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Protein kinase C inhibitors decrease endothelin ET_B receptor mRNA expression and contraction during organ culture of rat mesenteric artery

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Abstract

The effect of protein kinase C (PKC) inhibitors on the induction of endothelin ET_B receptors during organ culture was examined in isolated segments of the rat mesenteric artery. After 24 h of organ culture, the endothelin ET_B receptor agonist sarafotoxin 6c (S6c) induced a strong contraction compared to fresh segments. The contractile response after 24-h organ culture to S6c was studied in presence (30-min preincubation) or absence, after 24-h treatment, of the PKC inhibitors staurosporine, K252a and Ro31-7549. Exposure to staurosporine or K252a in presence and after 24-h treatment reduced the S6c contraction. In contrast, presence of 2-1[1-3(aminopropyl)indol-3-yl]-3(1-methyl-1*H*-indol-3-yl)maleimide (Ro31-7549), did not affect the S6c-induced contraction, whereas 24-h treatment abolished the increase of contraction. The PKA inhibitor *N*-(2-[bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide (H89) did not affect the S6c responses. The mRNA expressions of endothelin ET_B receptors (analysed with real-time PCR) were abolished after 24-h treatment with the PKC inhibitors. These results suggest that PKC is involved in the endothelin ET_B receptor upregulation following organ culture.

Keywords: PKC (protein kinase C); Endothelin; Sarafotoxin 6c; Endothelin ET_B receptor; Ro31-7549; Staurosporine; K252a; H89

1. Introduction

The endothelins are potent vasoconstrictor peptides (Yanagisawa et al., 1998). Their actions in mammals are mediated through two distinct G-protein coupled receptors: endothelin ET_A and ET_B receptors (Arai et al., 1990; Sakurai et al., 1990), both being seven transmembrane domain receptors. The endothelin ET_A receptor is the major contractile endothelin receptor subtype in vascular smooth muscle cells (SMC). The endothelin ET_B receptor is mainly found in the vascular endothelium where it induces transient reduction in vascular tone via release of nitric oxide (De Nucci et al., 1988; Szok et al., 2001). In addition, expression of both mRNA for endothelin ET_B receptors in the smooth muscle layer (Möller et al., 1997) and contractile responses to endothelin ET_B receptor stimulation have been observed in some vascular regions (Bigaud and Pelton, 1992; Maclean et al., 1994; Lodge et al., 1995; Adner et al., 1998b).

As the normal endothelin ET_B receptor response is weak in arteries, the physiological and pathophysiological importance of the contractile endothelin ET_B receptor response in the arterial system has been the subject of some debate. Recent reports have shown that the endothelin ET_B receptor, even though it only induces a very weak response, can modulate the endothelin ET_A induced responses (Mickley et al., 1997; Adner et al., 2001). Activation of endothelin ET_B receptors induces mitogenic effects in cultured smooth muscle cells (Eguchi et al., 1995) and causes intimal hyperplasia (Porter et al., 1998). In addition, the endothelin ET_B receptor has been shown to appear de novo during pathological conditions in coronary arteries of patients with atherosclerosis (Dagassan et al., 1996), in subcutaneous arteries of patients with coronary artery disease (Wenzel et al., 1996) and in cerebral arteries after experimental subarachnoid hemorrhage (Roux et al., 1995; Hino et al., 1996) and in ischemic stroke (Ahlrot et al., 2001).

In previous studies we have revealed that the endothelin ET_B receptor upregulation in smooth muscle cells occurs via de novo receptor transcription (Möller et al., 1997; Adner et

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al., 1998a). The present study was performed in order to further understand the cellular mechanisms behind the endothelin ET_B receptor upregulation. While cytokines have been reported to upregulate the bradykinin B_1 receptor via mitogen activated protein kinase kinase (MAPKK) kinases at the transcriptional level (Donaldsson et al., 1997), our studies have shown that cytokines enhance translation (White et al., 1999; Leseth et al., 1999). On the other hand, the histamine H_1 receptor has been found to be down-regulated by phorbol ester treatment, an effect that was blocked by the protein kinase C (PKC) selective inhibitor GF 109203X (Pype et al., 1998). Other studies have shown that blocking PKC inhibits beta-adrenergic receptor upregulation after treatment with angiotensin converting enzyme inhibitors (Yonemochi et al., 1998), and it has been speculated that PKC may be involved in the control of transcription factors (Wang and Dhalla, 2000).

The aim of the present study was to elucidate the role of protein kinases in the endothelin ET_B receptor upregulation as examined both with a sensitive in vitro system for studies of contractile phenotype and real-time polymerase chain reaction (PCR) for quantitation of mRNA levels. As tools to investigate the protein kinase pathways, we have used PKC inhibitors with different profiles of selectivity (staurosporine, K252a and 2-1[1-3(aminopropyl)indol-3-yl]-3(1-methyl-1*H*-indol-3-yl)maleimide (Ro31-7549)) and the selective PKA inhibitor *N*-(2-[bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide (H89).

2. Methods

2.1. Tissue preparation and organ culture procedure

A distal branch of the rat superior mesenteric artery (outer diameter <1 mm) was chosen as experimental target since we have in previous studies shown that this vessel upregulates endothelin ET_B receptors following 24 h of incubation with Dulbecco's modified Eagle's medium (DMEM) (Adner et al., 1998b). Male Wistar–Kyoto rats (200–300 g, M&B, Denmark) were anaesthetized with CO_2 and killed by exsanguination. The artery was removed, dissected free from adherent tissue and cut into 1-mm-long circular segments. Segments were placed in a well containing 1 ml of DMEM, supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), and incubated for 24 h at 37 °C in humidified 5% CO_2 in air (pH 7.4).

2.2. In vitro pharmacology

After incubation, the segments were immersed in temperature-controlled (37 °C) tissue baths containing a bicarbonate buffer solution. The solution was continuously aerated with 5% CO_2 in O_2 resulting in a pH of 7.4. Each segment was mounted on two L-shaped prongs, one of which was attached to a Grass FT-03 transducer (Grass Instr., Quincy, USA) connected to a MacLab (ADInstruments, Hastings, UK) unit for continuous recording of isometric tension. A tension of 2 mN was applied to each segment and the segments were allowed to stabilise at this tension for 1 h before being exposed to a K^+-rich (60 mM) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The K^+-induced contraction was used as a reference for the contractile capacity, and the segments were used only if K^+ elicited reproducible responses over 0.5 mN. Concentration–response curves for the S6c were obtained by cumulative administration of the peptide. To investigate the immediate effect of the PKC inhibitors, segments were incubated 30 min with respective inhibitor before administration of sarafotoxin 6c and during the experiments. To study the effect of the inhibitors on the ET receptor upregulation they were present during the entire incubation period. Paired segments were incubated in different concentrations of respective PKC inhibitor (for 24 h), or exposed to the PKC inhibitor for 30 min before the start of the in vitro contractile experiment. Concentration–response curves for the specific endothelin ET_B receptor agonist Sarafotoxin 6c were then performed. After each contractile experiment, a high concentration of endothelin-1 (0.1 μM) was added as a reference to the total endothelin receptor-induced response.

2.3. Buffer solutions and drugs

Standard buffer solution (mM): NaCl 119; $NaHCO_3$ 15; KCl 4.6; MgCl 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.5; glucose 5.5. Analytical grade chemicals and double-distilled water were used for preparing all solutions.

Dulbecco's modified Eagle's medium, penicillin and streptomycin were purchased from Gibco BRL, Paisley, Scotland. Staurosporine, K252a, H89 (Sigma, St. Louis, USA) and Ro31-7549 (Kind gift from Dr. H. Bergstrand, AstraZeneca, Lund, Sweden) were dissolved in dimethylsulphoxide (Labscan, Dublin, Ireland), and diluted in sterile H_2O. S6c and ET-1 (Auspep, Parkville, Australia) were dissolved in sterile water with bovine serum albumin (0.1% w/v).

2.4. Calculation and statistics

Data are expressed as mean values ± S.E.M. Contractile responses in each segment are expressed as a percentage of the K^+-induced contraction. E_{max} represents the maximal contraction induced by an agonist, and given either in absolute values or expressed as a percentage of the K^+-induced response for each vessel segment. The pEC_{50} value was calculated from the line between the concentrations above and below the midpoint of the concentration–response curve. Analysis of variance tests were used for paired analyses. Differences were considered significant at $P<0.05$.

2.5. Analysis of endothelin ET_B receptor mRNA

The vessels were snap frozen in −80 °C after removal, and total cellular RNA was extracted using the TRIzol reagent (Gibco BRL) following the suppliers' instructions. The resulting pellet was finally washed with 75% ethanol, air-dried and redissolved in 10 μl diethyl-pyrocarbonate (DEPC)-treated water. 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. First strand cDNA was synthesized from 1-μg total RNA in a 20-μl reaction volume using random hexamers as primers. The reaction mixture was incubated at 25 °C for 10 min, 42 °C for 15 min, heated to 99 °C for 5 min and chilled to 5 °C for 5 min. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System (Perkin-Elmer, Applied Biosystems) using the GeneAmp SYBR® Green kit (Perkin-Elmer) with the cDNA synthesized above as template in a 50-μl reaction volume. A no-template control was included in all experiments. The GeneAmp 5700 Sequence Detection System monitors the growth of DNA in real-time using an optics- and imaging system, via the binding of a fluorescent dye to double-stranded DNA. Specific primers were designed as follows:

ET_B receptor	forward	5′-GATACGACAACTTCCGCTCCA-3′
	reverse	5′-GTCCACGATGAGGACAATGAG-3′

Elongation factor-1 (EF-1) mRNA was used as a reference, since it is the product of a housekeeping gene, continuously expressed to a constant amount in cells. The EF-1 primers were designed as follows:

EF-1	forward	5′-GCAAGCCCATGTGTGTTGAA-3′
	reverse	5′-TGATGACACCCACAGCAACTG-3′

The real-time PCR was carried out with the following profile: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min. To prove that the cDNA of EF-1 and the endothelin ET_B receptor were amplified with the same efficacy during real-time PCR, a standard curve was made where the C_T-values were plotted against cDNA concentration based on the equation:

$$C_T = 1g\left(\frac{\text{concentration}}{(1+E)}\right)$$

where E is the amplification efficiency with the optimal value of 1. To prove that each primer-pair generates only one PCR product, an agarose gel electrophoresis with the PCR products was run.

The amount of endothelin ET_B receptor mRNA was calculated as relative to the amount of EF-1 mRNA in the same sample by the formula:

$$X_0/R_0 = 2^{(\mathrm{CtR}-\mathrm{CtX})}$$

where X_0 = original amount of ET receptor mRNA, R_0 = original amount of EF-1 mRNA, CtR = C_T-value for EF-1 and CtX = C_T-value for the ET receptor. Statistical analyses were performed with paired Students t-test, where $P < 0.05$ was considered significant.

3. Results

3.1. In vitro pharmacology

In fresh ring segments (not incubated) sarafotoxin 6c induced only a weak contraction (6 ± 3% in relation to 60 mM KCl, $n = 6$), compared to endothelin-1 (183 ± 22%) (Fig. 1). In segments incubated for 24 h there was a slight reduction in the K^+ response (not significant). A concomitant reduction in maximum endothelin-1-induced contraction was seen, thus, the contraction was unchanged in relation to the K^+ response. Following 1 day of organ culture, S6c induced a strong contraction that was similar to that of endothelin-1, reaching 137 ± 6% of the K^+ response.

3.2. Effect of protein kinase inhibitors

Since the main purpose of this study was to investigate the influence of PKC inhibitors on the regulation of endothelin ET_B receptors, the vessel segments were incubated with different inhibitors with specificity for different protein kinases. Furthermore, to differentiate the effects after culture, the responses to S6c were compared with those induced by the protein kinase inhibitors after 30-min incubation before and during the experiments.

3.2.1. Effect of general protein kinase inhibitors

Staurosporine, a general PKC inhibitor, was the first blocker studied due to its potency and low selectivity (Way et al., 2000). When staurosporine (100 nM) was present during the experiment in segments cultured for 24 h, the maximum contraction induced by sarafotoxin 6c was only half of that seen without inhibition (Fig. 2A). For

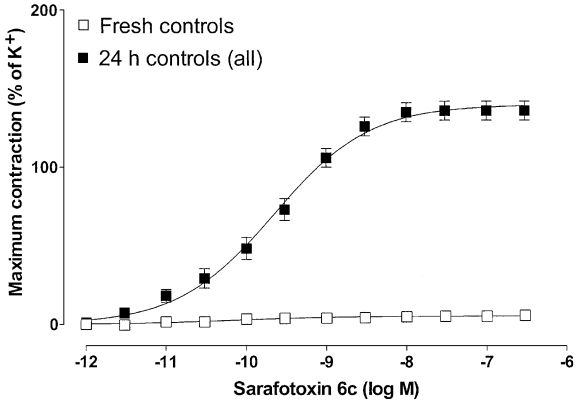

Fig. 1. Concentration response curves for S6c in fresh vessels or following 24 h organ culture. Data are shown as mean ± S.E.M.

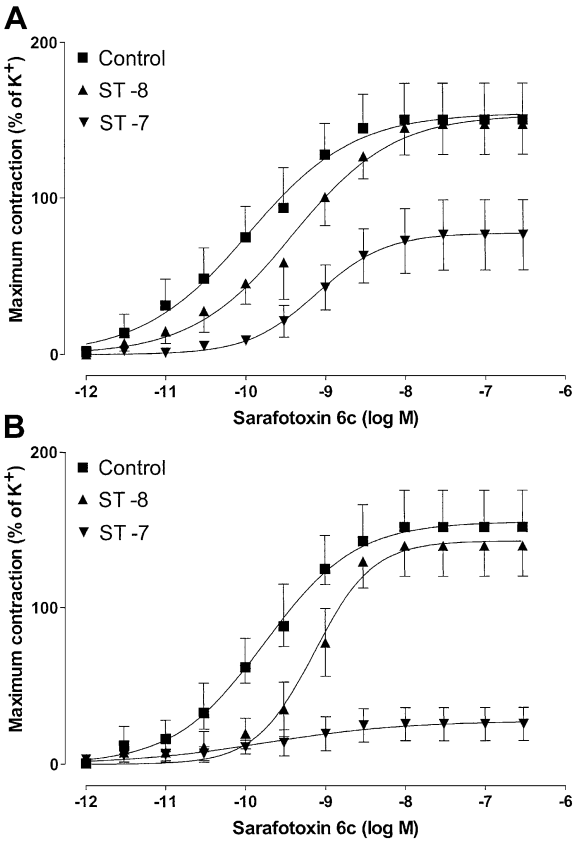

Fig. 2. Concentration response curves for S6c (A) in presence of the PKC antagonist ST (10–100 nM) or (B) following 24 h treatment with ST. Data are shown as mean ± S.E.M.

endothelin-1 the maximum effect was not altered. There was a decrease in potency for sarafotoxin 6c with 100 nM staurosporine in these experiments (Table 1).

In contrast, a decrease in maximum effect without altering the potency to sarafotoxin 6c was achieved after 24-h treatment with staurosporine (Fig. 2B). Moreover, after staurosporine treatment the single concentrations of endothelin-1 were increased in relation to the K^+ induced response, but not in absolute terms, due to a decrease in the K^+-induced response (Table 2).

The other potent and nonspecific PKC antagonist, K252a, was used to further evaluate the involvement of protein kinases. Incubation with 10 μM K252a present during the experiments (acute effect) induced a decrease in maximal response and potency of sarafotoxin 6c (Fig. 3A, Table 1).

In contrast, there was a concentration-dependent decrease in contractile response to sarafotoxin 6c in segments cultured for 24 h with 1 or 10 μM K252a (Fig. 3B). The K^+-induced response was only reduced at the highest concentration of K252a whereas the endothelin-1 induced response was unaffected in absolute terms but significantly increased in relation to K^+ (Table 2).

3.2.2. Effect of specific PKC inhibition

The more specific PKC inhibitor Ro31-7549 was used to further investigate the involvement of PKC on the process of endothelin ET_B receptor upregulation. In contrast to the staurosporine and K252a data, the presence of Ro31-7549 during the experiments did not affect the sarafotoxin 6c contraction (Fig. 4A; Table 1), whereas an almost complete inhibition of the sarafotoxin 6c response was seen after 24 h of treatment with the highest concentration (10 μM) (Fig. 4B). Moreover, K^+ and the endothelin-1 contractions were not affected by Ro31-7549 (Table 2).

3.3. Protein kinase A inhibition

Since both staurosporine and K252a also have been reported to inhibit PKA in high doses, additional experiments with the selective PKA inhibitor H89 were performed. The S6c-induced contraction was not affected by

Table 1
Contractile responses to S6c and ET-1 in cultured arteries incubated for 30 min in K252a, staurosporine (ST), Ro31-7549 (Ro) or H89

	N	K^+ mean	S6c			ET-1	
			E_{max} (mN)	E_{max} (%)	pEC_{50}	E_{max} (mN)	E_{max} (%)
Control	7	3.85 ± 0.91	4.88 ± 0.72	150 ± 24	10.23 ± 0.38	5.80 ± 0.80	185 ± 32
ST 10 nM	6	3.24 ± 1.08	3.99 ± 0.99	147 ± 19	9.39 ± 0.33	4.54 ± 1.28	161 ± 15
ST 100 nM	6	4.12 ± 1.02	2.63 ± 0.67[a]	76 ± 22[a]	8.86 ± 0.38[a]	4.28 ± 0.96	123 ± 29
Control	7	3.87 ± 1.10	4.93 ± 0.85	150 ± 24	10.23 ± 0.38	5.80 ± 0.80	185 ± 32
K252a 0.1 nM	5	2.81 ± 0.97	3.59 ± 1.15	132 ± 8	9.30 ± 0.36	4.40 ± 1.43	161 ± 11
K252a 1 μM	5	4.41 ± 0.97	4.76 ± 1.09	111 ± 10	9.02 ± 0.14	6.09 ± 1.46	139 ± 13
K252a 10 μM	5	3.95 ± 0.85	1.18 ± 0.64[a]	40 ± 25[a]	8.73 ± 0.35[a]	2.17 ± 0.92	74 ± 39
Control	11	2.08 ± 0.37	2.44 ± 0.52	119 ± 15	9.43 ± 0.18	3.28 ± 0.55	162 ± 11
Ro 0.1 μM	5	2.43 ± 0.68	3.76 ± 0.74	165 ± 12	9.25 ± 0.10	4.52 ± 0.90	197 ± 14
Ro 1 μM	5	2.90 ± 1.16	3.40 ± 1.33	118 ± 3	9.72 ± 0.32	4.00 ± 1.60	137 ± 6
Ro 10 μM	6	1.61 ± 0.24	2.28 ± 0.73	144 ± 54	9.44 ± 0.14	2.71 ± 0.87	174 ± 62
Control	8	2.65 ± 0.78	3.08 ± 0.58	140 ± 19	9.68 ± 0.36	4.34 ± 0.87	192 ± 21
H89 0.1 μM	5	3.03 ± 1.37	3.54 ± 0.62	163 ± 36	9.38 ± 0.30	4.21 ± 0.84	190 ± 42
H89 1 μM	5	1.73 ± 0.72	2.58 ± 0.95	193 ± 16	9.82 ± 0.28	3.48 ± 1.36	209 ± 19
H89 10 μM	5	2.93 ± 1.05	2.46 ± 0.73	93 ± 20	9.10 ± 0.25	3.37 ± 0.90	134 ± 28

Data are shown as mean ± S.E.M. *N* denotes the number of experiments.
[a] Denotes statistical significance [$P < 0.05$] vs. respective controls.

Table 2
Contractile responses to S6c and ET-1 in arteries incubated for 24 h in K252a, staurosporine (ST), Ro31-7549 (Ro) or H89

	N	K^+ mean	S6c			ET-1	
			E_{max} (mN)	E_{max} (%)	pEC_{50}	E_{max} (mN)	E_{max} (%)
Control	7	3.61 ± 1.02	4.63 ± 1.01	152 ± 23	9.69 ± 0.55	4.93 ± 0.89	171 ± 26
ST 10 nM	6	1.82 ± 0.37	2.63 ± 0.68	140 ± 20	9.27 ± 0.26	4.18 ± 0.86	238 ± 35
ST 100 nM	6	1.23 ± 0.28[a]	0.21 ± 0.05[a]	25 ± 11[a]	9.27 ± 0.26	4.09 ± 0.48	451 ± 147[a]
Control	7	4.26 ± 0.92	5.72 ± 1.33	135 ± 12	9.42 ± 0.21	5.91 ± 1.43	149 ± 18
K252a 0.1 nM	6	4.02 ± 0.59	5.50 ± 0.73	140 ± 8	9.14 ± 0.37	6.05 ± 0.79	173 ± 130
K252a 1 μM	6	3.01 ± 0.93	0.77 ± 0.33[a]	47 ± 28[a]	9.38 ± 0.63	4.99 ± 1.29	183 ± 19
K252a 10 μM	5	0.97 ± 0.16[a]	0.24 ± 0.06[a]	25 ± 6[a]	10.01 ± 0.19	5.41 ± 1.18	594 ± 167[a]
Control	11	2.08 ± 0.37	2.44 ± 0.52	119 ± 15	9.43 ± 0.18	3.28 ± 0.55	162 ± 11
Ro 0.1 μM	5	3.49 ± 1.11	4.70 ± 1.51	134 ± 12	9.41 ± 0.17	5.85 ± 1.82	168 ± 13
Ro 1 μM	4	3.32 ± 1.45	3.59 ± 1.54	112 ± 7	9.39 ± 0.27	5.30 ± 1.98	178 ± 19
Ro 10 μM	6	3.12 ± 0.83	0.16 ± 0.06[a]	10 ± 5[a]	9.47 ± 0.75	4.73 ± 1.21	155 ± 6
Control	8	2.65 ± 0.78	3.08 ± 0.58	140 ± 19	9.68 ± 0.36	4.34 ± 0.87	192 ± 21
H89 0.1 μM	5	2.68 ± 1.19	4.25 ± 1.72	183 ± 27	9.53 ± 0.25	4.47 ± 1.87	201 ± 34
H89 1 μM	5	1.91 ± 0.88	2.68 ± 1.17	142 ± 24	9.90 ± 0.26	3.59 ± 1.61	189 ± 27
H89 10 μM	5	1.96 ± 0.63	3.84 ± 1.35	192 ± 9	9.57 ± 0.47	4.92 ± 1.75	244 ± 10

Data are shown as mean ± S.E.M. *N* denotes the number of experiments.
[a] Denotes statistical significance [$P < 0.05$] vs. respective controls.

the presence of H89 in acute experiments nor after 24 h of treatment (Fig. 5). Interestingly, a tendency towards increased responses for both S6c and ET-1 was seen after 24 h of incubation with H89, and that was without any decrease in the K^+-induced contraction.

3.4. Endothelin ET_B receptor mRNA levels

The standard curves of each primer pair had almost similar slopes, indicating that the EF-1 and endothelin ET_B cDNAs were amplified with the same efficiency (Fig. 6). The

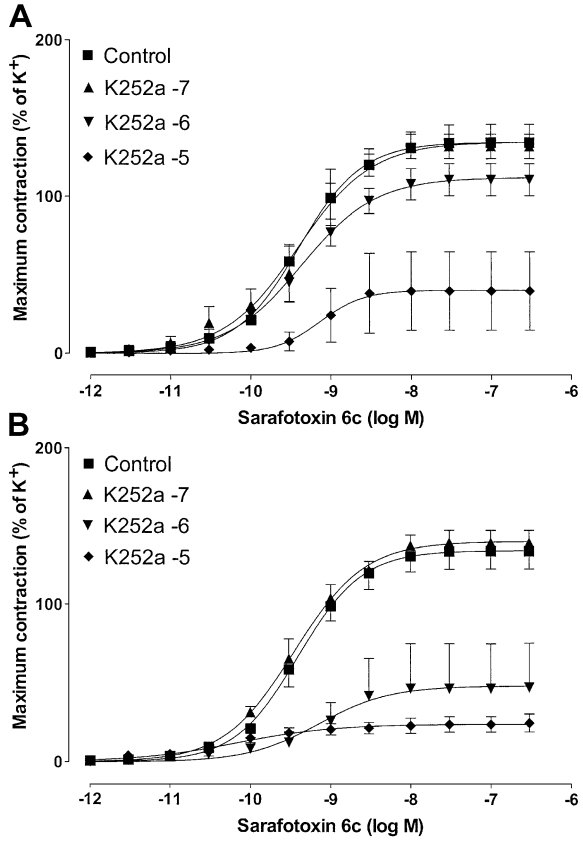

Fig. 3. Concentration response curves for S6c (A) in presence of the PKC receptor antagonist K252a (0.1–10 μM) or (B) following 24-h treatment with K252a. Data are shown as mean ± S.E.M.

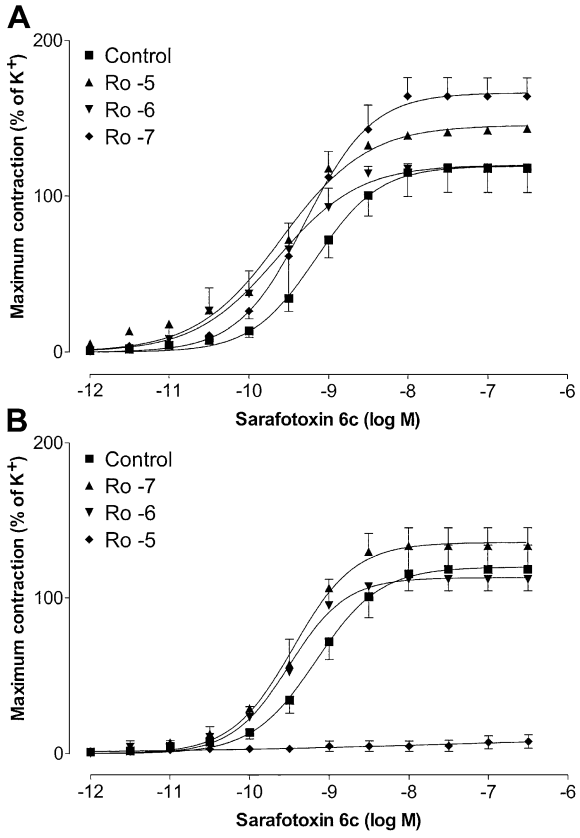

Fig. 4. Concentration–response curves for S6c (A) in presence of the PKC antagonist Ro31-7549 (0.1–10 μM) or (B) following 24-h treatment with Ro31-7549. Data are shown as mean ± S.E.M.

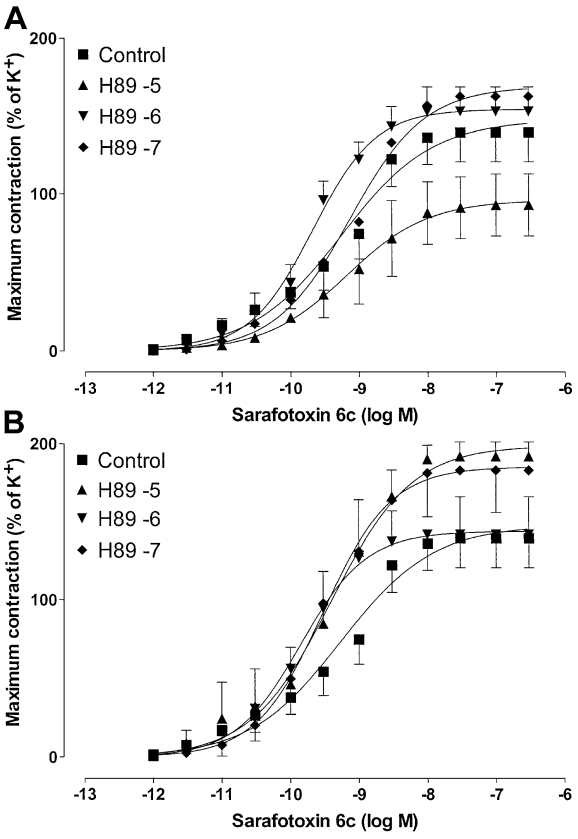

Fig. 5. Concentration response curves for S6c following (A) in presence of the PKA receptor antagonist H89 (0.1–10 μM) or (B) following 24-h treatment with H89. Data are shown as mean ± S.E.M.

values of each slope were close to 3.3, which means that the amplification efficiencies are almost optimal (E is very close to 1). Electrophoresis of the PCR products demonstrated that each primer pair generated only the expected product (Fig. 7). In each PCR experiment a no template control was included, and there were no signs of contaminating nucleic

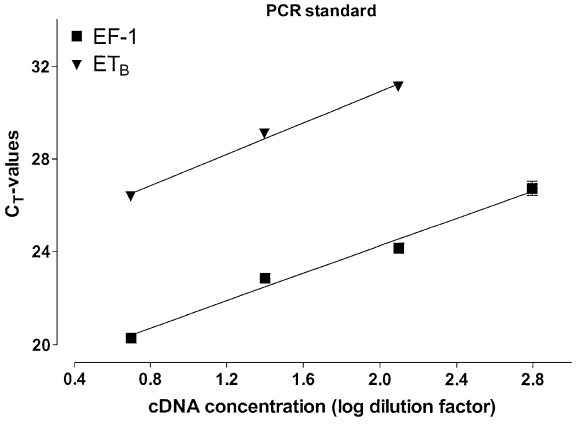

Fig. 6. Standard curves for each primer pair. X-axis = cDNA concentration (1g dilution factor), Y-axis = C_T-values. EF-1: slope = 2.96; ET_B: slope = 3.39.

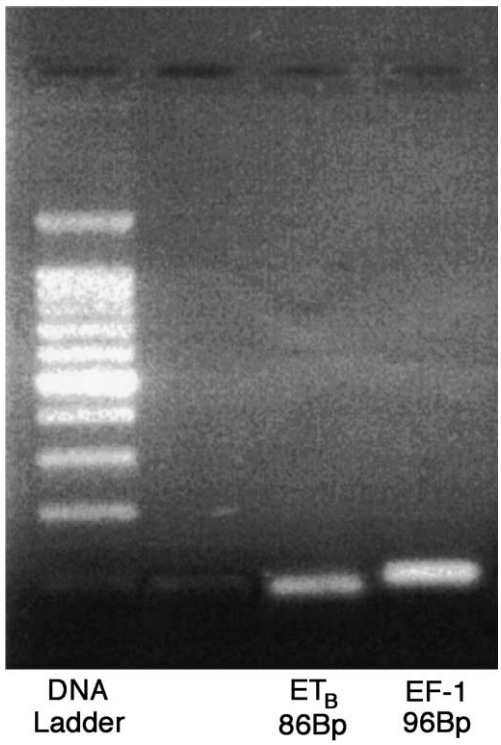

Fig. 7. Agarose gel electrophoresis showing the products from real time PCR. From left: 100 base-pair DNA-ladder, ET_B (86 bp) and EF-1 (96 bp).

acids in those samples. The results from real-time PCR showed significantly elevated levels of endothelin ET_B receptor mRNA relative to the amount of EF-1 mRNA in the mesenteric artery after organ culture for 24 h as compared to fresh segments ($ET_B = 0.052 \pm 0.020$ and 0.092 ± 0.002, $P < 0.05$). After incubation with either PKC inhibitor the increase in endothelin ET_B mRNA was markedly attenuated (Fig. 8). There were no differences in the endothelin ET_B receptor mRNA-levels when H89 was used (Fig. 8).

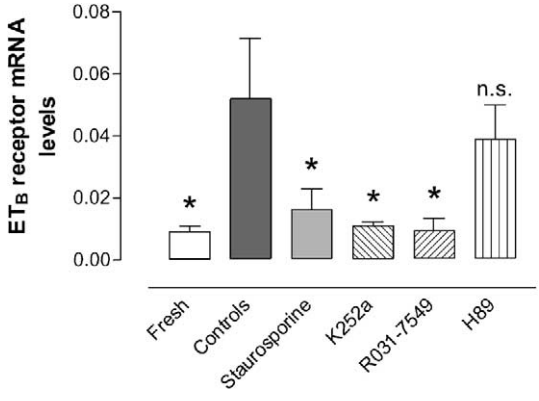

Fig. 8. Relative endothelin ET_B receptor mRNA levels in the fresh rat mesenteric artery and following organ culture with or without staurosporine (10 nM), K252a (10 μM), Ro31-7549 (10 μM) and H89 (− 5 M). Data are presented as mean ± S.E.M. relative to EF-1, $n = 3–5$. [* denotes statistical significance ($p < 0.05$) vs. the cultured control].

4. Discussion

In this study we have shown that the three PKC inhibitors staurosporine, K252a and Ro31-7549, but not the PKA inhibitor H89, attenuate the upregulation of contractile responses and mRNA for the endothelin ET_B receptors in rat mesenteric artery. Staurosporine and K252a could in high concentrations attenuate the contractile responses to both endothelin ET_A and ET_B receptors, indicating that the Gq-coupled pathway was inhibited. Further experiments with a selective PKA inhibitor indicated that the transcriptional upregulation of functional endothelin ET_B receptors was mediated through activation of PKC and not PKA.

In accordance with earlier receptor characterisations of the fresh rat mesenteric artery, sarafotoxin 6c induces negligible contractions as compared to the endothelin-1, suggesting that the endothelin ET_A receptor is the dominant contractile endothelin receptor in this artery (Adner et al., 1998b). Moreover, organ culture for 24 h induced a marked increase in the sarafotoxin 6c-induced contraction, suggesting an upregulation of endothelin ET_B receptors that is time-dependent and correlates with the increase in endothelin ET_B receptor mRNA (Möller et al., unpublished observations). The endothelin ET_B receptor-mediated contraction is in this situation approximately 80% of the endothelin-1 induced contraction, which indicates that endothelin ET_A receptors still are present after organ culture. Furthermore, experiments with vessel segments incubated without glucose abolished the endothelin ET_B receptor upregulation, but endothelin-1 induced the same contraction as before incubation, suggesting that the response induced by endothelin ET_A receptors is not markedly affected by organ culture for 24 h (Adner et al., 1998a). In addition, removal of the endothelium did not alter the responses, neither before nor after organ culture (Adner et al., 1998a).

In segments exposed to either staurosporine (100 nM) or K252a (10 μM), there was a reduction in the contractile response to sarafotoxin 6c as well as a rightward-shift of the concentration–response curve. In addition, the endothelin-1 induced responses were also reduced in segments exposed to staurosporine and K252a, suggesting that the decreased efficacy may be an effect through inhibition of protein kinases that are involved in the contractile pathways activated by the endothelin receptors. These results are consistent with a previous study in which Sudjarwo and Karaki (1995) observed that the PKC inhibitor calphostin C inhibited sarafotoxin 6c-induced contractions in the rabbit saphenous vein, which has a large population of endothelin ET_B receptors. The authors claimed that PKC plays an important part of smooth muscle cells induced by the endothelin receptors and is supported by others (see Karaki et al., 1997 for review). In contrast, the present results revealed that the selective PKC inhibitor Ro31-7549 did not affect the acute contractile responses to sarafotoxin6c or endothelin-1. This suggests that general protein kinase inhibitors such as staurosporine and K252a have effects on other protein kinases than PKC (Way et al., 2000).

The PKC isoenzymes are subdivided into three groups; the conventional, the novel and the atypical PKC isoforms with different cellular activities (Way et al., 2000). In the guinea-pig trachea, Inoue et al. (1989) did not observe any effect of calphostin C on S6c-induced increases of intracellular Ca^{2+} levels. It was observed that endothelin-1 translocated PKCα and that calphostin C caused a rightward shift in the endothelin ET_A-induced Ca^{2+} response. In addition, calphostin C is a PKC inhibitor which acts on the C_1 region that affects phorbol ester binding (Gordge and Ryves, 1994), and thus would not be expected to affect any of the atypical (ζ, λ and ι) PKC isoforms, whereas staurosporine, K252a and Ro31-7549 act on the ATP binding site and hence would be expected to affect this group of PKCs as well. Since Ro31-7549 did not affect the sarafotoxin 6c- or the endothelin-1-induced contractions, the atypical PKC isoforms seem not be involved in the contractile activity.

The long-term treatment with PKC inhibitors prevented the upregulation of endothelin ET_B receptors, suggesting that this upregulation of mRNA levels is mediated through a PKC pathway. In addition, the effects of the PKC inhibitors suggest that specific isoforms of PKC mediate the upregulation, however, we did not address this question presently.

The different PKC inhibitors used vary in their selectivity for different PKC isoforms, as well as in their selectivity for PKC compared to other similar kinases found in the cells. (Way et al., 2000). The most selective of the PKC inhibitors tested in this study was Ro31-7549, which completely prevented the upregulation of the endothelin ET_B receptor. It is worthwhile to note that the mRNA increase is equally inhibited by either of the PKC inhibitors, suggesting that they all prevent the upregulated process; however, to a varying degree as judged by the study of contractile phenotype experiments. Another similar "specific" PKC inhibitor, Ro31-8220, has recently been found to block the effects of mitogen activated protein kinase activated protein (MAPKAP) $K_{1β}$ and p70 S6 kinase as well, and thus the endothelin ET_B receptor upregulation may still be due to a non-PKC pathway (Alessi, 1996; Morreale et al., 1997). Since no data for such an interaction for Ro31-7549 yet is described, the present experiments suggest that the upregulation of endothelin ET_B receptors is mediated by one or more of the atypical PKC isoenzymes. The present data have demonstrated the importance of the PKC system in endothelin ET_B receptor induction.

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