# RhoA/Rho-kinase in erectile tissue: mechanisms of disease and therapeutic insights

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## A　B　S　T　R　A　C　T

Penile erection is a complicated event involving the regulation of corpus cavernosal smooth muscle tone. Recently, the small monomeric G-protein RhoA and its downstream effector Rho-kinase have been proposed to be important players for mediating vasoconstriction in the penis. RhoA/Rho-kinase increases MLC (myosin light chain) phosphorylation through inhibition of MLCP (MLC phosphatase) thereby increasing $Ca^{2+}$ sensitivity. This review will outline the RhoA/Rho-kinase signalling pathway, including the upstream regulators, guanine nucleotide exchange factors, GDP dissociation inhibitors and GTPase-activating proteins. We also summarize the current knowledge about the physiological roles of RhoA/Rho-kinase in both male and female erectile tissues and its aberrations contributing to erectile dysfunction in several disease states. Understanding the RhoA/Rho-kinase signalling pathway in the regulation of erection is important for the development of therapeutic interventions for erectile dysfunction.

## INTRODUCTION

ED (erectile dysfunction) is defined as the inability to achieve or maintain an erection of sufficient rigidity for vaginal penetration. Epidemiological studies suggest that ED is often associated with aging and chronic illnesses such as diabetes mellitus, hypogonadism and cardiovascular diseases. It is estimated that ED currently affects 30 million men in the United States and the number will increase to 322 million by 2025 [1,2]. In the past decade, knowledge of penile erection and the pathogenesis of ED has advanced dramatically. Penile erection ultimately depends on the states of contraction and relaxation of SMCs (smooth muscle cells) of the cavernosal arterioles and trabeculae of the corpora cavernosa. However, most studies have focused on vasodilatory mechanisms involving NO (nitric oxide), which is released from non-adrenergic and non-cholinergic nerve endings and endothelial cells in the penis. Impaired NO production or bioavailability leads to increased cavernosal smooth muscle contraction, resulting in ED. In contrast, understanding of contractile signalling pathways involved in erectile function has just emerged recently. In this review, we highlight the contractile mechanisms of erection through activation of the RhoA/Rho-kinase-mediated $Ca^{2+}$ sensitization pathway and provide an update on the

rapidly accumulated information about this pathway as it pertains to normal erection physiology and to vasculogenic ED.

## PHYSIOLOGY OF PENILE ERECTION

Penile erection is a complex neurovascular event which is initiated by stimulation of central and peripheral nervous systems [3,4]. In the flaccid state, sympathetic neuronal input is dominant, releasing neurotransmitters, such as noradrenaline (norepinephrine), that maintain cavernosal smooth muscle contraction. Following sexual stimulation, parasympathetic activity is increased, leading to increased release of NO synthesized by nNOS [neuronal NOS (NO synthase)] from nerve endings in the penis. NO activates the cGMP/PKG (protein kinase G) signalling cascade, inducing corporal smooth muscle relaxation. Reduced peripheral resistance of cavernosal arterioles allows blood flow into the penis under the driving force of systemic blood pressure. The increased blood flow causes shear stress which subsequently activates eNOS (endothelial NOS) to produce NO [5,6]. As a result, sinusoidal spaces are filled with blood, creating a pressure to compress the venules against the tunica albuginea and thus limiting venous outflow. Because of this venoocclusion mechanism, ICP (intracavernosal pressure) increases dramatically, resulting in penile erection.

## $Ca^{2+}$ SENSITIZATION PATHWAYS IN SMOOTH MUSCLE CONTRACTION

Vascular smooth muscle contraction involves $Ca^{2+}$-dependent mechanisms and $Ca^{2+}$ sensitization mechanisms, which are initiated by activation of receptors or depolarization of the plasma membrane [7,8]. In the classic $Ca^{2+}$-dependent pathway, agonist-induced GPCR (G-protein-coupled receptor) activation increases PLC (phospholipase C) activity, resulting in increased formation of $IP_3$ (inositol trisphosphate) and DAG (diacylglycerol). $IP_3$ subsequently induces $Ca^{2+}$ release from the sarcoplasmic reticulum, leading to a rise in intracellular $Ca^{2+}$ levels [9]. The binding of $Ca^{2+}$ to calmodulin causes a conformational change in the protein and enables $Ca^{2+}$/calmodulin to activate MLCK [MLC (myosin light chain) kinase]. Phosphorylation of the regulatory MLC by MLCK leads to the force generation. Meanwhile, DAG together with $Ca^{2+}$ activates PKC (protein kinase C), promoting contraction by phosphorylation of L-type $Ca^{2+}$ channels or other proteins that regulate crossbridge cycling. Finally, voltage-operated $Ca^{2+}$ channels in the membrane open in response to membrane depolarization brought on by vascular smooth muscle stretch.

Vascular smooth muscle is able to maintain contractile force for an extended period of time at a low cost of energy. The maintenance of force may not be tightly correlated with an elevation in intracellular $Ca^{2+}$ levels.

Early studies have demonstrated agonist-enhanced $Ca^{2+}$ sensitivity by simultaneous measurements of force and cytoplasmic $Ca^{2+}$. Intracellular levels of $Ca^{2+}$ are raised by the addition of an $\alpha$-adrenergic agonist only during the initial phase of force generation, while falling to near basal levels during the maintenance phase of force [10]. Furthermore, adrenergic agonists and GTP[S] (guanosine 5-[$\gamma$-thio]triphosphate), a non-hydrolysable analogue of GTP, have been shown to induce contractions without alterations in intracellular $Ca^{2+}$ levels in smooth muscle fibres permeabilized by $\alpha$-toxin [11]. The phenomenon of increased force at a low intracellular $Ca^{2+}$ level is termed $Ca^{2+}$ sensitization [12]. Regulation of MLCP (MLC phosphatase) has been suggested as one of the mechanisms to increase $Ca^{2+}$ sensitivity in various vascular tissues [13].

This $Ca^{2+}$-independent regulation involves the monomeric GTP-binding protein RhoA [14]. The Rho family belongs to the Ras superfamily of GTPases, including five subfamilies: Rho-like (RhoA, B and C), Rac-like (Rac1, 2 and 3 and RhoG), Cdc42-like, RhoBTB-like and Rnd-like (Rnd 1, 2 and 3) [15]. Although RhoA, RhoB and RhoC are highly related proteins that share more than 80 % amino acid homology, their cellular functions can be distinct [16,17]. Compared with that of RhoB and RhoC, the functions of RhoA have been well characterized. In addition to its important role in mediating smooth muscle contraction, RhoA is also involved in the regulation of various cellular processes, such as stress fibre formation, cell proliferation and migration and apoptosis [1,18,19]. The best characterized effector of RhoA is Rho-kinase, which is directly involved in smooth muscle contraction (discussed below). In addition, RhoA regulates the activities of other kinases, including citron, mammalian diaphanous protein and protein kinase N, whose functions are involved in cytokinesis, cell cycle, actin polymerization and endosomal trafficking [20,21].

## REGULATION OF RhoA GTPase

RhoA functions as a molecular switch, cycling between the inactive GDP-bound state and the active GTP-bound state (Figure 1). The activity of RhoA is tightly regulated by three proteins: RhoGEF (guanine nucleotide exchange factor), RhoGAP (GTPase-activating protein) and RhoGDI (GDP dissociation inhibitor) [19,22–24]. In the inactive state, RhoGDI binds to RhoA-GDP to form a complex, stabilizing RhoA-GDP and trapping the complex in the cytosol. The activation of RhoGEF facilitates the exchange of GTP for GDP on RhoA and dissociation from RhoGDI. The active RhoA-GTP then translocates from the cytosol to the plasma membrane and binds to the membrane through geranylgeranylation, initiating signalling transduction. RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP on RhoA-GTP thereby inactivating
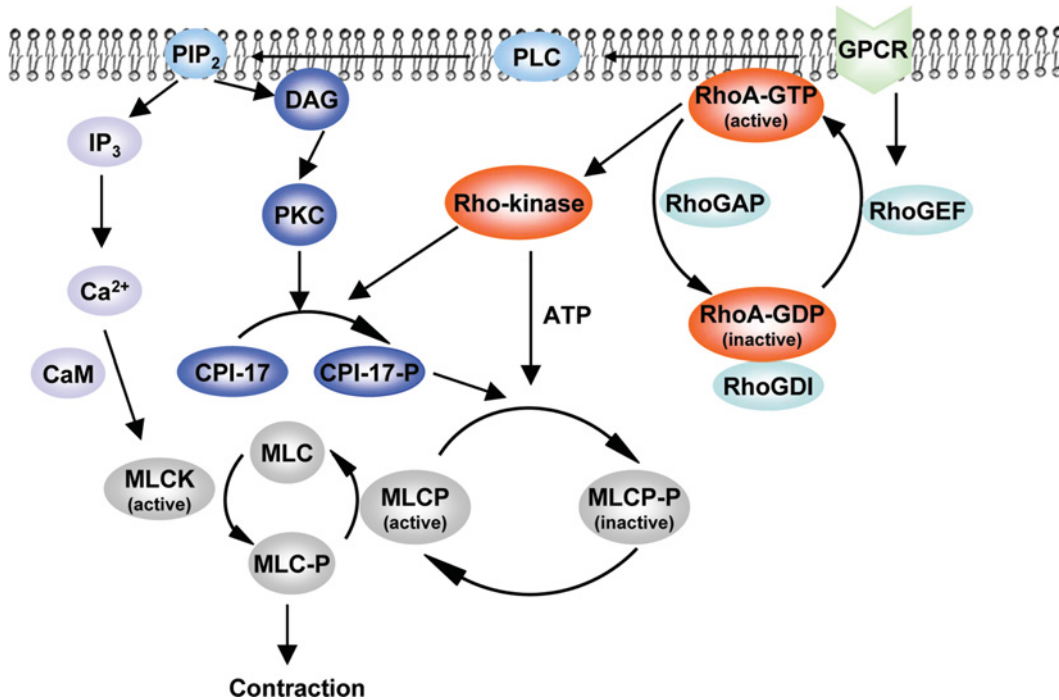
**Figure 1    Ca²⁺ sensitization and Ca²⁺-dependent pathways in penile smooth muscle**

Stimulation of GPCRs leads to the activation of both $Ca^{2+}$ sensitization and $Ca^{2+}$-dependent pathways. PLC catalyses $PIP_2$ into $IP_3$ and DAG. $IP_3$ increases intracellular $Ca^{2+}$ levels and, together with CaM (calmodulin), $Ca^{2+}$ activates MLCK. MLCK then phosphorylates MLC, resulting in smooth muscle contraction. After activation by DAG, PKC phosphorylates CPI-17. Phosphorylated CPI-17 has a high affinity for the catalytic subunit of MLCP and decreases MLCP activity through phosphorylation. Activation of GPCRs also stimulates RhoGEF activity, which facilitates the exchange of GTP for GDP on RhoA and dissociates RhoA from RhoGDI. The active RhoA-GTP translocates from the cytosol to the plasma membrane and activates Rho-kinase. Subsequently, Rho-kinase phosphorylates the targeting subunit of MLCP, leading to increased MLC phosphorylation. In addition, Rho-kinase has been shown to phosphorylate CPI-17. Meanwhile, RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP; thus, inactive RhoA-GDP re-associates with RhoGDI and relocates to the cytosol.

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## RhoGDI

Three isoforms of RhoGDI have been identified so far. $RhoGDI_\alpha$ appears to be universally expressed, whereas $RhoGDI_\beta$ and $RhoGDI_\gamma$ are restricted to haematopoietic tissues and the brain respectively [26]. RhoGDI acts as an endogenous inhibitor for RhoA through two mechanisms: stabilizing RhoA in its inactive GDP-bound form and translocating RhoA-GDP to the cytosol after extracting it from the membrane. Although RhoGDI is able to interact with both the GTP- and GDP-bound Rho, the binding of GDP to Rho increases the affinity for RhoGDI more than 10 times [27]. Overexpression or exogenous administration of high concentrations of RhoGDI in tissues inhibits Rho-mediated biological activities [28,29]. In a cell-free system derived from rat kidney, RhoA is extracted from the brush-border membrane after addition of a GST (glutathione S-transferase)–GDI fusion protein [30]. However, the effect of GST–GDI on membrane-bound

RhoA is abolished at physiological ionic strength of salt (150 mmol/l KCl) and normal body temperature, suggesting that the shuttling activity of RhoGDI may normally be down-regulated under physiological conditions.

The ability of RhoGDI to extract RhoA from the plasma membrane is not solely dependent on the exchange of nucleotide on RhoA. Phosphorylation of RhoA on $Ser^{188}$ by PKA (cAMP-dependent protein kinase A) has been shown to increase the RhoA interaction with RhoGDI even when it is in the GTP-bound state [31]. Forskolin, a cAMP activator, markedly reduces the amount of active RhoA in renal CD8 cells as determined by RhoA-GTP pull down assay and Western blot analysis. Forskolin treatment leads to increased phosphorylation of RhoA as well as decreased membrane fraction of GDI, which results in increased RhoGDI–RhoA complexes in the cytosol [32]. Transfection of human microvascular endothelial cells with adenovirus encoding the PKA inhibitor gene blocks the inhibitory effect of forskolin on RhoA [33]. These studies suggest that phosphorylation of RhoA is an alternative mechanism to inhibit RhoA activity, independently from its nucleotide state.

## RhoGEF

Various forms of RhoGEFs have been identified and among them p115RhoGEF, PDZRhoGEF and LARG (leukaemia-associated RhoGEF) are most studied. These RhoGEFs, as with other GEFs, contain a DH (Dbl homology) domain and a PH (pleckstrin homology) domain linked in tandem. Crystallographic studies provide the physical evidence that RhoGEFs directly interact with RhoA at both DH and PH domains [34]. The DH domain is thought to be responsible for catalysing the nucleotide exchange reaction, whereas the PH domain may stabilize the binding of DH domain to RhoA and direct the subcellular localization of RhoA to the membrane, allowing RhoA to interact with actin [35,36]. In some cases, the PH domain can also modulate the nucleotide exchange activity of the DH domain [37]. Kristelly et al. [38] have demonstrated that the DH/PH domains of LARG catalyse nucleotide exchange more efficiently than the DH domain only.

More importantly, p115RhoGEF, PDZRhoGEF and LARG have a unique motif known as the RGS (regulators of the G-protein signalling) domain at the N-terminal that serves as a binding site for the $\alpha$-subunits of the heterotrimeric G-proteins. Additionally, the RGS domain of these RhoGEFs functions as a GAP, accelerating G$\alpha$ intrinsic GTPase activity [39,40]. It has been reported that G$\alpha_{12}$ and G$\alpha_{13}$, members of the G$_{12}$ family, can physically associate with p115RhoGEF, PDZRhoGEF and LARG [41–43]. Mutation of G$\alpha_{13}$ on residues Glu[229], Lys[204] or Arg[232] attenuates the activation of p115RhoGEF and LARG and the subsequent RhoA activation [44,45]. In addition, the variant G$\alpha_q$ reportedly interacts with RhoGEFs, leading to the activation of RhoA [46,47]. In the inactive state of RhoGEFs, the RGS domain and the DH domain have reciprocal inhibitory effects. When G$\alpha$ is activated, the RGS domain of RhoGEF binds to G$\alpha$ and exposes the DH domain, which is able to interact with RhoA and initiate nucleotide exchange [48].

Activation of RhoGEFs may not only be dependent on $\alpha$-subunits of the G-protein, but also on the PKC pathway. In a study of p115RhoGEF-mediated thrombin-induced cytoskeletal rearrangement, direct phosphorylation of p115RhoGEF by PKC$\alpha$ is observed within 1 min of thrombin stimulation [49]. Inhibition of PKC$\alpha$ results in attenuation of thrombin-induced RhoA activation, along with decreased p115RhoGEF phosphorylation. The study suggests that G$_{12}$ and PKC$\alpha$ pathways may run in parallel to modulate RhoGEF activity.

## RhoGAP

RhoGAP inhibits RhoA through enhancing the intrinsic rate of GTP hydrolysis; thus active RhoA-GTP is converted into inactive RhoA-GDP. So far, the knowledge about RhoGAP and how RhoGAP regulates RhoA is incomplete. The mechanism of RhoGAP-facilitated GTP hydrolysis is predicted based on crystallographic studies demonstrating that Arg[85] on RhoGAP engages the GTPase active site and stabilizes the transition state of GTP hydrolysis [50]. Several GAPs have been identified to selectively interact with Rho such as Graf, p122RhoGAP and p190RhoGAP [51–54].

## Effectors of RhoA GTPase

Active RhoA binds to its downstream targets to initiate numerous cellular responses. Rho-kinase (ROCK or ROK), a serine/threonine kinase, is one of the most studied RhoA effectors and is involved in regulation of the actin cytoskeleton, cell proliferation, focal adhesion/stress fibre formation and smooth muscle contraction. Rho-kinase consists of two isoforms: Rho-kinase-$\alpha$ (ROCK-II/ROK$\alpha$) and Rho-kinase-$\beta$ (ROCK-I/ROK$\beta$). Rho-kinase contains a RhoA-binding site at the C-terminus and a catalytic site at the N-terminus [55]. Binding of RhoA to Rho-kinase causes a conformational change in Rho-kinase and its subsequent autophosphorylation, increasing Rho-kinase activity [55,56].

In smooth muscle, Rho-kinase induces vasoconstriction through phosphorylation of MLCP. MLCP is composed of three subunits: MYPT1 (a 110–130 kDa MLC-targeting subunit), PP1c (a 38 kDa catalytic subunit of protein phosphatase type 1) and a small 20 kDa subunit whose function is not clear [57]. MYPT1 is ubiquitously expressed with a relatively higher concentration in SMCs. With the PP1c-binding domain on the N-terminal region and the myosin-binding domain on the C-terminal region, MYPT1 targets MLCP to the myosin filaments and increases the catalytic activity of PP1c [58].

The state of MLC phosphorylation involving a balance between MLCK and MLCP activities determines the contractile activity of smooth muscle [59,60]. MLCP inhibition results in the maintenance of phosphorylated MLC at low intracellular Ca$^{2+}$ concentrations, thus promoting the binding of actin and myosin for force generation. Phosphorylation of MYPT1 at Thr[696] is thought to decrease PP1c catalytic activity, which inhibits MLCP and maintains MLC phosphorylation. Agonists such as prostaglandin F$_{2\alpha}$, lysophosphatidic acid and angiotensin II have been shown to increase phosphorylation of MYPT1 at Thr[696] and enhance Ca$^{2+}$ sensitivity through activation of Rho-kinase [61–63]. In chicken amnion smooth muscle and rat caudal arterial smooth muscle, Rho-kinase inhibits MLCP through phosphorylation of MYPT1 at Thr[855] or Thr[850], reducing the binding of MLCP to MLC [64–66].

It is noteworthy that Rho-kinase may be directly activated by arachidonic acid, independent of RhoA [67,68]. However, the detailed mechanism through which arachidonic acid activates Rho-kinase is not clear. In

addition, recent studies suggest that Rho-kinase can be inhibited by Rnd3 (RhoE), a member of the Rho family which does not have an intrinsic GTPase activity. Rnd3 is predominantly in a GTP-bound active form and it binds to the N-terminal of Rho-kinase. Studies have shown that Rnd3 competes with RhoA for binding Rho-kinase, although the binding sites for Rnd3 and RhoA on Rho-kinase are distinctively different [69,70]. Rho-kinase inactivation by Rnd3 may result from Rnd3 causing a conformational change of Rho-kinase or RhoA binding to part of the Rnd3-binding site of the enzyme [71]. In addition, Rnd3 stimulates RhoGAP activity, increasing the GDP-bound form of RhoA [72]. Functional studies demonstrated that overexpression of Rnd3 prevents phosphorylation of MLCP and stress fibre formation induced by Rho-kinase [71].

It has been reported that Rho-kinase may also increase $Ca^{2+}$ sensitization by phosphorylating CPI-17 (a 17 kDa PKC-potentiated phosphatase inhibitor). CPI-17 is a downstream target of PKC, which serves an important role in smooth muscle contraction [73]. Molecular and structural studies demonstrate that the phosphorylation of $Thr^{38}$ on CPI-17 by PKC enhances the affinity of CPI-17 and its inhibitory activity on MLCP more than 1000 times [74]. CPI-17 isolated from porcine aorta smooth muscle specifically inhibits PP1c as well as the MLCP holoenzyme [75,76]. It is suggested that phosphorylated CPI-17 selectively suppresses MLCP among various PP1 holoenzymes [77].

The complexities of the PKC/CPI-17 and the RhoA/Rho-kinase pathways have recently been demonstrated. The Rho-kinase inhibitor Y27632 [(+)-($R$)-trans-4-(1-aminoethyl)-$N$-(4-pyridyl) cyclohexanecarboxamide dihydrochloride] blocks CPI-17-mediated $Ca^{2+}$ sensitization in response to agonists of this pathway. The specificity of Y27632 for Rho-kinase may be questioned, since Y27632 inhibits phosphorylation of CPI-17 by purified PKC$\delta$ at a concentration similar to that required to inhibit Rho-kinase [78]. Nevertheless, there is evidence suggesting that Rho-kinase can directly phosphorylate native and recombinant CPI-17 at $Thr^{38}$ *in vitro*. Substitution of $Thr^{38}$ to alanine on CPI-17 significantly decreases its phosphorylation levels by Rho-kinase [79]. Overexpression of an active mutant RhoA or stimulation with thrombin in vascular SMCs increases CPI-17 protein expression as well as its phosphorylation. This phenomenon is attenuated by Y27632 or the RhoA inhibitor exoenzyme C3, but not a PKC inhibitor [80].

## REGULATION OF THE RhoA/Rho-KINASE PATHWAY BY NO

NO is the primary vasodilator in many vascular beds. NO diffuses into SMCs and activates guanylate cyclase, leading to an increase in cGMP levels. cGMP then activates PKG, thereby decreasing intracellular $Ca^{2+}$ concentration and inducing membrane hyperpolarization. Moreover, NO reduces $Ca^{2+}$ sensitization mediated by the RhoA/Rho-kinase pathway. Evidence has shown that Y27632 is less effective in causing relaxation if the endothelial cells (the primary source of NO) are removed in rat aorta strips [81]. If exogenous NO is added back to the aortic strip incubation, the inhibitory effect of Y27632 is restored. Furthermore, acute or chronic inhibition of NOS increases $\alpha_2$-adrenergic receptor agonist-induced RhoA/Rho-kinase activity [82]. The direct inhibitory effect of NO on RhoA/Rho-kinase activity is supported by molecular studies [83,84]. These studies demonstrated that PKG phosphorylates RhoA at $Ser^{188}$, destabilizing membrane binding of RhoA. Interestingly, administration of SNP (sodium nitroprusside) or a cGMP analogue enhances RhoA mRNA and protein expression, whereas long-term inhibition of NOS significantly reduces RhoA mRNA and protein expression [85]. This result suggests that the basal release of NO is required to maintain RhoA expression and RhoA-dependent functions.

The relationship between NO and RhoA/Rho-kinase becomes more complicated with the evidence that RhoA/Rho-kinase negatively regulates NO production by inhibition of NOS. Transfection of HUVECs (human umbilical vein endothelial cells) with constitutively active RhoA decreases eNOS phosphorylation at $Ser^{1177}$ through inhibition of PKB (protein kinase B) [86]. Additionally, eNOS gene expression is decreased by RhoA overexpression, but this is independent of PKB regulation [86]. Inhibition of Rho-kinase by Y27632 prevents up-regulation of the RhoA/Rho-kinase pathway and down-regulation of eNOS protein expression in the left ventricle from hypertensive rats [87]. Taken together, these studies suggest there is a homodynamic balance between the NO signalling pathway and the RhoA/Rho-kinase pathway to maintain smooth muscle function.

## TOOLS TO STUDY THE RhoA/Rho-KINASE PATHWAY

The C3 exoenzyme isolated from *Clostridium botulinum* is the most commonly used toxin for the examination of the functional role of RhoA, because it specifically inhibits RhoA through ADP-ribosylation at $Asn^{41}$ and has little effect on other proteins in the Rho family. The inhibition of RhoA by C3 exoenzyme is thought to block the dissociation of RhoA and RhoGDI complex and prevent the activation of RhoA by RhoGEF, as RhoA agonists GTP[S] and PIP$_2$ (phosphatidylinositol 4,5-bisphosphate) are unable to release RhoA from RhoGDI in the presence of C3 [88]. ADP-ribosylation of RhoA does not appear to interfere with its nucleotide binding, RhoGTPase activity or interaction with downstream effectors such as Rho-kinase [89–91]. On the other hand, CNF (cytotoxic necrotizing factor)-1 and -2, first isolated

from *Escherichia coli*, have been shown to turn RhoA into a constitutively active form by deamidating Gln[63] to glutamine, which blocks the RhoGAP-increased GTP hydrolysis [92,93]. CNF-1 and -2 also affect other RhoGTPases such as Rac and CDC42 [94]. Recently, CNF$_Y$ identified in *Yersinia pseudotuberculosis* strains has been demonstrated to selectively activate RhoA without activation of Rac or CDC42 [95].

Y27632, a highly selective inhibitor of Rho-kinase, has been a useful pharmacological tool for examining the role of Rho-kinase in various cellular functions [96]. It competes for the ATP-binding site on Rho-kinase, preventing the Rho-kinase-mediated phosphorylation of MLCP, resulting in relaxation of smooth muscle. Y27632 is transported into cells or tissue by a carrier facilitated diffusion mechanism to inhibit Rho-kinase activity; therefore the extracellular concentration of Y27632 is similar to the intracellular concentration. The $K_i$ value of Y26732 for Rho-kinase is about 200-fold lower than that for other protein kinases involved in smooth muscle contractility, such as PKC, PKA and MLCK [96,97]. Although Y27632 is relatively less selective for the $Ca^{2+}$-dependent PKC isoforms, it is a potent inhibitor of the $Ca^{2+}$-independent PKC$\varepsilon$ and PKC$\delta$ isoforms, which have been shown to mediate $Ca^{2+}$ sensitization [78,97]. The effects of Y27632 on $Ca^{2+}$ mobilization are concentration dependent. Y27632 has minimal effects on intracellular $Ca^{2+}$ at $10\,\mu$mol/l, whereas it significantly inhibits the rise of intracellular $Ca^{2+}$ levels induced by agonists at $100\,\mu$mol/l [98,99].

Another family of compounds derived from iso-quinolinesulphonamide shows selectivity for Rho-kinase. HA1077 (or fasudil hydrochloride), like Y27632, competes for the ATP-binding site on Rho-kinase with a $K_i = 0.3\,\mu$mol/l [100]. However, HA1077 is also a potent inhibitor of PKA and PKC with a $K_i = 1$ and $9.3\,\mu$mol/l respectively, which makes it difficult to separate activity of the Rho-kinase-mediated pathway from PKC-mediated $Ca^{2+}$ sensitization using this reagent [101]. H1152P is similar in structure to HA1077, except that it has two extra methyl groups, which significantly enhances its inhibitory effect and selectivity for Rho-kinase. Its $K_i$ value for Rho-kinase is approx. 1.6 nmol/l, 400 times more potent than that for PKA, PKC and MLCK [101].

The biological activities of RhoA and Rho-kinase can also be manipulated by overexpression of constitutively active and dominant-negative mutants of RhoA or Rho-kinase as well as the Rho GTPase regulators [102]. The dominant-negative RhoA (T19NRhoA) is generated by the substitution of Asp[19] for threonine, which leads to increased affinity for RhoGEF and decreased GTP binding, competing with endogenous RhoA for RhoGEF. The substitution of Leu[63] for glutamic acid results in a constitutively active RhoA (V14RhoA). Rho-kinase dominant-negative and active mutants are also used to in-

vestigate RhoA/Rho-kinase-mediated $Ca^{2+}$ sensitization [103,104]. Recently, siRNA (small interference RNA) has emerged as a new powerful tool to selectively silence expression of the target gene. This post-transcriptional gene silencing by these 21–25 nucleotides leads to degradation of target mRNAs. Anti-RhoA siRNA has been reported to successfully knock down RhoA expression in cells [105,106].

## RhoA/Rho-KINASE SIGNALLING IN PENILE ERECTION

Although the contribution of RhoA/Rho-kinase signalling to vasoconstriction and vascular abnormalities in peripheral vasculature has been extensively studied, it was not until recently that RhoA/Rho-kinase was identified to operate in the penis. The functional role of RhoA/Rho-kinase was first demonstrated in rat penile corpus cavernosum [107]. ICP is significantly increased after administration of Y27632 to the rat penis *in vivo* without nerve stimulation, suggesting RhoA/Rho-kinase is constantly active and plays an important role in maintaining the penis in the flaccid state. Inhibition of Rho-kinase also potentiates electrical stimulation-induced erections; however, this effect is independent of NO because pre-injection of a NOS inhibitor, *N*-nitro-L-arginine, and guanylate cyclase inhibitors Methylene Blue or ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1), does not block Y27632-induced penile relaxation.

Subsequently, RhoA/Rho-kinase was identified in rabbit and human penile tissue by immunohistochemistry and Western blot analysis [108,109]. Furthermore, RhoA protein concentration is 17 times greater in rabbit corpus cavernosum than that in ileum, suggesting that RhoA/Rho-kinase $Ca^{2+}$ sensitization may play a significant role in corpus cavernosum smooth muscle contraction [109]. In permeabilized rabbit corpus cavernosal strips, the addition of GTP[S] increases RhoA-GTP as an active form in plasma, suggesting that RhoA is functionally available for $Ca^{2+}$ sensitization in the penis. In addition, RhoGDI, MYPT1, PP1c and CPI-17 protein expression were detected in human and rabbit corpus cavernosum [109]. The localization of CPI-17 supports the possibility of PKC/CPI-17-mediated $Ca^{2+}$ sensitization in the penis. Finally, the significance of the RhoA/Rho-kinase pathway in penile erection was confirmed by adeno-associated viral gene transfer of a dominant-negative RhoA mutant (T19NRhoA) into the rat corpus cavernosum. T19NRhoA markedly increases basal ICP as well as cavernous nerve stimulation-induced ICP *in vivo* [110].

ET-1 (endothelin-1), $\alpha$-adrenergic agonists and angiotensin II are the primary vasoconstrictors responsible for keeping the penis in the flaccid state [111,112]. Evidence has shown that Y27632 abolishes corporal smooth muscle contractions induced by these vasoconstrictors, suggesting their actions involve the activation of the

RhoA/Rho-kinase pathway in the penis [107,113–115]. In rabbit cavernosum smooth muscle strips, Y27632 has no effect on intracellular $Ca^{2+}$ concentrations, but attenuates the contraction caused by phenylephrine [116]. The RhoA/Rho-kinase pathway was also shown to mediate the synergistic vasoconstrictive effects of ET-1 and phenylephrine [114]. These results underscore that the RhoA/Rho-kinase signalling pathway plays an important role in tonic contraction of the penis.

Limited information currently exists with regard to the functional relevance of PKC- or PKC/CPI-17-mediated $Ca^{2+}$ sensitization in penile erection. One study suggests that PKC isoforms may be involved in $\alpha$-adrenergic agonist-induced contraction in rat corpus cavernosum smooth muscle strips [117]. Another study has shown that a non-selective PKC inhibitor decreases contractions in response to an $\alpha$-adrenergic agonist while it does not affect intracellular $Ca^{2+}$ concentrations [116]. The authors suggested that PKC may mediate $Ca^{2+}$ sensitization in the penis. However, further studies are required in order to determine whether PKC is important in regulation of $Ca^{2+}$ sensitivity and whether the PKC/CPI-17 pathway interacts with the RhoA/Rho-kinase pathway in the penis.

Since RhoA/Rho-kinase is continuously active to contract the corporal smooth muscle during the flaccid state, it is plausible that NO may induce penile erection through inhibition of the RhoA/Rho-kinase pathway. Recent studies have demonstrated that NO suppresses RhoA/Rho-kinase-mediated vasoconstriction in the penis both *in vivo* and *in vitro*. Injection of Y27632 potentiates the rise in ICP resulting from intracorporal injection of a NO donor, NOR-1 [118]. In isolated rat corpus cavernosal smooth muscle strips, H1152P or Y27632 has a marked synergistic inhibition with SNP, an NO donor, on both $\alpha$-adrenergic agonist and electrical field stimulation-induced contractions [119]. On the other hand, RhoA/Rho-kinase may exert regulatory control of NO signalling in the penis. In streptozotocin-induced diabetic rat penes, RhoA/Rho-kinase is up-regulated in association with suppressed eNOS activity and protein expression [120]. Inhibition of RhoA using gene transfer of T19NRhoA restores eNOS expression and activity, demonstrating the regulatory basis of RhoA/Rho-kinase signalling.

## ROLE OF RhoA/Rho-KINASE IN ED AETIOLOGICAL CONDITIONS

Given the important role of RhoA/Rho-kinase-mediated $Ca^{2+}$ sensitization in smooth muscle contraction, irregular RhoA/Rho-kinase activity may lead to abnormal contractility of smooth muscle. Abundant evidence has shown that elevated RhoA/Rho-kinase activity contributes to the pathogenesis of diseases such as diabetes

and hypertension. Studies have suggested that ED is strongly associated with these chronic diseases. Below, we examine the role of the RhoA/Rho-kinase pathway in different diseases associated with ED.

## Hypertension

ED is associated with cardiovascular diseases such as hypertension [121,122]. The MMAS (Massachusetts Male Aging Study) showed that approx. 30 % of patients with ED have a history of hypertension [2,123]. The high prevalence of ED in hypertensive patients was also suggested in a study where 27 % of the hypertensive patients had ED compared with 4 % with ED in the general population living in the same area [124]. Among those hypertensive patients with ED, 89 % was caused by penile arterial dysfunction.

Since Uehata et al. [97] first demonstrated enhanced RhoA/Rho-kinase activity contributing to the increased peripheral vascular resistance in several models of hypertension, over the years substantial evidence has suggested the importance of RhoA/Rho-kinase signalling in the pathogenesis of hypertension. Structurally based studies of penile vascular beds in SHRs (spontaneously hypertensive rats) reveal that penile vasculature undergoes similar structural changes to other vascular beds [125,126]. Therefore increased activity of the RhoA/Rho-kinase pathway observed in hypertension may also be the cause of ED in hypertensive subjects. In stroke-prone SHRs, a well-recognized genetic model of hypertension, and DOCA (deoxycorticosterone)-salt-induced hypertensive rats, ICP/MAP (mean arterial pressure) was significantly lower than that of control rats in response to electrical stimulation of the major pelvic ganglion [127]. Administration of Y27632 into the corpus cavernosum improves erectile function in these hypertensive rats, but to a lesser extent when compared with normotensive rats [127]. The authors suggested that elevated RhoA/Rho-kinase activity contributed to hypertension-associated ED. In addition, impaired erectile function was reported in SHRs in which RhoA protein expression was found to be increased in SHR penes [128]. Intracavernosal injection of Y27632 restores the erectile function in SHR; however, the effect of Y27632 was not determined in normotensive WKY (Wistar–Kyoto) rats.

## Diabetes mellitus

Diabetes mellitus is a well-recognized risk factor for ED. The risk of ED is 3-fold greater for diabetic men and occurs at an earlier age than for non-diabetic men. In the MMAS, the age-adjusted prevalence of complete ED was 28 % in diabetic patients but only 9.6 % in the general population [2]. Consistently, the MALES (Men's Attitudes to Life Events and Sexuality) study also showed the prevalence of ED was 39 % in diabetic men compared with 16 % in the overall population [123]. The underlying

mechanisms causing the increased risk of ED in diabetes are complicated and remain unclear.

The RhoA/Rho-kinase pathway may be a major molecular mechanism involved in the pathogenesis of diabetes-related ED. Increased RhoA/Rho-kinase activity is observed in penile tissue in diabetic rodents. Chang et al. [129] reported enhanced sensitivity to ET-1 but not phenylephrine in diabetic rabbit corpus cavernosum smooth muscle. The molecular studies demonstrate that the increased sensitivity to ET-1 is due to increased $ET_A$ receptor expression as well as increased Rho-kinase $\beta$ protein expression. However, there are no significant differences between ET-1-induced contractions of diabetic and non-diabetic human corpus cavernosum [130]. The authors explained the discrepancy as relating to other pre-existing aetiologies in diabetic men. Furthermore, in vivo injection of Y27632 to corpus cavernosum increases ICP to a larger extent in streptozotocin-induced diabetic rats than in control rats, suggesting increased Rho-kinase activity in diabetic rat penes [120]. This increased Y27632 sensitivity is accompanied by increased membrane-fraction RhoA protein and MYPT1 phosphorylation levels in the penile tissue of diabetic rats [120]. However, in diabetic mice, although acetylcholine-induced corpus cavernosal tissue relaxation and erectile function are significantly decreased when compared with that of non-diabetic mice, Rho-kinase inhibitors cause a similar dose–response relaxation curve, indicating that RhoA/Rho-kinase signalling may not be altered in diabetic mouse penes [131].

In diabetes, insulin-mediated smooth muscle relaxation is significantly impaired. It has been reported that insulin inactivates Rho-kinase and decreases MYPT1 phosphorylation in aortic SMCs isolated from normal WKY rats, leading to activation of MLCP [132]. The inhibition of Rho-kinase by insulin is mediated by the PI3K (phosphoinositide 3-kinase)/NO/cGMP pathway. In aortic SMCs derived from Goto–Kakizaki rats, a Type II diabetic rat model, insulin loses its ability to inhibit Rho-kinase because of severely impaired IRS-1 (insulin receptor substrate-1) tyrosine phosphorylation induced by insulin [133]. The relationship between RhoA/Rho-kinase and insulin has not been investigated in penile tissue.

## Hypogonadism

Hypogonadism refers to primary testicular failure or insufficient testicular stimulation due to the lack of pituitary gonadotropins. Androgens are important sex hormones in the regulation of male sexual function, including sexual desire and erectile function [3]. It has been reported that hypogonadism is frequently related to ED and testosterone replacement has shown beneficial effects on erectile function [134–136].

Studies in the rat model of castration have suggested that up-regulation of the RhoA/Rho-kinase pathway in the penis could be an underlying mechanism in hypogonadism-associated ED. Contractile responses to phenylephrine are increased in corpus cavernosum muscle strips isolated from castrated rats in parallel with increased sensitivity to Y27632 and increased protein expression of RhoA and Rho-kinase in penes [137]. Testosterone replacement restores erectile function and decreases RhoA and Rho-kinase protein expressions in castrated rats, although these are still significantly higher than those in intact rats. This may be explained by the multifactorial effects of testosterone, including its stimulation of the production of the pro-erectile factor NO [137]. Topical application of Y27632 to the tunica albuginea of intact rat penis or the glans and surrounding skin of castrated rat penis also causes a significant increase in ICP [138].

## Aging

ED is strongly associated with aging. In the MMAS, it was estimated that the prevalence of ED is 52 % among men aged 40–70 years [2]. Previous studies have shown that age-associated ED results from decreased sex hormone levels, decreased NOS-containing nerve endings and eNOS expression, decreased VEGF (vascular endothelial growth factor) mRNA levels and increased superoxide production, which ultimately leads to diminished NO production or bioavailability [139–142]. In contrast, vasoconstrictors such as ET-1 in aged penile tissue are increased. Gene transfer of ecSOD (extracellular superoxide dismutase) or eNOS restores erectile function in aged rats [142,143].

The role of RhoA/Rho-kinase in age-related ED has just begun to be investigated. A recent study demonstrates that Y27632 significantly improves erectile responses to electrical stimulation of the major pelvic ganglion in aged Brown–Norway rats, suggesting the beneficial effects of Rho-kinase inhibition for correcting ED in the aged rat [144]. To evaluate whether increased RhoA/Rho-kinase activity is uniquely responsible for age-associated ED, we compared the effects of inhibition of RhoA/Rho-kinase by Y27632 and gene transfer of the dominant-negative RhoA on erectile responses in young and aged Fischer 344 rats [145]. Inhibition of RhoA/Rho-kinase signalling improves erectile responses to a much larger extent in aged rats, suggesting increased RhoA/Rho-kinase activity in aged rat penile tissue. These data are supported by molecular studies showing that MYPT1 phosphorylation is markedly increased in aged rat penes when compared with young rat penes, although the protein expressions of RhoA, Rho-kinase and MYPT1 are not altered.

## RhoA/Rho-KINASE IN FEMALE SEXUAL FUNCTION

In the past few decades, the majority of basic science studies in sexual medicine have focused on male ED

with little attention given to female sexual dysfunction. Studies have shown that decreased clitoral engorgement and lack of vaginal wall relaxation contributes to sexual dysfunction in women. The Rho-kinase inhibitor Y27632 reduced phenylephrine- and angiotensin II-induced contraction of vaginal and clitoral smooth muscle isolated from rabbits in a concentration-dependent manner [146,147]. The potency of Y27632 seems greater in female genital tissue than that of males; however, it is not clear whether the difference is due to increased protein expression levels of components of the RhoA/Rho-kinase signalling pathway or a high sensitivity of RhoA/Rho-kinase in response to agonists. Inhibition of NOS does not affect the $EC_{50}$ of Y27632, indicating the relaxation effect of Rho-kinase inhibition is independent of NO in rabbit vaginal wall and clitoral corpus cavernosum [147]. These studies provide evidence that the RhoA/Rho-kinase pathway is involved in the regulation of vaginal wall and clitoris smooth muscle tone. Although no study has been done to determine the role of RhoA/Rho-kinase signalling in female sexual dysfunction, one can speculate that increased activity of RhoA/Rho-kinase may contribute to this disorder.

## THE RhoA/Rho-KINASE PATHWAY AS A THERAPEUTIC TARGET FOR ED

Although there are a variety of pharmacological treatments available for ED, it is important to discover new therapies because current treatments are not universally suitable or efficacious in many patients. Much attention has focused on the development of therapies that increase corporal smooth muscle relaxation as a means to improve erectile function. A potential new approach for the treatment of ED may be based on directly inhibiting biochemical pathways that control smooth muscle contraction. Of particular interest is the activity of the RhoA/Rho-kinase-mediated $Ca^{2+}$ sensitization pathway which is relevant to the pathogenesis of ED. Studies have demonstrated that inhibition of tonic contraction of corporal smooth muscle by intracavernosal injection or topical application of Y27632 to the penis results in increased blood flow into erectile tissue causing an erection [107,115,127,137,138,144]. However, Rho-kinase inhibitors usually have profound systemic effects, lowering mean arterial blood pressure, when they circulate into the vascular system. Thus such pharmacological therapy would be restricted to local delivery only. The primary question about the potential clinical value of Rho-kinase inhibitors is whether the compounds can be developed to target the RhoA/Rho-kinase pathway in the vasculature of the penis while minimizing unwanted effects on other vascular beds.

An alternative way to inhibit the RhoA/Rho-kinase pathway in penile tissue would be to identify and manipulate tissue specific isoforms of RhoA regulatory proteins. Increasing genital-tissue-specific RhoGDI and RhoGAP activity or suppressing specific RhoGEF activity could be effective therapies for the treatment of ED.

Specifically targeting RhoA or Rho-kinase by gene transfer offers an additional possible treatment option for ED. Success in improving erection in animal models of ED using gene transfer of T19NRhoA strongly supports this possibility clinically [110,120]. Because the penis is easily accessible by its external location, it allows local application of therapeutic vectors with reduced risk of systemic effects. Studies have shown that the placement of a tourniquet around the base of the penis when introducing virus vectors encoding dominant-negative RhoA directly into the corpora cavernosa limits the virus entering the systemic circulation, having little effect on mean arterial blood pressure. Recently, siRNA has been employed to knock down RhoA protein expression *in vitro* [148]. Although some studies have demonstrated successful delivery of siRNA *in vivo*, the stability of siRNA expression still needs to be improved. Whether this technology can be employed specifically targeting the RhoA/Rho-kinase pathway in the penis remains to be investigated. Further studies in animal models may also clarify the beneficial effects and side effects of long-term inhibition of RhoA/Rho-kinase in the penis.

## CONCLUSIONS

A growing body of evidence has demonstrated that RhoA/Rho-kinase plays a pivotal role in the modulation of corpus cavernosum smooth muscle contraction and penile erection. Overactive RhoA/Rho-kinase signalling results in ED. Although the importance of RhoA/Rho-kinase in erectile function is well documented, the majority of studies are limited in using pharmacological inhibitors to reduce RhoA/Rho-kinase activity. The upstream regulation of RhoA/Rho-kinase in the penis is largely unknown. Recent studies has shown that RhoGEFs, including p115RhoGEF, LARG and PDZ-RhoGEF, are expressed in the rat penile tissue [119,149]; however, the functional roles of these RhoGEFs in the regulation of RhoA activity have not been investigated. Although physiological studies have suggested that NO inhibits RhoA/Rho-kinase activity, the detailed mechanisms by which this regulation occurs are yet to be determined. More studies are needed to determine whether NO regulates RhoA/Rho-kinase activity at transcriptional, translational or post-translational levels. In addition, improved understanding of the upstream regulation of the RhoA/Rho-kinase pathway and the discovery of regulators of RhoA/Rho-kinase uniquely expressed in penile tissue will probably foster the development of new therapeutic avenues for the treatment of ED.

## ACKNOWLEDGMENTS

## REFERENCES

1 Ayta, I. A., Mckinlay, J. B. and Krane, R. J. (1999) The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences. BJU Int. **84**, 50–56

2 Feldman, H. A., Goldstein, I., Hatzichristou, D. G., Krane, R. J. and McKinlay, J. B. (1994) Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. J. Urol. **151**, 54–61

3 Burnett, A. L. (2003) Neurophysiology of erectile function: androgenic effects. J. Androl. **24**, S2–S5

4 Andersson, K. E. and Wagner, G. (1995) Physiology of penile erection. Physiol. Rev. **75**, 191–236

5 Burnett, A. L. (2004) Novel nitric oxide signaling mechanisms regulate the erectile response. Int. J. Impot. Res. **16** (Suppl. 1), S15–S19

6 Burnett, A. L. (1995) Role of nitric oxide in the physiology of erection. Biol. Reprod. **52**, 485–489

7 Webb, R. C. (2003) Smooth muscle contraction and relaxation. Adv. Physiol. Edu. **27**, 201–206

8 Karaki, H., Ozaki, H., Hori, M. et al. (1997) Calcium movements, distribution, and functions in smooth muscle. Pharmacol. Rev. **49**, 157–230

9 Somlyo, A. P. and Somlyo, A. V. (2002) The sarcoplasmic reticulum: then and now. Novartis Found. Symp. **246**, 258–268

10 DeFeo, T. T. and Morgan, K. G. (1985) Calcium-force relationships as detected with aequorin in two different vascular smooth muscles of the ferret. J. Physiol. **369**, 269–282

11 Nishimura, J., Kolber, M. and van Breemen, C. (1988) Norepinephrine and GTP-$\gamma$-S increase myofilament $Ca^{2+}$ sensitivity in $\alpha$-toxin permeabilized arterial smooth muscle. Biochem. Biophys. Res. Commun. **157**, 677–683

12 Somlyo, A. P. and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. Nature (London) **372**, 231–236

13 Hartshorne, D. J., Ito, M. and Erdodi, F. (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. J. Muscle Res. Cell Motil. **19**, 325–341

14 Hirata, K., Kikuchi, A., Sasaki, T. et al. (1992) Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J. Biol. Chem. **267**, 8719–8722

15 Burridge, K. and Wennerberg, K. (2004) Rho and Rac take center stage. Cell **116**, 167–179

16 Chen, Z., Sun, J., Pradines, A., Favre, G., Adnane, J. and Sebti, S. M. (2000) Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation and suppress human tumor growth in nude mice. J. Biol. Chem. **275**, 17974–17978

17 Clark, E. A., Golub, T. R., Lander, E. S. and Hynes, R. O. (2000) Genomic analysis of metastasis reveals an essential role for RhoC. Nature (London) **406**, 532–535

18 Ridley, A. J. and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70**, 389–399

19 Somlyo, A. P. and Somlyo, A. V. (2003) $Ca^{2+}$ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. **83**, 1325–1358

20 Bishop, A. L. and Hall, A. (2000) Rho GTPases and their effector proteins. Biochem. J. **348**, 241–255

21 Schwartz, M. (2004) Rho signalling at a glance. J. Cell Sci. **117**, 5457–5458

22 Narumiya, S. (1996) The small GTPase Rho: cellular functions and signal transduction. J. Biochem. (Tokyo) **120**, 215–228

23 Takai, Y., Sasaki, T., Tanaka, K. and Nakanishi, H. (1995) Rho as a regulator of the cytoskeleton. Trends Biochem. Sci. **20**, 227–231

24 Van Aelst, L. and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks. Genes Dev. **11**, 2295–2322

25 Lamarche, N. and Hall, A. (1994) GAPs for Rho-related GTPases. Trends Genet. **10**, 436–440

26 Olofsson, B. (1999) Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. Cell. Signalling **11**, 545–554

27 Sasaki, T., Kato, M. and Takai, Y. (1993) Consequences of weak interaction of Rho GDI with the GTP-bound forms of Rho p21 and Rac p21. J. Biol. Chem. **268**, 23959–23963

28 Wei, L., Imanaka-Yoshida, K., Wang, L. et al. (2002) Inhibition of Rho family GTPases by Rho GDP dissociation inhibitor disrupts cardiac morphogenesis and inhibits cardiomyocyte proliferation. Development **129**, 1705–1714

29 Gong, M. C., Gorenne, I., Read, P. et al. (2001) Regulation by GDI of RhoA/Rho-kinase-induced $Ca^{2+}$ sensitization of smooth muscle myosin II. Am. J. Physiol. Cell Physiol. **281**, C257–C269

30 Bilodeau, D., Lamy, S., Desrosiers, R. R., Gingras, D. and Beliveau, R. (1999) Regulation of Rho protein binding to membranes by rhoGDI: inhibition of releasing activity by physiological ionic conditions. Biochem. Cell Biol. **77**, 59–69

31 Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M. and Bertoglio, J. (1996) Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J. **15**, 510–519

32 Tamma, G., Klussmann, E., Procino, G., Svelto, M., Rosenthal, W. and Valenti, G. (2003) cAMP-induced AQP2 translocation is associated with RhoA inhibition through RhoA phosphorylation and interaction with RhoGDI. J. Cell Sci. **116**, 1519–1525

33 Qiao, J., Huang, F. and Lum, H. (2003) PKA inhibits RhoA activation: a protection mechanism against endothelial barrier dysfunction. Am. J. Physiol. Lung Cell Mol. Physiol. **284**, L972–L980

34 Derewenda, U., Oleksy, A., Stevenson, A. S. et al. (2004) The crystal structure of RhoA in complex with the DH/PH fragment of PDZRhoGEF, an activator of the $Ca^{2+}$ sensitization pathway in smooth muscle. Structure **12**, 1955–1965

35 Zheng, Y. (2001) Dbl family guanine nucleotide exchange factors. Trends Biochem. Sci. **26**, 724–732

36 Cerione, R. A. and Zheng, Y. (1996) The Dbl family of oncogenes. Curr. Opin. Cell Biol. **8**, 216–222

37 Blomberg, N., Baraldi, E., Nilges, M. and Saraste, M. (1999) The PH superfold: a structural scaffold for multiple functions. Trends Biochem. Sci. **24**, 441–445

38 Kristelly, R., Gao, G. and Tesmer, J. J. G. (2004) Structural determinants of RhoA binding and nucleotide exchange in leukemia-associated Rho guanine-nucleotide exchange factor. J. Biol. Chem. **279**, 47352–47362

39 Hollinger, S. and Hepler, J. R. (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol. Rev. **54**, 527–559

40 Kurose, H. (2003) $G\alpha_{12}$ and $G\alpha_{13}$ as key regulatory mediator in signal transduction. Life Sci. **74**, 155–161

41 Suzuki, N., Nakamura, S., Mano, H. and Kozasa, T. (2003) $G\alpha_{12}$ activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. Proc. Natl. Acad. Sci. U.S.A. **100**, 733–738

42 Hart, M. J., Jiang, X., Kozasa, T. et al. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. Science **280**, 2112–2114

43 Fukuhara, S., Murga, C., Zohar, M., Igishi, T. and Gutkind, J. S. (1999) A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J. Biol. Chem. **274**, 5868–5879

44  Grabocka, E. and Wedegaertner, P. B. (2005) Functional consequences of $G\alpha_{13}$ mutations that disrupt interaction with p115RhoGEF. Oncogene 24, 2155–2165

45  Nakamura, S., Kreutz, B., Tanabe, S., Suzuki, N. and Kozasa, T. (2004) Critical role of lysine 204 in switch I region of $G\alpha_{13}$ for regulation of p115RhoGEF and leukemia-associated RhoGEF. Mol. Pharmacol. 66, 1029–1034

46  Booden, M. A., Siderovski, D. P. and Der, C. J. (2002) Leukemia-associated Rho guanine nucleotide exchange factor promotes $G\alpha$q-coupled activation of RhoA. Mol. Cell. Biol. 22, 4053–4061

47  Chikumi, H., Vazquez-Prado, J., Servitja, J.-M., Miyazaki, H. and Gutkind, J. S. (2002) Potent activation of RhoA by $G\alpha_q$ and $G_q$-coupled receptors. J. Biol. Chem. 277, 27130–27134

48  Fukuhara, S., Chikumi, H. and Gutkind, J. S. (2001) RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? Oncogene 20, 1661–1668

49  Holinstat, M., Mehta, D., Kozasa, T., Minshall, R. D. and Malik, A. B. (2003) Protein kinase $C\alpha$-induced p115RhoGEF phosphorylation signals endothelial cytoskeletal rearrangement. J. Biol. Chem. 278, 28793–28798

50  Rittinger, K., Walker, P. A., Eccleston, J. F. et al. (1997) Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. Nature (London) 388, 693–697

51  Haskell, M., Nickles, A., Agati, J., Su, L., Dukes, B. and Parsons, S. (2001) Phosphorylation of p190 on Tyr1105 by c-Src is necessary but not sufficient for EGF-induced actin disassembly in C3H10T1/2 fibroblasts. J. Cell Sci. 114, 1699–1708

52  Homma, Y. and Emori, Y. (1995) A dual functional signal mediator showing RhoGAP and phospholipase C-$\delta$ stimulating activities. EMBO J. 14, 286–291

53  Taylor, J. M., Hildebrand, J. D., Mack, C. P., Cox, M. E. and Parsons, J. T. (1998) Characterization of graf, the GTPase-activating protein for rho associated with focal adhesion kinase. Phosphorylation and possible regulation by mitogen-activated protein kinase. J. Biol. Chem. 273, 8063–8070

54  Billuart, P., Winter, C. G., Maresh, A., Zhao, X. and Luo, L. (2001) Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. Cell 107, 195–207

55  Amano, M., Fukata, Y. and Kaibuchi, K. (2000) Regulation and functions of Rho-associated kinase. Exp. Cell Res. 261, 44–51

56  Chen, X.-q., Tan, I., Ng, C. H., Hall, C., Lim, L. and Leung, T. (2002) Characterization of RhoA-binding kinase ROKα: implication of the pleckstrin homology domain in ROKα function using region-specific antibodies. J. Biol. Chem. 277, 12680–12688

57  Ito, M., Nakano, T., Erdodi, F. and Hartshorne, D. J. (2004) Myosin phosphatase: structure, regulation and function. Mol. Cell. Biochem. 259, 197–209

58  Tanaka, J., Ito, M., Feng, J. et al. (1998) Interaction of myosin phosphatase target subunit 1 with the catalytic subunit of type 1 protein phosphatase. Biochemistry 37, 16697–16703

59  Somlyo, A. P. and Somlyo, A. V. (2003) $Ca^{2+}$ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 83, 1325–1358

60  Somlyo, A. P. and Somlyo, A. V. (2000) Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J. Physiol. 522, 177–185

61  Feng, J., Ito, M., Ichikawa, K. et al. (1999) Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. J. Biol. Chem. 274, 37385–37390

62  Kimura, K., Ito, M., Amano, M. et al. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273, 245–248

63  Ito, K., Shimomura, E., Iwanaga, T. et al. (2003) Essential role of rho kinase in the $Ca^{2+}$ sensitization of prostaglandin $F_{2\alpha}$-induced contraction of rabbit aortae. J. Physiol. 546, 823–836

64  Wilson, D. P., Susnjar, M., Kiss, E., Sutherland, C. and Walsh, M. P. (2005) Thromboxane $A_2$-induced contraction of rat caudal arterial smooth muscle involves activation of $Ca^{2+}$ entry and $Ca^{2+}$ sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. Biochem. J. 389, 763–774

65  Stevenson, A. S., Matthew, J. D., Eto, M., Luo, S., Somlyo, A. P. and Somlyo, A. V. (2004) Uncoupling of GPCR and RhoA-induced $Ca^{2+}$-sensitization of chicken amnion smooth muscle lacking CPI-17. FEBS Lett. 578, 73–79

66  Velasco, G., Armstrong, C., Morrice, N., Frame, S. and Cohen, P. (2002) Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. FEBS Lett. 527, 101–104

67  Araki, S., Ito, M., Kureishi, Y. et al. (2001) Arachidonic acid-induced $Ca^{2+}$ sensitization of smooth muscle contraction through activation of Rho-kinase. Pflugers Arch. 441, 596–603

68  Fu, X., Gong, M. C., Jia, T., Somlyo, A. V. and Somlyo, A. P. (1998) The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTP$\gamma$S-, and phorbol ester-induced $Ca^{2+}$-sensitization of smooth muscle. FEBS Lett. 440, 183–187

69  Chardin, P. (2003) GTPase regulation getting aRnd Rock and Rho inhibition. Curr. Biol. 13, R702–R704

70  Riento, K., Villalonga, P., Garg, R. and Ridley, A. (2005) Function and regulation of RhoE. Biochem. Soc. Trans. 33, 649–651

71  Riento, K., Guasch, R. M., Garg, R., Jin, B. and Ridley, A. J. (2003) RhoE binds to ROCK I and inhibits downstream signaling. Mol. Cell. Biol. 23, 4219–4229

72  Wennerberg, K., Forget, M.-A., Ellerbroek, S. M., Arthur, W. T., Burridge, K., Settleman, J., Der, C. J. and Hansen, S. H. (2003) Rnd proteins function as RhoA antagonists by activating p190 RhoGAP. Curr. Biol. 13, 1106–1115

73  Li, L., Eto, M., Lee, M. R., Morita, F., Yazawa, M. and Kitazawa, T. (1998) Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. J. Physiol. 508, 871–881

74  Hayashi, Y., Senba, S., Yazawa, M., Brautigan, D. L. and Eto, M. (2001) Defining the structural determinants and a potential mechanism for inhibition of myosin phosphatase by the protein kinase C-potentiated inhibitor protein of 17 kDa. J. Biol. Chem. 276, 39858–39863

75  Eto, M., Senba, S., Morita, F. and Yazawa, M. (1997) Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. FEBS Lett. 410, 356–360

76  Eto, M., Ohmori, T., Suzuki, M., Furuya, K. and Morita, F. (1995) A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. J. Biochem. (Tokyo) 118, 1104–1107

77  Matsuzawa, F., Aikawa, S.-i., Ohki, S.-y. and Eto, M. (2005) Phospho-pivot modeling predicts specific interactions of protein phosphatase-1 with a phospho-inhibitor protein CPI-17. J. Biochem. (Tokyo) 137, 633–641

78  Eto, M., Kitazawa, T., Yazawa, M., Mukai, H., Ono, Y. and Brautigan, D. L. (2001) Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase $C\alpha$ and $\delta$ isoforms. J. Biol. Chem. 276, 29072–29078

79  Koyama, M., Ito, M., Feng, J., Seko, T., Shiraki, K., Takase, K., Hartshorne, D. J. and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. FEBS Lett. 475, 197–200

80    Pang, H., Guo, Z., Su, W., Xie, Z., Eto, M. and Gong, M. C. (2005) RhoA/Rho kinase mediates thrombin- and U-46619-induced phosphorylation of a myosin phosphatase inhibitor, CPI-17, in vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. **289**, C352–C360

81    Chitaley, K. and Webb, R. C. (2002) Nitric oxide induces dilation of rat aorta via inhibition of rho-kinase signaling. Hypertension **39**, 438–442

82    Carter, R. W., Begaye, M. and Kanagy, N. L. (2002) Acute and chronic NOS inhibition enhances $\alpha$2-adrenoreceptor-stimulated RhoA and Rho kinase in rat aorta. Am. J. Physiol. Heart Circ. Physiol. **283**, H1361–H1369

83    Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C. et al. (2000) Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced $Ca^{2+}$ sensitization of contraction in vascular smooth muscle. J. Biol. Chem. **275**, 21722–21729

84    Sawada, N., Itoh, H., Yamashita, J. et al. (2001) cGMP-dependent protein kinase phosphorylates and inactivates RhoA. Biochem. Biophys. Res. Commun. **280**, 798–805

85    Sauzeau, V., Rolli-Derkinderen, M., Marionneau, C., Loirand, G. and Pacaud, P. (2003) RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. J. Biol. Chem. **278**, 9472–9480

86    Ming, X. F., Viswambharan, H., Barandier, C. et al. (2002) Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. Mol. Cell. Biol. **22**, 8467–8477

87    Mita, S., Kobayashi, N., Yoshida, K., Nakano, S. and Matsuoka, H. (2005) Cardioprotective mechanisms of Rho-kinase inhibition associated with eNOS and oxidative stress-LOX-1 pathway in Dahl salt-sensitive hypertensive rats. J. Hypertens. **23**, 87–96

88    Genth, H., Gerhard, R., Maeda, A. et al. (2003) Entrapment of Rho ADP-ribosylated by clostridium botulinum C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex. J. Biol. Chem. **278**, 28523–28527

89    Sehr, P., Joseph, G., Genth, H., Just, I., Pick, E. and Aktories, K. (1998) Glucosylation and ADP ribosylation of rho proteins: effects on nucleotide binding, GTPase activity, and effector coupling. Biochemistry **37**, 5296–5304

90    Genth, H., Schmidt, M., Gerhard, R., Aktories, K. and Just, I. (2003) Activation of phospholipase D1 by ADP-ribosylated RhoA. Biochem. Biophys. Res. Commun. **302**, 127–132

91    Fujihara, H., Walker, L. A., Gong, M. C. et al. (1997) Inhibition of RhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. Mol. Biol. Cell **8**, 2437–2447

92    Aktories, K., Schmidt, G. and Just, I. (2000) Rho GTPases as targets of bacterial protein toxins. Biol. Chem. **381**, 421–426

93    Boquet, P. (1999) Bacterial toxins inhibiting or activating small GTP-binding proteins. Ann. NY Acad. Sci. **886**, 83–90

94    Aktories, K. and Schmidt, G. (2003) A new turn in Rho GTPase activation by *Escherichia coli* cytotoxic necrotizing factors. Trends Microbiol. **11**, 152–155

95    Hoffmann, C., Pop, M., Leemhuis, J., Schirmer, J., Aktories, K. and Schmidt, G. (2004) The Yersinia pseudotuberculosis cytotoxic necrotizing factor (CNFY) selectively activates RhoA. J. Biol. Chem. **279**, 16026–16032

96    Ishizaki, T., Uehata, M., Tamechika, I. et al. (2000) Pharmacological properties of Y-27632, a specific inhibitor of Rho-associated kinases. Mol. Pharmacol. **57**, 976–983

97    Uehata, M., Ishizaki, T., Satoh, H. et al. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature (London) **389**, 990–994

98    Ito, S., Kume, H., Honjo, H. et al. (2001) Possible involvement of Rho kinase in $Ca^{2+}$ sensitization and mobilization by MCh in tracheal smooth muscle. Am. J. Physiol. Lung Cell Mol. Physiol. **280**, L1218–L1224

99    Sakamoto, K., Hori, M., Izumi, M. et al. (2003) Inhibition of high K+-induced contraction by the ROCKs inhibitor Y-27632 in vascular smooth muscle: possible involvement of ROCKs in a signal transduction pathway. J. Pharmacol. Sci. **92**, 56–69

100    Nagumo, H., Sasaki, Y., Ono, Y., Okamoto, H., Seto, M. and Takuwa, Y. (2000) Rho kinase inhibitor HA-1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells. Am. J. Physiol. Cell Physiol. **278**, C57–C65

101    Sasaki, Y., Suzuki, M. and Hidaka, H. (2002) The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. Pharmacol. Ther. **93**, 225–232

102    Feig, L. A. (1999) Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. Nat. Cell Biol. **1**, E25–E27

103    Pawlak, G. and Helfman, D. M. (2002) Post-transcriptional down-regulation of ROCKI/Rho-kinase through an MEK-dependent pathway leads to cytoskeleton disruption in Ras-transformed fibroblasts. Mol. Biol. Cell **13**, 336–347

104    Morishige, K., Shimokawa, H., Eto, Y. et al. (2001) Adenovirus-mediated transfer of dominant-negative Rho-kinase induces a regression of coronary arteriosclerosis in pigs *in vivo*. Arterioscler., Thromb., Vasc. Biol. **21**, 548–554

105    Pille, J.-Y., Denoyelle, C., Varet, J. et al. (2005) Anti-RhoA and Anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells *in vitro* and *in vivo*. Mol. Ther. **11**, 267–274

106    Deroanne, C., Vouret-Craviari, V., Wang, B. and Pouyssegur, J. (2003) EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. J. Cell Sci. **116**, 1367–1376

107    Chitaley, K., Wingard, C. J., Webb, R. C. et al. (2001) Antagonism of Rho-kinase stimulates rat penile erection via a nitric oxide-independent pathway. Nat. Med. **7**, 119–122

108    Rees, R. W., Ziessen, T., Ralph, D. J., Kell, P., Moncada, S. and Cellek, S. (2002) Human and rabbit cavernosal smooth muscle cells express Rho-kinase. Int. J. Impot. Res. **14**, 1–7

109    Wang, H., Eto, M., Steers, W. D., Somlyo, A. P. and Somlyo, A. V. (2002) RhoA-mediated $Ca^{2+}$ sensitization in erectile function. J. Biol. Chem. **277**, 30614–30621

110    Chitaley, K., Bivalacqua, T. J., Champion, H. C. et al. (2002) Adeno-associated viral gene transfer of dominant negative RhoA enhances erectile function in rats. Biochem. Biophys. Res. Commun. **298**, 427–432

111    Andersson, K.-E. (2001) Pharmacology of penile erection. Pharmacol. Rev. **53**, 417–450

112    Jin, L., Linder, A. E., Mills, T. M. and Webb, R. C. (2003) Inhibition of the tonic contraction in the treatment of erectile dysfunction. Expert Opin. Ther. Targets **7**, 265–276

113    Rees, R. W., Ralph, D. J., Royle, M., Moncada, S. and Cellek, S. (2001) Y-27632, an inhibitor of Rho-kinase, antagonizes noradrenergic contractions in the rabbit and human penile corpus cavernosum. Br. J. Pharmacol. **133**, 455–458

114    Wingard, C. J., Husain, S., Williams, J. and James, S. (2003) RhoA-Rho kinase mediates synergistic ET-1 and phenylephrine contraction of rat corpus cavernosum. Am. J. Physiol. Regulatory Integrative Comp. Physiol. **285**, R1145–R1152

115    Mills, T. M., Chitaley, K., Wingard, C. J., Lewis, R. W. and Webb, R. C. (2001) Effect of Rho-kinase inhibition on vasoconstriction in the penile circulation. J. Appl. Physiol. **91**, 1269–1273

116 Takahashi, R., Nishimura, J., Hirano, K., Naito, S. and Kanaide, H. (2003) Modulation of $Ca^{2+}$ sensitivity regulates contractility of rabbit corpus cavernosum smooth muscle. J. Urol. **169**, 2412–2416

117 Husain, S., Young, D. and Wingard, C. J. (2004) Role of $PKC\alpha$ and $PKC\iota$ in phenylephrine-induced contraction of rat corpora cavernosa. Int. J. Impot. Res. **16**, 325–333

118 Mills, T. M., Chitaley, K., Lewis, R. W. and Webb, R. C. (2002) Nitric oxide inhibits RhoA/Rho-kinase signaling to cause penile erection. Eur. J. Pharmacol. **439**, 173–174

119 Teixeira, C. E., Ying, Z. and Webb, R. C. (2005) Pro-erectile effects of the Rho-kinase inhibitor (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl] homopiperazine (H-1152) in the rat penis. J. Pharmacol. Exp. Ther. **315**, 155–162

120 Bivalacqua, T. J., Champion, H. C., Usta, M. F. et al. (2004) RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: a mechanism for diabetes-associated erectile dysfunction. Proc. Natl. Acad. Sci. U.S.A. **101**, 9121–9126

121 Muller, S. C., el-Damanhoury, H., Ruth, J. and Lue, T. F. (1991) Hypertension and impotence. Eur. Urol. **19**, 29–34

122 Burchardt, M., Burchardt, T., Baer, L. et al. (2000) Hypertension is associated with severe erectile dysfunction. J. Urol. **164**, 1188–1191

123 Rosen, R. C., Fisher, W. A., Eardley, I., Niederberger, C., Nadel, A. and Sand, M. (2004) The multinational Men's Attitudes to Life Events and Sexuality (MALES) study: I. Prevalence of erectile dysfunction and related health concerns in the general population. Curr. Med. Res. Opin. **20**, 607–617

124 Jensen, J., Lendorf, A., Stimpel, H., Frost, J., Ibsen, H. and Rosenkilde, P. (1999) The prevalence and etiology of impotence in 101 male hypertensive outpatients. Am. J. Hypertens. **12**, 271–275

125 Toblli, J. E., Stella, I., Inserra, F., Ferder, L., Zeller, F. and Mazza, O. N. (2000) Morphological changes in cavernous tissue in spontaneously hypertensive rats. Am. J. Hypertens. **13**, 686–692

126 Okabe, H., Hale, T. M., Kumon, H., Heaton, J. P. and Adams, M. A. (1999) The penis is not protected: in hypertension there are vascular changes in the penis which are similar to those in other vascular beds. Int. J. Impot. Res. **11**, 133–140

127 Chitaley, K., Webb, R. C., Dorrance, A. M. and Mills, T. M. (2001) Decreased penile erection in DOCA-salt and stroke prone-spontaneously hypertensive rats. Int. J. Impot. Res. **13** (Suppl. 5), S16–S20

128 Wilkes, N., White, S., Stein, P., Bernie, J. and Rajasekaran, M. (2004) Phosphodiesterase-5 inhibition synergizes rho-kinase antagonism and enhances erectile response in male hypertensive rats. Int. J. Impot. Res. **16**, 187–194

129 Chang, S., Hypolite, J. A., Changolkar, A., Wein, A. J., Chacko, S. and DiSanto, M. E. (2003) Increased contractility of diabetic rabbit corpora smooth muscle in response to endothelin is mediated via Rho-kinase $\beta$. Int. J. Impot. Res. **15**, 53–62

130 Christ, G. J., Lerner, S. E., Kim, D. C. and Melman, A. (1995) Endothelin-1 as a putative modulator of erectile dysfunction: I. Characteristics of contraction of isolated corporal tissue strips. J. Urol. **153**, 1998–2003

131 Buyukafsar, K. and Un, I. (2003) Effects of the Rho-kinase inhibitors, Y-27632 and fasudil, on the corpus cavernosum from diabetic mice. Eur. J. Pharmacol. **472**, 235–238

132 Begum, N., Duddy, N., Sandu, O., Reinzie, J. and Ragolia, L. (2000) Regulation of myosin-bound protein phosphatase by insulin in vascular smooth muscle cells: evaluation of the role of Rho kinase and phosphatidylinositol-3-kinase-dependent signaling pathways. Mol. Endocrinol. **14**, 1365–1376

133 Sandu, O., Ragolia, L. and Begum, N. (2000) Diabetes in the Goto-Kakizaki rat is accompanied by impaired insulin-mediated myosin-bound phosphatase activation and vascular smooth muscle cell relaxation. Diabetes **49**, 2178–2189

134 Shabsigh, R. (2004) Testosterone therapy in erectile dysfunction. Aging Male **7**, 312–318

135 Albrecht-Betancourt, M., Hijazi, R. A. and Cunningham, G. R. (2004) Androgen replacement in men with hypogonadism and erectile dysfunction. Endocrine **23**, 143–148

136 Mills, T. M., Dai, Y., Stopper, V. S. and Lewis, R. W. (1999) Androgenic maintenance of the erectile response in the rat. Steroids **64**, 605–609

137 Wingard, C. J., Johnson, J. A., Holmes, A. and Prikosh, A. (2003) Improved erectile function after Rho-kinase inhibition in a rat castrate model of erectile dysfunction. Am. J. Physiol. Regulatory Integrative Comp. Physiol. **284**, R1572–R1579

138 Dai, Y., Chitaley, K., Webb, R. C., Lewis, R. W. and Mills, T. M. (2004) Topical application of a Rho-kinase inhibitor in rats causes penile erection. Int. J. Impot. Res. **16**, 294–298

139 Garban, H., Marquez, D., Cai, L., Rajfer, J. and Gonzalez-Cadavid, N. (1995) Restoration of normal adult penile erectile response in aged rats by long-term treatment with androgens. Biol. Reprod. **53**, 1365–1372

140 Carrier, S., Nagaraju, P., Morgan, D. M., Baba, K., Nunes, L. and Lue, T. F. (1997) Age decreases nitric oxide synthase-containing nerve fibers in the rat penis. J. Urol. **157**, 1088–1092

141 Rajasekaran, M., Kasyan, A., Jain, A., Kim, S.-W. and Monga, M. (2002) Altered growth factor expression in the aging penis: the Brown-Norway rat model. J. Androl. **23**, 393–399

142 Bivalacqua, T. J., Armstrong, J. S., Biggerstaff, J. et al. (2003) Gene transfer of extracellular SOD to the penis reduces $O_2^{\bullet-}$ and improves erectile function in aged rats. Am. J. Physiol. Heart Circ. Physiol. **284**, H1408–H1421

143 Champion, H. C., Bivalacqua, T. J., Hyman, A. L., Ignarro, L. J., Hellstrom, W. J. G. and Kadowitz, P. J. (1999) Gene transfer of endothelial nitric oxide synthase to the penis augments erectile responses in the aged rat. Proc. Natl. Acad. Sci. U.S.A. **96**, 11648–11652

144 Rajasekaran, M., White, S., Baquir, A. and Wilkes, N. (2005) Rho-kinase inhibition improves erectile function in aging male Brown-Norway rats. J. Androl. **26**, 182–188

145 Jin, L., Liu, T., Lagoda, G. A., Champion, H. C., Bivalacqua, T. J. and Burnett, A. L. (2005) Elevated RhoA/Rho-kinase activity in the aged rat penis: mechanism for age-associated erectile dysfunction. FASEB J., in the press

146 Cellek, S. (2003) The Rho-kinase inhibitor Y-27632 and the soluble guanylyl cyclase activator BAY41-B2272 relax rabbit vaginal wall and clitoral corpus cavernosum. Br. J. Pharmacol. **138**, 287–290

147 Park, J. K., Lee, S. O., Kim, Y. G., Kim, S. H., Koh, G. Y. and Cho, K. W. (2002) Role of rho-kinase activity in angiotensin II-induced contraction of rabbit clitoral cavernosum smooth muscle. Int. J. Impot. Res. **14**, 472–477

148 Bi, D., Nishimura, J., Niiro, N., Hirano, K. and Kanaide, H. (2005) Contractile properties of the cultured vascular smooth muscle cells: the crucial role played by RhoA in the regulation of contractility. Circ. Res. **96**, 890–897

149 Teixeira, C. E., Jin, L., Ying, Z., Palmer, T. and Webb, R. C. (2005) $Ca^{2+}$ sensitization and the regulation of contractility in rat anococcygeus and retractor penis muscle. Biochem. Pharmacol. **69**, 1483–1492