts with Gi/Go proteins to inhibit adenylyl cyclase (AC) activity and activates PLC. In DDT_1 MF-2 cells, it has been reported that the activation of A_1 receptors induced an increase in $[Ca^{2+}]_i$ from the Ca^{2+} store [17]. Our results also showed that activation of A_1 receptor induced a rise in $[Ca^{2+}]_i$ from Ca^{2+} store in MDCK cells. In regard to the role of intracellular Ca^{2+}, Rizzuto et al. showed that $Ins(1,4,5)P_3$-induced Ca^{2+} mobilization from intracellular Ca^{2+} stores caused an increase in $[Ca^{2+}]_{mito}$ [14,24], suggesting that mitochondria in close proximity to the endoplasmic reticulum play an important role in intracellular Ca^{2+} homeostasis. In the present study, we showed that the fluorescence intensity of Rhod-2/AM increased after the application of adenosine in MDCK cells and the images mostly overlapped with a mitochondrial marker. The staining sites of the mitochondrial marker were observed not only around nuclei but also near the plasma membrane. In fact, it has been shown that mitochondria located proximally to both the plasma membrane and the endoplasmic reticulum are prominently involved with mitochondrial Ca^{2+} sequestration in HeLa cells [14,24,25]. Therefore, we suggest that the intracellular signaling of adenosine is transmitted to mitochondria via Ca^{2+} release from intracellular Ca^{2+} stores, and that this phenomenon may occur near the plasma membrane in MDCK cells. Furthermore, the release of ATP triggered by adenosine was strongly inhibited by the mitochondrial modulators, such as oxidative phosphorylation modulators, mitochondrial ADP/ATP carrier inhibitors and a mitochondrial Na^+–Ca^{2+} exchange inhibitor. Taken together, these findings suggest that mitochondria may exert an influence on ATP efflux after the elevation of $[Ca^{2+}]_i$ by activating the A_1 receptor.

However, it is also thought that inhibition of ATP efflux is responsible for a decrease in the intracellular ATP content by mitochondrial modulators, because it is well known that

mitochondria are important in the synthesis of ATP. Most of the mitochondrial modulators significantly reduced the intracellular ATP content in our experimental condition. Next, we tested adenosine-induced ATP release from MDCK cells in the presence of a glycolysis inhibitor, 2-DG, that is known to reduce intracellular ATP [26]. 2-DG (5 mM) significantly reduced intracellular ATP content more than any of the mitochondrial modulators in our experiments. However, it is interesting that ATP was still released by adenosine with treatment of 5 mM 2-DG, and the release was inhibited by CCCP. These results indicate that the change in intracellular ATP content may not exert directly influence on adenosine-triggered ATP release. On the other hand, 10 mM 2-DG strongly reduced intracellular ATP content and completely blocked adenosine-induced ATP release. Since adenosine causes an increase in mitochondrial Ca^{2+}, the mitochondrion may have a role as a signaling element for ATP release induced by adenosine in MDCK cells.

On the other hand, adenosine did not induce an increase of intracellular ATP content (data not shown). As is well known, the intracellular ATP concentration is extremely high about 2–10 mM. It is likely that a change in ATP content induced by adenosine is masked by the high intracellular concentration of ATP.

The results of the present study indicate that mitochondria are involved in the intracellular signaling of A_1 receptor in MDCK cells. Furthermore, intracellular ATP, which originated from glycolysis or mitochondria, may play a distinct role in MDCK cells. In mouse sperm flagellar movement, glycolysis plays a major role in ATP supplementation [26]. In MDCK cells, ATP, which originates from mitochondria, plays a direct or indirect influence on adenosine-induced ATP release. Thus, MDCK cells have an interesting system that adenosine, a metabolite of ATP, may enhance ATP release into the extracellular space via the mitochondria. As adenosine and ATP have opposite effects in physiological functions, such as hyperalgesia [27,28], the system may act as a sensor to maintain homeostasis of nucleoside and nucleotide concentrations in MDCK cells.

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