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Buffering Contribution of Mitochondria to the $[Ca^{2+}]_i$ Increase by Ca^{2+} Influx through Background Nonselective Cation Channels in Rabbit Aortic Endothelial Cells

Young Chul Kim, Sang Jin Lee, and Ki Whan Kim[1]

Department of Physiology, Chungbuk National University, College of Medicine, Jeonju, Korea, [1]Department of Physiology and Biophysics, Seoul National University College of Medicine, Seoul 110–799, Korea

To prove the buffering contribution of mitochondria to the increase of intracellular Ca^{2+} level ($[Ca^{2+}]_i$) via background nonselective cation channel (background NSCC), we examined whether inhibition of mitochondria by protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) affects endothelial Ca^{2+} entry and Ca^{2+} buffering in freshly isolated rabbit aortic endothelial cells (RAECs). The ratio of fluorescence by fura-2 AM ($R_{340/380}$) was measured in RAECs. Biological state was checked by application of acetylcholine (ACh) and ACh (10 μM) increased $R_{340/380}$ by 1.1 ± 0.15 (mean ± S.E., n=6). When the external Na^+ was totally replaced by $NMDG^+$, $R_{340/380}$ was increased by 1.19 ± 0.17 in a reversible manner (n=27). $NMDG^+$-induced $[Ca^{2+}]_i$ increase was followed by oscillatory decay after $[Ca^{2+}]_i$ reached the peak level. The increase of $[Ca^{2+}]_i$ by $NMDG^+$ was completely suppressed by replacement with Cs^+. When 1 μM CCCP was applied to bath solution, the ratio of $[Ca^{2+}]_i$ was increased by 0.4 ± 0.06 (n=31). When 1 μM CCCP was used for pretreatment before application of $NMDG^+$, oscillatory decay of $[Ca^{2+}]_i$ by $NMDG^+$ was significantly inhibited compared to the control ($p < 0.05$). In addition, $NMDG^+$-induced increase of $[Ca^{2+}]_i$ was highly enhanced by pretreatment with 2 μM CCCP by 320 ± 93.7%, compared to the control (mean ± S.E., n=12). From these results, it is concluded that mitochondria might have buffering contribution to the $[Ca^{2+}]_i$ increase through regulation of the background NSCC in RAECs.

Key Words: Endothelial cell, Intracellular Ca^{2+} concentration, Nonselective cation channel, Mitochondria

INTRODUCTION

The regulation of endothelial $[Ca^{2+}]_i$ is composed of activating mechanisms that supply Ca^{2+} to the cytoplasm and homeostatic mechanisms that maintain a low level of $[Ca^{2+}]_i$ by removing cytoplasmic free Ca^{2+} after chemical or physical stimulation. The $[Ca^{2+}]_i$ of endothelial cells can be increased by several mechanisms such as Ca^{2+} release from endoplasmic reticulum (ER) through D-*myo*-inositol 1,4,5-trisphosphate [Ins (1,4,5) P_3]-sensitive Ca^{2+} channels activated by plasmalemmal receptors coupled to phospholipase C (PLC) and Ca^{2+}-dependent cation channels which is activated by a rise in $[Ca^{2+}]_i$ (Derian & Moskowitz, 1986; Lambert et al, 1986; Carter & Ogden, 1992; Lesh et al, 1993; Wang et al, 1995). It can also be increased by Ca^{2+} entry across the plasmalemma through nonselective Ca^{2+} leak pathway (Demirel et al, 1993), receptor-operated cation channels (ROCs) (Adams et al, 1989), "capacitative Ca^{2+} entry" (Putney, 1986; Putney, 1990) and stretch-activated channels (SACs) (Lansman et al, 1987).

To date, however, many studies on endothelial cells have been focused to elucidate Ca^{2+} entry pathways compared to Ca^{2+} removal mechanism. Therefore, only a few studies on Ca^{2+} removal in endothelial cells are reported (Goto et al, 1996; Sedova & Blatter, 1996). $[Ca^{2+}]_i$ can be decreased primarily by Ca^{2+}-ATPase located on the plasmalemmal and ER membrane to pump toward the extracellular space (Hagiwara et al, 1983). In addition, the Na^+-Ca^{2+} exchanger (NCX) on the plasmalemmal membrane may also possibly influence Ca^{2+} homeostasis (Li & Breemen, 1995). Since Ca^{2+} removal as well as Ca^{2+} entry are also important, underlying mechanism of Ca^{2+} extrusion and relation between influx and removal of Ca^{2+} should delicately be studied (Skarsgard et al, 2000). As a Ca^{2+} removing apparatus, mitochondria have also been known to regulate intracellular Ca^{2+} content in many types of cells, including endothelial cells (Ichas et al, 1997). Previously, it was assumed that mitochondria could participate only in Ca^{2+} sequestration, when intracellular Ca^{2+} concentrations approached cytotoxic levels (Gunter & Pfeiffer, 1990). However, mitochondria play a functional role as excitable organelles capable of generating and conveying Ca^{2+} signal (Ichas et al, 1997; Ward et al, 2000).

We earlier reported that background NSCC plays a crucial role in the regulation of membrane potential of RAECs (Park et al, 2000). Since the membrane potential of endothelium contributes to the driving force for Ca^{2+} entry

Corresponding to: Ki Whan Kim, Department of Physiology and Biophysics, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-799, Korea. (Tel) 82-2-2072-8223, (Fax) 82-2-763-9667, (E-mail) kimkw@plaza.snu.ac.kr

ABBREVIATIONS: NSCC, nonselective cation channel; RAECs, rabbit aortic endothelial cells; CCCP, carbonylcyanide-m-chlorophenylhydrazone; NCX, Na^+-Ca^{2+} exchanger; CPA, cyclopiazonic acid.

(Busse et al, 1993), further study on the relationship between background NSCC and regulation of $[Ca^{2+}]_i$ was needed. From these reasons, this study was designed to identify background NSCC and mitochondria as a Ca^{2+} regulating pathway and Ca^{2+} removing mechanism in RAECs.

METHODS

Preparation of cells

Rabbits of either sex (1~1.2 kg) were anaesthetized with ear vein injections of pentobarbital (50 mg/kg) and heparin (1,000 u/kg). A 3 cm-long segment of the aorta was isolated and placed in Ca^{2+}-free normal solution (composition given below). The connective tissue and fat layer were carefully removed under a dissecting microscope. Aorta was cut into small segment (3~4 mm) and transferred to 1 ml of Ca^{2+}-free normal solution containing 0.2% collagenase (Worthington type 1). After incubation at 37°C for 15 min in this solution, single endothelial cells were dispersed by gentle agitation of the digested segments with a wide-bore glass pipette. Freshly isolated endothelial cells were kept in KB storage solution at 4°C until use. All experiments were carried out within 12 h of harvesting cells. The freshly isolated rabbit aorta endothelial cells were spherical or oval with a characteristic granular surface.

Solutions and drugs

The normal physiological salt solution (PSS) contained (mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 HEPES (pH=7.4, titrated with NaOH). For high-K^+ solution (120 mM K^+), Na^+ was replaced by the corresponding amount of K^+ (pH=7.4, titrated with KOH). In all experiments recording nonselective cationic currents, the external cations were completely substituted with 160 mM Cs^+, Na^+, Li^+, $NMDG^+$, or 100 mM Ca^{2+} ions. For changing chloride concentration, aspartic acid was used as a substitute for Cl^-. The composition of the K^+ internal solution was (mM): 95 K-aspartate, 45 KCl, 10 HEPES (pH=7.2, titrated with KOH). The composition of the Cs^+ internal solution was (mM): 95 Cs-aspartate, 45 CsCl, 10 HEPES (pH = 7.2, titrated with CsOH), and 140 CsOH, 10 HEPES (pH = 7.2, titrated with CsOH). The NCX inhibitor, 2-[2-[4-(4- nitrobenzyloxy)phenyl]ethyl]isothiourea (KB-R7943), was kindly donated from Kanebo Inc (Japan), and was dissolved in DMSO as a stock solution (10 mM) and diluted in the final experimental solution. The fluorescence indicator fura 2-AM was purchased from Molecular Probes, Inc (USA) and all other chemicals and drugs used in this study were purchased from Sigma Chemical Co. (USA).

Electrophysiological recordings

Nystatin-perforated patch clamp technique was used to measure membrane potentials and membrane currents. The patch pipettes with a resistance of 3~4$M\Omega$ were used to make a gigaseal. pCLAMP v6.0.3, and Digidata-1200 (all from Axon Instruments) were used for the acquisition of data and applying command pulse. Whole-cell currents were recorded with a patch-clamp amplifier (RK-300, Biologic). The Ag/AgCl reference electrode was connected to the bath solution by a 3M KCl agar bridge. The liquid junction potential was corrected. Whole-cell current-voltage (I-V) relations were measured by applying either square or ramp pulses from a holding potential of −30 mV. The step pulse was 500 or 800 msec lasting with a range from −120 to 80 mV and sampled at a rate of 1 kHz. The ramp pulse was ascending from −120 to 80 mV during 3.0 sec and sampled at a rate of 250 Hz. Membrane potentials were continuously recorded in current-clamp mode. The compensation circuit of the amplifier indicated cell capacitance. The slope conductance and reversal potential were determined by calculating a regression line over an appropriate short segment of the I~V curve.

Fura-2 loading and $[Ca^{2+}]_i$ measurement

Isolated single cells were loaded with acetoxymethyl ester form of fura-2 (2 μM diluted from 1 mM stock in dimethyl sulfoxide) in Ca^{2+}-free normal solution for 15 min at room temperature. After then, the cell suspension was briefly centrifuged (800 r.p.m., 2min) and washed twice with Ca^{2+}-free normal solution. Fura-2 loaded cells were stored at 4°C until use. Experiments were done within 8 hours after isolation of cells. And all experiments were done at 32°C for better response from cells, including mitochondria. And to rule out Ca^{2+} release from ER by CCCP and $NMDG^+$, the cells were pretreated with cyclopiazonic acid (CPA, 10 μM) which is known as ER Ca^{2+}-ATPase blocker 15 min before application of CCCP and $NMDG^+$. In this condition, ACh (10 μM) was applied to bath solution repetitively to deplete Ca^{2+} from ER during 15 min pretreatment with CPA (10 μM). After confirming depletion of ER, CCCP and $NMDG^+$ were applied to bath solution in the presence of CPA.

The recording of $[Ca^{2+}]_i$ was performed with a microfluorometric system consisting of an inverted fluorescence microscope (Diaphot 300, Nikon, Japan) with a dry-type fluorescence objective lens (X40, NA0.85), a photomultiplier tube (type R 1527, Hamamatsu, Japan) and PTI-Deltascan illuminator (Photon Technology International Inc. USA). One drop of cell suspension was placed on a superfusion chamber (100 μl). Cells were allowed to settle down, thereafter, superfused at a constant flow of 2 ml/min. Light was provided by a 75 W xenon lamp (Ushino, Japan) and, to control excitation frequency, a chopper wheel alternated the light path to monochromators (340 and 380 nm) with a frequency of 5 or 10 Hz. A short-pass dichroic mirror passed emission light of < 570 nm onto the photomultiplier tube, and intensity at 510 nm was measured. A mechanical image mask was placed in the emission path, thus limiting measurement to a single cell. Both data acquisition and control of light application were done by using a computer software (Felix v. 1.1, PTI). Because of uncertainties in calibrating the fura-2 signals in intact cells, no attempt was made to calibrate $[Ca^{2+}]_i$, and all results were instead reported as changes in the 340 nm/380 nm signal ratio.

Statistics

Data are expressed as means±S.E.M., n=the number of cells tested.

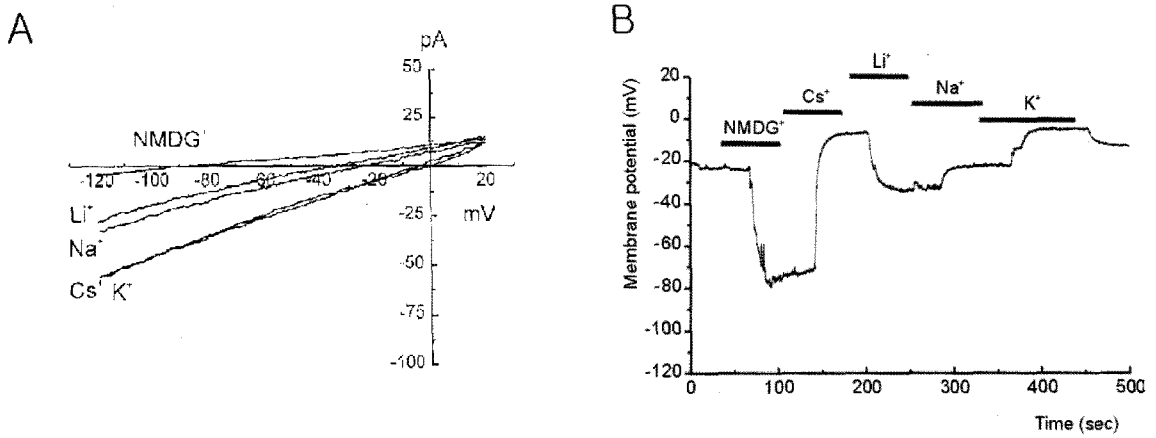

Fig. 1. Background nonselective cationic current in rabbit aortic endothelial cells (RAECs). The pipette solution contained 140 mM Cs^+ to suppress K^+ current. Ba^{2+} (100 mM) was applied to the bath to block the Ba^{2+}-sensitive K^+ current, when the K^+ conductance was examined. Averaged I-V curve which was obtained from the same cells is shown in A. B, Changes in resting membrane potential induced by totally substituting extracellular cations with $NMDG^+$, Cs^+, Li^+, Na^+ and K^+.

RESULTS

Background nonselective cationic current in rabbit aortac endothelial cell (RAEC)

In our previous study, we recorded background nonselective cationic current by total replacement of extracellular cations (Park et al, 2000). The intracellular solution contained 140 mM Cs^+ to suppress K^+ current. Ba^{2+} (100 mM) was applied to the bath to block the possible Ba^{2+}-sensitive K^+ currents, when the K^+ conductance was examined. Producing the inward currents in response to the ramp pulses (Fig. 1A) were 160 mM Li^+, Na^+, Cs^+ or K^+. The currents in each cation solution were compared with the current recorded in 160 mM $NMDG^+$ solution. The reversal potentials of each cationic current were -32.7 ± 0.8 (Li^+, n=17), -22.8 ± 0.6 (Na^+, n=17), -2.8 ± 0.8 (Cs^+, n=17), and 0.8 ± 0.6 mV (K^+, n=17). To confirm the role of the cation conductance for the regulation of resting membrane potentials, membrane potentials were recorded in a current-clamp mode. During the recording of membrane potential, extracellular cations were entirely replaced by each ion. $NMDG^+$, Li^+, Na^+, Cs^+ and K^+ (160 mM each) sequentially depolarized the membrane potential (Fig. 1B). Resting membrane potential (RMP) was -23.3 ± 1.1 mV (n=19). And membrane potential was hyperpolarized and depolarized by total replacement of $NMDG^+$ and Cs^+ for Na to -74 ± 2.9 mV and -5 ± 1.7 mV, respectively (n=3).

Ca^{2+} entry through regulation of background NSCC in RAECs

The contribution of background NSCC to the change of $[Ca^{2+}]_i$ was studied using photometry system. The fluorescence ratio of fura-2 AM ($R_{340/380}$) was measured in RAECs. In endothelium, Ca^{2+} plays an essential role in the release of biological substance such as NO, and ACh is well known agonist which provokes increase of $[Ca^{2+}]_i$ from ER in RAECs (Mayer et al, 1989; Wang et al, 1995). Therefore, the effect of acetylcholine (ACh) was studied to check the

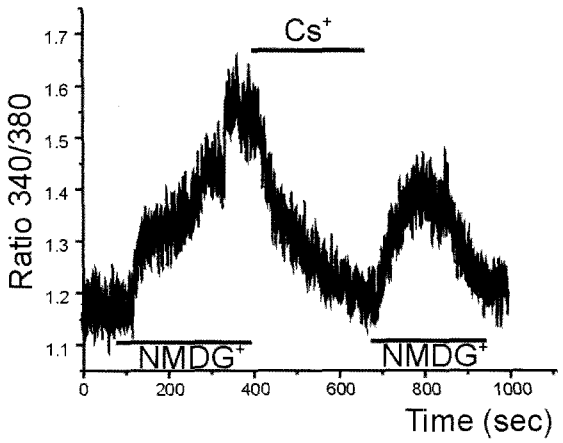

Fig. 2. The role of background NSCC in the change of $[Ca^{2+}]_i$ in RAECs. When the external Na^+ was totally replaced by $NMDG^+$, $R_{340/380}$ was increased by 1.19 ± 0.17 (n=27). And $NMDG^+$-induced increase of $[Ca^{2+}]_i$ was suppressed by substituting Cs^+ for $NMDG^+$.

functional biological state of RAECs. Although not shown, the ratio of $[Ca^{2+}]_i$ ($R_{340/380}$) was increased by 1.1 ± 0.15 (mean$\pm$S.E., n=6), when 10 μM ACh was applied to bath solution. In next step, the change in $[Ca^{2+}]_i$ by regulation of background NSC channels was studied. When the external Na^+ was totally replaced by $NMDG^+$, $R_{340/380}$ was increased in a reversible manner by 1.19 ± 0.17 (n=27). $NMDG^+$-induced increase in $[Ca^{2+}]_i$ was followed by slow oscillatory decay after $[Ca^{2+}]_i$ reached peak level. $NMDG^+$-induced increase in $[Ca^{2+}]_i$ was suppressed by total replacement of $NMDG^+$ by Cs^+ in a reversible manner (1.1 ± 0.15 of the maximum ratio by $NMDG^+$ replacement, Fig. 2, n=6). Although not shown, possible involvement of NCX in the increase of $[Ca^{2+}]_i$ during $NMDG^+$ replacement was studied using KB-R7943. KB-R7943 (10 μM) did not produce any significant change of $R_{340/380}$ in PSS ($p>0.05$). And $NMDG^+$

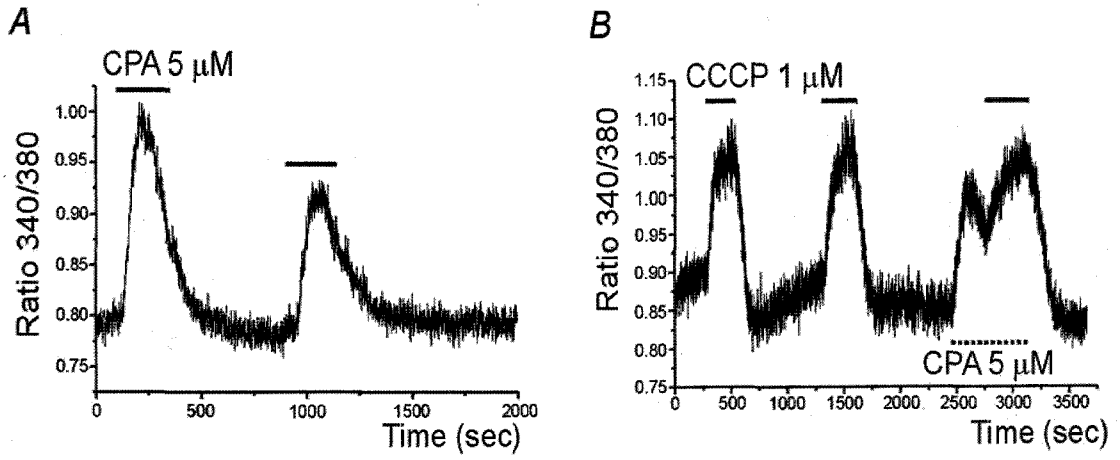

Fig. 3. The effects of CPA and CCCP on the change in $[Ca^{2+}]_i$ of RAECs. (A) CPA (5 μM) increased $R_{340/380}$ by 0.3±0.05 in a reversible manner (n=7). (B) When 1 μM CCCP was applied to bath solution, $R_{340/380}$ was increased by 0.4±0.06 (n=31) in a reversible manner. Pretreatment with 5 μM CPA did not affect the CCCP- induced $[Ca^{2+}]_i$ increase.

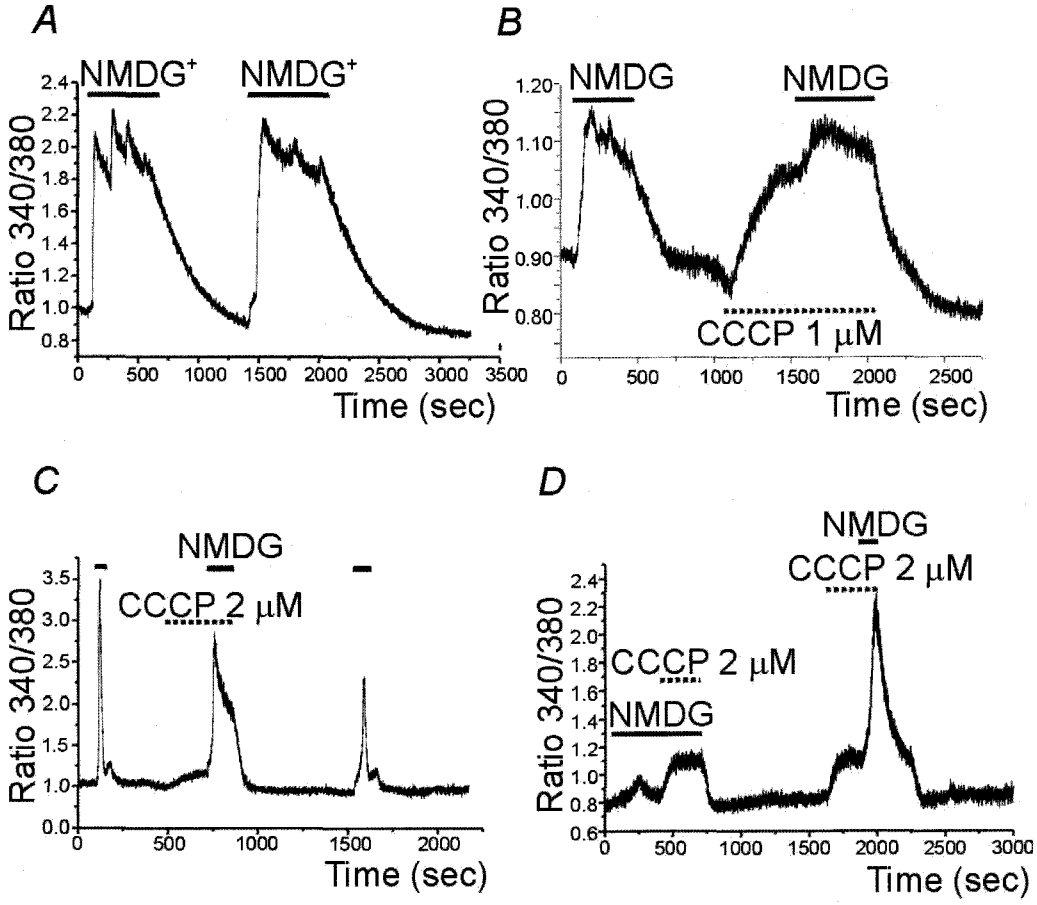

Fig. 4. The role of mitochondria in the regulation of $[Ca^{2+}]_i$ increase through background NSCC in RAECs. (A) Total replacement of extracellular Na^+ by $NMDG^+$ increased $[Ca^{2+}]_i$ which was followed by repetitive oscillatory decay. (B) The characteristic oscillatory decay was significantly suppressed by the pretreatment with 1 μM CCCP before the application of $NMDG^+$ ($p<0.05$). (C) In the case of transient $[Ca^{2+}]_i$ increase by $NMDG^+$, the pretreatment with 2 μM CCCP significantly reduced pattern of the $[Ca^{2+}]_i$ decay after it reached peak level. (D) The pretreatment with 2 μM CCCP highly enhanced the $NMDG^+$-induced $[Ca^{2+}]_i$ increase to 320±93.7%, compared to control (n=12).

replacement increased $R_{340/380}$ by 0.8 ± 0.1 and 0.75 ± 0.1 in the absence and presence of KB-R7943 in RAECs, respectively (n=3, data not shown, $p>0.05$). Since there was no significant difference between the two groups tested ($p>0.05$), NCX in this condition seemed not to significantly participate in the regulation of $[Ca^{2+}]_i$ in RAECs. Increasing effect of $NMDG^+$ on the regulation of $[Ca^{2+}]_i$ was dependent on the external concentration of Ca^{2+}. The increase in $[Ca^{2+}]_i$ by $NMDG^+$ was not observed in the absence of external Ca^{2+} (data not shown, n=3).

The roles of mitochondria and endoplasmic reticulum in the regulation of $[Ca^{2+}]_i$ in RAECs

The effect of protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) in the regulation of $[Ca^{2+}]_i$ was studied. Application of CCCP (1 μM) to bath solution increased $R_{340/380}$ by 0.4 ± 0.06 in a reversible manner (n=31, Fig. 3B). Since intracellular Ca^{2+} store in freshly isolated RAECs has already been reported, the change of mitochondrial $[Ca^{2+}]_i$ by CCCP was monitored using CPA. As shown in Fig. 3A, application of CPA (5 μM) increased $[Ca^{2+}]_i$ repetitively in a reversible manner. CPA (5 μM) increased ratio of $[Ca^{2+}]_i$ by 0.3 ± 0.05 (n=7). In Fig. 3B, possible contribution of mitochondria to buffer increased level of $[Ca^{2+}]_i$ from SR was studied. For this study, we focused to study whether the effect of CCCP on $[Ca^{2+}]_i$ in the presence of CPA was significantly enhanced, compared to that of CCCP. Therefore, CCCP was applied to bath solution during the recording of peak level of increase in $[Ca^{2+}]_i$ by application of CPA. As shown in Fig. 3B, repetitive application of CCCP increased $[Ca^{2+}]_i$. However, the effect of CCCP during the application of CPA did not change, compared to control ($p>0.05$), implying that increase of intracellular Ca^{2+} from ER by blocking Ca^{2+}-ATPase may not be captured as Ca^{2+} clearance by mitochondria in RAECs.

The buffering contribution of mitochondria to the increase of $[Ca^{2+}]_i$ through regulation of background NSCC

In RAECs, background NSCC is a major determinant for resting membrane potential and Ca^{2+} entry is also very dependent on driving force through these channels (Park et al, 2000). Therefore, next step of study was designed to verify whether mitochondria functionally regulate $[Ca^{2+}]_i$ which was increased via regulation of this channel. As shown in Fig. 4A, total replacement of $NMDG^+$ repetitively produced increase of $[Ca^{2+}]_i$ which was followed by oscillatory decay. Although not shown, the effect of CPA on $NMDG^+$-induced rise in $[Ca^{2+}]_i$ was studied to rule out involvement of $[Ca^{2+}]_i$ increase from ER during application of $NMDG^+$. ACh (10 μM) was repetitively applied to bath solution to deplete Ca^{2+} from ER during 15 min pretreatment with CPA (10 μM). CPA increased $R_{340/380}$ by 0.2 ± 0.06 (n=3). In the presence of CPA, second application of ACh (10 μM) produced negligible $[Ca^{2+}]_i$ increase by 0.08 ± 0.25 (n=3). In this condition, however, $NMDG^+$-induced rise in $[Ca^{2+}]_i$ was not affected and $R_{340/380}$ was increased by 0.75 ± 0.1 in the presence of CPA (n=3, $p>0.05$). $NMDG^+$- induced increase of $[Ca^{2+}]_i$ and following oscillatory decay were further studied using pretreatment of CCCP. This phenomenon was significantly suppressed by the pretreatment with CCCP (1 μM) before the application of $NMDG^+$ (Fig. 4B). $NMDG^+$-induced $[Ca^{2+}]_i$ increase was normalized against peak value of $[Ca^{2+}]_i$ and compared. In the absence and presence of CCCP (1 μM), normalized $R_{340/380}$ at 500 sec after $NMDG^+$ replacement were 0.6 ± 0.06 and 0.9 ± 0.03, respectively (n=6, $p<0.05$). In some cases, transient Ca^{2+} increase by $NMDG^+$ replacement was recorded as shown in Fig. 4C (n=4). In this case, decay of Ca^{2+} after reaching peak level by $NMDG^+$ replacement was changed to slow decay pattern by CCCP. In the absence and presence of CCCP (2 μM), normalized $R_{340/380}$ at 300 sec after $NMDG^+$ replacement were 0.07 0.02 and 0.6 0.05, respectively (n=4, $p<0.05$). And, further $NMDG^+$-induced increase of $[Ca^{2+}]_i$ was also highly enhanced to 320 ± 93.7 % by the pretreatment with 2 μM CCCP, compared to control (n=12, Fig. 4D).

DISCUSSION

Based on the results in this study, we suggest that mitochondria play an important role in the clearance of $[Ca^{2+}]_i$ in RAECs. Particularly, this is the first report that mitochondria had functional link for buffering $[Ca^{2+}]_i$ which was increased via regulation of background NSCC in resting RAECs.

In endothelial cells, rise in $[Ca^{2+}]_i$ is a critical event in the regulation of cell function, including many Ca^{2+}-dependent processes for producing and releasing biological substances (Busse et al, 1993). Owing to the absence of voltage-dependent calcium channel (VDCC) and the key role of the membrane potential in the Ca^{2+} entry in endothelial cells, passive Ca^{2+} entry pathways have been considered as an important Ca^{2+} source. In our previous study, we suggested that background NSCC plays an important role in the regulation of membrane potential in RAEC (Park et al, 2000). The conductance of background NSCC in RAECs also showed Ca^{2+} permeable characteristic. In addition, the change of this conductance was very well associated with membrane potential changes which are dependent on cation permeabilities ($P_K : P_{Cs} : P_{Na} : P_{Li}$=1 : 0.87 : 0.40 : 0.27). As we suggested in results, RMP was -23.3 ± 1.1 in normal condition, but it was hyperpolarized to -74 ± 2.9 mV by total replacement of Na^+ by $NMDG^+$. That implies more cation permeability and more depolarization in RAECs. From this aspect, changes of membrane potential by the regulation of background NSCC can be a candidate for the driving force of Ca^{2+} influx in RAEC. This hypothesis was verified by ion replacing experiments under the recording of $[Ca^{2+}]_i$ in RAEC using photometry system. As shown in Fig. 2, $[Ca^{2+}]_i$ in RAEC was increased by $NMDG^+$ replacement for Na^+. That implies that driving force via background NSCC might be one of important factors for influx of Ca^{2+} in RAECs. Although we suggested that Ca^{2+} increase was produced by regulation of background NSCC in RAECs, there is a possibility that NCX and ER might be involved in $[Ca^{2+}]_i$ increase by Na^+ removal (Li & Breemen, 1995; Wang et al, 1995). Therefore, we studied these possibilities using KB-R7943 (10 μM) and CPA (10 μM) as described in results. However, we could not find significant difference in KB-R7943 (10 μM) and CPA (10 μM) treated groups compared to control ($p>0.05$). Therefore, these findings suggest that increase of $[Ca^{2+}]_i$ by $NMDG^+$ replacement in RAECs might be the result from regulation of membrane potential via background NSCC, but not from regulation of NCX and ER.

And, for $[Ca^{2+}]_i$ homeostasis and production of biological substances, regulation of RMP and related resting level of

$[Ca^{2+}]_i$ in RAECs should be considered important. Therefore, relation between regulation of background NSCC and biological substance should be studied in the future. Similar to the effect of hyperpolarization by $NMDG^+$ replacement on $[Ca^{2+}]_i$ in RAEC, agonists such as ACh and ATP in endothelial cells are well known to increase $[Ca^{2+}]_i$ from hyperpolarization combined activation of outward current (Busse et al, 1988, Suh et al, 1999). In RAECs, ACh also increased $[Ca^{2+}]_i$, but this study was focused on $[NMDG^+]_{ext}$-induced increase of $[Ca^{2+}]_i$ related to background NSCC. Therefore, mechanism of regulation of $[Ca^{2+}]_i$ by ACh was not studied here.

Isolated mitochondria accumulate Ca^{2+} via Ca^{2+} uniporters that are driven by the large (−150 to −180 mV) negative potential across their inner membrane (Gunter & Pfeiffer, 1990). Protonophore such as CCCP, a mitochondrial uncoupler that abolishes mitochondrial Ca^{2+} uptake, dissipates the mitochondrial Ca^{2+} gradient by releasing accumulated Ca^{2+} and stopping further accumulation. In Fig. 3B, CCCP repetitively produced rise of $[Ca^{2+}]_i$, but there was a possibility that Ca^{2+} might have come from ER, since Ca^{2+} release through intracellular Ca^{2+} store (SR) has already been established in cultured and freshly isolated endothelial cells (Lambert et al, 1986; Carter & Ogden, 1992; Wang et al, 1995). To rule out possible Ca^{2+} release from ER, the effect of CCCP on $[Ca^{2+}]_i$ was also studied in RAECs pretreated with CPA for 15 min (data not shown). CPA (10 μM) increased $R_{340/380}$ by 0.2 ± 0.06, and ACh (10 μM) was repetitively applied to deplete Ca^{2+} from ER. In the presence of CPA, second application of ACh (10 mM) negligibly increased $[Ca^{2+}]_i$ by 0.09 ± 0.03 (n=3). In this condition, additive application of CCCP in the presence of CPA increased $[Ca^{2+}]_i$ by 0.4 ± 0.05 (n=3). This means that the source of $[Ca^{2+}]_i$ increased by CCCP might be mitochondria, but not ER. Therefore, we suggest that some fractions of Ca^{2+} in RAECs are already captured by mitochondria for the Ca^{2+} buffering in normal condition. For their wide distribution and large volume, mitochondria have already been suggested as a Ca^{2+} buffering system located just beneath the membrane (Budd et al, 1996; Herrington et al, 1996; Babcock et al, 1997). In addition to pathophysiological role, physiological participation of Ca^{2+} uptake was also reported in mitochondria (Miyata et al, 1991; Rizzuto et al, 1992; Werth & Thayer, 1994). Especially, Ca^{2+} decay after Ca^{2+} intrusion in rat adrenal chromaffin cells was markedly delayed by CCCP (Herrington et al, 1996). In ferret ventricular muscles, mitochondria were also regarded as a Ca^{2+} removal system, when $[Ca^{2+}]_i$ was increased in a slower time course (Tanaka & Kurihara, 1997). As shown in the results, we also found similar Ca^{2+} buffering function of mitochondria using CCCP(Fig. 4B, C & D), suggesting that mitochondrial Ca^{2+} uptake might play an important role in Ca^{2+} removal in RAECs.

In this study, protonophore such as CCCP was used for the study of functional role of mitochondria. There is another protonophore, carbonylcyanide p-trifluoromethoxyphenyl hydrazone (FCCP). However, FCCP is known to activate ionic currents and depolarization, which are strongly dependent on the plasmalemmal proton gradient and are likely to be mediated by both H^+ and Na^+ currents across the plasma membrane, as reported in bovine endothelial cells (Park KS et al, 2002). Protonophores-induced Ca^{2+} influx through VDCC has also been reported. However, endothelial cells do not express VDCC. There might be a possibility that CCCP activates some inward current, similar to the report with bovine endothelial cells. If CCCP activated some inward currents, that will produce membrane depolarization and reduce $[Ca^{2+}]_i$ increase. However, CCCP increased basal $[Ca^{2+}]_i$ and enhanced $NMDG^+$-induced $[Ca^{2+}]_i$ increase in RAECs. In addition, CCCP did not induce ionic currents such as NSCC in RAECs (data not shown, n=2). Therefore, the effects of CCCP in RAECs are very likely due to the release of mitochondrial $[Ca^{2+}]_i$.

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