Both Ca\(^{2+}\)-dependent and -independent pathways are involved in rat hepatic stellate cell contraction and intrahepatic hyperresponsiveness to methoxamine

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Laleman W, Van Landeghem L, Severi T, Vander Elst I, Zeegers M, Bisschops R, Van Pelt J, Roskams T, Cassiman D, Favery J, Nevens F. Both Ca\(^{2+}\)-dependent and -independent pathways are involved in rat hepatic stellate cell contraction and intrahepatic hyperresponsiveness to methoxamine. Am J Physiol Gastrointest Liver Physiol 292: G556–G564, 2007. First published September 28, 2006; doi:10.1152/ajpgi.00196.2006.—In chronic liver injury, hepatic stellate cells (HSCs) have been implicated as regulators of sinusoidal vascular tone. We studied the relative role of Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent contraction pathways in rat HSCs and correlated these findings to in situ perfused cirrhotic rat livers. Contraction of primary rat HSCs was studied by a stress-relaxed collagen lattice model. Dose-response curves to the Ca\(^{2+}\) ionophore A-23187 and to the calmodulin/myosin light chain inhibitor W-7 served to study Ca\(^{2+}\)-independent pathways. Y-27632, staurosporin, and calyculin (inhibitors of Rho kinase, protein kinase C, and myosin light chain phosphatase, respectively) were used to investigate Ca\(^{2+}\)-independent pathways. The actomyosin interaction, the common end target, was inhibited by 2,3-butanedione monoxime. Additionally, the effects of W-7, Y-27632, and staurosporin on intrahepatic vascular resistance were evaluated by in situ perfusion of normal and thioacetamide-treated cirrhotic rat livers stimulated with methoxamine (n = 25 each). In vitro, HSC contraction was shown to be actomyosin based with a regulating role for both Ca\(^{2+}\)-dependent and -independent pathways. Although the former seem important, an important auxiliary role for the latter was illustrated through their involvement in the phenomenon of “Ca\(^{2+}\) sensitization.” In vivo, preincubation of cirrhotic livers with Y-27632 (10\(^{-4}\) M) and staurosporin (25 nM), more than with W-7 (10\(^{-4}\) M), significantly reduced the hyperresponsiveness to methoxamine (10\(^{-4}\) M) by −66.8 ± 1.3%, −52.4 ± 2.7%, and −28.7 ± 2.8%, respectively, whereas in normal livers this was significantly less: −43.1 ± 4.2%, −40.2 ± 4.2%, and −3.8 ± 6.3%, respectively. Taken together, these results suggest that HSC contraction is based on both Ca\(^{2+}\)-dependent and -independent pathways, which were shown to be upregulated in the perfused cirrhotic liver, with a predominance of Ca\(^{2+}\)-independent pathways.

DURING CHRONIC LIVER INJURY, hepatic stellate cells (HSCs) undergo a process of activation from a quiescent “storing” to a highly proliferative “myofibroblast-like” phenotype. Besides a fibrogenic response, this activation process also leads to the acquisition of enhanced contractile properties. Both factors have been implied in the increased intrahepatic vascular resistance (IHVR) to portal flow, but especially the recognition that hypercontractile HSCs participate in the increased intrahepatic vascular tone provides the rationale for targeting these cells in the treatment of portal hypertension in advanced chronic liver disease (5, 12, 23). Due to the expression of smooth muscle proteins and the strategic anatomical location around hepatic sinusoids, a resemblance to pericytes or vascular smooth muscle cells has been proposed, leading to the assumption of a “smooth muscle-like” contraction pattern (12, 22–24). Yet, this supposed smooth muscle-like contraction pattern of activated HSCs conflicts with the alleged transition to a myofibroblast-like activated HSC phenotype. In smooth muscle cells, contraction is mainly powered by a Ca\(^{2+}\)-dependent myosin activation. Increased intracellular Ca\(^{2+}\) levels, either via inositol trisphosphate (IP\(_3\)) or Ca\(^{2+}\) influx through L-type channels, give rise to the activation of Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase (MLCK), which, in turn, results in increased phosphorylation of the regulatory light chain of myosin (rMLC). Phosphorylation of rMLC allows myosin ATPase to be activated by actin, leading to actomyosin cross-bridging and cell contraction (21, 28). In nonmuscle cells, including activated HSCs, increased phosphorylation of MLC has also been correlated with increased contractility (11, 26). However, in contrast to smooth muscle cells, several studies (10, 19, 27) favor non-Ca\(^{2+}\)-dependent contraction pathways to be the key event in force generation by myofibroblasts, of which the RhoA signaling and PKC-dependent pathway are the most relevant. Both these latter pathways inhibit the phosphatase activity of myosin light chain phosphatase (MLCP), leading to a delayed degradation of phosphorylated rMLC and thus promoting contraction (21, 27).

Since the contributions of these different pathways have not yet been fully elucidated for HSC contraction, we therefore aimed to 1) define the relative role of Ca\(^{2+}\)-dependent and -independent pathways in the actomyosin interaction of HSCs (Fig. 1), using a three-dimensional stress-relaxed collagen lattice contraction model; 2) compare the degree of Ca\(^{2+}\) dependency of vascular smooth muscle cells (VSMCs), cardiac myofibroblasts, and activated HSCs; and 3) determine the relevance of these in vitro findings in the increased IHVR associated with thioacetamide (TAA)-induced cirrhosis in the rat.

MATERIALS AND METHODS

Cell Isolation and Culture

HSCs were isolated from male Wistar rats, weighing 300–400 g (Animal House, Leuven, Belgium), as approved by the local Ethical Committee on Animal Research and as described previously (3). In brief, following in situ perfusion of the liver with collagenase type IV...
Biosciences, Roosendaal, The Netherlands). Depending on the exper-i
the resulting cell suspension was fractionated by density gradient
Viability and purity was systematically
/H11022
/H9251
/H9251
-smooth muscle actin (SMA)
by culturing enriched HSC fractions on plastic, was performed by
characterization of rat liver-derived myofibroblast-like cultures, established
beke, Belgium). The medium was renewed every 48 –72 h. Charac-
seeded on uncoated plastic culture dishes and cultured in William’s E
trypan blue exclusion and morphological characterization. Cells were
were washed twice with 1
PBS, followed by the addition of 1 ml
l
2 min

Ca2+
+ Calmodulin
A-23187

Calmodulin

Ca2+

PLC

PKC

Gq

PIP2

IP3

DAG

MLC

MLC-P + α-SMA

MLC

MLC

RhoA•GDP

RhoA•GTP

MLCP

MLCP

Y-27632

W-7

BDM

Contraction
Migration

Pro-contractile
Inhibition contraction

G557

CONTRACTION PATHWAYS OF HEPATIC STELLATE CELLS

Implant, presumed agonists and/or inhibitors were added. To initiate matrix contraction, mechanically stressed matrices were released by gentle circumferential dislodgement of the lattice using a micropipette tip (‘relaxation’). Cell-mediated contraction was measured by determining the relative partitioning of 3H2O between the gel phase and surrounding medium following 24 h of contraction, thereby allowing the estimation of gel phase volumes. More specifically, the separate tritium activities of the medium and gel phase were measured in 10 ml oscillation fluid (Perkin-Elmer) using a Beckmann liquid scintillation spectrometer. Control cell-free gels provided estimates for the pre-
contraction volume and allowed us to determine relative changes in volume (percent contraction). All data presented here are from experi-
ments using at least three sets of three collagen lattices using culture-activated HSCs from three different rat HSC isolations.

The same setup was used in the experiments comparing contrac-
tions of VSMCs, HSCs, and cardiac myofibroblasts. In these specific experiments, the extent of contraction was expressed relative to
FCS-induced contraction to allow comparisons between cell types.

Inhibitors and Agonists Affecting Contractility

Depending on the experiment, FCS (10%), the α1-adrenergic ago-
nist methoxamine (10−4 M, Sigma), or the Ca2+ ionophore A-23187 (Sigma) were used as agonists. Mostly, FCS was used because its ease of use, high availability, and ability to elicit as powerful a contraction as endothelin-1 (25). The actomyosin interaction, the end target of both Ca2+- and Ca2+-independent pathways, was studied with the use of 2,3-butanedione 2-monoxime (BDM), L-Ca2+ channel, L-type Ca2+ channel; PLC, phospholipase C; PIP2, inositol diaphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol.

(Σigma, St. Louis, MO) and pronase E (Merck, Darmstadt, Germany), the resulting cell suspension was fractionated by density gradient centrifugation using Optiprep (Nycomed). Cells were harvested at densities of <1.053 (9% Optiprep) according to Alpini et al. (1). Viability and purity was systematically >95%, as determined by trypan blue exclusion and morphological characterization. Cells were seeded on uncoated plastic culture dishes and cultured in William’s E medium supplemented with 10% FCS, 0.6 IU/ml insulin, 2 mM glutamine, and 1% antibiotic-antimycotic solution (Invitrogen, Merel-
bebke, Belgium). The medium was renewed every 48 –72 h. Charac-
terization of rat liver-derived myofibroblast-like cultures, established

(A-23187, calyculin (Caly), W-7, staurosporin (Stauro), Y-27632, and 2,3-butanedione 2-monoxime (BDM). L-Ca2+ channel, L-type Ca2+ channel; PLC, phospholipase C; PIP2, inositol diaphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol.

Three-Dimensional Stress-Relaxed Collagen Lattice Contraction Model

The ability of HSCs to contract three-dimensional collagen ma-
trixes was assessed as previously described with some slight modifi-
cations (29). In brief, hydrated collagen gels were prepared using rat
tail tendon collagen I (Becton Dickinson Labware, Becton Dickson,
Bedford, MA) and adjusted to physiological strength and pH with 1 N NaOH and 10× PBS at 4°C. Afterward, the collagen solution was mixed with a HSC suspension so that the final solution resulted in a collagen concentration of 1.5 mg/ml and 250,000 cells/ml. A 500-μl aliquot of the collagen solution was then cast into each well of a
24-well tissue culture plate (Falcon, Meylan, France) and, after 1 h,
aliquot of the collagen solution was then cast into each well of a

Ca2+-depleted and normal (Ca2+-containing) lattices. The former lattices were obtained by washing lattices with 1× Ca2+-free PBS (3 × 2 min each), followed by 1× Ca2+-free PBS + 3 mM EGTA (2 × 5 min each and then 14 min) and a final set of washes with 1× Ca2+-free PBS + 3 mM EGTA + 0.01 μM A-23187 (3 × 2 min each). The final washing step allowed depletion of intracellular Ca2+.
Western Blot Analysis for rMLC and Phosphorylated rMLC

HSCs, cultured for 24 h in the presence of W-7, Y-27632, staurosporine, or FCS alone, were harvested and homogenized in lysis buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 0.2 M DTT). After heat denaturation (100°C for 5 min) and centrifugation (13,000 g for 10 min), equal amounts of protein (6.5 µg) were run on a 7% SDS-PAGE gel and then transferred onto a nitrocellulose Protran membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% blocking solution (milk powder) in 1× PBS for 1.5 h at room temperature to avoid nonspecific binding. Thereafter, blots were incubated overnight with primary antibodies against rMLC and phosphorylated rMLC (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). The next day, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (SwAR-HRP, 1:500, Prosan, Merelbeke, Belgium), and immunoreactivity was visualized using chemiluminescence detection (ECL-plus, Amershams Biosciences). Membranes were stained with Ponceau staining to confirm equal protein loading and transfer between lanes. Densitometric quantification of Western blot signal intensity was performed with Un-Scan-IT Gel software (Silk Scientific) and calculated as percentages to mean values of HSCs treated only with FCS.

Fluo-4 Visualization of Intracellular Ca2+

HSCs, grown on glass coverslips, were incubated with 5 µM fluo-4 + 0.025% pleuronic acid (Molecular Probes) under FCS-free conditions. Coverslips were subsequently transferred to the coverglass chamber of a confocal scanning microscope (Nikon TE 300, Noran Oz). Cells were observed and photographed before, during, and after the addition of FCS-free medium containing 0.01 µM A-23187.

Animal Model of Cirrhosis

Male Wistar rats, weighing 200–250 g, were intoxicated with TAA in drinking water. The TAA concentration was adapted weekly to the initial 30-min stabilization period. After the inferior vena cava was transected, allowing the perfusate to escape, a thoracotomy was performed, and the upper right lobe was dissected out. An aortic clamp (1× PBS, 1.5 mm) was placed around the aorta, and the liver was perfused with oxygenated Krebs solution at 37°C. After the inferior vena cava was cannulated and perfused through a 14-gauge angiocath with oxygenated Krebs solution at 37°C, the liver was perfused with oxygenated Krebs solution at 37°C. The next day, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (SwAR-HRP, 1:500, Prosan, Merelbeke, Belgium), and immunoreactivity was visualized using chemiluminescence detection (ECL-plus, Amershams Biosciences). Membranes were stained with Ponceau staining to confirm equal protein loading and transfer between lanes. Densitometric quantification of Western blot signal intensity was performed with Un-Scan-IT Gel software (Silk Scientific) and calculated as percentages to mean values of HSCs treated only with FCS.

In Vitro Results

Relevance of intracellular Ca2+ and Ca2+-dependent pathways in HSC-mediated gel contraction. Increasing Ca2+ intracellularly under agonist-free conditions with A-23187, a Ca2+ ionophore, was demonstrated by means of confocal Ca2+ imaging, which showed a maximal rise in intracellular Ca2+ within seconds (Fig. 2). A dose-response curve with A-23187 showed maximal contraction of 54.7 ± 1.8% at 0.01 µM. Since this amounts to only 75.6 ± 1.8% of FCS-promoted contraction, increasing intracellular Ca2+ appears insufficient to exclusively cause contraction (Figs. 3 and 4). The equivalent maximal contraction relative to FCS-induced contraction generated in VSMCs and cardiac myofibroblasts amounted to 85.1 ± 2.3% and 34.6 ± 2.1%, respectively (P < 0.001 vs. the equivalent effect in HSCs; Fig. 4).

MLCK, the downstream effector of the Ca2+-dependent pathway, was inhibited using W-7, an inhibitor of calmodulin-mediated activation of MLCK. In FCS-promoted HSC contraction, this resulted in an attenuation to 17.6 ± 2% at 10−4 M (vs. 77.4 ± 1.2% for control, P < 0.001; Fig. 5).

When the effect of W-7 (10−4 M) was evaluated on maximal Ca2+-induced contraction (by the addition of 0.01 µM A-23187), Ca2+-induced contraction was inhibited (16.8 ± 2.7% vs. 54.7 ± 1.8%, P < 0.001), suggesting that calmodulin is indeed the downstream target of Ca2+-dependent pathways.

Relevance of Ca2+-independent pathways in HSC-mediated gel contraction. First, MLCP, the supposed converging end point of Ca2+-independent pathways, was inhibited using calycin, a type 1 phosphatase inhibitor. Calycin (10−9 M) was able to induce contraction of HSC-embedded collagen lattices in the absence of FCS (53.7% vs. 15.3% under FCS-free conditions, P < 0.05). To further specify the relative role of MLCP activity in HSC-mediated contraction, HSC contraction was examined under Ca2+-depleted and Ca2+-containing conditions in the presence or absence of calycin (10−9 M). Under similar Ca2+-depleted conditions, contraction occurred more efficient when MLCP was inhibited (4 ± 2.3% vs. 30 ± 2.7%, P = 0.002; Fig. 6). In Ca2+-containing lattices, a comparable effect was observed when MLCP was inhibited (15.3 ± 3.8% vs. 53.7 ± 5.2%, P = 0.004), but the degree of contraction was increased compared with Ca2+-depleted conditions. These data not only illustrate an auxiliary role for MLCP in the regulation of Ca2+ sensitivity.”

Subsequently, known inactivating upstream signaling pathways of MLCP, more specifically, Rho-associated kinase and PKC-mediated pathways, were evaluated. Y-27632 and staurosporin, their respective inhibitors, dose dependently attenuated FCS-promoted HSC contraction (Fig. 7, A and B). In contrast, Y-27632 (10−4 M) and staurosporin (25 nM), which both inhibited FCS-promoted contraction by >85%, inhibited calycin-promoted contraction by maximally 35% and 27%, respectively, strengthening the hypothesis that MLCP is their downstream target (Fig. 7C). Furthermore, W-7 (10−2 M), an inhibitor of calmodulin-mediated activation of MLCK, did not
change calyculin-promoted contraction at all, suggesting its unimportance in the regulation of MLCP (Fig. 7C).

Relevance of actomyosin interaction in HSC-mediated contraction. To assess whether the end point of Ca\(^{2+}\)/H\(_{11001}\) dependent and -independent pathways was indeed an actomyosin interaction, BDM, a nonmuscle myosin II-Ca\(^{2+}\)/H\(_{11001}\)-ATPase inhibitor, was added to the culture medium prior to release. This led to a dose-dependent decrease in FCS-promoted contraction, indicating the requirement of an actomyosin interaction in the contraction of stress-relaxed HSC-embedded collagen matrixes (Fig. 8A). To further substantiate the involvement of the calmodulin/MLCK-, Rho kinase-, and PKC-mediated pathways in this actomyosin interaction, we additionally examined the phosphorylation of MLC by Western blot analysis. Densitometric analysis of MLC and phosphorylated MLC (Fig. 8B) showed constitutive expression of MLC under all conditions. The addition of W-7 (10\(^{-4}\) M), Y-27632 (10\(^{-4}\) M), and staurosporin (25 nM) decreased the amount of phosphorylation of MLC compared with control conditions (FCS) (P < 0.001 vs. the degree of phosphorylation under control conditions).

Relevance of Ca\(^{2+}\)/H\(_{11001}\)-dependent and -independent pathways in the presence of the \(\alpha_1\)-adrenergic agonist methoxamine. To finally refute the possibility that these pathways were relevant exclusively to FCS stimulation, we repeated the experiments with W-7, Y-27632, and staurosporin in the presence of the \(\alpha_1\)-adrenergic agonist methoxamine, for which activated HSCs express the receptors and biosynthetic enzymes (18). Except for under basal conditions comparing only both agonists, we observed no differences to FCS-induced contraction in the presence of W-7, Y-27632, and staurosporin (Fig. 9).

Results in In Situ Perfused Normal and Cirrhotic Rat Livers

In agonist-free conditions, basal IHVR of cirrhotic rat livers was already increased compared with normal rat livers (0.22 ± 0.01 vs. 0.13 ± 0.01 mmHg·min·ml\(^{-1}\), respectively, P < 0.001). The addition of methoxamine (10\(^{-4}\) M) led to an increase in IHVR in both cirrhotic and normal perfused rat livers (0.65 ± 0.02 vs. 0.32 ± 0.02 mmHg·min·ml\(^{-1}\), respectively, P < 0.001). In the cirrhotic perfused rat liver, the response to methoxamine was aggravated compared with the normal perfused rat liver (change in increase in IHVR: 0.39 ± 0.03 mmHg·min·ml\(^{-1}\) for TAA vs. 0.21 ± 0.02 mmHg·min·ml\(^{-1}\) for control, P = 0.001), which is consistent with the phenomenon of “hyperresponsiveness” to vasocon-
strictors in cirrhosis. Preincubation with Y-27632 (10⁻⁴ M), staurosporin (25 nM), and W-7 (10⁻⁴ M) in the cirrhotic rat liver significantly decreased this hyperresponsiveness to methoxamine by −66.8 ± 1.3%, −52.4 ± 2.7%, and −28.7 ± 2.8%, respectively, suggesting a predominant involvement of Ca²⁺-independent pathways in the increased active IHVR associated with cirrhosis (Fig. 10). In the normal liver, we noted a reduction in IHVR only after inhibition of Ca²⁺-independent pathways and to a lesser extent than that found in cirrhosis: −43.1 ± 4.2% (P = 0.008 vs. TAA + Y-27632) and −40.2 ± 4.2% (P = 0.038 vs. TAA + staurosporin) (Fig. 10).

**DISCUSSION**

Accumulating evidence indicates that the contractile force generated by activated HSCs not only drives scar contraction in cirrhosis but also modulates the hepatic microcirculation (12, 19, 22–24, 26). Both aspects affect the increased IHVR to portal flow, and therefore make HSCs interesting targets for the treatment of portal hypertension. Because of their apparent key role, considerable effort has been made to elucidate the regulation that governs contractile force generation in these cells. At present, it is generally assumed that activated HSCs have a smooth muscle cell-like Ca²⁺-dependent contraction pattern (2, 5, 22–24). This contention is founded on different observations, such as 1) a functional and ultrastructural resemblance...
to pericytes (22); 2) the expression of smooth muscle proteins (like α-SMA and myosin II) (5, 26); 3) the expression of L-type voltage-operated Ca\(^{2+}\) channels (2); and 4) the fact that agonists that are known to cause contraction in HSCs are associated with increases in intracellular Ca\(^{2+}\) (22–24). Although persuasive, these observations are challenged heretofore by a lack of direct evidence proving that an increase in intracellular Ca\(^{2+}\) indeed mediates force generation and by an apparent contradiction in terminology, as witnessed in the smooth muscle cell-like contraction pattern of a myofibroblast-like activated HSC.

In contrast to previous studies, we applied a three-dimensional stress-relaxed collagen lattice contraction model to study the contractility of HSCs (16, 19, 29, 31). The reasons herefore
are twofold. First, cell adhesion, analogous to the in vivo situation, occurs three dimensionally to attachment sites made upon protein fibrils rather than two dimensionally along a protein-coated interface (like for contraction studies using confocal Ca\textsuperscript{2+} imaging or when HSCs are grown on top of polymerized collagen lattices). This model therefore mimics the reciprocal geometric and mechanical relationships with the surrounding matrix that HSCs entail in vivo (7, 16). Second, the relevance of stressed gels follows from the observation that in the cirrhotic liver, the injured liver tissue is tethered in a way that cell contraction will inevitably increase stress in the surrounding matrix, resulting in a mechanical feedback that is missing in classically used unrestrained gels (“floating gels”) as well as in the classical two-dimensional assay, as mentioned earlier.

Using this in vitro model, we first tested the premise that force generation in HSCs is Ca\textsuperscript{2+} dependent. We demonstrated that, although Ca\textsuperscript{2+}-/calmodulin-mediated MLCK activity is necessary for HSC contraction, elevation of intracellular Ca\textsuperscript{2+} was insufficient to cause maximal contraction. These results contrasted with the demonstrated properties of VSMCs, in which gradual elevation of intracellular Ca\textsuperscript{2+} ultimately led to a similarly efficient contraction as obtained after agonist stimulation, proving the dominance of Ca\textsuperscript{2+}-dependent pathways in these cells (19, 21, 27, 28). In cardiac myofibroblasts, quite the reverse phenomenon was observed, since these cells, like other types of myofibroblasts (19), appeared almost Ca\textsuperscript{2+} insensitive. These observations clearly refute any comparison for HSCs with smooth muscle cells and myofibroblasts, making terms like “smooth muscle cell like” and “myofibroblast like” inappropriate in this context.

The inability of increased intracellular Ca\textsuperscript{2+} to promote maximal contraction suggests that activated HSCs have an additional mechanism to Ca\textsuperscript{2+}/calmodulin-dependent MLCK that is critical in regulating contractility. We therefore tested Ca\textsuperscript{2+}-independent pathways. Since these pathways are thought to converge in the inactivation of MLCP, resulting in delayed degradation of phosphorylated rMLC and therefor decreased contraction, we first focused on this enzyme (8, 11, 19, 26, 27). We demonstrated that the phosphatase inhibitor calyculin, in the absence of any agonist, could promote HSC contraction, proving its involvement in the regulation of force generation additive to the Ca\textsuperscript{2+}-dependent pathway. To further specify its role in this regulation, we examined HSC contraction under Ca\textsuperscript{2+}-/depleted or Ca\textsuperscript{2+}-containing conditions in the presence or absence of calyculin. These experiments showed that inhibition of MLCP increased the amount of contraction independently of the level of intracellular Ca\textsuperscript{2+}, suggestive of a phenomenon known as Ca\textsuperscript{2+} sensitization (21, 27). In smooth muscle cells, this phenomenon has been extensively described and is mainly attributed to the inhibition of MLCP (21). Several mechanisms have been identified in these cells to inactivate MLCP, the most important of which are the phosphorylation of the regulatory subunit of MLCP by the Rho/Rho kinase pathway and the inhibition of the catalytic subunit of MLCP mediated by the PKC-dependent pathway (8, 17, 21, 27). For HSCs, to our knowledge, the concept of Ca\textsuperscript{2+} sensitization is novel. Reviewing the two aforementioned mechanisms, which are considered to be the main regulating factors in smooth muscle cells in this process, we observed surprising parallels in HSCs. First, we and others (10, 15, 30, 31), under different conditions, could document a role for the RhoA signaling pathway since preincubation with the Rho kinase inhibitor Y-27632 attenuated contraction and decreased phosphorylation of rMLC, the rate-limiting step in the actomyosin interaction. Furthermore, we also proved that the RhoA signaling pathway acts through MLCP, since preincubation with calyculin, a phosphatase inhibitor, largely counteracted inhibition of contraction by Y-27632. This suggests that Rho kinase, a serine/threonine kinase, phosphorylates MLCP and inhibits phosphatase activity, resulting in delayed degradation of phosphorylated rMLC and thus protracted contraction. Second, we also revealed a role for the PKC-dependent pathway. Stauro-

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**Fig. 9.** Relevance of Ca\textsuperscript{2+}-dependent [W-7 (10\textsuperscript{-4} M)] and -independent pathways [Y-27632 (10\textsuperscript{-4} M) and Stauro (25 nM)] in the presence of the \(\alpha\text{-adrenergic agonist methoxamine (MTX; 10\textsuperscript{-4} M)}\). *\(P < 0.05\), MTX vs. FCS.

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**Fig. 10.** Relevance of Ca\textsuperscript{2+}-dependent [W-7 (10\textsuperscript{-4} M)] and -independent pathways [Y-27632 (10\textsuperscript{-4} M) and Stauro (25 nM)] in situ perfused normal and thioacetamide (TAA)-induced cirrhotic rat livers \((n = 5\) per condition). Both Ca\textsuperscript{2+}-dependent and -independent pathways were shown to be involved in the hyperresponsiveness of the cirrhotic liver to MTX because inhibition of these pathways attenuated the increased intrahepatic vascular resistance (IHVR) with a predominance of Ca\textsuperscript{2+}-independent pathways, mediated through PKC- and RhoA-mediated signaling cascades. *\(P < 0.05\) vs. TAA + MTX; **\(P < 0.05\) vs. TAA + W-7; ***\(P < 0.05\) vs. TAA + Y-27632; 1\(P < 0.05\) vs. normal rat liver + MTX.
sporin, a PKC inhibitor, prevented FCS- and methoxamine-promoted contraction and decreased phosphorylation of rMLC. Similarly as for RhoA inhibition, calyculin also almost completely opposed inhibition of contraction by staurosporin, identifying MLCP as the end target of this pathway as well. These results indicate an auxiliary role for PKC to the RhoA pathway as a G protein-coupled effector of Ca$^{2+}$ sensitization. It should be taken into account that the inhibitors used to block the different pathways are highly specific but not exclusive to their intended target. In addition, a role for MLC phosphorylation-independent regulatory mechanisms is postulated in smooth muscle cells (21). Our data show that Ca$^{2+}$-dependent (MLCK related) and Ca$^{2+}$-independent (MLCP related) pathways are important in HSC contraction, but these findings do not exclude the presence or absence of alternative, more discrete pathways. The study of these will require more selective experimental tools and models that are lacking at the moment.

To correlate our in vitro findings to the in vivo situation, we reevaluated these pathways in the in situ perfused liver. We first confirmed the presence of an exaggerated response to methoxamine in the cirrhotic rat liver compared with the normal liver, illustrating the known phenomenon of intrahepatic hyperresponsiveness in the cirrhotic liver (6, 14). Both Ca$^{2+}$-dependent and -independent pathways were shown to be involved in this hyperresponsiveness of the cirrhotic liver since inhibition of these pathways attenuated the increased IHVR. A predominance of the Ca$^{2+}$-independent pathways, mediated through PKC- and RhoA-mediated signal cascades, was demonstrated.

In conclusion, we have shown that both Ca$^{2+}$-dependent and Ca$^{2+}$-independent pathways are necessary to raise a HSC-specific contraction pattern, which distinguishes them from both typical myofibroblasts and smooth muscle cells. These in vitro findings correlated with the data obtained in the cirrhotic liver where both pathways were involved, with Ca$^{2+}$-independent pathways predominating the picture. A better understanding of the intracellular signal transduction mechanisms leading to HSC contraction and the demonstration of their relevance in the establishment of the increased IHVR in the cirrhotic liver might lead to the identification of novel potential targets for the treatment of portal hypertension.

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