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Regulation of smooth muscle contraction by monomeric non-RhoA GTPases

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Smooth muscle contraction in the cardiovascular system, airways, prostate and lower urinary tract is involved in the pathophysiology of many diseases, including cardiovascular and obstructive lung disease plus lower urinary tract symptoms, which are associated with high prevalence of morbidity and mortality. This prominent clinical role of smooth muscle tone has led to the molecular mechanisms involved being subjected to extensive research. In general smooth muscle contraction is promoted by three major signalling pathways, including the monomeric GTPase RhoA pathway. However, emerging evidence suggests that monomeric GTPases other than RhoA may be involved in signal transduction in smooth muscle contraction, including Rac GTPases, cell division control protein 42 homologue, adenosine ribosylation factor 6, Ras, Rap1b and Rab GTPases. Here, we review these emerging functions of non-RhoA GTPases in smooth muscle contraction, which has now become increasingly more evident and constitutes an emerging and innovative research area of high clinical relevance.

1 | INTRODUCTION

Smooth muscle contraction in the cardiovascular system, airways, lower urinary tract and prostate is primarily involved in the pathophysiology of many diseases, which have a high mortality and are also causing high individual and socio-economic burden, such as in cardiovascular and obstructive lung diseases plus lower urinary tract symptoms. Accordingly, inhibition of smooth muscle contraction is an important strategy in medical treatment of these diseases, which together affecting billions of patients worldwide. In line with the prominent role of smooth muscle tone in health, disease and therapy

the molecular mechanisms of contraction and relaxation were and are the subject of continuous research.

Smooth muscle contraction is induced by receptors, by mechanical and other stimuli, which subsequently activate intracellular, contraction-mediating signalling pathways. These pro-contractile signalling pathways all end in a few final mechanisms, which are absolute prerequisites for smooth muscle contraction, including myosin light chain (MLC) phosphorylation, actin polymerization, organization and attachment of filaments to membranes and anchoring of cells to the extracellular matrix (Kim, Appel, Vetterkind, Gangopadhyay, & Morgan, 2008; Puetz, Lubomirov, & Pfitzer, 2009). Intracellular signalling pathways resulting in contraction include calcium-dependent mechanisms that parallel increases of calcium sensitivity, which was realized early during the exploration of smooth muscle contraction. The role of the small monomeric GTPase RhoA for signal transduction promoting vascular smooth muscle contraction by immediate increases of calcium sensitivity has been recognized since 1992

(Hirata et al., 1992). In 1997, Rho kinase was identified as an important mediator of smooth muscle contraction, which increases calcium sensitivity independently of PKC (Uehata et al., 1997). These discoveries were followed by numerous studies describing activation of Rho kinase by the small monomeric GTPase, RhoA, and on how this pathway results in smooth muscle contraction in different organs (Chiba, Matsusue, & Misawa, 2010; Christ & Andersson, 2007; Hennenberg, Trebicka, Sauerbruch, & Heller, 2008; Loirand & Pacaud, 2014; McMurtry, Abe, Ota, Fagan, & Oka, 2010; Rattan, Phillips, & Maxwell, 2010). Thus, receptor-dependent smooth muscle contraction is assumed to be mediated by activation of basically three intracellular signalling pathways, which are shared by smooth muscle in any smooth muscle-rich tissue (Somlyo & Somlyo, 2000, 2003):- (i) a calcium-dependent pathway activated by inositol-1,4,5-triphosphate (IP$_3$) resulting in receptor-dependent activation of phosphoinositide-specific PLC, (ii) increase of calcium sensitivity mediated by activation of PKC by DAG resulting from PLC activation and (iii) increase of calcium sensitivity mediated by receptor-dependent activation of RhoA/Rho kinase (Somlyo & Somlyo, 2000, 2003). Finally, these mechanisms promote contraction by increasing MLC phosphorylation, resulting from activation of MLC kinase (MLCK) by calcium-dependent pathways and from inactivation of MLC phosphatase by PKC- and Rho kinase-dependent pathways (Somlyo & Somlyo, 2000, 2003).

Apart from RhoA, the superfamily of small monomeric GTPases includes a panel of more than 100 further members, belonging to at least five different families (Takai, Sasaki, & Matozaki, 2001). Emerging evidence suggests that monomeric GTPases other than RhoA (herein referred to as "non-RhoA GTPases") may be involved in signal transduction in smooth muscle contraction as well, including Rac GTPases, the cell division control protein 42 homologue (Cdc42) and GTPases from the Ras, Rap and adenosine ribosylation factor (ARF) families. Here, we review the emerging function of these non-RhoA GTPases for smooth muscle contraction. As the role of RhoA for smooth muscle contraction has been already intensively described elsewhere, we will focus here on non-RhoA GTPases and only outline the already previously established mechanisms of smooth muscle contraction. For some organs, including urethral or vascular smooth muscle in the corpus cavernosum, a role of RhoA has been proven for smooth muscle contraction, but to the best of our knowledge not yet for other monomeric GTPases (Malmqvist, Hedlund, Sward, & Andersson, 2004; Teixeira, Jin, Priviero, Ying, & Webb, 2007; H. Wang, Eto, Steers, Somlyo, & Somlyo, 2002). Certainly, this does not reflect lack of functions of non-RhoA GTPases in smooth muscle contraction of these tissues. Rather, missing evidence is probably attributed lack of investigations, as these tissues are in fact relatively neglected in basic research.

2 | ESTABLISHED MECHANISMS OF SMOOTH MUSCLE CONTRACTION

In general, smooth muscle contraction depends essentially on several prerequisites, which are MLC phosphorylation, actin assembly to filaments, filament organization of and attachment to cell membranes,

and anchoring of cells in tissues and extracellular matrix (Kim et al., 2008; Puetz et al., 2009). These processes are endpoints of signalling pathways, which promote smooth muscle contractions and which are activated by receptors or receptor-independent stimuli (Puetz et al., 2009). Thus, these signalling pathways transduce extracellular signals induced by hormonal, neurogenic, autocrine, humoral, mechanical or other stimuli to the basic contraction machinery of smooth muscle cells.

Agonist-induced smooth muscle contraction starts with activation of GPCRs (Somlyo & Somlyo, 2000, 2003). Receptor activation by corresponding ligands causes conformational changes of the receptor, resulting in dissociation of receptor-associated heterotrimeric G proteins from receptors and of the Gα subunits from Gβ and Gγ subunits. Subsequently, Gα subunits (Gα$_{q/11}$, Gα$_{12}$ and/or Gα$_{13}$) of these G proteins activate PLC and RhoA. PLC hydrolyses phosphatidylinositol-4,5-bisphosphate, resulting in the formation of the second messengers IP$_3$ and DAG. IP$_3$ opens Ca^{2+} channels in the sarcoplasmic reticulum, leading to depolarization of the membrane potential by elevation of the otherwise low cytosolic Ca^{2+} concentration. Consequently, voltage-gated calcium channels in the cell membrane are opened, leading to a massive (capacitative) Ca^{2+} current from the extracellular space into the cell, according to the concentration gradient of Ca^{2+}. This causes activation of Ca^{2+}-dependent calmodulin and of calmodulin-dependent MLCK, finally resulting in contraction by enhanced MLC phosphorylation. This is paralleled by increases of calcium sensitivity, being accomplished by two mechanisms, which both increase MLC phosphorylation by inhibition of MLC phosphatase (MLCP). First, DAG activates PKC, which activates MLCP by direct phosphorylation of its myosin targeting subunit (MYPT1) or indirectly by phosphorylation-mediated inactivation of the MLCP inhibiting factor, CPI-17. Secondly, Gα subunits activate RhoA, which then activates Rho kinase, followed by phosphorylation-mediated inactivation of MLCP.

Activation and deactivation of monomeric GTPases underlie universal principles, although little is known about the precise players involved in the context of smooth muscle tone regulation (Cherfils & Zeghouf, 2013). Activation of monomeric GTPases is achieved by guanine nucleotide exchange factors (GEFs; Cherfils & Zeghouf, 2013; Puetz et al., 2009). It has been assumed that Gα$_{q/11}$, Gα$_{12}$ and Gα$_{13}$ subunits associated to contractile smooth muscle receptors activate RhoA by activation of guanine nucleotide exchange factors. However, the identity of these guanine nucleotide exchange factors still remains uncertain in the context of smooth muscle contraction (Puetz et al., 2009). Possible candidates may be LARG, PDZ-RhoGEF, p115 RhoGEF, Vsm-RhoGEF and SmgGDS (Puetz et al., 2009). These guanine nucleotide exchange factors occur in smooth muscle, whereas more than 70 Rho-activating guanine nucleotide exchange factors may exist in total in different cell types (Puetz et al., 2009). Guanine nucleotide exchange factors promote the exchange of guanosine diphosphate (GDP) bound to GTPases in their inactive state to GTP, what is a major step in GTPase activation (Cherfils & Zeghouf, 2013; Puetz et al., 2009). In addition, the activation step is associated with a translocation of the inactive GTPases being located to cytosolic

regions to the cell membrane (Puetz et al., 2009). This is accomplished by release of guanine nucleotide dissociation inhibitors from the inactive GTPase (Cherfils & Zeghouf, 2013; Puetz et al., 2009). GTPase-bound guanine nucleotide dissociation inhibitors keep the GTPase in cytosolic membranes by binding to the hydrophobic membrane anchors, so that their separation from the GTPase following activation by guanine nucleotide exchange factors permits the translocation to the membrane (Puetz et al., 2009). Finally, the GTPase will start to hydrolyze the bound GTP to GDP following its activation, resulting in its own inactivation (Puetz et al., 2009). GTP hydrolyzation and therefore inactivation of monomeric GTPases are accelerated by GTPase activating proteins (Cherfils & Zeghouf, 2013; Puetz et al., 2009).

3 | RAC GTPASES IN SMOOTH MUSCLE CONTRACTION

Of all non-RhoA GTPases taking part in regulation of smooth muscle contraction, Rac GTPases are the best characterized. Rac occurs in three different isoforms referred to as Rac1–3 (Takai et al., 2001). Rac GTPases regulate not only important cellular functions including cell cycle and growth in malignant and non-malignant cells but also cytoskeletal functions depending on correct actin assembly, such as lamellipodia formation, motility and migration and including smooth muscle cells (Marinkovic, Heemskerk, van Buul, & de Waard, 2015). Evidence supporting a pro-contractile role of Rac has been obtained, (i) for vascular, airway, lower urinary tract, prostate and gastrointestinal smooth muscle; (ii) for different species; (iii) using pharmacological and knockout approaches and (iv) using *in vitro* and *in vivo* models (Table 1). In addition to a contraction-mediating role of Rac, an anti-contractile role of Rac has been proposed for airway and vascular smooth muscle (Table 1).

Many findings suggesting a role of Rac1 in smooth muscle contraction were obtained using small molecule inhibitors with presumed specificity for Rac GTPases. Thus, increasing interest of Rac1 not only in oncological context but also in vascular diseases resulted in the development of Rac inhibitors. The first inhibitor with assumed specificity for Rac GTPases was NSC23766, which inhibits Rac GTPases by inhibition of Rac-specific guanine nucleotide exchange factors (Akbar, Cancelas, Williams, Zheng, & Zheng, 2006; Gao, Dickerson, Guo,

Zheng, & Zheng, 2004). Its selectivity may be higher for Rac1 than for Rac2 or Rac3, but it does not inhibit RhoA or Cdc42 (Akbar et al., 2006; Gao et al., 2004). Later on, it turned out that the specificity of NSC23766 is in fact limited, as it acts as a competitive antagonist at **muscarinic ACh receptors** (Levay et al., 2013). With EHT1864 and EHop-016, two other small molecule inhibitors with assumed Rac specificity were developed (Montalvo-Ortiz et al., 2012; Onesto, Shutes, Picard, Schweighoffer, & Der, 2008; Shutes et al., 2007). EHT1864 binds directly to Rac proteins and is unselective for all three isoforms (Onesto, Shutes, Picard, Schweighoffer, & Der, 2008; Shutes et al., 2007). Ehop-016 was developed on the basis of NSC23766 and consequently acts by inhibition of Rac guanine nucleotide exchange factors and preferentially inhibits Rac1 and Rac3 and also Cdc42 at higher concentrations (Montalvo-Ortiz et al., 2012). Rac inhibition by NSC23766 and EHT1864 is attained by different mechanisms, so that NSC23766 may inhibit Rac GTPases indirectly by prevention of Rac interaction with Rac-activating guanine nucleotide exchange factors, whereas EHT1864 may inhibit Rac directly (Akbar et al., 2006; Gao et al., 2004; Shutes et al., 2007). In addition to muscarinic receptor antagonism of NSC23766, Rac-independent effects of NSC23766 and EHT1864 were proposed, as both compounds showed effects in Rac1-deficient platelets if 100 μM were applied (Dütting et al., 2015). Thus, at least at higher concentrations, Rac1-independent effects may be expected by both of these compounds.

Rac-dependent actin organization and its involvement in actin-dependent functions have been described already before first reports suggested regulation of smooth muscle tone by Rac. Formation and organization of actin filaments by Rac were observed in vascular smooth muscle cells, so that the following discovery of Rac-dependent smooth muscle tone regulation may not be completely surprising (Bond, Wu, Sala-Newby, & Newby, 2008). The first study reporting the role of Rac for smooth muscle tone suggested Rac-mediated relaxation of airway smooth muscle (Roscioni et al., 2011). Findings of that study suggested that muscarinic receptors deactivate Rac during cholinergic airway smooth muscle contraction and that relaxation of airway smooth muscle by the cAMP effector exchange protein directly activated by cAMP (**Epac**) occurs by promotion of Rac activity (Figure 1; Roscioni et al., 2011).

These initial findings are opposed by later studies, suggesting that Rac promotes airway smooth muscle contraction rather than

TABLE 1 Summarized evidence for regulation of smooth muscle contractility by Rac GTPases

	Rac1			
	Role	Mechanism	Evidence from	Species
Vascular	Pro- and anti-contractile	PLC/Ca^{2+}, possibly others	Inhibitors, knockout	Mouse, rat
Airway	Pro- and anti-contractile	Pro-contractile: Ca^{2+} sensitization; anti-contractile: Epac	Inhibitors, knockout	Human, mouse, rat
Prostate	Pro-contractile	not MLC, possibly actin	Inhibitors	Human
Urinary bladder	Pro-contractile	—	Inhibitors, knockout	Human, mouse, rat
Gastrointestinal	Pro-contractile	—	Inhibitors, knockout	Mouse

Note. Available findings have been categorized to role, assumed mechanisms of Rac-dependent regulation, basis of findings and species.

counteracting it. Thus, EHT1864 and NSC23766 inhibited cholinergic contractions of isolated airway preparations from humans, mice and rats (Andre-Gregoire et al., 2017; Sakai, Kai, Sato, Ikebe, & Chiba, 2018). The inhibitory effect was not restricted to cholinergic

contractions, EHT1864 also inhibited 5-hydroxytryptamine-induced contractions of airway preparations from mice, and EHT1864 and NSC23766 inhibited endothelin-1-induced contractions of airway tissues from rats (Andre-Gregoire et al., 2017; Sakai et al., 2018). The
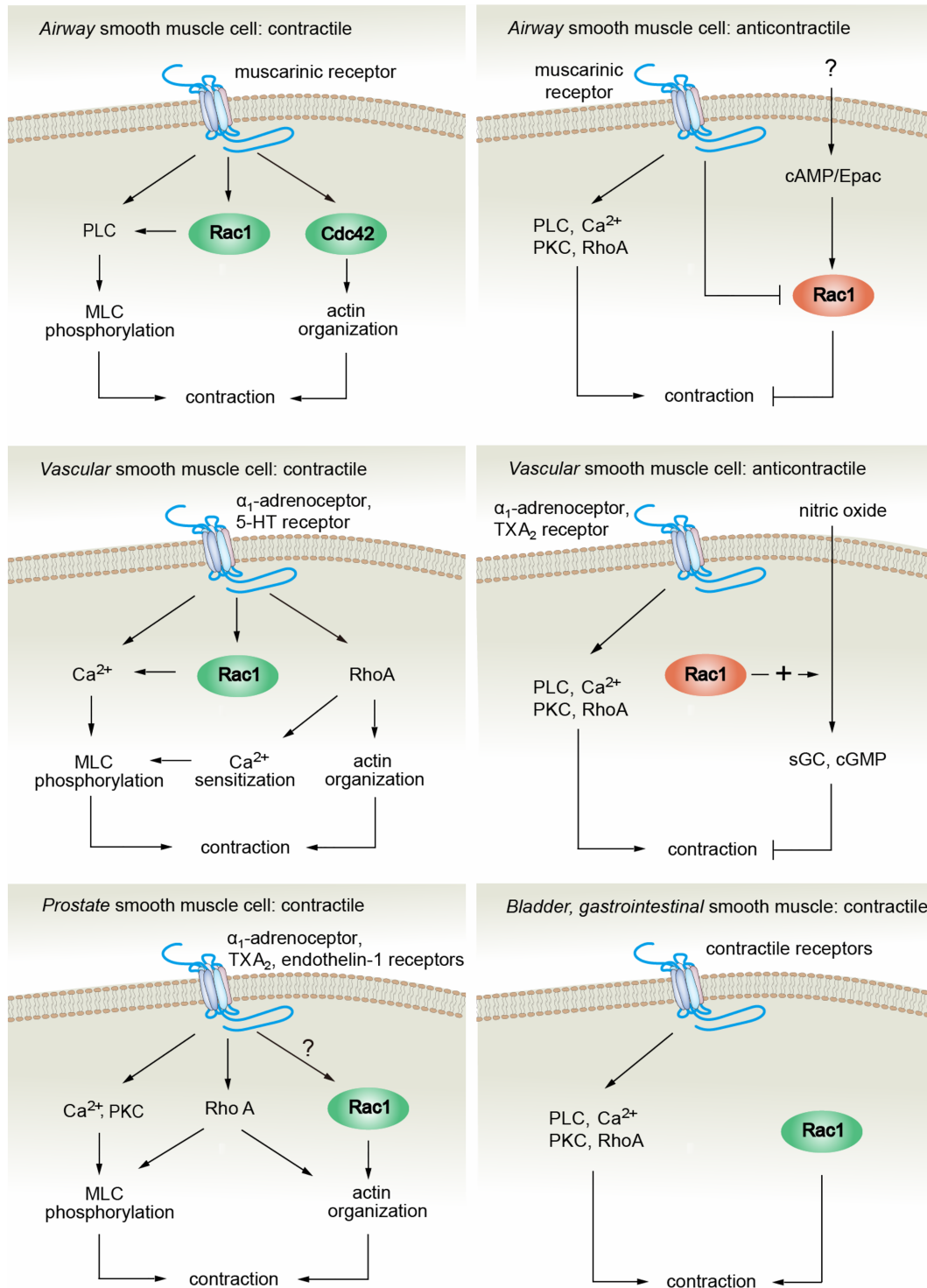

FIGURE 1 Proposed functions of Rac1 and Cdc42 in regulation of smooth muscle contraction. Rac1-mediated promotion of smooth muscle contraction has been suggested for airway, vascular, prostate, bladder, and gastrointestinal smooth muscle. Rac1-dependent inhibition of smooth muscle contraction has been suggested for airway and vascular smooth muscle. Illustration of proposed involved mechanisms has been simplified to the lowest common denominator if different studies revealed different results. GTPases with a pro-contractile role are marked by green colour, and GTPases with an anti-contractile function by red colour. Ca^{2+}, calcium; sGC, soluble guanylate cyclase

contraction-promoting role of Rac1 was confirmed by a knockout model, **methacholine**-induced contractions in airway preparations in mice with smooth muscle-specific knockout of Rac1 was reduced compared with that in wild types (Andre-Gregoire et al., 2017). Considering the possible limitations of available small molecule inhibitors outlined above, these findings using a knockout model may be of particular importance and point to the role of Rac1 as an isoform being involved in Rac-mediated airways smooth muscle contraction.

In addition to contractility studies in vitro, the role of Rac for airway smooth muscle contraction was confirmed *in vivo*. Although mice with smooth muscle-specific Rac1 deletion showed normal airway function under basal conditions, pulmonary resistance raised by aerosolized methacholine was lower in knock out mice compared with that in wild type animals (Andre-Gregoire et al., 2017). In wild type mice, but not in knockouts, the methacholine-induced airway response was decreased by inhalation of NSC23766 (Andre-Gregoire et al., 2017). Importantly, the efficacy of NSC23766 was similar to that of the β_2-adrenoceptor agonist **salbutamol**, the gold standard in experimental and clinical asthma treatment (Andre-Gregoire et al., 2017).

Similar to airway smooth muscle, evidence for the role of Rac1 in promotion of vascular smooth muscle is based on studies using inhibitors and knockout models *in vitro* and *in vivo*. In contractility studies performed with rat aortic rings, NSC23766 inhibited contractions induced by the **α_1-adrenoceptor** agonist **phenylephrine** and contractions induced by 5-hydroxytryptamine (Shibata et al., 2015). Again, in contractility assays, NSC23766 and EHT1864 inhibited contractions of mouse aorta, saphenous artery and mesenteric artery induced by phenylephrine (Rahman et al., 2014). Similarly, NSC23766 inhibited α_1-adrenoceptor mediated contractions induced by **noradrenaline** in rat resistance vessels of the cremaster, assessed by confocal microscopy (Staiculescu et al., 2013). The role of Rac1 in contraction of α_1- adrenoceptor vasocontraction was confirmed by inhibition of phenylephrine-induced contractions in contractility studies using aorta, saphenous artery and mesenteric artery from mice with smooth muscle-specific Rac1 knockout and after insertion of a dominant negative form of Rac1 into permeabilized rabbit femoral arteries (Rahman et al., 2014; Shibata et al., 2015).

Apart from a principal role of Rac GTPases for vascular smooth muscle contraction in non-diseased conditions, a role in vascular hypercontractility of stroke-prone spontaneously hypertensive rats has been suggested. Thus, whereas EHT1864 did not change phenylephrine contractions of mesenteric arteries from normotensive control rats, these were inhibited in vessels from hypertensive rats (Harvey et al., 2017).

In addition to airway smooth muscle, diverging results were reported concerning the role of Rac for vascular smooth muscle contraction. Whereas the studies summarized above suggest promotion of vascular smooth muscle by Rac1, another study reported increases of contractile responses to phenylephrine and the **TXA$_2$** analogue **U46619** in isolated mesenteric arteries from mice with inducible smooth muscle-specific Rac1 knockout (André et al., 2014). This was confirmed *in vivo*, as these mice developed hypertension, which was due to increased vascular resistance but not due to changes in diastolic BP or heart rate (André et al., 2014). Deeper analysis supported the idea that these effects were mediated by disruption of **nitric oxide** (NO)-induced cGMP production in vascular smooth muscle cells, which usually induces smooth muscle relaxation and may obviously be Rac-dependent (André et al., 2014).

In the lower urinary tract and prostate, the role of Rac GTPases for smooth muscle contraction has been examined for the prostate and bladder. NSC23766, EHT1864 and smooth muscle-specific Rac1 knockout all inhibited carbachol-induced bladder contractions in mice, as shown by contractility measurements (Rahman et al., 2014). In human prostate tissues, NSC23766 and EHT1864 inhibited α_1-adrenoceptor contractions induced by phenylephrine or noradrenaline and neurogenic contractions induced by electric field stimulation (Y. Wang et al., 2015). As α_1-adrenoceptor antagonists are the first-line option for medical treatment of lower urinary tract symptoms suggestive of benign prostatic hyperplasia, it is noteworthy that the degree of inhibition of neurogenic contractions by EHT1864 and NSC23766 resembles the efficacy of α_1 antagonists under the same conditions (Y. Wang et al., 2015). In addition to α_1-adrenoceptors, prostate smooth muscle contraction can be induced by activation of endothelin and TXA$_2$ receptors. In contrast to U46619-induced contractions, which were inhibited by EHT1864 and NSC23766, **endothelin-1** induced contractions of human prostate tissues were inhibited by EHT1864, but not by NSC23766 (Q. Yu, Gratzke, Wang, Wang et al., 2019). Consequently, it has been proposed that this may be attributed to divergent pharmacological profiles of both inhibitors and/or to divergent receptor-specific regulation of these contractions, which is incompletely understood (Q. Yu, Gratzke, Wang, Wang, et al., 2019). As most patients included in this study may show benign prostatic hyperplasia (BPH), it has been suggested that Rac-dependent smooth muscle contraction is relevant for the development of lower urinary tract symptoms suggestive of benign prostatic hyperplasia (Y. Wang et al., 2015). Examinations using knockout or transgenic models have not been performed to confirm the role of Rac GTPases for prostate smooth muscle contraction. Nevertheless, such role appears likely, considering the evidence from other organs summarized above.

Regarding the urinary bladder, a pro-contractile role of Rac1 in bladder smooth muscle has been confirmed using Rac1 knockout mice, where contractions induced by **carbachol** and high-molar potassium chloride were reduced to wild types (Rahman et al., 2014). In line with these findings, NSC23766 and EHT1864 inhibited cholinergic contractions of bladder tissues from wild type mice and neurogenic and carbachol-induced contractions of bladder tissues obtained from female and male patients undergoing radical cystectomy (B. Li et al., 2020; Rahman et al., 2014). In both studies, inhibitory effects of NSC23766 were, however, at least partially related to a Rac-independent antagonism of muscarinic receptors by NSC23766, in line with previous findings reporting this nonspecific feature of the presumed Rac inhibitor (Levay et al., 2013; B. Li et al., 2020; Rahman et al., 2014). Interestingly, the divergent pharmacological profiles of NSC23766 and EHT1863 became further manifest by different effects on TXA$_2$ induced contractions, which were inhibited by

EHT1864, but not NSC23766 in human bladder tissues from both genders (B. Li et al., 2020). In rats with experimentally induced diabetes, Rac1 expression was reported to be up-regulated in the detrusor, what was paralleled by pronounced inhibition of carbachol-induced contractions by NSC23766 (Evcim et al., 2015). According to the role of detrusor contraction for bladder emptying in micturition, a possible role of diabetes-related bladder dysfunction has been proposed (Evcim et al., 2015).

Evidence suggesting the role of Rac1 for smooth muscle contraction in the gastrointestinal tract is still limited. At least, it has been demonstrated that NSC23766, EHT1864 and smooth muscle-specific Rac1 knockout reduce contractions of the ileum in contractility studies (Rahman et al., 2014).

Findings suggesting Rac-mediated smooth muscle contractions were completed by addressing the underlying mechanisms. Several mechanisms have been suggested, which may diverge between smooth muscle types or even within the same organ but may also show overlapping features (Figure 1). The current understanding is certainly still incomplete, as most studies focussed on one or few mechanisms and did not systematically ruled out other possible mechanisms (Figure 1). Rac1 may be activated by contractile agonists, as shown by application of cholinergic agonists in airway smooth muscle cells and rat airway tissue or using phenylephrine in intact rat aortic tissue (Andre-Gregoire et al., 2017; Sakai et al., 2018; Shibata et al., 2015). Although Rac1 may activate p21 activated kinase 1 in airway smooth muscle, this is not responsible for Rac-mediated airway smooth muscle contraction, so that other mediators account for Rac-dependent contraction (Andre-Gregoire et al., 2017). Studies addressing relationship between Rac and calcium sensitization or calcium-mediated contractions revealed conflicting results. Rac inhibitors did not reduce MYPT1 phosphorylation in airway smooth muscle cells, so that interference of Rac with calcium sensitivity was excluded (Andre-Gregoire et al., 2017). Rather, experiments using inhibitors and Rac1-deficient airway smooth muscle cells suggested that Rac1 induces contraction by PLC activation and subsequent activation of calcium-dependent mechanisms (Andre-Gregoire et al., 2017). This was confirmed by findings from aorta, saphenous artery and mesenteric artery from mice, suggesting that Rac promotes vascular smooth muscle contraction by elevation of intracellular calcium, but not by PKC-dependent mechanisms and probably not via Rho kinase (Rahman et al., 2014). In contrast, contractility measurements and phosphorylation analyses performed with airway and aorta tissues from rats suggested the opposite, that is, that Rac inhibitors act by inhibition of MLC phosphatase and calcium sensitization, but not by inhibition of calcium-/MLCK-dependent mechanisms (Sakai et al., 2018; Shibata et al., 2015). In airway smooth muscle, this may involve PKC and Rho kinase, whereas involvement of PKC, but not Rho kinase, has been suggested for rat aortic smooth muscle (Sakai et al., 2018; Shibata et al., 2015). However, consistent with contradictory findings regarding calcium sensitization or calcium-dependent mechanisms, Rac inhibitors inhibited cholinergic and endothelin-1 induced MLC phosphorylation in rat airway tissues, as well as phenylephrine-induced MLC phosphorylation in rat aortic tissues

(Sakai et al., 2018; Shibata et al., 2015). In prostate smooth muscle, Rac-mediated contraction may be based on completely different mechanisms, as NSC23766 or EHT1864 did not alter MLC phosphorylation (Y. Wang et al., 2015). Rather, both inhibitors caused a breakdown of actin filaments, so that the role of Rac for regulation of actin organization was proposed for prostate smooth muscle (Y. Wang et al., 2015). Affecting contraction through different endpoint mechanisms may not be unique for Rac1, as the same has been reported for the RhoA/Rho kinase pathway. Thus, RhoA/Rho kinase are capable to promote smooth muscle contraction by increasing MLC phosphorylation and also via actin organization (Amin et al., 2013).

Another topic of investigations was the mechanisms of Rac activation by guanine nucleotide exchange factors in smooth muscle cells. Several proven and putative Rac guanine nucleotide exchange factors exist, which may be cell and/or pathway specific. Application of knock-out models in cell culture demonstrated that Rac1 is activated by p115 RhoGEF and kalirin, thus, at least two different guanine nucleotide exchange factors in vascular smooth muscle cells (Singh, Janjanam, & Rao, 2017; Wu et al., 2013). Whether this mechanism also takes place outside cell culture, that is, in vascular smooth muscle of intact vessels or also in smooth muscle cells of other organs still remains open.

4 | RAS GTPASES IN SMOOTH MUSCLE CONTRACTION

Ras GTPases are probably best characterized in oncologic context, according to their role as central players in tumour growth, based on Ras-mediated gene expression, proliferation and transformation (Takai et al., 2001). Numerous studies proved additional functions, including promotion of smooth muscle contraction (Table 2). First evidence suggesting the role of Ras for mediating vascular smooth muscle contraction has been provided surprisingly early. Thus, in 1993, it has been reported that activated H-Ras induces contraction at constant calcium levels in permeabilized mesenteric micro-arteries from guinea pigs (Satoh, Rensland, & Pfitzer, 1993). It was concluded that H-Ras may promote vasocontraction by immediately increasing calcium sensitivity (Figure 2; Satoh et al, 1993). Remarkably, this Ras function in signal transduction mediating smooth muscle contraction has not been pursued or confirmed thereafter. Instead, some later studies suggested Ras-dependent regulation of smooth muscle contraction at expression level rather than a direct, immediate impact of Ras-mediated signal transduction on contractile endpoints.

Thus, in spontaneously hypertensive rats, captopril treatment for 12–18 weeks resulted in decreased phospho-MLC levels in mesenteric arteries, which was paralleled by decreased Ras expression (Hu, Han, Gu, Piano, & de Lanerolle, 2007). The authors concluded that decreased Ras activity was the reason for the reduced MLC content (Hu et al., 2007). Certainly, such a causal relationship or correlation could not be excluded but was not proven and was definitely lacking after 24 weeks of captopril treatment (Hu et al., 2007).

Convincing evidence for Ras-mediated regulation of smooth muscle tone was provided by a study addressing Rho kinase-dependent

TABLE 2 Summarized evidence for regulation of smooth muscle contractility by GTPases other than RhoA and Rac

	Smooth muscle type	Role	Mechanism	Evidence	Species
Cdc42	Airway	Pro-contractile	Actin	Dominant negative Cdc42 expression	Dog
H-Ras	Vascular	Pro-contractile	Ca^{2+} sensitization	Recombinant Ras	Guinea pig
Ras	Internal anal sphincter, possibly also vascular	Anticontractile	Inhibition RhoA activity; suppression Rho kinase expression	Knockout (sphincter); inhibitors (vascular)	Mouse (sphincter), rat (vascular)
ARF6	Prostate	Pro-contractile	Not MLC, possibly actin	Inhibitors	Human
Rap1b	Vascular	Anti-contractile	MYPT1/MLC phosphorylation	Knockout	Mouse
Rab11A	Vascular	Anti-contractile	BK assembly	Silencing	Rat
Rab35	Airway	IL-induced hypercontractility (pro-contractile)	Actin	Silencing	Mouse

Note. Available findings for all indicated GTPases have categorized to smooth muscle type, role, assumed mechanims of GTPase-dependent regulation, basis of findings and species.

contraction in the internal anal sphincter of Ras knockout mice (de Godoy et al., 2007). Notably, these results suggested an opposing regulation, namely, antagonization of smooth muscle contraction by interference with RhoA activity and expression of Rho kinase (Figure 2; de Godoy et al., 2007). Thus, higher spontaneous tone of internal anal sphincter in Ras knockout mice was associated with decreased reactivity to the Rho kinase inhibitor Y27632, increased membrane translocation of RhoA and higher protein expression of Rho kinase 2 (de Godoy et al., 2007).

A similar concept suggested maintenance of Rho kinase expression by Ras activity, which was confirmed by reduced contractility following Rho kinase down-regulation and inhibition of Ras activity. In rat kidney cells, Rho kinase expression is post-transcriptionally down-regulated by sustained activation of the Ras/MAPK pathway (Pawlak & Helfman, 2002). This down-regulation was prevented by pharmacological Ras inhibition (Pawlak & Helfman, 2002). Post-transcriptional down-regulation of Rho kinase and subsequent loss of Rho kinase-mediated vasocontraction contributes to vascular hypocontractility, splanchnic vasodilation and finally portal hypertension in liver cirrhosis (Hennenberg et al., 2006). Treatment of rats with experimentally induced liver cirrhosis with sorafenib, which inhibits Ras and its downstream effectors, restored vascular Rho kinase expression and resulted in increased splanchnic and systemic vascular resistance and finally an improvement of portal hypertension (Hennenberg et al., 2009).

Together, these findings point to a Ras-dependent and organ-specific regulation of smooth muscle contraction, which includes promotion and reduction of contractility and takes place by Ras-mediated alterations of Rho kinase expression.

5 | CDC42 IN SMOOTH MUSCLE CONTRACTION

As RhoA and Rac GTPases, Cdc42 belongs to the Rho family of monomeric GTPases (Takai et al., 2001). Possibly due to its close relationship to RhoA, Cdc42 has been considered quite early in the context of

smooth muscle contraction. Transfection of permeabilized isolated dog tracheal rings with a dominant negative Cdc42 mutant reduced acetylcholine-induced contractions of these tissues, proving a role of Cdc42 for canine airway smooth muscle contraction (Table 2; Tang & Gunst, 2004). To the best of our knowledge, however, this was the only study directly reporting the role of CDC42 in smooth muscle contraction, that is, by showing smooth muscle contraction of intact tissues. Although other studies repeatedly suggested a similar role and suggested involved mechanisms, this evidence stayed preliminary and included demonstration of Cdc42 activation by contractile agonists and Cdc42 induced actin organization in smooth muscle cells (Choi et al., 2005; Fediuk, Sikarwar, Nolette, & Dakshinamurti, 2014; Q. F. Li & Tang, 2009; Tang & Gunst, 2004; Tang, Zhang, & Gunst, 2005; Zhang, Huang, & Gunst, 2016). Consequently, it has been assumed that Cdc42 promotes smooth muscle contraction by actin polymerization and filament organization but not via MLC phosphorylation (Figure 1), what involves most probably Wiskott–Aldrich Syndrome protein family members (Puetz et al., 2009).

6 | ADENOSINE RIBOSYLATION FACTOR (ARF) 6 IN SMOOTH MUSCLE CONTRACTION

The family of ARF GTPases contains six members referred to as ARF1–6. Various functions have been attributed to ARFs, including cytoskeletal organization and actin remodelling (Donaldson, 2002; Hongu & Kanaho, 2014; Humphreys, Davidson, Hume, Makin, & Koronakis, 2013; Klein, Franco, Chardin, & Luton, 2006; Luton et al., 2004; Schafer, D'Souza-Schorey, & Cooper, 2000). In fact, ARF6 promotes processes depending on correct actin assembly and cytoskeletal organization, including cell adhesion, migration and platelet activation (Charles, Namkung, Cotton, Laporte, & Claing, 2016; Choi, Karim, & Whiteheart, 2006; Hiroi, Someya, Thompson, Moss, & Vaughan, 2006; Torii et al., 2010; Urban, Quick, Miller, Krcmery, & Simon, 2016). In addition, ARF6 has been proposed as an intracellular effector and regulator of GPCRs, which mediates receptor-induced
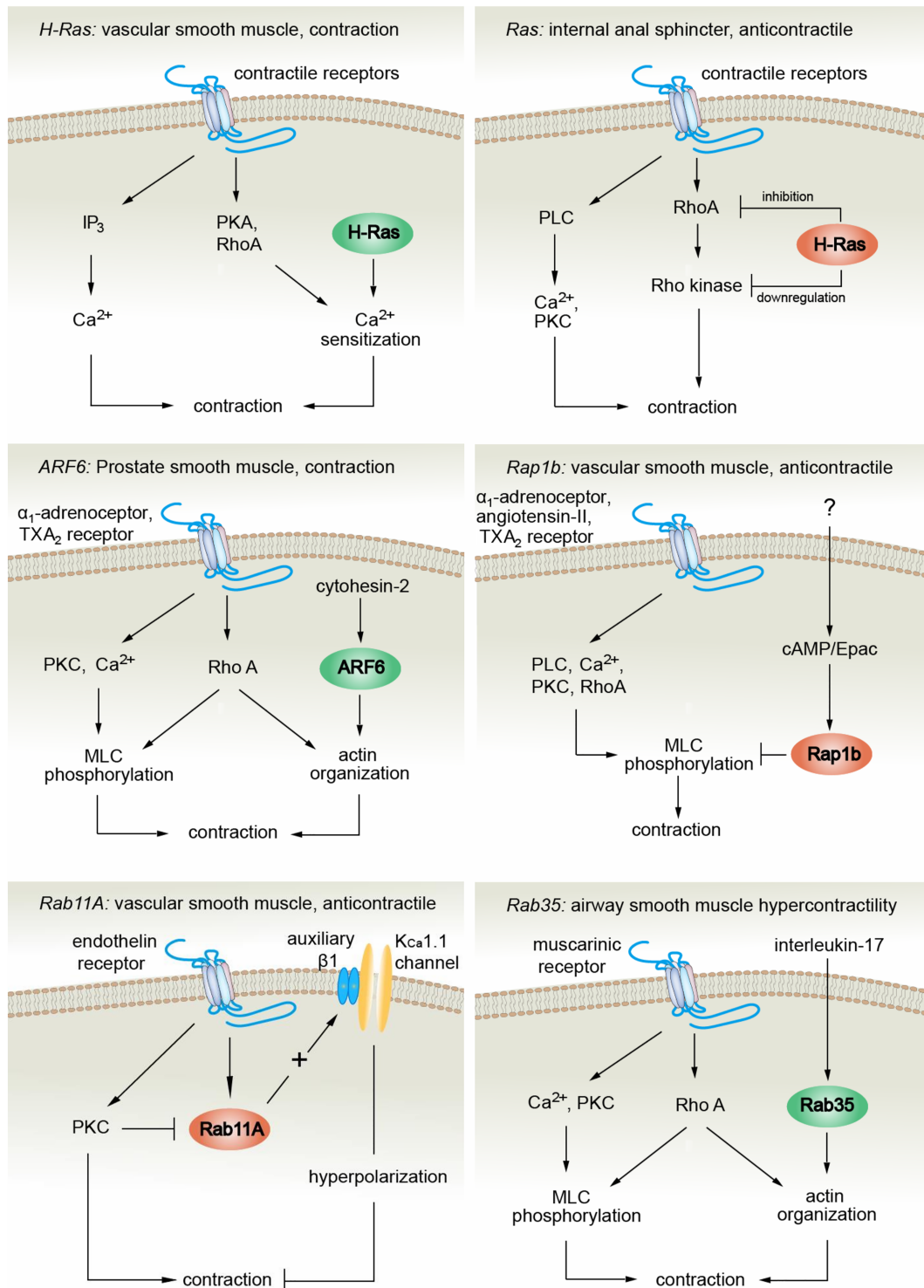
FIGURE 2 Proposed functions of Ras, ARF6, Rap1b, Rab11A, and Rab35 in regulation of smooth muscle contraction. Promotion of smooth muscle contraction has been suggested for Ras in vascular smooth muscle and ARF6 in prostate smooth muscle. Inhibition of smooth muscle contraction has been suggested for Ras in internal anal sphincter smooth muscle and for Rap1b and Rab11A in vascular smooth muscle. For Rab35, a role in IL-17-induced airway hypercontractility has been suggested. GTPases with a pro-contractile role are marked by green colour, and GTPases with an anti-contractile function by red colour. Ca^{2+}, calcium

effects and takes part in post-translational modification of receptors (Bourmoum, Charles, & Claing, 2016; Giguère et al., 2006; Hennenberg et al., 2013; Hunzicker-Dunn, Gurevich, Casanova, & Mukherjee, 2002). The best characterized and probably most common GEF for ARF6 is cytohesin-2 (Hongu & Kanaho, 2014; Torii et al., 2010; Torii et al., 2015). Effects of the cytohesin-2 inhibitor,

secinH3, on smooth muscle contraction have been recently examined in human prostate tissue. SecinH3 inhibited contractions of human prostate tissues induced by the α_1-adrenoceptor agonists phenylephrine and noradrenaline, as well as neurogenic contractions induced by electric field stimulation, or by endothelins 1 and 3 and U46619 (Herlemann et al., 2018). This was paralleled by inhibition of ARF6 activity in prostate tissues, whereas RhoA or Rac1 were not affected by secinH3 (Herlemann et al., 2018). This pointed for a first time to a role of ARF6 in promotion of smooth muscle contraction (Table 2).

With NAV2729, an inhibitor with assumed specificity for ARF6 has become available (Yamauchi, Miura, & Kanaho, 2017; Yoo et al., 2016). Its effects on smooth muscle contraction have been recently examined in human prostate tissues. NAV2729 inhibited neurogenic, α_1-adrenoceptor and endothelin induced smooth muscle contractions of these tissues (Q. Yu, Gratzke, Wang, Li, et al., 2019). Again, this was paralleled by inhibition of ARF6, but not of ARF1, Rac1 or RhoA (Q. Yu, Gratzke, Wang, Li, et al., 2019). A participation of ARF1 in regulation of smooth muscle contraction was furthermore excluded by lacking effects of brefeldin A, which may inhibit ARF1, but not ARF6 (Q. Yu, Gratzke, Wang, Li, et al., 2019). In addition to α_1-adrenoceptor and neurogenic contractions, NAV2729 inhibited U46619 but not endothelin-1 induced contractions, what may point to a divergent ARF6-mediated regulation of these contractions in human prostate smooth muscle (Q. Yu, Gratzke, Wang, Li, et al., 2019). ARF6 was not activated in prostate tissues by stimulation with α_1-adrenoceptor agonists, so that promotion of smooth muscle contraction by the NAV2729-sensitive mechanisms takes most likely place without ARF6 activation by contractile receptors (Q. Yu, Gratzke, Wang, Li, et al., 2019). NAV2729 did not affect phosphorylation of MYPT1 or MLCs, so that such mechanisms are unlikely to account for the inhibitory effects of NAV2729 on prostate smooth muscle contraction (Q. Yu, Gratzke, Wang, Li, et al., 2019). Rather, the effect may be caused by a breakdown of actin organization, which was observed in prostate stromal cells in response to NAV2729 (Q. Yu, Gratzke, Wang, Li, et al., 2019).

Together, it appears possible that a cytohesin-2/ARF6-dependent mechanism promotes smooth muscle contraction in the prostate (Figure 2). Evidence for a similar role in other organs is still missing at the present stage. Certainly, a definitive role of ARF6 for smooth muscle contraction still needs to be confirmed by knockout models. However, the anti-proliferative effect of NAV2729 was mimicked by silencing of ARF6 expression, so that similar results for contraction appear possible (Q. Yu, Gratzke, Wang, Li, et al., 2019).

7 | RAP GTPASES IN SMOOTH MUSCLE CONTRACTION

The role of Rap1b for vascular smooth muscle contraction has been suggested recently (Table 2). Rap1b knockout mice showed arterial hypertension, which was explained by increased vascular tone (Lakshmikanthan et al., 2014). In contractility studies, aortic tissues from Rap1b knockout mice showed enhanced contractile responses to phenylephrine, angiotensin II and U46619 compared with aortas from corresponding wild types (Lakshmikanthan et al., 2014). This was paralleled by increased MYPT1 phosphorylation (suggestive for inhibition of MLCP) and increased MLC phosphorylation (Lakshmikanthan et al., 2014). Accordingly, it has been concluded that Rap1 suppresses contraction of vascular smooth muscle cells by negative control of calcium sensitivity and that Rap1 may be activated through cAMP/Epac (Figure 2; Lakshmikanthan et al., 2014). It should be noted that this study proposed an endothelium-dependent mechanism of Rap1, which contributes to control of vascular tone in parallel to Rap1 in smooth muscle cells (Lakshmikanthan et al., 2014). Thus, in endothelial cells, Rap1 may be involved in production of NO, which subsequently contributes to vasorelaxation of adjacent smooth muscle cells (Lakshmikanthan et al., 2014). Further evidence from endothelium denuded pig coronary arteries and cultured smooth muscle cells suggested the role of Rap1 in endothelium independent vascular smooth muscle relaxation, by Epac-mediated inhibition of RhoA activity and subsequently reduced MLC phosphorylation in smooth muscle cells (X. Yu et al., 2017; Zieba et al., 2011). Certainly, the available chain of evidence in fact supports this role, although indirectly as it did not include Rap1-specific compounds or knockout models.

8 | RAB GTPASES IN SMOOTH MUSCLE CONTRACTION

Evidence has been provided that Rab GTPases may regulate smooth muscle contraction in vascular and airway smooth muscle (Table 2). Generally, Rab GTPases are central players in intracellular trafficking, including transport of proteins from intracellular compartments to the surface, or shuttling between vesicles and cytosolic destinations (Zhen & Stenmark, 2015). In rat arteries, Rab11A was suggested to play an anti-contractile role, which is based on assembly of large-conductance calcium-activated potassium channels ($K_{Ca}1.1$, syn. BK channels; Figure 2; Zhai, Leo, & Jaggar, 2017). Opening of $K_{Ca}1.1$ channels causes hyperpolarization and is involved in smooth muscle relaxation and counteracts contraction (Hennenberg, Trebicka, Sauerbruch, & Heller, 2008). The complete channel is composed of pore-forming BKα subunits and auxiliary β1 subunits (Zhai, Leo, & Jaggar, 2017). Trafficking of β1 subunits to the cell surface is required for assembly of $K_{Ca}1.1$ channels and was shown to depend on Rab11A in myocytes of rat cerebral arteries (Zhai et al., 2017). Phosphorylation of Rab11A serine 117 results in its inactivation and was found to be induced by endothelin-1 and subsequent PKC activation in these myocytes (Zhai et al., 2017). Consequently, transfection of rat cerebral artery tissue with a phosphorylation-resistant Rab11A mutant (i.e. a mutant, which cannot be inactivated by endothelin-1/PKC) resulted in reduced endothelin-1-induced contractions in pressurized myography (Zhai et al., 2017). Thus, endothelin-1-induced contraction of arterial smooth muscle may be (at least partially) mediated by inactivation of Rab11A and subsequently reduced current across BK channels.

In mice, Rab35 was suggested to mediate IL-17A induced airway hyperresponsiveness (Figure 2; Bulek et al., 2019). Thus, challenging

mice with IL-17A resulted in hyperresponsiveness of tracheal strips to the cholinergic agonist methacholine in organ bath studies, which was reflected by increased contractile forces during construction of concentration response curves for methacholine (Bulek et al., 2019). The development of this hyperresponsiveness by IL-17A was completely prevented by silencing of Rab35 expression by infection of mice with a Rab35 shRNA coding lentivirus (Bulek et al., 2019). Interestingly, this role of Rab35 seems to be rather specific for the development of airway hyperresponsiveness than representing a general regulatory mechanism of airway contraction, as the contractile responses to methacholine in mice with silenced Rab35 contraction was still preserved and resembled that of mice without IL challenge and without Rab35 silencing (Bulek et al., 2019).

9 | CONCLUSIONS

The role of monomeric GTPases other than RhoA for regulation of smooth muscle contraction has been suggested for vascular, airway, bladder, prostate and gastrointestinal smooth muscle. Non-RhoA GTPases with proposed roles in regulation of smooth muscle tone include Rac1, Cdc42, Ras, ARF6, Rap1, Rab11A and Rab35. Available evidence is based on functional studies addressing contractility *in vitro*, or organ-specific functions *in vivo*. The best characterized GTPase in this context is Rac1. Rac1 may promote vascular, airway, bladder, prostate and gastrointestinal smooth muscle contraction, whereas anti-contractile functions of Rac1 were suggested in airways and vessels. Roles of Cdc42 and Ras to promote airway and vascular smooth muscle contraction have been suggested early, whereas later studies suggested an opposing function of Ras at gene expression level, resulting in Ras-mediated reduction of smooth muscle contraction by suppression of Rho kinase expression. Evidence for regulation of smooth muscle contractility by ARF6, Rap1, Rab11A and Rab35 has been provided recently, pointing to a pro-contractile role of ARF6 in prostate smooth muscle, anti-contractile functions of Rap1 and Rab11A in vascular smooth muscle contraction and promotion of airway hypercontractility by Rab35. The role of non-RhoA GTPases for regulation of smooth muscle contractility constitutes a promising and innovative research field, which requires further attention to complete the understanding of these (and other) GTPases in regulation of smooth muscle tone and associated diseases. Opposing roles in different smooth muscle types may reflect the complexity and organ-specific diversity of molecular mechanisms underlying regulation of smooth muscle contraction. Divergent findings for Rac1 in the same smooth muscle type may highlight the character of GTPases as that what they are commonly termed molecular switches, being capable to change signalling in bidirectional functions.

Nomenclature of targets and ligands

Nomenclature of key protein targets and ligands in this article conform with the IUPHAR/BPS Guide to PHARMACOLOGY, as archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro, et al., 2019; Alexander, Kelly, et al., 2019; Alexander, Mathie, et al., 2019) or recommended by the IUPHAR/BPS Guide to PHARMACOLOGY database group.

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