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Development of Rho-kinase inhibitors for cardiovascular medicine

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Rho-kinase (ROCK) is one of the downstream effectors of the small G-protein Rho. The Rho–ROCK pathway has an important role in mediating various cellular functions, including contraction, actin cytoskeleton organization, cell adhesion and motility, proliferation, cytokinesis and gene expression, all of which are involved in the pathogenesis of cardiovascular disease. Indeed, vascular smooth muscle cells, endothelial cells, adventitial cells, cardiomyocytes and nerve cells all undergo pathophysiological changes through the ROCK pathway. Abnormal activation of this pathway is associated with the pathogenesis of various cardiovascular diseases such as hypertension, coronary and cerebral vasospasm, restenosis, atherosclerosis, stroke and heart failure, although the roles of the ROCK isoforms (ROCK1 and ROCK2) remain to be elucidated. In this article, we review the information about the therapeutic importance of the ROCK pathway and summarize the current status of the development of ROCK inhibitors.

Introduction

Families of small G-proteins such as Rho, Ras, Rab, Sarl/Arf and Ran are substantially involved in intracellular signaling [1]. The Rho family members, including Rho, Rac and Cdc42, regulate both cytoskeletal reorganization and gene expression. The effector domains of RhoA, RhoB and RhoC (collectively referred to here as Rho) have the same amino acid sequence, and these G proteins seem to have similar intracellular targets. As with other Rho GTPases, Rho acts as a molecular switch, cycling between an active GTP-bound state and an inactive GDP-bound state [2,3]. The exchange between the active and the inactive states is regulated by several regulatory proteins such as guanine dissociation inhibitor (GDI), guanine-nucleotide-exchange factor (GEF) and GTPase-activating protein (GAP) (Figure 1). In unstimulated cells, Rho resides predominantly in the cytosol in its inactive GDP-bound form, and Rho GDI binds to Rho-GDP and extracts it from the membrane to the cytosol. When cells are stimulated with certain agonists, Rho-GDP is converted to Rho-GTP through the action of Rho GEF. Rho-GTP is then targeted to the cell membrane, where it interacts with its specific targets. Rho GAP inactivates Rho by dephosphorylating GTP to GDP (Figure 1).

The best-characterized downstream effector of Rho is Rho-kinase (ROCK), which mediates various cellular functions [2]. ROCK was identified in the mid-1990s as one of the downstream effectors of Rho [1,2]. There are two isoforms of ROCK: ROCK1 and ROCK2 [1,2]. The genes expressing human ROCK1 and ROCK2 are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively [4,5]. ROCK1 and ROCK2 are highly homologous, sharing 65% homology in amino acid sequence and 92% homology in their kinase domains. Although both isoforms are ubiquitously expressed, ROCK2 is highly expressed in the brain and the heart, whereas ROCK1 is expressed preferentially in the lung, liver, spleen, kidney and testis [6]. However, evidence of the functional differences between ROCK1 and ROCK2 is lacking. ROCK1 activation by caspase-3 cleavage has an important role in cardiac myocyte apoptosis [7]. The consensus sequence for caspase-3 cleavage is absent from ROCK2, whereas the pro-apoptotic protease granzyme B that cleaves the C-terminus of ROCK-2 is absent from ROCK1 [8]. A recent study has also demonstrated that ROCK2 activation by caspase-2 cleavage is involved in thrombin-induced microparticle generation in response to cell activation [9]. These events are independent of cell death, and ROCK1 is not modulated by thrombin [9]. Mice lacking the gene encoding ROCK1 or ROCK2 have recently been generated; both types of mouse showed embryonic lethality, but for different reasons [10,11]. Only a small number of genetic approaches to the targeted deletion of ROCK isoforms has shown the distinction between the pathophysiological roles of the isoforms. ROCK1-deficient ($Rock1^{-/-}$) mice displayed a preserved compensatory hypertrophic response but showed reduced perivascular fibrosis and interstitial fibrosis in response to pressure overload [12]. This finding is supported by a recent study in which ROCK1-haploinsufficient ($Rock1^{+/-}$) mice showed cardiac fibrosis, but not cardiac hypertrophy, in response to pressure overload [13].

The substrates of ROCK have also been identified, including the myosin-binding subunit (MBS) of myosin light-chain phosphatase (MLCPh), the ERM (ezrin, radixin, moesin) family, adducin, intermediate filaments (e.g. vimentin and desmin), the Na^+–H^+ exchanger and LIM-kinase [1]. In addition to ROCK, several other proteins have been identified as effectors of Rho, including protein kinase N (PKN), rhophilin, rhotekin, citron, p140mDia and citron kinase [1]. However, the roles of these other effectors are largely unknown.

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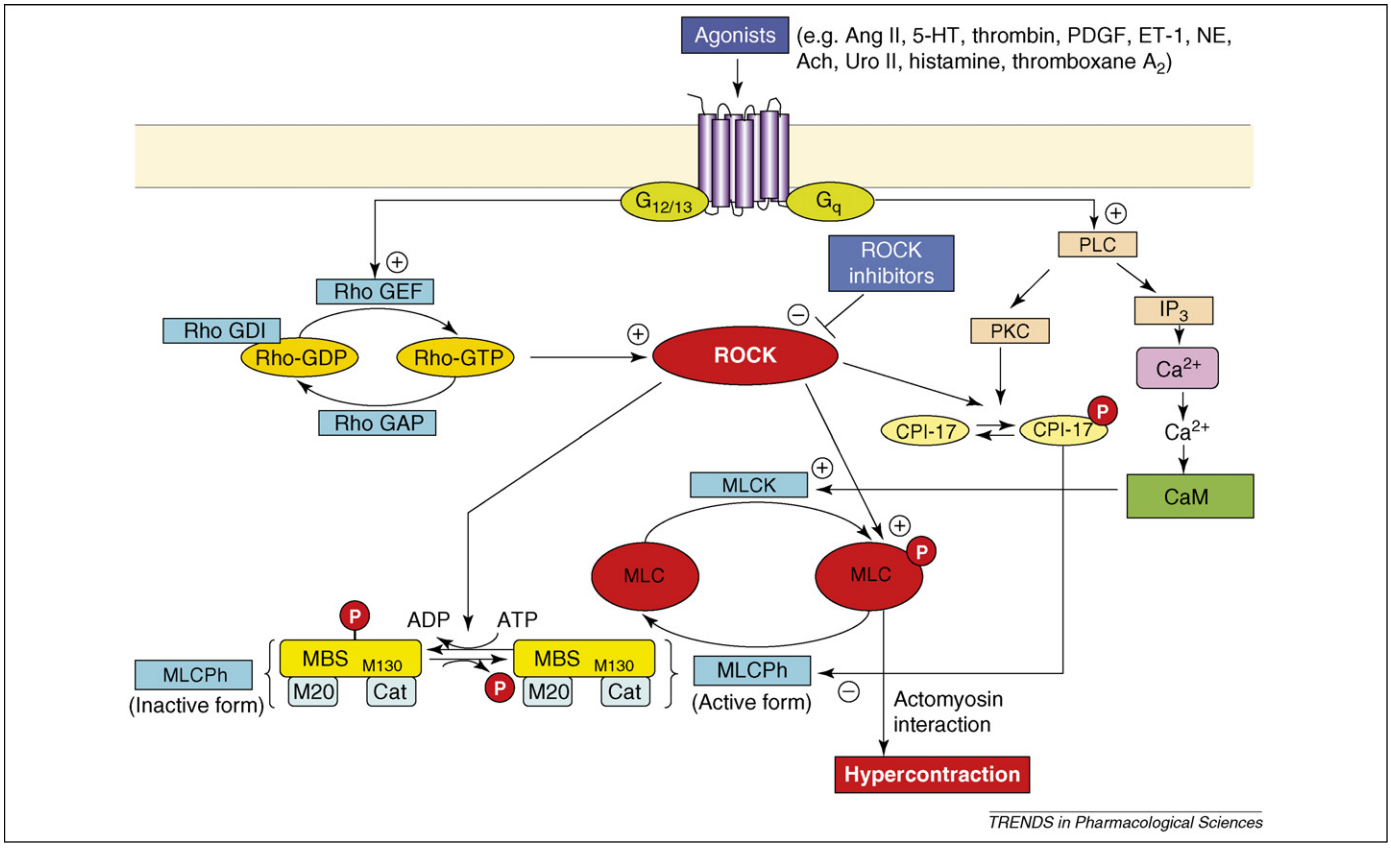

Figure 1. The Rho–ROCK signaling pathway in vascular smooth muscle cell contraction. Contraction is induced by the increased phosphorylation of MLC. The agonist-induced activation of G-protein-coupled receptors leads to the stimulation of MLCK through an increase in intracellular Ca^{2+} concentration, and inhibition of MLCPh. Following stimulation by various agonists, the Rho–ROCK-mediated pathway is activated, resulting in the inhibition of MLCPh (through phosphorylation of its MBS), with a resultant increase in MLC phosphorylation. This ROCK-mediated contraction can occur independently of intracellular Ca^{2+} changes and is known as Ca^{2+} sensitization. ROCK can also increase MLC phosphorylation and contractility by inactivating MLCPh after phosphorylation of CPI-17 or by direct phosphorylation of MLC. Abbreviations: Ach, acetylcholine; Ang II, angiotensin II; Cat, catalytic subunit; ET-1, endothelin-1; IP_3, inositol (1,4,5)-trisphosphate; M20, 20-kDa subunit; NE, norepinephrine; PLC, phospholipase C; PDGF, platelet-derived growth factor; Uro II, urotensin II. Stimulation is denoted by +; inhibition is denoted by −.

The Rho–ROCK pathway has recently attracted a great deal of attention in various research fields, particularly the cardiovascular research field, for several reasons [1]. First, the Rho–ROCK pathway has an important role in various cellular functions that are involved in the pathogenesis of cardiovascular disease (Figure 2). Second, this intracellular signaling pathway is substantially involved in the effects of many vasoactive substances, including angiotensin II, 5-hydroxytryptamine (5-HT), thrombin, platelet-derived growth factor (PDGF), extracellular nucleotides and urotensin II, all of which are implicated in the pathogenesis of cardiovascular disease (Figure 1). Third, it has been suggested that the so-called pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are mediated, at least in part, by their inhibitory effects on Rho and the resultant inhibition of ROCK. In this article, we review the information on the therapeutic importance of the ROCK pathway in cardiovascular medicine, with special reference to its effects on cardiovascular cells, and summarize the current status of the development of ROCK inhibitors.

Therapeutic importance of ROCK in cardiovascular medicine

ROCK and vascular smooth muscle cells

The RhoA–ROCK pathway is well known as a major regulator of not only vascular smooth muscle cell (VSMC) contraction but also cell migration, proliferation, differentiation, apoptosis and survival, and gene transcription [14] (Figure 2). VSMC contraction is regulated mainly by phosphorylation and dephosphorylation of myosin light chain (MLC) [15]. During agonist-induced VSMC contraction, MLC phosphorylation is a crucial step for force development. The extent of MLC phosphorylation is regulated in a dual manner by the Ca^{2+}/calmodulin (CaM)-activated MLC kinase (MLCK) and MLCPh [15] (Figure 1). ROCK, when activated by the small GTPase RhoA, inhibits MLCPh activity by phosphorylating its MBS and, thus, has a key role in agonist-induced Ca^{2+} sensitization and VSMC hypercontraction [16] (Figure 1). ROCK might also directly modulate VSMC contraction through MLC phosphorylation, independently of the CaM-dependent MLCK pathway [17]. Recently, the unrecognized Ca^{2+}-dependent VSMC contraction and differentiation modulated by Rho–ROCK stimulation have been demonstrated [18,19]. A recent study has also shown that ROCK activates and phosphorylates CPI-17 [protein kinase C (PKC)-potentiated phosphatase inhibitor of 17 kDa], which is a phosphorylation-dependent inhibitory protein of myosin phosphatase, resulting in the inhibition of MLCPh activity [20] (Figure 1).

ROCK and endothelial cells

The Rho–ROCK signaling pathway is involved in the regulation of endothelial barrier function, inflammation

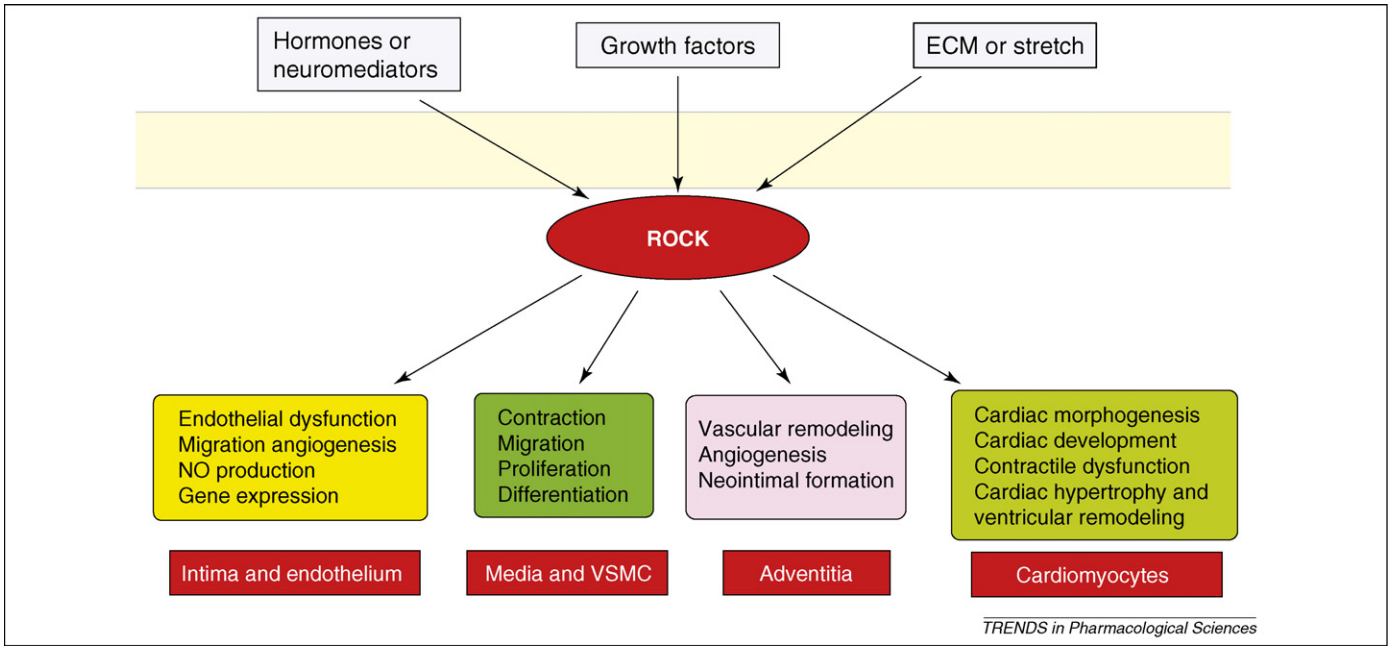

Figure 2. Regulation of various cellular functions by ROCK. ROCK is stimulated by hormones or neuromediators (vasoactive substances), growth factors, interaction with the extracellular matrix (ECM) or mechanical stress. ROCK activation leads to several cellular changes in vascular wall cells and in cardiomyocytes that directly and/or indirectly cause cardiovascular diseases. The various pathophysiological changes include endothelial dysfunction, migration and angiogenesis, nitric oxide (NO) production and gene expression in endothelial cells, contraction, migration, proliferation and differentiation in VSMCs, vascular remodeling, neointimal formation and angiogenesis in adventitia, cardiac morphogenesis, cardiac development, contractile function, cardiac hypertrophy and ventricular remodeling in cardiomyocytes.

and transendothelial leukocyte migration, platelet activation, thrombosis, gene expression and oxidative stress [21]. ROCK activation decreases the expression of endothelial nitric oxide synthase (eNOS) by reducing eNOS mRNA stability [22]. Direct inhibition of Rho by C3 transferase, or inhibition of ROCK by ROCK inhibitors, hydroxyfasudil or overexpression of dominant–negative ROCK prevented the downregulation of eNOS expression and eNOS mRNA stability [22].

In addition to lipid-lowering effects, statins might exert other, so-called 'pleiotropic' beneficial effects on the vascular wall, such as inhibition of vascular inflammation and atherosclerosis through Rho GTPases (Rho, Rac1 and Ras) [23]. Statins upregulate and activate eNOS expression through inhibition of Rho geranylgeranylation [23]. However, it remains to be determined to what extent clinical concentrations of statins inhibit RhoA activity and, thereby, ROCK activity.

ROCK and adventitia

In our porcine model of coronary arteriosclerosis with adventitial treatment of monocyte chemoattractant protein-1 and oxidized low-density lipoprotein, long-term treatment with fasudil (HA1077; see Chemical names) markedly inhibited macrophage accumulation in the adventitia and migration into the media with a subsequent inhibition of coronary lesion formation [24]. In addition, the fasudil treatment suppressed in-stent neointimal formation in porcine coronary arteries through multiple mechanisms, including reduced vascular inflammation, enhanced apoptosis and decreased collagen deposition [25]. Long-term treatment with fasudil or adenovirus-mediated transfer of dominant–negative ROCK induces a marked regression of both constrictive remodeling and coronary vasospastic activity in a porcine model with adventitial inflammation [26].

ROCK and cardiomyocytes

mRNA of both ROCK1 and ROCK2 is expressed in the developing heart [27]. The ROCK inhibitor Y27632 blocks migration of pre-cardiac mesoderm and cardiac tube fusion in cultured chick and mouse embryos [27]. The inhibition of ROCK in cultured mouse embryos decreases cell proliferation but does not cause any changes in programmed cell death, indicating that ROCK is not involved in cardiomyocyte apoptosis but regulates cardiomyocyte division during heart development [28]. This effect is mediated through the regulation of cell-cycle protein expression, cyclin D3, CDK6 and $p27^{Kip1}$ in cardiomyocytes [28]. ROCK also has an important role in endothelial cell proliferation and migration during endocardial cushion development, indicating the involvement of ROCK signaling in the pathogenesis of congenital heart disease [29].

Our recent study of ROCK inhibitors has demonstrated that the ROCK pathway has an important role in the pathogenesis of cardiovascular hypertrophy associated with enhanced oxidative stress by upregulation of endothelial NAD(P)H oxidase and endothelial dysfunction [30]. We have also reported that ROCK is substantially involved in the pathogenesis of left-ventricular remodeling after myocardial infarction (MI) associated with upregulation of proinflammatory cytokines, indicating the therapeutic importance of the molecule for preventing post-MI heart failure [31].

ROCK and the CNS

We have recently reported that the ROCK pathway contributes markedly to blood pressure regulation through

the sympathetic nervous system in the brain stem [32]. Fasudil is effective for the treatment of cerebral vasospasm after subarachnoid hemorrhage, indicating that the ROCK pathway is involved in the pathogenesis of this disorder [33]. The neuroprotective effect of ROCK inhibition is mediated, at least in part, by eNOS and indicates that ROCK is an important therapeutic target for ischemic stroke [32,34]. Fasudil increases cerebral blood flow in both ischemic and non-ischemic areas, reduces cerebral infarct size and improves neurological deficits [34].

Development of ROCK inhibitors

Therapeutic usefulness of ROCK inhibitors in cardiovascular medicine

Accumulating evidence indicates that ROCK inhibitors could cover the wide range of pharmacological effects of conventional cardiovascular drugs, including statins, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type 1 receptor blockers, calcium channel blockers, β-blockers and receptor blockers of thrombin, 5-HT and endothelin, with the apparent exception of inhibitory effects on cholesterol synthesis in the liver [1].

We and others have demonstrated the potential beneficial effects of ROCK inhibitors in animals and humans [1,32,35] (Figure 3). The available evidence indicates that these inhibitors are useful for the treatment of various disorders caused by VSMC hyperconstriction, including coronary and cerebral vasospasm [36,37], hypertension [38] and pulmonary hypertension [39]. They might also be useful for the treatment of various arteriosclerotic cardiovascular diseases, including arteriosclerosis–restenosis [25,26], ischemia–reperfusion injury [40], stroke [41], myocardial hypertrophy [30], heart failure [42], cardiac allograft vasculopathy [43] and vein graft disease [44]. Furthermore, they could be useful for the treatment of disorders associated with smooth muscle hyperreactivity, such as asthma and glaucoma [1]. Finally, recent studies indicate that ROCK inhibitors could be used to treat osteoporosis, renal disease, erectile dysfunction and cancers [1].

Our clinical studies of fasudil have indicated that ROCK inhibitors might be useful for treating cardiovascular diseases in humans, in addition to the current indication of cerebral vasospasm, including angina pectoris, hypertension, pulmonary hypertension, stroke and heart failure (Figure 3). Intracoronary administration of fasudil is effective at reducing coronary spasm and myocardial ischemia in patients with vasospastic angina and microvascular angina [45,46]. Long-term oral treatment with fasudil is also effective at ameliorating exercise tolerance in patients with stable-effort angina and adequate safety profiles [47]. Intra-arterial infusion of fasudil markedly enhances the vasodilator responses of forearm circulation in hypertensive patients [48]. Intravenous infusion of fasudil significantly reduces pulmonary vascular resistance in patients with pulmonary hypertension [49]. Intra-arterial infusion of fasudil causes a preferential increase in forearm blood flow in patients with heart failure, compared with control subjects [50]. However, the potential usefulness of the oral administration of ROCK inhibitors for the treatment of unstable angina, MI, pulmonary hypertension, hypertensive vascular disease and/or cardiac hypertrophy remains to be examined in humans. Another clinical trial of the intravenous administration of fasudil in the acute phase of stroke demonstrates that ROCK inhibitors exert beneficial effects on ischemic neuronal damage without causing serious adverse effects [51].

Current status of the development of selective ROCK inhibitors

A series of pyridine derivative compounds has been developed and, among them, Y27632 has shown a potent ROCK inhibitory action [16]. Y27632 selectively inhibits smooth muscle contraction by inhibiting Ca^{2+} sensitization, suppresses the ROCK-mediated formation of stress fibers in cultured cells and markedly corrects hypertension

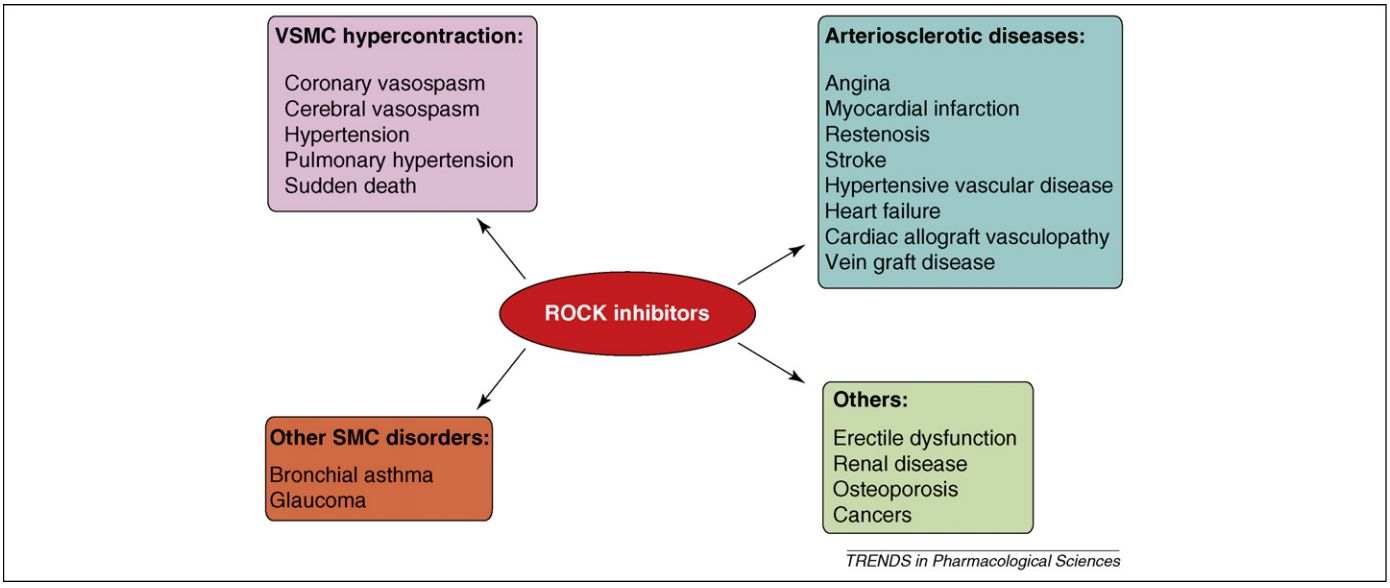

Figure 3. Possible indications of ROCK inhibitors. ROCK inhibitors seem to be useful for treating disorders caused by VSMC hypercontraction, arteriosclerotic diseases, other smooth muscle cell (SMC) disorders (e.g. bronchial asthma and glaucoma) and other diseases. The clinical usefulness of ROCK inhibitors remains to be fully elucidated.

in several hypertensive rat models [16]. Y27632, which has no specificity for ROCK isoforms, inhibits the kinase activity of both ROCK1 and ROCK2 by competing with ATP for binding to their catalytic sites [52]. Although Y27632 has a reasonably selective profile, it also inhibits Rho-dependent PKC-related kinase 2 with a similar potency to that with which it inhibits ROCK2 [53]. However, this pyridine derivative is used as an important pharmacological tool to examine the involvement of ROCK in the pathogenesis of cardiovascular and other diseases (Table 1).

Fasudil also selectively inhibits ROCK by competing with ATP for binding to the kinase [53]. It has been demonstrated that hydroxyfasudil, a major active metabolite of fasudil after oral administration, has a more selective inhibitory effect on ROCK than does the parent drug [30,36]. The IC_{50} values of hydroxyfasudil for ROCK1 and ROCK2 are 0.73 μmol/l and 0.72 μmol/l, respectively, whereas the IC_{50} values of fasudil for ROCK1 and ROCK2 are 1.2 μmol/l and 0.82 μmol/l, respectively [34]. In addition to their inhibitory effects on ROCK, fasudil and hydroxyfasudil exert nonspecific inhibitory effects on other serine/threonine kinases such as protein kinase A (PKA) and PKC. The IC_{50} values of fasudil and hydroxyfasudil for PKA are 5.3 μmol/l and 37 μmol/l, respectively, whereas the IC_{50} value of both is >100 μmol/l for PKCα [34]. Fasudil is currently the only ROCK inhibitor that is available for clinical use and has been used only in Japan for seven years to treat cerebral vasospasm after subarachnoid hemorrhage [32]. The clinical trials of the anti-anginal effects of fasudil in patients with stable-effort angina in Japan [47] and the USA [54] have demonstrated that long-term oral treatment with this ROCK inhibitor is effective at ameliorating exercise tolerance in patients with adequate safety profiles. This Phase II dose-finding trial in patients with stable angina showed that ST-segment depression was improved with fasudil at both peak and trough compared with placebo. In addition, fasudil improved Seattle Angina Questionnaire scores, was well tolerated and did not affect heart rate or blood pressure. No major adverse events were noted with fasudil treatment; most of the adverse events were mild and not considered to be related to study medication [47,54].

An effort has been made to develop more-specific and more-potent ROCK inhibitors [55]. Fasudil has been further modified [hexahydro-1-(isoquinoline-5-sulfonyl)-1H-1,4-diazepine] and H1152P (produced by methylation at 2-position of hexahydro-1H-1,4-diazpine), which has a better ROCK2 inhibitory profile than does fasudil, has been developed. The modification of H1152P with an ethenyl group at the 4-position of the isoquinoline moiety has provided potent and specific ROCK2 activity, and the IC_{50} value of this new H1152P derivative for ROCK2 is 6 nM, whereas its inhibitory effect against other kinases (e.g. PKA, PKC, PKG, AuroraA and CaM-dependent protein kinase II) is weaker [55]. Moreover, fasudil analogs have been designed on the basis of the complex structure of PKA and HA1077, and it was found that glycine derivatives of HA1077 are highly specific inhibitors of ROCK [55]. These inhibitors were applied to rabbit ocular hypertensive models, in which they reduced intraocular pressure – indicating that they might be useful for glaucoma, in addition to other ROCK-related diseases [55].

Recently, two aminofurazan-based inhibitors, GSK269962A and SB772077B, were characterized as members of a novel class of compounds [56]. These compounds have similar inhibitory effects on ROCK1 and

Table 1. Current status of the development of ROCK inhibitors[a]

Company	Drug	Development status	Indication
Asahi Kasei Pharma (Japan)	Fasudil	Launched	Cerebral vasospasm
		Registered	Acute cerebral ischemia
		Phase II clinical	Angina
		Phase I clinical	Pulmonary hypertension
Originally developed by Mitsubishi Pharma (Japan), now sold by Sigma-Aldrich (USA)	Y27632	Used as a pharmacological tool	None
Mitsubishi Pharma (Japan)	Y39983	Phase I clinical	Glaucoma
	Wf536	Discovered	Inhibition of tumor metastasis
	Patented	Discovered	Reperfusion injury, renal disease, ischemia
Kowa (Japan)	H1152P	Discovered	Glaucoma
GlaxoSmithKline (UK)	GSK269962A SB772077B	Discovered	Anti-inflammatory and vasodilator activity
Surface Logix (USA)	SLx2119 ROCK2 inhibitor	Discovered	Atherosclerosis
Alcon (USA)	Patented	**Possible indications**	
Bayer HealthCare (Germany)	Patented	Cardiovascular disease: angina, atherosclerosis, hypertension, cerebrovascular ischemia, congestive heart failure, reperfusion injury	
BioAxone Therapeutic (Canada)	Patented		
BioFocus (UK)	Patented		
Isis Pharmaceuticals (France)	Patented	Inflammatory disease: asthma, autoimmune disease, rheumatoid arthritis, HIV infection	
Kirin Brewery (Japan)	Patented		
Santen Pharmaceutical and UBE (Japan)	Patented		
Senju Pharmaceutical (Japan)	Patented	CNS disease: Alzheimer's disease, Huntington's chorea, spinal cord injury	
Sumitomo Pharmaceuticals (Japan)	Patented		
Vertex Pharmaceuticals (USA)	Patented	Ophthalmic disease: corneal disease, glaucoma, diabetic retinopathy, lacrimal gland disease	
Xcellsyz (UK)	Patented		
		Others: erectile dysfunction, hearing disorder, osteoporosis, fibrosis, renal disease, metabolic disorder, cancer	

[a]Source of patent information: Industrial Property Digital Library (www.ipdl.ncipi.go.jp) and IPDL (www.ipdl.ncipi.go.jp/links_e.htm).

ROCK2, and their potency is higher than that of Y27632 or fasudil [56]. They demonstrate a good correlation of the potency of ROCK inhibition with the efficacy of smooth muscle relaxation and they inhibit cytokine production in macrophages [56]. This study also demonstrated that these compounds have vasodilator activity and that they lower blood pressure in spontaneously hypertensive rats and deoxycorticosterone acetate (DOCA)-salt-treated hypertensive rats [56]. Recently, SLx2119, which is an orally bioavailable, potent and highly selective ROCK2 inhibitor, has been developed. SLx2119 attenuates arterial plaque formation in apolipoprotein-E-deficient mice, indicating that selective ROCK2 inhibition could limit atherosclerosis and avoid unwanted hemodynamic side-effects, compared with non-selective ROCK inhibitors [57]. Table 1 shows other ROCK inhibitors that are currently being developed, including Y39983 [58] and Wf536 [59]. Recently, the 3D crystal structure of ROCK and its binding site for fasudil has been determined, which should facilitate the development of selective ROCK inhibitors [60].

Chemical names

GSK269962A: *N*-(3-{[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1*H*-imidazo[4,5-*c*]pyridin-6-yl]oxy}phenyl)-4-{[2-(4-morpholinyl)ethyl oxy}benzamide
HA1077: 1-(5-isoquinolenesulfonyl)-homopiperazine
H1152P: (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolynyl)sulfonyl]homopiperazine
SB772077B: 4-(7-{[(3*S*)-3-amino-1-pyrrolidinyl]carbonyl}-1-ethyl-1*H*-imidazo[4,5-*c*]pyridin-2-yl)-1,2,5-oxadiazol-3-amine
Wf536: (+)-(*R*)-4-(1-aminoethyl)-*N*-(4-pyridyl) benzamide
Y27632: (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide
Y39983: 4-[(1*R*)-aminoethyl]-*N*-(1*H*-pyrrolo[2,3-b]pyridine-4-yl) benzamide

Concluding remarks

Accumulating evidence indicates that the ROCK pathway is substantially involved in the pathogenesis of various cardiovascular diseases and that ROCK inhibitors are useful for treating those diseases with a broad spectrum of pharmacological properties. However, further studies are required to understand better how ROCK1 and ROCK2 are activated and regulated, and how they cause pathophysiological changes in the cardiovascular system through their downstream effectors. Nonetheless, ROCK inhibitors could be the new class of drug for the treatment of cardiovascular diseases.

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