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ORIGINAL ARTICLE

Caffeine-inducible ATP release is mediated by Ca2+-signal transducing system from the endoplasmic reticulum to mitochondria

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Abstract Adenosine triphosphate (ATP) is released as an autocrine/paracrine signal from a variety of cells. The present study was undertaken to clarify the Ca^{2+}-signal pathway involved in the caffeine-inducible release of ATP from cultured smooth muscle cells (SMC). The release of ATP induced by caffeine (3 mM) was almost completely inhibited by ryanodine and tetracaine, but not by 2-APB, thus being mediated by ryanodine receptors (RyR). The expression of messenger RNA from only RyR-2 was detected in the cells. Furthermore, the induced release was attenuated by mitochondrial inhibitors, rotenone and oligomycin and by Cl^- channel blockers, niflumic acid, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid. Increase in Ca^{2+}-signals with fluo 4 and rhod-2 caused by caffeine were reduced by tetracaine and oligomycin plus carbonyl cyanide *m*-chlorophenylhydrazone, respectively. A close spatial relation between the endoplasmic reticulum (ER) and mitochondria was electromicroscopically observed in the SMC, supporting the existence of a Ca^{2+}-signaling bridge on both the organelli. These results suggest that caffeine stimulates ryanodine receptor (RyR-2) and facilitates a Ca^{2+}-signal transducing system from ER to mitochondria, and then, the signal appears to accelerate the ATP synthesis in mitochondria. In addition, the mitochondrial event may lead further cell signaling to the cell membrane and activates Cl^- channels, resulting in the extracellular release of cytosolic ATP.

Keywords ATP release · Caffeine · RyR-2 · Intracellular and mitochondrial Ca^{2+} concentrations · Ca^{2+}-signaling from ER to mitochondria · Cl^- channel · Vas deferens smooth muscle cells

Abbreviations

2-APB	(2-aminoethoxy)diphenylborane
BAPTA/AM	*O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester
$[Ca^{2+}]i$	intracellular Ca^{2+} concentrations
$[Ca^{2+}]mit$	mitochondrial Ca^{2+} concentrations
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CGP37157	7-chloro-5-(2-chloropheyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one
CFTR	cyctic fibrosis transmembrane regulator
ER	endoplasmic reticulum
$Ins(1,4,5)P_3$	inositol 1,4,5 trisphosphate
LDH	lactate dehydrogenase
MT-21	*N*-octyl-3-acethyl-4,5-dimethyl-5-hydroxy-3-pyrrolin-2-one

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NPPB	5-nitro-2-(3-phenylpropylamino)-benzoic acid
RyR	ryanodine receptor
SMC	smooth muscle cells
SN-6	2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethylester

Introduction

Adenosine triphosphate (ATP) is thought to play a crucial role as a functional mediator via activation of ion channel type-P2X receptors and G-protein-coupled type-P2Y receptors on a wide variety of cells (for review, see Ralevic and Burnstock 1998). Recent studies provide evidence that ATP as an autocrine/paracrine molecule is extracellularly released by mechanical and hypotonic stress from epithelial (Hazama et al. 1999; Walsh et al. 2000), hepatic (Schlosser et al. 1996; Feranchak et al. 2000), and red blood cells (Sprague et al. 1998) and by receptor-stimulation from glia (Queiroz et al. 1997) and smooth muscle cells (SMC; Katsuragi et al. 1991, 1996, 2002; Matsuo et al. 1997; Zhao et al. 2007). So far, however, the mechanism underlying the autocrine/paracrine-type release of ATP is still a matter of dispute, and the positive effects of ACh and noradrenaline on the release was clarified (Katsuragi et al. 1990). In cultured epithelial cells, it has been shown that the release of ATP induced by hypotonic stress is suppressed by NPPB and DIDIS, Cl^- channel blockers, and by Gd^{3+}, a blocker of stretch-activated Cl^- channels (Hazama et al. 1999; Braunstein et al. 2001). Therefore, it is likely that ATP is exported from cells through some kind of anionic channel such as maxi Cl^- channels after changing its form to ATP^{4-} or $[MgATP]^{2-}$ (Dutta et al. 2004).

A series of our studies on the extracellular release of ATP was started in 1990. At present, our research concern is to know the intracellular Ca^{2+} signaling pathway involved in the release of the nucleotide. We have found that stimulation with angiotensin II and bradykinin elicit the release of ATP from cultured tenia coli SMC, and the release is triggered by an accumulation of $Ins(1,4,5)P_3$ in the cells, resulting from a release of Ca^{2+} from the endoplasmic reticulum (ER; Katsuragi et al. 2002; Zhao et al. 2007). Furthermore, we demonstrated that adenosine also releases ATP via a G-protein-coupled $Ins(1,4,5)P_3$ signaling from MDCK cells and that there may be a positive feedback system acting on the ATP release caused by adenosine, which is an extracellular metabolite of ATP (Migita et al. 2005, 2007). In addition to $Ins(1,4,5)P_3$, we present in this paper evidence that caffeine causes an autocrine/paracrine-type release of ATP mediated by ryanodine receptors from cultured SMC. In the past few decades, Padova group (Rizzuto et al. 1992, 1993) found that, when mitochondria appear in close association with regions of ER, $Ins(1,4,5)P_3$-induced increases of $[Ca^{2+}]i$ trigger mitochondrial Ca^{2+} uptake, suggesting the existence of a characteristic Ca^{2+} signaling between ER and mitochondria. Furthermore, it has been reported that there is a functional coupling between the caffeine/ryanodine-sensitive Ca^{2+} store and mitochondria in rat aortic smooth muscle cells and H9c2 cells (Szalai et al. 2000; Vallot et al. 2001). Therefore, the present study was designed to clarify whether the intracellular Ca^{2+} signals between ER and mitochondria are involved in the release of ATP by caffeine from cultured vas deferens SMC from guinea pigs.

Materials and methods

Materials

Caffeine and oligomycin and BAPTA/AM were purchased from Nakalai Tesque (Kyoto, Japan) and Dojin (Kumamoto, Japan), respectively. MT-21, CGP37157, and thapsigargin were obtained from Calbiochem (Darmstadt, Germany), Tocris (Ellisville, MO, USA), and Alomone Labs (Jerusalem, Israel), respectively. Tetracaine and 2-APB were from Wako (Tokyo, Japan). Fluo 4/AM, rhod-2/AM, and Mito Tracker Green/FM were purchased from Molecular Probes (Eugene, OR, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA). SN-6 was generously supplied by Senju Pharmaceutical (Osaka, Japan).

Cell culture

The study protocols were approved by Fukuoka University, Animal Care Committee. Male guinea pigs (50–100 g) within 1 day after birth, which were purchased from KBT-Oriental (Tosu, Japan), were stunned and bled. Vasa deferentia were removed and dissected free of fascia and vascular tissue. Then, under a microscopy, the epithelial layer was carefully removed from the SMC layer with fine tweezers. For cell culturing, the tissue was minced into small pieces with fine scissors in phosphate-buffered saline supplemented with 0.25 mg/ml collagenase and kept at 4°C overnight. Small pieces were then moved to a CO_2 incubator (37°C) and maintained for 40 min. The culturing process was described previously (Katsuragi et al. 2002). After 4 days of culturing, the cell growth became confluent (approximately 10^5 cells per well). The cells in the dish were positively stained by anti-mouse a-actin with flourescein isothiocyanate-conjugated anti mouse IgG (Sigma; data not shown). Normal human dermal fibroblasts (NHDF 5787, Bio-Whittakar, Walkersville, MD, USA) were cultured for 2 days in the culture medium (FGM-2 Buller Kit, Takara, Tokyo, Japan), and the

resulting cells were used for measuring ATP levels in the superfusing experiment.

ATP release and luciferase assay

The cells collected from two dishes were trapped in a Millipore filter (pore size; 3 μM) and/or superfused at 0.5 ml/min using a peristaltic pump with oxygenated Ca^{2+}-free Krebs solution (37°C) of the following composition (mM): NaCl, 122; KCl, 5.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.6; D-glucose, 11; ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), 1.00; and ascorbic acid, 0.1. After a 5-min equilibration, superfusate was collected every 90 s for 15 min. Caffeine was applied to the superfusate for the fifth to the seventh fractions, and antagonists were present in the superfusate throughout the experiment. Then, 200 μl of the superfusate in each fraction was transferred into a microtube and the concentration of ATP was measured with 100 μl of ATP reagent solution (Lucifel-LU, Kikkoman, Chiba, Japan). The intensity of light produced by the reaction was measured with a lumitester (K-100, Kikkoman). The amount of ATP released was evaluated by a calibration curve using authentic samples of ATP and expressed as pmol ml^{-1} mg $protein^{-1}$ of cultured cells. The protein content of the cell lysate was measured after incubation (4°C) overnight with the filter paper in deionized water containing 0.1% Triton X-100.

Leakage of LDHS2

To examine whether the release of ATP by caffeine and hypotonic stress is due to leakage from the cell membrane, leakage of lactate dehydrogenase (LDH) from the cells into the extracellular solution was measured using a commercial kit, Iatrotec LDH rate (Diatron, Tokyo, Japan). The cells were cultured on a 12-well culture dish, and then, the medium was replaced with oxygenated Krebs solution (37°C). The dconversion of lactate into pyruvate by the LDH released was determined from the ratio of changes in absorbance at 340 and 415 nm with a full automatic analyzer (Hitachi 7350, Tokyo, Japan).

RT-PCR of RyR subtypes

Total RNA was isolated from the guinea pig samples using RNA Stat-60 according to the manufacturer's instructions (Tel-Test, TX, USA). Purified RNA was quantified and assessed for purity with a Gene Quant UV spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Polymerase chain reaction (PCR) primers for ryanodine receptors (RyR) were as follows: RyR-1, forward, 5′-GAAGGTTCTGGACAAACACGGG-3′, reverse, 5′-TCGCTCTTGTTGTTGTAGAATTTGCGC-3′, RyR-2, forward, GAATCAGTGAGTTACTGCATGG-3′, reverse 5′-CTGGTCTCTGAGTTCTCCAAAAGC-3′. Since there was no report on the guinea pig ryanodine subtype sequences, mouse sequences were referred to for the primers' design. Extracted RNA was reverse transcribed with multiscribe reverse transcriptase (RT) using the procedure recommended (Perkin-Elmer, Massachusetts, USA). The RT reaction mixture (10 μl) contained 0.5 mg of RNA, 2.5 mM oligo-d(T)16 primers, 1 mM of each deoxyribonucleotide triphosphate, 1 U/μl of RNase inhibitor, and 2.5 U/μl of reverse transcriptase. The RT reaction was performed at 42°C for 15 min, followed by heating at 99°C for 5 min in a GeneAmp 9600 thermal cycler (Perkin-Elmer).

PCR amplifications were performed using a GeneAmp 9600 thermal cycler (Perkin-Elmer). Reactions were performed in a mixture of 50 μl containing 5 μl of 10× PCR buffer (Perkin-Elmer), 0.25 U/μl of AmpliTaq, 2 mM $MgCl_2$, and 1 μM of each primer. The PCR protocol comprised a hold step of 2 min at 95°C followed by 45 s denaturation at 95°C, 1 min annealing at 57°C, and 1 min elongation at 72°C for 35 cycles. The PCR products were collected and run on a 2% (*w*/*v*) agarose/Tris-acetate-EDTA gel to confirm their size. The products were subcloned with a pGEM T vector (Promega, WI, USA) and then sequenced with an ABI Prism 310 automated sequencer (Perkin-Elmer). The sequence of PCR products of the guinea pig sample was more than 80% identical to that of mouse.

Measurements of $[Ca^{2+}]_i$

The cells were loaded with fluo 4/AM (10 μM) in the 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) solution without Ca^{2+} for 20 min at 25°C in the dark, rinsed, and then kept until the start of the experiment. The solution was of the following composition (in mM): NaCl, 136.9; KCl, 5.9; $CaCl_2$, 1.0; HEPES, 4.2, and glucose, 5.6 (pH 7.4). The experiment with the fluorescence microscope was undertaken with superfusion (1.2 ml/min) of the cells using the solution. Images were obtained (excitation, 470; emission, 520) using a cooled CCD camera (Dage 300 T, Michigan, USA) with an image intensifier unit (C-9016-01, Hamamatsu Photonics, Hamamatsu, Japan) attached to a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan). Fluorescence signals are expressed as pixel per pixel fluorescence ratios (F/F_0), where F is the fluorescence during a response and F_0 is the rest level fluorescence of the same pixel. Image processing and analysis were performed by using Metamorph software (Universal Imaging, West Chester, PA, USA).

Confocal laser microscopy

The cells cultured in a glass-bottomed dish were loaded with rhod-2/AM, 10 μM and/or Mito-Tracker Green/FM

1 μM (a marker of mitochondria; both from Molecular Probes) at room temperature for 20 min, rinsed, and then kept in the HEPES solution for an appropriate period of time. Microscopic observation was carried out with superfusion of the cells, as described above. The living cells were observed and photographed using a confocal scanner (Yokogawa CSU10, Tokyo, Japan) attached to the fluorescence microscopic system described above and further equipped with a krypton/argon laser as the light source. The rhod-2 images were captured with excitation at 545 nm and emission at 590 nm. The Mit-Tracker Green images were obtained with excitation at 470 nm and emission at 520 nm. The fluorescence ratio (F/F_0) was measured as described above.

Electromicroscopy

The cultured cells in the dish were fixed with 2.0% glutaraldehyde in 0.1% phosphate buffer (pH 7.4) at 4°C. The cells were washed three times with the buffer solution, post-fixed in osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812, which was polymerized at 60°C for 48 h. Ultrathin sections were double-stained with uranyl acetate and lead citrate and observed under an electron microscope (JEM 1200EX, JEOL, Japan).

Statistics

Differences between two means or multiple means were tested for statistical significance using Student's *t* test and the one-way analysis of variance followed by Dunnett's test. A value of $P<0.05$ was considered to be significant.

Results

Release of ATP evoked by caffeine

The effects of stimulation with caffeine on the release of ATP were evaluated in the superfusing experiment with cultured SMC from guinea pig vas deferens. Since there was no difference in the amount of ATP released by

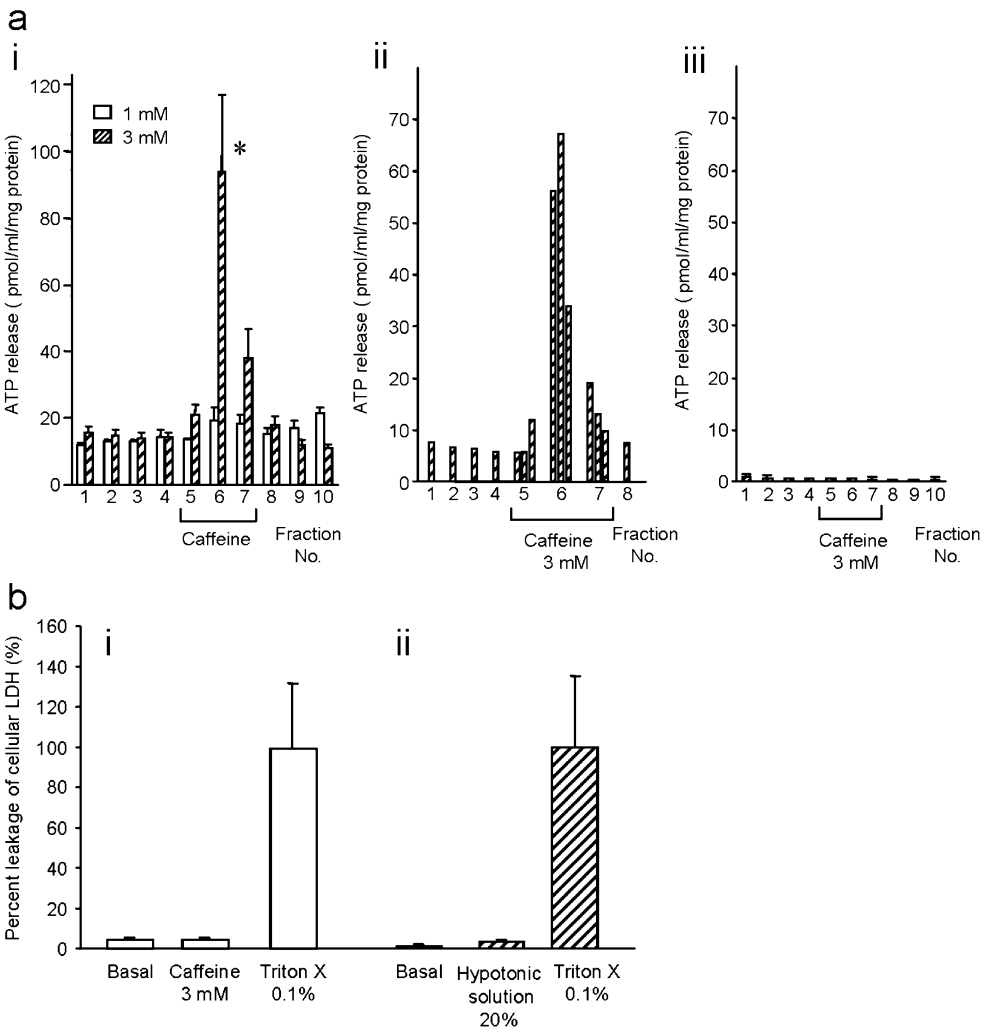

Fig. 1 **a** The release of ATP evoked by caffeine from cultured smooth muscle cells of guinea pig vas deferens (*i*, *ii*) and human dermal fibroblasts (*iii*). *i* Caffeine at 3 mM ($n=13$), not at 1 mM ($n=3$), releases ATP. *ii* A detailed release curve for ATP induced by 3 mM caffeine. The fifth, sixth, or seventh fraction consists of three-fractions collected every 0.5 min. *iii* Caffeine failed to induce any release of ATP from the fibroblasts ($n=4$). Values are expressed as the mean±SEM of pmol ml^{-1} mg $protein^{-1}$ of ATP. *$P<0.01$ compared with the control (fourth fraction). **b** Effects of caffeine and the 20% hypotonic solution on LDH's leakage. Basal—samples from the dish medium without any stimulant. Caffeine and hypotonic stress—from the dish medium 5 min after exposure to a stimulant. Triton X—from the dish medium after cell lysis with 0.1% TritonX-100 at the end of the experiment. Caffeine and hypotonic stress did not cause any significant increase in LDH in the medium. Values are expressed as means±SEM of the percentage from Triton X medium (100%) ($n=6$)

caffeine in the solution with or without Ca^{2+} (data not shown), the evaluation with caffeine was carried out in the Ca^{2+}-free solution containing 1 mM EGTA throughout the subsequent experiment. Caffeine at 3 mM, but not 1 mM, caused a substantial release of ATP from SMC (Fig. 1a, i). However, the drug failed to cause a measurable release of ATP from the fibroblasts, indicating that the amount of ATP released from fibroblasts as contaminants in a cultured dish was negligible (Fig. 1a, iii). The basal release (the fourth fraction before caffeine was added) and the peak release (the sixth fraction after caffeine was added) of ATP were 14.3±1.6 pmol ml^{-1} mg $protein^{-1}$ (n=13) and 93.8± 23.0 pmol ml^{-1} mg $protein^{-1}$ (n=13), respectively. The caffeine-evoked release of ATP was unaffected by verapamil (100 μM), a Ca^{2+} channel blocker, wortmannin (0.1 μM), a P13 kinase inhibitor, and SN-6 (10 μM), a Na^{+}–Ca^{2+} exchange inhibitor. In the presence of verapamil, wortmannin, and SN-6, the basal release and the peak release of ATP amounted to 20.7±2.4, 21.7±0.9, and 12.1± 0.1 pmol ml^{-1} mg $protein^{-1}$, and 111.0±16.5, 84.0±20.6, and 80.6±2.1 pmol ml^{-1} mg $protein^{-1}$ (n=6–10), respectively. To know the time course of ATP's efflux because of the transient release, the sample medium during the fifth to seventh fractions in the presence of caffeine was collected every 0.5 min as shown in Fig. 1a, ii. Caffeine, as well as the 20% hypotonic solution, which is known as an ATP releaser as described in "Introduction," did not induce any LDH from SMC (Fig. 1b).

Effects of various signal inhibitors on the evoked release of ATP

To evaluate the signal pathway triggering the response, we studied the effects of various signal inhibitors on the release of ATP elicited by caffeine. The release with 3 mM caffeine was completely abolished in the presence of 0.5 μM thapsigargin, a Ca^{2+} ATPase inhibitor, and 50 μM BAPTA/AM, an intracellular Ca^{2+} chelator (Fig. 2a). In addition, the evoked release of ATP was strongly inhibited by treatments with 30 μM ryanodine, a Ca^{2+} depleter from the ER store, and 100 μM tetracaine, a RyR antagonist. However, an $Ins(1,4,5)P_3$ receptor antagonist, 2-APB, failed to hamper the release of ATP (Fig. 2b). These findings show that the release is mediated by stimulation of the RyR but not the $Ins(1,4,5)P_3$ receptor. In further experiments, the release of ATP induced by caffeine was attenuated by mitochondrial inhibitors, rotenone and oligomycin at 30 μM and a proton-motive force inhibitor, CCCP at 3 μM (Fig. 3a). In addition, the evoked release subsided in the presence of a mitochondrial Na^{+}–Ca^{2+} exchange inhibitor, CGP37157, and an inhibitor of the ATP/adenosine diphosphate (ADP) translocator, MT-21 (Machida et al. 2002; Fig. 3b). Various kinds of Cl^{-} channel blockers,

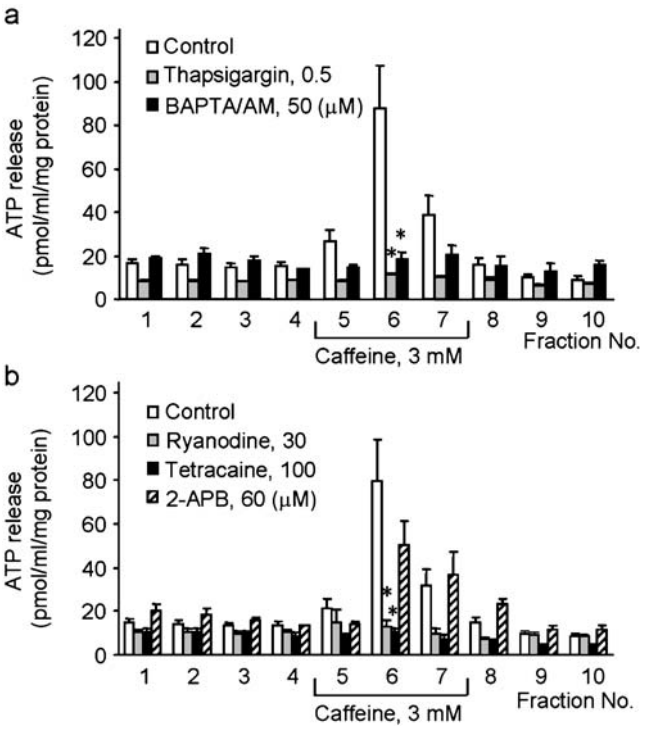

Fig. 2 Effects of intracellular signal inhibitors affecting ER on the release of ATP induced by caffeine. These inhibitors were administered to the superfusate 30 min before caffeine. Values are expressed as the mean±SEM (n=3–9) of pmol ml^{-1} mg $protein^{-1}$ of ATP. *P< 0.05 compared with the control (sixth fraction)

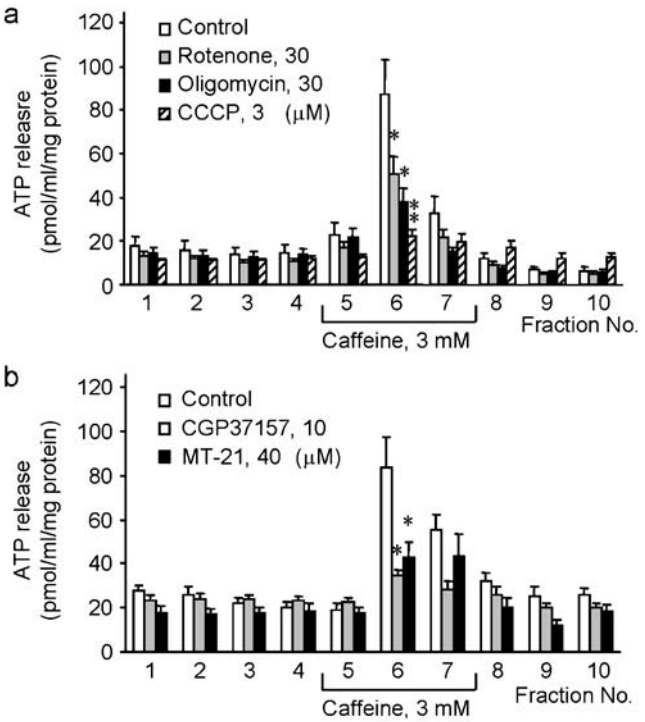

Fig. 3 Effects of mitochondrial inhibitors on the release of ATP induced by caffeine. For details, see Fig. 2. *CGP37157* A Na^{+}–Ca^{2+} exchange inhibitor, *MT-21* an inhibitor of ATP/ADP translocator. Values are expressed as the mean±SEM (n=4–7) of pmol ml^{-1} mg $protein^{-1}$ of ATP. *P<0.05, **P<0.01 compared with the corresponding control (sixth fraction)

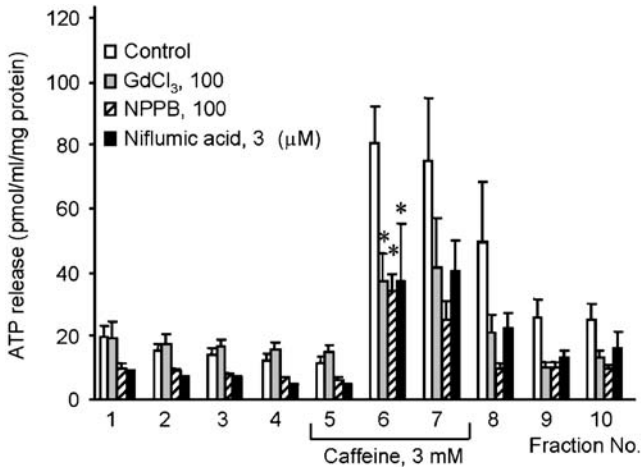

Fig. 4 Effects of Cl^- channel blockers on the release of ATP induced by caffeine. For details, see Fig. 2. Values are expressed as mean± SEM (n=3–8) of pmol ml^{-1} mg $protein^{-1}$ of ATP. *P<0.01 compared with the corresponding control (sixth fraction)

Gd^{3+} and NPPB at 100 μM and niflumic acid at 3 μM, attenuated the export of ATP across the cell membrane (Fig. 4).

Measurement of mRNA of RyR subtypes in SMC

In RT-PCR studies, unfortunately, the use of the primer of RyR-3 to guinea pig SMC failed to express a clear single band of messenger RNA (mRNA). However, the RT-PCR using the primers of RyR-1 and RyR-2 expressed mRNA of these receptors in skeletal muscles and cardiac muscles, respectively. Moreover, the expression of mRNA from RyR-2, but not from RyR-1, was observed at 520 bp in vas deferens SMC and ileal SMC from guinea pigs (Fig. 5).

Changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$ in the presence of caffeine

Measurement of $[Ca^{2+}]_i$ of the cells was fluoromicroscopically undertaken by using fluo 4, a Ca^{2+} indicator in a low Ca^{2+} medium. A transient and steep increase in $[Ca^{2+}]_i$ was elicited by administration of 3 mM caffeine into the medium. After the rinsing of the cells for 30 min in the medium without caffeine, the readdition of caffeine caused an increase of $[Ca^{2+}]_i$. The caffeine-induced increase was markedly blocked by tetracaine, thus indicating that the rise in $[Ca^{2+}]_i$ is mediated by stimulation of the RyR with caffeine (Fig. 6a). In a further experiment with confocal laser scanning microscopy, rhod-2/AM was used as an indicator of Ca^{2+} in the mitochondrial matrix, $[Ca^{2+}]_{mit}$. Whether the fluorescence spots obtained with rhod-2 were $[Ca^{2+}]_{mit}$ or not was confirmed by double staining with Mito-Tracker Green, a mitochondrial marker (data not shown). Caffeine (3 mM) enhanced gradually $[Ca^{2+}]_{mit}$ in the cells loaded with rhod-2, as reported by Drummond and Tuft (1999). The enhanced Ca^{2+} signal was reduced by the addition of mitochondrial inhibitors in combination with oligomycin and CCCP, thus indicating that the signal reflects Ca^{2+} coming from the mitochondrial matrix (Fig. 6b).

Electromicroscopic observation

The electron micrograph in Fig. 7 shows the distribution of mitochondria and the ER in vas deferens SMC in the cultured dish. We observed a close spatial relation between the mitochondria and the ER in the SMC as shown by Lawrie et al. (1996) in ECV 304 cells.

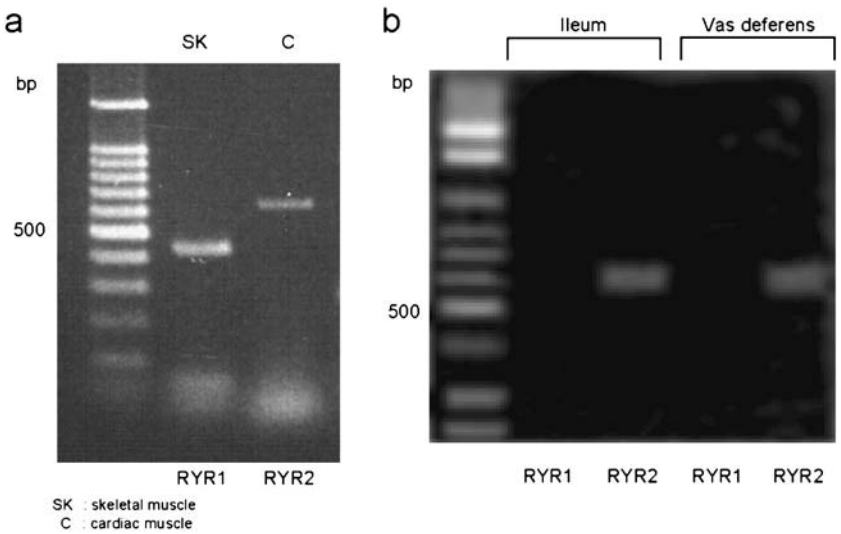

Fig. 5 Expression of RyR mRNA in various muscles. RyR-1 and RyR-2 were amplified by RT-PCR using RNA isolated from guinea-pig skeletal and cardiac muscles (**a**) and ileal and vas deferens smooth muscles (**b**). **a** Results indicate clear expression in skeletal (RyR-1) and cardiac (RyR-2) muscles, respectively. **b** RyR2 mRNA, not RyR1 mRNA, in vas deferens and ileum was obviously expressed

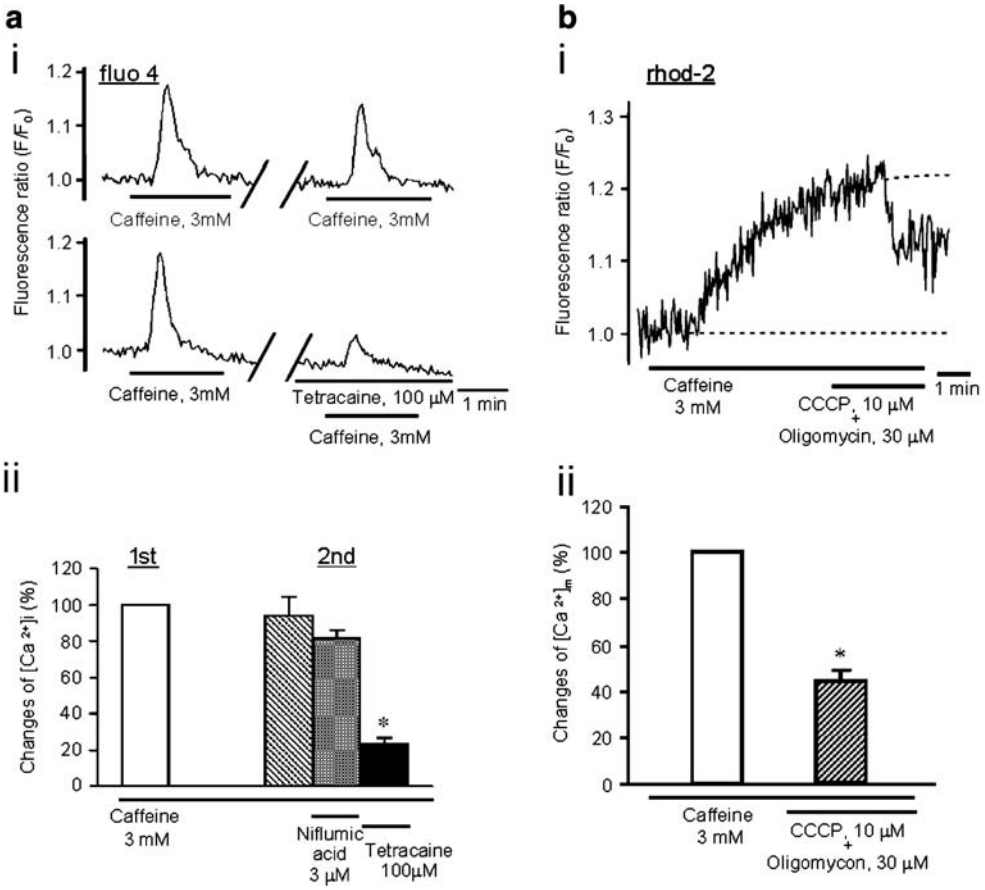

Fig. 6 Effects of inhibitors on Ca^{2+} signals induced by caffeine in the cells loaded with fluo-4/AM (**a**) and rhod-2/AM (**b**). **a**, *i*; **b**, *i* typical illustrations. **a**, *ii* A second dose of caffeine was introduced into superfusate 30 min after the first. Tetracaine was introduced 10 min before the second dose of caffeine. Values are expressed as the mean±SEM (n=4–14) of % Ca^{2+} signal after the first dose of caffeine (100%) (**a**, *ii*) and of the control (100%) (**b**, *ii*). *P<0.05 compared with the corresponding control

Discussion

In previous studies in cultured tenia-coli SMC, we presented findings that the release of ATP mediated by receptor stimulation with angiotensin II and bradykinin is triggered by the release of Ca^{2+} from the ER via stimulation of the Ins(1,4,5)P_3 receptor (Katsuragi et al. 2002; Zhao et al. 2007). In the present study, the relationship between the release of ATP and intracellular Ca^{2+} signals was evaluated by stimulating with caffeine in cultured vas deferens SMC of guinea pigs. Caffeine at 3 mM induced a transient and substantial release of ATP from the cells superfused with Krebs solution without Ca^{2+}. The release of ATP induced by caffeine is not due to inflow of outer Ca^{2+} because of the negative effects of verapamil and Ca^{2+} removal from outer medium. In addition, the evoked release seems to be unaffected by the contractile activity of the SMC from insensitivity to wortmannin, a myosin light chain kinase inhibitor. Further, caffeine did not cause any leakage of LDH from the SMC.

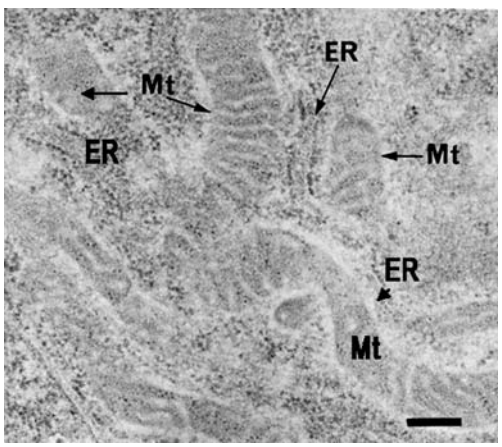

Fig. 7 Distribution of organella in a cultured cell. Scale bar means 200 nm. Notice that smooth endoplasmic reticulum is in vicinity of mitochondrion

The caffeine-inducible release of ATP was prevented by treatments with thapsigargin and BAPTA/AM and further by RyR antagonists such as tetracaine but not by an Ins(1,4,5)P_3 receptor antagonist, 2-APB. In our studies with cultured tenia-coli smooth muscle and MDCK cells, however, the release of ATP induced by Ins(1,4,5)P_3 and adenosine and the increase in [Ca2+] i by bradykinin and adenosine were largely diminished by 50 mM 2-APB (Migita et al. 2005; Zhao et al. 2007).

Thus, these suggest that the release of ATP triggered by caffeine is mediated by RyR. The RT-PCR-based experiment showed the expression of mRNA from RyR-2, not from RyR-1. In the experiment with fluo-4/AM, the

elevation in the internal concentration of Ca^{2+} ($[Ca^{2+}]_i$) in the cells exposed to caffeine was suppressed by tetracaine. There are some reports that Cl^- channel blockers such as niflumic acid attenuated the caffeine-induced increase in $[Ca^{2+}]$i (Cruickshank et al. 2003). However, we failed to show such inhibitable effect of niflumic acid as shown in Fig. 6b.

Therefore, as expected, these findings imply that the release of ATP evoked by caffeine is mediated by Ca^{2+} released from the ER via stimulation of RyR, not Ins(1,4,5)P_3 receptors.

Furthermore, the release of ATP evoked by caffeine was attenuated by inhibitors of ATP synthesis in mitochondria such as rotenone and by a proton-motive force inhibitor, CCCP. In addition, the release was reduced by Na^+–Ca^{2+} exchange inhibitors and ATP/ADP translocator inhibitors in mitochondria. In the experiment with rhod-2, caffeine increased the mitochondrial Ca^{2+} concentration, $[Ca^{2+}]_{mit}$, and the increase was reduced by a combination of oligomycin and CCCP.

In recent studies using the Ca^{2+}-sensitive photoprotein-equorin-targeting mitochondria, Rizzuto et al. (1992, 1993) postulated on the existence of a novel Ca^{2+}-signal transducing system operating between the ER and mitochondria. They further demonstrated that the kinetics and amplitude of the increase in $[Ca^{2+}]_m$ depend on the proximity of the mitochondria to the ER in some cells, e.g., an endothelial cell line (Hajnóczky et al. 1995; Lawrie et al. 1996; Robb-Gaspers et al. 1998a). Unfortunately, at present, the precise mechanism underlying the Ca^{2+}-signal transducing system between two organelli remains unknown.

Our findings on the increase in $[Ca^{2+}]_{mit}$ with caffeine are quite consistent with reports on a caffeine-induced increase in $[Ca^{2+}]_{mit}$ in rat pulmonary arterial SMC (Drummond and Tuft, 1999) and H9c2 cells (Szalai et al. 2000). From these observations, it is conceivable that a novel Ca^{2+}-signaling pathway is served as a signal regulator in the vas deferens SMC.

The hypothesis of such a characteristic Ca^{2+}-signal transducing system from the ER to mitochondria may be further supported by our functional and morphological studies.

The increase in $[Ca^{2+}]_{mit}$ activates the Ca^{2+}-sensitive mitochondrial dehydrogenases, e.g., pyruvate dehydrogenase and NAD^+-isocitrate dehydrogenase. This event leads to increased mitochondrial NAD(P)H levels (McCormack et al. 1990). From the result with CGP37157, a Na^+–Ca^{2+} exchanger, probably in the reverse mode, may play some role in the Ca^{2+}-intake into the inner matrix. Subsequently, the production of ATP in the organella was increased by augmentation of the maximal velocity of oxidative phosphorylation (Robb-Gaspers et al. 1998b; Putney and Thomas 2006). Finally, since the release of ATP was reduced by an inhibitor of ATP/ADP translocator, the overflow of ATP from the mitochondria may trigger further cell signaling and ATP efflux across the cell membrane.

In cultured epithelial cells, it has been shown that the release of ATP induced by hypotonic stress is suppressed by NPPB and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), Cl^- channel blockers, and by Gd^{3+}, a blocker of stretch-activated Cl^- channels.(Hazama et al. 1999; Braunstein et al. 2001). Furthermore, from cultured cardiomyocytes in hypotonic condition, ATP is released through some kind of Cl^- channels such as maxi-anion channels after changing its form to ATP^{4-} or $[Mg\text{-}ATP]^{2-}$ (Dutta et al. 2004). On the basis of the patch-clamp study, it was postulated that cyctic fibrosis transmembrane regulator (CFTR) is a dual ATP and Cl^- channel (Cantiello et al. 1998; Lader et al. 2000). In wild-type CFTR-expressing cells, the basal and hypotonic stress-evoked release of ATP was deteriorated by CFTR-Cl^- channel blockers, Gd^{3+} and DIDS (Schwiebert et al. 1995). In the present study, we provided the possibility that the export of ATP induced by caffeine is mediated by Cl^- channels. However, because the inhibitory effects of Cl^- channel blockers are moderate and partial, a multiple transport system including Cl^- channels may be involved in the extracellular release of ATP.

Molchanova et al. (2007) have exhibited that regulation of mitochondrial Ca^{2+} sequestration and activation of a Cl^- channel are involved in D-[^{3}H] aspartate release from murine corticostriatal slice. Our view on the mode of ATP release seems to be strengthened by this report.

Caffeine at high concentrations is known to inhibit phosphodiesterase and, thereby, to lead the intracellular cyclic AMP elevation. In our previous study with SMC, however, the release of ATP evoked by α,β-methylene ATP was not affected by forskolin (Katsuragi et al. 1996).

ATP is released neuronally as a co-transmitter with noradrenaline from sympathetic nerves of the vas deferens (Sneddon and Westfall 1984). In the vas deferens from purinergic P2X1-receptor-deficient mice, the contraction caused by the stimulation of sympathetic nerves was reduced by over 60%, resulting in a reduction in male fertility by 90% (Mulryan et al. 2000). Therefore, when male animals copulate, activation of the P2X1 receptor by ATP is essential to supply sufficient sperm during ejaculation.

Therefore, the nucleotide that is released from vas deferens SMC as an autocrine/paracrine molecule might serve to promote reproductivity.

In summary, caffeine stimulates RyR-2 and releases Ca^{2+} from the ER. The Ca^{2+} signals seem to reach mitochondria, and subsequently, an elevation in $[Ca^{2+}]_{mit}$ may accelerate the synthesis of ATP. The mitochondrial event could lead a further cell signaling to the cell membrane and activates Cl^- channels, resulting in the outflow of cytosolic ATP. This is the first report providing evidence that caffeine is capable of extracellularly releasing ATP.

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