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Inositol 1,4,5-Trisphosphate Receptor and Calcium Calmodulin-Dependent Protein Kinase are Involved in Endothelin Receptor Expression in Rat Cerebral Artery

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Abstract

Background: The present study was designed to examine the influence of inositol 1,4,5-trisphosphate receptor (IP3R) and calcium calmodulin-dependent protein kinase (CaMK) on endothelin receptor regulation during organ culture in rat middle cerebral arteries (MCA).

Methods: MCA segments were incubated with or without xestospongin C (XeC), an IP3R inhibitor, or KN93, a CaMKII blocker. The mRNA levels of the ET_A and ET_B receptors, nuclear factor of activated T cells activating protein (NFam1), CaMKII, IP3R and Downstream regulatory element antagonist modulator (DREAM) protein were determined by real-time PCR or immunohistochemistry. Contractile responses to endothelin-1 (ET-1) and sarafotoxin 6c (S6c) were studied by a sensitive myograph and the intracellular calcium levels $[Ca^{2+}]_i$ were evaluated by the FURA-2AM.

Results: The contractile responses to ET-1 and S6c, the ET_B receptors mRNA level and the baseline level of intracellular calcium $[Ca^{2+}]_i$ were all increased after 24 h organ culture. Incubation with XeC or KN93 attenuated ET_B receptor expression and increase in $[Ca^{2+}]_i$ induced by ET-1 or S6c. XeC decreased the NFam1 mRNA levels while KN93 decreased DREAM protein levels.

Conclusions: The study suggests that CaMKII and calcium release via IP3R are involved in the mechanisms regulate ET_B receptor putatively via DREAM and NFam1, respectively.

Keywords: IP3R; CaMKII; Endothelin receptors; Organ culture

Abbreviations: CAMK: Calcium-Calmodulin Dependent Protein Kinase; CREB: cAMP-Responsive Element Binding Protein; DREAM: Downstream Regulatory Element Antagonist Modulator; EF-1: Elongation Factor; ERK: Extracellular Signal-Regulated Kinase; IP3R: Inositol 1,4,5-Trisphosphate Receptor; MAPK: Mitogen Activated Protein Kinase; MCA: Middle Cerebral Artery; NFam1: Nuclear Factor of Activated T Cells Activating Protein; SAH: Subarachnoid Hemorrhage; S6c: Sarafotoxin 6c; XeC: Xestospongin C

Introduction

Cerebral ischemia after middle cerebral artery occlusion (MCAO) or after subarachnoid haemorrhage (SAH) is associated not only with elevated endothelin-1 (ET-1) levels but also with an increased number of endothelin receptors and stronger contractions in cerebral arteries [1-3]. Together, these changes lead to a powerful ET-1 mediated vasoconstriction following ischemic insult. We have found that when isolated middle cerebral artery (MCA) segments were organ cultured, some aspects of vascular alternation occur that mimic changes observed after stroke [4]. These changes include activation of mitogen activated protein kinase (MAPK) pathway, inflammation and ET receptor regulation [5-8]. Thus, organ culture of isolated cerebral arteries provides a way to study in detail the mechanisms involved in ET receptor regulation in cerebral arteries. We have reported that ET receptor expression is impacted by a number of intracellular signaling pathways and transcriptional regulation [1,9]. In addition to MAPK, calcium calmodulin-dependent protein kinase (CaMKII) is activated during organ culture [6,10-12]. Interestingly, co-incubation with a CaMKII inhibitor decreases ET_B receptor upregulation and S6c (ET_B receptor agonist) -induced contraction after organ culture in rat cerebral arteries [12]. Inhibition of CaMKII reduces ET_B receptor upregulation associated with experimental SAH, and it has a neuroprotective effect after experimental cerebral ischemia [13].

Based on our findings with CaMKII [12], we hypothesize that calcium mechanisms are involved in ET receptor regulation. To examine this, we used KN93, a CaMKII blocker, and the inositol 1.4.5-trisphosphate receptors (IP3R) inhibitor, xestospongin C (XeC), which inhibits calcium release from IP3R channels. In addition, we examined the effect of XeC and KN93 on transcription factor expression, including; (1)- downstream regulatory element antagonist modulator (DREAM) which is the Ca^{2+}-regulated transcription repressor [14] and binding to calcium ions inactivates it which permits activation of c-fos and CREB [15]; (2)- the cAMP-responsive element binding protein (CREB) which can be activated by contractile vasomotor agonists, growth factors, and (3)- the nuclear factor of activated T cells activating protein (NFam1), which is regulated by calcium and calcineurin [16]. We propose that calcium and calcium release from IP3R as well as CaMKII are involved in regulation of endothelin receptor expression during organ culture.

Material and Methods

Animals

The Animal Ethics Committee (Sweden) approved the experiments (2010, M217-03 and M161-07).

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The middle cerebral arteries (MCAs) from male Wistar rats weighing 250-300 g (Scanbur, Stockholm, Sweden) were incubated for 24 h in Dulbecco's modified Eagle's medium (DMEM; Gibco, Stockholm, Sweden) supplemented with 100 U/ml penicillin (Sigma, St Louis, MO, USA).

Segments were incubated for 24 h with or without the CaMKII inhibitor KN93 (10^{-5} M) [12], or inhibitor of IP3R-induced Ca^{2+} release from the endoplasmic reticulum xestospongin C (XeC) (25×10^{-6} M) [17] dissolved in DMSO. Control vessels were incubated with the same amount of DMSO.

In vitro pharmacology

The artery segments were mounted in a Mulvany-Halpern myograph (Danish Myo Technology A/S, Aarhus, Denmark). The vessel segments were immersed in temperature-controlled tissue baths (37°C) containing a bicarbonate buffer solution containing (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5, and glucose 5.6. The contractile capacity of each vessel was determined by exposure to an isotonic solution containing 63.5 mM potassium, obtained by a partial replacement of NaCl with KCl in the above buffer.

Concentration-response curves were obtained by the cumulative application of sarafotoxin 6c (S6c), an ET_B receptor agonist, at a concentration range of 10^{-11} to 10^{-7} M (Alexis Biochemicals, Farmingdale, NY, USA) and endothelin-1 (ET-1), an endothelin receptor A (ET_A receptor) agonist in the present study due to ET_B receptor desensitization, at a concentration range of 10^{-11} to 10^{-7} M. This protocol has been validated previously using the selective ET_A and ET_B receptor antagonists, FR133917 and BQ788, respectively [12].

Molecular biology

To examine the role of inhibitors on the mRNA expression of IP3R and CaMK, the arteries were cultured for 24 h in the presence or absence of KN93 (10^{-5} M) and XeC (25×10^{-6} M). All vessel segments were frozen and immediately stored at -80°C until use. Total cellular RNA was extracted using the FastRNA Pro Green kit (Qbiogene, France). Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) in a Perkin-Elmer DNA thermal cycler. Quantitative real-time PCR (qPCR) was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR® Green kit or TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). A sample without template served as a control. Specific primers were designed (Table 1). The IP3R has three types 1, 2 and 3 [18]. In this study we have evaluated the IP3R type 3 genes (Itpr3). IP_3R3 appeared to be located along the actin filaments in VSMCs [19]. In addition, we measured the NFAT activating protein with ITAM motif mRNA level because it not only represents the activation of NFAT but also the activation of intracellular calcineurin/NFAT signaling pathways [20,21]. In previous studies, we examined and compared elongation factor-1 (EF-1), β-actin, and GAPDH as housekeeping genes, and verified that EF-1 and β-actin are stable during organ culture [22].

Immunohistochemistry

Vessels segments were embedded in Tissue TEK (Gibco, Invitrogen A/S, Taastrup, Denmark), frozen at -80, and cryo-sectioned (10 µm, Cryo-star HM 560 M Thermo Scientific, Microm, Germany). The sections were fixed for 10 min in -20°C acetone and rehydrated 3×5 min in phosphate buffered saline (PBS, pH 7.2) containing 0.25% Triton X-100 (PBST). The sections were incubated overnight at 4°C with primary antibodies, anti-DREAM (1:250, FL-214, sc-9142 Santa Cruz Biotech., Inc. CA, USA) and rabbit polyclonal anti-CREB $pSpS^{129/133}$ phosphospecific (1:250, 44297G, Invitrogen, La Jolla, CA, USA) diluted in PBST containing 1% bovine serum albumin (BSA). After incubation, sections were rinsed in PBST for 2×15 min and incubated for 1 h at room temperature with secondary antibody FITC (1:100 Cayman Chemical, Ann Arbor, MI, USA) diluted in PBST containing 1% BSA. The sections were then rinsed in PBST for 2×15 min and mounted with anti-fading mounting medium (DAPI-containing Vectashield; Vector Laboratories Inc., Burlingame, CA, USA). The same experimental procedure was used for negative controls, where primary antibodies were omitted. This resulted in no immunoreactivity except for auto-fluorescence in the internal elastic lamina. Immunoreactivity was visualized at the appropriate wavelength with an epifluorescence microscope (Nikon 80i; Tokyo, Japan) and photographed with an attached Nikon DS-2Mv camera. The fluorescence intensity was measured with the Image J software (http://rsb.info.nih.gov/ij/) in a blinded fashion by a second unbiased person. Each treatment group contained of 4 rats, and 4-6 sections were evaluated for each rat. The fluorescence intensity was measured in four standardized areas in each section. The mean intensity per measured area was used as the fluorescence measurement. The intensity of each group is compared to fresh (not incubated) as a percent increase. Results are shown as mean ± SEM and n refers to the number of vessels in each group for each antibody.

Intracellular calcium measurements

MCAs were mounted as rings on two 40 µm stainless steel wires connected to a force transducer and a micrometer in a specially designed organ bath of a small vessel myograph (Danish Myo Technology A/S, Aarhus, Denmark). After mounting, the arteries were loaded with the fluorescent $[Ca^{2+}]_i$ indicator Fura-2/AM (an acetoxymethyl ester form of Fura-2) 10 µM, anhydrous dimethylsulfoxide (DMSO) 0.5% and 0.04% pluronic F-127 [23]. For Ca^{2+} measurements, the myograph was placed on the stage of an inverted microscope (Olympus IX70, America). The artery was illuminated with 340 nm and 380 nm light. Intracellular Ca^{2+}

Assay ID	Gene Symbol	Gene Name or Primer Sequence
Rn00667869-m1	Actb	Actin, beta
Rn01503888-m1	Nfam1	NFAT activating protein with ITAM motif
NM_175838	EF-1, Elongation factor 1	GCAAGCCCATGTGTGTTGAA TGATGACACCCACAGCAACTG
NM_012550	ET_A, Endothelin receptor A	ATTGCCCTCAGCGAACAC CAACCAAGCAGAAAGACGGTC
NM_017333	ET_B, Endothelin receptor B	GATACGACAACTTCCGCTCCA GTCCACGATGAGGACAATGAG
NM_012920.1	CaMKII	GGA AGC CTG CGG AAG CA TTA CAC TGA GTC TCC TGA GTC CAA A
Rn00565664-m1	Itpr3	Inositol 1,4,5-triphosphate receptor, type 3

Table 1: Primer sequences and accession number for the genes investigated by use of real time PCR.

concentration ($[Ca^{2+}]_i$) was calculated according to the equation: $[Ca^{2+}]_i = K_d \cdot ß \cdot [(R-R_{min})/(R_{max}-R)]$ [24] with the assumption that the dissociation constant of FURA-2-Ca^{2+} complex, K_d, is 225nM at 37°C. The parameter β is the ratio of emission at 380 nm excitation at maximum and minimum Ca^{2+} levels (corrected for background fluorescence signals). R_{min} and R_{max} were determined in each vessel at the end of the experiment by adding 40 μM ionomycin in calcium-free buffer solution and by using buffer solution containing 5 mM Ca^{2+} (see below for composition). Before calculating the ratio (R) between emission at 340 nm and emission at 380 nm, background fluorescence was measured by quenching the calcium-sensitive Fura-2 fluorescence with 20 mM Mn^{2+} ($MnCl_2$) at the end of each experiment [23]. PSS had the following composition (in mM): NaCl 125, $NaHCO_3$ 5, KCl 5, NaH_2PO_4 0.5, $MgCl_2 \cdot 6H_2O$ 2, $CaCl_2$ 1.8, BSA 0.5, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) 5, and glucose 10 with the pH adjusted to 7.4. Solutions used for determining R_{min} and R_{max} contained (in mM): HEPES 5, KCl 125, $MgCl_2 \cdot 6H_2O$ 1.17, and glucose 5.5, and either 2 mM EGTA or 10 mM $CaCl_2 \cdot 2H_2O$, respectively.

Statistics

The E_{max} values refer to the maximum contraction given as an absolute value (mN) or calculated as a percentage of the contractile capacity of ET-1-induced contraction. Results are given as mean ± SEM (standard error of the mean), and n refers to the number of vessel segments. The pEC_{50} value is the negative logarithmic value of the concentration of the agonist (S6c or ET-1) needed to produce half maximum contraction in the vessels. The Kruskal-Wallis nonparametric test with Dunn's post-hoc test and the nonparametric t-test with Mann-Whitney were used for statistical evaluation. The level of significance for all tests was set at $P<0.05$.

Results

Contractile responses

Potassium: The maximum contraction induced by K+ increased significantly after 24 h of organ culture (6.63 ± 0.47 mN, $P<0.001$) as compared to the Emax in freshly-isolated artery (3.49 ± 0.59 mN, Table 2). Incubation with XeC or KN93 decreased the K+-induced contraction, thus Emax % value, was measured as a percent of the maximal ET-1 response (Figure 1 and Table 2).

ETB receptor: S6c did not induce a significant contractile response in fresh MCAs whereas ET-1, in a concentration-dependent manner, induced a strong contraction (Table 2). In cultured arteries, however, S6c induced a strong contraction (E_{max}=6.07 ± 0.5 mN). The co-incubation with XeC ($P<0.001$) or KN93 ($P<0.01$) decreased the maximum contraction induced by S6c in cultured arteries (Figure 1 and Table 2).

ET_A receptor: The contractile response induced by ET-1 is mediated by the ET_A receptor in fresh vessels. This response is also seen following organ culture if the ET-1 induced contraction is preceded by desensitization or specific ET_B receptor blockade [1,12]. The 24 h organ culture resulted in increased ET-1-induced contraction from 5.6 ± 0.7 mN in fresh arteries to 8.5 ± 0.5 mN ($P<0.01$). Incubation with KN93 or XeC attenuated the contraction induced by ET-1 ($P<0.05$; Table 2 and Figure 1).

Intracellular calcium measurements

The baseline level of $[Ca^{2+}]_i$ was increased in arteries incubated for 24 h in DMEM (77.8 ± 9.8 nM, $P<0.05$) as compared to that seen in fresh arteries (20 ± 8 nM). Arteries incubated with different inhibitors (KN93, XeC) showed no difference in the baseline level of $[Ca^{2+}]_i$ compared to 24 h organ culture (Figure 2A).

K^+ administration induced stronger contraction and increase in $[Ca^{2+}]_i$ after organ culture as compared to that seen in fresh MCA segments. XeC and KN93 decreased this increase in $[Ca^{2+}]_i$ induced by K^+. It was significant for KN93 ($P<0.05$, Table 3).

In organ cultured arteries S6c administration caused a slow rise in $[Ca^{2+}]_i$ that was significant ($P<0.05$) as compared to that seen in fresh vessels (Figure 2B and Table 3). Administration of S6c did not increase the baseline level of $[Ca^{2+}]_i$ in arteries incubated with KN93 or XeC (Table 3).

In fresh segments ET-1 caused a rapid and strong rise in $[Ca^{2+}]_i$ (Figure 2C). This reached a maximum level within a few seconds and remained at a stable phase. This contractile response correlated well with the contraction recorded in parallel (Figure 1). Organ culture resulted in an elevated baseline level of $[Ca^{2+}]_i$: at this point ET-1 still caused a rapid rise in $[Ca^{2+}]_i$ but it was lower than in fresh vessel segments while the stable phase was almost the same. Incubation with KN93 significantly decreased the increase in $[Ca^{2+}]_i$ induced by ET-1 as well as the contraction ($P<0.05$, Table 3). XeC also attenuated the increase in $[Ca^{2+}]_i$ induced by ET-1.

The mRNA expression during organ culture

Endothelin receptors: Real-time PCR experiments were performed to understand whether the difference in the enhanced contraction by S6c and ET-1 in incubated MCAs involved endothelin receptor mRNA levels. Results show that co-incubation with XeC ($P<0.01$) attenuated the ET_A mRNA level (Figure 3A).

XeC and KN93 reduced ET_B receptor mRNA levels significantly ($P<0.001$ and $P<0.05$, respectively) (Figure 3B). These results explain the decrease in ET-1 and S6c induced contraction in arteries incubated with KN93 or XeC (Figure 1).

CaMKII and IP3R: To understand whether organ culture with increase in $[Ca^{2+}]_i$ has any effect on IP3R (Itpr3 gene) and CaMKII expression, their mRNA levels were measured. The results showed that the CaMKII mRNA level increased ($P<0.001$), while the IP3R mRNA level decreased ($P<0.01$) during organ culture (Figure 4).

Interestingly, KN93 increased the IP3R mRNA level ($P<0.05$) and

		K^+	S6c			ET-1	
	n	Emax,mN	Emax,mN	Emax, %	pEC_{50}	Emax,mN	pEC50
Fresh	6	3.49 ± 0.59***	0.02 ± 0.02***	0.17 ± 0.17**	N.A.	5.69 ± 0.70**	8.07 ± 0.13*
24 h	8	6.63 ± 0.47	6.07 ± 0.50	70.75 ± 4.80	8.69 ± 0.07	8.47 ± 0.46	8.32 ± 0.10
XeC	6	4.55 ± 0.39*	1.40 ± 0.27***	21.83 ± 4.22**	8.70 ± 0.03	6.96 ± 0.42*	8.47 ± 0.06
KN93	6	0.45 ± 0.16***	3.24 ± 0.60**	46.33 ± 10.54	8.77 ± 0.07	5.10 ± 0.68***	8.30 ± 0.12

E_{max} values are presented as absolute values in mN or as percent of the ET-1-induced contraction and pEC_{50} values. Values are presented as mean ± SEM. Inhibitor was added prior to incubation. Fresh represents non-incubated arteries. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to 24 h. N.A.= not available, XeC = xestospongin C.

Table 2: Contractile response induced by S6c and ET-1 in rat middle cerebral arteries.

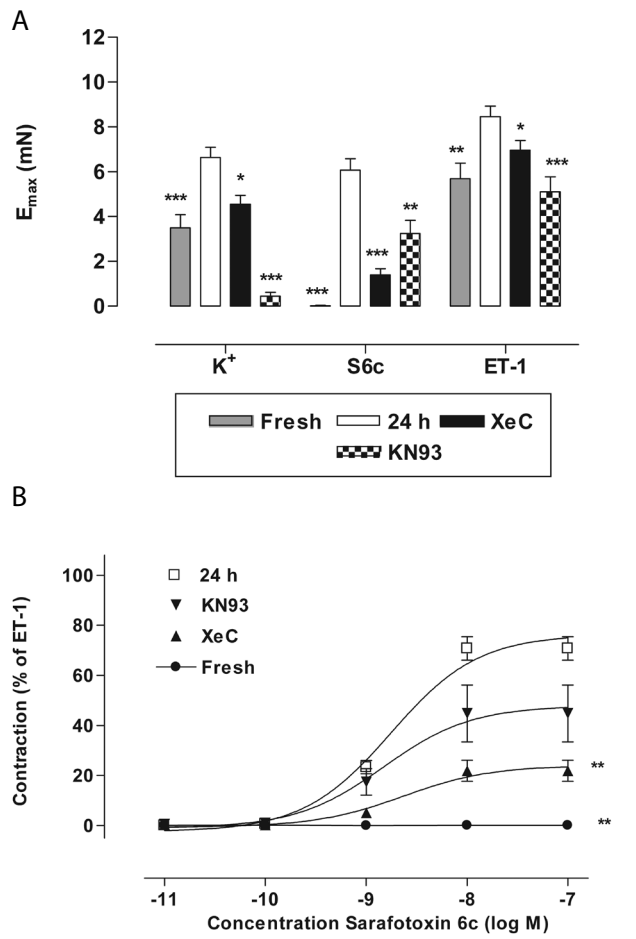

Figure 1: Contractile responses of cerebral arteries incubated in the absence (24 h) or presence of xestospongin C (XeC) or KN93. **A**, Maximum contractile responses (E_{max}) induced by K^+, S6c and endothelin (ET-1) in mN shows here. **B.** Concentration-response curve for ET_B receptor-mediated contraction induced by S6c calculated as a percentage of ET-1-induced contraction. *P<0.05, **P<0.01 and ***P<0.001 compared to 24 h. n = 6-8.

decreased the CaMKII mRNA level (P<0.0.5). Inhibition of calcium release from IP3R by XeC decreased CaMKII mRNA level (P<0.05) while it had no effect on the IP3R mRNA level (Figure 4).

Transcription factor

In order to understand mechanisms behind the responses to ET-1/S6c and the elevated levels of $[Ca^{2+}]_i$ we determined whether the inhibitors have effect at the transcriptional level.

The DREAM protein level increased during organ culture *per se* and was significant at 6 h (P<0.05). KN93 decreased DREAM protein significantly (P<0.01). On the other hand, XeC had no effect on the DREAM protein level (Figure 5A and 5B).

The protein level of p-CREB was increased during organ culture and it was significant at 6 h (P<0.05, Figure 5C and 5D). KN93 and XeC did not alter significantly the level of p-CREB.

The level of NFAT activating protein mRNA level was increased after 6 h of organ culture and had returned to control levels at 24 h. Incubation with KN93 had no effect on the level of NFam1 mRNA, while XeC resulted in a significant decrease (P<0.05, Figure 5E).

These results show that at the transcription level, CaMKII and IP3R have different targets and this is schematically illustrated in Figure 6.

Discussion

The present study was designed to evaluate the intracellular pathway of CaMK and the involvement of calcium and IP3R on endothelin receptor upregulation during organ culture. Schematic illustration of

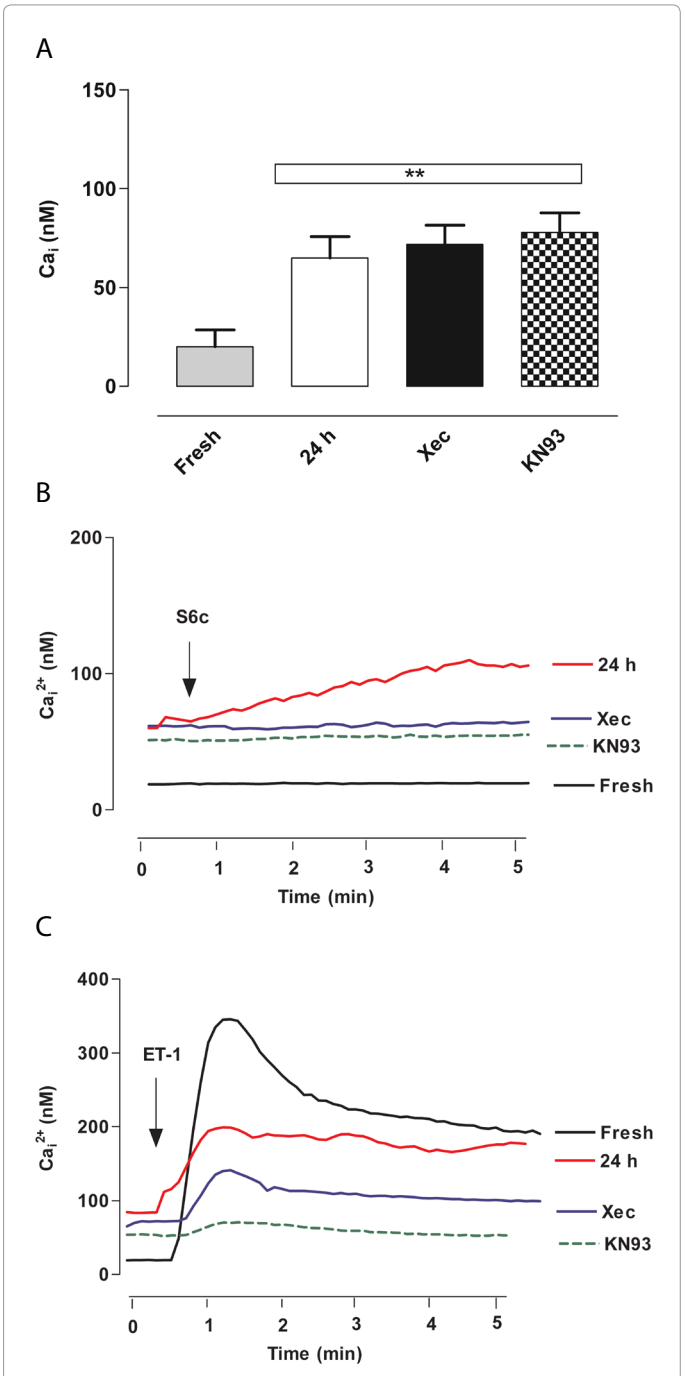

Figure 2: A. Significant increase in $[Ca^{2+}]_i$ after organ culture in vessels incubated with or without XeC or KN93 compared to non-incubated arteries (fresh), ** P < 0.01. . **B.** S6c induced increase in $[Ca^{2+}]_i$ after organ culture but not in fresh arteries and **C,** ET-1 induced increase in $[Ca^{2+}]_i$ in MCA in fresh and after 24 h organ culture. Data show as absolut value of $[Ca^{2+}]_i$ in nM. n = 4-8.

	n	Baseline	K+	S6c	ET-1
Fresh	5	20 ± 8	153.7 ± 53	16.3 ± 4	290 ± 31
24 h	3	77 ± 10*	228.3 ± 40	139.4 ± 40*	254.4 ± 81
KN93	5	77 ± 9*	95.8 ± 25^	55.5 ± 8	87.8 ± 23^
XeC	4	71 ± 9*	118 ± 46	68.7 ± 7	175.3 ± 5

Values are presented as absolute values of intracellular calcium in nM. Values are presented as mean ± SEM. Inhibitor was added prior to incubation. Fresh represent non-incubated arteries. * $P < 0.05$ represent significant compared to fresh. ^ $P < 0.05$ represent significant compared to 24 h, XeC = xestospongin C.

Table 3: Absolute value of intracellular calcium $[Ca^{2+}]_i$ induced by K^+, S6c and ET-1 in rat middle cerebral arteries.

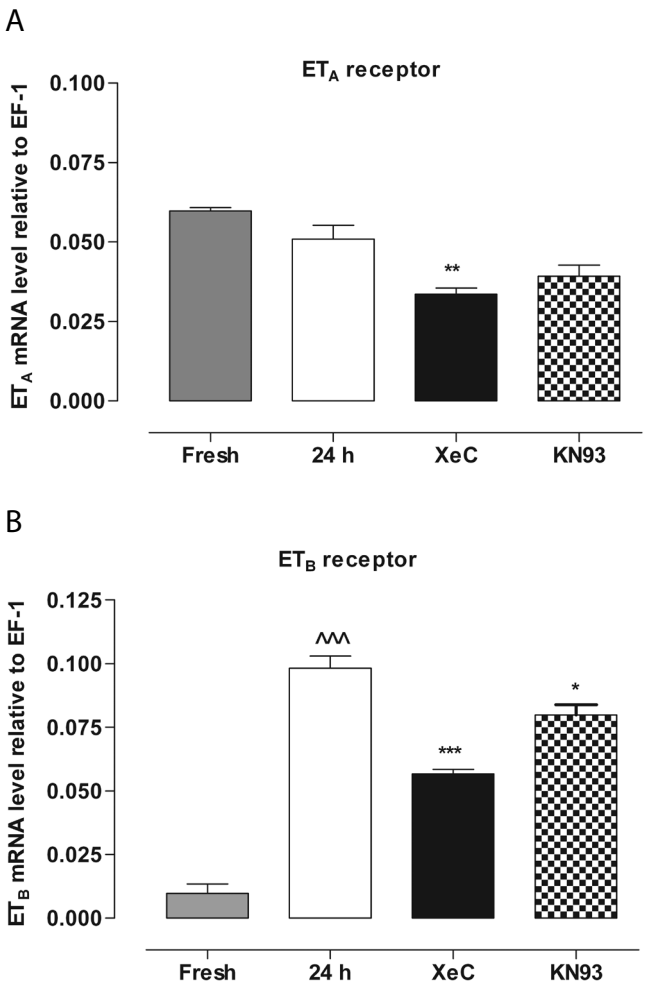

Figure 3: Endothelin receptors mRNA levels were evaluated by real time PCR. **A,** ET_A receptor mRNA level did not changed after 24 h organ culture but IP3R inhibitor (XeC) decreased significantly ET_A receptor mRNA level ** P < 0.01 compared to 24h, and **B**, ET_B receptor mRNA levels increased significantly after organ culture compared to fresh ^^^ P < 0.001. XeC and KN93 decreased significantly ET_B receptor mRNA levels compared to 24 h* P < 0.05, *** P < 0.001. n = 5.

the proposed interplay between organ culture, calcium, CaMKII and IP3R has been shown in Figure 6.

Incubation with KN93 or XeC decreased ET-1 and K^+-induced increase in $[Ca^{2+}]_i$ but not lower than 77 nM. This finding confirms a study showing that organ cultured arteries have more available intracellular calcium ions in the VSMCs, but this calcium might exist in a discrete compartment that is not involved in contraction [9].

There are many factors that may affect contraction during organ culture including effects on the contractile apparatus *per se* or calcium ion channels. In a previous study we have shown that components of the contractile apparatus such as the myosin light chain protein level did not change during organ culture [12]. In the present study we focused on calcium handling and on the IP3R channel. The results showed a difference between the pattern and the levels of increase in $[Ca^{2+}]_i$ induced by ET-1 and S6c. ET-1 induced strong increase in $[Ca^{2+}]_i$ while S6c induced a minor and slow increase in $[Ca^{2+}]_i$ that was parallel to the contraction pattern. These results are in agreement with studies showing that ET-1 and S6c activate different calcium channels and are dependent on calcium ions in different ways [25-27].

It has been shown that increase in $[Ca^{2+}]_i$ leads to activation of CaMK which in turn may stimulate intracellular pathways such as MAPK [28] and c-fos [29]. In addition, changes in calcium handling [30] and activation of IP3R-induced Ca^{2+} release [31] are involved in VSMC proliferation and remodeling. We evaluated the mRNA level of CaMKII and IP3R in arteries incubated with or without KN93 or XeC in order to understand the effect of organ culture and the increase in $[Ca^{2+}]_i$ on intracellular mediators. Results showed that organ culture induced an increase in CaMKII mRNA level and in ET_B receptor expression but a decrease in the IP3R mRNA level. Inhibition of CaMKII increased the IP3R expression, suggesting that CaMKII activation has an inhibitory effect on IP3R expression.

Inhibition of calcium release via IP3R during organ culture showed the following: 1- IP3R channels are active during organ culture. 2- Organ culture has effect on the IP3R mRNA.3- These channels have effect on gene regulation of ET receptors and CaMKII. We have illustrated this schematically in Figure 6.

Although CaMKII and IP3R had the same effect on contraction and endothelin receptor regulation, they did not have the same effect on transcriptional induction. KN93 decreased DREAM protein level and had a positive effect on p-CREB expression. DREAM is a transcription repressor and binding to calcium ions inactivates it which in turn permits activation of c-fos and CREB [15]. Also it has been shown that calcium and CaMKII cooperatively regulate the sub-cellular localization into the nucleus and thereby promotes DREAM- induced

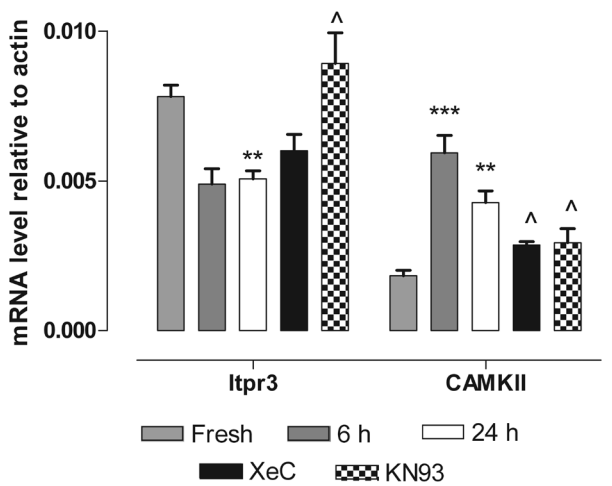

Figure 4: CaMKII and IP3R type 3 (Itpr3) mRNA level measured after 24 h incubation and compared to fresh that represents by ** P < 0.01, *** P < 0.001. Incuabtion with XeC or KN93 significantly affected CaMKII and IP3R type 3 (Itpr3) mRNA level (^ P < 0.05). . n=4-7.

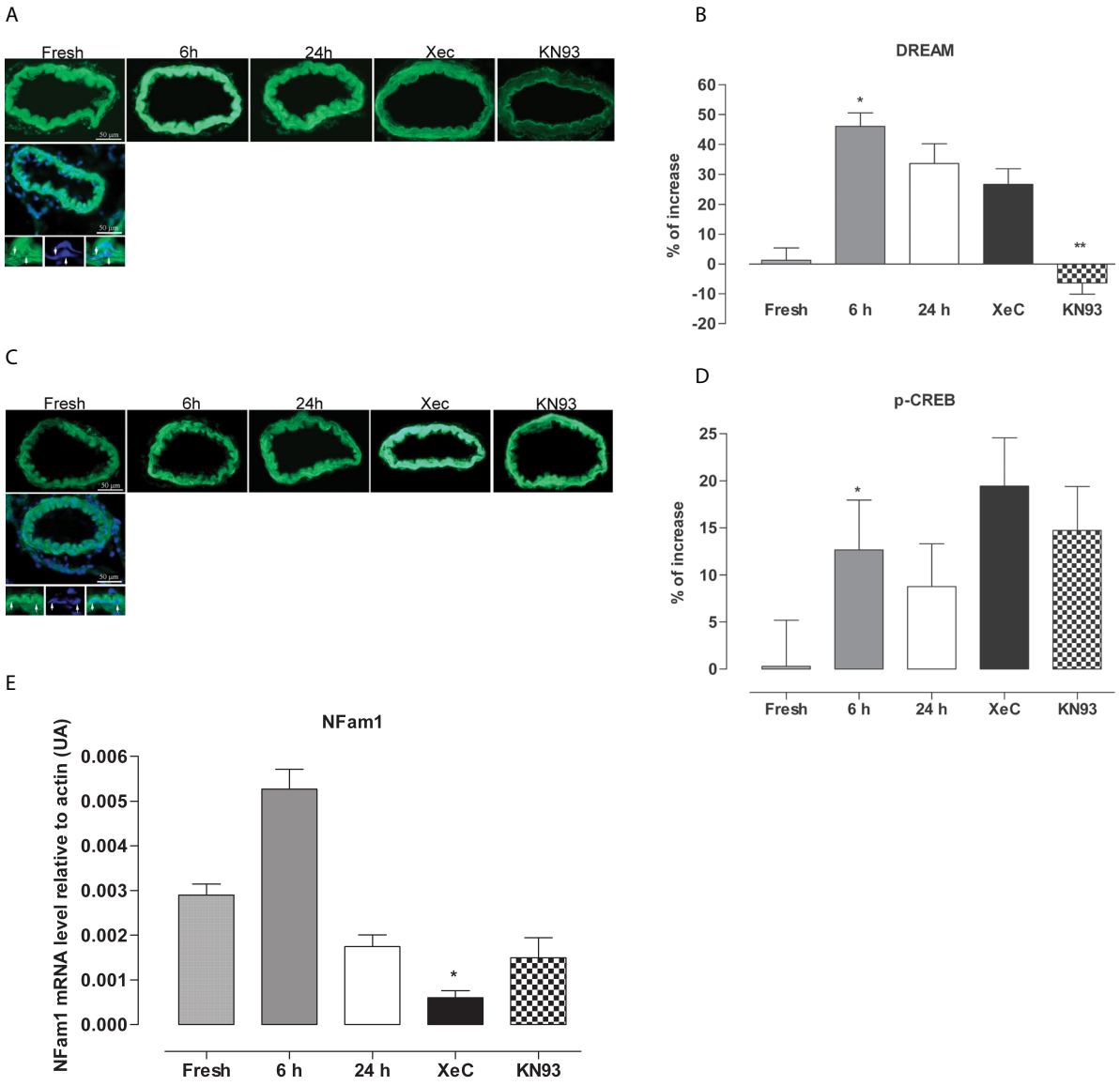

Figure 5: Protein and mRNA levels of transcription factors DREAM, p-CREB, and NFAT activating protein (NFam1) before (fresh) and after organ culture. **A and B,** Incubation with KN93 decreased DREAM protein level. **C and D,** Immunohistochemistry revealed that p-CREB protein level was slightly increased by KN93 or XeC incubation. Staining of DREAM and p-CREB was found in the cytoplasm (lower panel, arrows), nuclei staining (blue) is shown with DAPI, magnification 20X **E,** XeC decreased the NFam1 mRNA levels (n = 3-5) * P < 0.05, ** P < 0.01 compared to 24 h.

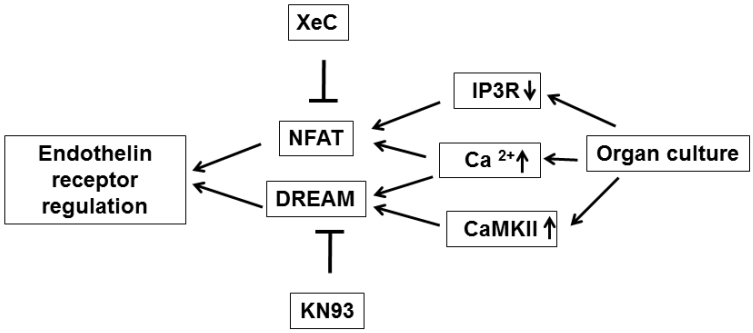

Figure 6: Schematic illustration of the proposed interplay between organ culture, calcium, CaMKII and IP3R that leads to regulation of endothelin receptors. Organ culture induce increase in intracellular calcium and CaMKII expression while decreased the IP3R expression. Inhibition of DREAM by CaMKII and NFAT by XeC attenuated the effect of organ culture on endothelin receptors.

transcriptional repression [32]. Our study is the first shows the effect of CaMKII on DREAM in endothelin receptor upregulation in VSMC.

In the present study we also evaluated the mRNA level of NFAT activating protein during organ culture. Our results show that XeC, but not KN93, has an inhibitory effect on NFAT activating protein, suggesting the possible intracellular signaling between IP3R and NFAT. This finding is in agreement with a study demonstrating that IP3R inhibitor prevented ET-1-induced nuclear accumulation of NFATc1 in adult atrial cardiomyocytes [33].

Conclusion

We report that organ culture induced increase in $[Ca^{2+}]_i$, ET_B receptor mRNA level and contractility induced by S6c or ET-1. Our results demonstrate for the first time that IP3R and CaMKII inhibitors decrease K^+, S6c and ET-1 induced contractions and attenuate protein levels of NFam1 and DREAM, respectively.

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