Involvement of Ca\(^{2+}\)/calmodulin-dependent protein kinase II in endothelial NO production and endothelium-dependent relaxation

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Received 25 October 2001; accepted in final form 8 January 2003

Nitric oxide (NO) is synthesized from L-arginine (L-Arg) by the enzyme NO synthase (NOS) isoform (eNOS). The present study assesses the role of Ca\(^{2+}\)/calmodulin-sensitive endothelial NO synthase (NOS) isoform (eNOS). The present study assesses the role of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK II) in endothelium-dependent relaxation and NO synthesis. The effects of three CaMK II inhibitors were investigated in endothelium-intact aortic rings of normotensive rats. NO synthesis was assessed by an NO sensor and chemiluminescence in culture medium of cultured porcine aortic endothelial cells stimulated with the Ca\(^{2+}\) ionophore A23187 and thapsigargin. Rat aortic endothelial NO synthesis was measured by the conversion of L-[\(^{3}H\)]arginine to L-[\(^{3}H\)]citrulline. Three CaMK II inhibitors, polypeptide 281–302, KN-93, and lavendustin C, attenuated the endothelium-dependent relaxation of endothelium-intact rat aortic rings in response to acetylcholine, A23187, and thapsigargin. None of the CaMK II inhibitors affected the relaxation induced by NO donors. In a porcine aortic endothelial cell line, KN-93 decreased NO synthesis and caused a leftward shift of the concentration-response curves to A23187 and thapsigargin. In rat aortic endothelial cells, KN-93 significantly decreased bradykinin-induced eNOS activity. These results suggest that CaMK II was involved in NO synthesis as a result of Ca\(^{2+}\)-dependent activation of eNOS.

endothelial function; nitric oxide; protein phosphorylation; signal transduction; thapsigargin

IN ENDOTHELIAL CELLS, stimulation of muscarinic M\(_{1}\) receptors by acetylcholine (ACh) results in the activation of phospholipase C-\(\gamma_{1}\), followed by a transient increase in the formation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_{3}\)] and diacylglycerol (2). The production of Ins(1,4,5)P\(_{3}\) is considered as an initial event leading to Ca\(^{2+}\) release from intracellular stores that precedes a steady or oscillating plateau phase resulting from a more prolonged transmembranous Ca\(^{2+}\) influx (16). A potential target of increased intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) is the endothelial nitric oxide (NO) synthase (NOS) isoform (eNOS). Ca\(^{2+}\)-mediated activation of eNOS requires the ubiquitous Ca\(^{2+}\)-binding protein calmodulin (49, 11) when calmodulin inhibitors do not influence the relaxant response to exogenous NO (42, 37, 50). Furthermore, the endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin, which induces an increase in [Ca\(^{2+}\)]\(_{i}\), triggers NO-dependent relaxation in vascular tissue (32). Thus the increase in [Ca\(^{2+}\)]\(_{i}\), results in the reversible formation of the Ca\(^{2+}\)/calmodulin complex, which binds to eNOS, stimulating its activity (19). Unstimulated endothelial cells continuously produce NO, suggesting that the intracellular Ca\(^{2+}\) level under resting conditions is sufficient for basal NO synthesis. In endothelial cells, the main signal transduction pathway of agonist-stimulated eNOS activation depends on Ca\(^{2+}\)/calmodulin. However, NO synthesis can be, at least in part, regulated by serine/threonine kinases, including cAMP-dependent protein kinase (9, 7), protein kinase C (36, 26), and serine/threonine kinases, including cAMP-dependent protein kinase (9, 7), protein kinase C (36, 26), and protein kinase B/Akt (20, 14). Although eNOS contains consensus sequences for phosphorylation by serine/threonine kinases, its regulation by Ca\(^{2+}\)-dependent and/or Ca\(^{2+}\)-independent phosphorylation remains to be specified. Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK II) is a ubiquitous Ca\(^{2+}\)/calmodulin-dependent enzyme involved in various Ca\(^{2+}\)-mediated mechanisms. Its relationship with neuronal NOS (nNOS) has been previously established by Nakane et al. (35). Although Deli et al. (13) showed that CaMK II was expressed in endothelial cells, the link between CaMK II and NO synthesis in the endothelium re-

Josette Dall’Ava-Santucci, and A. Tuan Dinh-Xuan. Nitric oxide (NO) is synthesized from L-arginine (L-Arg) by the enzyme NO synthase (NOS) isoform (eNOS). Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK II) is a ubiquitous Ca\(^{2+}\)-mediated enzyme involved in various Ca\(^{2+}\)-mediated mechanisms. Its relationship with neuronal NOS (nNOS) has been previously established by Nakane et al. (35). Although Deli et al. (13) showed that CaMK II was expressed in endothelial cells, the link between CaMK II and NO synthesis in the endothelium re-

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mains largely unknown. We hypothesized that CaMK II might modulate Ca\(^{2+}\)-mobilizing agent-dependent eNOS activity. The role of CaMK II in rat aorta endothelium-dependent relaxation was investigated by pre-treatment with CaMK II inhibitors. Furthermore, we studied the interaction between Ca\(^{2+}\)-induced NO production and CaMK II-dependent phosphorylation by measuring NO release and eNOS activity in a cultured porcine aortic endothelial cell (PAEC) line and rat aortic endothelial cells pretreated with CaMK II inhibitors.

**MATERIALS AND METHODS**

**Drugs.** Reagents were from Sigma and RBI, distributed by Sigma (Saint-Quentin Fallavier, France), unless otherwise stated. Polyethylene 281–302 (P281–302) bound calmodulin and was a potent inhibitor (IC\(_{50}\) = 80 nM) of exogenous substrate phosphorylation (12). KN-93 was an inhibitor of CaMK II activation (\(K_i = 0.37\ \mu M\)) (45). Its related compound, KN-92, does not show any CaMK II inhibitory activity. Lavendustin C (Calbiochem; Meudon, France) is a potent inhibitor of CaMK II (IC\(_{50}\) = 200 nM). Lavendustin C has noncompetitive inhibitory action on the tyrosine kinase ATP binding site and noncompetitive inhibitory action on the peptide binding site (3). P281–302 was dissolved in deoxygenated bidistilled water. KN-93, KN-92, and lavendustin C were dissolved in DMSO to prepare a stock solution of 0.01 M. Phenylephrine hydrochloride (\(\alpha_1\)-adrenoreceptor agonist), acetylcholine chloride (muscarinic agonist), Ca\(^{2+}\) ionophore A23187, sodium nitroprusside (NO donor), and N\(^{-}\)-nitro-L-arginine methyl ester hydrochloride (l-NAME; analog of arginine and competitive isozyme-nonselective NOS inhibitor) were made up in bidistilled deionized water. Thapsigargin (Calbiochem) was dissolved in DMSO. Each was made fresh daily and protected from light. All other drugs were dissolved in bidistilled deionized water except indomethacin, which was dissolved in ethanol. Further dilutions were made in Krebs solution for in vitro experiments. The drug solutions were prepared each day from dry powder.

**Tissue preparation and tension measurement.** All animal procedures were approved in accordance with the European Directive for Animal Experiments 86/609 (Centre National de la Recherche Scientifique; Paris, France). At the time of experimentation, adult male Sprague-Dawley rats (251–275 g, Charles River; Saint-Aubin Les Elbeufs, France) were anesthetized with thiopental sodium (Nesdonal; 80 mg/kg ip) and heparinized (100 IU ic). A thoracotomy was performed, and the rats were anesthetized with thiopental sodium (Nesdonal; 80 mg/kg ip) and heparinized (100 IU ic). A thoracotomy was performed, and the rings were suspended in a 20-ml tissue bath. One stainless steel wire was inserted into the arterial lumen, interspersed with the arterial wall, and was a potent inhibitor (IC\(_{50}\) = 302 nM) of exogenous substrate phosphorylation (12). KN-93 was an inhibitor of CaMK II activation (\(K_i = 0.37\ \mu M\)) (45). Its related compound, KN-92, does not show any CaMK II inhibitory activity. Lavendustin C (Calbiochem; Meudon, France) is a potent inhibitor of CaMK II (IC\(_{50}\) = 200 nM). Lavendustin C has noncompetitive inhibitory action on the tyrosine kinase ATP binding site and noncompetitive inhibitory action on the peptide binding site (3). P281–302 was dissolved in deoxygenated bidistilled water. KN-93, KN-92, and lavendustin C were dissolved in DMSO to prepare a stock solution of 0.01 M. Phenylephrine hydrochloride (\(\alpha_1\)-adrenoreceptor agonist), acetylcholine chloride (muscarinic agonist), Ca\(^{2+}\) ionophore A23187, sodium nitroprusside (NO donor), and N\(^{-}\)-nitro-L-arginine methyl ester hydrochloride (l-NAME; analog of arginine and competitive isozyme-nonselective NOS inhibitor) were made up in bidistilled deionized water. Thapsigargin (Calbiochem) was dissolved in DMSO. Each was made fresh daily and protected from light. All other drugs were dissolved in bidistilled deionized water except indomethacin, which was dissolved in ethanol. Further dilutions were made in Krebs solution for in vitro experiments. The drug solutions were prepared each day from dry powder.

**Histology.** Endothelium integrity was assessed by postpharmacological challenge histology analysis. Tissues were harvested, fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned. Histological sections (5 \(\mu\)m thick) were stained with hematoxylin and eosin (Sigma) and examined.

**PAEC culture.** All the reagents used for cell culture were from GIBCO-BRL (Cergy-Pontoise, France) if not otherwise specified. The PAEC line established by Malassagne et al. (27) was a gift from Dr. Bernard Weill (Laboratoire d’Immunologie Biologique, Hôpital Cochin; Paris, France). PAECs were cultured in 25-cm\(^2\) Primaria dishes (Polylabo; Paris, France) in RPMI 1640 medium-glutamax-1 supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml amphotericin B, and 10% fetal bovine serum. The cultures were incubated at 37°C in a humid atmosphere with 5% CO\(_2\). When cells reached confluence, they were detached by incubation with 0.05% trypsin-EDTA in RPMI 1640 medium-glutamax-1 for 3 min at 37°C, centrifuged at 300 g for 10 min, suspended in fresh complete medium, and further cultured under the same conditions. Cells were characterized as endothelial cells by their morphology, their ability to take up acetylated low-density lipoproteins (34), the detection of von Willebrand factor (41), and the expression of E-selectin (27).

**Cell stimulation.** At least four sets of transfected cells (passages 29 and 30) were tested. The cells were subcultured in 24-well flat-bottom culture plates (10\(^{5}\) cells/well) during 24 h to reach confluence. The culture medium was removed, and the cells were washed once and equilibrated in isotonic phosphate buffer (pH 7.4) containing (in mM) 8 Na\(_2\)HPO\(_4\), 1.5 KH\(_2\)PO\(_4\), 2.7 KCl, 0.9 CaCl\(_2\), 1 MgCl\(_2\), and 1 indomethacin oxygenated with 95% O\(_2\)-5% CO\(_2\) (Air Liquide Santé). For real-time NO measurements, the sensor probe was inserted vertically into a well with confluent cells, the sensor membrane was positioned 50 \(\mu\)m above the monolayer by using a manual micromanipulator, and the well was...
sealed. To investigate the response to the agonists, the cells were incubated with either A23187 (5 × 10⁻⁸–10⁻⁵ M) or thapsigargin (5 × 10⁻⁹–10⁻⁵ M). The effect of CaMK II was evaluated on the concentration-response curve for A23187 or thapsigargin of cells previously incubated with KN-93 during 30 min. For nitrate and nitrite (NOx) measurements, the cells were incubated with KN-93 (1 μM) or KN-92 (1 μM). After the cells were incubated for 30 min, they were stimulated by the Ca²⁺ ionophore A23187 (0.1, 1, and 10 μM) or thapsigargin (0.1, 1, and 10 μM). The samples of effluent were then withdrawn from the culture well and immediately centrifuged at 300 g and 0°C for 10 min. The NOx concentration was determined in the supernatant by chemiluminescence.

Rat aortic endothelial cell culture. The isolation of primary rat aortic cells was achieved according to the method of McGuire et al. (29). Cells were placed on a substrate including laminin and cultured in RPMI 1640 medium supplemented with 20% fetal calf serum. They were characterized by the detection of von Willebrand factor (41) and their ability to uptake acetylated low-density lipoproteins (34).

Cell stimulation. Cells were subcultured in six-well flat-bottom culture plates (4 × 10⁶ cells/well) and incubated with Ca²⁺ (1 μM) before stimulation with bradykinin (0.1 mM). The cells were detached by incubation with 0.05% trypsin-EDTA, centrifuged at 300 g for 10 min, and suspended in Tris-HCl (50 mM, pH 7.4) with EDTA (0.1 mM), leupeptin (1 μM), aprotinin (1 μM), and PMSF (1 μM) to measure eNOS activity. NOx measurement. The NOx content in the culture medium was determined by measuring NO based on a gas phase chemiluminescent reaction between NO and ozone with a NO analyzer (model 280, Sievers Instruments; Boulder, CO) (31). NO release in the headspace was purged through the gas-permeable membrane is oxidized at the working platinum electrode. The resulting redox current is proportional to the concentration of NO gas in the aqueous solution. The output current was recorded with the constant proportional to the concentration of NO gas in the aqueous solution. The output current was recorded with the constant stirring in glass vials sealed with a septum. Linear calibration curves were obtained from the resulting calibration plot.

Electrochemical detection of NO. S-nitroso-N-acetyl penicillamine (SNAP), CuCl, EDTA, and NaOH were obtained from Sigma-Aldrich and Fluka. All the solutions were dissolved in deoxygenated bidistilled water. Amperometric measurements of NO released were quantified with a Clark-type electrode (2 mm platinum disk NO sensor, Iso-NOP, World Precision Instruments; Stevenage, UK) connected to an Iso Mark II NO meter (World Precision Instruments). The newly developed electrode has a high selectivity for NO and measurement have previously been described (44). NO diffusion through the gas-permeable membrane is oxidized at the working platinum electrode. The resulting redox current is proportional to the concentration of NO gas in the aqueous solution. The output current was recorded with the constant laboratory temperature kept constant. Calibration of the electrode was performed daily according to the procedure described by Zhang et al. (52). The NO sensor was immersed in saturated CuCl solution. After stabilization, a known volume of the SNAP solution (final concentration of 10, 20, 50, 100, and 200 nM) was then added, and the response was monitored. Measurements of NO were performed under constant stirring in glass vials sealed with a septum. Linear calibration curves were obtained from the resulting calibration plot.

eNOS activity. NOS activity was measured by the conversion of L-[³H]arginine to L-[³H]citrulline according to the methods described by Bredt et al. (8). Enzyme extract (25 μl) was incubated in the buffer [50 mM HEPES (pH 7.4), 0.5 mM NADPH, 5 μM FAD, 5 mM tetrahydrobiopterin, 1.25 mM CaCl₂, and 10 μg calmodulin per ml] and 50 nM L-[³H]arginine. The enzymatic assay was terminated by the addition of 2 ml of ice-cold 20 mM HEPEs (pH 5.5)-2 mM EDTA and was applied to 1-ml columns of Dowex-50W X8 (Bio-Rad). L-[³H]citrulline was eluted with 2 ml of deionized water and quantified by liquid scintillation spectroscopy.

CaMK II-α immunoblot analysis. All reagents were from Bio-Rad (Marnes la Coquette, France). Whole cell lysate was prepared from nonstimulated cells. Cells were detached as previously described and suspended in PBS. After centrifugation, harvested cells were homogenized in lysis buffer [50 mM Tris (pH 8.1), 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 μM leupeptin and pepstatin, and 7 μM PMSF] and ultrasonicated. Whole cell lysates were added to SDS sample buffer [125 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 2% β-mercaptoethanol], boiled for 5 min, and separated on a SDS-PAGE gel according to the method of Laemmli (25). After the migration, the proteins were transferred on nitrocellulose membranes in semidy transfer buffer [25 mM Tris (pH 7.5), 200 mM glycine, and 20% methanol]. The blots were blocked in 1% bovine serum albumin, 25 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 and incubated with CaMK II antibodies (Transduction Laboratories; Lexington, UK) for 30 min at 37°C. After being washed, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG and developed according to the enhanced chemiluminescence immunodetection procedure (Amersham Pharmacia Biotech; Orsay, France).

Data analysis. All responses to phenylephrine were expressed as force (in g). Responses to vasodilator agents were expressed as a percentage of maximal relaxation. In the endothelium-intact vessels, the effects of CaMK II inhibitors were measured on two rings for each aorta and the values were averaged. For all experiments, n is the number of rat aorta studies. Data are expressed as means ± SE of n number of experiments. Results were analyzed using two-way ANOVA with repeated measures to compare the effects of inhibitors versus control with increasing concentrations of ACh. When the F-value for an effect was globally significant, comparisons were made using the Mann-Whitney U-test. The NOx concentration was normalized for cell number, and the results were expressed as the content of NO release (in nM) per 10⁶ cells. The cell counts were obtained manually using a hemocytometer (Neubauer type), with viabilities determined by trypan blue dye exclusion. The cells were counted after experimentation. NOx measurements were realized twice and averaged, and n corresponds to the number of sets analyzed. Statistical evaluation of the difference between aortic rings was achieved according to the method of McGuire et al. (29). Results were considered significant for an α-level below 0.05.

RESULTS

Effects of CaMK II inhibitors on endothelium-dependent relaxation to ACh. In precontracted endothelium-intact aortic rings, ACh (10⁻⁸–10⁻⁵ M) induced concentration-dependent relaxation (EC₅₀ = 2.57 ± 0.13 × 10⁻⁷ M, n = 8). CaMK II inhibitors (1 μM P281–302, 1 μM KN-93, and 1 μM lavendustin C) shifted to the right the ACh concentration-relaxation curves (EC₅₀ = 4.80 ± 0.24 × 10⁻⁷, 7.67 ± 0.31 × 10⁻⁷, and 5.98 ± 0.30 × 10⁻⁷ M, respectively, n = 5).
P281–302 (0.1 and 1 μM) partially reversed the ACh-induced relaxation by 14 ± 6% and 20 ± 4% for 0.1 and 1 μM, respectively, at an ACh concentration of 10⁻⁶ M and by 14 ± 6% and 19 ± 4% for 0.1 and 1 μM, respectively, at an ACh concentration of 10⁻⁵ M (P < 0.05, n = 6; Fig. 1).

Treatment of aortic rings with KN-93 (1 and 10 μM) decreased the ACh-induced relaxation by 23 ± 3% and 29 ± 3% for 1 and 10 μM, respectively, at an ACh concentration of 10⁻⁶ M and by 20 ± 2% and 28 ± 6% for 1 and 10 μM, respectively, at an ACh concentration of 10⁻⁵ M (P < 0.05, n = 5). Its inactive analog, KN-92 (1 μM), did not affect the ACh-induced relaxation (Fig. 2).

Lavendustin C (0.1 and 1 μM) elicited a concentration-dependent reduction of ACh-induced relaxation of aortic rings precontracted with phenylephrine by 17 ± 9% and 28 ± 3% for 0.1 and 1 μM, respectively, at an ACh concentration of 10⁻⁶ M and by 14 ± 7% and 14 ± 5% for 0.1 and 1 μM, respectively, at an ACh concentration of 10⁻⁵ M (P < 0.05, n = 5; Fig. 3).

Vehicle (0.01% DMSO) had no effect on both phenylephrine-induced contraction and ACh-induced relaxation.

Effects of CaMK II inhibitors on endothelium-dependent relaxation to A23187 and thapsigargin. In precontracted endothelium-intact aortic rings, A23187 (10⁻⁷–10⁻⁴ M) induced concentration-dependent relaxation (EC₅₀ = 9.33 ± 0.23 × 10⁻⁶ M, n = 6). KN-93 (1 μM) shifted to the right the A23187 concentration-relaxation curves (EC₅₀ = 6.46 ± 0.30 × 10⁻⁶ M, n = 5).

Treatment of aortic rings with KN-93 (1 μM) decreased the A23187-induced relaxation by 26 ± 5% at an A23187 concentration of 10⁻⁶ M and by 22 ± 3% at an A23187 concentration of 10⁻⁵ M (P < 0.05, n = 6). Its inactive analog, KN-92 (1 μM), did not affect the A23187-induced relaxation (Fig. 4).

In precontracted endothelium-intact aortic rings, thapsigargin (10⁻⁸–10⁻⁵ M) induced concentration-dependent relaxation (EC₅₀ = 1.91 ± 0.30 × 10⁻⁷ M, n = 5). KN-93 (1 μM) shifted to the right the thapsigargin concentration-relaxation curves (EC₅₀ = 2.45 ± 0.28 × 10⁻⁷ M, n = 5).

Treatment of aortic rings with KN-93 (1 μM) decreased the thapsigargin-induced relaxation by 22 ± 5% (P < 0.05; n = 5).

Effects of CaMK II inhibitors on endothelium-dependent relaxation to acetylcholine. Concentration-response curves for the vasorelaxation effect of acetylcholine (10⁻⁸–10⁻⁵ M) on phenylephrine-contracted aortic rings with endothelium are shown. Effects of 0.1 and 1 μM P281–302 are compared with Krebs buffer as a control. P281–302 was added at the plateau phase evoked by the α₁-adrenoceptor agonist and incubated 15 min before the acetylcholine challenge. Results are expressed as a percentage of the maximal relaxation and are presented as means ± SE; n = 6. *P < 0.05, significant difference between CaMK II inhibitor- and KN-92-treated preparations.

**Fig. 1.** Inhibitory effect of polypeptide 281–302 (P281–302) on endothelium-dependent relaxation to acetylcholine. Concentration-response curves for the vasorelaxation effect of acetylcholine (10⁻⁸–10⁻⁵ M) on phenylephrine-contracted aortic rings with endothelium are shown. Effects of 0.1 and 1 μM P281–302 are compared with Krebs buffer as a control. P281–302 was added at the plateau phase evoked by the α₁-adrenoceptor agonist and incubated 15 min before the acetylcholine challenge. Results are expressed as a percentage of the maximal relaxation and are presented as means ± SE; n = 6. *P < 0.05, significant difference between CaMK II inhibitor-treated preparations and control.

**Fig. 2.** Inhibitory effect of KN-93 on endothelium-dependent relaxation to acetylcholine. Concentration-response curves for the vasorelaxation effect of acetylcholine (10⁻⁸–10⁻⁵ M) on phenylephrine-contracted aortic rings with endothelium are shown. Effects of 1 and 10 μM KN-93 are compared with 1 μM KN-92 as a control. KN-93 and KN-92 were added at the plateau phase evoked by the α₁-adrenoceptor agonist and incubated 15 min before the acetylcholine challenge. L-NAME, N⁴-nitro-L-arginine methyl ester. Results are expressed as a percentage of the maximal relaxation and are presented as means ± SE; n = 5. *P < 0.05, significant difference between CaMK II inhibitor- and KN-92-treated preparations.
Fig. 4. Inhibitory effect of KN-93 on endothelium-dependent relaxation to Ca^{2+} ionophore A23187. Concentration-response curves for the vasorelaxation effect of A23187 (10^{-7}–10^{-4} M) on phenylephrine-contracted aortic rings with endothelium are shown. Effects of 1 μM KN-93 are compared with 1 μM KN-92 as a control. KN-93 and KN-92 were added at the plateau phase evoked by thapsigargin and incubated by 15 min before the endoplasmic reticulum Ca^{2+}-ATPase inhibitor challenge. Results are expressed as a percentage of maximal relaxation and are presented as means ± SE; n = 5. *P < 0.05, significant difference between CaMK II inhibitor- and KN-92-treated preparations.

8% at a thapsigargin concentration of 10^{-6} M and by 13 ± 5% at a thapsigargin concentration of 10^{-5} M (P < 0.05, n = 6). Its inactive analog, KN-92 (1 μM), did not affect the thapsigargin-induced relaxation (Fig. 5).

Effects of CaMK II inhibitors on exogenous NO-induced relaxation. Sodium nitroprusside (10 μM) totally relaxed the aortic ring (98 ± 2%). Neither CaMK II inhibitors nor the inactive analog KN-92 at all concentrations have inhibitory action on sodium nitroprusside (10 μM) relaxation (data not shown). None of the three CaMK II inhibitors evoked spontaneously relaxation in endothelium-intact or -denuded vascular rings.

Effect of CaMK II inhibitors on endothelium-denuded and -intact aortic ring contraction. In endothelium-denuded aortic rings, the three CaMK II inhibitors did not have an inhibitory action on phenylephrine (1 μM) contraction. In endothelium-intact aortic rings, both P281–302 (1 μM) and KN-93 (10 μM) did not alter phenylephrine (an α1-adrenoreceptor agonist) contraction. However, lavendustin C at a concentration of 10 μM induced an additional contraction of endothelium-intact aortic rings precontracted with phenylephrine by 18 ± 7% (P < 0.05, n = 5). The lavendustin C-induced increase in vascular tone was inhibited by l-NAME (10 μM).

Immunoblot characterization of CaMK II-α. After SDS-PAGE and semidy transfer, membrane extracts of PAECs were revealed with anti-CaMK II isotype IgG1. IgG1 bound to 52-kDa antigens. The same band was revealed on the control (rat brain lysate; Fig. 6).

Effect of the CaMK II inhibitor KN-93 on NO production. In cultured endothelial cells, A23187 (5 × 10^{-8}–10^{-5} M) and thapsigargin (5 × 10^{-9}–10^{-5} M) induced NO release (EC_{50} = 9.12 ± 7.24 × 10^{-7} M, respectively). Preincubation with l-NAME (10 μM) inhibited agonist-induced NO release. KN-93 (1 μM) shifted to the right the concentration-response curve of A23187- and thapsigargin-induced NO release (EC_{50} = 1.26 ± 10^{-6} and 9.77 ± 10^{-7} M, respectively; Fig. 7).

Decreased NOx concentration by KN-93 on cultured endothelial cells stimulated by A23187 or thapsigargin. Stimulation of endothelial cells with the Ca^{2+} ionophore A23187 (1 and 10 μM) increased the NOx concentration in PAEC culture medium by 1,477 ± 51 and 2,075 ± 111 nM/10^5 cells, respectively. l-NAME (10 μM) inhibited the NOx production. The background NOx concentration from the isotonic phosphate buffer was 114 ± 20 nM/10^5 cells.

KN-93 (1 μM) reduced the NOx concentration in culture medium of PAECs stimulated with A23187 (1 and 10 μM) by 26 ± 5% and 10 ± 4%, respectively (P < 0.05, n = 4; Fig. 7A). CaMK II inhibitor reduced the NOx concentration in the culture medium of PAECs stimulated with thapsigargin (1 and 10 μM) by 22 ± 4% and 17 ± 5%, respectively (P < 0.05, n = 4; Fig. 7B).

Fig. 5. Inhibitory effect of KN-93 on endothelium-dependent relaxation to the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin. Concentration-response curves for the vasorelaxation effect of thapsigargin (10^{-6}–10^{-5} M) on phenylephrine-contracted aortic rings with endothelium are shown. Effects of 1 μM KN-93 are compared with 1 μM KN-92 as a control. KN-93 and KN-92 were added at the plateau phase evoked by thapsigargin and incubated 15 min before the endoplasmic reticulum Ca^{2+}-ATPase challenge. Results are expressed as a percentage of maximal relaxation and are presented as means ± SE; n = 5. *P < 0.05, significant difference between CaMK II inhibitor- and KN-92-treated preparations.

Fig. 6. Western blot characterization of CaMK II in the porcine aortic endothelial cell line. Cell lysate SDS-PAGE analysis was performed on 12% polyacrylamide gels. After semidy transfer, the nitrocellulose membranes were incubated with anti-CaMK II isotype IgG1 (1/3,000) and developed according to the enhanced chemiluminescence immunodetection procedure. The subunit immunblotted was CaMK II-α. Control was rat brain lysate. Data presented are representative of three similar experiments with cells at different passages.
 CAMK II AND eNOS ACTIVATION

figure 7. Inhibitory effect of CaMK II inhibitors on nitric oxide (NO) release. Effects of CaMK II inhibitors on cultured porcine aortic endothelial cells stimulated with Ca²⁺ ionophore A23187 (5 × 10⁻⁶–10⁻⁵ M; A) and thapsigargin (5 × 10⁻⁶–10⁻⁵ M; B) are shown. The rightward shift of concentration-response curves for endothelial cell NO release by KN-93 (1 μM) was determined by repeated measures of NO concentration (in nM) and are presented as means ± SE; n = 4. Nitrite and nitrate (NOₓ) concentration was determined by chemiluminescence after 30-min preincubation with KN-93 (1 μM) and stimulation with A23187 and thapsigargin. Histograms represent means ± SE of at least four experiments. *P < 0.05, significant difference between CaMK II inhibitor-treated preparations and control.

**Decreased rat aortic endothelial cell eNOS activity by KN-93.** Bradykinin-dependent eNOS activity as assessed by the conversion of L-[³H]arginine to L-[³H]citrulline was reduced to 43 ± 3% by KN-93 (1 μM). Note that eNOS activity in rat aortic endothelial cells was totally inhibited by L-NAME (Fig. 8).

**DISCUSSION**

Our results provide evidence that three different CaMK II inhibitors decreased endothelium-dependent relaxation elicited by ACh in normotensive rats. None of the CaMK II inhibitors inhibited relaxation induced by NO donors. KN-93 inhibited both receptor-independent and -dependent agonist-induced relaxation. The effects of CaMK II inhibitors were confirmed in both the PAEC line and rat aortic endothelial primary cultured cells. In the former, KN-93 significantly decreased the NO release in response to both the Ca²⁺ ionophore A23187 and thapsigargin. In the latter, KN-93 markedly reduced bradykinin-stimulated eNOS activity. Our results suggest that NO synthesis is dependent on CaMK II. The involvement of CaMK II in porcine endothelial NO production and rat NO-dependent relaxation characterized by the response to P281–302, KN-93, and lavendustin C on endothelium-dependent relaxation may be due to 1) an interference with ACh-induced Ca²⁺ release, 2) a direct effect on eNOS and/or calmodulin phosphorylation, and 3) an inhibition of NO-independent vascular smooth muscle relaxation.

To avoid bias due to nonspecific inhibitory effects of the compounds, our experiments were repeated with three structurally different CaMK II inhibitors. The synthetic polypeptide P281–309 contains the calmodulin-binding site (amino acids 290–309) and the autophosphorylation (Thr²⁸⁶) of CaMK II and therefore inhibits CaMK II by blocking Ca²⁺/calmodulin activation and the enzyme active site. KN-93 and lavendustin C inhibit CaMK II in a competitive fashion against calmodulin by decreasing the autophosphorylation of CaMK II. The effects of KN-93 on other CaMK isoforms remain unknown. CaMK II and eNOS represent two Ca²⁺/calmodulin-dependent enzymes. Their activation follows the agonist-induced increase in [Ca²⁺]. Our results showing that CaMK II inhibitors decreased ACh-induced relaxation suggest that CaMK II inhibitors may alter the Ca²⁺ release induced by ACh. However, a specific interference at the level of the muscarinic M₁ receptors only may be excluded because KN-93 decreased receptor-independent Ca²⁺ ionophore A23187-induced rat aorta relaxation, and, in PAECs, the [Ca²⁺], increase-dependent eNOS activation elicited by A23187 was attenuated by pretreatment with CaMK II inhibitor. These findings are consistent with previous results showing that, in endothelial cells, KN-93 and lavendustin C did not modify basal [Ca²⁺], unlike the calmodulin antagonist-provoked dose-dependent increases in [Ca²⁺] (48). Furthermore, our results with thapsigargin, which induced an increase in the [Ca²⁺], by mobilization from Ins(1,4,5)P₃ Ca²⁺ stores (15), suggest that CaMK II was not involved in endoplasmic reticulum Ca²⁺-ATPase activity and did not inhibit Ca²⁺ release from

**Fig. 8.** Inhibitory effect of KN-93 on rat aortic endothelial NO synthase (NOS) activity. Endothelial NOS activity was determined by the conversion of L-[³H]arginine to L-[³H]citrulline, expressed as counts per minute (cpm) for 10⁵ cell. Rat aortic endothelial cells were stimulated by bradykinin (0.1 mM) after preincubation with KN-93 (1 μM). Histograms represent means ± SE of three experiments.
internal stores. In concert, our data support the hypothesis that CaMK II is involved downstream of the mobilization of intracellular Ca\(^{2+}\) stores directly on eNOS activation.

As with other NOS isoforms, eNOS might represent a direct protein substrate for CaMK II (43). However, Bredt et al. (7) reported no effect on soluble nNOS, whereas Nakane et al. (35) showed that CaMK II-induced phosphorylation of nNOS decreased its activity. Alternatively, Toda et al. (47) have shown that, in the cerebral artery, NO-mediated relaxation was attenuated by an inhibitor of CaMK II. This different behavior may be explained by the particular localization of eNOS in endothelial cells, where it is initially targeted to the membrane fraction (38) and subsequently translocated from the membrane to the soluble fraction after stimulation of the cells (30). This particular property of eNOS is partly due to the presence of NH\(_2\)-terminal myristoylation and palmitoylation (10, 39). However, these posttranslational modifications are not sufficient for membrane localization, and phosphorylation of the enzyme is an alternative mechanism for the reversible association of the enzyme with membrane phospholipids (28). Alternatively, in cultured endothelial cells, agonist-induced eNOS phosphorylation increases its sensitivity to activation by Ca\(^{2+}\) (17). Thus pretreatment of endothelial cells with CaMK II inhibitors might either inhibit the translocation of eNOS or enhance the desensitization to Ca\(^{2+}\). Our results showing the decrease of eNOS activity in parallel of the reduction of NO-dependent relaxation suggest that CaMK II directly phosphorylates eNOS protein. This hypothesis was recently confirmed by Fleming et al. (18), who demonstrated that porcine eNOS activity depends on serine (Ser\(^{1177}\)) eNOS phosphorylation by CaMK II. Their biochemical approach, together with our results, demonstrate the effect of CaMK II inhibitors on PAEC NO production and rat aorta endothelium-dependent relaxation after stimulation by Ca\(^{2+}\)-mobilizing agents.

Our results with sodium nitroprusside suggest that CaMK II did not affect soluble guanylyl cyclase activity. These findings are consistent with those of Toda et al. (47), who showed that the response to exogenous NO was unaffected by CaMK II inhibitors, whereas the agonist-dependent NO-induced increase in cGMP concentration was reduced. Thus ACh-induced relaxation was attenuated by CaMK II inhibitors, indicating their roles in NO synthesis. Furthermore, CaMK II inhibitors did not spontaneously relax endothelium-denuded rat aortic rings, suggesting that an endothelium-insensitive effect may be ruled out.

CaMK II has been implicated in the regulation of tonic vascular smooth muscle contractility mediated by myosin light chain phosphorylation (1, 46, 40). However, the CaMK II inhibitor KN-93 did not impair the contraction in response to the \(\alpha_1\)-adrenoceptor agonist phenylephrine (24). Our results with endothelium-denuded aortic rings confirm that vascular smooth muscle was insensitive to KN-93. However, a high concentration of lavendustin C slightly increased endothelium-intact contraction, suggesting that lavendustin C can increase vasoconstrictor tone by inhibiting basal NO activity. This hypothesis was confirmed by the absence of the lavendustin C contractile effect in the presence of L-NAME.

In conclusion, P281–302, KN-93, and lavendustin C, three structurally different CaMK II inhibitors, decreased the endothelium-dependent relaxation of isolated vessels and NO production by endothelial cells. These results suggest that, in addition to protein kinases A, B, and C, CaMK II was involved in NO synthesis. The agonist-induced [Ca\(^{2+}\)]\(i\) increase leads to Ca\(^{2+}\)/calmodulin-mediated eNOS activation but may also potentiate the NO synthesis by CaMK II-dependent phosphorylation of eNOS. Thus CaMK II modulates, at least in part, the activity and/or the intracellular localization of eNOS and may add another level of regulation in endothelium-dependent relaxation, which is of pharmacological interest in cardiovascular therapy.

The authors are grateful to S. Chouzenoux for technical assistance.

This work was supported by a grant from Air Liquide Santé.

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