British Journal of Pharmacology (2002) 137, 118–126
www.nature.com/bjp

Protein kinase mediated upregulation of endothelin A, endothelin B and 5-hydroxytryptamine 1B/1D receptors during organ culture in rat basilar artery

*[1,2]Jacob Hansen-Schwartz, [1]Carl-Lennart Svensson, [1]Cang-Bao Xu & [1,2]Lars Edvinsson

[1]Department of Internal Medicine, Lund University Hospital, Lund, Sweden and [2]Department of Clinical Experimental Research, Glostrup University Hospital, Glostrup, Denmark

1 Organ culture has been shown to upregulate both endothelin (ET) and 5-hydroxytryptamine 1B/1D (5-$HT_{1B/1D}$) receptors in rat cerebral arteries. The purpose of the present study was to investigate the involvement of protein kinases, especially protein kinases C (PKC) and A (PKA) in this process.

2 The effect of inhibiting protein kinases during organ culture with staurosporine (unspecific protein kinase inhitor), RO 31-7549 (specific inhibitor of classical PKC's) and H 89 (specific inhibitor of PKA) was examined using *in vitro* pharmacological examination of cultured vessel segments with ET-1 (unspecific ET_A and ET_B agonist), S6c (specific ET_B agonist) and 5-CT (5-HT_1 agonist). Levels of mRNA coding for the ET_A, ET_B, 5-HT_{1B} and 5-HT_{1D} receptors were analysed using real-time RT–PCR.

3 Classical PKC's are critically involved in the appearance of the ET_B receptor; co-culture with RO 31-7549 abolished the contractile response ($6.9 \pm 1.8\%$) and reduced the ET_B receptor mRNA by $44 \pm 4\%$ as compared to the cultured control. Correlation between decreased ET_B receptor mRNA and abolished contractile function indicates upstream involvement of PKC.

4 Inhibition of PKA generally had an enhancing effect on the induced changes giving rise to a 7–25% increase in E_{max} in response to ET-1, S6c and 5-CT as compared to the cultured control.

5 Staurosporine inhibited the culture induced upregulation of the response of both the ET_A and the 5-$HT_{1B/1D}$ receptors, but had no significant effect on the mRNA levels of these receptors. This lack of correlation indicates an additional downstream involvement of protein kinases.

British Journal of Pharmacology (2002) **137,** 118–126. doi:10.1038/sj.bjp.0704838

Keywords: Endothelin receptors; 5-HT_1 receptors; cerebral arteries; rats; protein kinases; organ culture; *in vitro* pharmacology; real-time PCR

Abbreviations: 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; bp, base pairs; EF1, elongation factor 1; ET, endothelin; MLCK, myosin light chain kinase; PKA, protein kinase A; PKC, protein kinase C; S6c, sarafotoxin 6c

Introduction

Organ culture of isolated whole segments of cerebral arteries result in an upregulation of both the endothelin (ET) (Hansen-Schwartz & Edvinsson, 2000; Hansen-Schwartz *et al*., 2002; Leseth *et al*., 1999) and the 5-hydroxytryptamine 1B/1D (5-$HT_{1B/1D}$) receptors (Hoel *et al*., 2001). Inhibition of gene transcription and gene translation effectively inhibits these phenotypical changes brought about through organ culture (Hansen-Schwartz *et al*., 2002; Möller *et al*., 1997) indicating that the changes are the result of gene induction and subsequent transcription and translation. In addition, depriving the vessels of glucose as well as warmth (37°C) during the process of organ culture also prevents the phenotypic changes (Adner *et al*., 1998) clearly indicating the change as a result of an active metabolic process. Thus, organ culture represents a stimulus to the smooth muscle cells and also, this stimulus must therefore activate a signal transduction pathway.

Given the almost ubiquitous involvement of both protein kinases A (PKA) and C (PKC) in intracellular signal transduction, we have aimed to characterize the involvement of these protein kinases in the events leading to the phenotypic changes in the ET and 5-HT_1 receptor function during organ culture. The study was carried out using the basilar artery from the rat using a sensitive *in vitro* pharmacology method to test the functional status of receptors studied, and quantitative real-time reverse transcriptase polymerase chain reaction for studies of receptor mRNA expression. The involvement of the protein kinases in the process was tested by coculturing the cerebral arteries with protein kinase inhibitors, notably staurosporine (unspecific protein kinase inhibitor), RO 31-7549 (specific inhibitor of classical PKC's) and H 89 (specific inhibitor of PKA).

*Author for correspondence at: Department of Clinical Experimental Research, EB 4th floor, Glostrup University Hospital, DK-2600 Glostrup, Denmark; E-mail: jacob.schwartz@dadlnet.dk

Methods

Tissue preparation and organ culture procedure

All animal procedures were carried out strictly within national laws and guidelines and approved by the University Animal Ethics Committee. Male Wistar-Kyoto rats (250–300 g) were anaesthetized using CO_2 and then killed by decapitation and the brain removed. Under microscope the basilar artery was carefully dissected free from the brain, cleared of connective tissue and cut into 1 mm long cylindrical segments with intact endothelial cell layer.

The segments were cultured in humidified air supplemented with 5% CO_2 for a period of 20 h in 1 ml serum free Dulbecco's modified Eagles' medium containing D-glucose 5 mM, $NaHCO_3$ 44 mM and N-acetyl-L-alanyl-L-glutamine 4 mM, supplemented with 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. To test the involvement of protein kinases in phenotypical changes of the receptor population, especially PKC and PKA, some of the vessel segments were cultured in the presence of different protein kinase inhibitors. Staurosporine is a potent inhibitor of a wide range of tyrosine and serine/threonine kinases with an IC_{50} of 10^{-8} M (Hoffman & Newlands, 1991). Among the inhibited kinases, some of the more important are PKC, PKA, MAP kinase, calmodulin dependent protein kinase and protein kinase G (Way *et al*., 2000). The concentration used was $10^{-7.5}$ M providing effective inhibition of most protein kinases. To provide more discrete analysis of the relative contributions of PKC and PKA, RO 31-7549, a selective PKC inhibitor with some isozyme specificity, primarily inhibiting classical PKC's (IC_{50} for PKC_{α} $10^{-7.3}$ M, $PKC_{\beta I}$ $10^{-6.7}$ M, $PKC_{\beta II}$ $10^{-6.8}$ M, PKC_{γ} $10^{-6.7}$ M, and PKC_{ε} $10^{-6.8}$ M (Wilkinson *et al*., 1993)), and H 89, a selective PKA inhibitor, ($K_i = 10^{-7.3}$ M (Chijiwa *et al*., 1990)) were used. Both of the latter inhibitors were employed at a concentration of $10^{-5.5}$ M.

In vitro *pharmacology*

The segments were mounted on two metal wires 40 μm in diameter (Myograph®, J.P. Trading, Denmark), one connected to a micrometer screw for adjustment of passive tension, and the other connected to a force displacement tranducer. The vessels were mounted submerged in a temperature controlled buffer solution (37°C) of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, MgCl 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. The buffer was continuously aerated with oxygen enriched with 5% CO_2 resulting in a pH of 7.4. Tensions were recorded by a PowerLab® unit (ADInstruments, Hastings, U.K.) using the program Chart®.

The vessels were stretched to an initial resting tone of 2 mN and then allowed to stabilize at this tone for 1 h. The viability of the vessels were tested by exposing them to an isotonic solution containing 60 mM K^+, obtained by partial change of NaCl for KCl in the above buffer. The contraction induced by K^+ was used as a measure of tissue contractile capability and as reference for subsequent contractile experiments.

The presence of an intact functional endothelium was tested by precontracting the vessel using 5-HT ($10^{-5.5}$ M) and subsequently exposing it to acetylcholine (10^{-5} M). A relaxant response of over 70% of the precontracted tension was considered indicative of a functional endothelium. The vessels were allowed to rest for 20 min before commencing experiments. Concentration-response curves were constructed with ET-1 in the concentration range 10^{-14}–10^{-7} M, sarafotoxin 6c (S6c) in the range 10^{-12}–10^{-7} M and 5-carboxamidotryptamine (5-CT) in the range 10^{-10}–$10^{-4.5}$ M, all as agonists. ET-1 is a general endothelin receptor agonist, S6c is a selective ET_B agonist (Hansen-Schwartz & Edvinsson, 2002) and 5-CT is selective for the $5\text{-}HT_1$ receptor (Hoyer *et al*., 1994).

Real time reverse transcriptase polymerase chain reaction method

To quantify mRNA for the ET_A, ET_B, $5\text{-}HT_{1B}$ and $5\text{-}HT_{1D}$ receptors, RT–PCR with real-time detection was employed.

Cellular RNA from the vessels was extracted using the TRIzol® agent after mechanical homogenization. The resulting RNA pellet was finally washed with 70–75% ice-cold ethanol, air-dried and re-dissolved in 20–80 μl diethyl-pyrocarbonate-treated water. The purity of RNA was assessed by spectrophotometry (DU64, Beckman, U.S.A.).

The reverse transcriptase synthesis of cDNA was done using 1 μg total RNA in a 20 μl reaction volume. The reaction mixture was incubated at 25°C for 10 min, 42°C for 15 min, heated to 99°C for 5 min and afterwards cooled to 5°C.

Quantitative real-time PCR was performed in a GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) using the GeneAmp SYBR® Green with the cDNA synthesized above as template in a 50 μl reaction volume. A blank control containing just water was included in all experiments. The detection system is based on an imaging system that, *via* the binding of fluorescent dye to the double-stranded DNA, monitors the total amount of DNA produced (Qing *et al*., 2001). Specific primers for the rat ET_A, ET_B, $5\text{-}HT_{1B}$ and $5\text{-}HT_{1D}$ receptors were designed using Primer Express® software (Applied Biosystems, U.S.A.) and had the following sequences:

ET_A receptor forward : 5′-ATT GCC CTC AGC GAA CAC-3′
reverse : 5′-CAA CAA AGC AGA AAG ACG ACG GTC-3′
ET_B receptor forward : 5′-GAT ACG AC AAC TTC CGC TCC-3′
$5\text{-}HT_{1B}$ receptor forward : 5′-TCC GGG TCT CCT GTG TAC GT-3′
reverse : 5′-GGC GTC TGA GAC TCG CAC TT-3′
$5\text{-}HT_{1D}$ receptor forward : 5′-CAC CCT CCC TCT ACG GGA AG-3′
reverse : 5′-AGC CCG TGA TAA GCT GTG CT-3′

mRNA for Elongation factor 1 (EF1) was used as reference because it is expressed constantly and independently of cell type (in a pilot study we found no difference in expression as compared to β-actin (data not shown)). The EF1 primers were designed as follows:

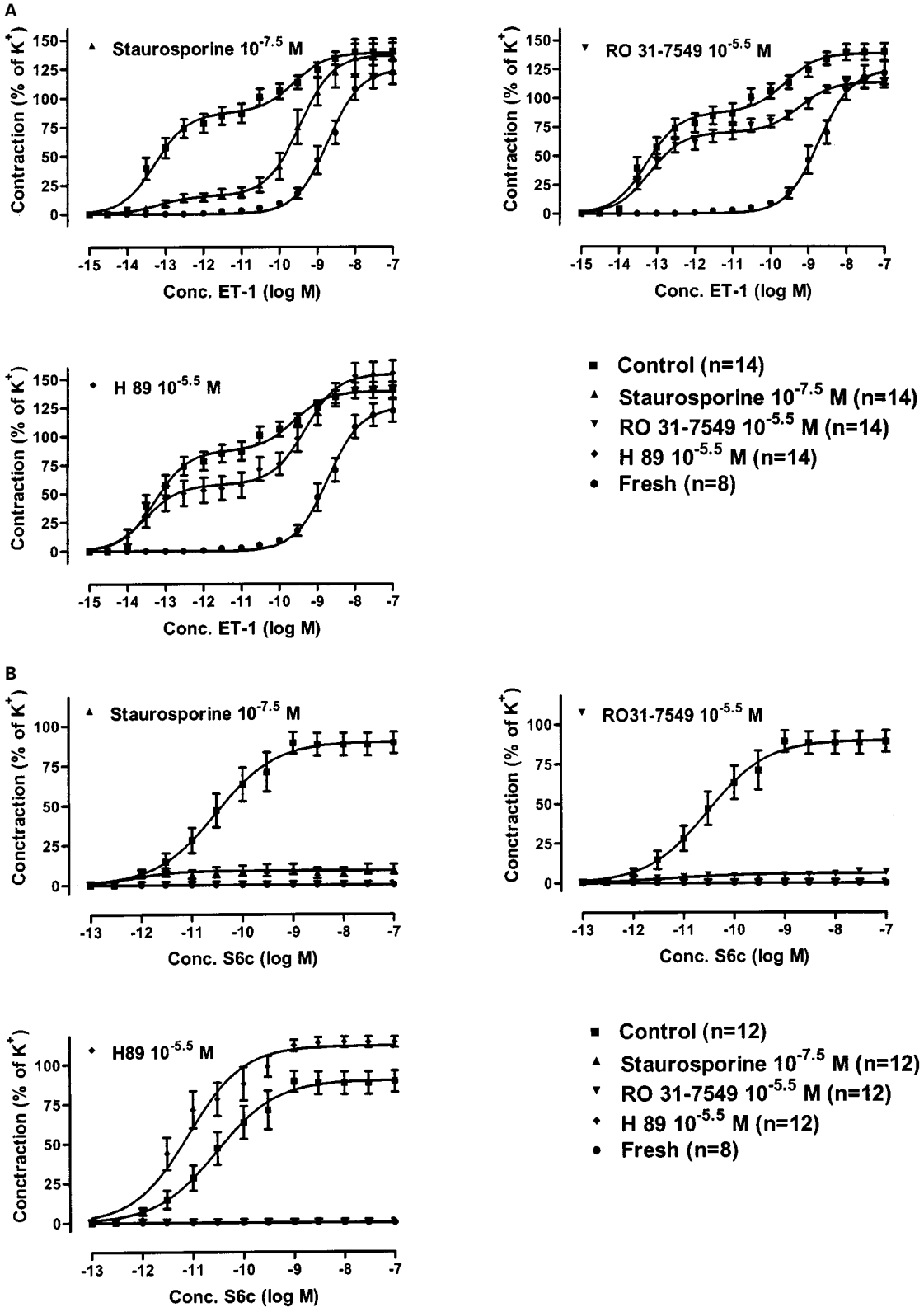
EF1 forward : 5′-GCA AGC CCA TGT GTG TTG AA-3′
reverse : 5′-TGA TGA CAC CCA CAG CAA CTG-3′

The real-time PCR was carried out with the following profile: The first cycle at 50°C for 2 min and 95°C for 10 min. The subsequent 40 cycles were done at 95°C for 15 s and 60°C for 1 min. To verify that each primer-pair only generated one PCR product at the expected size, the real-

time PCR products were separated electrophoretically in a 1.5% agarose gel containing 0.5 μg ml^{-1} ethidium bromide and subsequently viewed in a UV-box. The expected sizes of the PCR products were: ET_A 64 base pairs (bp), ET_B 86 bp, 5-HT_{1B} 51 bp, 5-HT_{1D} 51 bp, and EF1 96 bp.

Calculation and statistics

In vitro pharmacology Data are expressed as mean ± s.e.mean. Contractile responses in each segment are expressed as a percentage of the contraction induced in that segment



by 60 mM K^+. In a given experiment E_{max} denotes the maximum contractile response elicited and pD_2 the negative logarithm of the concentration that elicits half the maximum response. For biphasic responses, $E_{max(1)}$ and $pD_{2(1)}$ are used to describe the high affinity phase and $E_{max(2)}$ and $pD_{2(2)}$ to describe the low affinity phase.

Vessel segments cultured in the presence of staurosporine showed a significantly lower K^+ response compared to the cultured control. In order to be able to compare the contractile responses of these vessel segments with other segments, a correction factor was calculated. The factor was derived from the ratio of contraction induced by K^+ in arteries cultured with staurosporine to the contraction induced by K^+ in the cultured controls.

Differences in contractile responses were tested for statistical significance by comparing respective E_{max} and pD_2 values using Student's *t*-test. *P*-values below 0.05 were considered significant.

Real-time PCR C_T denotes the number of cycles for a given sequence to be amplified to a predefined detection threshold. The amount of cDNA for a given primer is expressed as a percentage of the amount of cDNA for EF1 as given by the equation

$$X = (1 + E)^{\Delta C_T}$$

where E is the replication efficiency of the PCR reaction, and ΔC_T is the difference in number of cycles to reach the detection limit between the reaction to amplify the cDNA in question and the reaction to amplify cDNA for EF1. Only responses above 0.1% were considered relevant. The efficiency of the amplification reaction was deduced from a so-called standard curve, a semi-logarithmic plot of C_T for a given primer against the logarithm of the concentration of total cDNA (measured arbitrarily). Given a straight regression line (which in itself is indicative of an amplification reaction without the presence of non-specific inhibitors), the efficiency was calculated by the equation

$$E = 0.1^{1/a} - 1$$

where a is the slope. The equation was derived by simple rearrangement of the equation for cumulative increase.

Measurements were repeated thrice, and differences were tested for statistical significance employing Student's *t*-test employing a probability level of 0.05.

Drugs

Staurosporine, RO 31-7549 and H 89 were obtained from Calbiochem, Sweden; endothelin-1 and sarafotoxin 6c from Auspep, Australia; 5-hydroxytryptamine and 5-carboxamidotryptamine from Sigma, Denmark; Dulbecco's modified Eagle's medium from Kebolab, Denmark. For molecular biology TRIzol® was obtained from Invitrogen, Denmark,

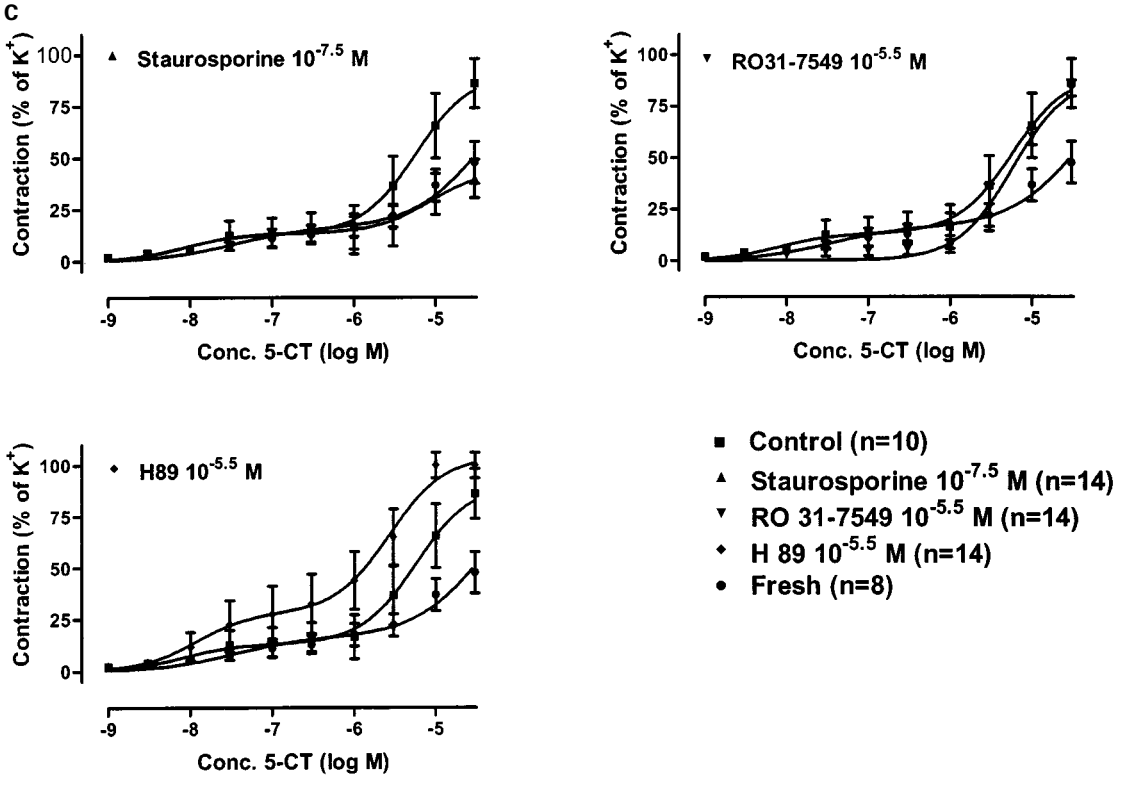


Figure 1 Concentration–contraction curves with (A) ET-1, (B) S6c and (C) 5-CT as agonists. The effect of co-culturing with staurosporine, RO 31-7549 and H 89, respectively, is displayed. Data are displayed as mean ± s.e.mean, *n* indicates number of rats in each experiment. The data for the curves displaying results after co-culture with staurosporine have been corrected due to lower K^+ contractility (see Methods). Staurosporine was in general capable of inhibiting changes approximating the concentration–response curves to the curves derived from experiments conducted on fresh arterial segments. Ro 31-7549 effectively inhibited the appearance of a contractile ET_B response, and H 89 had in general an augmenting effect on the changes induced by organ culture. For more details see Table 1 and the results section.

primers were custom synthesized by GibcoBRL Custom Primers (Life Technologies, U.K.); all other RT–PCR reagents were obtained from Applied Biosystems, Sweden. All chemicals were obtained from Merck Eurolab, Denmark. Only double-distilled water was used throughout all experiments.

Results

In vitro *pharmacology*

Contractile response to potassium; assessment of viability The contractile response of fresh arterial segments (4.2±0.3 mN), cultured controls (4.0±0.6 mN) or segments cultured in the presence of either RO 31-7549 (3.7±0.7 mN) or H 89 (3.9±0.5 mN) to 60 mM K^+ was not significantly different. However, the contraction produced in vessel segments cultured in the presence of staurosporine were on average 3.5 times less powerful (1.2±0.3 mN, $P<0.01$) compared to the cultured controls. The absolute maximum response to ET-1, however, was not different between cultured controls (4.5±0.8 mN) and segments cultured in the presence of staurosporine (4.1±0.7 mN). Therefore, to make comparisons possible between E_{max} values of vessels segments cultured in the presence of staurosporine with other vessel segments, the E_{max} values of the staurosporine cultured segments were corrected with a factor 3.5.

Contractile response to ET-1 Organ culture over 20 h induced an increased contractile response to ET-1 as compared to fresh vessels (Figure 1). The response was biphasic with a $pD_{2(1)}$ of 13.3±0.1, a $pD_{2(2)}$ of 9.6±0.2 and an $E_{max(1)}$ of 81.7±4.6% and an $E_{max(2)}$ of 140.6±7.1%. This clearly indicates an enhanced response to ET-1 as compared to fresh vessel segments where a contraction with a pD_2 of 8.7±0.1 and an E_{max} of 122.2±10.9% was obtained (normal sigmoidal shape). Organ culture in the presence of staurosporine significantly inhibited the first part of the biphasic response ($P<0.01$) whilst it had no effect on the second phase. Organ culture in the presence of RO 31-7549 did not affect the shape of the concentration response curve, though the final $E_{max(2)}$ (113.5±4.3%) was similar to the E_{max} value obtained in fresh vessels being significantly lower than the $E_{max(2)}$ of the cultured control ($P<0.05$). H 89 significantly attenuated the first part of the biphasic response, $E_{max(1)}$ 55.0±4.0% as compared to the cultured control ($P<0.05$). The second phase, however, was not affected by H 89 although there was a non-significant tendency of these vessels to have a higher final E_{max} compared to the cultured controls.

Contractile response to S6c In fresh vessels, application of S6c did not give rise to any contraction. In contrast, in the cultured control, S6c yielded a contraction with an E_{max} 89.3±6.7% and a pD_2 of 10.5±0.1 (Figure 1). Upon co-culture with either staurosporine or RO 31-7549, this upregulation was almost completely attenuated; E_{max} 9.8±3.1% and 6.9±1.8%, respectively, and pD_2 11.8±0.3 and 11.3±0.3, respectively ($P<0.01$). Interestingly, organ culture in the presence of H 89 potentiated the response with

Table 1 Overview of E_{max} and pD_2 values

		Fresh			*Cultured control*			*Incubated for 20 h with* *Staurosporine* ($10^{-7.5}$ M)			*RO 31-7549* ($10^{-5.5}$ M)			*H 89* ($10^{-5.5}$ M)	
Agonist	n	pD_2	E_{max} (% of K^+)	n	pD_2	E_{max} (% of K^+)	n	pD_2	E_{max} (% of K^+)	n	pD_2	E_{max} (% of K^+)	n	pD_2	E_{max} (% of K^+)
ET-1	8	8.7±0.1	122.2±10.9%	14	13.3±0.1	81.7±4.6%	14	13.2±0.2	17.0±3.5%**	14	13.2±0.1	71.2±2.8%	14	13.5±0.1	55.0±4.0%
					9.6±0.2	140.6±7.1%		9.5±0.1	136.2±15%		9.2±0.2	113.5±4.3%		9.3±0.1	155.3±11.0%
S6c	8	–	–	12	10.5±0.1	89.3±6.7%	12	11.8±0.3	9.8±3.1%**	12	11.3±0.3	6.9±1.8%**	12	11.1±0.1	114.1±3.5%**
5-CT	8	7.5±0.4	13.8±4.5%	10	7.8±0.3	17.4±4.6%	14	8.8±0.3	14.2±7.1%	14	(5.3±0.1)	(83.9±4.0%)	14	7.9±0.3	30.0±11.7%
		(4.4±0.1)	(47.8±10.2%)		(5.4±0.1)	(86.1±12.1%)		(5.0±0.2)	(40.0±8.9%)					5.6±0.1	100.1±6.2%

Two values in a cell indicates biphasic course of the concentration-contraction curve. Each vale corresponds to the respective phase. Differences compared to the respective cultured controls were tested using Student's *t*-test; *indicates $P<0.05$, and **$P<0.01$. *n* indicates number of rats in each experiment. Note: For the agonist 5-CT, it was, except for one experiment, not possible to employ a concentration at which the concentration-contraction curve levelled out, a criterion necessary for a valid estimate of E_{max} and pD_2. The values obtained are therefore quoted in parentheses and are based on the highest contraction possible with concentration range employed.

an E_{max} of 114±3.5% (significantly higher than the cultured control ($P<0.01$)) and a pD_2 of 11.1±0.1.

Contractile response to 5-CT In fresh vessels application of 5-CT gave rise to a biphasic contractile response with an $E_{max(1)}$ of 13.8±4.5%, an estimated $E_{max(2)}$ of 47.8±10.2%, a $pD_{2(1)}$ of 7.5±0.4 and an estimated $pD_{2(2)}$ of 4.4±0.1, respectively (Figure 1). Upon organ culture the contractile profile was still biphasic with an $E_{max(1)}$ of 17.4±4.6%, an estimated $E_{max(2)}$ of 86.1±12.1%, a $pD_{2(1)}$ of 7.8±0.3, and an estimated $pD_{2(2)}$ of 5.4±0.1. Co-culture with staurosporine significantly reduced $E_{max(2)}$ ($P<0.01$) making the curve similar to the one obtained in fresh vessels. RO 31-7549 changed the biphasic response to an ordinary sigmoidal curve which followed the second phase of the cultured control. Again, as with the response to S6c, organ culture in the presence of H 89 augmented the contractile response, $E_{max(1)}$ was 30.0±11.7%, $E_{max(2)}$ 100.1±6.2%, $pD_{2(1)}$ 7.9±0.3 $pD_{2(2)}$ 5.6±0.1, though not significantly. E_{max} and pD_2 values are summarized in Table 1.

Real time RT–PCR.

Relative amounts of mRNA for the ET_A, ET_B and 5-HT_{1B} receptors are presented in Table 2. mRNA for the 5-HT_{1D} receptor could not be detected. In Figure 2 the effect of co-culturing with the various inhibitors compared to the cultured control is shown. The mRNA level for the ET_A receptor was reduced by RO 31-7549, though statistically only borderline significant ($P=0.07$), whereas almost no effect was noted in the case of staurosporine or H 89. The levels of ET_B receptor mRNA was significantly reduced by both staurosporine ($P<0.01$) and RO 31-7549 ($P<0.05$) whereas again, H 89 was without effect. However, co-culture with H 89 significantly and drastically increased mRNA for the 5-HT_{1B} receptor ($P<0.01$), but the transcription of the same receptor was not affected by neither staurosporine nor RO 31-7549. The standard curve was straight for all primers employed yielding slopes between 3.1 and 3.2 indicating replication efficiencies close to 1 (a replication efficiency of unity would yield a slope of 3.3). All standard curves regressed with correlations between 0.98 and 0.99 indicating a uniform PCR product throughout, independent of cDNA concentration (Figure 3). Electrophoresis verified only one product for each primer pair at the expected size (data not shown).

Discussion

In the present study we have examined the influence of protein kinases, more specifically PKA and PKC, on the culture induced changes in the function of the ET_A, ET_B and 5-$HT_{1B/1D}$ receptors as well as on the mRNA levels for the respective receptors in the rat basilar artery.

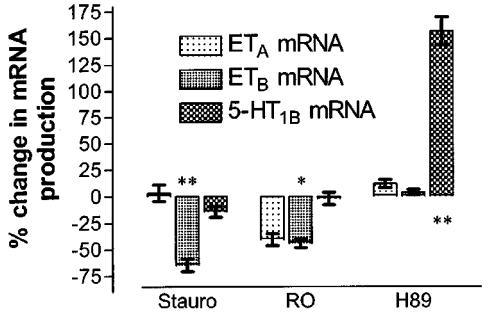


Figure 2 Quantitative analysis with real-time PCR of the levels of receptor mRNA when co-culturing with inhibitors compared to the amount in cultured controls. The bars represent mean±s.e.mean of three separate measurements. Statistically significant changes are indicated with *$P<0.05$ and **$P<0.01$. The level of ET_A receptor mRNA is not affected significantly through the inhibitors employed, the level of ET_B receptor mRNA is significantly reduced when co-culturing with staurosporine and RO 31-7549, and the level of 5-HT_{1B} receptor mRNA was greatly augmented when co-culturing with H 89. Results for mRNA for the 5-HT_{1D} receptor are not displayed since the levels were below detection limit.

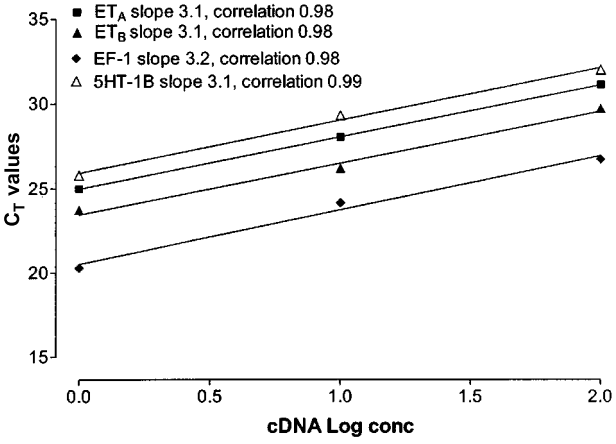


Figure 3 Standard curves for the primers employed for real-time PCR. All primers used had an efficiency of approaching unity, both in terms of amplification efficiency as well as purity of reaction.

Table 2 Amounts of mRNA (mean±s.e.m. for three separate measurements) for the ET_A, ET_B and 5-HT_{1B} receptors relative to the amount of mRNA for the reference protein, EF1

	Cultured control	*Staurosporine*	*RO 31-7549*	*H89*
ET_A mRNA	0.43±0.09%	0.46±0.13%	0.25±0.03%	0.49±0.12%
ET_B mRNA	2.03±0.30%	0.69±0.02%**	1.11±0.08%	2.09±0.02%
5-HT_{1B} mRNA	0.13±0.01%	0.11±0.01%	0.13±0.01%	0.33±0.04%**

Differences compared to the respective cultured controls were tested using Student's *t*-test; *indicates $P<0.05$, and **$P<0.01$.

Functional results

Only co-culture with staurosporine was able to inhibit the culture induced changes in all three receptor-systems; complete inhibition of receptor upregulation was observed both with 5-$HT_{1B/1D}$ and ET_B, and partial inhibition of ET_A receptor upregulation.

Organ culture in the presence of RO 31-7549, a specific inhibitor of the PKC isozymes PKC_α, $PKC_{\beta I+II}$, PKC_γ and PKC_ε (Way *et al*., 2000), completely abolished the upregulation of the contractile ET_B receptor when compared to the cultured control. Compared to the cultured control, a lower $E_{max(2)}$ was noted with ET-1 whereas no effect was observed in the response to 5-CT. Thus, it may be concluded that the classical PKC's are critically involved in the appearance of the contractile ET_B receptor, whereas the upregulation of 5-HT_{1B} and of ET_A receptors seems not dependent on a classical PKC related mechanism, though of course a role for novel and atypical classes of PKC cannot be ruled out (Way *et al*., 2000). Co-culture with H 89, a specific inhibitor of PKA, had in general an augmenting effect on the response of all the receptor systems studied, though most pronounced on the 5-$HT_{1B/1D}$ and ET_B responses. The response to ET-1 (mixed ET_A and ET_B response), was augmented in so far as $E_{max(2)}$ was slightly elevated. Apparently and interestingly, PKA therefore seems to have an inhibitory effect on receptor upregulation.

Another point to be discussed is the biphasic response of fresh vessels to 5-CT and the biphasic response of cultured vessels to ET-1 and to 5-CT. Usually a biphasic course of a concentration–contraction curve may be taken as indicative of the presence of two receptors with different affinity to the agonist employed. Hoel *et al*. (2001) showed, using the specific 5-$HT_{1B/1D}$ antagonist GR 55562, that the first part of the fresh curve was a 5-$HT_{1B/1D}$ receptor response whereas the latter part could be identified as a degenerate 5-HT_{2A} receptor mediated response. It was shown that the 5-$HT_{1B/1D}$ receptor was selectively upregulated as a result of organ culture. 5-CT is in addition an agonist at the 5-HT_7 receptor (Krobert *et al*., 2001), a receptor shown to mediate vasodilatation (Centurion *et al*., 2000; Terron & Falcon-Neri, 1999). Although the presence and functionality of 5-HT_7 receptors in rat cerebral arteries have to our knowledge not been explored, the influence of this receptor on the results obtained is an open question.

In a previous study performed on the rat basilar artery, it was shown that the enhanced response to ET-1 after organ culture was due to an upregulation of both the ET_A and the ET_B receptors (Hansen-Schwartz & Edvinsson, 2000). In the same study it was not possible to determine the relative contribution of each receptor to the total response to ET-1; it was demonstrated that selective ET_B antagonism had no effect on the ET-1, response whereas high doses of the selective ET_A antagonist FR 139317 ($10^{-5.5}$ M) had some antagonizing effect. Even so, the biphasic nature of the ET-1 curves is probably not exclusively caused by a double receptor relationship; when vessel segments were cultured in the presence of staurosporine or RO 31-7549, both observed to prevent the appearance of a contractile ET_B receptor, a biphasic course was still observed. For half a decade several studies have examined in some detail the culture induced changes in the vascular receptor phenotype of arterial segments (Adner *et al*., 1995; 1996; Hansen-Schwartz & Edvinsson, 2000; Hansen-Schwartz *et al*., 2002; Möller *et al*., 1997). Using cyclohexamide and Actinomycin D, it was shown that the appearance of a contractile ET_B receptor in mesenteric arterial segments after organ culture was dependent on transcription and translation, and concluded that it was due to *de novo* synthesis of receptors (Möller *et al*., 1997). In view of the biphasic response to ET-1, and on the presupposition that in the case of organ culture in the presence of either RO 31-7549 or staurosporine only the ET_A receptor is responsible for the contractile action of ET-1, another mechanism than a simple increase in the absolute number of receptors must be responsible for the enhanced response, unless possible new ET_A receptors were of a different nature as compared to the existing receptors. Other possible mechanisms responsible for the enhanced response could include the switching on of alternate and more efficient signal transduction pathways leading to contraction of the vascular smooth muscle cells, although at this point, it would be purely speculative to point at possible candidates.

In our primary studies on the effect of organ culture on rat basilar arteries (Hansen-Schwartz & Edvinsson, 2000), the vessel segments were cultured for over 48 h. In the present study we only cultured for 20 h to avoid toxic effects of the protein kinase inhibitors. In these primary studies only normal sigmoidal curves (but leftward shifted compared to fresh responses) were observed after organ culture. The reason for this difference could be that after 48 h phenotypic alterations have been completed, thus, at 20 h an intermediate stage is observed.

Effect on transcription

The results of the quantitative RT–PCR analysis revealed some discrepancy compared to the function, i.e. there is not complete congruence between amount of mRNA and functional status of the receptor. The highest degree of congruence is observed with the function of the ET_B receptor; when mRNA production is decreased there is also decreased function. This points to the involvement of classical PKC's upstream from transcription in the signal transduction pathway leading to the appearance of a contractile ET_B receptor. The correlation between transcription and appearance of a contractile ET_B receptor substantiates the idea of culture induced *de novo* synthesis of this receptor as previously suggested (Adner *et al*., 1996). mRNA for the ET_A receptor at large seems unaffected by the various inhibitors employed. The fact that staurosporine does not affect the mRNA levels of the ET_A receptor, yet able functionally to inhibit the organ culture induced changes, clearly points at possible downstream modulation of either translation or post-translational modification of receptors. In terms of both ET_A and ET_B receptor function, the results also suggest a downstream inhibitory effect of PKA, since the changes observed in function when co-culturing with H 89 are not reflected in the mRNA levels. PKA has an inhibitory effect upstream with regard to transcription of the 5-HT_{1B} receptor as judged from the increased levels of mRNA for this receptor upon inhibiting PKA. It may not be ruled out that this increased transcription has an effect on the functional responses. Clearly, vessel segments cultured with H 89 gives rise to the most powerful contractile response (though not statistically significant) to 5-CT. Given the

employment of a higher concentration of 5-CT, the difference may indeed reach statistical significance.

Usage of staurosporine as inhibitor of protein kinases

The use of staurosporine to elucidate intracellular signal transduction has been somewhat disapproved due to its wide range of biological effects, and certainly, it is no longer recommended for research into the specific role of PKC (Nixon, 1997; Way *et al*., 2000). Yet the use of staurosporine is still justified; staurosporine serves as a basic compound from which many of the more specific PKC inhibitors have been developed, including the specific PKC inhibitor RO 31-7549 used in the present study and in addition, the effect of staurosporine cannot be substituted by the collective effect of specific inhibitors (Condrescu *et al*., 1999). This latter fact alludes to the existence of as yet unknown protein kinases. The use of staurosporine is not uncomplicated as emphasised by our observation that the absolute response to K^+, but not ET-1, was decreased in vessels cultured in the presence of staurosporine. The contractile effect of K^+ is due to depolarisation of the cell membrane opening voltage controlled Ca^{2+} channels in the cell membrane. Since staurosporine did not affect E_{max} of ET-1, which would have been expected if staurosporine in general had affected the viability of the vessel, it is reasonable to conclude that co-culturing with staurosporine leads to an impairment of the function or number of voltage controlled Ca^{2+} channels.

Relevance to cerebrovascular pathophysiology

Organ culture induced changes are as such *in vitro* phenomena. Even so, there are preliminary data to suggest that alteration of the vascular receptor phenotype may also be relevant in the understanding of cerebrovascular diseases. In a rat model of stroke using temporary occlusion of the middle cerebral artery, the appearance of a contractile ET_B receptor has been demonstrated in the middle cerebral artery (Ahlrot *et al*., 2001), and in a rat model of subarachnoid haemorrhage, increased potency toward ET-1 in the basilar artery was observed (Alafaci *et al*., 1990). Therefore, it might be suggested, that the changes induced in the culture cabinet to some extent mimics events taking place in the course of cerebrovascular diseases (although certainly, more experimental data are needed to substantiate such a claim). Knowledge of the involvement of protein kinases in short-term receptor changes in vascular smooth muscle cells may thus have a bearing on providing future pharmacological targets for treatment of these diseases. When considering the involvement of protein kinases, the complexity of intracellular signal transduction and, indeed the interaction between different pathways, must be taken into account (Garrington & Johnson, 1999; Schaeffer & Weber, 1999). Therefore, although only PKA and PKC have been considered, it is likely that other pathways are also involved, e.g. the MAPkinase pathway.

This work was supported by Swedish Research Council (grant no. 5958), King Oscar V and Queen Victoria Foundation (Sweden), the Leo Foundation and the Foundation for Research in Neurology (the latter two both in Denmark). Karen Eskesen is thanked for her technical assistance in carrying out some of the real time RT–PCR parts of the study.

References

ADNER, M., CANTERA, L., EHLERT, F., NILSSON, L. & EDVINSSON, L. (1996). Plasticity of contractile endothelin-B receptors in human arteries after organ culture. *Br. J. Pharmacol.,* **119,** 1159–1166.

ADNER, M., ERLINGE, D., NILSSON, L. & EDVINSSON, L. (1995). Upregulation of a non-ET_A receptor in human arteries in vitro. *J. Cardiovasc. Pharmacol.,* **26** (Suppl. 3): S314–S316.

ADNER, M., GEARY, G.G. & EDVINSSON, L. (1998). Appearance of contractile endothelin-B receptors in rat mesenteric arterial segments following organ culture. *Acta Physiol Scand,* **163,** 121–129.

AHLROT, E., UDDMAN, E., MALMSJÖ, M., MÖLLER, S., GIDÖ, G., WIELOCH, T. & EDVINSSON, L. (2001). Ischemic stroke upregulates vascular endothelin receptors in rats. *J. Cereb. Blood. Flow. Metab.,* **21,** S140–S140.

ALAFACI, C., JANSEN, I., ARBAB, M.A., SHIOKAWA, Y., SVENDGAARD, N.A. & EDVINSSON, L. (1990). Enhanced vasoconstrictor effect of endothelin in cerebral arteries from rats with subarachnoid haemorrhage. *Acta. Physiol. Scand.,* **138,** 317–319.

CENTURION, D., SANCHEZ-LOPEZ, A., ORTIZ, M.I., DE VRIES, P., SAXENA, P.R. & VILLALON, C.M. (2000). Mediation of 5-HT-induced internal carotid vasodilatation in GR127935- and ritanserin-pretreated dogs with $5\text{-}HT_7$ receptors. *Naunyn. Schmiedebergs. Arch. Pharmacol.,* **362,** 169–176.

CHIJIWA, T., MISHIMA, A., HAGIWARA, M., SANO, M., HAYASHI, K., INOUE, T., NAITO, K., TOSHIOKA, T. & HIDAKA, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J. Biol. Chem.,* **265,** 5267–5272.

CONDRESCU, M., HANTASH, B.M., FANG, Y. & REEVES, J.P. (1999). Mode-specific inhibition of sodium-calcium exchange during protein phosphatase blockade. *J. Biol. Chem.,* **274,** 33279–33286.

GARRINGTON, T.P. & JOHNSON, G.L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Opin. Cell. Biol.,* **11,** 211–218.

HANSEN-SCHWARTZ, J. & EDVINSSON, L. (2000). Increased sensitivity to ET-1 in rat cerebral arteries following organ culture. *Neuroreport,* **11,** 649–652.

HANSEN-SCHWARTZ, J. & EDVINSSON, L. (2002). Endothelin. In *Cerebral Blood Flow and Metabolism*. ed. Edvinsson, L. & Krause, D. pp. 339–353. New York: Lippincott, Williams & Wilkins.

HANSEN-SCHWARTZ, J., NORDSTROM, C.H. & EDVINSSON, L. (2002). Human endothelin subtype A receptor enhancement during tissue culture via de novo transcription. *Neurosurgery,* **50,** 127–135.

HOEL, N.L., HANSEN-SCHWARTZ, J. & EDVINSSON, L. (2001). Selective up-regulation of $5\text{-}HT_{1B/1D}$ receptors during organ culture of cerebral arteries. *Neuroreport,* **12,** 1605–1608.

HOFFMAN, R. & NEWLANDS, E.S. (1991). Role of protein kinase C in adriamycin-induced erythroid differentiation of K562 cells. *Cancer Chemother. Pharmacol.,* **28,** 102–104.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.,* **46,** 157–203.

KROBERT, K.A., BACH, T., SYVERSVEEN, T., KVINGEDAL, A.M. & LEVY, F.O. (2001). The cloned human 5-HT_7 receptor splice variants: a comparative characterization of their pharmacology, function and distribution. *Naunyn. Schmiedebergs. Arch. Pharmacol.,* **363,** 620–632.

LESETH, K.H., ADNER, M., BERG, H.K., WHITE, L.R., AASLY, J. & EDVINSSON, L. (1999). Cytokines increase endothelin ET_B receptor contractile activity in rat cerebral artery. *Neuroreport,* **10,** 2355–2359.

MÖLLER, S., EDVINSSON, L. & ADNER, M. (1997). Transcriptional regulated plasticity of vascular contractile endothelin ET_B receptors after organ culture. *Eur. J. Pharmacol.,* **329,** 69–77.

NIXON, J.S. (1997). The biology of Protein Kinase C Inhibitors. In *Protein Kinase C*. ed. Parker, P.J. & Dekker, L.V. pp. 205–236. Austin: R.G. Landes Company.

QING, X., SVAREN, J. & KEITH, I.M. (2001). mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. *Am. J. Physiol. Lung. Cell. Mol. Physiol.,* **280,** L547–L554.

SCHAEFFER, H.J. & WEBER, M.J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.,* **19,** 2435–2444.

TERRON, J.A. & FALCON-NERI, A. (1999). Pharmacological evidence for the 5-HT_7 receptor mediating smooth muscle relaxation in canine cerebral arteries. *Br. J. Pharmacol.,* **127,** 609–616.

WAY, K.J., CHOU, E. & KING, G.L. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends. Pharmacol. Sci.,* **21,** 181–187.

WILKINSON, S.E., PARKER, P.J. & NIXON, J.S. (1993). Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.,* **294** (Pt 2), 335–337.

(Received April 9, 2002
Revised May 16, 2002
Accepted June 10, 2002)