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Store-operated Calcium Entry in Vascular Endothelial Cells Is Inhibited by cGMP via a Protein Kinase G-dependent Mechanism*

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Store-operated Ca^{2+} entry in vascular endothelial cells not only serves to refill the intracellular Ca^{2+} stores, but also acts to stimulate the synthesis of nitric oxide, a key vasodilatory factor. In this study, we examined the role of cGMP in regulating the store-operated Ca^{2+} entry in aortic endothelial cells. Cyclopiazonic acid (CPA) and thapsigargin, two selective inhibitors of endoplasmic reticulum Ca^{2+}-ATPase, were used to induce store-operated Ca^{2+} entry. 8-Bromo-cGMP, an activator of protein kinase G, inhibited the CPA- or thapsigargin-induced Ca^{2+} entry in a concentration-dependent manner. An inhibitor of protein kinase G, KT5823 (1 μM) or H-8 (10 μM), abolished the inhibitory action of 8-bromo-cGMP and resumed Ca^{2+} entry. Addition of *S*-nitroso-*N*-acetylpenicillamine (a nitric oxide donor) or dipyridamole (a cGMP phosphodiesterase inhibitor) during CPA treatment elevated cellular cGMP levels, stimulated protein kinase G activity, and at the same time reduced Ca^{2+} influx due to CPA. Patch clamp study confirmed the existence of a CPA-activated Ca^{2+}-permeable channel sensitive to cGMP inhibition. These results suggest that cGMP via a protein kinase G-dependent mechanism may play a key role in the regulation of the store-operated Ca^{2+} entry in vascular endothelial cells.

Ca^{2+} influx in nonexcitable cells regulates such diverse processes as gene regulation, contraction, exocytosis, and apoptosis. In these cells, the predominant Ca^{2+} entry pathway is the store-operated one (1), in which Ca^{2+} entry is governed by Ca^{2+} content of intracellular Ca^{2+} stores. Release of Ca^{2+} from intracellular stores causes store depletion, which then activates Ca^{2+} entry from extracellular space. This Ca^{2+} entry, which has been termed the store-operated Ca^{2+} entry, may be the basis by which these cells maintain elevated $[Ca^{2+}]_i$ [1] and replenish their intracellular Ca^{2+} stores in response to agonist stimulation (1). The importance of this signaling pathway has been recognized in numerous investigations and has received a great deal of attention (1). However, up to now, the mechanism by which this Ca^{2+} entry pathway is regulated has still been poorly understood.

At least in some cell types, activation of store-operated Ca^{2+} entry appears to involve a soluble messenger that is generated at the endoplasmic reticulum and that is capable of modulating plasma membrane Ca^{2+} permeability. A putative mediator termed the Ca^{2+} influx factor has been identified in Jurkat T lymphocytes (2). Store-operated Ca^{2+} entry may also be regulated by several other factors, including small molecular weight G proteins (3, 4), tyrosine kinase (5), and tyrosine phosphatase (6).

There is a controversy as to whether cGMP plays a key regulatory role in store-operated Ca^{2+} entry. Several research groups proposed that cGMP could activate the store-operated Ca^{2+} entry in pancreatic acinar cells (7, 8) and colonic epithelial cells (9). However, subsequent studies by Putney and coworkers (10, 11) failed to observe any effect of cGMP on thapsigargin-evoked Ca^{2+} influx in both pancreatic acinar cells and Jurkat T cells. Similarly, in *Xenopus* oocytes and rat basophilic leukemia cells, cGMP had no effect on store-operated Ca^{2+} influx (4, 12, 13).

Vascular endothelial cells *in vivo* form an interface between flowing blood and vascular tissue, responding to numerous humoral and physical stimuli to secrete relaxing and contracting factors, which modulate the contractility of vascular smooth muscle cells. In many cases, the initial response of endothelial cells to these diverse signals involves Ca^{2+} release from intracellular stores (14). Like in other nonexcitable cells, depletion of intracellular stores activates Ca^{2+} entry in vascular endothelial cells (15–18). An increase in intracellular Ca^{2+} may then elevate cellular cGMP levels in endothelial cells (19–21). However, it is not known whether this elevation in cellular cGMP may in any way influence the store-activated Ca^{2+} entry in vascular endothelial cells.

This study was performed to assess the role of cGMP and protein kinase G (PKG) in the regulation of the store-operated Ca^{2+} entry in vascular endothelial cells. We found that an elevated cGMP level attenuated the store-operated Ca^{2+} entry and that this inhibitory effect might be mediated by a PKG-dependent mechanism. These results suggest that cGMP and PKG may play a key role in the regulation of the store-operated Ca^{2+} entry in vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—8-Br-cGMP, 8-Br-cAMP, SKF-96365, KT5823, H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide), thapsigargin, and cyclopiazonic acid (CPA) were obtained from Calbiochem. Primary antibody against von Willebrand factor and fluorescein isothiocyanate-labeled secondary antibody were from Dako (Glostrup, Denmark). RPMI 1640 medium and fetal bovine serum were supplied by Life Technologies, Inc. Fluo3/AM and pluronic F-127 were obtained from Molecular Probes, Inc. (Eugene, OR). The ^{125}I-cGMP assay kit was from Amersham Pharmacia Biotech (Bucks, United Kingdom). The protein assay kit, EGTA, EDTA, trypsin, $NiCl_2$, and collagenase were purchased from Sigma.

Preparation and Culture of Aortic Endothelial Cells—Primary aortic endothelial cells were isolated from rat aorta and cultured as described elsewhere (22). Briefly, male Harlan Sprague-Dawley rats were decapitated. The thoracic aorta was removed and washed twice in sterile

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[1] The abbreviations used are: $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; PKG, protein kinase G; Br, bromo; CPA, cyclopiazonic acid; PBS, phosphate-buffered saline; N-PSS, normal physiological saline solution; $0Ca^{2+}$-PSS, calcium-free physiological saline solution; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

PBS. Fat and connective tissues were then trimmed off. The aorta was cut into small sheets and treated with 0.2% collagenase in PBS for 15 min at 37 °C. The suspension after the enzyme digestion was centrifuged at 800 × *g* for 5 min. The cells were then cultured in 90% RPMI 1640 medium and 10% fetal bovine serum and incubated in T-25 tissue culture flasks in air with 5% CO_2 atmosphere at 37 °C. Confluent cell monolayers were passaged using 0.25% trypsin containing 2.5 mM EDTA. Only cells from the first two passages were used for experiments.

Immunofluorescence—The identity of the primary cultured rat aortic endothelial cells was confirmed by immunostaining using an antibody against von Willebrand factor. The cultured cells were fixed in 4% formaldehyde in PBS for 2 h and were blocked with 1% bovine serum albumin in PBS for 30 min. The cells were then stained with a polyclonal antibody against human von Willebrand factor (diluted 1:400 in PBS with 1% bovine serum albumin) overnight at 4 °C. The slides were washed in PBS and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG for 1 h. For controls, some slides were incubated in 1% bovine serum albumin in PBS without the primary antibody. Some slides were counterstained with 0.00003% 4,6-diamidino-2-phenylindole in 0.9% NaCl. After washing in PBS, the slides were mounted in glycerol and examined under a fluorescence microscope. The results showed that >98% of the cells were positively stained, indicating that they were of endothelial origin.

$[Ca^{2+}]_i$ Measurement—Cells were prepared and loaded with the fluorescence dye Fluo3/AM as described (23). Briefly, the cells were grown in culture medium on circular discs overnight at 37 °C. For loading of Fluo3/AM, cells were incubated for 1 h in the dark at room temperature with 10 μM membrane-permeant Fluo3/AM and 0.02% pluronic F-127 in normal physiological saline solution (N-PSS) that contained 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM Hepes, pH 7.4. Cells were then pretreated with 10 μM CPA for 40 min or with 4 μM thapsigargin for 20 min in N-PSS. Cells were washed in and maintained briefly in a medium ($0Ca^{2+}$-PSS) that contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 2 mM EGTA, and 5 mM Hepes, pH 7.4. Ca^{2+} influx was initiated by replacing $0Ca^{2+}$-PSS with N-PSS, which contained 1 mM $CaCl_2$. Unless stated otherwise, the cells were pretreated with or without 8-Br-cGMP, KT5823, H-8, SKF-96365, or Ni^{2+} for 5 min. For the experiments in Fig. 5, the cells were pretreated with 100 μM SNAP for 5 min or with 10 μM dipyridamole for 20 min in the presence of CPA. These cells were then divided into three portions: one for Ca^{2+} influx study, one for cGMP measurement, and one for PKG activity measurement. In some experiments, cells were treated directly with 8-Br-cGMP or KT5823 without CPA or thapsigargin pretreatment. The fluorescence signal was monitored and recorded by an MRC-1000 laser scanning confocal imaging system with MRC-1000 software. Data analysis was performed with Confocal Assistant and Metaflour.

cGMP Measurement—Cellular cGMP content was measured with the ^{125}I-cGMP assay kit following the manufacturer's protocol. Briefly, cultured cells were extracted with ice-cold 65% ethanol. Debris was removed by centrifuging the sample at 2000 × *g* for 15 min at 4 °C. The extracts were then lyophilized and dissolved in assay buffer. Radioimmunoassay was performed by mixing the samples with a fixed quantity of ^{125}I-labeled cGMP and a cGMP-specific antibody. After incubation for 15 h at 4 °C, radioactive cGMP bound by antibody was separated by magnetic separation, and the amount of radioactivity was counted in a COBRA II γ-counter. Protein concentrations of all samples were measured by the standard method of Lowry *et al.* (24). Cellular cGMP content in picomoles/mg of protein was calculated.

PKG Activity Assay—Endogenous PKG activity was estimated by measuring cGMP kinase activity ratios (*i.e.* activity in the absence of added cGMP divided by activity in the presence of a saturated amount of cGMP) using a modification of the method described by Fiscus *et al.* (25, 26). Activity ratio measurements are considered to be more consistent from sample to sample than kinase activity measurements based on tissue protein (25, 26). In the method, the measured endogenous activity was expressed as a percentage of its maximal possible cellular activity. In brief, the procedure was as follows. Cells were treated with lysis buffer that contained 20 mM K_2PO_4, pH 7.0, 10 mM EDTA, 0.5 mM isobutylmethylxanthine, 6 mM dithiothreitol, and 1% Triton X-100. The cell lysates were rapidly transferred to cold microcentrifuge tubes and centrifuged at 8000 × *g* in a microcentrifuge at 4 °C for 30 s, and the supernatant fractions were used for kinase assay. To minimize cGMP dissociation from the enzyme during the sample extract preparation, each sample was homogenized, centrifuged, and assayed individually with a total time of 2.0 min from the beginning of homogenization to the beginning of the assay. Kinase activity was determined by measuring the amount of ^{32}P transferred from [γ-^{32}P]ATP to histone H2b during a 2.5-min incubation at 0 °C. The assays were initiated by adding 7 μl of supernatant fraction of tissue homogenates to 35 μl of reaction mixture containing 15 mM K_2PO_4, pH 7.0, 7 mM magnesium acetate, 0.5 mg/ml histone H2b, 20 mM NaF, 0.25 mM isobutylmethylxanthine, 30 μM ATP with [γ-^{32}P]ATP (5000 cpm/pmol), and 0.3 μM KT5720 (a highly specific inhibitor of cAMP kinase). The reaction was stopped by transferring 35 μl of final reaction mixture to Whatman No. 3MM chromatography paper squares (1.6 × 1.6 cm) and immediately immersing them in ice-cold 10% trichloroacetic acid with 2.5% pyrophosphate. The paper squares were washed in 5% trichloroacetic acid with 2.5% pyrophosphate twice for 15 min at 90 °C, followed by two washes at room temperature for 20 min each. After a final wash in 95% ethanol for 10 min, the paper squares were dried and counted in an LS6000 liquid scintillation counter.

Single Channel Recording—Single channel currents were measured by standard methods (27, 28) with an EPC-9 patch clamp amplifier. The signal was sampled at 5.0 kHz and filtered at 300 Hz for data analysis. Data were analyzed by TAC and TAC-fit software. Amplitude histograms were based on continuous recordings of 1 min and fitted with gaussian functions. Ca^{2+} saline contained 100 mM $CaCl_2$ and 10 mM Hepes, pH 7.4; KCl saline contained 140 mM KCl, 2.5 mM NaCl, 1 mM $CaCl_2$, and 10 mM Hepes, pH 7.4. The results are presented as means ± S.E. (n = number of experiments). All experiments were conducted at room temperature.

RESULTS

CPA is a mycotoxin from *Aspergillus* and *Penicillium*. It selectively inhibits endoplasmic reticulum Ca^{2+}-ATPase and prevents Ca^{2+} re-uptake, thereby stimulating Ca^{2+} entry by a mechanism independent of receptor stimulation and inositol 1,4,5-trisphosphate formation. This chemical has become a major tool for depleting intracellular Ca^{2+} stores and activating store-operated Ca^{2+} entry (1, 15). In this experiment, CPA was applied to induce store depletion. Ca^{2+} influx was then initiated by changing the extracellular medium from a Ca^{2+}-free solution ($0Ca^{2+}$-PSS) to a Ca^{2+}-containing solution (N-PSS). Control cells without CPA pretreatment were washed in and then maintained briefly in $0Ca^{2+}$-PSS. For the control cells, there was no change in $[Ca^{2+}]_i$ when external Ca^{2+} was elevated (Fig. 1*A*). In contrast, for those cells pretreated with 10 μM CPA, an elevation in external Ca^{2+} drastically increased $[Ca^{2+}]_i$ (Fig. 1*A*). These results suggest that depletion of intracellular Ca^{2+} stores activates Ca^{2+} influx. A prolonged exposure of cultured cells to a Ca^{2+}-free medium may also result in store depletion. We examined the relationship between cell exposure time in $0Ca^{2+}$-PSS and the subsequent Ca^{2+} influx triggered by elevation of extracellular Ca^{2+}. Elevation of extracellular Ca^{2+} caused no change in $[Ca^{2+}]_i$ for those cells incubated in $0Ca^{2+}$-PSS for up to 30 min. An incubation of 60 min in $0Ca^{2+}$-PSS resulted in the Ca^{2+} influx evoked by extracellular Ca^{2+}. However, such a prolonged incubation in $0Ca^{2+}$-PSS often led to cell detachment from cultureware.

To confirm that the CPA-induced increase in $[Ca^{2+}]_i$ is indeed caused by Ca^{2+} influx instead of Ca^{2+} release from CPA-insensitive intracellular stores, we used two known blockers of Ca^{2+} entry. Ni^{2+} (3 mM), a potent blocker of Ca^{2+} entry that competes for Ca^{2+}-binding sites (29), completely blocked the increase in $[Ca^{2+}]_i$ (Fig. 1*B*). SKF-96365 (50 μM), an inhibitor of receptor-mediated Ca^{2+} entry (30), also abolished the rise in $[Ca^{2+}]_i$ (Fig. 1*B*). We also tested the effect of membrane depolarization on the rise in $[Ca^{2+}]_i$. 80 mM extracellular K^+ completely suppressed the $[Ca^{2+}]_i$ rise due to CPA (Fig. 1*B*). Membrane depolarization is known to reduce the driving force for Ca^{2+} entry (31). Therefore, these results are consistent with the concept that the CPA-induced rise in $[Ca^{2+}]_i$ was caused by Ca^{2+} influx.

8-Br-cGMP was used to examine the effect of cGMP on store-operated Ca^{2+} entry. Application of 8-Br-cGMP reduced the $[Ca^{2+}]_i$ rise due to CPA in a concentration-dependent manner with an IC_{50} of 180 μM (Fig. 2*B*). 8-Br-cGMP at 2 mM com-

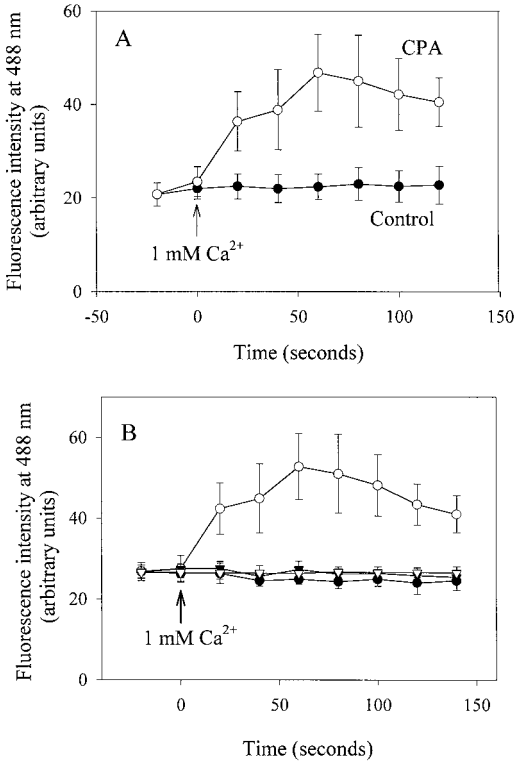

FIG. 1. **CPA-induced rise in $[Ca^{2+}]_i$.** $[Ca^{2+}]_i$ was monitored in Fluo3/AM-loaded rat aortic endothelial cells. Cells were placed in $0Ca^{2+}$-PSS with or without 10 μM CPA for 40 min. At the time indicated by the *arrow*, the media were changed to the respective media containing 1 mM $CaCl_2$ without EGTA. *A*, CPA-induced $[Ca^{2+}]_i$ change. ○, CPA; ●, without CPA. *B*, effects of blockers or membrane depolarization. Inhibitors or depolarization was introduced 5 min prior to the experiments. ○, CPA; ●, CPA + 3 mM Ni^{2+}; ▼, CPA + 50 μM SKF-96365; ▽, CPA + 80 mM K^+. Each point represents the mean ± S.E. (n = 4–8).

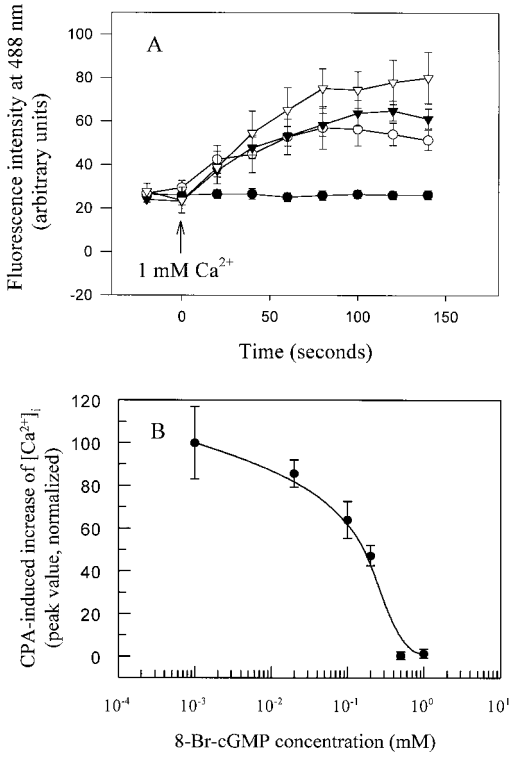

FIG. 2. **Effects of PKG modulators on CPA-induced rise in $[Ca^{2+}]_i$.** Cells were placed in $0Ca^{2+}$-PSS with CPA for 40 min. Chemicals were introduced 5 min prior to the experiments. At the time indicated by the *arrow*, the media were changed to the respective media containing 1 mM $CaCl_2$ without EGTA. *A*, effects of PKG modulators. ○, control; ●, 2 mM 8-Br-cGMP; ▽, 2 mM 8-Br-cGMP + 1 μM KT5823; ▼, 2 mM 8-Br-cGMP + 10 μM H-8. *B*, concentration-dependent inhibition by 8-Br-cGMP of the CPA-induced rise in $[Ca^{2+}]_i$. The peak amplitude of $[Ca^{2+}]_i$ was plotted *versus* 8-Br-cGMP concentration. The peak value in the absence of cGMP was normalized to 100. Each point represents the mean ± S.E. (n = 6–12).

pletely blocked the Ca^{2+} entry (Fig. 2, *A* and *B*). Since cGMP is an intracellular second messenger that activates PKG, we next examined the possible involvement of PKG. KT5823, a potent and highly specific PKG inhibitor (32), and H-8, another PKG inhibitor, were used for this purpose. 1 μM KT5823 or 10 μM H-8 abolished the inhibitory action of 8-Br-cGMP and resumed the Ca^{2+} entry due to CPA (Fig. 2*A* and *B*). These results suggest that store-operated Ca^{2+} entry in endothelial cells is regulated by a PKG-dependent mechanism. It appears that when PKG activity is blocked by KT5823 or H-8, 8-Br-cGMP is no longer able to stimulate PKG. The overall effect is an inhibition of PKG by KT5823 or H-8. The inhibition of PKG may then open the pathway for store-operated Ca^{2+} entry.

We then tested whether CPA-induced Ca^{2+} influx could be augmented by prior inhibition of PKG. In the presence of 1 μM KT5823 or 10 μM H-8, CPA-induced Ca^{2+} influx was significantly increased (Fig. 3). For those cells treated with PKG inhibitors, an initial $[Ca^{2+}]_i$ peak was followed by a rapid decrease in $[Ca^{2+}]_i$ (Fig. 3). We speculate that quicker refillings of intracellular Ca^{2+} stores under this condition may subsequently suppress Ca^{2+} influx.

It has been reported that PKG and protein kinase A have similarities in structure and substrate specificity (33). We thus tested the effect of 8-Br-cAMP on CPA-induced Ca^{2+} entry. Unlike cGMP, 2 mM 8-Br-cAMP had no effect on CPA-induced Ca^{2+} entry (n = 15), suggesting that PKA is not involved.

Store depletion has been reported to increase cellular cGMP in other nonexcitable cells (8). To further explore the possible regulatory role of cGMP in store-operated Ca^{2+} entry, we tested the effect of CPA on cellular cGMP levels in vascular

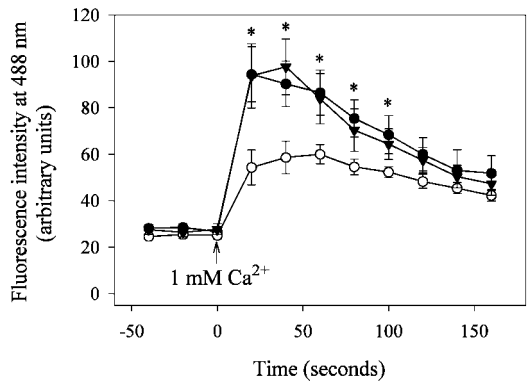

FIG. 3. **Effects of KT5823 and H-8 on CPA-induced rise in $[Ca^{2+}]_i$.** Cells were placed in $0Ca^{2+}$-PSS in the presence of CPA + KT5823 or CPA + H-8 for 40 min. At the time indicated by the *arrow*, the media were changed to the respective media containing 1 mM $CaCl_2$ without EGTA. ○, control; ●, 1 μM KT5823; ▼, 10 μM H-8. Each point represents the mean ± S.E. (n = 8–12). *, $p < 0.05$ compared with the control.

endothelial cells. Fig. 4 illustrates that CPA (10 μM) stimulated cGMP production in vascular endothelial cells. Maximal stimulation occurred at 10 min after addition of CPA. At this time point, CPA increased cGMP levels by 13-fold. SNAP (100 μM), a NO donor (34), and dipyridamole (10 μM), an inhibitor of cGMP phosphodiesterase (35), were also used to elevate cellular cGMP levels. Addition of SNAP or dipyridamole during CPA treatment further raised cellular cGMP (Fig. 5*B*) and almost abolished the Ca^{2+} influx due to CPA (Fig. 5*A*). Endogenous

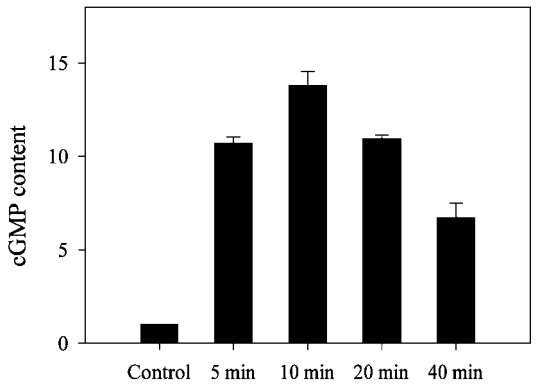

FIG. 4. **Effect of CPA on cGMP content of rat aortic endothelial cells.** Cells were treated with CPA. At the indicated times, samples were extracted with ethanol to determine cGMP content. The cGMP content in the control cells without CPA treatment was normalized to 1. Values represent means ± S.E. (n = 6).

PKG was also stimulated, with its activity raised to ~95% of its maximal activity (Fig. 5*C*).

For all the above experiments, we also used thapsigargin, another selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (1, 36), as an independent tool to evaluate our findings obtained from CPA experiments. When thapsigargin was used to activate Ca^{2+} influx, results similar to those with CPA were observed (data not shown).

These data strongly suggest that a PKG-sensitive Ca^{2+} entry pathway may play an important role in store-operated Ca^{2+} entry. However, they give no indication as to whether this pathway plays a role in the maintenance of $[Ca^{2+}]_i$ under basal unstimulated conditions. An attempt was therefore made to address this issue. Cultured endothelial cells were bathed in a normal physiological solution containing 1 mM Ca^{2+} without CPA pretreatment. Application of 2 mM 8-Br-cGMP caused only a slight decrease in intracellular Ca^{2+} (n = 10). On the other hand, treatment of the cells with 1 μM KT5823 caused a large increase in $[Ca^{2+}]_i$ (Fig. 6*A*). The KT5823-induced rise in $[Ca^{2+}]_i$ resulted from Ca^{2+} influx since it was rapidly blocked by 3 mM Ni^{2+} (Fig. 6*B*). These data suggest that the basal Ca^{2+} entry through the PKG-sensitive pathway may be low. On the other hand, opening of this Ca^{2+} entry pathway can significantly increase $[Ca^{2+}]_i$.

We also tested the effect of H-8 on resting $[Ca^{2+}]_i$ of the cells bathed in normal physiological solution without CPA pretreatment. However, unlike KT5823, H-8 (10 μM) did cause any immediate rise in $[Ca^{2+}]_i$. One possibility is that the inhibition of PKG by H-8 might be slow, probably due to slow diffusion of the chemical across the plasma membrane to its targeted site. Any increase in $[Ca^{2+}]_i$ due to slow opening of this PKG-sensitive Ca^{2+} influx pathway may be cancelled out by other $[Ca^{2+}]_i$-reducing mechanisms such as plasma membrane Ca^{2+}-ATPase and endoplasmic reticulum Ca^{2+}-ATPase. Therefore, the net change in $[Ca^{2+}]_i$ might be very little.

A patch clamp technique was used to search for store depletion-activated channels in isolated aortic endothelial cells. A Ca^{2+}-permeable channel was identified in the cell-attached mode with the bath solution being KCl saline and the pipette solution being $CaCl_2$ saline. Fig. 7 (*A–C*) shows the single channel current traces recorded in a typical experiment. Fig. 7 (*E–G*) shows the corresponding amplitude histograms. The current traces were recorded with the patch potential held at −100 mV. A very low level of channel activity could be observed immediately after the formation of a gigaohm seal (Fig. 7, *A* and *E*). CPA treatment drastically increased the channel activity (Fig. 7, *B* and *F*). A subsequent application of 1 mM 8-Br-cGMP in the presence of CPA completely abolished the

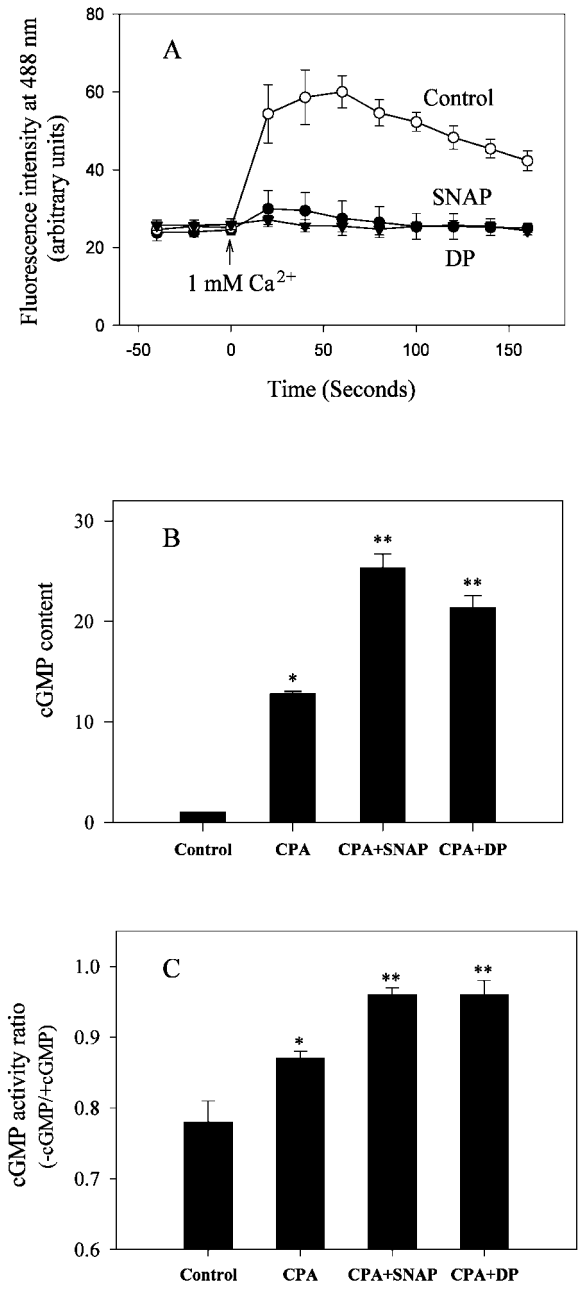

FIG. 5. **Effect of SNAP and dipyridamole on CPA-induced $[Ca^{2+}]_i$ rise, cellular cGMP, and cellular PKG activity.** Cells were placed in 0Ca^{2+}-PSS with CPA for 40 min. Chemicals were introduced 5 min (SNAP) or 20 min (dipyridamole) prior to the experiments. Samples were divided into three portions: one for Ca^{2+} influx study, one for cGMP measurement, and one for PKG activity assay. *A*, CPA-induced $[Ca^{2+}]_i$ rise. ○, control; ●, 100 μM SNAP; ▼, 10 μM dipyridamole (*DP*). *B*, cellular cGMP levels. The cGMP content in control cells without CPA treatment was normalized to 1. *C*, cellular PKG activity ratio. Values are the means ± S.E. (n = 6–15). *, $p < 0.05$ compared with the control; **, $p < 0.05$ compared with CPA alone.

channel activity (Fig. 7, *C* and *G*). A unitary current amplitude of −1.0 ± 0.5 pA was calculated by fitting with two gaussian functions. The single channel *I-V* relationship from the same patch is displayed in Fig. 7*D*. This CPA-activated channel was observed in 11 out of 62 cell-attached membrane patches. Among these 11 patches, seven had long-lasting good seals, which made the subsequent cGMP treatment possible. cGMP treatment abolished CPA-induced channel activity in all seven membrane patches. For three membrane patches that lasted even longer, we applied 1 μM KT5823. KT5823 resumed the

channel activity in all three patches. The slope conductance and the extrapolated reversal potential were obtained by linear regression. The average slope conductance and the reversal potential from seven different patches were 9.1 ± 1.4 picosiemens and 22 ± 5 mV, respectively. The conductance of this channel was not changed after application of CPA. Since this channel reversed at a positive voltage under our experimental conditions, it should be a Ca^{2+}-permeable channel.

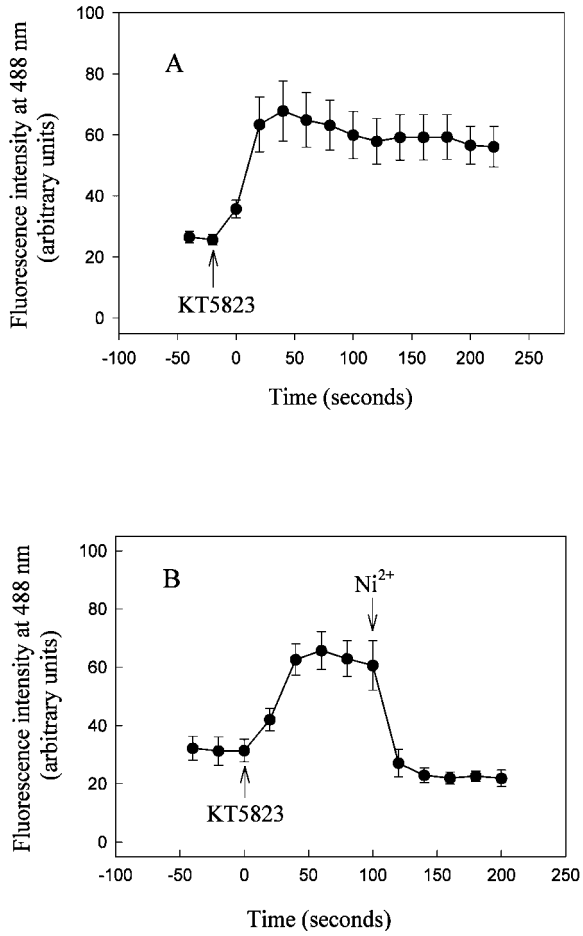

FIG. 6. **Effect of KT5823 on resting $[Ca^{2+}]_i$.** Cells were placed in N-PSS, which contained 1 mM $CaCl_2$. At the time indicated by the *upward arrows*, 1 μM KT5823 was added. The *downward arrow* illustrates the point at which 3 mM $NiCl_2$ was added. *A*, effect of KT5823; *B*, effect of Ni^{2+} on KT5823-induced rise in $[Ca^{2+}]_i$. Each point represents the mean ± S.E. (n = 4–12).

DISCUSSION

In this study, we used CPA and thapsigargin, two potent inhibitors of endoplasmic reticulum Ca^{2+}-ATPase, to deplete intracellular Ca^{2+} stores. CPA or thapsigargin treatment activated Ca^{2+} entry from extracellular space as indicated by an elevation in intracellular Ca^{2+} (Fig. 1*A*). Several lines of evidence suggest that the rise in $[Ca^{2+}]_i$ is caused by Ca^{2+} influx instead of Ca^{2+} release from CPA- or thapsigargin-insensitive intracellular stores. 1) The increase in $[Ca^{2+}]_i$ is evoked by application of Ca^{2+} to extracellular solution. 2) It is blocked by Ni^{2+} and SKF-96365. 3) It is abolished by membrane depolarization induced by 80 mM external K^+ (Fig. 1*B*).

Fig. 2 illustrates that the store-operated Ca^{2+} entry in endothelial cells can be inhibited by 8-Br-cGMP in a concentration-dependent manner. PKG inhibitors KT5823 and H-8 reverse the inhibitory effect of 8-Br-cGMP. These data suggest that the store-operated Ca^{2+} entry is subjected to modulation by cGMP through a PKG-dependent mechanism. We propose that cGMP and PKG may be crucial components in controlling store-operated Ca^{2+} entry at least in aortic endothelial cells.

In pancreatic acinar cells, depletion of intracellular Ca^{2+} stores is known to stimulate production of NO, which subsequently activates guanylate cyclase, leading to an elevation of cellular cGMP (8). The results from Fig. 4 indicate that store depletion elevates cellular cGMP levels in cultured endothelial cells as well. Our proposed model is that the elevated cGMP may in turn inhibit Ca^{2+} entry via a PKG-dependent pathway, therefore providing a negative feedback mechanism through which Ca^{2+} influx is finely regulated depending upon intracellular contents of Ca^{2+}, cGMP, and NO. NO is a crucial signal in the regulation of vascular tone. It is conceivable that vascular endothelial cells may need such a fine regulatory mechanism for a better control of vascular tone. This feedback mechanism also allows the cells to avoid detrimental effects of excessive $[Ca^{2+}]_i$ and/or NO. Excessive $[Ca^{2+}]_i$ or NO may lead to apoptosis and cell death (37, 38). Our model predicts that the inhibition of PKG will substantially augment CPA-induced Ca^{2+}

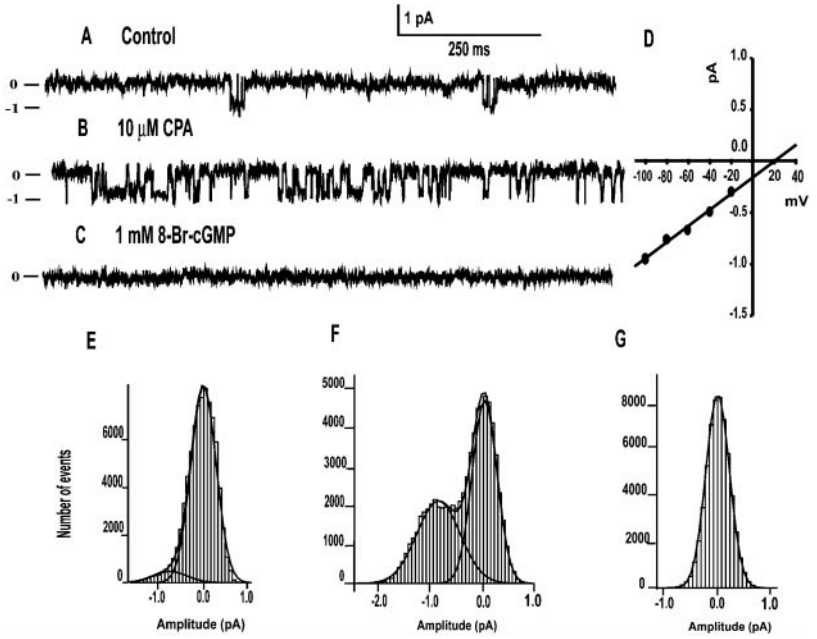

FIG. 7. **A CPA-inducible Ca^{2+}-permeable channel sensitive to the inhibition of cGMP.** The pipette/bath solutions contained $CaCl_2$ saline/KCl saline. *A–C* are single channel current traces. *E–G* are current amplitude histograms for *A–C*, respectively. Each histogram was derived from current activities during a period of 60 s. *A* and *E*, control; *B* and *F*, CPA treatment; *C* and *G*, 8-Br-cGMP treatment in the presence of CPA. The patch potential was held at −100 mV. *D*, single channel current-voltage relationship.

influx. This is indeed confirmed by the experiments in Fig. 3, which show that, in the presence of KT5823 (1 μM) or H-8 (10 μM), CPA-induced Ca^{2+} influx is augmented.

Although CPA elevates cellular cGMP levels and stimulates PKG activity in endothelial cells, PKG is not maximally activated (Fig. 5*C*). The elevated PKG activity is not enough to offset the Ca^{2+} influx triggered by CPA-induced store depletion. SNAP and dipyridamole were used to further increase the cellular cGMP levels in the presence of CPA. These treatments cause additional increases in cellular cGMP and PKG activity (Fig. 5, *B* and *C*) and almost abolish the Ca^{2+} influx due to CPA (Fig. 5*A*).

It is likely that this PKG-sensitive Ca^{2+} entry plays only a limited role in the maintenance of $[Ca^{2+}]_i$ under basal unstimulated conditions. PKG activity is substantial even without CPA treatment (Fig. 5*C*). Treatment of cultured endothelial cells with 2 mM 8-Br-cGMP causes no significant change in $[Ca^{2+}]_i$, whereas the inhibition of PKG by 1 μM KT5823 causes a rapid and significant increase in Ca^{2+} entry (Fig. 6. *A* and *B*). These results suggest that this Ca^{2+} influx pathway is mostly closed by PKG phosphorylation under basal unstimulated conditions. However, this Ca^{2+} influx pathway may open under the condition of store depletion, presumably through a PKG-independent pathway such as production of the Ca^{2+} influx factor or activation of tyrosine kinase (2–6). On the other hand, store depletion may stimulate the production of cGMP (Fig. 4), which then initiates the PKG-dependent feedback mechanism. The presence of two separate control mechanisms, one that positively stimulates Ca^{2+} entry via the Ca^{2+} influx factor or tyrosine kinase and the other that negatively attenuates Ca^{2+} entry through PKG, allows intracellular Ca^{2+} to be finely regulated.

With the use of the patch clamp technique, we have recorded a CPA-activated Ca^{2+}-permeable channel from isolated endothelial cells in the cell-attached mode (Fig. 7). Its properties appear to be similar to those of the channel previously reported by Zhang *et al.* (39). Both channels are stimulated by CPA. When the pipette is filled with high concentrations of $CaCl_2$, both channels reverse at a positive voltage with the single channel conductance around 7–9.5 picosiemens. In this study, we demonstrated that the activity of this channel is inhibited by bath application of 1 mM 8-Br-cGMP (Fig. 7, *C* and *G*). A subsequent application of KT5823 (1 μM) can resume the channel activity. It is likely that this particular Ca^{2+}-permeable nonselective cation channel might be the target for CPA stimulation and PKG phosphorylation.

Our conclusion that cGMP inhibits the store-operated Ca^{2+} entry in vascular endothelial cells is not consistent with previously published data in other nonexcitable cells. Available reports on other nonexcitable cells suggest that an increase in cGMP from the basal level either activates store-operated Ca^{2+} entry or has no effect (4, 7–13). In pancreatic acinar cells, Xu *et al*. (8) described a "biphasic response," in which they proposed that a low concentration of cGMP enhanced Ca^{2+} entry, whereas a high concentration inhibited Ca^{2+} entry. No quantitative concentration of cGMP was given in their report. However, their hypothesis was not supported by Bahnson *et al.* (7) and Bischof *et al.* (9), who reported that a high concentration of 8-Br-cGMP (1 mM) activated store-operated Ca^{2+} entry in pancreatic acinar cells (7) and colonic epithelial cells (9). We did not observe any biphasic response in cultured aortic endothelial cells. Addition of 8-Br-cGMP to the cultured cells causes a concentration-dependent decrease in store-operated Ca^{2+} entry. Complete inhibition is achieved when the concentration of 8-Br-cGMP is raised to 1 mM. Based on these data, we suggest that store-operated Ca^{2+} entry is regulated by different mechanisms in different cell types.

In conclusion, this study provides evidence that store-operated Ca^{2+} entry is regulated by cGMP and PKG in vascular endothelial cells. The control of Ca^{2+} entry by this mechanism may allow Ca^{2+} entry to be finely regulated depending upon the contents of intracellular Ca^{2+}, cGMP, and NO in vascular endothelial cells.

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