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CNGA2 Contributes to ATP-Induced Noncapacitative Ca^{2+} Influx in Vascular Endothelial Cells

Hiu-Yee Kwan[a–c] Kwong-Tai Cheng[a–c] Yan Ma[a–c] Yu Huang[a–c]
Nelson L. Tang[a, d] Shan Yu[a–c] Xiaoqiang Yao[a–c]

[a]Li Ka Shing Institute of Health Sciences, [b]Institute of Vascular Medicine, [c]School of Basic Medical Sciences, and [d]Department of Chemical Pathology, The Chinese University of Hong Kong, Hong Kong, SAR, China

Key Words
CNGA2 channels · ATP · cAMP · Endothelial cells · Ca^{2+}

Abstract
Background/Aims: ATP can activate several Ca^{2+} influx channels in vascular endothelial cells. For example, it stimulates TRPC channels via capacitative and noncapacitative Ca^{2+} entry (CCE and non-CCE, respectively) mechanisms; it also directly acts on P2X purinoceptors, resulting in Ca^{2+} influx. In the present study, we tested the hypothesis that cyclic nucleotide-gated (CNG) channels also contribute to ATP-induced non-CCE. ***Methods:*** Two selective inhibitors of CNG channels, L-*cis*-diltiazem and LY-83583, and CNGA2-specific siRNA were used to study the involvement of CNGA2 in ATP-induced non-CCE in endothelial cells. Ca^{2+} influx was studied using Ca^{2+}-sensitive fluorescence dyes Fluo-3 and Fluo-4. ***Results/Conclusion:*** L-*cis*-diltiazem and LY-83583 markedly reduced ATP-induced non-CCE in 3 types of endothelial cells including the H5V endothelial cell line, the primary cultured bovine aortic endothelial cells and the endothelial cells within isolated mouse aortic strips. The CNGA2-specific siRNA also reduced the ATP-induced non-CCE in H5V endothelial cells. The Ca^{2+} influx was inhibited by Rp-8-CPT-cAMPS, MDL-12330A, SQ-22536 and MRS-2179, but not by ODQ or NF-157. Taken together, the present study demonstrated that CNGA2 channels contribute to ATP-induced non-CCE in vascular endothelial cells. It is likely that ATP acts through $P2Y_1$ receptors and adenylyl cyclases to stimulate CNGA2.

Introduction

Extracellular ATP exerts dual effects on blood vessels. ATP can be released as a cotransmitter with noradrenaline from perivascular sympathetic nerves. The released ATP then acts on P2X receptors in vascular smooth muscle cells, resulting in vascular contraction [1–3]. ATP can also be released from red blood cells, platelet and endothelial cells into circulating blood. The circulating ATP acts on vascular endothelial cells to induce release of relaxing factors [4], causing subsequent vascular dilation [1, 3, 4].

ATP elicits a $[Ca^{2+}]_i$ rise in endothelial cells. The ATP-induced $[Ca^{2+}]_i$ rise consists of a large initial transient followed by a small sustained phase [5]. The initial $[Ca^{2+}]_i$ rise is mostly due to Ca^{2+} release from intracellular Ca^{2+} stores, whereas the sustained elevation is due to Ca^{2+} influx. After ATP binds to P2Y receptors, phospholipase C

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Prof. Xiaoqiang Yao
School of Basic Medical Sciences
The Chinese University of Hong Kong
Hong Kong, SAR (China)
Tel. +852 2609 6877, Fax +852 2603 5022, E-Mail yao2068@cuhk.edu.hk

is activated, resulting in the production of inositol (1,4,5)-trisphosphate and diacylglycerol [6]. Inositol (1,4,5)-trisphosphate triggers Ca^{2+} release from intracellular Ca^{2+} stores. Resultant depletion of intracellular Ca^{2+} stores then induces Ca^{2+} influx via capacitative Ca^{2+} entry (CCE) [7]. In addition to CCE, ATP may stimulate Ca^{2+} influx via noncapacitative Ca^{2+} entry (non-CCE) [8–10]. For example, stimulation of P2Y receptors increases the production of diacylglycerol, which activates TRPC3/6/7 channels [8, 9]. ATP may also elevate the nitric oxide level, which activates TRPC5 via S-nitrosylation [10]. ATP can also act on P2X receptors, which are ATP-gated Ca^{2+}-permeable channels [11]. Regardless of detailed pathways, an overall endothelial $[Ca^{2+}]_i$ rise in response to ATP serves as a key signal affecting the production and release of endothelium-derived vasodilators. Therefore, ATP-induced Ca^{2+} influx plays an important role in vascular tone control [12].

Cyclic nucleotide-gated (CNG) channels are a group of Ca^{2+}-permeable channels activated by cyclic AMP (cAMP) and/or cGMP [13]. Among 3 functional CNG isoforms (CNGA1–3), CNGA2 has a higher sensitivity to cAMP than other CNG isoforms [13]. Functionally, Zhang et al. [14] suggested that CNGA2 may mediate endothelial Ca^{2+} influx in response to Ca^{2+} store depletion. Our recent studies indicated that CNGA2 mediates endothelial Ca^{2+} influx in response to cAMP [15] and cAMP-elevating agents such as adenosine and β-adrenergic agonists [15, 16]. Because ATP can stimulate cAMP production [17–19] in vascular endothelial cells, we hypothesize that ATP can also activate CNGA2 in these cells. The results from the present study showed that CNGA2 contributes to ATP-induced non-CCE in endothelial cells and that the signaling pathway involves adenylyl cyclases and $P2Y_1$ receptors.

Methods

Cell Culture and Aortic Strip Preparation

H5V cells, which were derived from murine heart microvessel endothelium, were a generous gift from Dr. Vecchi, Italy [20]. H5V cells were grown in 90% DMEM and 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin. The primary cultured bovine aortic endothelial cells (BAECs) were isolated from bovine aorta. Briefly, bovine aortic segments were cut open longitudinally. The intima layer was peeled off with the help of a scalpel blade, and the segments were then digested with 0.1% collagenase in PBS (in mM: 140 NaCl, 3 KCl, 25 Tris; pH 7.4) for 15 min at 37°C under vigorous shaking. Dissociated cells were centrifuged, resuspended and then grown in a culture medium that contained 90% RPMI-1640 and 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin. Only the cells from the first 4 passages were used for experiments.

For mouse aortic strip preparation, thoracic aorta were dissected from male C57 mice and cut into small strips (3 × 5 mm) and then mounted onto an experimental chamber with the endothelial surface facing the objectives [15].

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

$[Ca^{2+}]_i$ was measured as described elsewhere [15]. Briefly, cultured cells were loaded with 10 μM Fluo-3/AM and 0.02% pluronic F-127 for 1 h in the dark at 37°C in a physiological saline solution (PSS) that contained in mM: 140 NaCl, 5 KCl, 1 $CaCl_2$, 10 glucose, 5 Hepes; pH 7.4. Isolated aortic strips were loaded with 10 μM Fluo-4/AM under the same condition. The experimental chambers containing either cultured endothelial cells or isolated aortic strips were placed on the stage of an inverted microscope (Olympus IX81). For cultured cells, $[Ca^{2+}]_i$ fluorescence of individual cells was measured at room temperature in PSS. For aortic strips, average $[Ca^{2+}]_i$ fluorescence from 20–30 adjacent endothelial cells was measured at 37°C in Krebs solution, which contained (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.2 $NaHCO_3$, 11.1 glucose, pH 7.4, and bubbled with 95% O_2/5% CO_2. $[Ca^{2+}]_i$ fluorescence was measured using the FV1000 laser scanning confocal imaging system. The excitation wavelength was at 488 nm and the fluorescence signals were collected using a 515-nm-long pass emission filter. Data analysis was performed with FV1000 software. Changes in $[Ca^{2+}]_i$ were displayed as a ratio of fluorescence relative to the fluorescence before the application of ATP (F1/F0).

Unless described otherwise, ATP-induced non-CCE was measured by pretreating the cultured cells with 4 μM thapsigargin for 30 min in PSS or pretreating the aortic strips with 4 μM thapsigargin for 5 min in Krebs solution to deplete intracellular Ca^{2+} stores, followed by ATP (100 μM) application to initiate the non-CCE. For measuring CCE, the cells in $0Ca^{2+}$-PSS were treated with 4 μM thapsigargin to deplete intracellular Ca^{2+} stores, followed by application of extracellular Ca^{2+} (2 mM) to initiate CCE. When needed, inhibitors were added 10 min before the application of extracellular ATP or Ca^{2+}. $0Ca^{2+}$-PSS contained in mM: 140 NaCl, 5 KCl, 0.2 EGTA, 10 glucose, 5 Hepes; pH 7.4.

siRNA and Transfection

Vector-based siRNA was used to knock down the expression of CNGA2 [15]. A 19-nt siRNA sequence against mouse CNGA2 gene was designed using Ambion siRNA Target Finder. A pair of inverted repeat sequences containing the 19-nt siRNA was then synthesized. The sequence for strand 1 was 5′-*TGGCAAAGATGACCACAGG*TTCAAGAGA*CCTGTGGTCATCTTTGCCA*TTTTTT-3′, and that for strand 2 was 5′-AATTAAAAAA*TGGCAAAGATGACCACAGG*TCTCTTGAA*CCTGTGGTCATCTTTGCCA*GGCC-3′. The CNGA2-specific nucleotides are italicized. This sequence is specific to CNGA2 only, and it does not cross-react with other CNG isoforms. These 2 strands were annealed and then cloned into a self-constructed siRNA expression vector pcDU6C [5]. pcDU6C contains a U6 RNA polymerase III promoter and a blasticidin resistance gene. The insertion of siRNA sequence was verified by DNA sequencing using the ABI autosequencer (Perkin Elmer, Shelton, Conn., USA). Vector-based scrambled siRNA was purchased from Santa Cruz Biotech (Santa Cruz, Calif., USA; sc-108060).

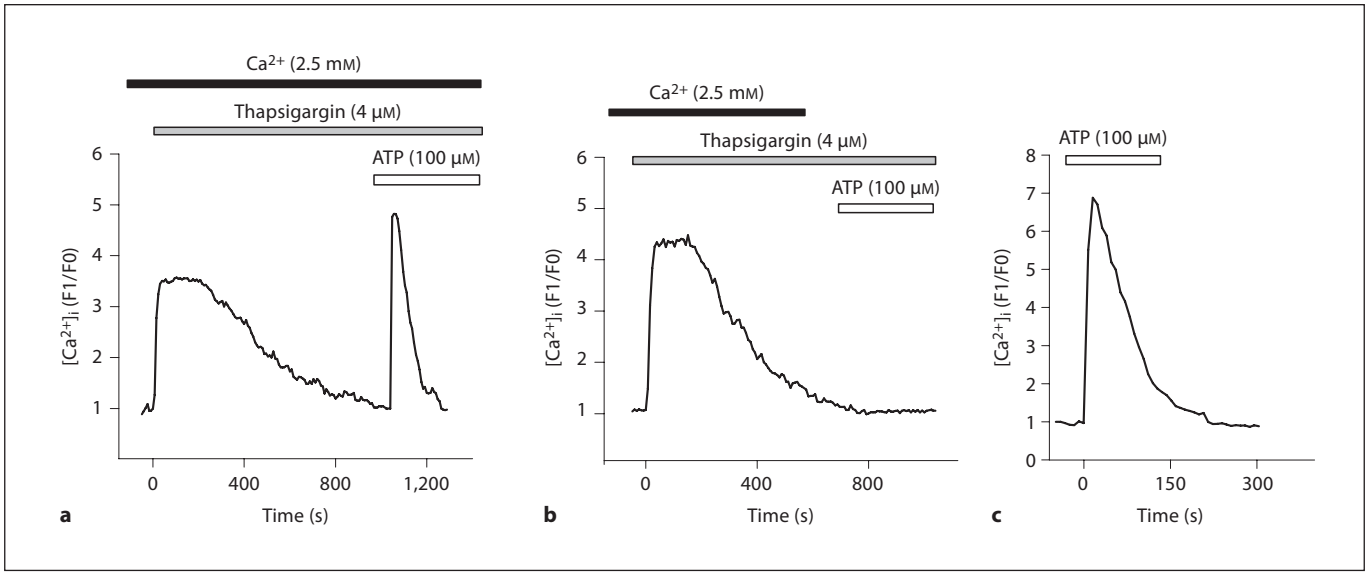

Fig. 1. ATP-induced non-CCE in H5V cells. Representative traces of $[Ca^{2+}]_i$ change in Fluo-3/AM-loaded H5V cells. Cells were placed in PSS, treated with (**a**, **b**) or without thapsigargin (4 μM) (**c**), followed by ATP (100 μM) challenge in the presence (**a**) or absence (**b**, **c**) of extracellular Ca^{2+}. n = 3–5 experiments, 10–15 cells per experiment.

H5V cells were transfected with CNGA2-specific siRNA or scrambled siRNA. The transfection was carried out using lipofectamine 2000.

Materials

Fluo-3/AM, Fluo-4/AM and pluronic F-127 were obtained from Molecular Probes Inc. (Eugene, Oreg., USA). L-*cis*-diltiazem was from Biomol (Plymouth Meeting, Pa., USA). DMEM, FBS, blasticidin and lipofectamine 2000 were from Invitrogen (Carlsbad, Calif., USA). Thapsigargin, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) and NF-157 were from Tocris. LY-83583, MDL-12330A, SQ-22536 and MRS-1754 were from Calbiochem (Darmstadt, Germany). Rp-8-CPT-cAMPS, UTP, MRS-2179, EGTA, Hepes and Tris-HCl were from Sigma (St. Louis, Mo., USA).

Results

ATP-Induced Non-CCE in H5V Cells

H5V cells were first treated with thapsigargin (4 μM) in PSS to deplete intracellular Ca^{2+} stores. Thapsigargin treatment caused a transient rise in $[Ca^{2+}]_i$ due to the release of Ca^{2+} stores and subsequent CCE (fig. 1a). The $[Ca^{2+}]_i$ level returned to its basal level within 10–20 min (fig. 1a), presumably due to compensatory mechanisms from Na^+-Ca^{2+} exchanger and plasma membrane Ca^{2+}-ATPase, both of which reduce the $[Ca^{2+}]_i$ level [21]. After $[Ca^{2+}]_i$ returned to its basal level, ATP (100 μM) was applied, which elicited another $[Ca^{2+}]_i$ rise (fig. 1a). Because intracellular Ca^{2+} stores had been emptied by thapsigargin, ATP would not cause further store Ca^{2+} release and CCE. Thus, we assigned this $[Ca^{2+}]_i$ rise as non-CCE. Control experiments were performed to verify the effectiveness of thapsigargin treatment. In these control experiments, after thapsigargin treatment, the cells were transferred to $0Ca^{2+}$-PSS followed by ATP challenge (100 μM; fig. 1b). The ATP challenge failed to elicit a rise in $[Ca^{2+}]_i$ in these cells, indicating that thapsigargin treatment indeed emptied the intracellular Ca^{2+} stores. As expected, for cells bathed in $0Ca^{2+}$-PSS but without thapsigargin treatment, ATP elicited a $[Ca^{2+}]_i$ rise (fig. 1c).

Role of CNGA2 Channels in ATP-Induced Non-CCE in H5V Cells

We then explored the possible involvement of CNG channels in ATP-induced non-CCE. L-*cis*-diltiazem (50 μM), a selective inhibitor for CNG channels [13], caused a marked reduction in ATP-induced non-CCE (fig. 2a, b, d). Another CNG channel blocker LY-83583 (20 μM) [22] had a similar inhibitory effect (fig. 2a, c, d). RNA interference was used to identify the specific CNG isoform that is involved in ATP-induced non-CCE in H5V endothelial cells. CNGA2 was chosen as a possible candidate be-

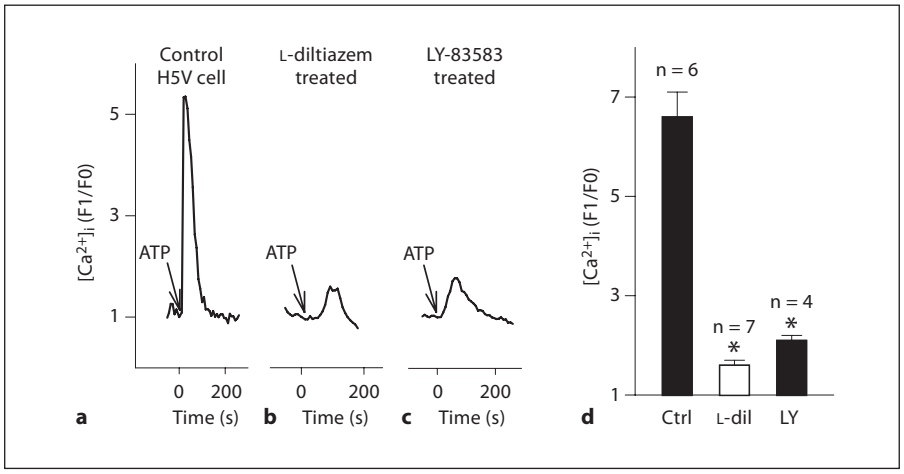

Fig. 2. Effect of CNG channel inhibitors on ATP-induced non-CCE in H5V cells. **a–c** Representative traces of ATP (100 μM)-induced non-CCE in control (**a**), L-*cis*-diltiazem (50 μM)-treated (**b**) and LY-83583 (20 μM)-treated (**c**) cells. **d** Summary of data as in **a–c**. Ctrl = Control; L-dil = L-*cis*-diltiazem; LY = LY-83583. Mean ± SEM (n = 4–7 experiments, 10–15 cells per experiment). * $p < 0.05$ compared with the control.

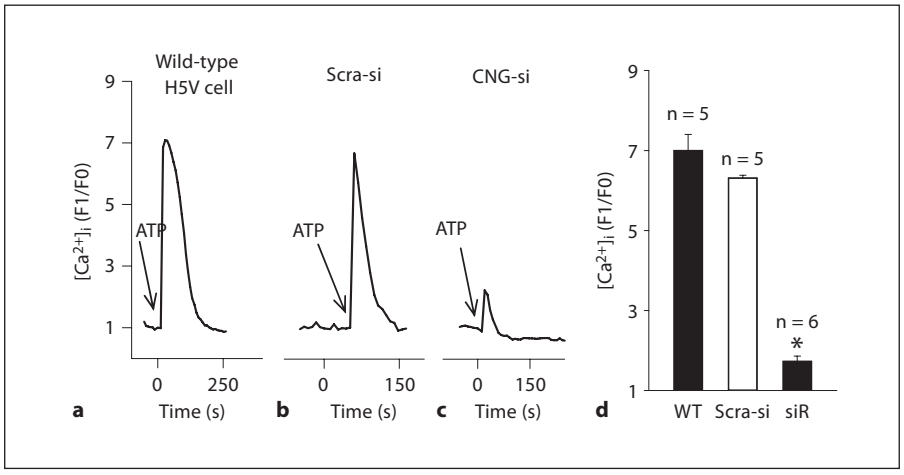

Fig. 3. Effect of CNGA2-specific siRNA on ATP-induced non-CCE in H5V cells. **a–c** Representative traces showing ATP (100 μM)-induced non-CCE in wild-type (**a**), scrambled siRNA-transfected (**b**) and CNGA2-siRNA-transfected H5V cells (**c**). **d** Summary of data as in **a–c**. WT = Wild-type; Scra-si = scrambled siRNA transfected; CNG-si = CNGA2-siRNA transfected; siR = CNGA2-siRNA transfected. Mean ± SEM (n = 6–7 experiments, 10–15 cells per experiment). * $p < 0.05$ compared with vector-transfected cells.

cause CNGA2 is known to have functional importance in vascular endothelial cells [14–16, 23, 24]. A CNGA2-specific siRNA was developed previously and had been shown to selectively reduce the expression of CNGA2 in H5V cells by 80 ± 2% (n = 5) as determined by immunoblot using an anti-CNGA2 antibody from Alpha-Diagnostic Int. (San Antonio, Tex., USA) [15, 16]. In the present study, this CNGA2-specific siRNA markedly reduced ATP-induced non-CCE (fig. 3a, c, d). In contrast, transfection with a scrambled siRNA had no effect on ATP-induced non-CCE (fig. 3a, b, d).

Involvement of Adenylyl Cyclases and P2Y$_1$ Receptors in ATP-Induced Non-CCE in H5V Cells

Because CNGA2 is a cAMP-activated channel, we examined the possible involvement of adenylyl cyclases. MDL-12330A (10 μM) and SQ-22536 (300 μM), 2 selective inhibitors for adenylyl cyclases, markedly reduced ATP-induced non-CCE (fig. 4a). In contrast, a guanylyl cyclase inhibitor ODQ (50 μM) had no effect on this Ca^{2+} influx (fig. 4a). Furthermore, Rp-8-CPT-cAMPS (30 μM), an agent that competitively inhibits cAMP binding to the CNG channel and protein kinase A, also suppressed ATP-induced non-CCE (fig. 4a) [25]. These results support the notion that ATP activates adenylyl cyclases to elevate cAMP, which in turn activates CNGA2.

ATP may bind to P2Y$_1$, P2Y$_2$ or P2Y$_{11}$ to elevate cAMP level [26–28]. We found that ATP-induced non-CCE could not be mimicked by a P2Y$_2$ agonist UTP (1 μM; fig. 4a) [6]. Furthermore, a P2Y$_{11}$ antagonist NF157 (2 μM) [29] had no effect on ATP-induced non-CCE (fig. 4a). In contrast, ATP-induced non-CCE was almost completely abolished by MRS-2179 (10 μM), which is a selective inhibitor of the P2Y$_1$ receptors [28]. These data suggest that the action of ATP on non-CCE is mediated through P2Y$_1$ receptors but not through P2Y$_2$ or P2Y$_{11}$.

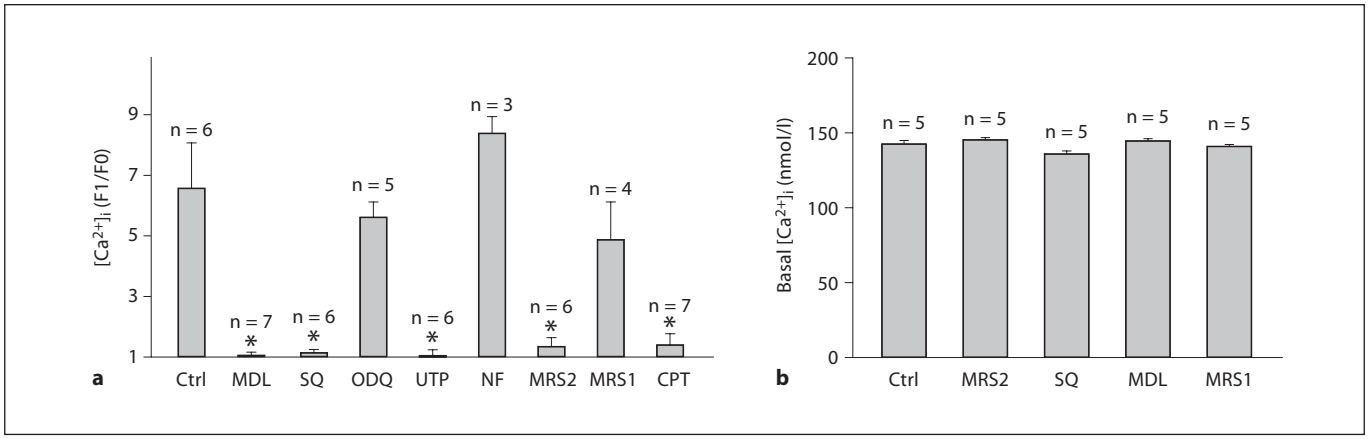

Fig. 4. Effect of a panel of pharmacological agents on ATP-induced non-CCE and the basal $[Ca^{2+}]_i$ level in H5V cells. **a** ATP-induced non-CCE. **b** Basal $[Ca^{2+}]_i$ level. Ctrl = Control; MDL = MDL-12330A (10 μM); SQ = SQ-22536 (300 μM); ODQ (50 μM); UTP (1 μM); NF = NF-157 (2 μM); MRS2 = MRS-2179 (10 μM); MRS1 = MRS-1754 (1 μM); CPT = Rp-8-CPT-cAMPS (30 μM). Mean ± SEM (n = 3–7 experiments, 10–15 cells per experiment). * $p < 0.05$ as compared with the control.

ATP can be degraded into adenosine by ecto-nucleotidases to generate adenosine, which may activate adenylyl cyclases and CNGA2 channels via a P1 receptor A_{2B} [15]. There is also a possibility that ATP at high concentration can directly act on P1 receptors [27]. Therefore, we examined the possible involvement of P1 receptors. Blockage of the main P1 receptors (A_{2B} receptors) in H5V cells with MRS-1754 (1 μM) was shown to have no effect on the ATP-induced non-CCE (fig. 4a). These data argue against an involvement of P1 receptors in ATP-induced non-CCE in H5V cells.

Effect of Adenylyl Cyclase Inhibitors and P2Y Receptor Inhibitors on the Basal $[Ca^{2+}]_i$ Level in H5V Cells

CNGA2 channels are highly permeable to Ca^{2+} [13]. Our previous studies have confirmed an important role of CNGA2 in maintaining a basal $[Ca^{2+}]_i$ level in H5V cells [16], because L-*cis*-diltiazem, LY-83583 and CNGA2-specific siRNA all reduced the basal $[Ca^{2+}]_i$ level in H5V cells [16]. In the present study, we further tested the role of adenylyl cyclases and P2Y purinoceptor in the maintenance of the basal $[Ca^{2+}]_i$ level. As shown in figure 4b, inhibiting adenylyl cyclases with MDL-12330A (10 μM) or SQ-22536 (300 μM) had no effect on the basal $[Ca^{2+}]_i$ level in these cells (fig. 4b). $P2Y_1$ antagonist MRS-2179 (10 μM) and P1 (especially A_{2B}) antagonist MRS-1754 (1 μM) also had no effect on the basal $[Ca^{2+}]_i$ level (fig. 4b). These results suggest that, in the absence of exogenous ATP, basal activity of adenylyl cyclases and $P2Y_1$ receptors is very low, and they do not significantly influence the basal $[Ca^{2+}]_i$ level in these cells. These data also suggest that, under normal nonstimulated condition, CNGA2 contributes to the maintenance of the endothelial basal $[Ca^{2+}]_i$ level via a cAMP-independent mechanism. In agreement, other studies have already demonstrated that CNGA2 channels have spontaneous basal activity in the absence of cyclic nucleotides [30].

ATP-Induced Non-CCE in BAECs

H5V is a cell line. There is concern that prolonged cell culture may alter its endothelial phenotype. Therefore, the primary cultured BAECs were used to verify the above findings. It was found that ATP-induced non-CCE in BAECs was inhibited by L-*cis*-diltiazem (50 μM) and LY-83583 (20 μM; online suppl. fig. 1; for all suppl. material see www.karger.com/doi/10.1159/000235969). Similar to H5V cells, inhibition of adenylyl cyclases by MDL-12330A (10 μM) or SQ-22536 (300 μM) reduced this Ca^{2+} influx in BAECs (online suppl. fig. 2). Furthermore, MRS-2179 (10 μM), an inhibitor of $P2Y_1$ receptors, markedly reduced this Ca^{2+} influx, whereas MRS-1754 (1 μM) had no effect. These data are consistent with the role of CNG channels, adenylyl cyclases and $P2Y_1$ receptors in ATP-induced non-CCE in H5V cells.

Role of CNG Channels in CCE

Zhang et al. [14] reported that CNGA2 contributes to CCE in pulmonary artery endothelial cells. In the present

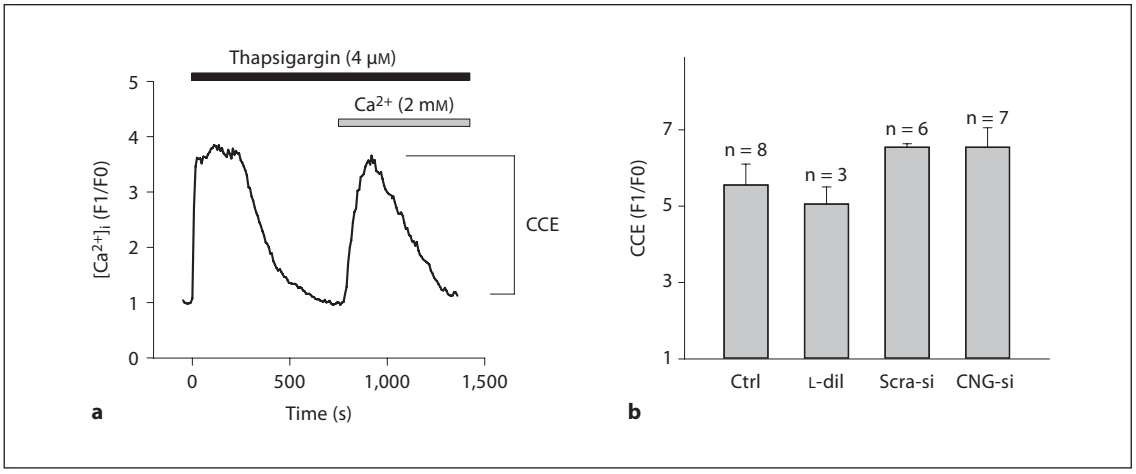

Fig. 5. Effect of L-*cis*-diltiazem and the CNGA2-specific siRNA on CCE in H5V cells. **a** A representative trace of $[Ca^{2+}]_i$ change in H5V cells. Cells were bathed in $0Ca^{2+}$-PSS, treated with thapsigargin (4 μM), followed by application of extracellular Ca^{2+} (2 mM) to induce CCE. **b** Summary of data. Ctrl = Control; L-dil = L-*cis*-diltiazem (50 μM); Scra-si = scrambled siRNA transfected; CNG-si = CNGA2-siRNA transfected. Mean ± SEM (n = 3–8 experiments, 10–15 cells per experiment).

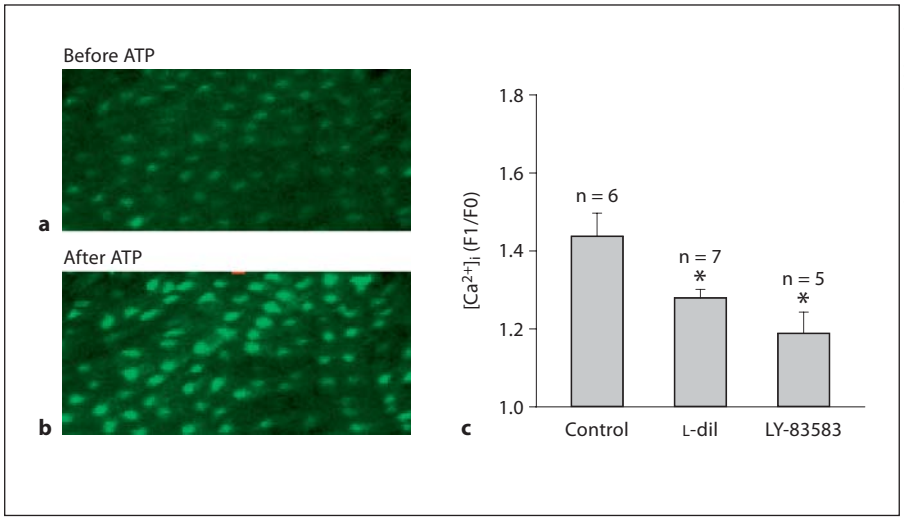

Fig. 6. Effect of CNG channel inhibitors on ATP-induced non-CCE in endothelial cells within mouse aortic strips. **a**, **b** Representative endothelium fluorescence images before (**a**) and after (**b**) 200 μM ATP. **c** Summary of data showing the effect of L-*cis*-diltiazem (100 μM) and LY-83583 (10 μM) on the peak magnitude of $[Ca^{2+}]_i$ transient in response to 200 μM ATP. Mean ± SEM (n = 5–7 experiments). * $p < 0.05$ as compared with the control.

study, H5V cells were treated with thapsigargin (4 μM) in $0Ca^{2+}$-PSS to deplete intracellular Ca^{2+} stores. After the thapsigargin-induced $[Ca^{2+}]_i$ transient returned to its basal level, extracellular Ca^{2+} was then applied to induce CCE (fig. 5a). We found that this CCE was not affected by L-*cis*-diltiazem (50 μM) or CNGA2-specific siRNA (fig. 5b). These results argue against an involvement of CNGA2 in CCE.

Role of CNG Channels in ATP-Induced Non-CCE in Endothelial Cells of Mice Aortic Strips

There is concern that the endothelial phenotype may change during cell culture and cell passage conditions. Thus, we studied $[Ca^{2+}]_i$ response in endothelial cells within isolated mouse aortic strips to verify the above findings. After store depletion by treating the aortic strips with 4 μM thapsigargin for 5 min, subsequent application of ATP (200 μM) elicited a $[Ca^{2+}]_i$ rise in the majority of

endothelial cells in isolated mouse aortic strips (fig. 6). This ATP-induced $[Ca^{2+}]_i$ rise was significantly reduced in aortic strips that were pretreated with L-*cis*-diltiazem (50 μM) or LY-83583 (10 μM) for 10 min (fig. 6). Note that we previously observed that LY-83583 at 20 μM facilitated the spreading of Ca^{2+} signals to neighboring cells [15]. In the present study, we used 10 μM LY-83583, with which the facilitating effect of L-*cis*-diltiazem on intercellular Ca^{2+} spreading became much lower. For those tissues that still had an increased intercellular Ca^{2+} spreading in response to LY-83583, we waited for these Ca^{2+} spreading to subside before ATP application.

Discussion

ATP can act on endothelial cells to induce a $[Ca^{2+}]_i$ rise, which is a key signal for endothelium-dependent vascular dilation [1, 12]. The mechanism for this $[Ca^{2+}]_i$ rise includes CCE and non-CCE. CCE is a common Ca^{2+} influx mechanism activated by diverse groups of Ca^{2+}-mobilizing agonists [7] and is believed to be mediated by STIM1 and Orai proteins [31] and/or TRPC channels [7, 12]. In contrast, the activation mechanisms for non-CCE are diverse and may differ with agonist types. Previous studies have demonstrated that ATP may activate TRPC3/6 [9], TRPC5 [10] and $P2X_4$ [11] independent of Ca^{2+} store depletion in endothelial cells. In the present study, we demonstrated that ATP-induced non-CCE can be markedly inhibited by LY-83583 and L-*cis*-diltiazem, 2 blockers of CNG channels, in H5V endothelial cells and the primary cultured BAECs. Furthermore, a CNGA2-specific siRNA reduced the ATP-induced non-CCE in H5V endothelial cells, suggesting an involvement of CNGA2. On the other hand, in disagreement with Zhang et al. [14], we found no evidence for an involvement of CNG channels in CCE. Note that even after L-*cis*-diltiazem or CNGA2-specific siRNA, ATP was still able to evoke residual Ca^{2+} influx (fig. 2, 3, online suppl. fig. 2), probably reflecting the participation of other Ca^{2+} influx channels such as TRPC channels in ATP-induced non-CCE. Taken together, these results demonstrated for the first time that CNGA2 contributes to ATP-induced non-CCE in vascular endothelial cells. This scheme provides an alternative pathway for endothelial Ca^{2+} influx in response to ATP.

CNG channels are a group of Ca^{2+}-permeable cation channels sensitive to activation by cyclic nucleotides [13]. Most published data show that ATP stimulates the production of cGMP [32] and cAMP [18–20] in vascular endothelial levels. Furthermore, the stimulating effect of ATP on cAMP production is more prominent in the presence of a cAMP-elevating agent forskolin [18, 19]. However, there is one exception in which ATP treatment was found to reduce the cAMP level in rat brain capillary endothelial cells [33]. This discrepancy could be due to the differential expression of P2Y receptor subtypes under different culture conditions [33]. In the present study, we found that ATP-induced non-CCE was inhibited by 2 selective adenylyl cyclase inhibitors MDL-12330A and SQ-22536, but not by a guanylyl cyclase inhibitor ODQ. These results support the notion that ATP elevates the cAMP level in endothelial cells. The elevated cAMP may then stimulate CNGA2 activity.

Efforts were made to determine the membrane receptors that are involved in ATP-induced CNG activation. ATP might bind to P2X and/or P2Y receptors. Two lines of evidence suggest that P2X is not involved. Firstly, P2X is directly gated by ATP. Thus, the action of ATP on P2X should not be affected by CNGA2-specific siRNA, CNG channel inhibitors or adenylyl cyclase inhibitors. Secondly, Ca^{2+} influx through P2X, if any, should only inhibit CNGA2, which is known to be inhibited via the Ca^{2+}-calmodulin pathway [34]. These predictions are contradictory to our findings that (1) ATP elicited a Ca^{2+} influx and (2) the influx was inhibited by a CNGA2-specific siRNA, CNG channel inhibitors and adenylyl cyclase inhibitors. We next examined the possible involvement of P1 receptors, because ATP could be degraded by ectonucleotidases to generate adenosine, which may bind to P1 receptors. Furthermore, at the concentration we used, ATP may directly act on P1 receptors. In this regard, we used a P1 (A_{2B} subtype) receptor blocker MRS-1754, which was previously shown to completely abolish P1-mediated Ca^{2+} influx in endothelial cells [15]. In the present study, we found that MRS-1754 had no effect on ATP-induced non-CCE, suggesting that P1 receptors are not involved in ATP-induced non-CCE.

We then explored the possible involvement of $P2Y_1$, $P2Y_2$ and $P2Y_{11}$, each of which is known to stimulate adenylyl cyclases at least under certain conditions [26–28]. We found that the effect of ATP could not be mimicked by a $P2Y_2$ agonist UTP and was not inhibited by a $P2Y_{11}$ antagonist NF157. In contrast, the action of ATP on $[Ca^{2+}]_i$ was almost completely abolished by MRS-2179, which is a selective inhibitor of the $P2Y_1$ antagonist. These data suggest that ATP activates $P2Y_1$ receptors to stimulate Ca^{2+} influx in H5V cells and BAECs.

We also investigated the effect of adenylyl cyclase inhibitors (MDL-12330A and SQ-22536), P1 receptor in-

hibitor (MRS-1754) and $P2Y_1$ receptor inhibitor (MRS-2179) on the basal $[Ca^{2+}]_i$ level. These inhibitors had no effect on the basal $[Ca^{2+}]_i$ level in H5V endothelial cells. These results suggest that, in the absence of exogenous ATP, the basal activity of adenylyl cyclases and $P2Y_1$ receptors is very low, and that they do not significantly influence the basal $[Ca^{2+}]_i$ level. However, as we have reported previously, CNGA2 channels have basal activity in the absence of any agonist. This basal activity may contribute to the maintenance of the basal $[Ca^{2+}]_i$ level in endothelial cells [16]. Taking this into consideration, caution needs to be taken for the above conclusion that ATP, via cAMP, stimulates CNGA2, resulting in an increased non-CCE. In our experiments, suppression of CNGA2 by LY-83583, L-*cis*-diltiazem or a CNGA2-specific siRNA resulted in a reduction in ATP-induced non-CCE. A straightforward interpretation of the data, as we already mentioned above, is that ATP stimulated CNGA2, resulting in an increased non-CCE. However, we cannot completely exclude an alternative possibility that the basal $[Ca^{2+}]_i$ level, which is partly maintained by basal CNGA2 activity, could play a permissible role for another Ca^{2+} influx pathway activated by ATP.

In conclusion, CNGA2 contributes to ATP-induced non-CCE in vascular endothelial cells. It appears that ATP binds to $P2Y_1$ to activate adenylyl cyclases. The resultant production of cAMP activates CNGA2 to cause Ca^{2+} influx. This signaling pathway may contribute to endothelium-dependent vascular dilation to ATP.

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