Effect of Organ Culture on Noradrenaline-Evoked Contraction, Calcium Signalling and TRPC Expression in Rat Mesenteric Artery

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Key Words

Mesenteric artery • Organ culture • Contractile phenotype • Ca²⁺ signal • Noradrenaline • Transient receptor potential canonical • Src kinase

Abstract

The aim of this study was to explore the changes evoked by organ culture in the signalling pathways activated by noradrenaline in rat resistance mesenteric artery. Contractile responses and calcium signalling were significantly more sensitive to noradrenaline in arteries cultured for 48–72 h in the absence of growth factors compared to fresh arteries. Both calcium release activated by noradrenaline in calcium-free solution and calcium entry measured after the addition of external calcium were higher in cultured arteries than in fresh tissue. Blockers of non-selective cation channels (SKF-96365, flufenamic acid, Gd³⁺) more potently inhibited noradrenaline contraction in cultured arteries than in fresh ones. The src kinase inhibitors genistein or PP2 normalised the increased contraction and the increased calcium release evoked by noradrenaline in cultured arteries. In cultured arteries, trpc1 (transient receptor potential canonical 1) mRNA expression was decreased by $47 \pm 8\%$ ($n = 5$, $p < 0.05$), while trpc6 mRNA expression was increased by $92 \pm 24\%$ ($n = 5$, $p < 0.05$) in comparison with non-cultured arteries. Immunofluorescence analysis of protein expression confirmed the

up-regulation of TRPC6 protein after culture. These results indicate that mesenteric artery culture results in src kinase-dependent increase in the responses to noradrenaline and in a change in cation channel activity, which could contribute to the increased contraction.

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Introduction

Organ culture of isolated artery is frequently used as an experimental model for vascular pathologies characterised by growth and proliferation of vascular smooth muscle cells (VSMC), such as atherosclerosis, hypertension or balloon injury. Arteries cultured in the absence of added growth factors maintain their contractile phenotype for several days [1, 2]. However, culture has been reported to evoke diverse cellular changes. Cultured porcine coronary artery displays increased contractile and Ca²⁺ responses to endothelin-1, which are attributed to ETA receptors [3]. In rat basilar artery, organ culture increases the contraction to endothelin-1, an effect associated with the up-regulation of ETB receptor without change in ETA receptor subtype [4]. Organ culture of rat mesenteric artery has been reported to enhance the contraction evoked by noradrenaline, and to increase α_1 -adrenoceptor mRNA expression, through a signalling

pathway involving PKC and ERK1/2 [5]. Organ culture of vascular tissue also evokes changes in the Ca^{2+} handling, as suggested by the increase in store-operated Ca^{2+} entry in cultured cerebral arteries, which has been reported to be related to the increased expression of TRPC1 (transient receptor potential canonical 1) [1]. The latter observation is of particular interest since calcium is known to play a major role in cell proliferation.

The aim of the present study was to characterize the effect of 48 h of organ culture on the Ca^{2+} signal and contraction in response to noradrenaline in resistance mesenteric arteries of the rat. We also investigated the effect of culture on the signalling pathways involved in these responses. Vasoconstriction of peripheral arteries in response to the sympathetic neurotransmitter noradrenaline plays an important role in the regulation of arterial blood pressure. The activation of the $\alpha 1$ -adrenoreceptor leads to the production of IP₃ and diacylglycerol (DAG), the release of internal Ca^{2+} stores and Ca^{2+} influx. The experiments were designed to determine whether culture affects noradrenaline-activated Ca^{2+} signalling in resistance mesenteric arteries, in relation to contractile responses. Results indicated that organ culture of mesenteric artery increased its contractile sensitivity to noradrenaline and to the G protein activator AlF_4 , without changing the maximum level of contraction. Pharmacological and molecular tools were used to investigate the molecular basis of this effect.

Material and Methods

Organ Culture

All protocols were in accordance with institutional guidelines for the use of experimental animals. The rats were anaesthetised and sacrificed by decapitation at a body weight of 150–200 g. The mesenteric artery was rapidly removed and immersed in phosphate-buffered saline (PBS; composition, in mmol/l: NaCl 136.9; KCl 2.7; KH_2PO_4 1.5; Na_2PO_4 8; pH 7.4, with Tris). Third-order branches of the superior mesenteric artery (mean internal diameter normalised at 100 mm Hg was $304 \pm 5.7 \mu\text{m}$) were carefully cleaned of all fat and connective tissue, and the endothelium was gently removed using a sterile hair. Segments, about 2 mm in length, were either immediately used for contraction or kept at -80°C for further analysis, or cultured for 24, 48 or 72 h in an incubator at 37°C with humidified 5% CO_2 in Dulbecco's modified Eagle's medium without serum, with 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin and 2 mmol/l glutamine.

Measurement of Contractile Response

Freshly isolated (condition 0 h) or cultured segments of mesenteric artery were mounted in a wire myograph (Model 500A, Danish Myo Technology A/S, Aarhus, Denmark) filled with physio-

logical solution (composition, in mmol/l: NaCl 122; NaHCO_3 15; KCl 5.9; CaCl_2 1.25; MgCl_2 1.2; glucose 11) bubbled with 95% O_2 /5% CO_2 and maintained at 37°C . The vessel was set at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mm Hg. After an equilibration period, each preparation was contracted by changing the physiological solution in the bath to a depolarizing 100 mmol/l KCl solution (composition, in mmol/l: NaCl 27; KCl 100; NaHCO_3 15; MgCl_2 1.25; CaCl_2 1.25; glucose 11). Effective removal of the endothelium was tested by measuring the relaxation evoked by acetylcholine (1 $\mu\text{mol}/\text{l}$). After the KCl-evoked contraction, the NO synthase inhibitor N ω -nitro-L-arginine (L-NNA; 100 $\mu\text{mol}/\text{l}$) was added to the physiological solution.

Arteries were thereafter stimulated by cumulative additions of noradrenaline (10 nmol/l to 10 $\mu\text{mol}/\text{l}$, final concentration). After washout, the vessels were incubated with inhibitors or equal volume of solvent for 30 min, and the noradrenaline concentration-response curve was resumed in the continuous presence of the inhibitor/solvent. Contractile response to noradrenaline was expressed either in mN/mm (by dividing the force developed by the artery by twice the length of the artery segment) or as a percentage of the maximum response recorded during the first stimulation.

Measurement of Cytosolic Ca^{2+} Concentration and Contractile Tension

Freshly isolated or cultured mesenteric arteries were incubated for 3–3.5 h at room temperature in physiological solution (composition as above) containing 5 $\mu\text{mol}/\text{l}$ fura-2 acetoxymethyl ester (fura-2-AM) and 0.05% Cremophor EL [6]. After the loading period, the arteries were mounted in a myograph continuously perfused with oxygenated physiological solution supplemented with L-NNA (100 $\mu\text{mol}/\text{l}$) at 37°C . The myograph was part of a fluorimeter (CAF, Jasco, Tokyo, Japan) which allowed estimation of the Ca^{2+} signal. The muscle tone was measured using an isometric force transducer. Arteries were first stimulated with 100 mmol/l KCl solution. After washout, arteries were incubated with or without inhibitor or solvent and stimulated with noradrenaline. To measure Ca^{2+} release evoked by noradrenaline, arteries were perfused with Ca^{2+} -free solution prepared from physiological solution without Ca^{2+} supplemented with EGTA (0.1 mmol/l). At the end of the experiment, the fura-2- Ca^{2+} signal was calibrated: the maximum fluorescence ratio F340/F380 was measured in saturating calcium medium after addition of ionomycin (10 $\mu\text{mol}/\text{l}$) and the minimum ratio was obtained in the presence of EGTA (10 mmol/l). The Ca^{2+} signal was corrected for the autofluorescence measured at 340 and 380 nm after the addition of MnCl_2 (5 mmol/l). Cytosolic Ca^{2+} concentration was calculated as described previously [6, 7]. Responses to noradrenaline were normalised to the response evoked by the high-KCl solution in the same artery segment.

Total RNA Isolation

Total RNA was isolated from freshly isolated and from cultured mesenteric arteries using the Trizol isolation reagent (Invitrogen), and treated with DNase I (Invitrogen). The RNA sample was additionally purified by ethanol precipitation and its concentration was determined by measuring absorbance at 260 nm.

Table 1. Oligonucleotide sequences of rat primers used for RT-PCR

mRNA	Accession number	Orientation	Primer sequence (5'–3')	Amplicon, bp
rtrpc1	NM_053558	forward	CTGTGGTATGAAGGGTTGGAAG	425
		reverse	GTGTCGTTGCTTTGCTGTTTAC	
rtrpc3	NM_021771	forward	TGCTTGTGTTCAACGCCTCA	146
		reverse	CCCGAGAACCCAGACCATAAT	
rtrpc4	NM_053434	forward	CGTGGAAGGGGGGACTATG	181
		reverse	CGCATCGCCAACATAGACATTGAA	
rtrpc5	NM_080898	forward	AACCTACTTCGGAGCCAATGTAC	158
		reverse	CCGGATTTTACCTAACTCTACCTC	
rtrpc6	NM_053559	forward	GTCGGTGGTCATCAACTACAATC	155
		reverse	CCACATCCGCATCATCTCAATT	
rtrpc7	XM_225159	forward	ATCATCTCGGAAGGGCTGTAC	209
		reverse	GCACCTCGGTAGTAGGAGTAC	
rpl	X06483	forward	AGGCACCAGTCGGACCGATA	159
		reverse	GAAGCCGCTAGGCAGCATGT	

trpc = Transient receptor potential canonical; rpl = ribosomal protein L32.

PCR Primers

PCR primers for the genes chosen for classic PCR were designed based on published sequences in GenBank (table 1).

Polymerase Chain Reaction

DNase-treated RNA samples were reverse transcribed into cDNA by using Superscript II (Invitrogen). Reverse transcriptase-negative controls were prepared without the addition of Superscript II to exclude the presence of contaminating genomic DNA. PCR reactions were performed using the AccuPrime™ SuperMix I (Invitrogen) with a tracer amount (approximately 25 kBq) of α -³²P-dCTP (Amersham) and primer pairs based on known rat trpc sequences specific for the different trpc isoforms (table 1). The Techne Progene PCR cycloer was programmed to perform initial denaturation at 95°C for 5 min followed by 30 cycles for trpc and 24 for rpl32 (ribosomal protein L32) of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 2 min, followed by prolonged extension at 68°C for 10 min. PCR products were separated on polyacrylamide gels. The radiolabelled bands in dried gels were detected and quantitated by means of a Cyclone Storage Phosphor System (Perkin-Elmer, Boston, Mass., USA). mRNA expression was normalised to the level of expression of rpl32 mRNA.

Immunohistochemistry

After dissection or culture, mesenteric segments were immediately washed twice in PBS (containing, in mmol/l: NaCl 137; KCl 6; CaCl₂ 2; MgCl₂ 1.2; glucose 10; HEPES 10; pH 7.4, with Tris), pH 7.3, opened longitudinally and fixed with 4% formaldehyde in PBS for 10 min. Arteries were then permeabilised by treatment with 1% Triton X-100 for 15 min.

Arteries were incubated for 60 min in PBS containing 5% bovine serum albumin at 37°C, followed by incubation in PBS containing 1% bovine serum albumin and rabbit polyclonal anti-TRPC6 (Alomone, Jerusalem, Israel) at 1:250 dilution. After

washing with PBS, segments were treated with Alexa 568 anti-rabbit IgG (Molecular Probes) at 1:1,000 and TO-PRO-3 (1 μ mol/l, Molecular Probes) for DNA staining, washed again and placed luminal-side up on a glass slide and mounted in Mowiol (Calbiochem). Arteries were then visualised using a Zeiss Axiovert microscope coupled to a MRC-1024 confocal scanning laser imaging system (BioRad). In each artery, 10 optical sections of 238 μ m² were taken every 0.5 microns on the z-axis. The average pixel fluorescence value was calculated using the formula:

$$\text{Average} = \frac{\sum i(p)}{n(p)} (\text{I.U./pixel}),$$

where $i(p)$ is the intensity of a pixel and $n(p)$ is the number of pixels.

Drugs Used

PD 98059, RHC-80267, GF-109203, PP2, genistein and fura-2 acetoxymethylester (fura-2-AM) were purchased from Calbiochem. Gö 6976, flufenamic acid and GdCl₃ were obtained from Sigma. AlF₄ was produced by the combination of 5 mmol/l sodium fluoride (NaF) and 30 mmol/l aluminium chloride (AlCl₃). Stock solutions of PP2, genistein, Gö 6976, RHC-80267, GF-109203 and PD 98059 were prepared in DMSO at 10 mmol/l. Gd³⁺ and SKF-96365 were dissolved in water. Flufenamic acid was dissolved in ethanol.

Statistical Analysis

Data are presented as means $\pm$ SEM. The noradrenaline concentration producing 50% of the maximal effect (EC₅₀), or pD₂ value (–logEC₅₀), was calculated by non-linear curve fitting (Prism, GraphPad). Log values were used for statistical analysis. Comparisons were tested with Student's *t* test or ANOVA. $p < 0.05$ was considered significant.

Fig. 1. Effect of src kinase, PKC, DAG lipase and MAPK inhibitors on the contractile response to noradrenaline in fresh and cultured mesenteric artery. **a** Control concentration-response curves to noradrenaline in freshly isolated arteries ($n = 62$) or in arteries cultured for 24 h ($n = 6$), 48 h ($n = 62$) or 72 h ($n = 6$). Data are expressed as percent of the maximum contraction. **b–f** Noradrenaline concentration-response curves in freshly isolated mesenteric arteries (squares) and in those cultured for 48 h (tri-

angles) determined after incubation with different antagonists (solid lines) or their solvent (dotted lines). **b** PD 98059 ($10 \mu\text{mol/l}$, fresh $n = 4$, cultured $n = 4$). **c** Genistein ($1 \mu\text{mol/l}$, fresh $n = 8$, cultured $n = 6$). **d** PP2 ($1 \mu\text{mol/l}$, fresh $n = 6$, cultured $n = 8$). **e** Gö 6976 ($10 \mu\text{mol/l}$, fresh $n = 4$, cultured $n = 4$). **f** RHC-80267 ($10 \mu\text{mol/l}$, fresh $n = 4$, cultured $n = 4$). Data are expressed as percent of the maximum contraction evoked by noradrenaline before incubation with antagonists/solvents.

Table 2. Effect of organ culture on contractile responses of mesenteric resistance artery

	Fresh artery ($n = 62$)	48 h cultured artery ($n = 62$)
<i>Noradrenaline</i>		
Maximum contraction (mN/mm)	3.8 ± 0.2	3.7 ± 0.2
pD2	5.87 ± 0.03	6.12 ± 0.03^a
<i>KCl</i>		
Maximum contraction (mN/mm)	3.2 ± 0.2	3.2 ± 0.2

Noradrenaline pD2 values were calculated by nonlinear regression of individual concentration-contraction curves. Each value represents the mean $\pm$ SEM from n determinations. Statistical analyses were performed with Student's t test.

^a $p < 0.05$ vs. freshly isolated arteries.

Results

Cultured Arteries Are More Sensitive to Noradrenaline than Are Freshly Isolated Mesenteric Arteries

In freshly isolated mesenteric arteries mounted in a wire myograph, cumulative increase in the concentration of noradrenaline induced a concentration-dependent contraction. After 2 days of organ culture, we observed a significant increase in the sensitivity of the contraction to noradrenaline, which did not further change after 3 days of culture. At 48 h of culture, the concentration-response curve was shifted to the left, as indicated by the significant increase in the noradrenaline pD2 value, from 5.87 ± 0.03 ($n = 62$) in freshly isolated arteries to 6.12 ± 0.03 ($n = 62$) in cultured arteries ($p < 0.01$; fig. 1a, table 2). Culturing the artery segments for 24 h did not evoke a significant change in the contractile response to noradrenaline (pD2 was 5.89 ± 0.14 , $n = 6$). The maximum

contraction evoked by 10 $\mu\text{mol/l}$ noradrenaline was not affected by culture (table 2). Culture also did not change the contractile response to 100 mmol/l KCl depolarizing solution (table 2).

Noradrenaline-Evoked Contraction Requires Extracellular Ca^{2+} Entry in Both Fresh and Cultured Mesenteric Arteries, but Cultured Arteries Are More Sensitive to Blockers of TRPC Channels

The increase in cytosolic Ca^{2+} responsible for the noradrenaline-evoked contraction is known to originate from the intracellular and the extracellular compartments, through Ca^{2+} selective voltage-dependent channels (VOC) and non-selective cationic channels [8]. In order to investigate the contribution of Ca^{2+} entry to the contractile response to noradrenaline, we tested the effect of several inhibitors of Ca^{2+} channels in arteries mounted in the wire myograph.

To determine the involvement of VOC channels in noradrenaline-induced contraction, we used the dihydropyridine VOC inhibitor nimodipine. At 1 $\mu\text{mol/l}$, nimodipine inhibited the contractile response of mesenteric arteries to 1 $\mu\text{mol/l}$ noradrenaline by $94.6 \pm 0.1\%$ ($n = 3$), suggesting that VOC channels play a prominent role in the contraction (fig. 2). The effect of nimodipine was not affected by culture.

Recently, non-voltage-dependent cation channels belonging to the TRP family have been reported to be involved in artery contraction evoked by agonists [9]. To investigate their contribution to the Ca^{2+} entry pathways activated by noradrenaline and to the change evoked by artery culture, 3 blockers of non-voltage-dependent cation channels were used [10]. Lower concentrations of the inhibitors (flufenamic acid 10 $\mu\text{mol/l}$, SKF-96365 3 $\mu\text{mol/l}$ and Gd^{3+} 0.1 $\mu\text{mol/l}$) did not affect noradrenaline-evoked contraction in freshly isolated arteries but significantly displaced noradrenaline concentration-response curves in cultured arteries, indicating that cultured arteries were more sensitive to these compounds than freshly isolated arteries (fig. 3). Higher concentrations depressed noradrenaline contraction in fresh and in cultured arteries, but inhibition was more pronounced in cultured arteries.

The Increased Sensitivity to Noradrenaline in Cultured Mesenteric Artery Is Abolished in the Presence of src Kinase Inhibitors, but Is Unaffected by PKC, Diacylglycerol Lipase and MAPK Inhibitors

Ca^{2+} entry evoked by α -adrenergic agonists has been reported to result from the activation of TRPC6 by phos-

Fig. 2. Effect of nimodipine on contractile tension evoked by noradrenaline in mesenteric artery. Typical traces of the contraction induced by noradrenaline (1 $\mu\text{mol/l}$) in freshly isolated resistance mesenteric artery. Nimodipine (Nimo; 1 $\mu\text{mol/l}$, black trace) or ethanol (0.01%, shaded trace) were added at the plateau of the contraction, as indicated. The experiment was repeated 3 times with the same result.

pholipase C-mediated generation of diacylglycerol (DAG) [11, 12]. In contrast to reports showing the activation of Ca^{2+} entry by OAG, a synthetic analogue of DAG [13–16], we did not detect any contraction in response to OAG (100 $\mu\text{mol/l}$) in mesenteric arteries either before or after organ culture. To further test the potential involvement of DAG in the response to noradrenaline, we pre-treated vessels with the DAG lipase inhibitor RHC-80267 to prevent DAG degradation. Pre-incubation of the mesenteric arteries with RHC-80267 did not affect the contraction evoked by noradrenaline either in cultured or in fresh arteries (fig. 1f, table 3).

The pre-incubation of the mesenteric arteries with the specific PKC inhibitor Gö 6976 (10 $\mu\text{mol/l}$) [16, 17] depressed the contractile response to noradrenaline. The maximum response was decreased by $20.9 \pm 8.9\%$ ($n = 4$) and $35 \pm 6.3\%$ ($n = 4$), in fresh and cultured mesenteric arteries, respectively. However, Gö 6976 did not affect the increase in noradrenaline sensitivity observed in cultured arteries compared to fresh tissue (fig. 1e, table 3). A similar effect was observed with another PKC inhibitor, GF-109203 (table 3).

Src kinases are highly expressed in smooth muscle cells and have been suggested to play a role in the regulation of arteriolar contractility [18]. In order to investigate their potential role in the change in noradrenaline-evoked contraction in cultured mesenteric artery, we used the src kinase family inhibitors genistein and PP2. Genistein (1 $\mu\text{mol/l}$) displaced the noradrenaline concentration-response curve to the right, and depressed the maximum

Fig. 3. Effect of cation channel inhibitors on noradrenaline-induced contraction. Freshly isolated mesenteric arteries (**a, c, e**) and those cultured for 48 h (**b, d, f**) were stimulated by cumulative concentrations of noradrenaline in the absence or in the presence of SKF 96365 (**a, b**), flufenamic acid (FFA; **c, d**), or Gd^{3+} (**e, f**), or their solvent. Data are expressed as percent of the maximum contraction evoked by noradrenaline in the absence of antagonist/solvent and are shown as means $\pm$ SEM ($n = 4$). * $p < 0.05$ versus curves measured in the presence of the solvent (ANOVA).

Table 3. Effect of antagonists on noradrenaline pD₂ value in fresh and cultured mesenteric arteries

Antagonists	Fresh artery		48 h cultured artery	
	solvent	with antagonist	solvent	with antagonist
PP2 (1 $\mu\text{mol/l}$)	5.9 ± 0.09 ($n = 8$)	5.71 ± 0.09 ($n = 6$)	6.10 ± 0.02^a ($n = 8$)	5.81 ± 0.12 ($n = 8$)
Genistein (1 $\mu\text{mol/l}$)	5.83 ± 0.10 ($n = 6$)	5.71 ± 0.21 ($n = 8$)	6.23 ± 0.09^a ($n = 7$)	5.78 ± 0.10 ($n = 6$)
PD 98059 (10 $\mu\text{mol/l}$)	5.75 ± 0.04 ($n = 5$)	5.64 ± 0.05 ($n = 4$)	6.26 ± 0.11^a ($n = 4$)	5.89 ± 0.07^b ($n = 5$)
Gö 6976 (10 $\mu\text{mol/l}$)	5.78 ± 0.08 ($n = 4$)	5.64 ± 0.10 ($n = 4$)	6.19 ± 0.06^a ($n = 4$)	6.95 ± 0.04^b ($n = 4$)
GF109203 (10 $\mu\text{mol/l}$)	5.76 ± 0.03 ($n = 4$)	5.10 ± 0.05^a ($n = 4$)	6.29 ± 0.06^a ($n = 4$)	5.46 ± 0.08^b ($n = 4$)
RHC-80267 (10 $\mu\text{mol/l}$)	5.77 ± 0.05 ($n = 7$)	5.78 ± 0.09 ($n = 4$)	6.13 ± 0.14^a ($n = 5$)	6.09 ± 0.01^b ($n = 4$)

pD₂ values were calculated by nonlinear regression of individual experimental data. Each value represents the mean $\pm$ SEM from n determinations. Statistical analyses were performed with ANOVA.

^a $p < 0.05$ vs. untreated fresh arteries; ^b $p < 0.05$ vs. fresh arteries incubated in the presence of the same antagonist.

Fig. 4. Ca^{2+} signal and contraction evoked by noradrenaline were increased in cultured mesenteric artery, while KCl-induced responses were not affected. **a** Ca^{2+} signal (upper panel) and simultaneous contraction (lower panel) evoked by cumulative concentrations of noradrenaline in fresh (open squares) and cultured (closed triangles) mesenteric arteries. Data (means $\pm$ SEM from 5 determinations) are expressed as percent of the responses to KCl in the same artery segment. * $p < 0.05$ (ANOVA). **b** Increase in Ca^{2+} signal (upper panel) and simultaneous contraction (lower panel) evoked by 100 mmol/l KCl solution in the same segments of fresh and cultured mesenteric artery. Data are means $\pm$ SEM from 5 determinations. * $p < 0.05$ versus freshly isolated arteries (t test).

response. It was more potent in cultured arteries than in freshly isolated ones so that in the presence of genistein the difference between fresh and cultured arteries was abolished (fig. 1c, table 3). The same effect was observed when arteries were incubated with PP2 (1 $\mu\text{mol/l}$), a more specific inhibitor of src kinases [13]. PP2 did not affect the contraction to noradrenaline in freshly isolated arteries but displaced the concentration-response curve to noradrenaline to the right in cultured arteries so that, in the presence of PP2, noradrenaline concentration response curves in fresh and cultured arteries were superimposed (fig. 1d, table 3). PP2 did not affect the maximum contraction to noradrenaline (fig. 1d).

Phosphorylation of ERK1/2 has been reported to be required for mesenteric artery contraction to ET-1 [19]. Moreover, the presence of MAPK inhibitors in the culture medium of mesenteric artery prevents the increase in artery contraction to noradrenaline [5]. In order to test the involvement of MAPK in the contraction evoked by noradrenaline, we measured the response of the arteries to noradrenaline in the presence of the MAPK inhibitor PD 98059 (10 $\mu\text{mol/l}$). Figure 1b shows that noradrena-

line-evoked contraction was depressed in both cultured and freshly isolated mesenteric arteries. However, PD 98059 did not affect the increase in sensitivity to noradrenaline observed in cultured arteries compared to freshly isolated arteries (fig. 1b, table 3).

Organ Culture Increases the Sensitivity of Ca^{2+} Signal to Noradrenaline

Fura-2 fluorescence measurement showed that the increase in contractile tension evoked by noradrenaline was associated with a parallel increase in cytosolic Ca^{2+} (fig. 4). Measurement of cytosolic Ca^{2+} in freshly isolated and cultured arteries showed that culture similarly increased the sensitivity of the Ca^{2+} signal and the contraction to noradrenaline (fig. 4), without affecting the resting cytosolic Ca^{2+} concentration, which was estimated to be 143 ± 12 nmol/l Ca^{2+} ($n = 15$) and 120 ± 10 nmol/l Ca^{2+} ($n = 17$), in freshly isolated and cultured mesenteric artery segments, respectively. In fura-2 loaded arteries, the increase in cytosolic Ca^{2+} and the contraction evoked by 100 mmol/l KCl solution in freshly isolated and in cultured arteries were not statistically different (fig. 4).

Fig. 5. The increased Ca^{2+} release evoked by noradrenaline in cultured mesenteric arteries was normalised in the presence of the src kinase inhibitor, PP2. The left panels show typical traces of Ca^{2+} signal (upper traces) and contraction (lower traces) evoked by noradrenaline ($3 \mu\text{mol/l}$) in resistance mesenteric artery perfused with Ca^{2+} -free solution and after re-addition of Ca^{2+} to the solution. The right panels show mean values of the increase in Ca^{2+} signal (upper graphs) and contraction (lower graphs) induced by noradrenaline ($3 \mu\text{mol/l}$) in the absence and presence of external Ca^{2+} ($n = 6$ artery segments) in control artery and in the presence of the src kinase inhibitor PP2 ($1 \mu\text{mol/l}$). Data are expressed as percentage of the responses evoked by KCl in the same artery segment. ^a $p < 0.05$ versus untreated artery; ^b $p < 0.05$ versus freshly isolated arteries (ANOVA). **a** Freshly isolated mesenteric artery. **b** Cultured mesenteric artery.

Src Kinase Is Involved in the Increased Ca^{2+} Signal Evoked by Noradrenaline in Cultured Arteries

To further dissect the vascular response to noradrenaline after organ culture, we studied the effect of noradrenaline in the absence and presence of Ca^{2+} in the external solution. In the absence of Ca^{2+} , an increase in Ca^{2+} signal and contractile tension in response to noradrenaline were hardly detected in freshly isolated mesenteric arteries ($6.3 \pm 4.1 \text{ nmol/l } \text{Ca}^{2+}$, $n = 7$). However, after 48 h of organ culture, noradrenaline produced a sig-

nificant increase in Ca^{2+} signal in arteries bathed in the absence of Ca^{2+} ($39 \pm 11 \text{ nmol/l } \text{Ca}^{2+}$, $n = 7$, $p < 0.05$ compared to freshly isolated artery segments) accompanied by a small increase in contractile tension ($0.6 \pm 0.1 \text{ mN/mm}$ compared to $0.2 \pm 0.1 \text{ mN/mm}$ in freshly isolated arteries; fig. 5b). The Ca^{2+} release evoked by noradrenaline in cultured arteries in Ca^{2+} -free solution was significantly inhibited by PP2 ($10 \pm 6 \text{ nmol/l } \text{Ca}^{2+}$, $n = 4$, $p < 0.05$ vs. control), suggesting the involvement of src kinase in this response. PP2 also inhibited the contrac-

Fig. 6. Contractile response evoked by AIF₄ in fresh (black line) and cultured (shaded line) mesenteric arteries. Results are expressed as percentage of maximum contraction evoked by 10 μ mol/l AIF₄ in fresh and cultured arteries. Traces are mean values from 7 experiments. Vertical bars represent SEM values, shown at selected time points. * $p < 0.05$ between the contractile responses of fresh and cultured arteries (ANOVA).

tion evoked by noradrenaline in Ca²⁺-free solution, but this effect did not reach a statistically significant level.

After the addition of Ca²⁺ to the physiological solution in the continuous presence of noradrenaline, Ca²⁺ signal and contraction rapidly increased in fresh and in cultured arteries, reflecting the activation of Ca²⁺ entry. The increase in Ca²⁺ signal was larger in cultured arteries compared to freshly isolated tissue (312 ± 98 nmol/l Ca²⁺, $n = 6$, vs. 260 ± 29 nmol/l Ca²⁺, $n = 6$, respectively; fig. 5). These responses to noradrenaline were not significantly affected by PP2, either in freshly isolated or in cultured mesenteric arteries (fig. 5b).

Contraction of Cultured Mesenteric Arteries Is More Sensitive to AIF₄

We used AIF₄ (10 μ mol/l), a potent activator of G-proteins, in order to bypass receptor activation and find out whether the sensitization of contractile response after organ culture was related to a change at the level of the adrenergic receptor. AIF₄ induced a slowly developing tonic contraction in fresh mesenteric arteries. In cultured mesenteric arteries, the contractile response evoked by AIF₄ developed more rapidly than in fresh arteries (time to reach 50% of maximal contraction was 431 ± 128 s and 768 ± 51 s for fresh and cultured mesenteric arteries, respectively, $p < 0.05$; fig. 6). The maximum contractions

Fig. 7. TRPC expression in fresh and cultured rat mesenteric arteries. **a** mRNA expression of trpc1 and trpc3–7. The mRNA levels were adjusted to rpl32 expression. Data are means $\pm$ SEM from 5 independent experiments. **b** Immunofluorescence detection of TRPC6 protein expression in fresh and cultured mesenteric arteries. 10 scan images were analyzed in each artery and the results are expressed as means $\pm$ SEM of 5 different arteries. * $p < 0.05$ between freshly isolated and cultured artery (t test).

evoked by AIF₄ were not significantly different between fresh and cultured arteries (3.33 ± 0.58 and 3.08 ± 0.37 mN/mm, $n = 7$, respectively).

Up-Regulation of TRPC6 Expression after Organ Culture

We investigated the expression of TRPC in mesenteric arteries before and after organ culture. RT-PCR performed with mesenteric artery mRNA showed the expression of trpc 1 and 3–7 isoforms in fresh and cultured mesenteric artery. trpc1 and 6 were the most abundant

isoforms in freshly isolated arteries, *trpc4* was expressed at an intermediate level and *trpc5* and 7 mRNAs were practically undetectable (fig. 7a). In arteries cultured for 2 days, *trpc1* mRNA expression decreased by $47 \pm 8\%$ ($n = 5$, $p < 0.05$), while *trpc6* mRNA expression increased by $92 \pm 24\%$ ($n = 5$, $p < 0.05$) in comparison with non-cultured arteries. The protein expression of TRPC6 was investigated by confocal microscopy in whole mounts of fresh and cultured mesenteric arteries using anti-TRPC6 antibodies. Average pixel fluorescence was significantly higher in cultured artery segments compared to freshly isolated arteries (13.8 ± 0.54 , $n = 5$ artery segments, vs. 7.7 ± 0.52 , $n = 5$ artery segments, in cultured and fresh mesenteric arteries, respectively; fig. 7b).

Discussion

The present results show that cultured arteries exhibit an increased sensitivity to noradrenaline, as indicated by a significant shift to the left of the noradrenaline concentration-contraction curve in comparison with non-cultured mesenteric arteries. This sensitization was completely inhibited by 2 different src kinase inhibitors.

Noradrenaline-evoked contraction is associated with the release of intracellular Ca^{2+} and the entry of Ca^{2+} from the extracellular compartment. These 2 components in the response to noradrenaline were identified by perfusing the artery with Ca^{2+} -free and Ca^{2+} -containing solutions. Activation of intracellular Ca^{2+} release and contraction by noradrenaline in the absence of external Ca^{2+} were hardly detected in freshly isolated mesenteric arteries. The addition of Ca^{2+} to the perfusion solution produced a rapid increase in cytosolic Ca^{2+} and in contraction. The Ca^{2+} signal evoked by the addition of Ca^{2+} to the perfusion solution was completely inhibited by nimodipine (data not shown), which also completely inhibited the contraction evoked by noradrenaline. This observation indicates that Ca^{2+} entry through VOC plays a prominent role in the contractile response of mesenteric artery to noradrenaline.

Culture of mesenteric artery for 48 h in the absence of serum resulted in a significant increase in the sensitivity of contraction to noradrenaline, without change in the maximal amplitude of the contraction. The increase in contraction was associated with an increase in Ca^{2+} signal both in the absence of Ca^{2+} and after addition of Ca^{2+} in the solution. The increase in intracellular Ca^{2+} release and in capacitative Ca^{2+} entry has been reported in rat basilar and tail arteries after 3–4 days in culture [20]. In-

terestingly, we found that, in cultured arteries, src family tyrosine kinase inhibitor PP2 and the less selective tyrosine kinase inhibitor genistein abolished the increased sensitivity of mesenteric artery contraction to noradrenaline. In fura-2-loaded cultured mesenteric arteries, PP2 completely abolished noradrenaline-induced Ca^{2+} release measured in artery perfused with Ca^{2+} -free solution, whereas it did not affect Ca^{2+} entry.

Src kinases form a family of widely expressed non-receptor tyrosine kinases. Among the numerous src-related kinases, c-src has been reported to be highly expressed in VSMCs. It has been implicated in the regulation of vascular smooth muscle tone [21, 22], and has been reported to modulate L-type Ca^{2+} channel activity [23, 24], and to regulate TRPC6 channel activity by direct phosphorylation [13].

The present results indicate that the increased Ca^{2+} entry evoked by noradrenaline in cultured arteries was not affected by PP2. This observation is not consistent with the involvement of src kinases in the activation of Ca^{2+} entry, either through L-type Ca^{2+} channels or TRPC6 channels [11]. It implies that the kinases might regulate SR Ca^{2+} content or permeability. This result is in line with the reported inhibition of agonist-evoked Ca^{2+} release by src tyrosine kinase inhibitors in several cell types, which has led to the suggestion that tyrosine phosphorylation of the Gq/11 subunit by tyrosine kinases contributes to receptor-mediated activation of Gq [25]. PP2 and phospholipase C inhibition have also been reported to abolish the Ca^{2+} oscillations induced by OAG in various cell types in Ca^{2+} -free solution, suggesting that src kinases are required for the stimulation of phospholipase C activity by OAG [26]. Src tyrosine kinases also activate phospholipase C $\gamma 2$ [27] and regulate the levels of phospholipids [28].

At the light of these reports, we may suggest that the activation of phospholipase C by src kinase may contribute to the increased Ca^{2+} release evoked by noradrenaline in cultured artery. However, we did not detect any effect of OAG on the contractile tension of freshly isolated and cultured mesenteric arteries, what suggests that DAG is not involved in the increased reactivity to noradrenaline in cultured artery.

Artery culture was also associated with an increase in the Ca^{2+} entry evoked by noradrenaline. The importance of Ca^{2+} entry for the contractile response to noradrenaline in resistance mesenteric artery was confirmed by the inhibition of contraction by the voltage-dependent Ca^{2+} channel inhibitor nimodipine, which indicated that most of the Ca^{2+} entry occurs through VOC. Noradrenaline-

evoked contraction was abolished not only by nimodipine, but also by flufenamic acid or SKF-96365, known to modulate agonist-evoked Ca^{2+} entry in several cell types, through their interaction with TRPC channels [29, 30]. This is compatible with the hypothesis that non-selective cation channels could mediate cell membrane depolarization leading to the opening of VOC. In portal vein, Inoue et al. [11] reported, using an antisense strategy, that TRPC6, which is highly expressed, is an important component of α -adrenoceptor signalling pathway. The activity of the TRPC6 channel has been reported to be increased by flufenamic acid [11], which also inhibits other TRPC channel proteins [31, 32]. The inhibitory effect of flufenamic acid on noradrenaline contraction in mesenteric artery thus suggests that heterotetrameric TRPC channels might be involved in the contractile response to noradrenaline, in agreement with PCR data indicating that most TRPC isoforms are expressed in this tissue.

Interestingly, the increased contractile response to noradrenaline was more sensitive to cation channel inhibitors in cultured arteries than in freshly isolated artery segments, suggesting that the contribution of non-selective cation channels in the response to noradrenaline is higher after culture. This observation may be related to the up-regulation of TRPC6 expression in cultured arteries compared to freshly isolated arteries. Changes in TRPC expression with organ culture have been reported by Bergdahl et al. [1] in cerebral arteries, where culture was associated with increased expression of TRPC6 and of TRPC1. However, we did not confirm the increased expression of *trpc1* in mesenteric artery after organ culture [1]. This discrepancy might be due to vessel difference (mesenteric vs. cerebral) or the different culture conditions. Whether increased expression of TRPC6 is responsible for the potentiation of noradrenaline responses after culture cannot be definitively determined from the present data. In the absence of a selective pharmacologic inhibitor, inhibition of protein expression will be required to address this question. Alternatively, altered membrane potential regulation, or a change in the signalling pathway activated by noradrenaline might contribute to the increased Ca^{2+} entry evoked by noradrenaline in cultured arteries.

Increased contraction of cultured rat mesenteric artery in response to noradrenaline has been ascribed to the up-regulation of α 1-adrenoreceptor [5]. Although a change in the expression of adrenergic receptor with organ culture cannot be ruled out, it cannot explain all the observations reported in the present study, and in particular, the selective inhibition of noradrenaline-evoked

responses by PP2. Moreover, the contraction evoked by several other agonists (unpubl. data) and by AlF_4 , which directly activates G protein [33–35], were also enhanced after organ culture, in comparison with freshly isolated arteries. Nevertheless, a change in the distribution of receptor sub-types or in the localisation of the receptors might occur with culture. Further study should test this possibility.

The present data indicate that the increased reactivity to noradrenaline observed after organ culture of resistance mesenteric artery is associated with increase in Ca^{2+} entry through non-selective calcium channel and with increased Ca^{2+} release, which involves src family kinase. Increased vascular reactivity is reported in several vascular diseases like hypertension [36] or atherosclerosis [37]. Enhanced TRPC6 expression and ROC currents have been described in VSMCs from DOCA-salt hypertensive rats, and is proposed to contribute to altered vascular reactivity and to hypertension [38]. On the other hand, src has been reported to be involved in the cellular growth response to pressure stimulus [39], and to mediate VSMC growth in response to angiotensin [40]. The present results thus suggest that organ culture may reproduce vascular alterations characterising hypertension.

Acknowledgements

This work was supported by a grant from the Ministère de l'Éducation et de la Recherche Scientifique (Action Concertée No. 06/11-339) and from the FRSM (grant No. 3.4601.06).

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