Journal of Molecular and Cellular Cardiology 45 (2008) 437–445

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Epinephrine-induced Ca^{2+} influx in vascular endothelial cells is mediated by CNGA2 channels

Bing Shen [a,b,c], Kwong-Tai Cheng [a,b,c], Yuk-Ki Leung [a,b,c], Yuk-Chi Kwok [a,b,c], Hiu-Yee Kwan [a,b,c], Ching-On Wong [a,b,c], Zhen-Yu Chen [d], Yu Huang [a,b,c], Xiaoqiang Yao [a,b,c,*]

[a] Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, China
[b] Department of Physiology, The Chinese University of Hong Kong, Hong Kong, China
[c] Institute of Vascular Medicine, The Chinese University of Hong Kong, Hong Kong, China
[d] Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China

ARTICLE INFO

Article history:
Received 12 February 2008
Received in revised form 27 May 2008
Accepted 13 June 2008
Available online 24 June 2008

Keywords:
CNGA2 channels
Epinephrine
Isoprenaline
Endothelial cells
Ca^{2+}

ABSTRACT

Epinephrine, through its action on β-adrenoceptors, may induce endothelium-dependent vascular dilation, and this action is partly mediated by a cytosolic Ca^{2+} ($[Ca^{2+}]_i$) change in endothelial cells. In the present study, we explored the molecular identity of the channels that mediate epinephrine-induced endothelial Ca^{2+} influx and subsequent vascular relaxation. Patch clamp recorded an epinephrine- and cAMP-activated cation current in the primary cultured bovine aortic endothelial cells (BAECs) and H5V endothelial cells. L-*cis*-diltiazem and LY-83583, two selective inhibitors for cyclic nucleotide-gated channels, diminished this cation current. Furthermore, this cation current was greatly reduced by a CNGA2-specific siRNA in H5V cells. With the use of fluorescent Ca^{2+} dye, it was found that epinephrine and isoprenaline, a β-adrenoceptor agonist, induced endothelial Ca^{2+} influx in the presence of bradykinin. This Ca^{2+} influx was inhibited by L-*cis*-diltiazem and LY-83583, and by a β_2-adrenoceptor antagonist ICI-118551. CNGA2-specific siRNA also diminished this Ca^{2+} influx in H5V cells. Furthermore, L-*cis*-diltiazem and LY-83583 inhibited the endothelial Ca^{2+} influx in isolated mouse aortic strips. L-*cis*-diltiazem also markedly reduced the endothelium-dependent vascular dilation to isoprenaline in isolated mouse aortic segments. In summary, CNG channels, CNGA2 in particular, mediate β-adrenoceptor agonist-induced endothelial Ca^{2+} influx and subsequent vascular dilation.

1. Introduction

Epinephrine is the primary catecholamine released from the adrenal medulla in response to low blood glucose, exercise and stress. It exerts a profound effect on vascular system. Depending on vascular beds and chemical concentration, epinephrine may either induce vascular dilation or contraction [1,2]. While the contractile action of epinephrine is mainly mediated through α-adrenoceptors in vascular smooth muscle cells, the relaxant effect of epinephrine is mainly mediated through β-adrenoceptors, which are located in both vascular smooth muscle and endothelial cells [1]. In many vascular beds, the dilation to β-adrenoceptor agonists is, at least partly, endothelium-dependent and can be attributed to an increased production of nitric oxide (NO) in endothelial cells [3–7].

cAMP is an important second messenger that participates in the endothelium-dependent vascular dilation. Activation of β-adrenoceptors stimulates adenylyl cyclases, causing subsequent production of cAMP [1]. Elevated cAMP then activates endothelial nitric oxide synthase (eNOS) either via a Ca^{2+}-independent pathway that involves protein kinase A [8] or via a Ca^{2+}-dependent pathway that involves Ca^{2+}-calmodulin [9–11]. In the latter case, β-adrenoceptor agonists act on endothelial cells to elevate cAMP, which either increases cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) by itself [9] or enhances agonist-induced $[Ca^{2+}]_i$ rise [10,11], both of which result in an increased NO biosynthesis [7,10,11].

Little is known about the molecular identity of channels that mediate epinephrine-induced Ca^{2+} influx in endothelial cells. Vascular endothelial cells express multiple Ca^{2+}-permeable channels, which include transient receptor potential (TRP) and cyclic nucleotide-gated (CNG) channels [12–14]. CNG channels are activated by cAMP and cGMP [15], the levels of which are elevated when endothelial cells are exposed to β-adrenoceptor agonists [4,10,16,17]. Therefore, CNG channels could be a potential candidate that mediates β-adrenoceptor agonist-induced Ca^{2+} influx in endothelial cells.

In the present study, we used the methods of patch clamp, Ca^{2+}-sensitive fluorescent dye and myograph to study the role of CNG channels in vascular endothelial cells. Our data demonstrated that CNG channels, especially CNGA2, mediate the endothelial Ca^{2+} influx in response to epinephrine and β-adrenoceptor agonists. Furthermore,

* Corresponding author. Department of Physiology, The Chinese University of Hong Kong, Hong Kong, China. Fax: +852 26035022.
E-mail address: yao2068@cuhk.edu.hk (X. Yao).

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doi:10.1016/j.yjmcc.2008.06.005

inhibition of CNG channels greatly reduced isoprenaline-induced vascular dilation in mouse aortic segments.

2. Materials and methods

2.1. Cell culture and aortic strip preparation

The animal study was conducted in conformity with the *Guide for animal Care and Use of Laboratory Animals* published by the US National Institute of Health. The primary cultured BAECs were isolated from bovine aorta as described elsewhere [18]. Briefly, bovine aortic segments were cut open longitudinally. The intima layer was peeled off and then digested with 0.1% collagenase in PBS (in mmol/L: 140 NaCl, 3 KCl, 25 Tris, pH 7.4) for 15 min at 37 °C under vigorous shaking. Dissociated cells were centrifuged, re-suspended and then grown in a culture medium that contained 90% RPMI-1640 and 10% FBS. Only the cells from the first four passages were used for experiments. H5V cells, which were derived from murine embryonic heart endothelium, were a generous gift from Dr. Vecchi A, Italy [19]. Both H5V and a bronchial epithelial cell line 16HBE were grown in 90% DMEM and 10% FBS.

For mouse aortic strip preparation, thoracic aorta were dissected from male C57 mice and cut into small strips (3 mm width×5 mm long), and then mounted onto an experimental chamber with endothelial surface facing the objectives [18].

2.2. siRNA, CNGA2 clone and transfection

The vector-based siRNA strategy was used to allow H5V cells to stably express the CNGA2-specific siRNA [18]. 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 the strand 1 was 5′-TGGCAAAGATGACCACAGGTTCAAGAGACCTGTGGTCATCTTTGCCATTT-TTT-3′, and that for strand 2 was 5′-AATTAAAAAA TGGCAAAGATGAC-CACAGGTCTCTTGAACCTGTGGTCATCTTTGCCAGGCC-3′. The CNGA2-specific nucleotides are underlined. This sequence is specific to CNGA2 only, and it does not cross-react with other CNG isoforms in Genbank. These two strands were annealed and then cloned into a self-constructed siRNA expression vector pcDU6C [18]. CNGA2 gene was a gift from Dr. Ko WH, the Chinese University of Hong Kong. It was subcloned in pcDNA6.

H5V cells were stably transfected with either the siRNA-containing construct or the control plasmid vector pcDU6C using Lipofectamine 2000 [18]. 16HBE was stably transfected with the CNGA2-containing pcDNA6.

2.3. Immunoblots

Immunoblots were performed as described elsewhere [18]. Briefly, whole-cell lysates were extracted with protein extraction buffer, which contained 50 mmol/L Tris–HCl, 150 mmol/L NaCl, 50 mmol/L NaF, 2 mmol/L EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.5, with addition of the protease inhibitor cocktail tablets (Roche). 100 μg proteins was loaded onto each lane and separated on a SDS/PAGE gel. Proteins were transferred to a PVDF membrane, and blotted with the primary anti-CNGA2 (1:1000) or anti-CNGA4 (2 μg/ml) antibody (1:100). Immunodetection was accomplished with horseradish peroxidase-conjugated secondary antibody, followed by ECLÔ Plus western blotting detection system. Immunoblots with anti-β-tubulin antibody were used to confirm that an equal amount of proteins was loaded onto each lane. The intensity of the bands was analyzed by FluorChem 8000 imaging system.

2.4. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured as described elsewhere [18]. Briefly, cultured cells or isolated aortic strips were loaded with 10 μmol/L Fluo-4/AM and 0.02% pluronic F-127 for 1 h in dark at 37 °C in a normal physiological saline solution (NPSS) that contained in mmol/L: 140

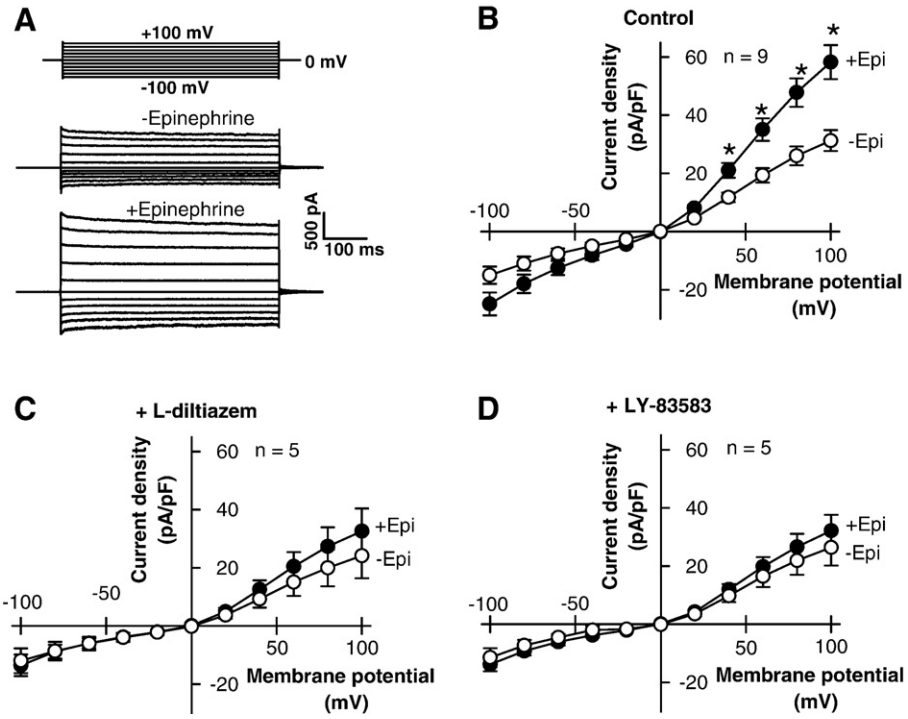

Fig. 1. Effect of CNG channel inhibitors on epinephrine-activated whole-cell cation current in BAECs. (A), representative traces showing voltage protocol (upper) and the corresponding whole-cell current before (middle) and after (lower) epinephrine (1 μmol/L) application. (B), steady state current–voltage relationships as in A. (C, D), steady state current–voltage relationship for cells that were pretreated with 100 μmol/L L-*cis*-diltiazem (C) or 20 μmol/L LY-83583 (D). +Epi, with epinephrine; −Epi, without epinephrine. Mean ± SEM (n=5–9 independent experiments). *P<0.05 as compared to −epinephrine.

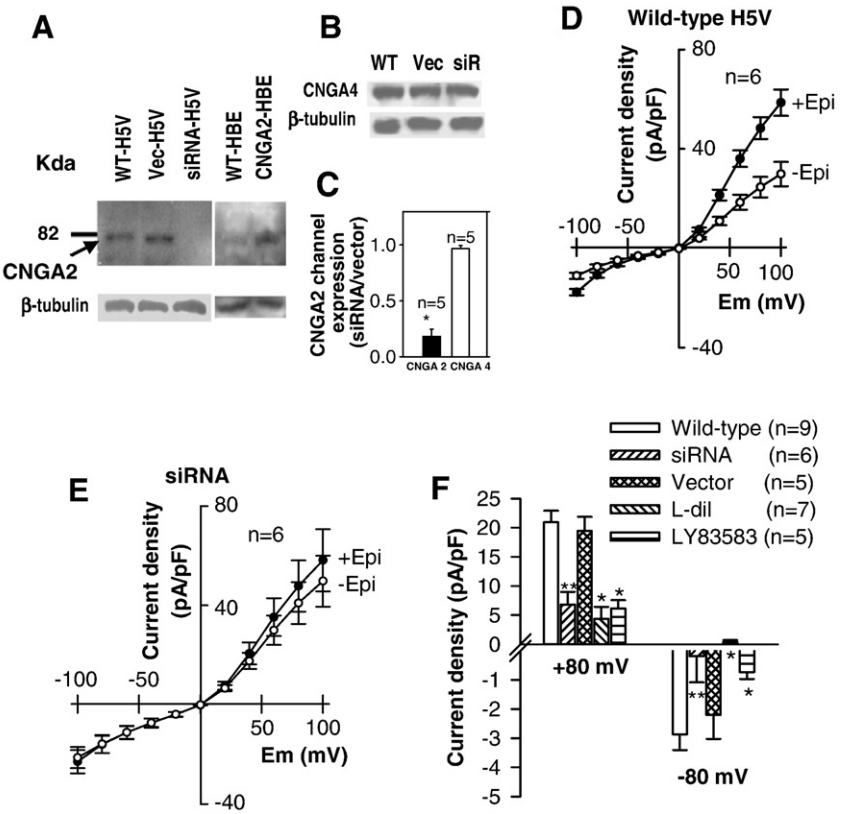

Fig. 2. Effect of CNGA2-specific siRNA and CNG channel inhibitors on epinephrine-activated cation current in H5V cells. (A and B): representative images (A, B) and summary (C) of immunoblot experiments. The protein levels of CNGA2 (A) and CNGA4 (B) were compared between wild-type, vector-transfected, and siRNA-expressing H5V cells. CNGA2 heterologously expressed in 16HBE cells was blotted together as a control. (D and E): current–voltage relationships before and after epinephrine (1 μmol/L) treatment in wild-type (D) and siRNA-expressing H5V cells (E). (F), summary of data showing the effect of CNGA2-specific siRNA, L-*cis*-diltiazem (100 μmol/L), and LY-83583 (20 μmol/L) on epinephrine-activated whole-cell cation current at −80 mV and +80 mV. The epinephrine-activated current was obtained by subtracting the current before epinephrine treatment from that after the treatment. WT-H5V, wild-type H5V; Vec-H5V, vector-transfected H5V; siR-H5V, siRNA-transfected H5V; WT-HBE, wild-type 16HBE; CNGA2-HBE, CNGA2-transfected 16HBE. +Epi, with epinephrine; −Epi, without epinephrine; L-dil, L-*cis*-diltiazem. Mean ± SEM (n = 5–9 independent experiments). *P < 0.05 as compared to wild-type (F) or CNGA4 expressing H5V cells (C). **P < 0.05 as compared to vector-transfected H5V cells.

NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5 Hepes, pH 7.4. 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 NPSS. 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 mmol/L: 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 epinephrine or isoprenaline (F1/F0).

Unless stated otherwise, cultured cells bathed in NPSS were first treated with bradykinin (10 nmol/L) and then challenged with epinephrine (1 μmol/L) or isoprenaline (10 μmol/L). For aortic strips, no bradykinin was added. The $[Ca^{2+}]_i$ response was continuously monitored. When appropriate, 100 μmol/L L-*cis*-diltiazem, 20 μmol/L LY-83583, or 1 μmol/L ICI-118551 was added 10 min before epinephrine or isoprenaline.

2.5. Electrophysiology

Currents were recorded in whole-cell or excised inside-out mode using an EPC9 patch clamp amplifier (HEKA) controlled by Pulse software. Patch pipette solution contained in mmol/L: 145 Na^+-glutamate, 5 CsCl, 5 EGTA, 5 Hepes, pH 7.4 adjusted with TEA-OH. Bath solution contained in mmol/L: 145 Na^+-glutamate, 5 Hepes, 10 glucose, pH 7.4 adjusted with TEA-OH. Whole-cell currents were recorded in response to successive voltage pulses of 100 ms duration, increasing in 20 mV increments from −100 to +100 mV. The recordings were made before and 5 min after the epinephrine (1 μmol/L) or 8-BrcAMP (100 μmol/L for BAECs, 300 μmol/L for H5V cells) application. For the excised inside-out patches, patch currents were recorded in response to voltage pulses of −60 mV and +60 mV for 500 ms duration. The recordings were made before and ~1 min after cAMP perfusion to the bath. All currents were sampled at 50 kHz and filtered at 5 kHz, and the data were analyzed with PulseFit. Electrophysiological experiments were performed at room temperature. If needed, L-*cis*-diltiazem and LY-83583 were added 10 min before epinephrine application.

2.6. Arterial tension measurement

The thoracic aorta was dissected from male C57 mice and cut into rings of 3 mm in length. The vessels were mounted in a myograph (model 610M, DMT, Denmark) under a normalized tension as previously described [18]. The aortic rings were preconstricted with 11-deoxy prostaglandin $F_{2\alpha}$ to achieve sustained contractions. The concentration of 11-deoxy prostaglandin $F_{2\alpha}$ varied from 20 to 300 nmol/L in order to achieve similar degree of constriction in different arteries. Isoprenaline was then added in a cumulative fashion to the bath to obtain the concentration–response curves. If

needed, L-*cis*-diltiazem (100 μmol/L) was added 30 min before isoprenaline application. L-*cis*-diltiazem did not cause a significant change in contraction of aortic rings to 11-deoxy prostaglandin $F_{2\alpha}$ The relaxation response to isoprenaline was expressed as % of 11-deoxy prostaglandin $F_{2\alpha}$-induced tone. In some experiments, the endothelial layer was mechanically disrupted with a small piece of plastic tubing. Endothelium integrity or functional removal was verified by the presence or absence, respectively, of the relaxant response (over 80% relaxation) to 1 μmol/L acetylcholine at the start of each experiment. All tension experiments were performed at 37 °C in Krebs solution.

2.7. Materials

Fluo-4/AM and pluronic F-127 were obtained from Molecular Probes Inc. The primary antibodies against CNGA2 and CNGA4 were from Alpha-Diagnostic Int., USA. ECLÔ Plus western blotting detection system was from Amersham Pharmacia. Protease inhibitor cocktail tablets were from Roche. L-*cis*-diltiazem was from Biomol, USA. RPMI-1640, DMEM, FBS, blasticidin, and lipofectamine 2000 were from Invitrogen. LY-83583 was from Calbiochem. Epinephrine, isoprenaline, ICI-118551, Nonidet P-40, sodium deoxycholate, TEA, SDS, EDTA, and Hepes were from Sigma.

3. Results

3.1. Role of CNG channels in epinephrine-induced cation current

Whole-cell voltage clamp was used to study the epinephrine-activated cation current in BAECs. The recorded current (Fig. 1A) and the corresponding current–voltage (I–V) relationship (Fig. 1B) showed that the current had slight outward rectification. Epinephrine treatment (1 μmol/L, 5 min) increased the magnitude of the whole-cell current in both inward (negative) and outward (positive) directions (Figs. 1A and B). It is known that epinephrine, via its action on β-adrenergic receptors, causes an elevation in cytosolic cAMP. Thus, we tested the possible involvement of CNG channels. In cells pretreated with CNG channel blockers, either L-*cis*-diltiazem (100 μmol/L) or LY-83583 (20 μmol/L) [23,24], epinephrine was unable to cause a significant increase in cation current (Figs. 1C and D). These data suggest an involvement of CNG channels in the epinephrine-activated cation current.

Because both L-*cis*-diltiazem and LY-83583 inhibit multiple CNG isoforms, they cannot be used to differentiate specific CNG isoform involved. Thus, we next used siRNA strategy. CNGA2 was chosen as the possible target based on its high sensitivity to cAMP [15] and its expression in vascular endothelial cells [13,22,23]. Because bovine CNGA2 gene is still not cloned and its nucleotide sequence is not available in Genbank, we were unable to design siRNA against bovine CNGA2. Instead, we designed siRNA against mouse CNGA2 and studied its effect in the mouse endothelial cell line H5V. In immunoblot experiments, the CNGA2-specific antibody recognized a protein band with molecular size of ~80 kDa, which correlates well with that of mouse CNGA2 proteins in Genbank (NM_007724) [24]. This antibody was also able to detect the CNGA2 proteins over-expressed in a human bronchial epithelial cell line 16HBE, confirming the specificity of this antibody (Fig. 2A). Stable expression of CNGA2-specific siRNA reduced the CNGA2 protein level by 82±6% (n=5) (Figs. 2A and C). In contrast, transfection with control vector had no effect on the CNGA2 protein level (Fig. 2A). The effect of

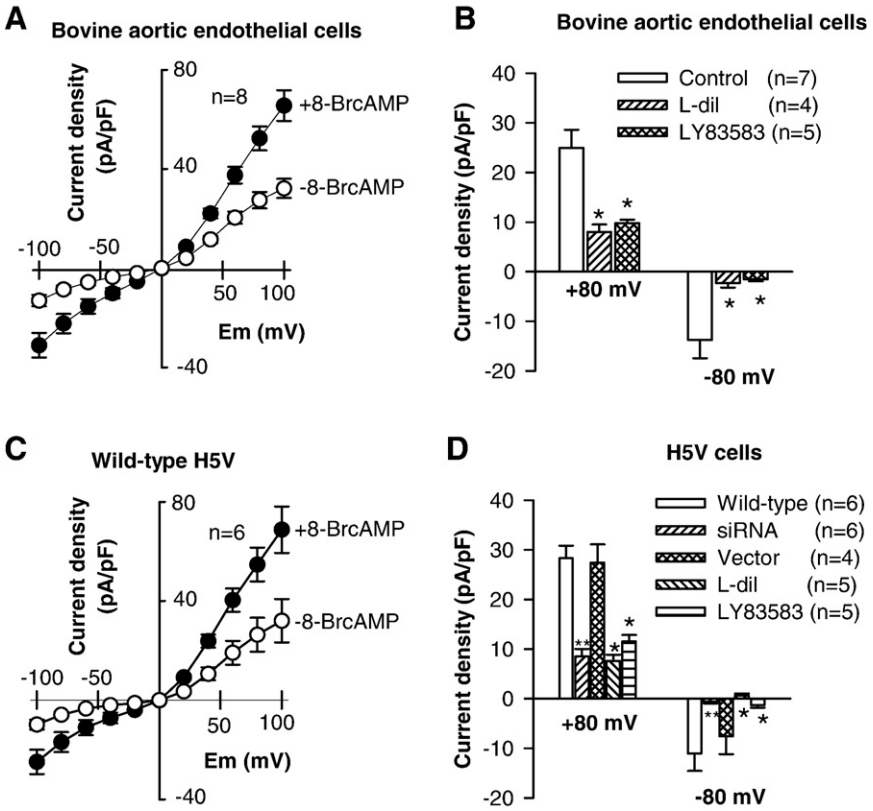

Fig. 3. Effect of CNG channel inhibitors and CNGA2-specific siRNA on cAMP-activated whole-cell cation current. (A and C): current–voltage relationships before and after 8-BrcAMP treatment in BAECs (A) and H5V cells (C). (B and D): summary of data showing the effect of , L-*cis*-diltiazem (100 μmol/L), LY-83583 (20 μmol/L), and CNGA2-specific siRNA on cAMP-activated whole-cell cation current at −80 mV and +80 mV in BAECs (B) and H5V cells (D). The cAMP-activated current was obtained by subtracting the current before 8-BrcAMP treatment from that after the treatment. +8-BrcAMP, with 8-BrcAMP (100 μmol/L for BAECs, 300 μmol/L for H5V cells); −8-BrcAMP, without 8-BrcAMP; L-dil, L-*cis*-diltiazem. Mean ± SEM (n=4–8 independent experiments). *P<0.05 as compared to wild-type cells. **P<0.05 as compared to vector-transfected H5V cells.

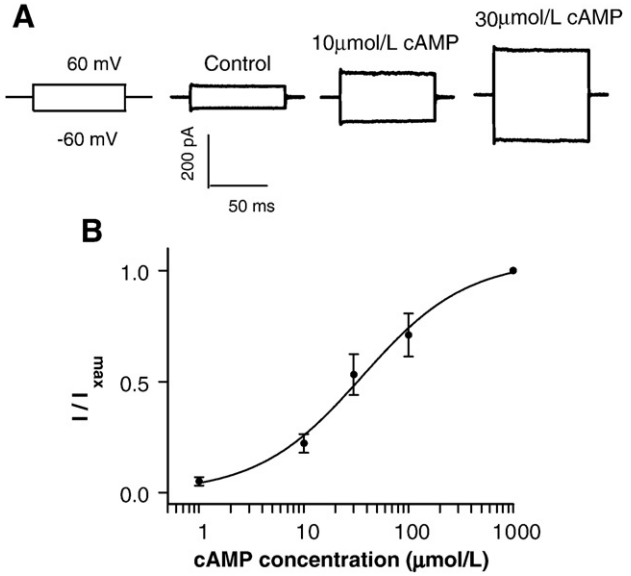

Fig. 4. Dose–response relationship between cAMP concentrations and its induced cation current in excised inside-out patches from BAECs. (A), representative traces showing voltage protocol (left) and the corresponding patch currents before (middle left) and after cAMP (10 μmol/L, middle right; 30 μmol/L, right) application. (B), summary of data showing the relationship between cAMP concentrations and cAMP-induced peak inward currents at −60 mV. The cAMP-induced currents were obtained by subtracting the peak currents in the absence of cAMP from the values in the presence of cAMP. The currents were plotted as the percentage of maximal current in response to 1 mmol/L cAMP. Mean ± SEM (n = 3–4 experiments).

siRNA was CNGA2-specific, because it had no influence on the expression of another CNG isoform CNGA4, which has the molecular size of ~66 kDa (NM_001033317) (Figs. 2B and C). Functionally, the expression of siRNA diminished the epinephrine-activated cation current (Figs. 2D–F), while the control vector had no effect (Fig. 2F). The effect of L-*cis*-diltiazem and LY-83583 was also tested. As expected, both inhibitors diminished the magnitude of epinephrine-activated cation current in both inward and outward directions (Fig. 2F). Taken together, these data strongly suggest that CNGA2 are the main channels responsible for epinephrine-activated cation current in endothelial cells.

3.2. Role of CNG channels in cAMP-activated cation current

CNGA2 channels are cAMP-activated channels. Therefore, we next examined cAMP-activated cation current in endothelial cells. 8-BrcAMP, which is a membrane-permeant analog of cAMP, increased the magnitude of whole-cell cation current in both BAECs (Figs. 3A and B) and H5V cells (Figs. 3C and D). Furthermore, the cAMP-activated cation current was markedly reduced by L-*cis*-diltiazem (100 μmol/L) and LY-83583 (20 μmol/L) (Figs. 3B and D). Importantly, CNGA2-specific siRNA diminished the cAMP-activated cation current in H5V cells (Fig. 3D). These data suggest that CNGA2 are the main channels responsible for cAMP-activated cation current in endothelial cells.

A cAMP-activated current was also recorded in the excised inside-out patches of endothelial cells (Figs. 4A and B). It was found that bath application of cAMP increased the cation currents in the inside-out patches in a dose-dependent manner (Fig. 4B) with EC_{50} at ~36 μmol/L.

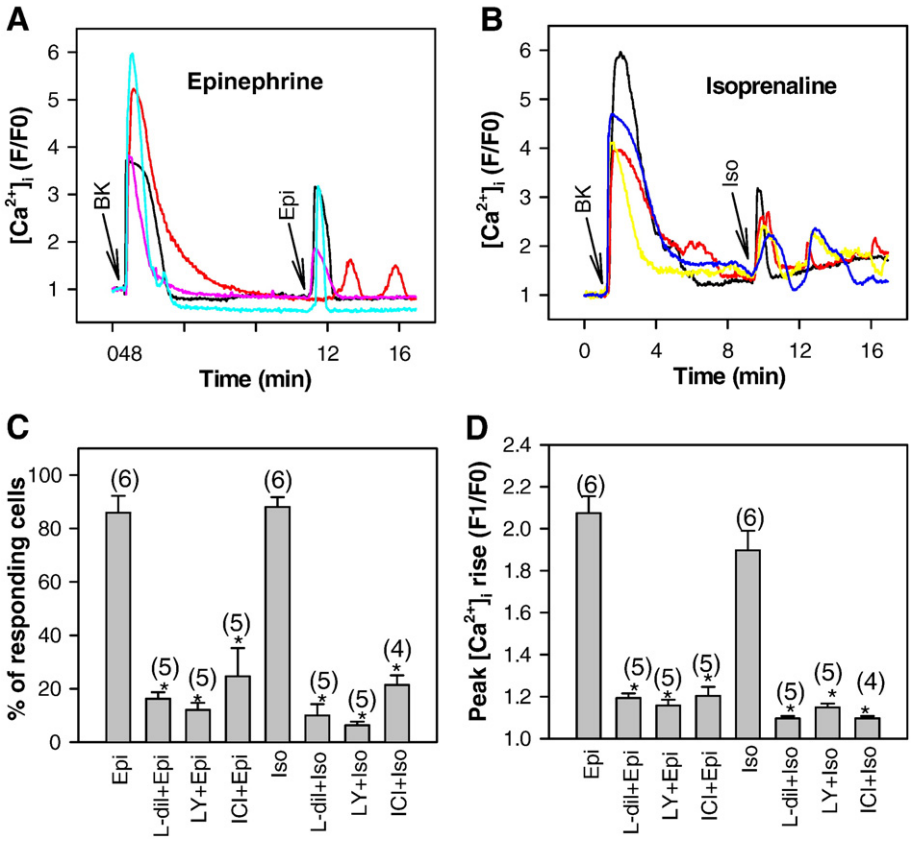

Fig. 5. Effect of CNG channel inhibitors on epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises in BAECs. (A and B), representative traces of the $[Ca^{2+}]_i$ rises in response to epinephrine (Epi, 1 μmol/L) and isoprenaline (Iso, 10 μmol/L) in the presence of bradykinin (BK, 10 nmol/L). (C and D), summary of data showing the effect of inhibitory agents on the percentage of cells displaying epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises (C) and on the magnitude of first $[Ca^{2+}]_i$ transient among the responding cells (D). L-dil, L-*cis*-diltiazem (100 μmol/L); LY, LY-83583 (20 μmol/L); ICI, ICI-118551 (1 μmol/L). Mean ± SEM (n = 4–6 independent experiments, 15 to 40 cells per experiment). *P<0.05 as compared to epinephrine or isoprenaline alone.

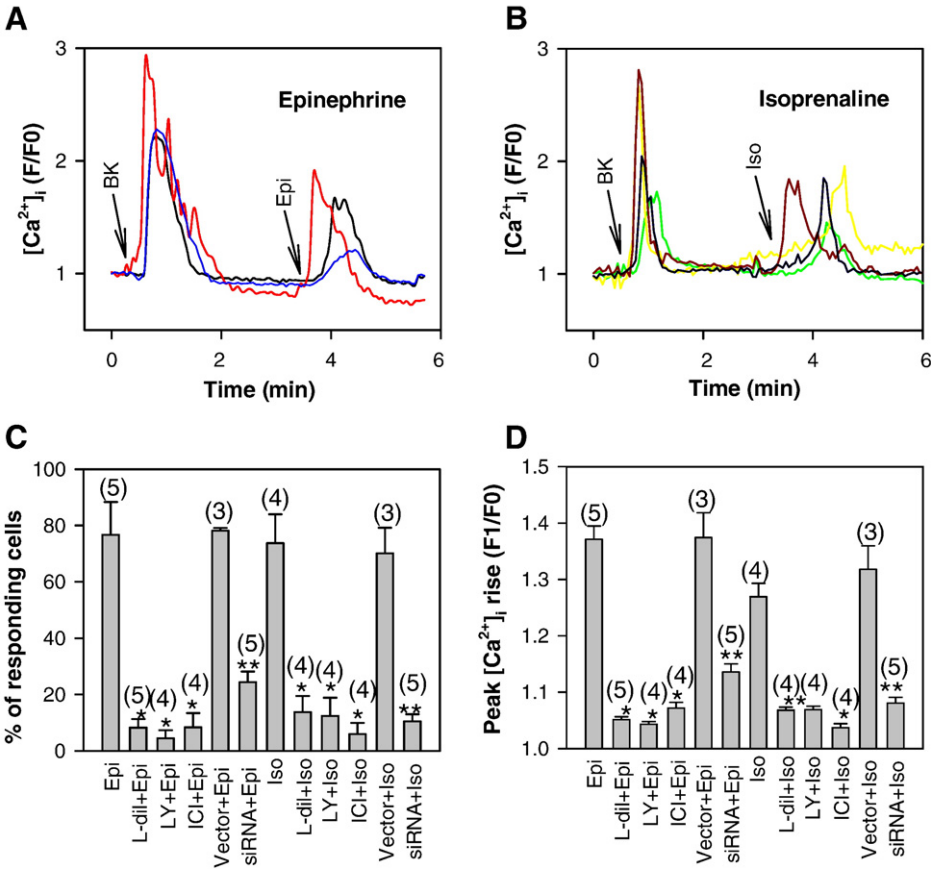

Fig. 6. Effect of CNG channel inhibitors and CNGA2-specific siRNA on epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises in H5V cells. (A and B), representative traces of the $[Ca^{2+}]_i$ rises in response to epinephrine (Epi, 1 μmol/L) and isoprenaline (Iso, 10 μmol/L) in the presence of bradykinin (BK, 10 nmol/L). (C and D), summary of data showing the effect of inhibitors and CNGA2-specific siRNA on the percentage of cells displaying epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises (C) and on the magnitude of first $[Ca^{2+}]_i$ transient among the responding cells (D). L-dil, L-*cis*-diltiazem (100 μmol/L); LY, LY-83583 (20 μmol/L); ICI, ICI-118551 (1 μmol/L); siRNA, CNGA2-specific siRNA. Mean±SEM (n=3–5 independent experiments, 15 to 40 cells per experiment). *P<0.05 as compared to epinephrine or isoprenaline alone. **P<0.05 as compared to vector-transfected H5V cells.

3.3. Role of CNG channels in epinephrine- and isoprenaline-induced Ca^{2+} influx in cultured endothelial cells

We then explored the functional role of CNG channels in endothelial cells. Previous reports showed that epinephrine and isoprenaline were able to elicit a $[Ca^{2+}]_i$ rise in the endothelial cells that were pretreated with Ca^{2+}-mobilizing agonists such as bradykinin or thrombin [10,11]. It is speculated that, in these cases, Ca^{2+}-mobilizing agonists serve to potentiate the epinephrine and isoprenaline-induced $[Ca^{2+}]_i$ responses [10,11]. Here, we explored the possibility of whether epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises are mediated by CNG channels. BAECs were challenged with epinephrine (1 μmol/L) or isoprenaline (10 μmol/L). In the absence of bradykinin, only ~10–20% of cells responded with a small rise in $[Ca^{2+}]_i$ (data not shown). However, in the presence of bradykinin (10 nmol/L), both epinephrine (1 μmol/L) (Fig. 5A) and isoprenaline (10 μmol/L) (Fig. 5B) were able to elicit a $[Ca^{2+}]_i$ rise in majority of BAECs (86±6%, n=6 experiments). In some cells, this $[Ca^{2+}]_i$ rise displayed an oscillatory pattern, whereas in other cells only a single $[Ca^{2+}]_i$ transient could be observed (Figs. 5A and B). This $[Ca^{2+}]_i$ rise required the presence of extracellular Ca^{2+}, because epinephrine or isoprenaline failed to elicit the $[Ca^{2+}]_i$ rise in cells bathed in 0Ca^{2+}-PSS. Importantly, L-*cis*-diltiazem (100 μmol/L) and LY-83583 (20 μmol/L) markedly reduced the percentage of cells displaying epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises (Fig. 5C), and they also suppressed the magnitude of peak $[Ca^{2+}]_i$ rises among the cells displaying epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises (Fig. 5D). The effect of epinephrine and isoprenaline was likely to be mediated by β_2-adrenoceptors, because ICI-118551 (1 μmol/L), a selective antagonist for β_2-adrenoceptors [1], effectively blocked the $[Ca^{2+}]_i$ rises (Figs. 5C and D).

Experiments were also performed in H5V cells. Similar to BAECs, both epinephrine (1 μmol/L) (Fig. 6A) and isoprenaline (10 μmol/L) (Fig. 6B) induced a $[Ca^{2+}]_i$ rise in H5V cells that were pretreated with bradykinin (10 nmol/L). L-*cis*-diltiazem (100 μmol/L) and LY-83583 (20 μmol/L) markedly diminished epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises (Figs. 6C and D). These $[Ca^{2+}]_i$ rises were inhibited by ICI-118551 (1 μmol/L). Importantly, CNGA2-specific siRNA also suppressed the $[Ca^{2+}]_i$ rises, whereas the control vector had no effect (Figs. 6C and D). We also tested the effect of CNG channels inhibitors and CNGA2-specific siRNA on bradykinin-induced $[Ca^{2+}]_i$ rise, which is known to be mainly resulted from Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores [25]. As expected, L-*cis*-diltiazem, LY-83583 and CNGA2-specific siRNA had no effect on bradykinin (10 nmol/L)-induced $[Ca^{2+}]_i$ rise (Supplemental Fig. 1), Taken together, these data support a key role of CNGA2 channels in epinephrine- and isoprenaline-induced Ca^{2+} influx.

CNGA2 channels are highly permeable to Ca^{2+} [15]. Therefore we also examined the role of CNGA2 in the maintenance of basal $[Ca^{2+}]_i$ level in endothelial cells. As shown in Supplemental Fig. 2, L-*cis*-diltiazem (100 μmol/L) or LY-83583 (20 μmol/L) treatment for 1 h caused a significant reduction in basal $[Ca^{2+}]_i$ level for both BAECs and H5V cells (Supplemental Figs. 2A and B). Furthermore, CNGA2-specific siRNA also decreased the basal $[Ca^{2+}]_i$ level in H5V cells (Supplemental Fig. 2B). These data suggest an important role of CNGA2 in the maintenance of basal $[Ca^{2+}]_i$ level in endothelial cells under normal non-stimulated condition.

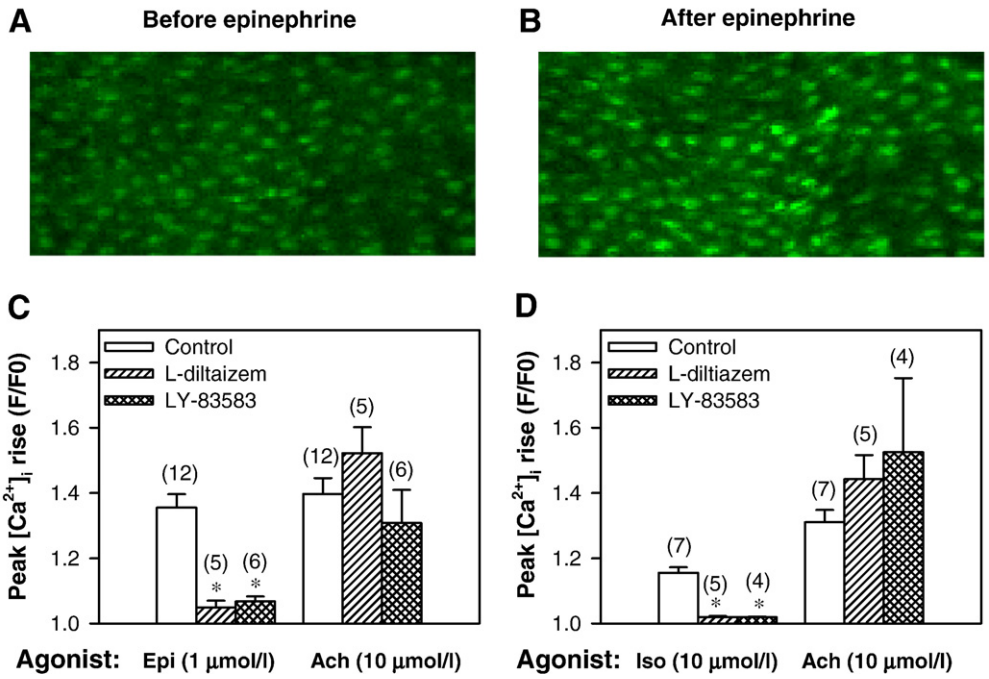

Fig. 7. Effect of CNG channel inhibitors on epinephrine- and isoprenaline-induced endothelial $[Ca^{2+}]_i$ rises within mouse aortic strips. (A and B), representative endothelium fluorescence images before (A) and after (B) 1 μmol/L epinephrine. (C and D), summary of data showing the effect of L-*cis*-diltiazem (100 μmol/L) and LY-83583 (10 μmol/L) on the magnitude of first $[Ca^{2+}]_i$ transient in response to 1 μmol/L epinephrine (Epi, C), 10 μmol/L isoprenaline (Iso, D), and 10 μmol/L acetylcholine (Ach, C and D). Mean±SEM (n=4–12 experiments). *P<0.05 as compared to epinephrine or isoprenaline alone.

Note that, although Ca^{2+} influx was required for epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises, it was apparent that store Ca^{2+} release also contributed to this $[Ca^{2+}]_i$ rise probably via the mechanism of Ca^{2+}-induced store Ca^{2+} release. This was evident because in cells pretreated with thapsigargin (4 μM) for 30 min to deplete intracellular Ca^{2+} stores, the epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ responses became much smaller (Supplemental Fig. 3). Furthermore, in cells after Ca^{2+} store depletion, only a single $[Ca^{2+}]_i$ transient (without $[Ca^{2+}]_i$ oscillation) could be observed in response to epinephrine and isoprenaline.

3.4. Role of CNG channels in epinephrine- and isoprenaline-induced endothelial Ca^{2+} responses in mice aorta

There is a concern that endothelial phenotype may change during cell culture and cell passage conditions. Thus, we studied the $[Ca^{2+}]_i$ changes in endothelial cells within isolated mouse aortic strips to verify the above findings. Application of epinephrine (1 μmol/L) or isoprenaline (10 μmol/L) elicited a transient $[Ca^{2+}]_i$ rise in more than 90% of endothelial cells in the isolated mouse aortic strips (Figs. 7A and B). Surprisingly, unlikely cultured endothelial cells, the endothelial cells within the isolated aortic strips were capable of responding to epinephrine or isoprenaline in the absence of exogenous bradykinin. We speculate that the microenvironment within isolated aortic tissues might already contain sufficient amount of Ca^{2+}-mobilizing agonists, so that exogenous addition of agonists was no longer needed. Importantly, epinephrine- and isoprenaline-induced endothelial $[Ca^{2+}]_i$ rises were almost completely abolished in aortic strips that were pretreated with L-*cis*-diltiazem (100 μmol/L) or LY-83583 (10 μmol/L) for 10 min (Fig. 7C and D). In contrast, L-*cis*-diltiazem or LY-83583 had no effect on acetylcholine-induced peak $[Ca^{2+}]_i$ rise (Fig. 7C and D), which is known to be mainly resulted from store Ca^{2+} release [25].

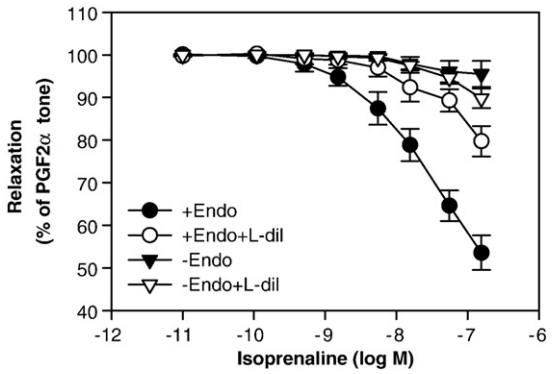

Fig. 8. Effect of L-*cis*-diltiazem on isoprenaline-induced vascular relaxation in mouse aortic segments. Mouse aortic segments were preconstricted with 11-deoxy prostaglandin $F_{2\alpha}$ in the absence or presence of 100 μmol/L L-*cis*-diltiazem. Isoprenaline was then added in a cumulative fashion to the bath to induce vascular relaxation. +Endo, endothelium-intact; −Endo, endothelium-denuded; L-dil, L-*cis*-diltiazem. Mean±SEM (n=5 independent experiments).

3.5. Role of CNG channels in β-adrenoceptor-mediated vascular relaxation

We further examined the functional role of CNG channels in β-adrenoceptor-mediated vascular relaxation. Mouse aortic segments were preconstricted with 11-deoxy prostaglandin $F_{2\alpha}$, and in agreement with the results from other groups [26], isoprenaline induced concentration-dependent vascular relaxation (Fig. 8). The relaxation was mostly endothelium-dependent, because removal of the endothelium almost completely abolished isoprenaline-induced relaxation (Fig. 8). Importantly, L-*cis*-diltiazem (100 μmol/L) markedly inhibited the relaxation to isoprenaline in endothelium-intact aortic rings (Fig. 8), but it had no effect on the residual small relaxation to isoprenaline in endothelium-denuded aortic rings (Fig. 8). These data suggest that CNG channels play a key role in the endothelium-dependent relaxation induced by β-adrenoceptor agonists.

4. Discussion

The major findings of this study are as follows: Firstly, with the use of whole-cell patch clamp, we recorded a current that was activated

by cAMP and epinephrine in the primary cultured BAECs and H5V endothelial cells. We demonstrated that this current was sensitive to selective CNG channel blockers L-*cis*-diltiazem and LY-83583. Furthermore, a CNGA2-specific siRNA diminished this current in H5V cells. These results strongly suggest that epinephrine activates CNG channels, CNGA2 in particular, in vascular endothelial cells. Secondly, we studied the functional role of CNGA2 in endothelial Ca^{2+} influx. We found that in the presence of bradykinin, both epinephrine and isoprenaline, via their action on β_2-adrenoceptors, could stimulate Ca^{2+} influx in cultured endothelial cells. This Ca^{2+} influx was markedly reduced by L-*cis*-diltiazem, LY-83583, and CNGA2-specific siRNA. The role of CNGA2 in epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ responses in endothelial cells were also confirmed using isolated mouse aortic strips. Thirdly, inhibition of CNG channels by L-*cis*-diltiazem reduced the endothelium-dependent vascular dilation to isoprenaline in isolated mouse aortic segments. Taken together, these data provide compelling evidence that CNG channels, CNGA2 in particular, are the channels responsible for epinephrine- and β-adrenoceptor agonist-induced Ca^{2+} influx in endothelial cells and subsequent vascular dilation.

With the use of RT-PCR and immunoblot, several groups have found the expression of several CNG isoforms, including CNGA1, CNGA2, and CNGA4, in vascular endothelial cells [13,14,22,23]. Regarding functional role of CNG channels, Zhang et al. [23] proposed that CNGA2 contributes to the store-operated Ca^{2+} influx in pulmonary artery endothelial cells, whereas Wu et al. [22] suggested that CNGA2 mainly allows Na^+ entry, resulting in membrane depolarization, which reduces the driving force for Ca^{2+} entry. However, in general there is a lack of convincing evidence for the functional role of CNG channels in vascular endothelial cells. In the previous functional studies by Wu et al. and Zhang et al., the primary evidence for the functional involvement of CNG were mainly based on CNG-selective pharmacological blockers L-*cis*-diltiazem and LY-83583. While L-*cis*-diltilzem has good selectivity to CNG channels [20], LY-83853 is not very selective and it also acts on other targets such as guanylate cyclases [21]. Up to the present, there is still no report employing the gene "knocking-down" technique, which is far more selective to its target molecules, to study CNG function in vascular tissue. Furthermore, no report has demonstrated "a cyclic nucleotide-activated current in excised inside-out patches" in endothelial cells, which is considered to be a hallmark of CNG channels. In the present study, immunoblot experiments demonstrated the expression of CNGA2 protein in endothelial cells, and furthermore excised inside-out patch clamp experiments showed a cAMP-activated cation current. In addition, CNGA2-specific siRNA reduced the cAMP-activated cation current. These studies give unequivocal support for an important functional role of CNGA2 channels in vascular endothelial cells.

Previous studies have well demonstrated that stimulation of endothelial β-adrenoceptors results in endothelium-dependent vascular dilation [1,3–7], and that the dilation is mediated by cAMP [1,5–7]. However, there is controversy on whether $[Ca^{2+}]_i$ plays a role in cAMP-induced endothelium-dependent vascular dilation. On the one hand, there is evidence that cAMP can activate eNOS in a Ca^{2+}-independent manner via protein kinase A phosphorylation on Ser-1179 and Ser-635 [8]. On the other hand, Graier et al. provided the evidence that elevated cAMP following the stimulation of β-adrenoceptors elicits a $[Ca^{2+}]_i$ rise in endothelial cells [10]. The rise in endothelial $[Ca^{2+}]_i$ would certainly activate eNOS, which is an enzyme sensitive to Ca^{2+}-calmodulin activation [10]. In the present study, we confirmed that both β-adrenoceptor agonists and cAMP can induce a $[Ca^{2+}]_i$ rise in endothelial cells. Importantly, we were able to identify CNGA2 as the channel mediating β-adrenoceptor agonist-induced endothelial Ca^{2+} influx and vascular dilation. These findings provide a mechanistic support for the involvement of endothelial $[Ca^{2+}]_i$ in β-adrenoceptor-mediated vascular dilation.

Our findings also fit well with a unique property of CNGA2, i.e. its higher sensitivity to cAMP than other CNG isoforms [15]. Interestingly, vascular endothelial cells *in vivo* are also the targets of several other physiologically important cAMP-elevating agents, which include calcitonin gene-related peptide [27], adrenomedullin [27] and adenosine [28]. Like β-adrenoceptor agonists, these cAMP-elevating agents induce endothelium-dependent relaxation via two different mechanisms [27–29]. The first mechanism is well characterized and it involves Ca^{2+}-independent activation eNOS by cAMP and protein kinase A [29]. The second mechanism is Ca^{2+}-sensitive and the underlying mechanism is largely unclear [30]. Recently, we have demonstrated an important role of CNGA2 in adenosine-induced Ca^{2+} influx in endothelial cells [18]. Together with the present data, we hypothesize that the adenylyl cyclase-cAMP-mediated activation of CNGA2 could be a pathway shared by many other cAMP-elevating agents in vascular endothelial cells.

In conclusion, we demonstrate that CNG channels, especially CNGA2, are the main channels responsible for epinephrine- and isoprenaline-induced Ca^{2+} influx in vascular endothelial cells. The effect of epinephrine and isoprenaline is mediated through β_2-adrenoceptors.

Acknowledgments

We thank the financial support of the Hong Kong Research Grant Council (CUHK4526/06M), Focused Investment Scheme of Chinese University of Hong Kong, and Li Ka Shing Institute of Health Sciences.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2008.06.005.

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Supplemental results:

Effect of CNG channel inhibitors and CNGA2-specific siRNA on bradykinin-induced $[Ca^{2+}]_i$ rise in BAECs and H5V cells

In order to verify the specificity of pharmacological blockers and CNGA2-specific siRNA, we examined the possibility of whether L-*cis*-diltiazem, LY-83583, and CNGA2-specific siRNA could have non-specific effect on bradykinin-induced $[Ca^{2+}]_i$ transient. It has been well demonstrated that bradykinin-induced $[Ca^{2+}]_i$ transient is mainly due to Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores [Busse *et al.* 1989]. CNG channels are not expected to be involved in the process. Indeed, we found that the pretreatment with L-*cis*-diltiazem (100 μmol/L) or LY-83583 (20 μmol/L) for 10 min had no effect on the peak amplitude of bradykinin-induced $[Ca^{2+}]_i$ transient in BAECs (supplemental figure IA) and H5V cells (supplemental figure IB). In addition, expression of CNGA2-specific siRNA had no effect on the bradykinin-induced $[Ca^{2+}]_i$ transient in H5V cells (supplemental figure IB). These data gave more support to the specificity of CNG channel inhibitors and CNGA2-siRNA.

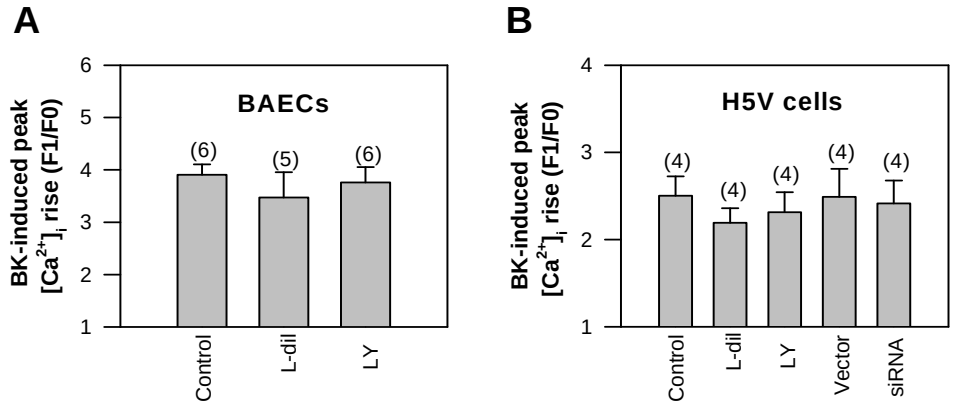
Effect of CNG channel inhibitors and CNGA2-specific siRNA on basal $[Ca^{2+}]_i$ level in BAECs and H5V cells

CNGA2 channels are highly permeable to Ca^{2+} [15]. Therefore we also examined the role of CNGA2 channels in the maintenance of basal $[Ca^{2+}]_i$ level in endothelial cells. To measure basal $[Ca^{2+}]_i$ level, we utilized the Molecular Probes' Calcium Calibration Buffer Kits to calibrate Fura-2 fluorescence ratio (340 nm/380 nm) into free Ca^{2+} concentration according to Molecular Probe's technical instruction. As shown in supplemental figure II, L-*cis*-diltiazem (100 μmol/L) or LY-83583 (20 μmol/L) treatment for 1 hr caused a significant reduction in basal $[Ca^{2+}]_i$ level for both BAECs (supplemental fig. IIA) and H5V cells (supplemental fig. IIB). Furthermore, CNGA2-specific siRNA also decreased the basal $[Ca^{2+}]_i$ level in H5V cells (supplemental fig. IIB). These data suggest an important role of CNGA2 in contributing to basal $[Ca^{2+}]_i$ level in endothelial cells under normal non-stimulated condition. These results are consistent with notion that CNGA2 channels have spontaneous basal activity in the absence of cyclic nucleotides (Keene 2000).

Effect of Ca^{2+} store depletion on epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ responses in BAECs and H5V cells

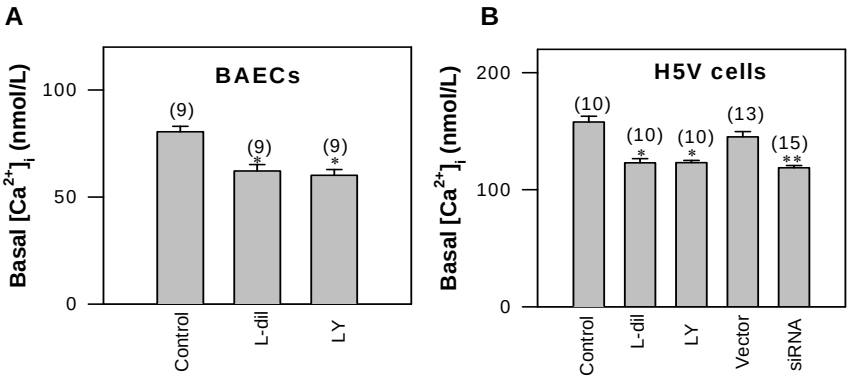
Ca^{2+} influx was required for epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises. In the absence of extracellular Ca^{2+}, epinephrine or isoprenaline failed to elicit the $[Ca^{2+}]_i$ rise. Next we explored the possible contribution of store Ca^{2+} release towards epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ rises. We found that treatment of cells with thapsigargin (4 μM) for 30 min, which depletes intracellular Ca^{2+} stores, caused a marked reduction in epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ responses in BAECs (supplemental fig. IIIA) and H5V cells (supplemental fig. IIIB). Furthermore, in the cells after Ca^{2+} store depletion, only a single $[Ca^{2+}]_i$ transient (without $[Ca^{2+}]_i$ oscillation) could be observed in response to epinephrine and isoprenaline. These data support an important contribution of store Ca^{2+} release towards overall $[Ca^{2+}]_i$ rises in response to epinephrine and isoprenaline.

Supplemental fig. I



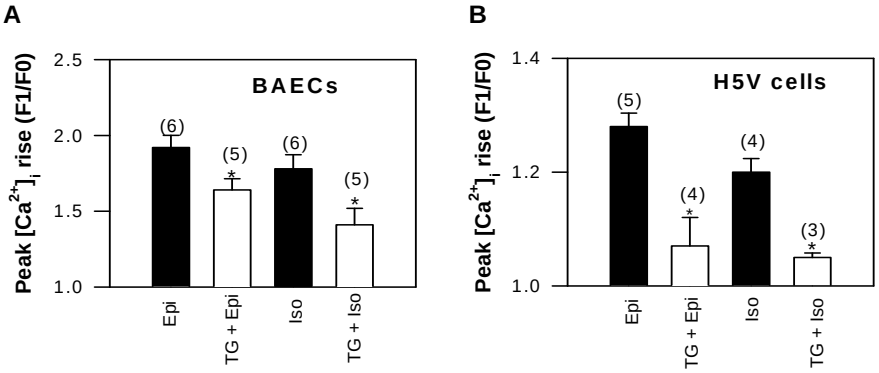
Supplemental fig. I. Effect of CNG channel inhibitors and CNGA2-specific siRNA on bradykinin-induced $[Ca^{2+}]_i$ rise. Summary of data showing the effect of different treatments on bradykinin (10 nmol/L)-induced $[Ca^{2+}]_i$ rise in BAECs (A) and H5V cells (B). Control, non-treated cells; L-dil, 100 μmol/L L-*cis*-diltiazem; LY, 20 μmol/L LY-83583; Vector, vector-transfected H5V cells; siRNA, CNGA2-siRNA-transfected H5V cells. Mean ± SE (*n* = 4-6 experiments, 15 to 40 cells per experiment).

Supplemental fig. II



Supplemental fig. II. Effect of CNG channel inhibitors and CNGA2-specific siRNA on basal $[Ca^{2+}]_i$ level. Summary of data showing the effect of different treatments on basal $[Ca^{2+}]_i$ level in BAECs (A) and H5V cells (B). Control, non-treated cells; L-dil, 100 μmol/L L-*cis*-diltiazem; LY, 20 μmol/L LY-83583; Vector, vector-transfected H5V cells; siRNA, CNGA2-siRNA-transfected H5V cells. Mean ± SE (*n* = 9-15 experiments, 15 to 40 cells per experiment). **P*< 0.05 as compared to control. ***P*< 0.05 as compared to vector-transfected H5V cells.

Supplemental fig. III



Supplemental fig. III. Effect of Ca^{2+} store depletion on epinephrine- and isoprenaline-induced $[Ca^{2+}]_i$ responses. Summary of data showing the effect of 30 min thapsigargin (TG) pretreatment on the magnitude of peak $[Ca^{2+}]_i$ rises in response to epinephrine (Epi, 1 μmol/L) and isoprenaline (Iso, 10 μmol/L) in BAECs (A) and H5V cells (B). 10 nmol/L bradykinin was added before epinephrine or isoprenaline challenge. Mean ± SEM (*n* = 3-6 independent experiments, 15 to 40 cells per experiment). **P*< 0.05 as compared to the cells without TG treatment.

Supplemental references

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