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Negative-feedback regulation of ATP release: ATP release from cardiomyocytes is strictly regulated during ischemia

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**Key words: ATP release; cardiomyocytes; maxi-anion channel; hemichannel;
purinoceptor**

Abstract

Extracellular ATP acts as a potent agonist on cardiomyocytes, inducing a broad range of physiological responses via P2 purinoceptors. Its concentration in the interstitial space within the heart is elevated during ischemia or hypoxia due to its release from a number of cell types, including cardiomyocytes. However, the exact mechanism responsible for the release of ATP from cardiomyocytes during ischemia is not known. In this study, we investigated whether and how the release of ATP was strictly regulated during ischemia in cultured neonatal rat cardiomyocytes. Ischemia was mimicked by oxygen-glucose deprivation (OGD). Exposure of cardiomyocytes to OGD resulted in an increase in the concentration of extracellular ATP shortly after the onset of OGD (15 min), and the increase was reversed by treatment with blockers of maxi-anion channels. Unexpectedly, at 1 and 2 hours after the onset of OGD, the blocking of maxi-anion channels increased the concentration of extracellular ATP, and the increase was significantly suppressed by co-treatment with blockers of hemichannels, suggesting that ATP release via maxi-anion channels was involved in the suppression of ATP release via hemichannels during persistent OGD. Here we show the possibility that the release of

ATP from cardiomyocytes was strictly regulated during ischemia by negative-feedback mechanisms; that is, maxi-anion channel-derived ATP-induced suppression of ATP release via hemichannels in cardiomyocytes.

1. Introduction

Extracellular adenosine 5'-triphosphate (ATP) acts as a potent agonist on a variety of different cell types, including cardiomyocytes [1], inducing a broad range of physiological responses. We have recently revealed in cultured cardiomyocytes that the extracellular ATP-purinoceptor system is responsible for the intercellular synchronization of Ca^{2+} oscillation probably via the activation of G-protein-coupled P2Y_1 receptors [2,3]. ATP is known to be released into the interstitial spaces within the heart in response to a variety of physiological and pathological stimuli [4], including hypoxia or ischemia [5,6]. In neonatal rat cardiomyocytes, exposure of cultures to ischemic stress mimicked by oxygen-glucose deprivation (OGD) results in the release of ATP via maxi-anion channels [7]. In addition, Shintani-Ishida et al. [8] have recently demonstrated that brief ischemic stress to neonatal cardiomyocytes transiently opens Cx43 hemichannels, leading to the release of ATP via hemichannels. At present, however, it is almost completely unknown whether the release of ATP from cardiomyocytes during ischemia is strictly regulated, and if so, how cardiomyocytes

regulate the release. In fact, we have recently demonstrated the possible existence of a negative-feedback loop for regulating the release of ATP via hemichannels during ischemia in cultured astrocytes [9]. Such a negative-feedback system for the closure of astrocytic hemichannels during ischemia might contribute to the protection of astrocytes themselves as well as neurons from ischemia-induced cell damage.

In this study, we investigated whether and how the release of ATP was regulated under ischemic condition mimicked by OGD in cultured neonatal rat cardiomyocytes. Here we show the possibility that the release of ATP from cardiomyocytes was strictly regulated during ischemia by a negative-feedback mechanism; that is, maxi-anion channel-derived ATP-induced suppression of ATP release via hemichannels in cardiomyocytes.

2. Materials and methods

The animal experiments conformed to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1996), as well as the "guide for the care and use of laboratory animals", Hokkaido University School of Medicine.

2.1 Isolation and culture of cardiomyocytes

The method of culturing cardiomyocytes is described in detail in our previous papers [10-13]. In brief, cardiomyocytes were prepared from the ventricles of Wistar rats aged 2-3 days, removed after decapitation. The ventricles were rinsed in a 25 mM HEPES buffered minimum salt solution (MSS) to remove contaminating blood cell components and then minced with scissors into fragments to be digested with 0.1% collagenase (Wako Chemical, Tokyo, Japan) in MSS at 37°C for 10 min. The digested fragments were centrifuged at 1000 rpm for 4 min (LC-100, TOMY, Tokyo, Japan) and precipitated cell components were washed twice with MSS to terminate the effects of the collagenase. The cell components were suspended in MCDB 107 (Research

Institute for Functional Peptides, Yamagata, Japan) containing 5% heat-inactivated fetal calf serum (MBL, Nagoya, Japan), and then passed through a wire mesh screen (90 μm porosity) to remove large aggregates of cells; the filtered suspension contained cardiomyocytes and cardiac fibroblasts. To separate cardiomyocytes from cardiac fibroblasts based on the selective adhesion technique, the cell suspension was poured into petri dishes (ϕ 60 mm, CELL STAR[®], Greiner Bio-one), and incubated for 60 min at 37°C, in 5% CO₂ and 95% air. By virtue of the procedure, most cardiac fibroblasts adhere to the dish. After the incubation, the suspension, mostly containing cardiomyocytes, was collected. Cultures incubated for 4-5 days were used in this study.

2.2 Oxygen-glucose deprivation (OGD)

OGD procedure is described elsewhere in detail [11,14]. In short, serum-containing incubation medium was exchanged with serum-free DMEM (Gibco BRL, Invitrogen Corp., Carlsbad, CA) 2 h before the start of experiments. Near anoxic conditions were achieved using an Anaero-Pack System (Mitsubishi Gas

Chemical, Tokyo, Japan). After pre-gassing with 95% N₂-5% CO₂ for at least 5 min, ischemia buffer was added to the cells, which were then placed in a sealed chamber containing the deoxygenation reagent (Kenki for Cells, Mitsubishi Gas Chemical). The catalytic reaction of the reagent resulted in the consumption of O₂ and in the production of CO₂. This Anaero-Pack System provided near anaerobic conditions with an O₂ concentration of < 1% and a CO₂ concentration of about 5% within 1 h of incubation at 37 °C.

2.3 Measurement of extracellular and intracellular ATP concentrations

ATP concentrations of the cultures were analyzed with a CheckLite™ 250 (Kikkoman, Chiba, Japan) or ATP-assay kit (Toyo B-net, Tokyo, Japan) according to each manufacturer's instructions, based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin [3,11]. For the measurement of the concentration of intracellular ATP, the cells were placed in 400 µl of Tris-HCl buffer, and lysis reagent was added 20 min later. The lysate was mixed with a micropipette, and transferred to a microcentrifuge tube. Samples were read using a TD-20/20

Luminometer (Turner Designs, Sunnyvale, CA). A standard curve was obtained prior to all experiments using ATP (Oriental Yeast, Tokyo, Japan).

2.4 Immunofluorescence staining

Cardiomyocytes were stained with antibodies against sarcomeric α -actinin (Sigma, St. Louis, MO), Cx43 (Sigma), ENTPD2 (Santa Cruz), and ENTPD3 (Santa Cruz). First, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then rinsed three times with 0.02 mol phosphate-buffered saline (PBS), pH 7.4. Fixed cells were permeabilized in 0.1% triton X-100 in PBS for 15 min, blocked with 1% normal goat serum in PBS for 30 min, rinsed three times with 0.02 M PBS, pH 7.4, and incubated with a primary antibody over a 24-hour period. After being washed with 0.02 mol PBS, they were incubated with a secondary antibody (Alexa fluor 532, 488, 405 (Molecular Probes), 1:200) for 1h at room temperature. After again being washed with 0.02 mol PBS, the specimens were mounted in PBS and sealed with nail polish. They were then observed under a confocal laser microscope (FLUOVIEW FV300, OLYMPUS) at the OPEN FACILITY, Hokkaido University Sousei Hall,

Sapporo, Japan.

2.5 Western blotting

Lysed cells were sonicated and centrifuged at $20,000 \times g$ for 20 min at 4°C, and protein concentrations were measured using the BCA (Bicinchoninic Acid) methods. Proteins (50 µg per lane) were electrophoresed on a 12% SDS-polyacrylamide gel and transferred onto a difluoride membrane (Bio-Rad, Hercules, CA). Nonspecific binding sites were blocked with 5% non-fat milk or Blocking Buffer (Thermo Scientific, SuperBlock[®] Blocking Buffer in PBS) for 60 min, and the membrane was incubated overnight at 4°C with antibodies: Cx43, sarcomeric α -actinin (Sigma, St. Louis, MA), ENTPD2 (Santa Cruz), ENTPD3 (Santa Cruz), and then goat anti-rabbit IgG Horseradish peroxidase (HRP)-conjugated antibody for Cx43, ENTPD2, and ENTPD3, or goat anti-mouse IgG HRP-conjugated antibody for sarcomeric α -actinin (Cell Signaling Technology). The immunoreactive bands were detected with an enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA). Quantification of the expression of Cx43, ENTPD2, ENTPD3, and sarcomeric α -actinin was performed

by densitometric analysis using Image J 1.42q for Windows (NIH, USA).

2.6 Measurement of intracellular Ca^{2+}

Changes in the cytosolic concentration of free Ca^{2+} were measured using fluo 4. Cardiac myocytes in culture were loaded with the fluorescent calcium indicator during a 30-min incubation with acetoxymethyl ester of fluo 4 (fluo 4/AM, 5 μ M; Invitrogen, Karlsruhe, Germany) in MCDB medium at room temperature. Fluo 4 was excited at 490 nm, and emission intensity was measured at 525 nm. Fluorescent images were acquired at about 200-ms intervals with a cooled CCD camera (C4880-80; Hamamatsu Photonics, Hamamatsu, Japan). An analysis of the acquired images was made with an image processing and measuring system (AQUACOSMOS; Hamamatsu Photonics). Fluorescent intensity (F) was normalized with the initial value (F_0), and F/F_0 was used to assess the changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$).

2.7 Statistical analysis

The data are expressed as the mean \pm standard error (S.E.). Group comparisons

were made using an analysis of variance (ANOVA) with Fisher's test. A *P*-value of less than 0.01 or 0.05 was considered significant.

3. Results

The basal concentration of extracellular ATP from cultured cardiomyocytes was measured to be 25.2 ± 13.6 pM (mean \pm SE, n=6) by a luciferin-luciferase assay. This level of ATP did not change significantly for up to 3 hours in cultures without OGD stress (Fig. 1A, open circle). In contrast, when cardiomyocytes were transferred to OGD conditions, the bulk ATP in the incubation medium rapidly increased from the basal level to a peak level of approximately 3.5 nM within 15 min (Fig. 1A, closed circle and Fig. 1B1). The ATP level, however, rapidly decreased after the peak, and the bulk ATP concentration did not differ significantly from the level in cultures without OGD at more than 60 min after the onset of OGD (Fig. 1A and B). This characteristic OGD-induced transient ATP increase raises a question as to why the amount of ATP released from cardiomyocytes decreased to the control level as the duration of OGD increased. There are at least three possible explanations. The first is that the release of ATP from cardiomyocytes is strictly regulated at low levels, since ATP is a crucial energy source for maintaining homeostasis and the survival of cells during persistent ischemic stress. The second is that the concentration of intracellular ATP decreased

due to ischemia, leading to a reduction in the ATP gradient across the plasma membrane and the resultant decrease in ATP release. The third is that once released, the ATP is rapidly hydrolyzed due to the OGD-induced enhanced expression and/or activity of ecto-ATPases. We first investigated the underlining mechanisms for this transient ATP increase caused by the exposure to OGD. The concentration of intracellular ATP did not decrease, but rather increased probably due to a reduction in ATP utilization, since spontaneous and rhythmic contractions were almost completely abolished at 1 h and 2 h after the onset of OGD [11]. However, the intracellular ATP was markedly decreased at 3 h OGD due to continued suppression of the production of ATP. Previous studies have revealed that the plasma membrane of cardiomyocytes primarily expresses ENTPD2 (ecto-ATPase) [15] and ENTPD3 [16]. OGD for 2 h significantly decreased the expression of ENTPD2 (Fig. 2C2), suggesting the rapid hydrolysis of ATP not to be a primary cause of the observed low concentration of ATP during 2 and 3 h OGD. The expression of ENTPD3 was also decreased, but not significantly (Fig. 2C3). All these results suggested that the release of ATP from cardiomyocytes during OGD was strictly regulated at low levels via unknown cellular mechanisms.

We then investigated the mechanisms responsible for the OGD-induced release of ATP in cardiomyocytes at 15 min after the start of OGD (Fig. 1C). Previous studies have suggested that cardiomyocytes release ATP in response to cell stress via a variety of factors such as maxi-anion channels [7], cystic fibrosis transmembrane conductance regulator (CFTR) [17], and connexin and/or pannexin hemichannels [8,18]. In addition, in cultured astrocytes, mechanical stress to cells results in the release of ATP via exocytosis (vesicular ATP release) [19]. The OGD-induced increase in ATP release was significantly suppressed by GdCl₃ and NPPB, but not by blockers of hemichannels, CFTR, and exocytotic vesicular transport (Fig. 1C), suggesting that OGD-induced ATP increase was mainly caused by the release of ATP via Gd³⁺-sensitive maxi-anion channels. The possibility, however, that treatment with Gd³⁺ itself decreased the luciferin-luciferase chemiluminescence [20] cannot be completely excluded. Therefore, we tested it (Suppl. Fig. 1). In the cell-free system, administration of GdCl₃ at up to 300 μM did not significantly decrease the luciferin-luciferase chemiluminescence (Suppl. Fig. 1A), indicating that 50 μM GdCl₃ used to inhibit the activity of maxi-anion channels did not affect the ATP assay itself.

A question then arises as to whether ATP released via maxi-anion channels plays some role in the regulation of cell function. Therefore, we next measured changes in the concentration of extracellular ATP during OGD when the activity of maxi-anion channels was inhibited by treatment with GdCl_3 (Fig. 3A). Unexpectedly, as the duration of OGD increased, treatment with GdCl_3 significantly increased the ATP concentration. The GdCl_3 -induced increase was significantly antagonized by co-treatment with CBX, a blocker of hemichannels (Fig. 3B), suggesting that ATP released via maxi-anion channels early after the onset of OGD was involved in the feedback suppression of the further release of ATP via hemichannels at later OGD. Actually, when the cultures were not treated with GdCl_3 during OGD, treatment with blockers of hemichannels (SBX, BBG, and LaCl_3) did not increase the concentration of extracellular ATP (Fig. 3C), further supporting our inference that ATP released via maxi-anion channels was involved in the feedback suppression of ATP release via hemichannels later during OGD. In our cultures, neonatal rat cardiomyocytes showed a distinct punctate expression of Cx43 mainly at the border between cells (Fig. 3D1), but showed an ambiguous cytosolic distribution of pannexin 1 (Fig. 3D2). Therefore,

pannexin 1 in the cultured cardiomyocytes possibly did not form functional hemichannels as was reported in cultured astrocytes by Huang et al. [21].

Based on the present findings, we supposed that ATP released via maxi-anion channels resulting from the OGD-induced activation of the channels was involved in the feedback suppression of ATP release via hemichannels. However, the possibility that factors other than ATP released via maxi-anion channels were responsible for the feedback suppression cannot be excluded. Therefore, we next indirectly investigated this possibility. If ATP-purinoceptor signaling was involved in the feedback suppression of ATP release, treatment with antagonists of P2 purinoceptors during OGD would paradoxically increase the concentration of extracellular ATP. Expectedly, treatment with either PPADS (100 μ M), a nonspecific antagonist for P2Y and P2X receptors [22,23], or MRS2500 (10 μ M), a potent specific antagonist of P2Y₁ purinoceptors [24], during OGD significantly increased the release of ATP (Fig. 4A). In addition, treatment with 2-APB, an inhibitor of IP3 receptors at the sarcoplasmic reticulum (SR) [25], dose-dependently increased the concentration of extracellular ATP (Fig. 4B). Treatment with either MRS2500 (10 μ M) or 2-APB (100 μ M) itself did not

affect the luciferin-luciferase chemiluminescence (Suppl. Fig. 1B). However, PPADS (100 μ M) itself significantly decreased the chemiluminescence (Suppl. Fig. 1B), suggesting that the PPADS-induced increase in the ATP concentration was possibly underestimated. Treatment with U73122 (1 μ M), a potent PLC inhibitor, also significantly increased the release of ATP (data not shown). Furthermore, the OGD-induced increase in the concentration of intracellular Ca^{2+} was significantly suppressed by treatment with either GdCl_3 or PPADS (Fig. 4C-G). MR2500 treatment during OGD also significantly decreased the concentration of intracellular Ca^{2+} (Suppl. Fig. 2). All these findings suggested that maxi-anion channel-derived ATP-induced activation of purinoceptors, especially P2Y_1 receptors, was responsible for the feedback suppression of ATP release during OGD.

4. Discussion

The present study indicated the release of ATP from cardiomyocytes to be strictly regulated during ischemia by negative-feedback mechanisms; that is, maxi-anion channel-derived ATP-induced suppression of ATP release via hemichannels in cardiomyocytes.

In this study, the extracellular ATP-purinoreceptor system was possibly involved in the negative-feedback regulation of the release of ATP via hemichannels during OGD in cultured cardiomyocytes; that is, maxi-anion channel-derived ATP activated P2 purinoreceptors, especially P2Y₁ receptors, resulting in the activation of PLC, enhanced production of IP3, and an increase in the release of Ca²⁺ from sarcoplasmic reticulum (IP3-induced Ca²⁺ release, IICR), leading to an increase in intracellular Ca²⁺. A question then arises as to what signaling mechanisms are involved in the suppression of the release of ATP via hemichannels downstream of the OGD-induced increase in intracellular Ca²⁺. The exact mechanisms are currently unknown, but one possibility is the OGD-induced activation of Ca²⁺-dependent nitric oxide synthase (NOS), and the

resultant enhanced production of nitric oxide (NO). We have previously revealed in cultured neonatal cardiomyocytes that treatment with either L-NMMA, a non-specific inhibitor of NOS, or carboxy-PTIO, a scavenger of NO, during OGD resulted in a decrease in intracellular ATP, leading to increased ischemia/reperfusion injury in cardiomyocytes [11]. In addition, Feron et al. [26] have revealed that eNOS is found in caveolin-3-containing lipid rafts in cardiomyocytes, and that elevations in intracellular Ca^{2+} cause the activation of eNOS, further supporting our inference that NO was involved in the suppression of ATP release via Cx43 hemichannels. At present, however, no convincing evidence that NO suppresses the release of ATP via hemichannels during ischemia has been reported. Further studies are needed to clarify whether NO is crucially involved in the suppression of the release of ATP via hemichannels.

In this study, treatment with $GdCl_3$ during OGD did not increase the intracellular Ca^{2+} concentration, but significantly decreased it (Fig. 4C-4G). A critical question then arises as to why the Gd^{3+} -induced increase in the release of ATP via hemichannels at 1 and 2 h OGD did not activate P2Y receptors and did not increase the concentration

of intracellular Ca^{2+} . There are at least two possible explanations for this phenomenon. One possibility is that hemichannels and P2Y receptors are physically separated from each other and the ATP released via hemichannels does not activate P2Y receptors; e.g., hemichannels are located within caveolae while P2Y receptors are at the surface of plasma membranes, or vice versa. The other possibility is that the exposure of cardiomyocytes to long-lasting OGD decreased the expression of P2Y receptors. Our preliminary immunofluorescence analyses using anti-caveolin-3 (Cav-3), anti-Cx43, and anti-P2Y₁ antibodies revealed that Cx43 and P2Y₁ were partially co-localized with Cav-3 in neonatal cardiomyocytes (Suppl. Fig. 3). Cx43 expressed in the caveolae may not be able to form gap junctions, but form hemichannels, since the caveolae are invaginations of the plasma membrane without physical contact between cells [27]. Therefore, the first possibility seems unlikely, since some portions of both Cx43 and P2Y₁ were co-localized in the caveolae. A preliminary experiment concerning the second possibility revealed that OGD for 2 h also decreased the expression of P2Y₁ (Suppl. Fig. 4). Therefore, the OGD-induced decrease in the expression of P2Y₁ was probably one of the reasons why the Gd^{3+} -induced increase in the concentration of ATP

released via hemichannels did not activate P2Y₁ receptors leading to an increase in the concentration of intracellular Ca²⁺ and the recurrent activation of P2Y₁ receptors themselves during OGD.

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Figure legends

Figure 1

OGD-induced increase in the concentration of extracellular ATP. The concentration of extracellular ATP ([ATP]_e) increased transiently, and peaked at around 15 min after the onset of OGD (A). Closed circles indicate the ATP concentration during OGD, and open circles are the control without OGD. The ATP concentrations at 60 and 120 min were not significantly different from that in the control (B). Data are expressed as the mean \pm SE (n=15). ** p <0.01 vs. Control. The OGD-induced increase in [ATP]_e was significantly suppressed by treatment with either GdCl₃ (50 μ M) or NPPB (100 μ M), but not with either CBX (50 μ M), N-Phenyl (400 μ M), or BFA (10 μ M) (C). The cultures were pre-treated with these drugs 30 min before the start of OGD, and treated throughout OGD. Data are expressed as the mean \pm SE (n=6).

** p <0.01 vs. Control.

Figure 2

OGD-induced ATP release was regulated during OGD. Immunofluorescence analyses using either anti-ENTPD2 (A) or anti-ENTPD3 (B) antibody under control conditions (A1, B1), and after 2 h OGD (A2, B2). Western blotting revealed that the expression of ENTPD2 and ENTPD3 in cardiomyocytes was significantly decreased after 2 h OGD (C1-C3). Data are expressed as the mean \pm SD (n=5). * p <0.05 vs. Control. The concentration of intracellular ATP was not decreased, but rather increased at 1 h and 2 h after the onset of OGD (D). However, the concentration was significantly reduced at 3 h after the onset of OGD. Data are expressed as the mean \pm SE (n=4). ** p <0.01, * p <0.05 vs. Control.

Figure 3

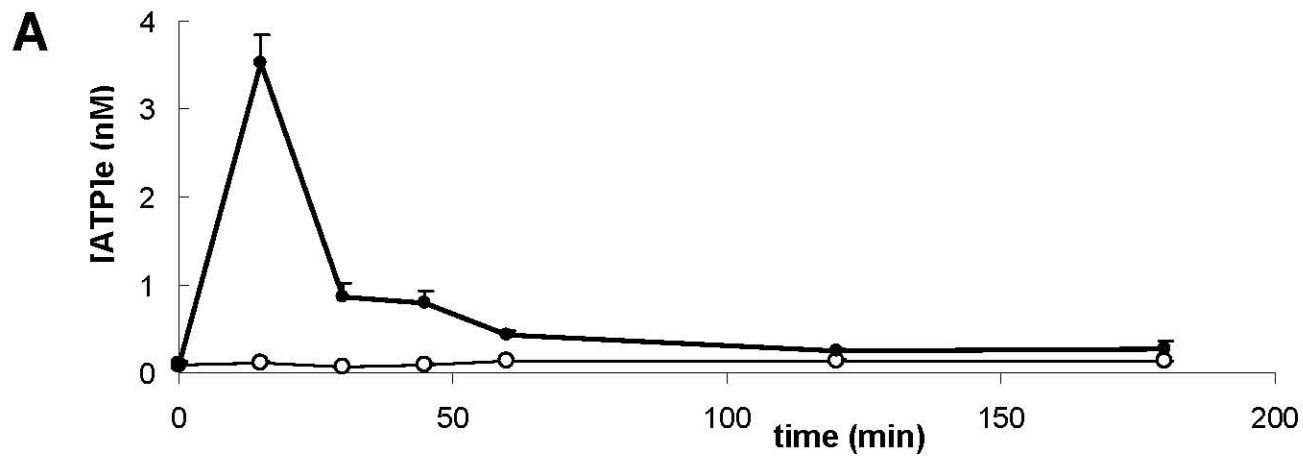
Suppression of the activity of Gd-sensitive maxi-anion channels increased ATP release during ischemia. Treatment with GdCl₃ (50 μ M) significantly increased the concentration of extracellular ATP at 60 and 120 min after the onset of OGD (A). Data are expressed as the mean \pm SE (n=16). * p <0.05 vs. OGD of each duration. The

Gd-induced increase in the concentration of extracellular ATP was significantly reversed by co-treatment with CBX (50 μ M) (B1, B2). Data are expressed as the mean \pm SE (n=5). ** p <0.01, * p <0.05 vs. OGD 60 min or OGD 120 min. Treatment with blockers of hemichannels; CBX (50 μ M), BBG (10 μ M), or LaCl₃ (100 μ M), during 2 h OGD did not significantly change the concentration of extracellular ATP (C). Data are expressed as the mean \pm SE (n=4). Immunofluorescence analyses were conducted on cultured cardiomyocytes using anti-Cx43 (D1) or anti-pannexin1 (D2) antibody.

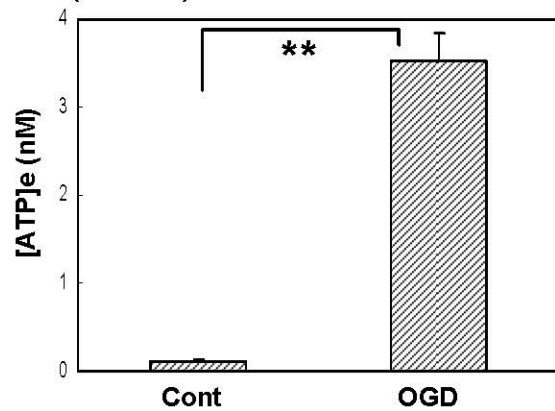
Figure 4

Inhibition of P2-purinoceptors increased the concentration of extracellular ATP during OGD, but suppressed the OGD-induced increase in intracellular Ca²⁺. The cultures were treated with PPADS (100 μ M) or MRS2500 (10 μ M) during 2h OGD (A), resulting in a significant increase in the concentration of extracellular ATP (A). Data are expressed as the mean \pm SE (n=6). ** p <0.01, * p <0.05 vs. OGD for 2 h. Treatment with 2-APB dose-dependently increased the concentration of extracellular ATP, but not that with 1 % DMSO, the vehicle for 2-APB (B). Data are expressed as

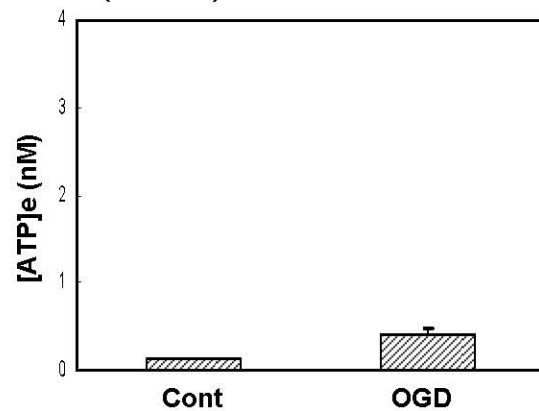
the mean \pm SE (n=5). $**p<0.01$ vs. OGD for 2 h. OGD increased the concentration of free intracellular Ca^{2+} in cardiomyocytes. Figures C1-E3 illustrate the images of fluo-4 fluorescence reflecting intracellular Ca^{2+} during OGD (C1-C3), OGD with GdCl_3 (50 μM) (D1-D3), and OGD with PPADS (100 μM) (E1-E3), respectively. Changes in the intensity of relative fluo-4 fluorescence during OGD with time (F). OGD: squares, OGD with GdCl_3 : circles, OGD with PPADS: triangles. OGD-induced Ca^{2+} increase was significantly suppressed by treatment with GdCl_3 or PPADS at 57 min after the onset of OGD (G). Data are expressed as the mean \pm SE (n=24 cells from 4 different cultures). $**p<0.01$ vs. OGD for 57 min.



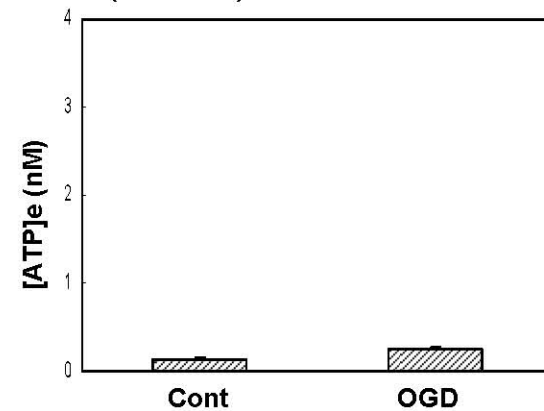
B1 (15 min)



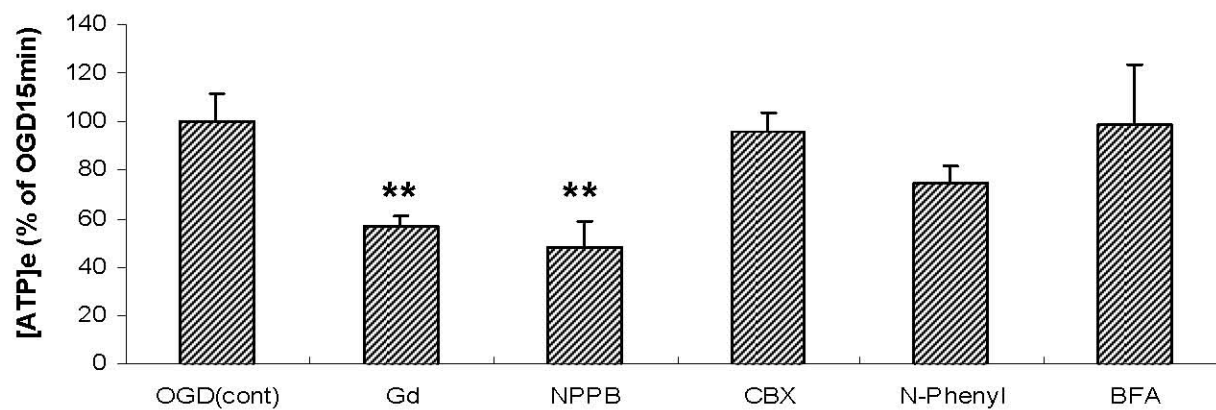
B2 (60 min)



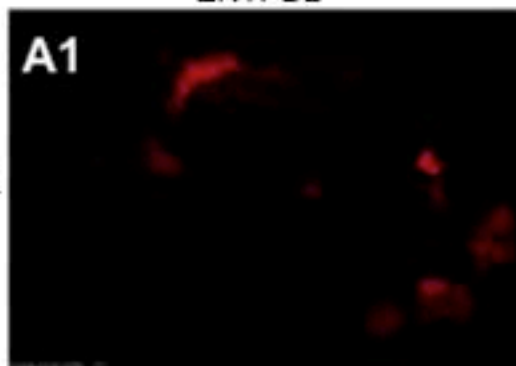
B3 (120 min)



C

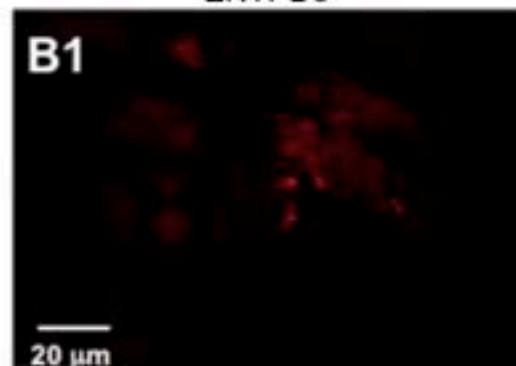
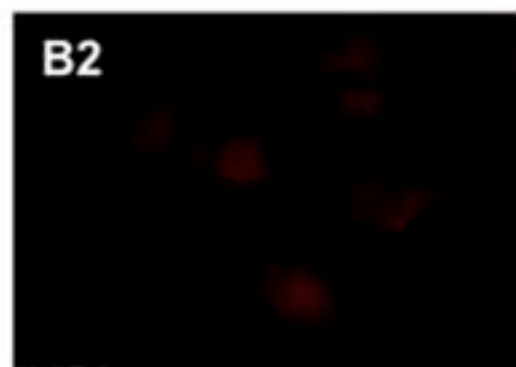
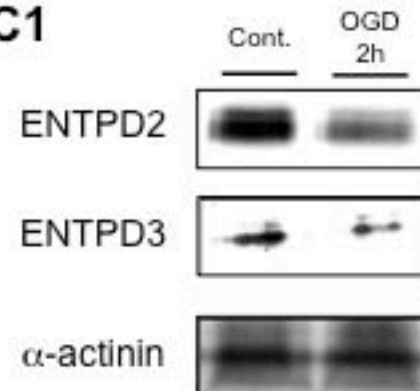
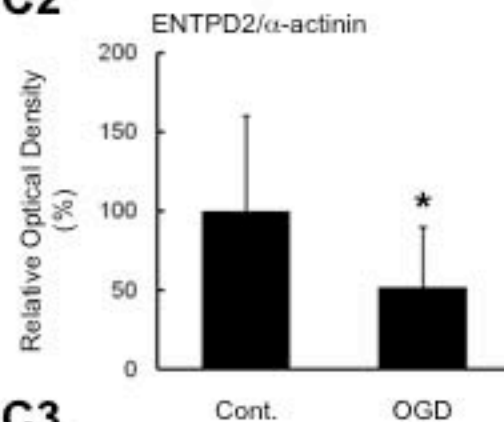
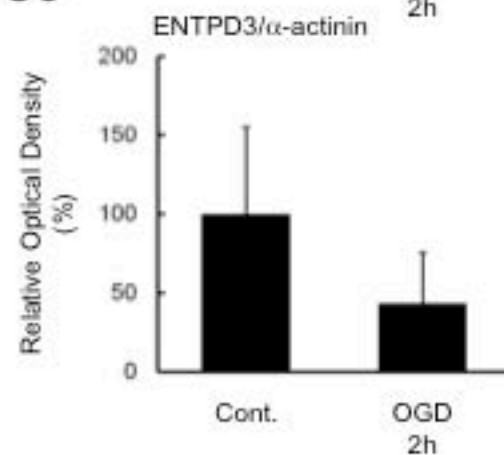


ENTPD2



Cont.

ENTPD3

OGD
2h**C1****C2****C3****D**