Involvement of calcium-calmodulin-dependent protein kinase II in endothelin receptor expression in rat cerebral arteries

Roya Waldsee, Hilda Ahnstedt,* Sajedeh Eftekhari,* and Lars Edvinsson
Division of Experimental Vascular Research, Department of Clinical Sciences, Lund University and Lund University Hospital, Lund, Sweden

Submitted 14 August 2009; accepted in final form 7 December 2009

Waldsee R, Ahnstedt H, Eftekhari S, Edvinsson L. Involvement of calcium-calmodulin-dependent protein kinase II in endothelin receptor expression in rat cerebral arteries. Am J Physiol Heart Circ Physiol 298: H823–H832, 2010. First published December 11, 2009; doi:10.1152/ajpheart.00759.2009.—Experimental cerebral ischemia and organ culture of cerebral arteries result in the enhanced expression of endothelin ETB receptors in smooth muscle cells via increased transcription. The present study was designed to evaluate the involvement of calcium-calmodulin-dependent protein kinase (CAMK) in the transcriptional expression of endothelin receptors after organ culture. Rat basilar arteries were incubated for 24 h with or without the CAMK inhibitor KN93 or ERK1/2 inhibitor U0126. The contractile responses to endothelin-1 (ET-1; ETA and ETB receptor agonist) and sarafotoxin 6c (S6c; ETB receptor agonist) were studied using a sensitive myograph. The mRNA levels of the ETA and ETB receptors and CAMKII were determined by real-time PCR, and their protein levels were evaluated by immunohistochemistry and Western blot. The mRNA levels of CAMKII and the ETB receptor increased during organ culture, but there was no change in the expression of the ETA receptor. This effect was abolished by coincubation with KN93 or U0126. In functional studies, both inhibitors attenuated the S6c-induced contraction. Incubating the arteries with KN93, but not U0126, decreased the amount of phosphorylated CAMKII. The inhibitors had no effect on the levels of myosin light chain during organ culture, as measured by Western blot. CAMKII is involved in the upregulation of the endothelin ETB receptor and interacts with the ERK1/2 pathway to enhance receptor expression. CAMKII has no effect on the contractile apparatus in rat cerebral arteries. extracellular signal-regulated kinase 1/2; organ culture

ENDOTHELIN-1 (ET-1) is a regulatory peptide with paracrine and autocrine functions. The peptide is produced in the endothelium of blood vessels and is a potent contractile agent. Responses to ET-1 are mediated by two G protein-coupled receptors, the endothelin ETA and the ETB receptors (20). ETA receptor predominates vasoconstrictor receptor throughout the vascular system and is the primary mediator of the ET-1 induced response and the maintenance of vascular tone (28), whereas the role of ETB receptor remains under debate. ETB receptor localized on the endothelium may play a role in the release of prostaglandins and nitric oxide from endothelium leading to vasodilation (27). A number of stimuli can affect the endothelin receptor expression. During culture, vascular smooth muscle cells (VSMC) demonstrate a shift from ETA toward ETB receptor expression. In human blood vessels after ischemic heart disease and in rat cerebral arteries after ischemic stroke, the ETB receptor upregulation has been shown (25). It has also been shown that smaller arteries have more plasticity for ETB receptor upregulation (2). Endothelin receptor upregulation with increase in the circulating ET-1 after ischemic stroke together induced more ischemia. Our laboratory previously reported that contractile ETB receptors in VSMC are upregulated via de novo transcription following organ culture (2, 21). However, details about the intracellular signaling pathways responsible for this upregulation remain elusive. Present evidence suggests that protein kinase C (PKC) (10, 27) and mitogen-activated protein kinase (MAPK) (12, 14) are involved in this process in cerebral arteries. The MAPKs are a group of serine/threonine kinases that play an important role in intracellular signaling (11). There are three major MAPK pathways: extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun NH2-terminal kinase (JNK).

We have shown that the ERK1/2 pathway is primarily involved in ETB receptor upregulation after organ culture (12, 14) and in cerebral ischemia (19). The organ culture model is seen as a method that allows for a more detailed study of the mechanisms involved in ETB receptor regulation. Inhibition of ERK1/2 activity attenuates ETB receptor upregulation following organ culture of rat cerebral arteries (14). On the other hand, ETB receptors have the ability to activate ERK1/2 and JNK via parallel pathways that are differentially regulated by PKC and calcium ions (4).

To further our understanding of the intracellular mechanisms triggering the ETB receptor upregulation, we investigated the involvement of calcium-calmodulin-dependent protein kinase (CAMK). CAMKII is a multifunctional serine/threonine kinase with various biological targets, such as gene expression, cell cycle control, and hormone production (15, 26), and it has been implicated in the regulation of VSMC contraction. On the other hand, the roles of CAMK in endothelin receptor upregulation and mechanistically in the MEK/ERK1/2 pathway are not known. In the present study, we set out to evaluate the time dependency of an ERK1/2 inhibitor [U0126; a MEK1/2 blocker (13)] and CAMK inhibitor (KN93) on ETB receptor upregulation after organ culture.

The aim of this study was to investigate the intracellular pathways involved in endothelin receptor regulation. We hypothesized that CAMKII activity plays an important role in the initiation and activation of protein kinase cascades and that it is involved in the transcriptional regulation of the endothelin receptor.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250–300 g (Scanbur, Stockholm, Sweden) were used for this study. The animals were anesthetized with CO2 and killed by decapitation. The basilar arteries were

* H. Ahnstedt and S. Eftekhari contributed equally to this work.
Address for reprint requests and other correspondence: R. Waldsee, Div. of Experimental Vascular Research, Wallenberg Neurocenter, BMC A13, Lund Univ., SE-221 85 Lund, Sweden (E-mail: Roya.Waldsee@med.lu.se).

http://www.ajpheart.org 0363-6135/10 $8.00 Copyright © 2010 the American Physiological Society
CAMK AND ERK1/2 ARE INVOLVED IN ENDOTHELIN RECEPTOR REGULATION

isolated and placed in cold bicarbonate buffer. One basilar artery (diameter, ~0.5 mm) was divided into 4–6 ring segments (length, 1.5–2 mm). The vessels used for in vitro pharmacology studies were incubated for 24 h in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Stockholm, Sweden) supplemented with 100 μg/ml penicillin (Sigma, St. Louis, MO) at 37°C in humidified 5% CO2. During incubation, the CAMK inhibitor KN93 (10−5 M), ERK1/2 inhibitor U0126 (10−5 M), and the inactive analog of KN93 (KN92; 10−5 M) dissolved in DMSO was added at 0, 3, and 6 h after the start of incubation. Segments incubated in DMSO without inhibitor were used as control. After the incubation period, the segments were mounted in myographs as described previously (1).

The Animal Ethics Committee, Lund University (Sweden), approved the experiments (M217-03 and M161-07).

**In vitro pharmacology.** Sensitive myographs were used for recording the isometric tension in isolated basilar artery segments. The segments were threaded on two stainless steel wires (40 μm diameter) and mounted in Mulvany-Halpern myographs (Danish Myo Technology A/S, Aarhus, Denmark). The potassium and ET-1-induced contractions were used as references for the contractile capacity. Contraction-response curves for the specific endothelin ETα receptor agonist sarafotoxin 6c (S6c) were obtained by cumulative administration of the peptide (10−11–10−7 M) before ET-1-induced contraction-response curves (10−11–10−7 M) were performed. Further methodological details were previously published (1, 27). The contractile response is shown as a percentage of ET-1-induced contraction or in absolute milliNewton values.

To be certain that the contraction induced by ET-1 is only mediated via the ETα receptors in the rat basilar arteries, we evaluated the time for desensitization of the ETα receptors after administering S6c. The vessels organ cultured for 24 h were mounted in myographs, followed by incubation with ETα receptor antagonist FR139317 (Tocris; Cookson, Bristol, UK) for 15 min, and 10−7 M S6c then induced the first contraction. After the vessels were washed, S6c was added at 5, 10, 15, 30, 90, or 150 min in the presence of 10−5 M FR139317. The contractile response is shown as a percentage of potassium or ET-1-induced contraction or in absolute milliNewton values.

**Molecular biology.** To evaluate the time dependency of endothelin receptors and CAMKII expression, basilar artery segments were incubated in DMEM for 3, 6, or 24 h. To examine the role of CAMK on endothelin receptor upregulation, the basilar artery segments were cultured for 24 h in the presence or absence of KN93 (10−5 M) or U0126 (10−5 M) at 0, 3, and 6 h after initiating incubation. Arteries incubated 24 h with 10−5 M KN92 were studied to evaluate the effect of a closely related drug that does not have CAMK inhibitory activity. All vessel segments were frozen and immediately stored at −80°C until use.

Total cellular RNA was extracted using the FastRNA Pro Green kit (Qiogene, Illkirsh, France) for 60 s in the FastPrep FP120 instrument (Qiogene) following the suppliers’ instructions. The reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (PE Applied Biosystems, Foster City, CA) in a Perkin-Elmer DNA thermal cycler.

Specific primers were designed as follows: ETα receptor, 5′-ATGCGCCTCAGGAAACAC-3′ as the forward and 5′-CAACACGACGAGAGAAGGCAGTGTC-3′ as the reverse; ETβ receptor, 5′-GATGACGAGACCTTCGGCTCA-3′ as the forward and 5′-GTCCACAGTGAGGACATGAG-3′ as the reverse; and CAMKII, 5′-GGAAGCCTGCGGAAGCA-3′ and 5′-GGAAGCCTGCGGAAGCA-3′ as the forward and 5′-TGATGACCCACCGAGAAGCTG-3′ as the reverse.

**Western blot analysis.** Lysates were dissolved in Laemmli sample buffer (Bio-Rad) supplemented with 2-mercaptoethanol (Bio-Rad) and boiled for 4 min at 95°C. Equal amounts of protein (50 μg/lane) were loaded on a 4–15% linear gradient Tris- HCl gel (Bio-Rad) and separated by SDS-PAGE. Before primary antibody incubation, the membranes were blocked for unspecific binding. The primary anti-CAMKII, 5′-CGCTCCA-3′ was the forward and 5′-GGAAGCCTGCGGAAGCA-3′ as the reverse. The housekeeping genes were elongation factor-1 (EF-1) with 5′-GCAAGCCCATGTTGTTGAA-3′ as the forward and 5′-TGATGACCCACCGAGAAGCTG-3′ as the reverse.

In previous studies, we examined and compared EF-1, β-actin, and GAPDH as housekeeping genes and verified that EF-1 is stable during the organ culture procedure (27). Further methodological details have been published (14).

**Immunohistochemistry.** Segments to be used for immunohistochemistry were cultured for 24 h, and KN93 or U0126 was administered at 0 and 6 h. Vessels incubated for 24 h without inhibitor were used as control. The basilar artery segments were embedded in Tissue Tek (Gibco, Invitrogen A/S, Taastrup, Denmark), frozen at −80°C, and subsequently sectioned into 10-μm-thick slices in a cryostat (Cryo-star HM 560 M Thermo Scientific, Microm, Germany). The sections were fixed for 10 min in ice-cold acetone (−20°C) and rehydrated in PBS (pH 7.2) containing 0.25% Triton X-100 (PBST) for 5 min. Primary antibodies were used as follows: rabbit anti-CaMKII (1:100; Abcam, Cambridge, UK) or rabbit anti-ETα (1:400; Abcam), goat anti-ETα (1:100; Santa Cruz Biotechnology), or rabbit anti-phospho-ERK p44/42 MAPK (Cell Signaling Technology, Beverly, MA). The antibody against phosphorylated CAMKII did not work for immunohistochemistry; therefore, we had to choose an antibody against total CAMKII. Sections were subsequently incubated with secondary antibody for 1 h at room temperature. The secondary antibodies used were Cy3-conjugated donkey anti-rabbit (1:200; Jackson ImmunoResearch, West Grove, PA) or Cy5-conjugated donkey anti-goat (1:100; Jackson ImmunoResearch) diluted in PBST and 1% BSA. The sections were subsequently washed with PBST and mounted with Crystal mounting medium (Sigma). To determine the cellular localization of the endothelin receptor in VSMC, double immunofluorescence was performed by adding a mouse anti-smooth muscle actin antibody (Santa Cruz). Texas Red-conjugated donkey anti-mouse (Jackson ImmunoResearch) diluted 1:200 in PBST and 1% BSA was used as the secondary antibody. Immunoreactivity was visualized and photographed with an Olympus Microscope (BX 60) at the appropriate wavelengths. Negative controls for all antibodies were performed by excluding the primary antibodies; in all cases, this resulted in no specific staining. Only autofluorescence was seen in the lamina elastic interna. Double staining for actin and the endothelin ETα and ETβ receptors were done to show the colocalization of the receptors and VSMC in our samples, and the measurement was done only in the vascular media layer of the vessels. The fluorescence intensity was measured with the software program ImageJ (http://rsb.info.nih.gov/ij/). There were four rats in each group, and 4–6 sections from each rat were evaluated. The fluorescence intensity was measured in four areas in each section. The mean value of the intensity per measured area was used for the fluorescence measurements (7).

**Tissue lysis and protein content determination.** Basilar artery segments were incubated in DMEM for 0, 3, 6, or 24 h to examine the time dependency of phosphorylated CAMKII, myosin light chain (MLC), and phosphorylated MLC during organ culture. To understand whether CAMK and ERK1/2 effect MLC to decrease the contraction induced by endothelin receptors during organ culture, basilar artery segments were cultured for 24 h in the presence or absence of KN93 (10−5 M) or U0126 (10−5 M) at 0, 3, and 6 h after initiating incubation. All vessel segments were frozen and immediately stored at −80°C until use.

**AJP-Heart Circ Physiol • VOL 298 • MARCH 2010 • www.ajpheart.org**

Downloaded from http://alpha.ajpheart.org by 10.220.33.3 on June 27, 2017
bodies were mouse monoclonal anti-CAMKII phospho-specific (1:1,000; Santa Cruz Biotechnology), mouse monoclonal anti-MLC-2 phospho-specific (1:1,000; Cell Signaling Technology), rabbit polyclonal anti-MLC-2 (1:1,000; Cell Signaling Technology), or mouse monoclonal anti-β-actin (1:5,000; Santa Cruz Biotechnology). Subsequently, the membranes were incubated with the appropriate secondary antibody for 1 h at room temperature: IgG-horseradish peroxidase horse anti-mouse or goat anti-rabbit (1:2,000; Cell Signaling). The protein levels of β-actin were used to confirm equal loading. The membranes were developed using the Supersignal West Dura kit (Pierce, Rockford, IL) and visualized using a Fujifilm LAS-1000 Luminescent Image Analyzer (Stamford, CT).

Band densities were quantified using the ImageJ program. The optical density values for the immunoblots were presented as fold changes in activity in the treated groups.

Drugs and solutions. The physiological saline solution buffer used for myograph studies of the basilar artery had the following composition (in mM): 119 NaCl, 15 NaHCO3, 4.6 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 1.2 CaCl2, and 5.5 glucose. The solution was gassed with 5% CO2 in O2, resulting in a pH of 7.4. Analytical grade chemicals and double-distilled water were used in the preparation of all solutions. ET-1 and S6c were purchased from Sigma and Alexis Biochemical (Lausen, Switzerland), respectively, and dissolved in 0.9% saline with 0.1% BSA (Kabi, Stockholm, Sweden). KN93 and KN92 were dissolved in DMSO. Chemical ingredients were purchased from Sigma.

Statistics. The maximal systolic elastance (Emax) values refer to the maximum contraction given as an absolute value (in mN) or calculated as a percentage of the contractile capacity of ET-1 because KN93 inhibited the potassium-induced contraction.

Results are given as means ± SE, and n refers to the number of vessel segments. There were 3–6 rats in each group with 4–6 vessel segments from each rat. The pEC50 value is the negative logarithmic value of the stimulator concentration (here, S6c or ET-1) needed to produce half maximum contraction in the vessels. When vessels were incubated with inhibitor, the pEC50 decreased, meaning that higher concentrations of S6c are required to induce the same maximal contraction. This value was calculated from the line between the concentrations above and below the midpoint of the concentration-response curve.

The Kruskal-Wallis nonparametric test with Dunn’s post hoc test was used for PCR and Western blot results. The control for time-dependent activity is fresh arteries, and for arteries incubated with different inhibitors, the control is 24 h incubation with DMSO in all experiments. The nonparametric t-test with Mann-Whitney was used for immunohistochemistry results, and the control is the 24-h incubation with DMSO. The level of significance for all tests was set at P < 0.05.

RESULTS

Alteration in CAMKII and endothelin receptor mRNA expression during organ culture. With the use of basilar artery segments, real-time PCR showed that there was a significant increase in CAMKII (P < 0.02) and ETB receptor mRNA levels (P < 0.001) after 24 h of incubation in DMEM compared with fresh vessels (Fig. 1). There was no significant change in ETA receptor mRNA. These data show that organ culture induces CAMKII activation and ETB receptor upregulation but not ETA receptor upregulation.

Effect of KN93 and U0126 on CAMKII and endothelin receptor mRNA levels. Coincubation of the basilar artery segments with KN93, administered at 0, 3, or 6 h, after initiation of organ culture reduced significantly the enhanced CAMKII and ETB receptor mRNA expression during organ culture for 24 h (Fig. 2). Incubation in DMEM without KN93 (24 h) was considered the control; a 24-h incubation with KN92, the inactive analog of KN93, had no antagonist effect and was considered the control; a 24-h incubation with KN93 inhibited the potassium-induced contraction.

Fig. 1. Relative CAMKII (A), ETA receptor (B), and ETA receptor (C) mRNA levels compared with housekeeping gene elongation factor-1 (EF-1) in organ cultured rat basilar arteries. The arteries were incubated for 0 (fresh), 3, 6, or 24 h in DMEM. Nonincubated segments (fresh) were used as control. Data are presented as means ± SE; n = 4–7. *P < 0.05; **P < 0.001.
Incubation with KN92 did not result in any change in the parameters studied, and the results did not differ from incubation with DMSO for 24 h (Fig. 2D).

Incubation of the vessels with U0126 attenuated the increase in ET<sub>B</sub> receptor mRNA induced by organ culture and diminished the level of ET<sub>A</sub> receptor mRNA, but had no effect on the CAMKII mRNA level (P < 0.07) (Fig. 2). Arteries incubated for 24 h in the presence of U0126, added at 3 h, had significantly decreased ET<sub>B</sub> receptor expression (P < 0.01), and ET<sub>A</sub> receptor mRNA levels (P < 0.019; Fig. 2, B and C). On the other hand, the addition of U0126 at 6 h after start of organ culture significantly attenuated the increase in ET<sub>B</sub> receptor mRNA (P < 0.001) and reduced ET<sub>A</sub> receptor (P < 0.01) mRNA levels.

These data show that both KN93 and U0126 have an inhibitory effect on ET<sub>B</sub> receptor upregulation after organ culture; in addition, they may diminish the constitutional ET<sub>A</sub> receptor mRNA level. Interestingly, U0126 was more effective when added at 6 h after initiating incubation compared with KN93, whereas U0126 had no effect on CAMKII expression.

KN93 and U0126 attenuate contractions induced by potassium and endothelin receptors. Contractile responses to the cumulative administration of S6c and ET-1 were evaluated in myograph studies using isolated ring segments. In fresh vessel segments, S6c showed no contractile response, whereas the effect of ET-1 was strong and concentration dependent. The effect induced by ET-1 was mediated by the ET<sub>A</sub> receptor, as previously shown (8). After organ culture, a S6c-mediated contractile effect occurs via upregulated smooth muscle cell ET<sub>B</sub> receptors (8). Our laboratory previously showed that S6c can desensitize the ET<sub>B</sub> receptors, and ET-1 then solely activates the ET<sub>A</sub> receptors (2). To be certain that the contraction induced by ET-1 occurred only via the ET<sub>A</sub> receptors, we initially evaluated the desensitization time of the ET<sub>B</sub> receptors after administering a high dose of S6c (10<sup>-7</sup> M). Figure 3D shows that the ET<sub>B</sub> receptors were not resensitized at 5 min; hence, ET-1 could induce contraction only via ET<sub>A</sub> receptors at this time point. In our experiments, ET-1 was added directly after washing the vessels of S6c and attaining baseline tone. Table 1 shows that the contraction induced by ET-1 did not differ by S6c application time. This observation suggests that, despite some ET<sub>B</sub> receptor resensitization after 5 min, and up to 150 min, the ET<sub>B</sub> receptor has no meaningful effect on the maximum level of ET-1-induced contraction.

The concentration-dependent contraction induced by S6c was significantly attenuated (P < 0.001) in arteries incubated with KN93 (Fig. 3A) or U0126 (Fig. 3B) when added at various time points compared with control, but KN92 had no significant effect on S6c-induced contraction (Fig. 3C). These results indicate that the addition of inhibitors after initiating incubation (at 3 and 6 h) has a strong inhibitory effect on S6c-induced contraction. Vessels incubated with U0126 or KN93 had a reduced contractile response to S6c at 6 h compared with 0 h, which was more apparent for U0126 than KN93 (Fig. 3). No changes in the pEC<sub>50</sub> of S6c were observed, suggesting that the number of receptors is altered, not their characteristics (1, 12).

ET-1-induced contraction decreased significantly (P < 0.01) in vessels incubated with KN93 only when added at 6 h but not upon U0126 treatment (Table 2). To perform an in-depth pharmacological test, basilar arteries were incubated for 24 h in DMEM and KN93 was administered at 0, 3, and 6 h after initiating incubation. Concentration-response curves for ET<sub>A</sub> receptors were obtained by cumulative administration of ET-1 (10<sup>-11</sup>–10<sup>-7</sup> M), and arteries were incubated with specific ET<sub>A</sub> inhibitors.
receptor antagonist before administration of ET-1. Results show (Fig. 4) that the ETA receptor antagonist caused rightward shift in contractions induced by ET-1 in arteries incubated with KN93 added at 6 h (pEC50, 7.54 ± 0.01) more than when it added at 0 (pEC50, 7.78 ± 0.07) and 3 (pEC50, 7.70 ± 0.01) h. This suggests that ET receptors are more sensitive to their antagonist when KN93 was added at this time point. The Emax value induced by ET-1 is also lower in this group (4.04 ± 0.65 mN) compared with other groups (Table 2 and Fig. 4). This could be dependent on lower number of ETA receptors. There is significant decrease in mRNA levels of ETA receptors in arteries incubated with KN93 added at 0 h, but functional studies did not shown decrease in Emax for ET-1-induced contraction in these groups. We cannot explain this discrepancy, but experiments with the ETA antagonist show that incubation with KN93, added at 6 h, has an effect on both ETA receptors and actin confirmed the receptor localization in the smooth muscle cell. The reported measurements were obtained only in the media layer (Fig. 5C).

Table 1. The absolute value of ET-1 and potassium-induced contractions in basilar arteries

<table>
<thead>
<tr>
<th>S6c Addition, min</th>
<th>ET-1</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emax, mN</td>
<td>Emax, %</td>
<td>n</td>
<td>K</td>
<td>Emax, mN</td>
</tr>
<tr>
<td>5</td>
<td>9.13 ± 1.3</td>
<td>148.6 ± 2.73</td>
<td>6</td>
<td>6.35 ± 1.19</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>7.71 ± 1.22</td>
<td>132.8 ± 3.03</td>
<td>7</td>
<td>6.05 ± 1.04</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>12.24 ± 2.1</td>
<td>134.6 ± 1.99</td>
<td>8</td>
<td>9.09 ± 1.24</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>10.06 ± 1.34</td>
<td>140.8 ± 1.8</td>
<td>6</td>
<td>7.21 ± 1.01</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>13.81 ± 3.8</td>
<td>137.4 ± 2.71</td>
<td>5</td>
<td>9.45 ± 0.76</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>9.34 ± 0.85</td>
<td>137.4 ± 5.2</td>
<td>5</td>
<td>6.07 ± 0.76</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Arteries were incubated for 24 h in DMEM. The vessels were precontracted with sarafotoxin 6c (S6c), and S6c was added again 5–150 min after the first contraction. ET-1, endothelin-1. analog KN92 were not significantly different from control (Fig. 3C and Table 2). At this time, we cannot explain the effect of inhibitors on potassium-induced contraction in our model. Because KN93 decreased contraction induced by endothelin receptors and potassium, we want to evaluate the nonspecific effect of this inhibitor. We have examined other vasoconstrictors like 5-HT (serotonin) and U46619 (9, 11-diepoxymethanopy prostaglandin F2α; Fluka Chemie AG, Switzerland), which induce contraction in our vessels, and KN93 had no inhibitory effect to these vasoconstrictors (data not shown).

KN93 decreased endothelin receptors, CAMKII, and p-ERK1/2 protein levels. The protein levels of CAMKII, p-ERK1/2, and the ETA receptors were evaluated by immunohistochemistry using specific monoclonal antibodies. Vessels incubated for 24 h with or without KN93 (added at 0 h) expressed reduced protein levels of CAMKII (18 ± 3.9%; P < 0.05), p-ERK1/2 (26.3 ± 1.8%; P < 0.001), ETB receptor (28.5 ± 4.0%; P < 0.0003), and ETA receptor (25.5 ± 4.7%; P < 0.0001; Fig. 5B). Image analysis (Fig. 5B) showed that CAMKII is inhibited if KN93 is given at 0 h but not at 6 h. KN93 also inhibited the p-ERK1/2 activity and abolished the increase in ETB and ETA receptors at both 0 and 6 h. Incubation with KN93, applied after 6 h of organ culture, decreased the ETB receptor (16 ± 2.8%; P < 0.01), ETA receptor (13.3 ± 5.9%; P < 0.01), and p-ERK1/2 (24.3 ± 2.3%; P < 0.001) protein levels compared with control, but there was no difference in the CAMKII protein level. Thus KN93 has an inhibitory effect on the expression of endothelin receptors, p-ERK1/2, and CAMKII proteins after incubation for 24 h; it has a lesser effect when added 6 h after starting incubation. Double immunostaining for endothelin ETA or ETB receptors and actin confirmed the receptor localization in the smooth muscle cell. The reported measurements were obtained only in the media layer (Fig. 5C).
U0126 decreased the endothelin receptor protein levels. Our laboratory previously showed that ERK1/2 phosphorylation is involved in the enhanced upregulation of ETB receptors (12, 13). Here we demonstrated that KN93 has an inhibitory effect on p-ERK1/2 in the organ culture model. To evaluate the effect of ERK1/2 on the expression of CAMKII and endothelin receptors, we examined the effect of the selective MEK1/2 inhibitor U0126. The ETB (27.5 ± 6.1%; P < 0.05) and the ETA (37.4 ± 5.6%; P < 0.001) receptor protein levels were reduced in arteries coincubated with U0126 for 24 h (Fig. 6). Incubation with U0126 applied at 6 h also showed decreased protein levels for ETB receptors (37.2 ± 6%; P < 0.001) and ETA receptors (31.7 ± 6.1%; P < 0.001) compared with control, but there was no significant change in vessel wall CAMKII expression (P > 0.05).

These results show that the ERK1/2 inhibitor has no effect on CAMK, but the CAMK inhibitor has an effect on p-ERK1/2, and both have an effect on endothelin receptor expression during the organ culture procedure.

To show the phosphorylation of CAMK targets in VSMC layer, arteries were incubated for 0, 3, 6, and 24 h in DMEM. Samples were evaluated by immunohistochemistry to show the CAMK activation during organ culture and by Western blot that is shown in Fig. 8.

Western blot results. Because organ culture resulted in marked changes in the expression of the endothelin receptors, we wanted to know whether there was any change in MLC20 during organ culture. The level of phosphorylated MLC and total MLC in the presence and absence of KN93 or U0126 was measured by Western blot to determine whether endothelin

Table 2. Absolute contractile values induced by S6c, ET-1, and potassium after organ culture and upon coincubation with different inhibitors

<table>
<thead>
<tr>
<th></th>
<th>S6c</th>
<th>ET-1</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emax, mN</td>
<td>Emax, %</td>
<td>rEC⁵₀</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>5.22 ± 1.13</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>KN93</td>
<td>3</td>
<td>3 ± 0.65</td>
<td>35 ± 7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.92 ± 0.42</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>U0126</td>
<td>0</td>
<td>0.6 ± 0.13</td>
<td>16 ± 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.90 ± 0.93</td>
<td>29 ± 6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.27 ± 0.43</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>KN92</td>
<td>0</td>
<td>0.25 ± 0.25</td>
<td>2 ± 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.64 ± 0.54</td>
<td>64 ± 5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.76 ± 0.8</td>
<td>51 ± 4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.96 ± 1.13</td>
<td>42 ± 4</td>
</tr>
</tbody>
</table>

*Values are means ± SE. Arteries incubated for 24 h were control. Inhibitor KN93, U0126, or KN92 was added at 0, 3, or 6 h after initiating incubation. *P < 0.05; †P < 0.001; ‡P < 0.0001.
receptor-induced contractions involve changes in MLC. The level of p-MLC decreased after 3 h and reached almost zero after 24 h of incubation; on the other hand, the level of total MLC did not differ during organ culture (Fig. 7). In addition, incubation with the inhibitors had no effect on MLC expression (Fig. 7C). These results show that KN93 and U0126 have no effect on these aspects of the contractile apparatus and that their effects must thus involve the receptor contraction pathway in a different manner (e.g., receptor upregulation).

The level of p-CAMKII was measured during organ culture in the presence and absence of KN93 or U0126. The p-CAMKII level decreased at 3 h and remained at the same level during organ culture that shows in Fig. 8A by Western blot and in Fig. 8B by immunohistochemistry. CAMK activation started early and incubation of the arteries with KN93, but not U0126, decreased p-CAMKII. This finding was confirmed by real-time PCR and immunohistochemistry.

**DISCUSSION**

Under normal conditions there is a balance between the distribution of contractile $\text{ET}_\text{A}$ receptors in cerebrovascular smooth muscle cells and relaxant $\text{ET}_\text{B}$ receptors; however, in stroke or after a subarachnoid hemorrhage this is altered with significant rise in contractile $\text{ET}_\text{B}$ receptors in the smooth muscle cells (6).

Our research is focused on mechanisms involved in the expression of $\text{ET}_\text{B}$ receptors in cerebral arteries and their

---

**Fig. 5.** Immunohistochemistry (A) and fluorescence intensity (B) of the vascular smooth muscle layer of the rat basilar arteries incubated for 24 h in the presence or absence of CAMK inhibitor (KN93; 10$^{-5}$ M), which was added at the start of incubation (0 h) or 6 h after initiating incubation. C: double immunostaining of the vessels shows the colocalization of the vascular smooth muscle cells (actin) and endothelin receptor in media layer. Negative controls for all antibodies in A and C were made by excluding primary antibodies and in all cases resulted in no specific staining; only autofluorescence in lamina elastic interna was seen. A: negative control; a: cy3-conjugated donkey anti rabbit; b: cy3-conjugated donkey anti goat; c: Texas Red-conjugated donkey anti mouse. *P < 0.05; **P < 0.001; ***P < 0.0001.

**Fig. 6.** Effect of the MEK/ERK1/2 inhibitor on endothelin receptor and CAMKII protein levels in rat basilar arteries incubated for 24 h. Controls were incubated with DMSO for 24 h. Immunohistochemistry (A) and the fluorescence intensity (B) of the measured areas in the vessel walls are shown. The pictures were taken with an Olympus microscope (×20 objective). Data are shown as means ± SE; n = 4. *P < 0.05; **P < 0.001; ***P < 0.0001.
upregulation, as seen in experimental cerebral ischemia (9, 25) and in human stroke (23). The upregulation or enhanced expression of endothelin receptors after stroke may be due to changes in perfusion pressure (shear stress) during and after the occlusion (12). This hypothesis is supported by other studies that have shown that ETB receptor mRNA levels in rat aortic smooth muscle cells are increased by up to 10-fold after periodic stretch (5) and by the finding that increased perfusion pressure in isolated rat mesenteric arteries resulted in enhanced

**Figure 7.** Vessels were incubated for 0 (fresh), 3, 6, or 24 h, and the level of myosin light chain (MLC; A) and phosphorylated MLC (p-MLC; B) was measured by Western blot to determine whether organ culture affects the MLC level. C: effect of KN93 (10^{-5} M) or U0126 (10^{-5} M) was measured after adding the inhibitors to the incubated vessels at different time points (0, 3, and 6 h). The 24-h incubation was used as control. Data are shown as means ± SE; n = 3.

**Figure 8.** The vessels were incubated for 0, 3, 6, or 24 h, and the level of phosphorylated CAMKII (A) was measured by Western blot and by immunohistochemistry evaluation (B) showing localization and augmentation of CAMK in the media layer of basilar artery during organ culture. C: effect of KN93 (10^{-5} M) or U0126 (10^{-5} M) on p-CAMKII was evaluated by incubating the vessels for 24 h and adding the inhibitors at different time points (0, 3, and 6 h) after initiating incubation. Data were compared with 24 h incubation with DMSO. Data are shown as means ± SE; n = 3. **P < 0.001.
expression of ET$_B$ receptor mRNA and protein (17). Furthermore, several studies have shown that circulating ET-1 levels are increased in ischemic stroke (16), which, together with more endothelin receptors in contractile smooth muscle cells, suggests a role in cerebral ischemia. We hypothesize that ET-1 and endothelin receptor regulation are important in cerebrovascular pathophysiology, whereby cerebral blood vessels are modified after ischemic stroke and possibly participate in the development of the penumbra (6). In support, systemic administration of U0126 reduces cerebrovascular ET$_B$ receptor expression and infarct volume after 2 h of ischemia followed by 48 h of reperfusion in rat (19).

Our laboratory previously showed that endothelin receptor expression is controlled by the MEK/ERK1/2 pathway (13). Here we make a novel demonstration that the expression of p-ERK1/2 is modified by CAMKII activity; inhibition of CAMKII reduced the receptor expression induced by organ culture. Blockade of MEK/ERK1/2 pathway with U0126 did not alter CAMKII, but it altered the endothelin receptor expression. This is the first study to show that CAMKII inhibition reduces organ culture-induced expression of endothelin receptors in cerebrovascular smooth muscle cells.

Extracellular calcium influx and the binding of calcium to intracellular calmodulin is an important pathway for inducing smooth muscle cell contraction. CAMK has been implicated in the regulation of VSMC contraction. Intracellular contractile proteins, such as MLC kinase, MLC20, caldesmon, and calponin, are substrates of CAMK (15, 24). However, our goal was not to study the direct contraction-calcium homeostasis but to evaluate whether CAMKII is involved in ET$_B$ receptor expression as a long-term effect.

The organ culture model provides a method for mimicking changes that occur in cerebral ischemia (29) and for studying the intracellular mechanisms involved in more detail, such as the time course of both mRNA and functional receptor changes (22).

Incubation of the basilar arteries with KN93 or U0126, but not KN92, had significant effects on S6c-induced contraction (Fig. 3). When the inhibitors were added at 3 or 6 h into the incubation, they had a greater effect on the S6c-induced contraction. The reason for this may be that the appropriate timing of the inhibitors is after the process leading to ET$_B$ receptor mRNA expression has been initiated (Fig. 1B). On the other hand, we have shown that the ERK1/2 inhibitor is most effective when added 6 h after initiating incubation, but KN93 was more effective when added at 0 h. This finding suggests that ERK1/2 activity starts slightly later than that of CAMK. This may suggest that ERK1/2 is downstream of CAMK when considering the immunohistochemistry, PCR, and Western blot data. We have previously revealed that organ culture per se induces ET$_B$ receptor upregulation, which has been used as a way to mimic the in vivo upregulation and shown in detailed studies to occur via the MEK/ERK pathway (12, 28). This conclusion is, in part, supported by data showing that CAMKII mediates ERK1/2 activation in response to calcium-mobilizing stimuli and cell adhesion in VSMC (3). Immunohistochemistry showed a significant effect of KN93 on ERK1/2 (26.32 ± 1.8%; $P < 0.001$), which confirms the results from earlier studies (18). The present results show for the first time in cerebrovascular smooth muscle cells that CAMK is involved in ET$_B$ receptor upregulation and may activate the ERK1/2 pathway.

Surprisingly, KN93 also decreased the ET$_A$ receptor mRNA and protein levels and, in the functional tests, the ET$_A$-mediated contraction (Table 1). This effect seems robust and suggests a role of CAMKII in the constitutional expression of ET$_A$ receptors. This effect is different from U0126, which had effect on the mRNA and protein level of ET$_A$ but no effect on the ET$_A$-mediated contraction, which is important in vasoconstriction. The E$_{\text{max}}$ value of ET-1-induced contraction was decreased, and the rightward shift induced by ET$_A$ receptor antagonist was increased by incubation of arteries with KN93 (added at 6 h). This suggests that KN93 has effect on both the number and the sensitivity of the receptors. The results make the CAMK pathway interesting for the understanding of contraction induced by endothelin receptors.

On the other hand, KN93 decreased the potassium-induced contraction in basilar arteries that was significant when added at 3 and 6 h into the incubation, whereas U0126 and KN92 decreased slightly the potassium-induced contraction only when added at 6 h and was not significant. As demonstrated in Table 2, the ET-1-induced contraction was not altered by all inhibitors and only when KN93 was added at 6 h ($E_{\text{max}} = 4.04 ± 0.65$ mN); therefore, we could use it as a reference instead of potassium in this part of the study.

The tissue baths were washed completely of KN93 and incubated in buffer without KN93 for at least 2 h before performing the pharmacological study. Thus the attenuated contractions of arteries in response to potassium and ET$_B$ receptor activation by KN93 suggest an effect on the intracellular signaling. The incubation of porcine carotid arteries for a short time (30 min) with KN93 decreased potassium- and histamine-induced contraction, suggesting that CAMKII has a role in regulating smooth muscle cell contractility by modulating MLC kinase activity (20). In ferret aortas, KN93 decreased the potassium-induced contraction. This observation revealed that CAMKII activates the MAPK pathway, resulting in phosphorylation of MLC20 via MLC kinase (13). Our results confirm these data and show that KN93 also reduced the potassium-induced contraction after a long incubation time (24 h).

The other question was whether the level of MLC20 is altered during organ culture. Four days after subarachnoid hemorrhage in canine cerebral arteries, the level of p-MLC increased (23) and a high level of phosphorylated MLC plays a role in the development of cerebral vasospasm. We showed that the p-MLC level decreased to zero during organ culture after 24 h of incubation, but the level of MLC did not change. In control arteries incubated for 24 h, a low level of p-MLC had no effect on S6c-induced contraction. Moreover, KN93 and U0126 had no significant effect on the level of MLC during organ culture. Thus the attenuation of S6c-induced contraction could be due to receptor expression in our vessels.

In conclusion, our results show that CAMK is associated with ERK1/2 in endothelin receptor regulation and contraction. A CAMK inhibitor could be used to inhibit ET$_A$ receptor upregulation, possibly via interaction with an ERK1/2 inhibitor (12). The results from this study need more experimental evaluation with regard to both the intracellular calcium changes and the relationship between endothelin receptors and CAMKII.

**GRANTS**

This study was supported by grants from the Swedish Research Council (Grant No. 5958) and the Swedish Heart and Lung Foundation.
REFERENCES


