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Evolving Mechanisms of Vascular Smooth Muscle Contraction Highlight Key Targets in Vascular Disease

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Abstract

Vascular smooth muscle (VSM) plays an important role in the regulation of vascular function. Identifying the mechanisms of VSM contraction has been a major research goal in order to determine the causes of vascular dysfunction and exaggerated vasoconstriction in vascular disease. Major discoveries over several decades have helped to better understand the mechanisms of VSM contraction. Ca^{2+} has been established as a major regulator of VSM contraction, and its sources, cytosolic levels, homeostatic mechanisms and subcellular distribution have been defined. Biochemical studies have also suggested that stimulation of Gq protein-coupled membrane receptors activates phospholipase C and promotes the hydrolysis of membrane phospholipids into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates initial Ca^{2+} release from the sarcoplasmic reticulum, and is buttressed by Ca^{2+} influx through voltage-dependent, receptor-operated, transient receptor potential and store-operated channels. In order to prevent large increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), Ca^{2+} removal mechanisms promote Ca^{2+} extrusion via the plasmalemmal Ca^{2+} pump and Na^+/Ca^{2+} exchanger, and Ca^{2+} uptake by the sarcoplasmic reticulum and mitochondria, and the coordinated activities of these Ca^{2+} handling mechanisms help to create subplasmalemmal Ca^{2+} domains. Threshold increases in $[Ca^{2+}]_c$ form a Ca^{2+}-calmodulin complex, which activates myosin light chain (MLC) kinase, and causes MLC phosphorylation, actin–myosin interaction, and VSM contraction. Dissociations in the relationships between $[Ca^{2+}]_c$, MLC phosphorylation, and force have suggested additional Ca^{2+} sensitization mechanisms. DAG activates protein kinase C (PKC) isoforms, which directly or indirectly via mitogen-activated protein kinase phosphorylate the actin-binding proteins calponin and caldesmon and thereby enhance the myofilaments force sensitivity to Ca^{2+}. PKC-mediated phosphorylation of PKC-potentiated phosphatase inhibitor protein-17 (CPI-17), and RhoA-mediated activation of Rho-kinase (ROCK) inhibit MLC phosphatase and in turn increase MLC phosphorylation and VSM contraction. Abnormalities in the Ca^{2+} handling mechanisms and PKC and ROCK activity have been associated with vascular dysfunction in multiple vascular disorders.

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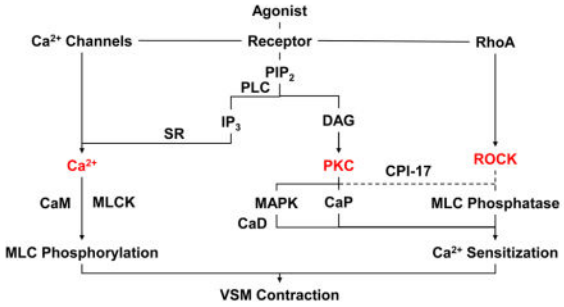
CONFLICT OF INTEREST

None

Modulators of $[Ca^{2+}]_c$, PKC and ROCK activity could be useful in mitigating the increased vasoconstriction associated with vascular disease.

Graphical Abstract



Keywords

blood vessels; calcium; channels; protein kinase; sarcoplasmic reticulum; signaling

1. Introduction

Vascular smooth muscle (VSM) is a major component of the tunica media of blood vessels, and an important regulator of vascular function. VSM contraction plays an important role in the regulation of peripheral vascular resistance and blood pressure, and vascular dysfunction, excessive vasoconstriction, and vasospasm could lead to major cardiovascular disorders such as hypertension and coronary artery disease. Over the past decades important studies and major discoveries have helped to better understand the mechanisms of VSM contraction. Under physiological conditions, agonist activation of VSM causes an initial contraction followed by a tonic contraction that can be maintained with minimal energy expenditure. Ca^{2+}-dependent myosin light chain (MLC) phosphorylation and subsequent formation of crossbridges between actin and myosin have been recognized as a major mechanism of VSM contraction. Various sources of intracellular Ca^{2+} and both Ca^{2+} mobilization and Ca^{2+} removal mechanisms have been identified. VSM contraction is triggered by an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) due to Ca^{2+} release from the intracellular stores in the sarcoplasmic reticulum (SR) and Ca^{2+} influx from the extracellular space through plasma membrane Ca^{2+} channels [1, 2]. The Ca^{2+} concentration is several-fold higher in SR and the extracellular space than in the cytosol, and the opening of Ca^{2+} channels in SR or cell surface membrane causes Ca^{2+} mobilization into the cytosol and increases $[Ca^{2+}]_c$. Ca^{2+} then binds calmodulin (CaM) to form a Ca^{2+}–CaM complex, which activates MLC kinase and causes MLC phosphorylation, actin–myosin interaction, and VSM contraction (Fig. 1). VSM relaxation is initiated by a decrease in $[Ca^{2+}]_c$ due to Ca^{2+} uptake by SR Ca^{2+} pump and Ca^{2+} extrusion via the plasmalemmal Ca^{2+} pump and Na^+–Ca^{2+} exchanger. The decrease in $[Ca^{2+}]_c$ causes dissociation of the Ca^{2+}–CaM complex, and the phosphorylated MLC is dephosphorylated by MLC phosphatase [1, 2]. However, dissociations in the relationships between $[Ca^{2+}]_c$, MLC phosphorylation and force have been observed, and Ca^{2+}-dependent MLC phosphorylation could not explain all modalities

of VSM contraction. That prompted the development of better techniques to measure $[Ca^{2+}]_c$ and further research into its intracellular distribution and subcellular domains. Several bioluminescent and fluorescent probes have been developed for accurate measurements of $[Ca^{2+}]_c$, but have shown different Ca^{2+} sensitivities. Also, the previously thought uniformity of intracellular Ca^{2+} has been challenged by the discovery of uneven intracellular distribution of Ca^{2+} in different subcellular domains, and nanojunctions between SR, the plasma membrane and other cell organelles [3, 4]. Other mechanisms of VSM contraction have also been proposed. Activation of protein kinase C (PKC) has been suggested to increase the myofilament force sensitivity to $[Ca^{2+}]_c$ and MLC phosphorylation, and thereby maintain VSM contraction with smaller increases in $[Ca^{2+}]_c$. PKC is now recognized as a family of various Ca^{2+}-dependent and Ca^{2+}-independent isoforms with different tissue and subcellular distribution, substrates and function. PKC translocation to the cell surface may trigger a cascade of protein kinases that ultimately interact with the contractile myofilaments and cause VSM contraction. Additional signaling pathways involving the small GTP-binding protein RhoA, RhoA-mediated increase in Rho-kinase (ROCK) activity, inhibition of MLC phosphatase and increased MLC phosphorylation and the myofilament force sensitivity to Ca^{2+} have also been proposed. In this review, we will discuss how the role of these Ca^{2+}-dependent and Ca^{2+}-sensitization pathways has evolved to better understand the mechanisms underlying the development and maintenance of VSM contraction [5–7]. We will also discuss how understanding the mechanisms of VSM contraction has helped to understand the pathogenesis of vascular disorders, and how modulators of Ca^{2+}-dependent and Ca^{2+}-sensitization pathways of VSM contraction could provide potential tools in the management of vascular disease.

2. Ca^{2+} Mobilization Mechanisms

The role of Ca^{2+} in muscle function was first suggested in 1883, when Ringer observed that Ca^{2+} was necessary for maintaining the activity of the isolated heart [8]. Seven decades later, Heilbrunn and colleges supported the role of intracellular Ca^{2+} in muscle contraction [9]. The sources of intracellular Ca^{2+} have later been identified as Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular space. Advances in electrophysiology and voltage-clamp techniques provided evidence that the Ca^{2+} channel is a physiologically distinct entity that plays an important role in excitation-contraction coupling [10–12]. Further methodological advances and tight-seal single channel measurements led to the recording of Ca^{2+} movement through single Ca^{2+} channel in cardiac cells [13]. In the 1980s, the field of Ca^{2+} channels rapidly expanded with the discovery of multiple types of Ca^{2+} channels with different biophysical properties, and the molecular purification of the channels and characterization of their structure, function, activators and inhibitors.

3. Ca^{2+} Release from SR

Ca^{2+} release from the intracellular stores contributes to agonist-induced VSM contraction [1, 2]. In the absence of extracellular Ca^{2+}, agonists often produce a transient VSM contraction [1, 2]. Also, in vascular preparations pretreated with Ca^{2+} channel blockers the maintained agonist-induced contraction and $^{45}Ca^{2+}$ influx are inhibited substantially, but a smaller

transient contraction can still be observed [1, 14, 15]. Also, in $^{45}Ca^{2+}$ loaded vascular preparations and incubated in a Ca^{2+}-free medium, agonists stimulate Ca^{2+} efflux [16].

Ultrastructure studies and electron probe X-ray microanalysis in smooth muscle revealed structures consistent with the SR that can accumulate Ca^{2+} from solutions containing micromolar Ca^{2+} concentrations [17]. The SR is an intracellular system of tubules or flattened cisternae [17, 18], that occupy 1.5% to 7.5% of the smooth muscle cell volume [19]. In large elastic arteries such as the rabbit aorta and main pulmonary artery, the SR occupies a larger volume, and therefore these vessels elicit a large contraction in Ca^{2+}-free solution. In contrast, in phasic smooth muscle preparations such as the rabbit mesenteric vein and guinea pig taenia coli, the SR occupies 1.5 to 2.5% of the cell volume [20], and therefore these preparations show very small contraction in the absence of extracellular Ca^{2+}.

Using advanced fractionation techniques, the SR has been isolated as a microsomal fraction. Isolated smooth muscle SR microsomes accumulate $^{45}Ca^{2+}$ and release it in response to Ca^{2+}-releasing agents such as caffeine and ryanodine. Also, Ca^{2+} release channels have been identified in SR vesicles planted in planar lipid bilayer [21]. Studies in smooth muscle preparations chemically permeabilized by saponin or α-toxin have avoided the loss of essential cellular components that occur during isolation and purification of SR vesicles, and thereby helped to assess the Ca^{2+} release mechanism under more physiological conditions [18, 22–25]. Ca^{2+} release from the SR can be triggered by inositol 1,4,5-trisphosphate (IP_3) or by Ca^{2+}.

3.1. IP_3-Induced Ca^{2+} Release

Agonist–receptor interaction activates membrane-associated phospholipase C (PLC), which breaks down the plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and 1,2-diacylglycerol (DAG) [26]. Because IP_3 is water-soluble, it diffuses in the cytosol and stimulates Ca^{2+} release from SR [27–30] (Fig. 2). On the other hand, DAG is lipophilic and therefore remains in the plasma membrane where it activates PKC [6, 31]. In saponin-skinned smooth muscle cells IP_3 induces large and rapid Ca^{2+} release. IP_3 has a half maximal effective concentration (EC_{50}) of ~1 μM which is low enough to account for the transient smooth muscle contraction [27, 29, 32]. Also, in accordance with the criteria of a second messenger, endogenous IP_3-specific 5-phosphatase activity, that rapidly inactivates IP_3 and converts it to inositol 1,4-bisphosphate (IP_2) [26], has been identified in smooth muscle [33]. IP_3 binds to IP_3 receptor and activates Ca^{2+} release channels in SR. Heparin, through its electronegative charge, competes with IP_3 and blocks IP_3 receptor and IP_3-induced Ca^{2+} release from SR [34]. Deletion of IP_3 receptor in mice suppresses aortic contraction to the vasoconstrictor agonists phenylephrine, U46619, serotonin, and endothelin-1, reduces U46619-induced phosphorylation of MLC-20 and myosin phosphatase target subunit 1 (MYPT1), and attenuates the pressor response to chronic infusion of angiotensin II (AngII), supporting a role of IP_3 receptor-mediated Ca^{2+} release in regulating VSM contraction and blood pressure [35]. IP_3 also binds and activates plasmalemmal transient receptor potential-3 (TRPC3) channels and in turn promotes Ca^{2+} influx in airway smooth muscle cells [36].

3.2. Ca^{2+}-Induced Ca^{2+} Release

Studies in skinned skeletal [18, 37], cardiac [38], and VSM [22] have shown that small concentrations of Ca^{2+} induce additional release of Ca^{2+} from SR. Ca^{2+}-induced Ca^{2+} release (CICR) is a regenerative process that can be facilitated by Ca^{2+}-releasing drugs such as caffeine and ryanodine [18]. A threshold 3 μM increase in Ca^{2+} concentration near SR. is required to trigger CICR. CICR is augmented by 3′,5′-cyclic adenosine monophosphate (cAMP) and inhibited by Mg^{2+} and procaine [22]. An initial IP_3-induced Ca^{2+} release raises Ca^{2+} concentration near SR above the 3 μM threshold, and in turn stimulates additional Ca2+ release through CICR channels [39]. This Ca^{2+} release amplification mechanism is supported by the observation that Ca^{2+} enhances IP_3-induced Ca^{2+} release from SR in skinned smooth muscle of guinea-pig taenia caeci [32]. Also, studies using calsequestrin-targeted Ca^{2+} indicator have shown that endothelin-1 (ET-1) stimulates waves of Ca^{2+} depletion from VSM SR. A transient elevation in SR luminal Ca^{2+} concentration was observed both at the site of wave initiation, just before regenerative Ca^{2+} release commences, and at the advancing wave front, during propagation. These observations suggest a role for SR luminal Ca^{2+} in the activation of IP_3 receptor during agonist-induced Ca^{2+} waves, and that these waves are due to regenerative CICR by the IP_3 receptor [40]

4. Ca^{2+} Influx from the Extracellular Space

Ca^{2+} enters VSM through non-specific Ca^{2+} leak and more selective channels including voltage-dependent, receptor-operated, *transient receptor potential (TRP),* store-operated, and stretch-activated Ca^{2+} channels (Fig. 2).

4.1. Ca^{2+} Leak

Because of the high electrochemical Ca^{2+} gradient across the plasma membrane, Ca^{2+} enters continuously into the resting VSMCs through Ca^{2+} leak. The Ca^{2+} leak pathway is lined with phosphate and carboxyl groups, partially blocked by low pH and high H^+ concentration, and blocked by ~66% by cobalt or lanthanum [1]. While Ca^{2+} leak is thought to involve non-specific Ca^{2+} movement across the plasma membrane, electrophysiological studies have suggested that a divalent cation-selective channel that displays occasional spontaneous openings contributes to Ca^{2+} leak [41]. The Ca^{2+} leak channel opens at holding potentials below the threshold for activation of voltage-dependent Ca^{2+} channel and has a higher conductance than the adenosine triphosphate (ATP)-sensitive Ca^{2+} channel, a receptor-operated Ca^{2+} channel. In rabbit aorta under resting conditions, the $^{45}Ca^{2+}$ leak amounts to ~14 μmole/kg/min [2]. This large Ca^{2+} leak does not cause VSM contraction because it is constantly balanced by Ca^{2+} uptake by SR and Ca^{2+} extrusion by the plasmalemmal Ca^{2+} pump. However, in conditions associated with compromised Ca^{2+} removal mechanisms or increased myofilament force sensitivity to Ca^{2+}, the Ca^{2+} leak could cause VSM contraction.

4.2. Voltage-Dependent Ca^{2+} Channels

Extracellular Ca^{2+} is necessary for maintained contraction in most blood vessels [1]. In rabbit aorta incubated in the absence of extracellular Ca^{2+}, contraction to membrane depolarization by high KCl solution is abolished, and norepinephrine-induced contraction is

inhibited substantially. High KCl stimulates $^{45}Ca^{2+}$ influx that is sensitive to organic Ca^{2+} antagonists such as dihydropyridines [14], and Ca^{2+} antagonist-induced blockade of $^{45}Ca^{2+}$ influx is associated with inhibition of vascular contraction [1]. Also, the Ca^{2+} channel agonist Bay-K8644 stimulates Ca^{2+} influx and promotes vascular contraction. These observations have suggested a distinct plasma membrane Ca^{2+} entry pathway that is activated by membrane depolarization, and has been termed voltage-dependent Ca^{2+} channels (VDCCs) [42–44]. Voltage-clamp and patch-clamp studies have identified two components of voltage-activated Ca^{2+} current, long-lasting L-type current activated by relatively large depolarizations and inactivates relatively slowly, and transient T-type current activated by relatively small depolarizations and inactivates relatively rapidly [45]. Both L and T Ca^{2+} currents are blocked by cadmium, cobalt and lanthanum [46–49], but show different sensitivities to dihydropyridines. While the L current is blocked by nifedipine, nimodipine, nisoldipine and nitrendipine and augmented by Bay-K8644 and Bay-R5417, the T current is not affected by these dihydropyridines [45, 46, 48]. Also, while physiological agonists are often thought to not stimulate voltage-activated Ca^{2+} current [45, 46, 48], norepinephrine, acting via a non-α non-β receptor, stimulates the L-type but not T-type current in rabbit ear artery [50], and increases the open probability of VDCCs in rabbit mesenteric artery [44].

In 1990, the vascular L-type $Ca_V1.2$ channel (LTCC) was first sequenced from rabbit lungs and showed 65% amino acid sequence homology with its skeletal muscle counterpart [51]. LTCC is comprised of pore-forming α_{1c} and auxiliary β, $\alpha_2\delta$, and γ subunits that modulate the channel function [52]. The α_{1c} contains the voltage sensor, gating system, and the Ca^{2+}-permeable pore and comprises four homologous I, II, III, IV domains, each of which is composed of six transmembrane S1–S6 segments and intracellular NH_2- and COOH-termini. The S5 and S6 segments of each of the homologous domains form the channel pore, two glutamate residues at the pore loop determine the Ca^{2+} selectivity, and the S1–S4 segments form the voltage sensor that rotates to open the channel pore [52, 53]. $Ca_V1.2$ function is prominent at more depolarized VSM membrane potentials (~ −45 to −36 mV) observed at greater intraluminal vascular pressures [54],

T-type Ca^{2+} channels (TTCCs) were first identified as a separate VDCC in guinea pig ventricular myocytes having a transient conductance of ~8 pS with Ba^{2+} as the charge carrier. T-type currents are activated at membrane potentials ~−30 mV, and dihydropyridines at nanomolar range have little effects on TTCCs. TTCCs have three isoforms; $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ channels. $Ca_V3.1$ and $Ca_V3.3$ predominantly contribute to myogenic tone at lower intraluminal vascular pressures (20–40 mmHg) at which VSM membrane potential is ~ −60 to−50 mV [55]. $Ca_V3.2$ contributes to negative feedback regulation of pressure-induced vascular tone by modulating the ryanodine receptor-large conductance Ca^{2+} activated K^+ channel (BK_{Ca}) axis [56].

Ca^{2+}-dependent inactivation of LTCC plays a crucial feed-back role in limiting increases in $[Ca^{2+}]_c$, likely through binding of the Ca^{2+}/CaM complex to the C-terminus of the pore-forming α_{1c} subunit. Studies have also examined the biophysical properties of Ca^{2+} current through the three TTCC isoforms, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ using whole cell patch clamp and internal solutions containing 27 nM or l μM $[Ca^{2+}]_c$. Both activation and inactivation

kinetics of $Ca_v3.3$ current were more rapid at l μM $[Ca^{2+}]_c$ than 27 nM $[Ca^{2+}]_c$ solution. In contrast, the biophysical properties of $Ca_v3.1$ and $Ca_v3.2$ isoforms were not different between the two $[Ca^{2+}]_c$. Overexpression of CaM1234, a calmodulin mutant that doesn't bind Ca^{2+}, prevented the effects of l μM $[Ca^{2+}]_c$ on $Ca_v3.3$. Yeast two-hybrid screening and co-immunoprecipitation revealed direct interaction of CaM with the carboxyl terminus of Cav3.3. These findings have suggested that T-type $Ca_v3.3$ channel is also regulated by $[Ca^{2+}]_c$ via interaction of Ca^{2+}/CaM with the carboxyl terminus of $Ca_v3.3$, and represents another negative feedback mechanism restricting excessive increases in Ca^{2+} entry through VDCCs. [57].

4.3. Receptor-Operated Ca^{2+} Channels

Physiological agonists activate other Ca^{2+} entry pathways separate from those activated by membrane depolarization. Norepinephrine causes further contraction in rabbit aorta maximally activated by high KCl depolarizing solution. $^{45}Ca^{2+}$ influx induced by combined stimulation with maximal concentrations of norepinephrine and KCl equals the sum of that stimulated by each one alone, suggesting that norepinephrine and KCl-induced Ca^{2+} influx are additive [1, 58]. $^{45}Ca^{2+}$ influx stimulated by the Ca^{2+} channel agonist Bay-K8644 is additive to that induced by maximal norepinephrine concentration, but not KCl. Also, while high KCl-induced VSM contraction and Ca^{2+} influx are sensitive to Ca^{2+} channel antagonists, norepinephrine-induced VSM contraction and Ca^{2+} influx are refractory to organic Ca^{2+} antagonists [1, 58]. These observations have suggested that receptor stimulation by physiological agonists activate Ca^{2+} channels that are different from those activated by membrane depolarization, and have been termed receptor-operated Ca^{2+} channels (ROCs) [42, 43]. Electrophysiological studies provided direct evidence for ROCs and showed that ATP activates a distinct Ca^{2+} current in rabbit ear artery VSM [41]. The ATP-sensitive channel displays a 3:1 selectivity for Ca^{2+} over Na^+ at near physiological ionic conditions and can be distinguished from VDCCs by its insensitivity to nifedipine or cadmium, its opening at high negative potentials, and its unitary conductance of ~5 pS in 110 mM Ca^{2+} or Ba^{2+}. Also, the channel is not activated when ATP is added outside the cell-attached patch pipette, suggesting that it is directly coupled to receptor activation by ATP rather than ATP-induced generation of a freely diffusible messenger [59].

The term ROCs is not commonly used now, and is often referred to as a type of transient receptor potential (TRP) channels [60].

4.4. Transient Receptor Potential (TRP) Channels

TRP channels are a superfamily of cationic channels with 28 encoding genes. Based on their sequence homology, TRP channels have been categorized into six subfamilies; TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPA (ankyrin), and TRPML (mucolipin). Multiple TRP channels are expressed in VSM and contribute to the regulation of VSM membrane potential and contraction, and the development of myogenic tone. Additionally, certain TRP channels contribute to vascular mechanosensitivity via G-protein coupled signaling in resistance arteries. While most TRP channels are permeable to Ca^{2+}, TRPM4 and TRPM5 are Ca^{2+} activated, but not permeable to Ca^{2+} [61].

Vasoconstrictor agonists stimulate Ca^{2+} entry through VDCCs activated by membrane depolarization, and non-selective cation channels, most of them members of the TRPC channels family. TRPC channels are activated following receptor occupancy (ROCs) or secondary to internal Ca^{2+} stores depletion that induces capacitative Ca^{2+} entry (store-operated cation channels or SOCs). TRPCs simultaneously induce Na^{+} and Ca^{2+} entry thus triggering cell membrane depolarization and increasing $[Ca^{2+}]_c$ [60, 62]. With the exception of TRPC2 and TRPC7, all other TRPC isoforms are found in VSM at varying levels depending on the vessel type. TRPC1 and TRPC6 are highly expressed in VSM. TRPC4 is detected at a lower level than TRPC1 and TRPC6 in rat aorta, cerebral, mesenteric, and renal artery, and is not detected in caudal artery. TRPC3 level is higher in rat cerebral, renal, and caudal artery than in the aorta. TRPC5 shows a slight signal in rat aorta and renal artery, but is not detected in mesenteric artery [60].

4.5. Store-Operated Ca^{2+} Channels

During cell activation, the initial Ca^{2+} release from the intracellular stores is followed by maintained Ca^{2+} entry from the extracellular space. Depleted Ca^{2+} stores in sarcoplasmic/endoplasmic reticulum could act as a capacitor for “capacitative” or “store-operated” Ca^{2+} entry [63–65]. Studies have identified store-operated Ca^{2+} release-activated Ca^{2+} current [66, 67]. The functional significance of store-operated Ca^{2+} channels (SOCs) has been supported by experiments using inhibitors of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-adenosine triphosphatase (Ca^{2+}-ATPase) (SERCA) such as cyclopiazonic acid and thapsigargin. These compounds deplete SR Ca^{2+} stores by inhibiting Ca^{2+} uptake without activating guanosine triphosphate (GTP)-binding proteins and thereby differentiate between Ca^{2+} entering through SOCs and ROCs. In cultured VSMCs, depletion of SR Ca^{2+} stores with thapsigargin activates Ca^{2+} influx that is independent of the generation of IP_3 and resistant to the L-type VDCC blocker nicardipine [68]. Ca^{2+} influx induced by SERCA inhibitors is dependent on extracellular Ca^{2+} and sufficient to maintain vascular tone [69, 70].

Members of the canonical TRPCs such as TRPC1 and TRPC5 play a role in store-operated Ca^{2+} entry in VSM [71–74]. TRPC1 is linked to TRPP2 (polycystin-2) Ca^{2+} permeable channel [75] and TRPC5 represents another component of SOCCs [74]. Other members of the TRPC family, including TRPC3, TRPC4, and TRPC7, have been associated with store-operated Ca^{2+} entry in nonvascular cells [76, 77].

In mouse aortic VSMCs, depletion of Ca^{2+} stores triggers the release of a Ca^{2+} influx factor (CIF), which activates SOCCs [78]. Other studies have identified a 3-pS Ca^{2+}-conducting channel that is activated by 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and thapsigargin. The 3-pS channel is also activated in inside-out membrane patches from smooth muscle cells when stimulated by CIF extracted from mutant yeast cell line [79]. CIF has been partly purified in a stable form, but its molecular structure has not been well-characterized [80, 81]. A membrane-spanning protein termed stromal-interacting molecule 1 (STIM1) also plays a role in the activation of SOCCs. STIM1 serves as a sensor of Ca^{2+} within the stores, interacts with TRPC1 and promotes store-operated Ca^{2+} entry [82, 83]. Other studies have suggested that Orai1 is a pore subunit of SOCCs [84]. Studies have

suggested an interaction between STIM1 and Orai1 that leads to a gain in SOCC function [85, 86]. STIM1 senses depletion of intracellular Ca^{2+} stores in response to physiological stimuli, and relocalizes within the sarcoplasmic/endoplasmic reticulum to plasma-membrane-apposed junctions, where it recruits and gates open plasma membrane Orai1 Ca^{2+} channels. Septins are cytoskeletal proteins capable of self-association, polymerization and binding to cell membranes [87]. Septin has been suggested as a potential coordinator of store-operated Ca^{2+} entry [88]. Septin filaments and phosphatidylinositol-4,5-bisphosphate rearrange locally at the endoplasmic reticulum-plasma membrane junction before and during formation of STIM1-Orai1 clusters, thus facilitating STIM1 targeting to these junctions and promoting stable recruitment of Orai1, and efficient STIM1-Orai1 communication and Ca^{2+} entry [89].

4.6. Stretch-Activated Ca^{2+} Channels

During “autoregulation” of blood flow, an elevation of intravascular pressure and stretch of the vascular wall can cause maintained increase in VSM tone [90]. Stretch-stimulated vascular tone is highly dependent on extracellular Ca^{2+} through stretch-activated Ca^{2+} channel [91]. Stretch-activated Ca^{2+} channels differ from VDCCs and ROCCs in their sensitivity to Ca^{2+} antagonists, being more sensitive to diltiazem but insensitive to dihydropyridines. Mechanical stretch stimulates $^{45}Ca^{2+}$ influx in smooth muscle membranes [92]. A role of the endothelium in myogenic vascular response to stretch has also been suggested [93]. In cannulated cat cerebral arteries with intact endothelium, elevation of transmural pressure was associated with membrane depolarization, action potential generation, and reduction in internal diameter. Perfusing the vessels briefly with collagenase and elastase to disrupt the endothelium without damaging the smooth muscle cells, abolished the responses to elevation of transmural pressure, suggesting that endothelial cells serve as a transducer in the vascular autoregulatory response to pressure [93].

TRP vanilloid type 2 (TRPV2) is a Ca^{2+} permeable stretch-activated channel [94]. TRPV4 is also stimulated by mechanical stress, including sheer stress and cell swelling, and has been implicated in regulation of myogenic tone [52]. TRPC6 channels are activated by mechanosensation such as sheer-stress and cell swelling and promote Ca^{2+} entry into VSM and vasoconstriction [95]. TRPP are other mechanosensitive Ca^{2+}-permeable channels [61]

5. Mechanisms of Ca^{2+} Removal

In addition to their role in Ca^{2+} mobilization, the smooth muscle plasma membrane and intracellular organelles play a role in maintaining Ca^{2+} in. The plasmalemmal Ca^{2+}-Ca^{2+}-ATPase (PMCA) plays a role in maintaining $[Ca^{2+}]_c$ close to the basal levels, and the Na^+–Ca^{2+} exchanger contributes to removal of excess cytosolic Ca^{2+} (Fig. 2). Also, two intracellular organelles, namely the SR and mitochondria, regulate $[Ca^{2+}]_c$. These organelles have pump-leak system that involves active uptake of Ca^{2+} from the cytosol and passive leak of Ca^{2+} back to the cytosol.

5.1. Plasmalemmal Ca^{2+}-ATPase (PMCA)

Metabolic inhibition of the smooth muscle of guinea pig taenia coli using iodoacetic acid or 2,4-dinitrophenol causes a net Ca^{2+} uptake similar in magnitude to the passive Ca^{2+} leak [96, 97]. These observations have suggested that an ATP-dependent Ca^{2+} extrusion pump contributes to smooth muscle Ca^{2+} homeostasis and that inhibition of the ATP-dependent Ca^{2+} pump causes accumulation of Ca^{2+} inside the cell [98]. The smooth muscle plasmalemmal Ca^{2+} pump [99] shares some properties of the better studied Ca^{2+} pump in the squid axon and red blood cells. The Ca^{2+} pump has a molecular weight of 130 kDa, and is stimulated by CaM and inhibited by vanadate. Vanadate causes maximal VSM contraction, suggesting that the plasmalemmal Ca^{2+} pump plays a major role in the regulation of $[Ca^{2+}]_j$ and vascular tone [100]. Also, certain agonists such as oxytocin and prostaglandins promote smooth muscle contraction in part by inhibiting the plasmalemmal Ca^{2+} pump [101, 102].

PMCA can be distinguished from other ATPases in the plasmalemma and endoplasmic reticulum by its insensitivity to ouabain (distinction from Na^+/K^+-ATPase), high sensitivity to inhibition by vanadate (more sensitive than SERCA), sensitivity to K^+ (less sensitive than SERCA), and sensitivity to CaM antagonists [103]. Molecular biology studies have been successful in cloning. purification and amino acid sequencing of the plasmalemmal Ca^{2+} pump from several cell types including smooth muscle [104–106].

5.2. The Sodium–Calcium Exchanger

The Na^+–Ca^{2+} exchanger (NCX) is an alternative plasma membrane pathway through which excess intracellular Ca^{2+} is removed to the extracellular space against a large Ca^{2+} gradient. NCX contributes to Ca^{2+} removal in many cell types including smooth muscle [107, 108]. In membrane vesicles, NCX activity copurifies with plasma membrane markers, suggesting a plasmalemmal activity. Studies have succeeded in the isolation and functional reconstitution of the plasmalemmal NCX [109, 110], and distinguished it from the mitochondrial NCX by its markedly different specificity and stoichiometry [111].

NCX is driven by the transmembrane Na^+ and Ca^{2+} gradients and the membrane potential. The energy derived from either Na^+ or Ca^{2+}, moving down its electrochemical gradient, is balanced by an antiport movement of the coupled ion. This transport mechanism is electrogenic with $3Na^+$:Ca^{2+} stoichiometry [112, 113]. NCX plays a role in Ca^{2+} extrusion in VSM, but its contribution varies in different blood vessels [114, 115]. Also, depending on the membrane potential and the transmembrane Na^+ and Ca^{2+} gradients, NCX contributes to either Ca^{2+} extrusion or Ca^{2+} influx (reverse-mode NCX). The role of NCX as a source of intracellular Ca^{2+} may be increased in vascular disorders such as hypertension [116].

5.3. Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA)

The role of SERCA in Ca^{2+} homeostasis has long been recognized in skeletal and cardiac muscles [117]. SERCA has a molecular weight of 100 kDa and a 2:1 stoichiometry of Ca^{2+} transport to ATP hydrolysis. The ability of SR to accumulate Ca^{2+} is markedly less in smooth muscle compared with skeletal and cardiac muscles [118]. However, smooth muscle SR microsomes show energy-dependent Ca^{2+} uptake. Also, Ca^{2+} electron probe X-ray

microanalysis of saponin-permeabilized smooth muscle has demonstrated a nonmitochondrial ATP-dependent Ca^{2+}-pump activity that is blocked by vanadate [17]. SERCA affinity for Ca^{2+} (K_m = 0.2–0.6 μM) is sufficient to take up Ca^{2+} and promote muscle relaxation. Calsequestrin is a high-capacity low-affinity Ca^{2+}-binding protein that increases SR Ca^{2+} storage capacity in skeletal and smooth muscle [119, 120]. Because of the limited capacity of SR to accumulate Ca^{2+}, the mitochondria become the major Ca^{2+} pool during repeated and excessive Ca^{2+} loads [121].

Cyclopiazonic acid is a specific inhibitor of SERCA that causes slowly developing contractions in VSM, and a second application of cyclopiazonic acid causes smaller repeatable contraction that depends on the vessel type. In rat aorta, cyclopiazonic acid-induced contractions are decreased upon the second application, but are completely repeatable in the presence of PMCA inhibitor vanadate, but not the Na^+/K^+ pump inhibitor ouabain. The contractions are also completely repeatable in the presence of the forward mode NCX inhibitor 2′,4′-dichlorobenzamil, but not the reverse mode NCX inhibitor KBR7943. These findings indicate that cyclopiazonic acid by inducing a transient rise in $[Ca^{2+}]_c$ causes a long-lasting stimulation of plasma membrane Ca^{2+} extrusion mechanisms and leading to a diminished contraction upon its second application, and thereby suggest a functional coupling between SERCA and plasma membrane Ca^{2+} extruders and in rat aortic VSMCs [122]. SERCA is now known to form nanojunctions with the plasma membrane and other cell organelles including lysosomes, mitochondria, and the nucleus [3, 4].

5.4. Mitochondria and Ca^{2+}

Mitochondria occupy ~5% of smooth muscle cell volume [8], but their role in the regulation of intracellular Ca^{2+} has not been fully examined, and the concentration of free Ca^{2+} in the mitochondrial matrix space is unclear. Separate Ca^{2+} influx and Ca^{2+} efflux pathways affect Ca^{2+} movement across the mitochondrial membrane [111, 123]. Ca^{2+} influx operates as a Ca^{2+} uniporter driven by the large mitochondrial membrane potential (150 mV, inside negative), and Ca^{2+} efflux involves a Ca^{2+}:$2H^+$ or Ca^{2+}:$2Na^+$ antiporter [124, 125]. The Ca^{2+} efflux has lower capacity than Ca^{2+} influx [123]. Under physiological conditions, the major cellular cytosolic anion is phosphate (HPO_4^{2-}). When Ca^{2+} is taken up by mitochondria, HPO_4^{2-} is also taken up via: HPO_4^{2-}:$2OH^-$ exchange and calcium phosphate is formed. According to Mitchell's hypothesis of mitochondrial energy transfer [126], the primary event is the development of an electrochemical proton gradient across the mitochondrial membrane with the pH gradient greater in mitochondria than the cytoplasm. In an alkaline environment, the solubility of calcium phosphates is extremely low. Thus, the major determinants of the free Ca^{2+} concentration within the mitochondrial matrix space are the extra- and intramitochondrial phosphate concentration, the intramitochondrial pH, and the K_m and V_{max} of the efflux pathway [124]. The role of mitochondria in cellular Ca^{2+} homeostasis can be easily understood by considering the rate of Ca^{2+} uptake into mitochondria as a function of $[Ca^{2+}]_c$. The rate of mitochondrial Ca^{2+} uptake increases dramatically as $[Ca^{2+}]_c$ rises to abnormally high levels. Since the Ca^{2+} efflux out of the mitochondria is saturable [123], the rate of mitochondrial Ca^{2+} uptake will exceed Ca^{2+} efflux and a net accumulation of Ca^{2+} by the mitochondria occurs [124]. The accumulated Ca^{2+} then deposits into a nonionic pool of calcium phosphate. Thus, the mitochondria

function as a sink for Ca^{2+} during Ca^{2+} overload. The mitochondrial free Ca^{2+}, however, is in equilibrium with the large nonionic calcium pool. This arrangement means that $[Ca^{2+}]_c$ is coupled to the nonionic calcium pool in the mitochondria. Consequently, when $[Ca^{2+}]_c$ is lower than the mitochondrial free Ca^{2+}, the nonionic calcium pool is released to stabilize $[Ca^{2+}]_c$. On the other hand, when $[Ca^{2+}]_c$ is within the normal basal level (~0.1 μM), the mitochondrial free Ca^{2+} will have a similar value and the plasma membrane and the SR will be largely responsible for maintaining the cellular Ca^{2+} homeostasis. Also, because the capacity of mitochondria, although large, is finite, it is presumed that they slowly release their stored calcium during periods of cellular quiescence when it can be handled by the plasmalemmal and SR Ca^{2+} pumps. The apparent K_m of mitochondria for Ca^{2+} uptake is ~10–17 μM, which is higher than that of SR (K_m ~1 μM). Thus, SR is the major Ca^{2+} storage site under physiological conditions, and mitochondria accumulate Ca^{2+} only when $[Ca^{2+}]_c$ is abnormally high, exceeding 5 μM [17, 127]. In other words, smooth muscle mitochondria are minimally loaded with Ca^{2+} under physiological conditions, and the mitochondrial large Ca^{2+} buffering capacity plays a role mainly under pathological conditions when "Ca^{2+} overload" occurs and the cell viability is threatened by massive Ca^{2+} influx. The high Ca^{2+} content of mitochondria isolated from atherosclerotic blood vessels may reflect damaged smooth muscle cells, and such cells may represent the initial sites of vascular calcification [17].

6. Ca^{2+}-Dependent Myosin Light Chain Phosphorylation

6.1. Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_c$)

$[Ca^{2+}]_c$ is regulated by a balance between the Ca^{2+} mobilization and Ca^{2+} removal mechanisms. $[Ca^{2+}]_c$ was first measured in large cells by microinjection of the cells with metallochromic dyes such as arsenazo III and antipyralzo III [128] or bioluminescent proteins such as aequorin [129, 130], or by impalement of the cell with Ca^{2+}-sensitive microelectrodes [131]. VSMCs are very small and are not suitable for the microinjection or impalement techniques. This problem was first circumvented by administering aequorin into VSM preparations using a transient membrane permeabilization technique [132]. Thereafter, several fluorescent Ca^{2+} indicators including quin-2, fura-2, and indo-1 have been developed for measuring $[Ca^{2+}]_c$ in many cell types including VSM [133–137]. The nonpolar acetoxymethyl ester of Ca^{2+} indicators is more lipophilic and diffuses into the cell where it is hydrolyzed by intracellular esterases into the more hydrophilic free acid that does not cross the plasma membrane and is trapped inside the cell. Regardless of the technique used, the physiological VSM $[Ca^{2+}]_c$ is in the range between 0.1 and 1 μM.

6.2. Myosin Light Chain Phosphorylation

Ca^{2+}-dependent MLC phosphorylation is a major determinant of smooth muscle contraction [138, 139]. The thick-filament regulation hypothesis of smooth muscle contraction predicts that Ca^{2+} binds CaM to form a Ca^{2+}–CaM complex, which activates MLC kinase, and results in the phosphorylation of the 20-kDa MLC [138, 139]. The phosphorylated MLC increases the activity of actin-activated Mg^{2+}-ATPase leading to actin–myosin interaction and smooth muscle contraction (see Fig. 1). Smooth muscle relaxation is initiated by a decrease in $[Ca^{2+}]_c$ due to Ca^{2+} uptake by SR and Ca^{2+} extrusion by the plasmalemmal Ca^{2+}

pump and NCX. The decrease in $[Ca^{2+}]_c$ causes dissociation of the Ca^{2+}–CaM complex and the phosphorylated MLC is dephosphorylated by MLC phosphatase.

6.3. Evidence for Other Mechanisms of Smooth Muscle Contraction

Agonist-induced vascular tone can not be explained only by Ca^{2+}-dependent MLC phosphorylation. In rabbit aortic rings incubated in Ca^{2+}-free solution, the α-adrenergic receptor agonist phenylephrine causes an initial transient contraction likely due to Ca^{2+} release from SR followed by a smaller but maintained contraction [2], which in the absence of extracellular Ca^{2+} may be due to other Ca^{2+} sensitization mechanisms. Simultaneous measurements of force and $[Ca^{2+}]_c$ in VSM preparations have suggested agonist-induced increases in myofilament force sensitivity to Ca^{2+} [132, 140]. In rabbit inferior vena cava loaded with fura-2, norepinephrine causes an initial contraction followed by a maintained contraction in parallel with a rapid $[Ca^{2+}]_c$ spike followed by a smaller increase in $[Ca^{2+}]_c$ above basal levels. In contrast, membrane depolarization by high KCl causes sustained increases in contraction and $[Ca^{2+}]$c. Also, for approximately the same increase in $[Ca^{2+}]_c$, norepinephrine causes greater contraction than that induced by high KCl. Also, when the relationship between $[Ca^{2+}]_c$ and force was constructed by maximally stimulating inferior vena with norepinephrine or high KCl in Ca^{2+}-free solution, then increasing extracellular Ca^{2+} stepwisely, the norepinephrine $[Ca^{2+}]_c$–force curve was enhanced and located to the left of that induced by high KCl, suggesting that norepinephrine increases the myofilament force sensitivity to Ca^{2+} [140].

Dissociations in the $[Ca^{2+}]_c$-force relationship were observed during agonist stimulation of various smooth muscle preparations including ferret aorta [141], rabbit pulmonary artery [142], and swine carotid artery [143]. Also, dissociations in the $[Ca^{2+}]_c$-MLC phosphorylation relationship were observed during agonist-induced activation of smooth muscle and were related to G protein-mediated change in the MLC kinase/MLC phosphatase activity ratio [144]. Agonist-induced dissociations between MLC phosphorylation and force have also been reported [145, 146], and have been explained by the “latch bridge” hypothesis, which proposes that the dephosphorylation of myosin may generate a slowly cycling cross-bridge that supports force maintenance [147]. However, Ca^{2+}-dependent MLC phosphorylation may not be the only determinant of agonist-induced VSM contraction and other mechanisms that increase the myofilament force sensitivity to $[Ca^{2+}]_c$ and MLC phosphorylation have been proposed.

7. Protein Kinase C

The interaction of vasoconstrictor agonists such as phenylephrine, angiotensin II (AngII), and endothelin-1 (ET-1) with their Gq protein-coupled receptors (GPCRs) activates a GTP-binding protein and PLCβ, which stimulates the hydrolysis of PIP_2 into IP_3 and DAG [148]. IP_3 stimulates Ca^{2+} release from SR, while DAG activates protein kinase C (PKC). PKC is a ubiquitous enzyme found in almost all cell types including the vascular endothelium, VSM and fibroblasts. PKC is a serine/threonine kinase that phosphorylates a large number of substrates and is widely implicated in numerous physiological and pathological processes. PKC was discovered by Nishizuka and colleagues in rat brain extract [149] as a Ca^{2+}/

phospholipid protein kinase that is activated by DAG [150] and the tumor promoter phorbol ester [151]. PKC was then found to be a family of several isoforms with different subcellular localization, substrates and functions.

7.1. PKC Structure and Isoforms

The PKC molecule comprises a N-terminal regulatory domain, a hinge region and a C-terminal catalytic domain [152] (Fig. 3). The conventional PKC isoforms α, βI, βII, and γ have four conserved regions (C1, C2, C3 and C4) and five variable regions (V1, V2, V3, V4, and V5). The regulatory domain contains two conserved C1 and C2 regions. The C1 region contains cysteine-rich zinc finger-like motifs and lipid-binding sites surrounded by a band of hydrophobic residues that penetrate the lipid bilayer and anchor PKC to DAG-containing membranes. PKC also stably associates with membranes through the C2 region [153]. The C1 and possibly C2 region also bind the PKC cofactor phosphatidylserine (PS) [154, 155]. An autoinhibitory pseudosubstrate sequence immediately precedes the C1 region, and comprises a 19–36 amino acid residues that resemble the PKC substrate phosphorylation site [156]. The PKC catalytic domain contains the conserved C3 region, an ATP/Mg-binding site in a narrow hydrophobic pocket and a binding site for the phospho-acceptor sequence in the substrate [157]. The C4 region comprises the substrate-binding part of PKC [158]. The catalytic domain also contains three key phosphorylation and autophosphorylation sites in the C-terminal activation loop, turn-motif and hydrophobic-motif.

PKC is a large serine/threonine kinase family that comprises ~2% of the human kinome [159] and encodes nine different genes and 10 isoforms [160]. Based on the structure of the N-terminal domain, PKC isoforms are classified into conventional cPKCs α, βI, βII, and γ; novel nPKCs δ, ε, η and θ; and atypical aPKCs ζ and ι/λ isoforms [6] (Fig. 3). The cPKCs consist of four conserved regions (C1–C4) and five variable regions (V1–V5), and are activated by Ca^{2+}, DAG, and PS. The N-terminal regulatory domain contains a highly homologous 60–80% C1 region among different PKC isoforms [157]. The C1 region contains the recognition site for PS, DAG, and phorbol esters. The C2 region is rich in acidic residues and contains the binding site for Ca^{2+} [158]. In cPKCs, the C2 region comprises 105 to 130 residue eight-stranded anti-parallel β-sandwich structures with three inter-strand Ca^{2+}-binding loops responsible for Ca^{2+}-dependent anionic phospholipid binding [161]. Both cPKCs and nPKCs have twin C1 regions (C1A and C1B) and a C2 region, but the ordering of the C1 and C2 regions is reversed in nPKCs compared to cPKCs [161]. Also, nPKCs have a variant C2 region that lacks the critical Ca^{2+}-coordinating aspartic acid residues that are highly conserved in cPKCs, making it insensitive to Ca^{2+} [156]. The C1 region of nPKCs has a higher affinity for DAG than that of cPKCs, and functions as a lipid-binding membrane-targeting module in a Ca^{2+}-independent manner [162]. The C2 region of PKCδ does not bind lipids, but has a protein–protein interaction domain that binds phospho-tyrosine residues flanked by the consensus sequence (Y/F)-(S/A)-(V/I)-pY-(Q/R)-X-(Y/F). PKCδ contains several tyrosine phosphorylation sites throughout its structure, including the regulatory and catalytic domains and the hinge region [163]. The aPKCs do not have a C2 region but have a variant form of C1 and are therefore activated by PS but not Ca^{2+} or DAG [156]. However, aPKCs do retain lipid-binding activity, and the C1 region confers DAG binding that is not duplicated, unlike the C1A–C1B tandem repeat found in cPKCs and

nPKCs [164]. The aPKCs also uniquely encode the protein–protein-interacting Phox and Bem 1 (PB1) region in the N-terminal domain, which binds ZIP/p62, Par6, or MEK5 through a PB1-PB1 domain interaction that controls the localization of aPKCs [165]. PKCμ and PKCν are often considered a fourth class of PKC or members of protein kinase D (PKD) family [155, 166].

The greatest homology among PKC isoforms is in the highly conserved catalytic domain (~70%). Also, similar to other Ser/Thr kinases, PKC isoforms have a highly conserved ATP-binding site. The exception to the catalytic domain homology is the variable V5 region, consisting of 60–70 different amino acids. PKC isoforms also differ in the V3 hinge region [157]. PKCβI and βII are generated by alternative splicing from a single gene, but differ in their C-terminal 50 residues (βI) or 52 residues (βII) [158]. The amino acid in each phosphorylation site also varies in different PKCs. For example, the activation loop contains a phosphorylatable T497 in PKCα, T500 in PKCβII, T505 in PKCδ and T538 in PKCθ, and the turn motif contains a T638 in PKCα, T641 in PKCβI and PKCβII, S643 in PKCδ and S676 and S685 in PKCθ, while the hydrophobic motif contains a S657 in PKCα, S660 in PKCβII, S662 in PKCδ and S695 flanked by bulky hydrophobic residues in PKCθ [153, 167].

7.2. PKC Distribution and Translocation

PKCs are found in numerous tissues and vascular beds. PKCα, δ and ζ are expressed in most blood vessels, and other PKCs show specific distribution in certain blood vessels [168, 169] (Table 1). PKCα, β, δ and ε, but not PKCζ, are highly expressed in human VSMCs [170]. Fluorescent-tagged PKC and live imaging techniques allowed the study of PKC localization in real-time [171, 172]. In resting cells, PKCα, β and γ are localized mainly in the cytosolic fraction, and activated PKC translocates from the cytosolic to the particulate and membrane fraction [168, 173] (Fig. 4). Simple diffusion and other physico-chemical forces may drive PKC movement inside the cell, and targeting mechanisms including conformation changes, altered hydrophobicity, lipid modification, protein-protein interaction, targeting sequences, and phosphorylation allow its translocation and tight binding to different cell membranes [168, 174].

PKC binding to Ca^{2+} or DAG causes conformational changes and unfolding of the PKC molecule, leading to exposure of the substrate region, increased PKC hydrophobicity and binding to membrane lipids [158]. Changes in the plasma membrane lipid domains influence PKC distribution. The plasma membrane is composed of several domains of focal adhesions alternating with zones rich in caveolae, and both harbor a subset of membrane-associated proteins. PKCα exhibits binding activity in caveolae, and may not bind to non-caveolae membranes [175]. Localized [Ca^{2+}] gradients affect the amount of PKC retained in caveolae. For instance, caveolae contain PKCα only in the presence of Ca^{2+}, while retention of PKCε and PKCλ in caveolae is Ca^{2+} independent [175]. Caveolins are scaffold proteins that help PKCα and ζ localize to the caveolar microdomains where they are subsequently activated [176]. In rabbit femoral and renal arteries at rest, PKCζ is localized in punctate plasma membrane aggregates alternating with vinculin and in a perinuclear location, and such locations are conducive to regulating VSM $[Ca^{2+}]_c$ [177]. Plasma membrane lipids are

also segregated into cholesterol-rich lipid rafts and glycerophospholipid-rich non-raft regions, an arrangement that is critical for preserving the membrane architecture and for translocation of proteins. In VSMC membranes, lipid segregation is supported by annexins that target membrane sites of distinct lipid composition, and each annexin requires different [Ca^{2+}] for its translocation to the plasma membrane, thus allowing a spatially confined graded response to external stimuli and plasmalemmal localization of PKC [178]. Several members of the annexin family function as PKC substrates and promote membrane association of PKC [179, 180] (Table 2). Annexin A1, A2, A5 and A6 (or anexxin I, II, V, and VI) display specific abilities to interact and promote membrane targeting of distinct PKCs. Also, because of the ability of annexins to create specific membrane microenvironments, they could allow PKCs to phosphorylate certain substrates and regulate their downstream effector pathways in specific subcellular locations [160]. PKC isoforms interact with specific members of the annexin family, and PKCβ, ε and α interact with annexin I, II and VI, respectively. Also, interaction between annexin V and PKCδ occurs in cells after PKCδ stimulation, but before its translocation to the membrane fraction, suggesting that PKCδ requires binding to annexin V for its translocation [181]. Whether other PKCs require annexin binding before translocation is unclear.

Myristoylated alanine-rich C kinase substrate (MARCKS) plays a role in PKC membrane binding. MARCKS is a major PKC substrate that binds F-actin and cross-bridges between the plasma membrane and cytoskeletal actin [182]. Phosphorylation of MARCKS by PKC has an electrostatic effect that affects its affinity to the plasma membrane and interferes with its actin cross-linking, leading to its displacement from the plasma membrane. MARCKS and CaM are co-distributed in SMCs and co-targeted simultaneously to the cell interior upon cell stimulation. PKC activation triggers the translocation of CaM which facilitates the translocation of MARCKS and its subsequent phosphorylation at multiple sites [183]. Dephosphorylation of MARCKS causes its re-association with the plasma membrane via its stably attached myristic acid membrane-targeting moiety [184].

Protein-protein interactions are crucial in signal transduction, and binding sites for arginine-rich polypeptides have been identified in the PKC molecule distal to its catalytic site and may allow targeting of PKC to precise substrates at specific cellular locations. Scaffold proteins such as receptor for activated C kinase (RACK), substrates that interacts with C kinase (STICK), receptor for inactive C kinase (RICK), and A-kinase activating proteins (AKAPs) assist in PKC translocation to the membrane [185]. RACKs and STICKs bind to active PKCs, whereas RICKs and AKAPs interact with inactive PKCs. Binding of a specific activated PKC to its RACK provides access to, and phosphorylation of, its substrates [186]. Binding of RACK increases the phosphorylation capacity of PKC several-fold independently from the substrate identity [187]. RACKs also target PKC to cytoskeletal elements [187]. The interaction of PKC and RACK is isoform specific and is largely mediated by the C2 region of cPKCs [188], and peptide fragments of this region serve as modulators of PKC activity [189]. These short peptides induce activation and translocation of the PKC isoform by mimicking the action of RACK on the isoform and, therefore, are termed ‘pseudo RACKs’ (ψRACK) [190, 191]. Disruption of the interaction between ψε RACK and the RACK-binding site is a critical rate-limiting step in translocation of PKCε [192]. Other scaffold proteins including 14-3-3, heat shock protein (HSP), importins, and even actin can

tether PKC isoforms to different membranes and organelles [193–197]. Protein-protein interactions between PKC isoforms and their substrates provide further anchoring to specific subcellular sites. For PKCε, protein-protein interactions may involve a myofilament-binding site in the C2 region [198], an intra-SR calsequestrin-binding site [199], a neurocytoskeletal elements-binding site [200], an actin-binding site in the C1 region, and a Golgi-binding site [191, 197, 201]. PKCε association with Golgi membranes via its zinc finger domain can modulate Golgi function [202]. The PKC pseudosubstrate and hinge regions can facilitate its plasma membrane and cytoskeletal association [203]. Also, the V5 region can contribute to the regulation of PKCα activity by multiple mechanisms involving stabilizing the kinase through direct interaction with its N-terminal, interacting with the pseudosubstrate in the N-terminal regulatory domain, and interaction with RACK [204].

While the interaction of cPKCs at the plasma membrane has been well-studied, less is known about the activity of nPKCs and aPKCs at the plasma membrane and other membranes in the nucleus, mitochondria, endoplasmic reticulum (ER) and Golgi. For instance, c-src-dependent phosphorylation of tyrosine Y256 in PKCι, through enhanced interaction with the nuclear transporter protein importin-β, results in its translocation to the nucleus [205]. Also, the ER membrane is a major target for PKCδ recruitment. PKCε displays a similar translocation pattern to the ER following ATP binding. The localization of nPKCs in the ER membrane suggests possible role in protein synthesis and modification [206].

The allosteric model for PKC activation by lipid cofactors and the concept that membrane translocation is essential for PKC activation have been challenged. For instance, the model predicts that the cellular actions of PKC will be limited to the membranes where lipid cofactors facilitate PKC translocation. However, immunohistochemical studies have shown that the distribution of PKCα does not differ in the longitudinal and circular layers of the swine stomach under resting conditions, being predominantly localized near the plasma membrane, and stimulation with PDBu or carbachol does not alter this peripheral PKCα distribution [207]. Also, PKC is found in other cell compartments like the mitochondria, and in the soluble fraction of cells subjected to oxidative stress, a known activator of PKC [161, 208]. PKCs in the soluble fraction of VSM also phosphorylate contractile proteins located distant from the membrane lipids [161]. PKC translocation may also be dependent on cytoskeletal elements and transport along the cytoskeleton through protein-protein interactions [209–211]. Another misconception of the canonical model of PKC activation is that PKC catalytic activity is an inherent property of the enzyme that is not altered by the activation process; a model that does not explain the diverse and often opposing actions of certain PKCs [161].

7.3. PKC Phosphorylation

In the inactive PKC, both the regulatory and catalytic domains are folded together and the pseudosubstrate binds the catalytic site in the C4 region [212]. In the activated state, PKC unfolds, the pseudosubstrate dissociates from the C4 region, and PKC is ready to target its true substrate (Fig. 4). Before it becomes catalytically competent and able to respond to its allosteric activators, nascent PKCs undergo phosphorylation by a PKC kinase and

autophosphorylation at three conserved Ser/Thr phosphorylation sites in the activation loop, turn motif, and hydrophobic motif of the C-terminal domain [158, 213, 214]. Phosphorylation changes PKC protein conformation and electric charge and affects its lipid affinity and binding to the plasma membrane. Phosphorylation keeps PKC in a catalytically competent and protease resistant conformation. Full activation of PKC by allosteric activators induces an open conformation that makes it susceptible to phosphatases and proteases, leading to either repeated autophosphorylation/dephosphorylation cycles, or proteolytic degradation of the PKC molecule and *de novo* synthesis of the enzyme [214, 215].

The first critical and rate-limiting phosphorylation of the activation loop at the conserved threonine is catalyzed by phosphoinositide-dependent kinase (PDK) [156, 216]. Mutation of phosphorylatable Thr-residues in the activation loop abolishes PKC activity [217, 218], and in the absence of PDK-1, PKC is prone to rapid degradation [219]. Phosphorylation of the activation loop introduces a negative charge that properly aligns residues to form a competent catalytic domain and facilitates the subsequent autophosphorylation of the ‘turn motif’ (which corresponds to a phosphorylation site in protein kinase A (PKA) localized at the apex of a turn), and the hydrophobic motif [220]. The hydrophobic motif is important for PKC stability, functioning as a docking-site for PDK-1 through its repeated negatively charged aspartate sequence termed PDK-1 interacting fragment [213, 219]; an interaction that allows PDK-1 to access the activation loop [221]. PDK-1 and mTOR are upstream kinases that promote PKC phosphorylation in different motifs [216, 222, 223]. Phosphorylation of the turn motif by the mTORC2 complex triggers autophosphorylation of the hydrophobic motif [224, 225]. In VSMCs, α-adrenergic receptor agonists induce translocation of the actin-binding protein calponin (CaP) from the contractile filaments to VSMC cortex, and promote CaP-dependent phosphorylation of PDK at S241, PKCα phosphorylation at the activation loop T497, and autophosphorylation at the hydrophobic motif [154]. Autophosphorylation of the turn motif contributes to relative stability of PKCδ. The aPKCs are phosphorylated at the activation loop and turn motif, and contain glutamate ‘phosphomimetic’ residues in their hydrophobic motif [213, 214, 226], while the hydrophobic motif of nPKCs contains an aspartate residue [226].

PKC phosphorylation may occur only during maturation of the newly synthesized enzyme, as with PKCα, or is dynamically regulated, as with nPKCs [227–229]. Phosphorylation of multiple sites is required for activation of mature PKCs, e.g. during H_2O_2-induced tyrosine phosphorylation of PKCδ [230]. Also, in cardiomyocytes, PKCδ and PKCε undergo phosphorylation of the activation loop and hydrophobic motif even in the absence of allosteric regulators [227], supporting that the regulatory pathways of PKC are isoform- and cell-specific.

The scaffold protein 14-3-3 serves as a partner of phosphorylated PKCε in mammalian cells. Phosphorylation of PKCε on Ser346 and Ser368 is required for binding to 14-3-3, and locks the enzyme in an open, active and lipid-independent conformation [164, 231]. On the other hand, direct interaction between PKCθ and 14-3-3 tau has been observed in T cells, and 14-3-3 overexpression inhibits PKCθ translocation and function [232].

Other phosphorylation patterns may be specific to certain PKC isoforms. PKCδ has tyrosine phosphorylation sites, and tyrosine-phosphorylated PKCδ is constitutively active and does not require DAG as a cofactor [208]. Tyrosine phosphorylation also underlie redox control of PKCδ activity. A Src family kinase (Lck)-driven phosphorylation of PKCδ at Tyr311 in rodents (Tyr313 in human) mediates H_2O_2-dependent increase in PKCδ activity [161, 208].

PKC phosphorylation has been used as a marker of its activation [233, 234]. S299-phosphorylated PKCδ is localized at both the plasma and nuclear membranes, making it the best marker of the activated enzyme [233]. However, PKCδ is phosphorylated at other sites and undergoes autophosphorylation at three sites in its V3 region (S299, S302, S304), each of which is evolutionarily conserved and unique to PKCδ. S643 is another PKCδ autophosphorylation site [235] that may not be an ideal marker of activation because it is relatively resistant to dephosphorylation and remains phosphorylated even when PKCδ releases DAG and adopts a ‘closed’ conformation [214, 233].

PKC kinase activity is terminated by dephosphorylation, when PKC is in an “open” conformation unbound by the pseudosubstrate or constitutively active [236–238]. For cPKCs and nPKCs, dephosphorylation is carried out by the PP2C member pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) at the hydrophobic motif, which drives PKC to be further dephosphorylated by PP1/PP2A protein phosphatases at the turn motif [215, 237, 239, 240]. Phosphatases also indirectly affect PKC, e.g. dephosphorylation of the PKCθ downstream molecules CARMA1 by PP2A leads to PKCθ deactivation [241]. Dephosphorylation predisposes “naked” protein kinases to ubiquitination and degradation [242]. Partial inhibition of phosphorylation is caused by binding with HSP70, thus promoting rephosphorylation of PKCs and their subsequent reactivation [155, 243].

PKC-priming phosphorylation is also influenced by the inferred allosteric behavior caused by ATP binding. Nucleotide pocket occupation promotes a PKC conformation that is conducive to upstream kinases and protective from phosphatases. When the PKC kinase domain is compromised through mutation of the highly conserved lysine residue responsible for coordination of the α-β phosphates of ATP, it fails to be primed, but can be fully primed upon binding an ATP-competitive PKC inhibitor. Expression of inactive PKCε K437M mutant in HEK cells led to accumulation of inactive PKCε lacking phosphorylation at the priming sites, while wild-type PKCε expressed under the same conditions was constitutively phosphorylated at all priming sites. The PKC inhibitor bisindolylmaleimide induced rapid phosphorylation of the priming sites of PKCε K437M–expressing cells, but did not increase phosphorylation of wild-type PKCε, suggesting that the conformation induced by occupation of the nucleotide pocket of PKCε K437M with an inhibitor was sufficient to promote priming. Similarly, the active site PKC inhibitor Gö 6983 locks PKC in a conformation in which the priming phosphorylation sites are resistant to dephosphorylation and down-regulation by phorbol esters [244]. These findings have suggested that autophosphorylation is not critical for PKC priming, and that ATP pocket occupation is sufficient for maturation and activity of the kinase [164, 245].

7.4. PKC Activators

PKCs are activated by hormones such as epinephrine and AngII, growth factors including epidermal growth factor and insulin, and neurotransmitters like dopamine and endorphin through the hydrolysis of PIP_2 and the generation of DAG [157]. High $[Ca^{2+}]_c$ can also activate PLC and lead to PKC activation [157]. PKC isoforms respond differently to Ca^{2+}, PS, DAG, and other phospholipids. cPKCs bind Ca^{2+} in a phospholipid-dependent manner, and Ca^{2+} may form a “bridge” holding the protein and phospholipid complex together at the membrane [246]. PS is indispensable for activation of PKC. Phosphatidylinositol and phosphatidic acid activate PKC at high Ca^{2+} concentrations. DAG activates PKC by reducing its Ca^{2+} requirement and enhancing its membrane association [247]. Lipids derived from sources other than glycerolipid hydrolysis such as cis-unsaturated free fatty acids and lysophosphatidylcholine, ceramide (a sphingomyelinase product), phosphatidylinositol 3,4,5-trisphosphate, and cholesterol sulfate can also activate PKC [248].

Phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) activate PKC and stabilize its membrane association by reducing its apparent K_m for Ca^{2+} [169]. PMA binds to PKC 1000-fold more strongly than DAG [249, 250]. PMA binding to the PKC C1B domain alone does not induce sufficient conformational change or release the pseudosubstrate from the catalytic core, but generates a hydrophobic cap covering polar groups that helps PKC to insert into membrane lipids [251].

DAG analogs and phorbol esters are not specific for a particular PKC isoform, and have other effects unrelated to PKC. For example, PMA recruits both cPKCs and nPKCs to the plasma membrane [206]. PKC activators often activate cPKCs isoforms to the greatest degree, then the nPKCs and aPKCs [252]. Also, the DAG analog 1,2-dioctanoyl-sn-glycerol (DiC8) blocks K_v, BK_{Ca} and K_{ATP} channels of mesenteric artery VSM in a PKC-independent manner. 1-oleoyl-2-acetyl-sn-glycerol (OAG) is a related compound that activates PKC without blocking K^+ channels, and is a preferred over DiC8 as a pharmacological tool to study PKC [253].

Post-translational modifications affect PKC activity. Proteolysis in the hinge region activates PKCδ [254]. Oxidation, acetylation, nitration and phosphorylation also activate PKC [153]. Oxidants such as H_2O_2 activate PKC by oxidative modification of both the regulatory and catalytic domains [255]. The zinc-binding cysteine-rich motifs of the N-terminal regulatory domain are particularly susceptible to oxidative modification [256]. Hydroquinone, catechol, and whole cigarette smoke condensate activate PKC in Lewis lung carcinoma cells [257].

7.5. PKC Substrates

When PKC is not catalytically active, the basic autoinhibitory pseudosubstrate is protected from proteolysis by an acidic patch in the substrate-binding site. When PKC is activated, it phosphorylates arginine-rich protein substrates, which neutralize the acidic patch and displace the pseudosubstrate from the kinase core [158, 212]. The amino acid sequence near the substrate phosphorylation site assist in PKC substrate recognition. Several PKC substrates have been identified (Table 2). PKCα, β, and γ are potent histone IIIS kinases,

while PKCδ, ε, and η have a poor capacity to phosphorylate histone [169]. PKC isoforms show overlapping specificities for substrates derived from modification of their pseudosubstrate regions. For example, the PKC targeting protein AKAP79 binds the catalytic core of all PKCs through a pseudosubstrate-like mechanism [258, 259]

PKC substrates include the anchoring proteins STICKs such as MARCKs, MacMARCKs, α-, β-, and γ-adducin, clone 72 (SseCKs), GTP-binding proteins and cytoskeletal proteins [185, 187, 221]. PKC causes phosphorylation of the inhibitory GTP-binding protein Gi, facilitating the dissociation of its αi subunit from adenylyl cyclase and thus relieves it from inhibition [169]. PKC phosphorylates and activates adhesion molecules such as focal adhesion kinase, paxillin, and vinculin [260–263]. PKC phosphorylation of the cytoskeletal protein vinculin affects cell shape and adhesion [264]. PKC also phosphorylates substrates involved in protein trafficking. Recycling of β1-integrins to the plasma membrane requires PKCε-mediated phosphorylation of vimentin, an intermediate filament protein upregulated upon epithelial cell transformation. Inhibition of PKC and vimentin phosphorylation causes integrins to become trapped in vesicles and attenuates directional cell motility. *In vitro* reconstitution assays showed that PKCε dissociates from integrin containing endocytic vesicles in a selectively phosphorylated vimentin-containing complex. Mutations of PKC-regulated sites on vimentin lead to accumulation of intracellular PKCε/integrin positive vesicles, while introduction of wild-type vimentin promotes cell motility in a PKCε-dependent manner, supporting that PKC-mediated phosphorylation of vimentin is a key process in integrin trafficking and cell motility [265]. PKC also plays a role in phosphorylation and nucleo-cytoplasmic shuttling of S6KβII, one of the forms of the ribosomal protein S6 kinase (S6K) involved in the regulation of protein synthesis and the G1/S transition in the cell cycle, and this PKC-mediated phosphorylation is induced by PMA, EGF, IGF-1, and platelet-derived growth factor (PDGF) [266]. Myosin binding protein-C slow (MyBP-C slow) is a thick filament-associated protein, that plays a role in the formation of actomyosin cross-bridges, and its activity is regulated by PKC-mediated phosphorylation at Ser-83 and Thr-84 [267]. The list of PKC substrates is growing and many of these substrates play a role in VSM contraction and growth.

7.6. PKC Inhibitors

Several PKC inhibitors have been developed (Table 3). The first generation PKC inhibitors such as H7 and staurosporine are nonspecific pan-PKC inhibitors that inhibit all PKC isoforms [268]. H7 and staurosporine bind to and compete with ATP in the catalytic domain. Because the ATP hydrophobic pocket is conserved throughout the kinome, ATP-binding PKC inhibitors have poor selectivity and intact with other ATP-binding kinases and cause severe side effects *in vivo* [157, 269]. Some PKC inhibitors targeting the ATP-binding site such as indolcarbazole and bisindoylmaleimide have shown selectivity, e.g. ruboxistaurin is a relatively selective PKCβ inhibitor [270, 271]. PKC inhibitors competing at the DAG/phorbol ester or the PS binding site are more specific. Calphostin C binds to the C1 domain, mimicking DAG-binding [157]. Extended exposure to phorbol esters downregulate PKCα, β and γ [272], but phorbol esters have tumor-promoting properties.

Peptides that interfere with the intramolecular interactions within PKC have been developed [191]. For instance, myr-ψPKC, a myristoylated peptide based on the substrate motif of PKCα and PKCβ, inhibits TPA-induced PKC activation and phosphorylation of MARCKS [273]. Other peptides disrupt protein-protein interactions between the PKC regulatory domain and RACK [157]. The interaction of PKC and RACK is isoform selective and largely involves the C2 region of cPKC, and peptide fragments of this region may serve as selective cPKCs inhibitors [188]. Also, a peptide derived from the PKC binding proteins annexin I and RACKI inhibits translocation of PKCβ [187].

The autoinhibitory role of the PKC pseudosubstrate has been suggested from the observation that deletion of the pseudosubstrate site abrogates the inhibitory effect of the regulatory domain of PKCα on the full-length enzyme [274]. Synthetic oligopeptides based on pseudosubstrate sequence are specific PKC inhibitors because they exploit its substrate specificity and do not interfere with ATP binding [212, 258, 273] (Table 3). The synthetic peptide (19–36) inhibits PKC autophosphorylation and protein substrate phosphorylation. Replacement of Arg-27 with alanine in the peptide [Ala-27]-PKC(19–31) increases the IC_{50} for inhibition of substrate phosphorylation. A structure-function study of the PKC pseudosubstrate sequence R19FARK-GALRQKNV31 examined the role of specific residues using an alanine substitution scan. Arg-22 was the most important determinant in the inhibitor sequence, since its substitution by alanine led to a 600-fold increase in its IC_{50}. Substitutions of other basic residues with Ala-19, Ala-23 and Ala-27 increased the IC_{50} 5-, 11- and 24-fold, respectively. The importance of basic residues in determining the potency of the pseudosubstrate peptide reflects the requirement of these residues in peptide substrate phosphorylation. Gly-24, Leu-26 and Gln-28 residues are also important for pseudosubstrate inhibitor potency. The large increase in the IC_{50} for the [A22]PKC(19–31) peptide makes it a valuable control in studies utilizing the pseudosubstrate peptide to examine functional roles of PKC [275]. Pseudosubstrate inhibitors are also more specific because the pseudosubstrate region provides a large interface for multiple points of contact with the PKC molecule [191, 258]. However, a cell-penetrating myristoylated PKCζ pseudosubstrate inhibitor peptide (ZIP) shows affinity for all PKC isoforms and disrupts PKC translocation, suggesting that some pseudosubstrates have well-conserved residues [258]. Also, mutation of alanine in the pseudosubstrate with serine or glutamate, mimics the charge of a phosphorylated residue and in effect activates PKC [209, 274, 276].

Some compounds like β-adrenoceptor activators and antioxidants counteract the effects of PKC. In portal vein, stimulation of β-adrenoceptors activates cAMP-dependent protein kinase (PKA) and in turn opposes the effects of PKC, causes vasodilatation and inhibits store-operated Ca^{2+} entry [277, 278]. The PKC catalytic domain contains several reactive cysteines that can be targeted by antioxidants such as selenocompounds, vitamin E, and curcumin [256, 279, 280]. Also, α-tocopherol inhibits the expression, activity, and phosphorylation of PKCα and decreases VSM proliferation, and these effects are not mimicked, and even be opposed, by β-tocopherol [281, 282]. In animal models, hyperglycemia-induced retinal vascular dysfunction is prevented by α-tocopherol likely through inhibition of DAG-PKC [282]. Vitamin E decreases hyperglycemia-induced DAG and PKC activity and reverses the changes in the retinal and renal vessels in diabetes [283]. Glutathione inhibits PKC via a nonredox mechanism [284]. Tamoxifen is an estrogen

receptor antagonist and a PKC inhibitor, and some tamoxifen analogs have high affinity and selectivity to PKC [285]. PKCZI195.17 is a novel inhibitor with high efficacy and specificity to PKCζ [286], and CGX1037 is a specific PKCδ inhibitor in platelets [287].

Post-translational modifications of PKC affect its activity. S-nitrosylation, a ubiquitous protein modification in redox-based signaling that forms S-nitrosothiol from nitric oxide (NO) on cysteine residues, decreases PKC activity and contraction in mouse aorta, and may represent a key mechanism in conditions associated with decreased vascular reactivity [288].

Transgenic animals, knockout mice and antisense techniques have been used to study the effects of PKC downregulation *in vivo*. PKC knockout mice have shown a critical role of PKC in the endocrine and immunological systems, and further characterization of the PKC knockout vascular phenotype should shed more light on the role of PKC in the vascular system. Also, antisense and siRNA for specific PKC isoforms can be used to study the role of PKC in various cell functions. ISSI-3521 is a phosphorothioate antisense oligonucleotide that targets the 3′-untranslated region of PKCα mRNA, and causes reduction of PKCα expression in cancer cell lines and human tumor xenograft models [269, 289].

Thus, while the ~70% homologous structure of the PKC catalytic domain poses a challenge in the development of specific inhibitors of PKC isoforms, the C2 region is less conserved among different PKCs, and pharmacological tools that target the C2 region are more selective [157]. The V5 region may also be a good target for isoform-specific PKC inhibitors. The PKC-substrate interaction can be selectively disrupted by peptide inhibitors that share the same substrate sequence. Also, protein-protein interactions can regulate the localization and activity of PKC isoforms [157].

7.7. PKC and VSM Contraction

PKC isoforms show diverse effects in different cell types including VSM. The role of PKC in vascular responses has been supported by measuring PKC mRNA expression, protein levels and activity, and by testing the effects of PKC inhibitors and the vascular changes in PKC knockout mice and transgenic rats [290]. PKC can affect VSM contraction through regulation of ion channels, pumps and $[Ca^{2+}]_c$, Ca^{2+} sensitization of the contractile proteins, and activation of Ca^{2+} independent contraction pathways. PKC translocation to the cell surface could also trigger a cascade of protein kinases that ultimately interact with the contractile myofilaments and cause VSM contraction. In some instances, PKC inhibits VSM contraction.

7.8. PKC, Ion Channels, and $[Ca^{2+}]_c$

PKC modulates the activity of plasmalemmal K^+ and Ca^{2+} channels, and in turn $[Ca^{2+}]_c$. K^+ channels play a role in the regulation of the resting membrane potential, and inactivation of K^+ channels causes membrane depolarization, elevation of $[Ca^{2+}]_c$ and VSM contraction [291]. Membrane depolarization activates Ca^{2+} entry through L-type VDCCs and could facilitate Ca^{2+} release from IP_3- and ryanodine-sensitive Ca^{2+} stores [292, 293]. Large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are the predominant K^+ channels in VSMCs [291, 294]. PKC activation by PDBu inhibits BK_{Ca} and increases vascular tone [295–298] in pulmonary [296], coronary [299], cerebral [300], and uterine vessels [301].

PKC activators inhibit BK_{Ca} by phosphorylation of the channel protein and decreasing its sensitivity to activation by cGMP-dependent protein kinase [302, 303].

Voltage-gated K^+ channels (K_v) can be modulated by vasoconstrictors such as arginine-vasopressin, ET-1 and AngII through activation of PKC. In rat mesenteric artery VSMCs, vasopressin regulates Kv7.4 and Kv7.5 subunits of Kv7 channels via activation of PKC. PKCα-dependent phosphorylation of the K^+ channel proteins on serine residues is sufficient to reduce Kv7 channel activity, and the extent of PKC-mediated Kv7.4 and Kv7.5 phosphorylation and K^+ current suppression depends on the subunit composition of the channel proteins [304]. Also, thromboxane A2 induces pulmonary vasoconstriction by a mechanism involving PKCζ and inhibition of Kv [305]. In rabbit coronary arterial VSMCs, ET-1 and AngII inhibit Kv currents by activating PKCε, and inhibit K_{IR} channel by activating PKCα [306, 307].

Vasoconstrictor agonists also inhibit K_{ATP} through PKC signaling [291, 308]. Phorbol esters inhibit K_{ATP} currents in mesenteric arteries [309]. In human embryonic kidney cells (HEK293) AngII and PDBu induce PKC-mediated inhibition of K_{ATP} channel through the formation of channel complexes comprising four Kir6.1 and their associated SUR2B subunits [310]. PKC-mediated phosphorylation of K_{ATP} alters the channel properties, kinetics and/or number at the cell membrane [311, 312]. Protein trafficking studies have suggested that PKC initiates internalization and inactivation of K_{ATP} channel complex [313].

7.9. PKC, Ion Pumps, Co-transporters, and $[Ca^{2+}]_c$

PMCA and SERCA are important Ca^{2+} removal mechanisms in VSM. PKC activates PMCA or SERCA, leading to Ca^{2+} extrusion and re-uptake and decreased VSM $[Ca^{2+}]_c$. In isolated cardiac SR preparations, PKC activates SERCA [314]. Also, PKC-mediated inhibition of the α1 subunit of Na^+/K^+-ATPase affects the intracellular concentrations of Na^+ and K^+ and in turn membrane potential [315]. PKC activation by phorbol esters and DAG analogs phosphorylates and activates the Na^+/H^+ antiport exchanger and thereby increases the cytoplasmic pH, leading to alkalinization which generally increases vascular contraction [316–319].

7.10. PKC and Ca^{2+}-Sensitization of Contractile Proteins

PKC activation increases the myofilament force sensitivity to $[Ca^{2+}]_c$, thereby maintaining VSM contraction with smaller increases in $[Ca^{2+}]_c$. In pulmonary arteries, PKC inhibitors attenuate ET-1 induced constriction and $[Ca^{2+}]_c$ and the vasoconstrictor responses associated with store-operated Ca^{2+} entry, suggesting that PKC contributes to both Ca^{2+} influx and Ca^{2+} sensitization [320]. The nPKC isoforms play an important role in mediating VSM contraction through a Ca^{2+} sensitizing pathway, and inhibition of nPKCs attenuates norepinephrine-induced VSM contraction [321]. PKC phosphorylates CPI-17, which in turn inhibits MLC phosphatase, increases MLC phosphorylation, and enhances VSM contraction [322]. PKC also inhibits MLC-phosphatase through phosphorylation of myosin phosphatase target subunit 1 (MYPT1) [323]. PKC may contribute to VSM contraction in a MLC phosphorylation-independent manner. In rat middle cerebral artery, PKC activation by PDBu is associated with sustained vasoconstriction that is much larger than that expected with the

same level of MLC phosphorylation achieved by 5-HT [323]. Also, activation of PKCα causes phosphorylation of the actin-binding protein CaP, and thereby reverses its inhibition of actin-activated myosin ATPase, and enhances VSM contraction. Interestingly, CaP activates PKC *in vitro* in the absence of lipid cofactors, and knockdown of CaP inhibits PKC-dependent contraction in ferret arterial VSM [5, 324].

PKC may also be involved in stretch-induced vascular myogenic response. In rat cerebral artery VSMCs, DAG, PMA and cell swelling activate a cation current through stretch-activated TRP channels, and these effects are blocked by PKC inhibitors. It has been suggested that myogenic tone involves mechanotransduction through stimulation of PLC, hydrolysis of phosphoinositides, DAG production, and PKC activation, which increase cation channel activity, and the associated depolarization activates L-type VDCCs, leading to increased $[Ca^{2+}]_c$ and vasoconstriction [277, 325]. PKCδ, PKCθ and PKCμ participate in VSM mechanotransduction in response to mechanical and cyclic stretch [263, 326].

AngII, ET-1, serotonin, norepinephrine, and neuropeptide Y activate PKC-dependent pathways and cause VSMC membrane depolarization and contraction [308, 327, 328]. Of note, AngII activates multiple PKC isoforms in VSM [329] and PKC increases VSM contraction via other pathways involving downregulation of atrial natriuretic peptide (ANP) receptor and decreased ANP-induced inhibition of contraction [330]. PKC affects not only arterial but also venous contraction. In deoxycorticosterone salt-sensitive rat model of hypertension ET-1 increases venomotor tone, suggesting a role of the venous system in regulation of blood pressure likely through increases in venous return and cardiac output [331]. The PKC inhibitor chelerythrine attenuates ET-1-induced contraction in both the aorta and vena cava. However, in the aorta, ET-1-induced contraction is dependent on PLC activation and IP_3-mediated Ca^{2+} release, while in the vena cava ET-1 induced contraction is unaffected by the IP_3 receptor antagonist 2-APB. Also, only the vena cava contracts in response to the DAG analog OAG, highlighting the differences in the venous and arterial pathways of contraction [331]. Endothelium-derived NO also regulates VSM tone by activating guanylate cyclase, increasing cGMP and promoting vasodilation, and PKC inhibits NO-mediated vasodilation by inhibiting guanylate cyclase, leading to increased vasoconstriction [332].

7.11. PKC and Cytoskeletal Proteins

Studies in cerebral microvessels have shown that PKC mediates myogenic constriction through dynamic reorganization of the cytoskeleton and increased actin polymerization [333]. Also, both in the presence and absence of Ca^{2+}, PKC promotes cerebral vasoconstriction by increasing the phosphorylation of paxillin and HSP27, reducing G-actin content, and promoting actin cytoskeleton reorganization [323]. In rat middle cerebral arteries, PDBu-induced constriction is more sensitive to disruption of actin cytoskeleton compared to inhibition of cross-bridge cycling, supporting the pivotal contribution of PKC-mediated cytoskeletal actin polymerization to force generation in cerebral arteries [323].

PKC modulates certain genes that code for structural proteins such as fibronectin and type IV collagen by changing the binding of nuclear transcription factors to the promoter regions on responsive genes [268]. PKC affects the expression of the regulator of G-protein

signaling 2 (RGS2), which affects vascular tone. In cultured VSMCs, adrenotensin increases RGS2 expression, while the PKC inhibitor chelerythrine reduces RGS2 expression, suggesting that adrenotensin increases RGS2 expression via a PKC-mediated pathway [334].

7.12. PKC-Dependent Signaling Cascades

PKC activation may trigger a cascade of protein kinases that ultimately stimulate VSM contraction. PKC affects Akt signaling [260, 335]. Also, mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK), p38 and JNK are common downstream effectors of PKC [336, 337]. MAPK is a Ser/Thr kinase that is activated by its dual phosphorylation at Thr and Tyr residues. PKC, MAPK, and c-Raf-1 have been implicated in VSM growth. In cultured VSMCs, MAPK is mainly cytosolic, and translocates to the nucleus during activation by mitogens [338]. Tyrosine kinase and MAPK activities have also been identified in differentiated contractile VSM. During VSM activation, MAPK transiently translocates to the plasma membrane, then undergoes redistribution to the cytoskeleton during maintained VSM contraction [339]. DAG promotes translocation of cytosolic PKCɛ to the plasma membrane, where it is fully activated. Activated PKCɛ then stimulates the translocation of cytosolic MAPK kinase (MEK) and MAPK to the plasmalemma, where they form a kinase complex. PKC phosphorylates and activates MEK, which in turn phosphorylates MAPK at both Thr and Tyr residues. Tyrosine phosphorylation targets MAPK to the cytoskeleton, where it phosphorylates the actin-binding protein caldesmon (CaD) and reverses its inhibition of Mg^{2+}-ATPase activity, thus increasing actin-myosin interaction and VSM contraction [5, 339]. Also, in aortic VSM, phenylephrine promotes CaP-dependent PKC autophosphorylation and activation, followed by a delayed ERK activation, CaD phosphorylation and VSM contraction [154]. These PKC-dependent pathways function in parallel with the spike in $[Ca^{2+}]_c$ and MLC phosphorylation to enhance VSM contraction [154]. Biochemical studies have shown that PDBu does not directly change the phospho-content of CaP or CaD [323], supporting that other kinases downstream of PKC cause phosphorylation of CaP or CaD [154]. In, esophageal smooth muscle, PKC-dependent resting tone and contraction are associated with ERK and HSP27-linked p38 MAPK phosphorylation [340], supporting that MAPK serves as a link in the signal transduction cascade between membrane-bound PKC and smooth muscle contraction.

7.13. PKC and Vasodilation

PKC affects Ca^{2+} channel permeability in VSM. In VSMCs, GPCR agonists affect ROCs, TRPCs, and SOCs activity via activation of PKC. While low levels of DAG activate TRPC6 via a PKC-independent mechanism, high levels of DAG inhibit TRPC6 SOCs activity in a PKC- and Ca^{2+}-dependent manner [277, 341]. In mesenteric artery and ear artery VSMCs, DAG inhibits TRPCs through a PKC-dependent pathway, and such mechanism may limit ROC activity at high agonist concentrations [342].

The 20-kDa MLC and MLC kinase also serve as substrates for PKC, and their phosphorylation counteracts Ca^{2+}-induced actin-myosin interaction and VSM contraction [343]. In human VSMCs, PKC activation stimulates secretion of C-type natriuretic peptide (CNP), a known endogenous vasodilator [344, 345]. PMA increases CNP expression via PKCα- and PKCδ-mediated pathways, and PDGF increases CNP in SMCs via a PKCδ-

dependent pathway [346]. PKC also regulates the activity of endothelial NO synthase, and in turn NO production and vasodilation [321].

8. Rho Kinase

GPCR agonists, particularly those coupling to Ga12/13 proteins, can also activate the small G-protein RhoA. In its active GTP-bound form, RhoA activates Rho-associated coiled-coil protein kinase or Rho-kinase (ROCK), which then phosphorylates and inhibits MLC phosphatase, increases MLC phosphorylation and promotes VSM contraction. The ROCK-mediated enhancement of VSM contraction often occurs in the absence of substantial increases in $[Ca^{2+}]_c$ and is therefore considered a Ca^{2+} sensitization mechanism [347–350].

8.1. ROCK Structure and Isoforms

ROCK was initially described in the mid 1990s as a member of AGC family of protein kinases [351, 352], and a major target of the small GTPase RhoA. ROCKs are Ser/Thr kinases with a molecular mass of ~160 kDa. Two ROCK isoforms encoded by two different genes have been identified: ROCK-1 (ROCK-I, ROKα) and ROCK-2 (ROCK-II, ROKβ) [351, 353–356]. Human ROCK-1 and ROCK-2 genes are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively. ROCK structure comprises a kinase domain located at the N-terminus of the protein (amino acids 1–420), a coiled-coil region containing the Rho-binding domain, and a pleckstrin-homology domain with a cysteine-rich domain at the C-terminus that helps in ROCK binding to the plasma membrane (Fig. 5). ROCK-1 and ROCK-2 are highly homologous, with 65% overall amino acid sequence identity; 92% in the kinase domain and 58% in the Rho-binding domain [355].

8.2. Tissue Expression of ROCK

ROCK-1 and ROCK-2 are ubiquitously expressed. Both ROCK-1 and ROCK-2 are expressed in VSM and the heart [357], and ROCK-2 mRNA is highly expressed in the brain and skeletal muscle [355, 358]. ROCK-1 and ROCK-2 expression is up-regulated by AngII via angiotensin type 1 receptor and by interleukin-1β [359], possibly through PKC- and nuclear factor κB-dependent pathways. Chronic administration of AngII in mice causes up-regulation of ROCK in the coronary artery [359].

8.3. Subcellular Distribution of ROCK

Similar to other GTPases, RhoA cycles between an inactive guanosine diphosphate (GDP)-bound form and an active GTP-bound form. In unstimulated cells, RhoA resides mainly in the cytosol bound to GDP, but upon receptor stimulation it undergoes translocation to the plasma membrane where GDP–GTP exchange takes place [350]. RhoA translocation to the plasma membrane is facilitated by the hydrophobic geranylgeranyl tail that is attached to its C-terminal during post-translational modification of the protein. ROCKs are also essentially distributed in the cytosol and are partially translocated to the plasma membrane during activation by RhoA [351, 354]. The mechanisms responsible for translocation of ROCKs could be similar to those involved in the translocation of PKC.

8.4. Regulation of ROCK Activity

The C-terminal of ROCK contains an autoinhibitory region [360], including the pleckstrin-homology domain and Rho-binding domain, which binds to the N-terminal kinase domain and inhibits ROCK activity [361] (Fig. 5). During ROCK activation, the binding of RhoA disrupts the interaction between the C-terminal autoinhibitory region and kinase domain and yields an active kinase. Truncated forms of ROCK lacking the C-terminal region that contains the Rho-binding domain and pleckstrin-homology domain are constitutively active. However, when the C-terminal region of ROCK is expressed in cells, it acts as dominant negative [362], suggesting that the C-terminal region is a negative regulatory region responsible for autoinhibition of kinase activity in resting cells [363]. Binding of active GTP-bound form of RhoA to Rho-binding domain increases the phosphotransferase activity of ROCK 1.5- to 2-fold [364]. Arachidonic acid is a metabolic by-product of the agonist–receptor interaction produced from the hydrolysis of membrane phospholipids by phospholipase A2 or through the transformation of DAG to arachidonic acid by DAG lipase. At micromolar concentrations, arachidonic acid inhibits MLC phosphatase and Ca^{2+}-dependent MLC phosphorylation [365]. Arachidonic acid and sphingosine phosphorylcholine interact with the regulatory region and pleckstrin-homology domain of ROCK, disrupt their inhibitory action on the catalytic domain, and increase ROCK activity 5- to 6-fold independently of RhoA [363, 364, 366].

Dimerization affects ROCK activity and affinity for ATP [367]. ROCK2 harbors a C-terminal extension within the kinase domain that contains a hydrophobic cluster of phenylalanine and tyrosine residues surrounding a key threonine residue. The hydrophobic motif at Thr405 of ROCK is essential for substrate phosphorylation and kinase domain dimerization. Mechanistically, both ROCK2 activity and dimerization are dependent upon the interaction between Thr405 of the hydrophobic motif and Asp39 of the N-terminal extension. The observation that ROCK2 hydrophobic motif requires association with the N-terminal extension for kinase activity provides the rationale for the development of small-molecule inhibitors designed to block ROCK activation by selectively interfering with hydrophobic motif-mediated activation and dimer formation [368].

Other Rho proteins exert negative control on ROCK activity. The small G-protein RhoE binds to the N-terminal region of ROCK1 containing the kinase domain, thus preventing RhoA from binding to Rho-binding domain and inhibiting ROCK [369]. Other small G proteins such as Gem and Rad bind and inhibit ROCK through an unclear mechanism [370].

8.5. ROCK Substrates

Activated RhoA interacts with the Rho-binding domain of ROCK and induces a conformational change that allows the interaction of the Ser/Thr kinase with its substrates [371]. The consensus sequence of ROCK phosphorylation site is RXXS/T or RXS/T [372–375]. ROCKs require basic amino acids such as Arg (R) close to its phosphorylation site. Several ROCK substrates have been identified, and the functional consequence of their ROCK-mediated phosphorylation is often related to actin filament formation and organization, and cytoskeleton rearrangements [376, 377]. Proteomics analysis has identified more than 100 proteins as potential substrates of ROCK [378].

ROCK targets include the myosin phosphatase target subunit-1 (MYPT-1) [379], CPI-17 [380], the 20-kDa MLC [372], and CaP [381], which are known effectors of VSM contraction. MYPT-1 is a major effector of ROCK-mediated Ca^{2+} sensitization of smooth muscle contraction. Cardiac troponin is another ROCK substrate, but ROCK-mediated phosphorylation of troponin reduces contraction in cardiac myocytes [382].

Phosphatase and tensin homologue (PTEN) is another ROCK substrate [383]. PTEN dephosphorylates the phosphatidylinositol 3-kinase (PI_3-kinase)/Akt pathway involved in the regulation of mRNA transcription, protein synthesis, and cell growth and survival. Phosphorylation of PTEN by ROCK stimulates its phosphatase activity, and ROCK inhibitors reduce ROCK-mediated PTEN phosphorylation and enhance Akt signaling in endothelial cells [384]. Activated ROCK also phosphorylates the insulin receptor substrate-1 (IRS-1) in VSMCs, leading to inhibition of insulin-induced IRS-1 tyrosine phosphorylation and PI_3-kinase activation [385]. The interaction between ROCK and IRS-1 is increased, and insulin signaling is markedly decreased in VSMCs from hypertensive rats [385].

Because the kinase domains of ROCK-1 and ROCK-2 are nearly identical, it has been thought that they share the same substrates. The N-terminal region that precedes the kinase domain determines the substrate specificity of the ROCK isoforms [369]. ROCK-1, but not ROCK-2, binds to and phosphorylates RhoE [386], while elongation initiation factor-1-α-1 is a ROCK-2 substrate [387].

8.6. ROCK and VSM Function

A large body of evidence suggests important functions of ROCK in VSMCs. A major role of ROCKs is in the organization of actin cytoskeleton, a process involved in multiple cell functions including proliferation, apoptosis, contraction, migration and adhesion [388–390]. ROCK plays a role in phosphorylation and inhibition of MLC phosphatase [391], activation of LIM-kinase 2 and formation of stress fibers, focal adhesions and membrane blebs [392], phosphorylation of ezrin/radixin/moesin [374] and phosphorylation of adducin and regulation of cell motility [375]. RhoA-mediated ROCK activation phosphorylates MYPT-1, the regulatory subunit of MLC phosphatase, and thereby inhibits MLC phosphatase, causes Ca^{2+} sensitization of the contractile proteins, and enhances VSM contraction. While both ROCK-1 and ROCK-2 have been implicated in microfilament bundle assembly and smooth muscle contraction, ROCK-2, but not ROCK-1, binds to and is sensitive to phosphatidylinositol 3,4,5-P_3 [393]. Also, some studies suggest that ROCK-2 is the major isoform regulating VSMC contraction through direct binding to MLC phosphatase [394].

ROCK also regulates cell migration, proliferation, apoptosis/survival, gene transcription, and differentiation. ROCK-1-deficient mice have open eyelids at birth [395]. ROCK-2-deficient mice show placental dysfunction and intrauterine fetal growth restriction [396, 397]. The vascular phenotype of ROCK-1 and ROCK-2 knockout mice needs to be further analyzed.

8.7. ROCK Inhibitors

ROCK activation involves RhoA translocation to the plasma membrane, RhoA binding to ROCK, and ATP-dependent phosphorylation of various substrates. Disruption of prenylation by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) or protein

prenyltransferase inhibitors prevents the membrane translocation and activation of RhoA and impairs its ability to activate ROCK.

Some ROCK inhibitors compete with ATP for binding at the kinase domain, or “active site” of the enzyme. Commonly known ROCK inhibitors include Y-27632 and fasudil. Y-27632 is a synthetic pyridine derivative that inhibits ROCK by competing with ATP for the kinase active site. Y-27632 has ~200- and ~2000-fold higher affinity for ROCK than the structurally similar kinases PKC and MLC kinase, respectively. Y-27632 lowers blood pressure in animal models of experimental hypertension, supporting a link between VSM ROCK and increased blood pressure [397]. Experiments with Y-27632 have also suggested that ROCK activity might be enhanced in the cerebral circulation during chronic hypertension [398–401]. Despite the utility of Y-27632 in numerous studies and its promise as an antihypertensive agent [397, 402, 403], its specificity and safety profile have not been fully verified. Y-27632 inhibits PKC-related kinase (PRK2) with a similar potency to that of ROCK-2 [404]. At 10 μM concentration Y-27632 inhibits PKC-dependent vasoconstriction of the aorta and superior mesenteric artery [405], suggesting that ROCK activation occurs downstream of PKC, or that Y-27632 causes nonselective inhibition of PKC.

Fasudil is an isoquinoline derivative that inhibits ROCK by competing with ATP for the kinase active site. Fasudil has been used to assess the role of ROCK in vascular function in small-scale clinical studies [406–408]. The good safety profile of fasudil contributed to its approval for treatment of cerebral vasospasm following subarachnoid hemorrhage in Japan. Fasudil is a prodrug, and after its oral administration, it is metabolized to the more selective ROCK inhibitor hydroxyfasudil. In porcine model of coronary vasospasm, hyroxyfasudil potently inhibited vasospasm and hypercontraction [409]. Of note, the active site of ROCK is similar to that of protein kinase A (PKA), the target of cAMP that mediates vasodilation and inhibits platelet aggregation, and therefore fasudil inhibits ROCK and PKA with equal potency and is prone to cause clinical side-effects. Although hydroxyfasudil is 15-fold more selective for ROCK than for PKA, the possibility that fasudil could exert unwanted vascular effects if its metabolism is compromised should be considered. Studies have identified amino acid sequence differences between ROCK and PKA, which could be used to further improve the selectivity of ROCK inhibitors [410].

Other ROCK inhibitors include PT-262 which inhibits RhoA-ROCK-MLC pathway by interacting with the ATP-binding site of ROCK protein [411]. DJ4 is a multi-kinase inhibitor of ROCK and MLC kinase in an ATP competitive manner [412]. FSD-C10 and K-115 showed superior neuroprotective effects and lower cytotoxicity compared to fasudil [413, 414]. L-F001 also showed inhibitory effect on ROCK activity [415].

9. VSM Dysfunction and Vascular Disease

Identification of the mechanisms of VSM contraction has helped to understand the mechanisms of vascular disease and to develop new tools for the management of vascular disorders. Increased Ca^{2+} permeability of plasma membrane channels and $[Ca^{2+}]_c$ have been demonstrated in VSMCs isolated from animal models of hypertension and coronary vasospasm [137, 416, 417], and Ca^{2+} channel blockers could be useful in these conditions.

Ca^{2+} antagonist-insensitive forms of hypertension and coronary vasospasm require other treatment modalities that target other pathways such as PKC and ROCK

PKC plays a role in the pathogenesis of vascular restenosis, coronary artery disease, cerebral vasospasm, hypertension, and vascular complications of diabetes [6, 418, 419]. PKC contributes to vascular restenosis following vascular bypass and angioplasty procedures by promoting thrombosis and inflammation and subsequent VSMC migration and proliferation [420]. PKCα, β, δ and θ are expressed in platelets, and cPKCs promote while nPKCs inhibit platelet aggregation and thrombus formation [421]. Knocking out PKCδ or PKCθ potentiates murine platelet aggregation, and the PKCδ inhibitor rottlerin potentiates human platelet aggregation [422]. PKCα, β and ζ also potentiate the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), a key step in leucocyte recruitment and VSMC migration [423–425]. PKCα, β and δ further affect VSMC migration by promoting actin polymerization and cell adhesion [426–428]. PKCα is involved in VSMCs migration induced by ROS [429], and PKCε promotes VSMC migration by upregulating matrix metalloproteinases MMP-2 and MMP-9 [420, 430, 431]. PKC also contributes to VSMC proliferation. PKCβ mediates synergistic proliferative effect of PDGF and high glucose in human coronary VSMCs [432], and the PKCβ inhibitor LY-379196 attenuates DNA synthesis and cell growth [260]. In rat models of aortic balloon injury, the PKCε activator ψεRACK promotes neointimal development, while the PKCε inhibitor εV1-2 reduces luminal narrowing, neointimal proliferation, VSMC ERK phosphorylation, and PDGF-induced VSMC proliferation/migration [433]. Repeated stimulation with phorbol myristate acetate (PMA) downregulates PKCε and inhibits VSMC proliferation [434].

PKCδ contributes to coronary artery disease through decreased ATP generation and increased ROS formation, apoptosis and necrosis [157, 435, 436]. On the other hand, PKCε protects mitochondrial functions and proteasomal activity, activates aldehyde dehydronage 2 (ALDH2) and reduces aldehyde load [157, 189, 437, 438]. A combination of a PKCδ inhibitor and PKCε activator could be useful for organ preservation, and prevention of ischemia-reperfusion injury and graft coronary artery disease in cardiac transplantation [439].

PKCδ has a deleterious effect in cerebral reperfusion by promoting neutrophil migration into ischemic tissue. In a model of transient middle cerebral artery occlusion, PKCδ-null mice showed decreased neutrophil migration into ischemic tissues and a 70% reduction in stroke size compared with wildtype. Transplantation of bone marrow from PKCδ-null mice into wildtype mice reduced infarct size, while bone marrow transplantation from wildtype donors increased infarction size and worsened neurological scores in PKCδ-null mice [440, 441]. Inhibition of PKCδ also improves microvascular pathology and function in transient focal ischemia and reduces ischemic damage in normotensive and hypertensive animals. Therefore, PKCδ could be a therapeutic target for the preservation of cerebrovascular function following stroke, and its inhibition may reduce stroke risk in hypertensive patients [442]. PKCζ mRNA is also induced in the cerebral cortex after focal brain ischemia, and inhibiting PKCζ prevents N-methyl-D-aspartate (NMDA)-induced excito-toxic neuronal cell death [440, 443]. In contrast, PKCε mediates ischemic tolerance in response to cerebral ischemic stress. Systemic delivery of PKCε activator ψεRACK immediately prior to stroke

confers neuroprotection against a subsequent cerebral ischemic event, and decreases microvascular cerebral blood flow thus further contributing to cerebral protection [444].

PKC plays a role in hypertension, and mutations of PKC may influence the individual susceptibility to vascular hyper-reactivity and hypertension. A consistent association has been found between the single nucleotide polymorphism (SNP) rs9922316 in PKCβ gene (*PRKCB*) and inter-individual variation in the constriction responses of dorsal hand vein to the α2-adrenergic receptor agonist dexmedetomidine [445]. PKCα plays a role in Ca^{2+}-dependent contraction of VSM [446], and overexpression of PKCα has been implicated in the pathogenesis of hypertension [447]. In normotensive rats, PKCα is localized mainly in the cytosol of VSMCs, and is hyperactivated and concentrated at VSM plasma membrane in hypertensive animals [447]. Also, PKCδ expression is increased in VSM from spontaneously hypertensive rats, and increases VSM contraction by decreasing BK_{Ca} channel conductance, as the PKC inhibitor chelerythrine restores K^+ channel activity [298].

Sleep apnea could cause systemic and pulmonary hypertension [448, 449]. Chronic hypoxia attenuates store-operated Ca^{2+} entry in pulmonary arteries from Sprague-Dawley rats, while augmenting this response in Wistar rats. PKC inhibition restored store-operated Ca^{2+} entry in Sprague-Dawley arteries, and had no effect in Wistar arteries, suggesting rat strain difference in the role of PKC and store-operated Ca^{2+} entry in the pulmonary arteries following chronic hypoxia [450]. Rat models of sleep apnea produced by exposure to eucapnic intermittent hypoxia show increased circulating ET-1 levels and ET-1-dependent systemic hypertension that is likely mediated by PKCδ-dependent VSM Ca^{2+} sensitization in systemic arteries [449, 451]. On the other hand, intermittent hypoxia mediates a PKCβ-dependent increase in the pulmonary arterial reactivity to multiple vasoconstrictors including ET-1 [452]. In fawnhooded rat model of pulmonary hypertension, PKC inhibits BK_{Ca}, resulting in indirect activation of VDCCs and pulmonary vasoconstriction [453]. PKC-mediated Ca^{2+} sensitization is also demonstrated by an increase in phosphorylated CPI-17 in pulmonary arteries from newborn swine exposed to hypoxia [454]. PKCε may have divergent effects in pulmonary hypertension. PKCε null mice show decreased acute hypoxic pulmonary vasoconstriction, increased Kv3.1b channel expression and membrane hyperpolarization [455]. However, PKCε null mice exposed to chronic hypoxia show a greater pulmonary arterial pressure compared to wild-type mice, and the increase in pressure is reversed by inhaled NO, suggesting that PKCε mediates hypoxic downregulation of NO synthase [456].

Hypertension in pregnancy and preeclampsia are major complications of pregnancy, and placental ischemia/hypoxia could be an initiating event. Hypoxia during pregnancy attenuates the effects of sex steroids, and enhance PKC activity and vascular tone in uterine arteries of pregnant sheep [457]. Increased BK_{Ca} channel activity inhibits PKC-mediated contraction in ovine uterine arteries during pregnancy, and gestational hypoxia upregulates PKC and inhibits BK_{Ca} [458]. Hypoxia also inhibits K_{IR} channels via PKC-dependent mechanism, and contributes to the maladaptation of uterine vascular hemodynamics in preeclampsia [327]. In cultured rat cardiomyocytes, IgG obtained from preeclamptic women enhances angiotensin type 1 receptor-mediated response, which is ameliorated with the PKC inhibitor calphostin C, supporting a role of PKC in preeclampsia [459].

PKC plays a role in diabetes-related vascular pathology by promoting cell growth and proliferation, cell permeability, oxidative stress, and phospholipase A2 activity; inhibition of K^+ channels and Na^+/K^+-ATPase; increasing vascular reactivity, extracellular matrix and remodeling; and increasing vascular inflammation and pro-inflammatory cytokines [247, 460, 461]. In diabetes, PKC is activated by advanced glycation end (AGE) products and polyol pathway flux [270, 293, 462]. Activated PKC increases endothelial cell permeability and angiogenic factors, which could contribute to the loss of capillary pericytes, and increased retinal capillary permeability, ischemia, and neovascularization associated with diabetes [463–467]. High glucose stimulates DAG production and activates PKC in bovine aortic endothelial cells and VSMCs [468], and PKC activation alters the expression of vascular endothelial growth factor (VEGF), PDGF, and transforming growth factor-β [467, 469], and in turn affect extracellular matrix proteins and vascular remodeling [470]. PKC is also activated by ROS generated by different oxidases and the mitochondrial electron transport chain, and following AGE:RAGE (AGE receptor) interactions [471], and PKC in turn activates NADPH oxidases and further increases ROS [293, 472, 473]. High glucose via PKC activation and oxidative stress also reduces VSMC K_v current resulting in VSMC depolarization, activation of L-VDCCs and increased vasoconstriction [474–477]. PKC promotes Ca^{2+} sensitization in VSM myofilaments and vascular reactivity in diabetes [293]. Diabetic patients have reduced nocturnal dip in blood pressure and increased vascular complications, partly due to lack of diurnal PKC inhibition [478, 479]. Unsaturated fatty acids and their coenzyme A esters work synergistically with DAG to activate PKC [268], while long-chain omega-3 polyunsaturates from fish oil limit PKC activity. Fish oil-rich diets reduce blood pressure [480] and vasoconstriction to AngII and norepinephrine in humans [481], likely through decreased DAG production and PKC activity, and treatment of rat VSMCs with eicosapentaenoic acid prevents vasopressin-induced increase in DAG production [482]. In VSMCs, high glucose activates PKCα, β, δ, and ε, but not PKCζ [467, 483, 484]. PKCβ and δ are the dominant PKCs in diabetic vasculopathy. In rat VSMCs, high glucose increases PKCβ and δ in the membrane fraction, and p38 MAPK phosphorylation [270, 484]. PKCβ is implicated in insulin resistance, and transgenic mice overexpressing PKCβII exhibit decreased insulin-induced Akt activation in vascular cells [270, 485]. PKC inhibits insulin's anti-atherosclerotic mechanisms by inhibiting the PI_3K/Akt pathway at the insulin receptor substrate (IRS) level [486], but accentuates insulin's pro-atherosclerotic mechanisms via the $ERK_{1/2}$ signaling pathway [270, 487]. PKCβ activation by hyperglycemia may mediate the diabetic microvascular complications of retinopathy, nephropathy, and neuropathy. Hyperglycemia-induced activation of PKCβ causes abnormal signaling, cytokine activation, vascular alterations, cell cycle and transcriptional factor dysregulation, and abnormal angiogenesis [157, 270]. PKCβ mediates diabetic retinopathy by affecting VEGF expression through the mRNA-stabilizing human embryonic lethal abnormal vision protein HuR in the retina [488, 489]. In arteries of streptozotocin-induced diabetic mice and human coronary artery VSMCs, high glucose increases the expression of PKCβ which in turn increases vascular contraction by reducing the expression of the $BK_{\beta I}$ channel subunit and BK_{Ca} channel activity, and PKCβ inhibition restores BK_{Ca}-mediated vasodilation in diabetic mice [293, 490]. The nPKCs also contribute to insulin resistance through phosphorylation and inhibition of IRS1 [491, 492]. PKCδ plays a role in islet cell function and insulin response, and changes in PKCδ expression/activity in mice correlate

with insulin resistance and glucose intolerance. Also, mice with global or liver-specific downregulation of PKCδ show increased hepatic insulin signaling and improved glucose tolerance with aging. Conversely, mice with liver-specific overexpression of PKCδ develop hepatic insulin resistance and decreased insulin signaling [493]. Diabetes-induced PKCδ activation also decreases responsiveness to PDGF leading to pericyte apoptosis, acellular capillaries, and retinopathy [494]. PKCδ may also be involved in poor collateral vessel formation, as the ischemic adductor muscles of diabetic *PRKCD* knockout mice show increased blood flow and capillary density compared with diabetic *PRKCD*$^{+/+}$ mice. The poor angiogenesis response in ischemic diabetic muscles could be caused by PKCδ-induced expression of Src homology-2 domain-containing phosphatase-1 (SHP-1), which contributes to VEGF and PDGF unresponsiveness [467]. PKCδ also inhibits K^+ current in aortic VSMCs, and PKCδ gene silencing by siRNAs restores VSMCs K^+ current and endothelium-dependent vasodilatation in aorta of streptozotocin-induced diabetic rats [293, 495]. Endothelium-independent vasoconstriction mediated by prostaglandin E_2 EP1-/EP3-receptor activation is enhanced in mesenteric arteries of diabetic rats and is highly sensitive to PKCδ inhibition [293, 496]. Indolylmaleimide and its derivatives are nonselective PKC inhibitors that reduced diabetic nephropathy, cardiomyopathy and neuropathy in clinical trials [293, 497], but their lack of specificity raises safety concerns. Ruboxistaurin (LY333531) is an oral PKCβII inhibitor that is well-tolerated in diabetic retinopathy, nephropathy and neuropathy [297, 498, 499]. Ruboxistaurin decreases vessel permeability and diabetic macular edema, improves retinal condition, and prevents reduction of visual acuity in diabetic patients [270, 489]. Some antidiabetic drugs have inhibitory effects on PKC. In cultured human endothelial cells, combined use of metformin and liraglutide (a glucagon like peptide-1) inhibits high glucose-induced PKCβII translocation and phosphorylation, oxidative stress through inhibition of PKC-NADPH oxidase, p47phox translocation and NADPH oxidase activation, and high glucose-induced production of DAG and phosphorylation of AMP-activated protein kinase [500].

ROCK plays a role in metabolic and neurological disorders, cancer, and systemic and pulmonary hypertension [501–503]. Animal models of hypertension show increased RhoA/ROCK activity [504], and the ROCK inhibitors Y-27632 and fasudil normalize arterial pressure in experimental hypertension [397]. Chronic inhibition of ROCK suppresses vascular media hypertrophy and perivascular fibrosis in coronary arteries of spontaneously hypertensive rats [505], and in a rat model of hypertension induced by chronic inhibition of NO synthesis, where RhoA/ROCK activity is increased [506]. In hypertensive rats, inhibition of angiotensin type 1 receptor decreases RhoA/ROCK activity, suggesting that ROCK activation is likely due to increased AngII activity [506]. Long-term infusion of AngII increases RhoA/ROCK activity, media thickness and perivascular fibrosis in coronary arteries, and ROCK inhibitors inhibit AngII-induced coronary hypertrophy and fibrosis [507], and reduce production of superoxide anion [507], and monocyte chemoattractant protein-1 and PAI-1 [508, 509]. Hypertension is associated with increased mechanical strain on the vessel wall which in turn stimulates VSMC proliferation [510], and ROCK inhibition inhibits stretch-induced activation of MAPK and VSMC growth [511, 512]. Pulmonary hypertension involves sustained vasoconstriction and structural remodeling of pulmonary arteries leading to narrowing of the pulmonary microvessels and increased pulmonary

vascular resistance. Reduced endothelium-derived NO in pulmonary arteries has been implicated in pulmonary hypertension [513], and ROCK mediates hypoxia-induced decrease in eNOS expression in human pulmonary endothelial cells [514]. RhoA/ROCK also contributes to both vasoconstriction and vascular remodeling in pulmonary hypertension [515, 516]. Chronic hypoxia in rats is associated with 2-fold increase in ROCK expression and enhanced ROCK-dependent Ca^{2+} sensitization in small pulmonary arteries [517]. The ROCK inhibitor Y-27632 attenuates hypoxia-induced pulmonary vasoconstriction, hypertension and vascular remodeling [518]. Inhibition of ROCK by oral or inhaled fasudil also improves monocrotaline-induced pulmonary hypertension in rats by inhibiting VSMC proliferation and macrophage infiltration, and improving endothelium-dependent pulmonary artery relaxation [516, 519, 520].

10. Summary

In the past decades, great advances have been made in our understanding of the mechanisms of VSM contraction and their role in the pathogenesis of vascular disease. $[Ca^{2+}]_c$ is a major determinant of VSM contraction, and is controlled by Ca^{2+} channels and Ca^{2+} pumps in the plasma membrane and intracellular organelles. The balance between Ca^{2+} mobilizing and Ca^{2+} removal mechanisms maintains resting $[Ca^{2+}]_c$ constant. Vasoconstrictor agonists and pathological states such as hypertension disrupt the balance between Ca^{2+} mobilizing and Ca^{2+} removal mechanisms leading to increased $[Ca^{2+}]_c$, Ca^{2+}-dependent MLC phosphorylation, and vasoconstriction. PKC is also a major regulator of VSM function. PKC is a family of conventional, novel and atypical isoforms with different Ca^{2+} and phospholipid dependency, cellular localization, and substrates. PKC is mainly cytosolic, and upon VSMC activation it undergoes phosphorylation, maturation and translocation to the plasma membrane, nucleus, endoplasmic reticulum, and other cell organelles; a process facilitated by scaffold proteins. Activated PKC phosphorylates different substrates including ion channels, pumps and nuclear proteins. PKC phosphorylates CPI-17 leading to inhibition of MLC phosphatase, increased MLC phosphorylation and enhanced VSM contraction. PKC also activates a cascade of protein kinases leading to phosphorylation of the actin-binding proteins CaP and CaD, increased actin-myosin interaction, and VSM contraction. Agonists also increase RhoA/ROCK activity leading to inhibition of MLC phosphatase and enhancement of the myofilament force sensitivity to Ca^{2+}. Knockout animals lacking a specific channel subunit, PKC isoform, or ROCK have supported their role in the regulation of vascular function. Of note, cyclic nucleotides such as cAMP and cGMP activate PKA and PKG, respectively, which in turn affect Ca^{2+} handling mechanisms and $[Ca^{2+}]_c$, smooth muscle membrane potential, and the sensitivity of the contractile machinery to Ca^{2+}, and thereby cause opposing effects and decrease smooth muscle contraction [521].

Increases in $[Ca^{2+}]_c$, PKC and ROCK activity play a role in the exaggerated vasoconstriction, VSM growth and proliferation, and vascular remodeling associated with coronary artery disease, hypertension, and diabetic vasculopathy. The subcellular location of PKC may determine the state of VSM activity, and could be useful in the diagnosis/prognosis of hypertension [6]. Ca^{2+} channel blockers have been used in treatment of hypertension and coronary vasospasm. PKC may represent an alternative target for treatment of vascular disease. The first generation of PKC inhibitors such as staurosporine and

chelerythrine have shown mixed results in experimental and clinical trials [157], but isoform-specific PKC inhibitors show some promise. PKC inhibitors could be useful in Ca^{2+} antagonist-resistant forms of hypertension [6]. Inhibitors of PKCβ and δ improve glucose tolerance and reduce fat accumulation, hepatosteatosis, and foam cell formation in obesity and hyperlipidemia-induced atherosclerosis [493, 522, 523]. A PKCβ inhibitor or PKCε activator may reduce vascular damage secondary to endothelial dysfunction and VSMC proliferation in patients with atherosclerosis [522, 524–526]. PKCε activators may be useful in coronary artery disease, and the PKCε activator acadesine reduced the 2 year mortality in patients with postoperative acute myocardial infarction after coronary bypass grafting [157]. Ruboxistaurin is a PKCβII inhibitor that has been tested in diabetic retinopathy, nephropathy and neuropathy [297, 498, 499]. PKC siRNA could target specific PKC isoforms in vascular disease. PKCδ gene silencing with the short hairpin RNAs (shRNAs)-plasmid delivery system administered intravenously normalizes vascular function and blood pressure in spontaneously hypertensive rats [298]. Also, PKCδ siRNA attenuates the proinflammatory effect of human C-reactive protein in diabetic rats [527]. Target-delivery of PKC pseudosubstrate inhibitory peptides may be useful in localized vascular disease. PKC inhibitors could be coated onto stents and directly released at effective concentrations in vasospastic areas. PKCβII and PKCδ inhibitors coated stents or balloons showed efficacy in experimental trials [260]. ROCK inhibitors such as fasudil have shown some promise in pulmonary hypertension and cerebral vasospasm, but more specific inhibitors are needed for clinical use in humans.

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List of Abbreviations

AGE	Advanced Glycation End products
ALDH2	aldehyde dehydrogenase 2
AngII	angiotensin II
BK_{Ca}	large conductance Ca^{2+}-activated K^+ channel
Ca^{2+}	calcium
$[Ca^{2+}]_c$	cytosolic free Ca^{2+} concentration
CaD	caldesmon
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CaP	calponin
cGMP	cyclic guanosine monophosphate
CICR	Ca^{2+}-induced Ca^{2+} release

CNP	C-type natriuretic peptide
CPI-17	PKC-potentiated phosphatase inhibitor protein-17
DAG	diacyglycerol
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
HSP	heat shock protein
ICAM-1	intercellular adhesion molecule-1
IP_3	inositol 1,4,5-trisphosphate
IRS1	insulin receptor substrate 1
K_v	voltage-gated K^+ channel
LTCC	L-Type $Ca_V1.2$ channel
MARCKS	myristoylated alanine-rich C kinase substrate
MLC	myosin light chain
PDBu	phorbol 12,13-dibutyrate
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PMA	phorbol 12-myristate 13-acetate
PMCA	plasmalemmal Ca^{2+}-ATPase
PLC	phospholipase C
PS	phosphatidylserine
RAGE	AGE receptor
ROC	receptor-operated Ca^{2+} channel
ROCK	Rho-kinase
ROS	reactive oxygen species
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

SOC	store-operated Ca^{2+} channel
SR	sarcoplasmic reticulum
TRP	transient receptor potential channel
TTCC	T-type $Ca_V3.1/3.2/3.3$
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VDCC	voltage-dependent Ca^{2+} channel
VSM	vascular smooth muscle

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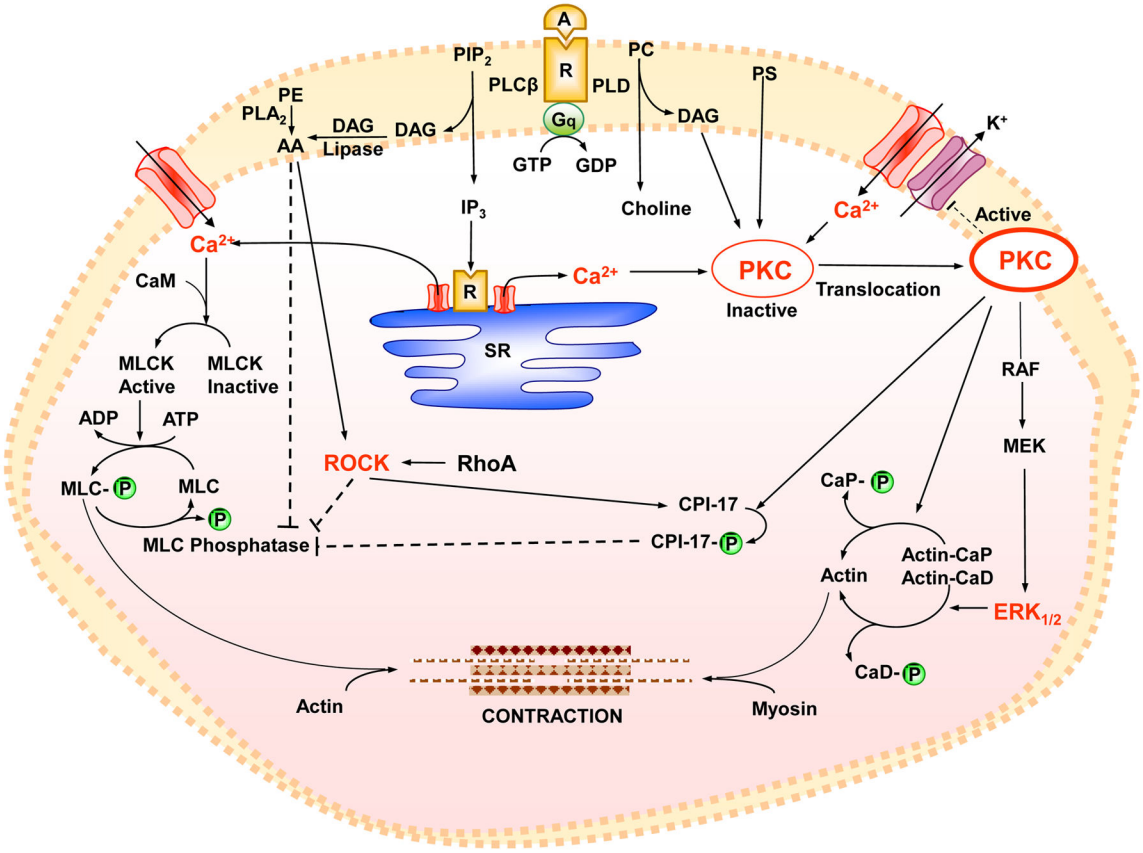

Fig. 1.

Mechanisms of VSM contraction. A vasoconstrictor agonist (A) binding to its receptor (R) is coupled to heterotrimeric GTP-binding protein (Gq) and activates phospholipase C (PLCβ) which stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The agonist also activates phospholipase D (PLD) which hydrolyzes phosphatidylcholine (PC) into choline and DAG. IP_3 stimulates Ca^{2+} release from sarcoplasmic reticulum (SR). The agonist also stimulates Ca^{2+} influx through Ca^{2+} channels. Ca^{2+} binds calmodulin (CaM), activates MLC kinase (MLCK), causes MLC phosphorylation, and initiates VSM contraction. DAG, phosphatidylserine (PS), and Ca^{2+} (for cPKCs) cause activation and translocation of PKC. PKC inhibits K^+ channels leading to membrane depolarization and activation of voltage-dependent Ca^{2+} channels. PKC phosphorylates CPI-17, which in turn inhibits MLC phosphatase and enhances the myofilament force sensitivity to Ca^{2+}. PKC phosphorylates calponin (CaP), allowing more actin to bind myosin. PKC may activate a protein kinase cascade involving Raf, MAPK kinase (MEK), and MAPK ($ERK_{1/2}$), leading to phosphorylation of the actin-binding protein caldesmon (CaD). DAG is transformed by DAG lipase into arachidonic acid (AA), and activation of phospholipase A2 (PLA2) increases the hydrolysis of phosphatidylethanolamine (PE) into AA, which in turn inhibits MLC phosphatase. Agonist-induced activation of RhoA/ROCK also inhibits MLC phosphatase and further enhances Ca^{2+} sensitivity of the contractile proteins. Dashed line indicates inhibition.

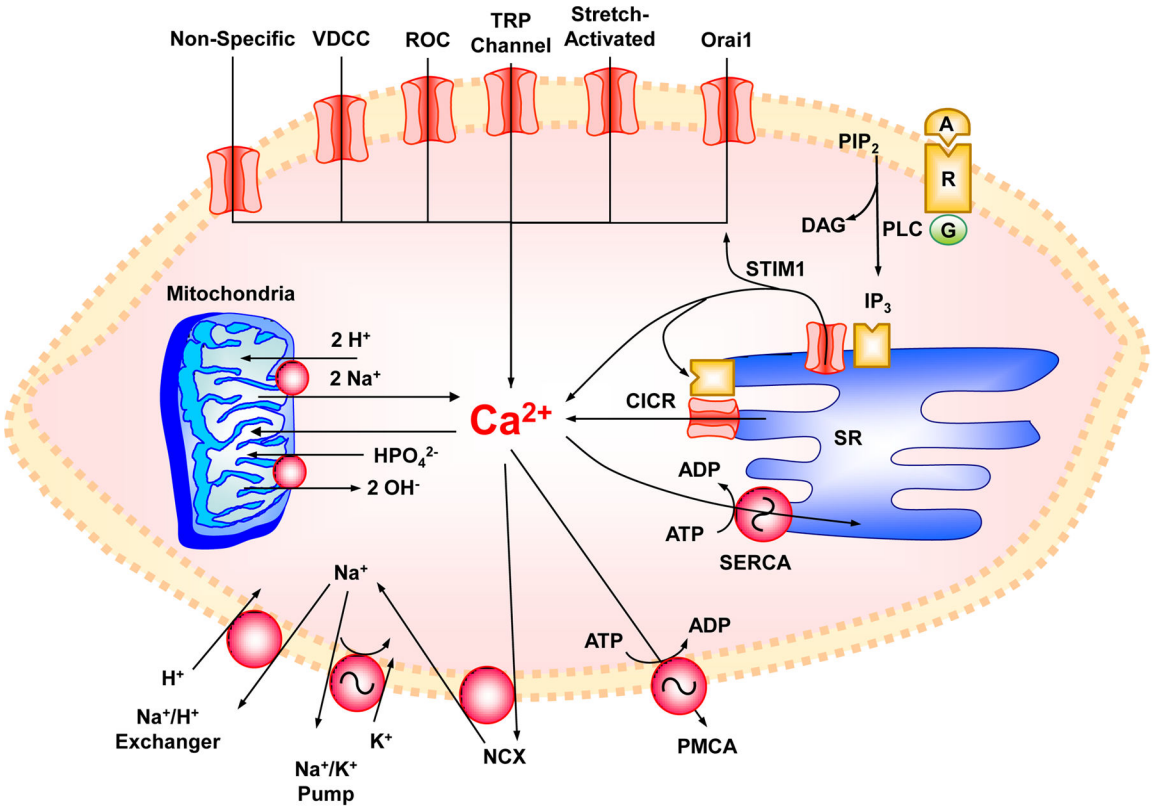

Fig. 2.

Ca^{2+} mobilization and Ca^{2+} removal mechanisms in VSM. Agonist (A)-receptor (R) interaction causes Ca^{2+} release from sarcoplasmic reticulum (SR) in response to 1,4,5-inositol trisphosphate (IP_3) and to Ca^{2+} via Ca^{2+}-induced Ca^{2+} release (CICR). VSMC activation also stimulates Ca^{2+} influx through nonspecific Ca^{2+} leak, voltage-dependent Ca^{2+} channels (VDCC), receptor-operated channels (ROC), transient receptor potential (TRP) channels, and stretch-activated channels. Depletion of intracellular Ca^{2+} stores in SR causes the release of stromal interaction molecule (STIM1) which in turn stimulates Orai1 store-operated Ca^{2+} channels. The increased intracellular Ca^{2+} is taken up by SR Ca^{2+}-ATPase (SERCA) or extruded by the plasmalemmal Ca^{2+}-ATPase (PMCA) or Na^+-Ca^{2+} exchanger (NCX), and the resulting excess Na^+ is extruded via Na^+/K^+ pump and Na^+/H^+ exchanger. At very high and pathological increases in intracellular Ca^{2+}, the mitochondria play a role in Ca^{2+} uptake and homeostasis. When Ca^{2+} is taken up by mitochondria, HPO_4^{2-} is also taken up via HPO_4^{2-}:$2OH^-$ exchange and calcium phosphate is formed. Under favorable conditions and when Ca^{2+} can be handled by SERCA, PMCA and NCX, mitochondrial Ca^{2+} is slowly released via a Ca^{2+} efflux pathway involving a Ca^{2+}:$2H^+$ or Ca^{2+}:$2Na^+$ antiporter. PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DAG, diacyglycerol

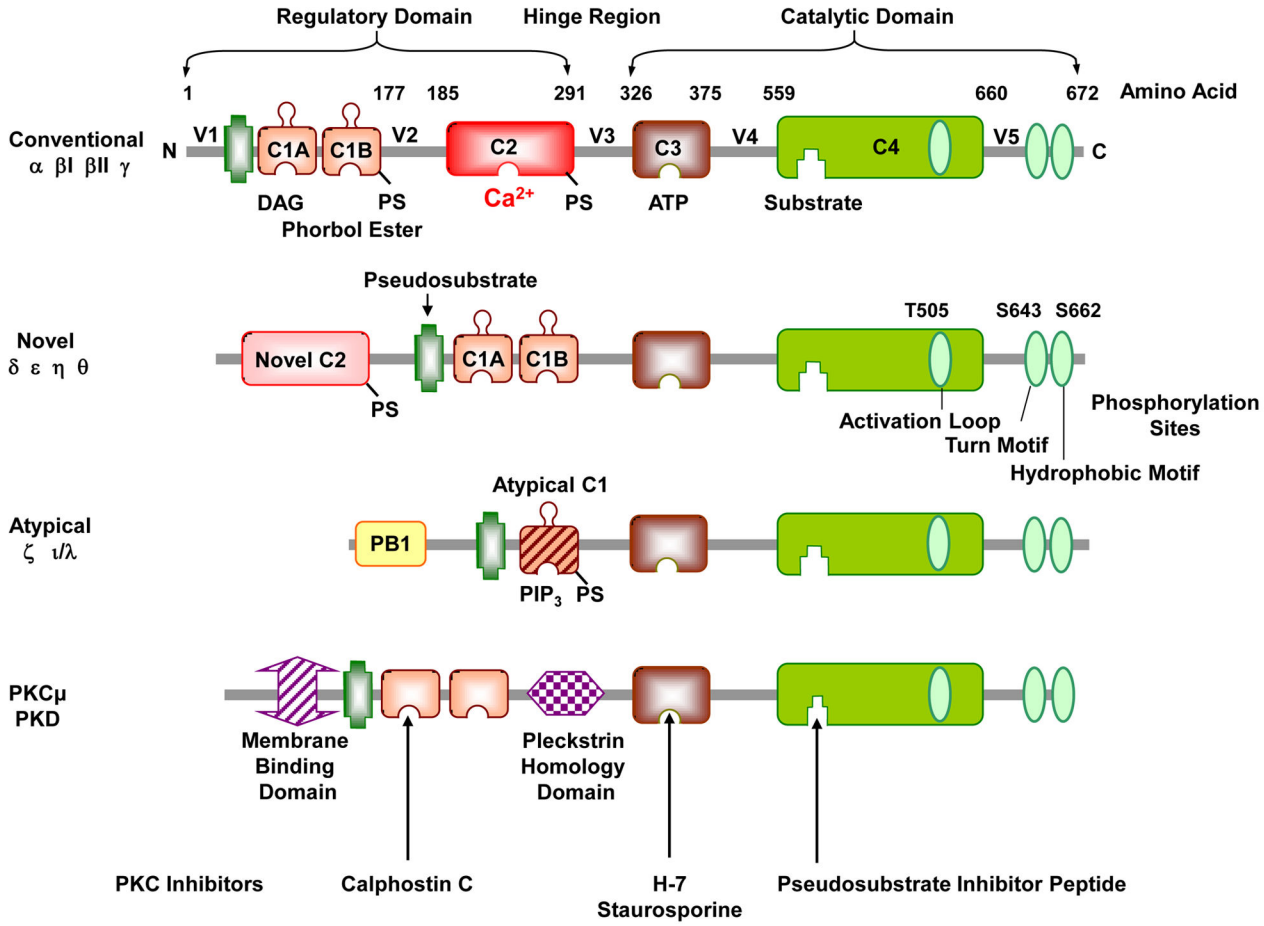

Fig. 3.

PKC structure and isoforms. PKC comprises a N-terminal regulatory domain and a C-terminal catalytic domain, connected by a V3 hinge region. The regulatory domain contains two conserved C1 and C2 regions, and the pseudosubstrate region. The catalytic or kinase activity domain contains a C3 ATP-binding site and a C4 binding site for the substrate. The catalytic domain also contains phosphorylation sites in the activation loop, turn-motif and hydrophobic-motif (The figure illustrates PKCδ phosphorylation sites, which vary in different PKCs). The PKC family is classified into conventional cPKCs α, βI, βII, and γ; novel nPKCs δ, ε, η and θ; and atypical aPKCs ζ and ι/λ isoforms. cPKCs consist of 4 conserved (C1–C4) and 5 variable regions (V1–V5) and are activated by DAG, PS and Ca^{2+}. The C1 region binds phosphatidylserine (PS), DAG, and phorbol esters, and the C2 region contains the binding site for Ca^{2+}. PS can also bind to the C2 region. Both cPKCs and nPKCs have twin C1 regions (C1A and C1B) and a C2 region, but the order of C1 and C2 regions is switched in nPKCs compared to cPKCs. The nPKCs have a variant form of C2 region that is insensitive to Ca^{2+}, but still binds lipids. The aPKCs do not have a C2 region and hence not activated by Ca^{2+}, and have a variant form of C1 that is not duplicated, but retains lipid-binding activity and sensitivity to PS. The aPKCs also have a protein–protein-interacting region Phox and Bem 1 (PB1) that controls their cellular localization. Other related kinases include PKCμ (PKD). PKC inhibitors compete with DAG at the C1 region

(calphostin C), ATP at the ATP-binding site (H-7, staurosporine) or the PKC true substrate (pseudosubstrate inhibitor peptide).

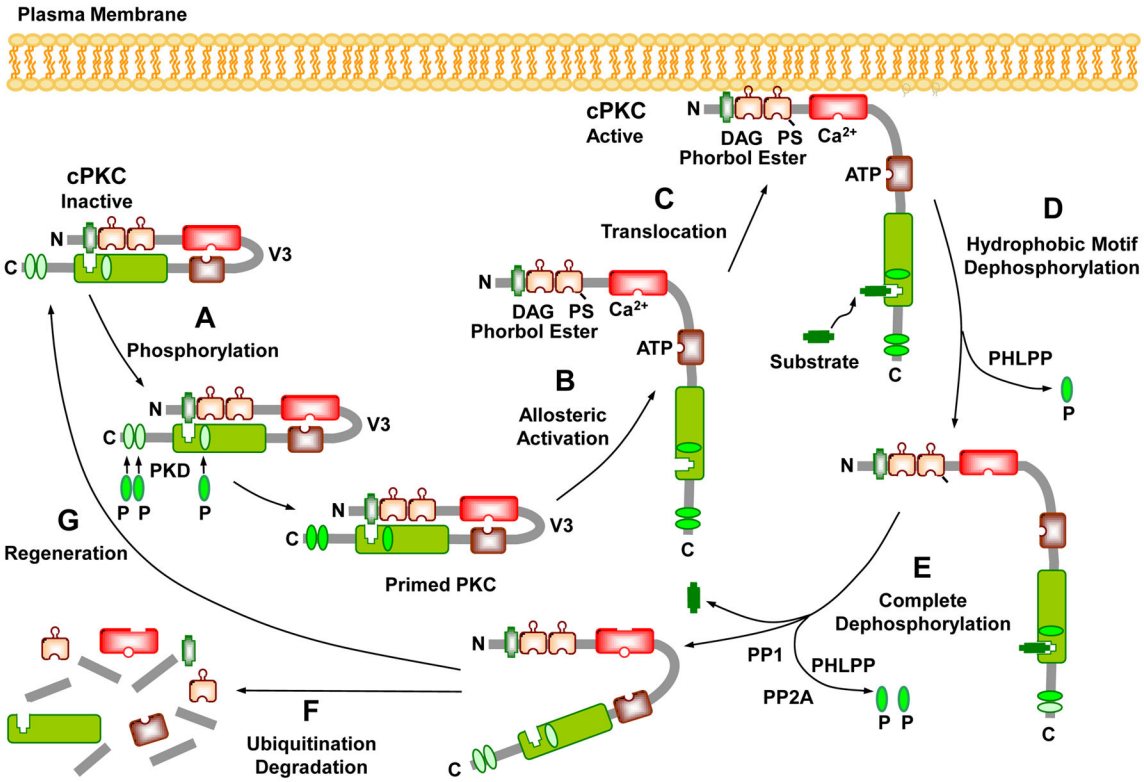

Fig. 4.

Activation, translocation, substrate interaction and deactivation of cPKCs. In the PKC cytosolic and inactive state, the pseudosubstrate binds the catalytic site in the C4 region, and the regulatory and catalytic domains are folded. Before it becomes catalytically competent, nascent PKC undergoes phosphorylation at three phosphorylation sites. Phosphorylation of the activation loop by phosphoinositide-dependent kinase (PDK) introduces a negative charge that properly aligns residues to form a competent catalytic domain, facilitate subsequent autophosphorylation at the turn motif and hydrophobic motif, and keep PKC in a catalytically competent and protease resistant conformation. Phosphate groups are indicated as green ovals labeled “P”. PKC activators such as PS, DAG, phorbol esters, and Ca^{2+} promote allosteric activation, translocation of PKC to the plasma membrane, and subsequent interaction with the substrate. Allosteric activation also induces an open conformation state, making PKC susceptible to phosphatases and proteases and allows PKC to either enter an autophosphorylation/dephosphorylation cycle, or undergo proteolytic degradation. PKC dephosphorylation terminates its kinase activity and is carried out by the PP2C member pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) at the hydrophobic motif, which starts the process that consequently drives further dephosphorylation of PKC by PP1/PP2A protein phosphatases at the turn motif. Dephosphorylation also predisposes “naked” PKC to ubiquitination and degradation, leading to *de novo* synthesis and regeneration of the enzyme.

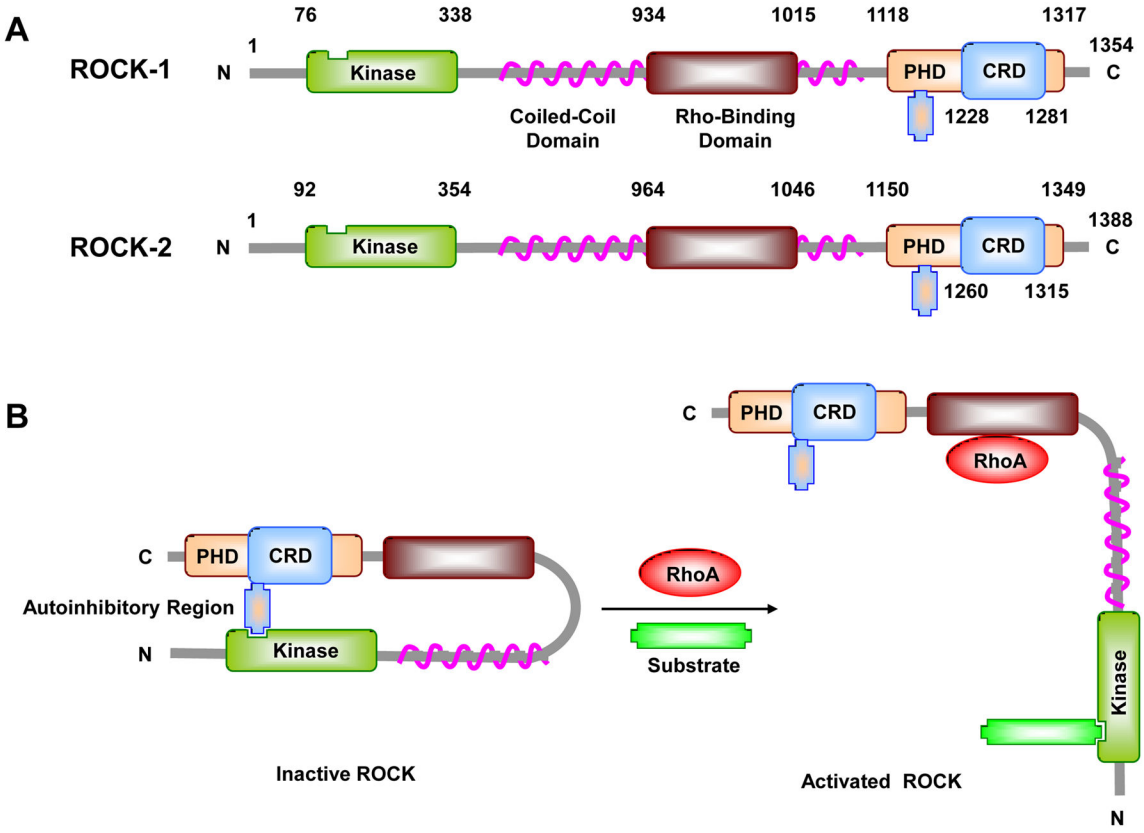

Fig. 5.

Structure and activation of ROCKs. (A) ROCK amino acid sequence comprises a kinase domain located at the N-terminus, a coiled-coil region containing the Rho-binding domain, and a pleckstrin-homology domain (PHD) with a cysteine-rich domain (CRD). ROCK-1 and ROCK-2 are highly homologous with an overall amino acid sequence identity of 65%. (B) In the inactive form, the C-terminus region of ROCK is folded over the N-terminus region, allowing the autoinhibitory region to block the kinase site. Binding of activated GTP-bound RhoA causes unfolding and activation of ROCK, and thereby exposes the kinase domain and allows phosphorylation of the true substrate.

Table 1

Distribution and scaffold proteins of PKC in representative tissues and blood vessels

PKC	MW (kDa)	Major Tissue Distribution	Blood vessel	Location Inactive PKC	Location Active PKC	Scaffold Protein	Ref
cPKC α	74–82	Universal	Rat aorta	Cytosol	Nuclear	RACK1	[160, 272, 446, 528–531]
			Rat mesenteric artery	Cytosol/Membrane	Cytosol/Membrane	p32	
			Rat carotid, ferret portal vein, porcine coronary, bovine aorta	Cytosol	Plasma membrane	RACK1, AKAPs, HSP, p32	
β	80–82	Adipose tissue, liver, kidney, spleen, skeletal muscle, brain	Rat aorta	Cytosol	Nuclear	RACK1, p32	[160, 290, 529]
			Rat carotid	Cytosol	Membrane	RACK1, AKAPs, HSP, p32, 14-3-3	
γ	70–82	Adrenal gland, brain	Rat mesenteric artery	Cytosol	Cytosol	RACK1, AKAPs, HSP, 14-3-3, Importins	[160, 528, 530]
nPKC δ	76–82	Universal	Rat aorta	Cytoskeleton/Organelles	Cytoskeleton/Organelles	RACK1, p32	[160, 447, 530, 532]
			Rat mesenteric artery	Membrane	Membrane	AKAPs, HSP, p32, 14-3-3	
ε	90–97	Pancreas, kidney, brain	Rat mesenteric artery, porcine coronary artery	Cytosol/Membrane	Cytosol/Membrane	AKAPs, p32	[160, 272, 528, 530, 533]
			Ferret aorta	Cytosol	Surface membrane	RACK1, RACK2 AKAPs, HSP, p32, 14-3-3	
η	80	Lung, skin, brain	NIH 3T3 fibroblasts	Cytosol/Membrane Golgi	Membrane		[160, 534, 535]
θ	79	T cells, hematopoetic cells, skeletal muscle	cerebral microvascular endothelium	cytosol	Membrane Lipid rafts	AKAPs, HSP, p32, 14-3-3, Importins, CARMA1, Vav1	[160, 221]
aPKC ζ	64–82	Universal	Rat aorta, ferret aorta and portal vein	Perinuclear	Intranuclear	AKAPs, HSP, p32, 14-3-3, Importins	[160, 447, 528, 530, 533]
			Rat mesenteric artery	Cytosol	Cytosol		
ι/λ	70	Kidney, testis, ovary, brain	Rabbit femoral artery and portal vein	Cytosol	Cytosol	AKAPs, HSP, 14-3-3, Importins	[160, 536, 537]

MW, molecular weight

Table 2

Representative PKC substrates and the effect of their phosphorylation

Substrate	Effect of Substrate Phosphorylation	Reference
Histones: H3T45	DNA fragmentation, apoptosis	[538]
H3T6	Prevents LSD1 from demethylating H3K4 during androgen receptor-dependent gene activation. Promotes cell proliferation	[539]
Membrane-bound proteins: MARCKS (myristoylated, alanine-rich C kinase substrate)	MARCKS binds F-actin. Functions as cross-bridge between cytoskeletal actin and plasma membrane	[182]
Inhibitory GTP-binding protein Gi	Facilitates the dissociation of the αi subunit from adenylyl cyclase and thereby relieves it from inhibition.	[169]
Ion Channels BK_{Ca} channels	Inhibition, leading to membrane depolarization, activation of L-type VDCCs, and increased $[Ca^{2+}]_c$ and vascular tone, e.g. in pulmonary artery and porcine coronary artery.	[295, 296, 299, 300, 302, 303, 327]
Voltage-dependent K^+ channel	Inhibition. Increases vascular tone	[298, 304, 305, 327]
K_{ATP} channels	Inhibition. Alters the channel kinetics and/or number at the cell membrane, e.g. in mesenteric artery	[309, 311, 312, 327]
Store-operated Ca^{2+} channel	Inhibition, e.g. in HEK293 cells.	[341]
Ion Pumps & Exchangers: PMCA	Activation. Promotes Ca^{2+} extrusion. Explains transient nature of agonist-induced increase in $[Ca^{2+}]_c$	[6]
α1 subunit of Na^+/K^+-ATPase	Inhibition. Alters membrane potential and intracellular concentrations of Na^+ and K^+	[315]
Na^+/H^+ antiport exchanger	Activation. Increases cytoplasmic pH and alkalinization, leading to increased contraction	[316–318]
Regulatory Proteins: CPI-17	Inhibits MLC phosphatase, increases MLC phosphorylation and enhances myofilament force sensitivity to Ca^{2+} and VSM contraction, e.g. in rabbit femoral artery	[322]
Calponin	Allows actin-myosin interaction and enhances VSM contraction	[540]
Raf	Initiates a cascade involving MAPK kinase (MEK) and MAPK, and phosphorylation of the actin-binding protein caldesmon which allows actin–myosin interaction and VSM contraction	[5, 339]
20-kDa MLC and MLCK	Counteracts Ca^{2+}-induced actin–myosin interaction and force development, e.g. in rabbit mesenteric artery	[343]
Cytoskeletal Proteins: Vinculin	Controls cell shape, and adhesion	[264]
Vimentin	Recycles β1-integrins to plasma membrane	[265]
Ribosomal Protein Kinases: S6KβII	Nucleo-cytoplasmic shuttling of S6KβII. Regulates protein synthesis and G1/S transition in the cell cycle	[266]
Other: Arginine-rich protein substrates	Neutralizes the acidic patch in the substrate binding site. Displaces PKC pseudosubstrate from the kinase core	[158, 212]

Table 3

Representative PKC Inhibitors

Class/Inhibitor	Chemistry	Site of Action	Selectivity	K_d or IC_{50}	Ref
Isoquinolines H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazines	ATP-binding site	Pan-PKC	PKCβI 3.5 μM PKCζ 6 μM	[541]
Benzophenones Chelerythrine	1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridin-12-ium	ATP-binding site	Pan-PKC	0.66 μm	[260]
Indolocarbazoles Gö6976	5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile	Catalytic domain	PKCα, βI	PKCα 2.3, βI 6.2 nM	[542, 543]
Gö6983	1H-Pyrrole-2,5-dione, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-	ATP-binding site. Suppreses PKCμ auto-phosphorylation	Pan-PKC PKCα, β, γ, δ > PKCζ	PKCα 7, β 7, γ 6, δ 10, ζ 60 nM	[544, 545]
Enzastaurin (LY317615)	3-(1-methyl-1H-indol-3-yl)-4-(1-(1-(pyridin-2-ylmethyl)piperidin-4-yl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione	ATP-binding site	PKCβ > PKCα, γ, ε	PKCα 39, β 6, γ 83, ε 110 nM	[546, 547]
LY379196		ATP-binding site	PKCβ	3– 6 μM	[548]
Staurosporine (CGP41251)	9,13-Epoxy-1H,9H-diindolo[1,2,3-gh:3′,2′,1′-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one, 2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-11-(methylamino)-, [9S-(9α,10β,11β,13α)]-	ATP-binding site	Pan-PKC PKCα, γ, η > PKCδ, ε	PKCα 2, γ 5, δ 20, η 4 nM	[549, 550]
CGP53353	5,6-bis[(4-Fluorophenyl)amino]-1H-isoindole-1,3(2H)-dione	ATP-binding site	PKCβ	PKCβI 3.8, βII 0.41 μM	[551]
UCN-01	7-hydroxystaurosporine	ATP-binding site	cPKCs	25–50 nM	[552]
Sotrastaurin (AEB071)	3-(1H-indol-3-yl)-4-(2-(4-methylpiperazin-1-yl)quinazolin-4-yl)-1H-pyrrole-2,5-dione	ATP-binding site	Pan-PKC, especially PKCθ	PKCα 0.95, βI 0.64, δ 2.1, ε 3.2, η 1.8, θ 0.22 nM	[553, 554]
Staurosporine Analogs Ruboxistaurin (LY333531)	(9S)-9-[[(Dimethyl-d6)amino]methyl]-6,7,10,11-tetrahydro-9H,18H-5,21:12,17-Dimethenodibenzo[e, k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione Hydrochloride	ATP-binding site	PKCβI, βII	PKCβI 4.7, βII 5.9 nM	[499]
Midostaurin (PKC412, CGP41251)	(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3′,2′,1′-lm]pyrrolo[3,4-j][1,7]benzodiamzonine-1-one	ATP-binding site	Pan-PKC	12 nM	[555]
Bisindolylmaleimid e (GF 109203X, Gö 6850)	3-(1-(3-(Dimethylamino)propyl)-1H-indol-3-yl)-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione	ATP-binding site	Pan-PKC, especially PKCα, β, γ	PKCα 8.4, βI 18, βII 16,	[556, 557]

Class/Inhibitor	Chemistry	Site of Action	Selectivity	K_d or IC_{50}	Ref
				γ 20, δ 210, ε 132, ζ 5800 nM	
Ro 31-8220	Carbamimidothioic acid, 3-[3-[2,5-dihydro-4-(1-methyl-1H-indol-3-yl)-2,5-dioxo-1H-pyrrol-3-yl]-1H-indol-1-yl]propyl ester, methanesulfonate	Catalytic domain	Pan-PKC, PKCα, βI, βII, γ, ε	PKCα 5, βI 24, βII 14, γ 27, ε 24 nM	[404, 558]
SCH47112		ATP-binding site			[559]
Dicationic, lipophilic drugs Dequalinium Cl	Quinolinium, 1,1′-(1,10-decanediyl)bis[4-amino-2-methyl-, chloride (1:2)	Covalently modifies the C2-domain	Pan-PKC	7–18 μM	[269, 560, 561]
Flavonoid Myricitrin	4H-1-Benzopyran-4-one, 3-[(6-deoxy-α-L-mannopyranosyl)oxy]-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-	DAG/Phorbol ester-binding site	PKCα, ε		[562]
Quercetin	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-		Weak PKC inhibitor		[563]
Benzothiazole Riluzole	6-(trifluoromethoxy)benzothiazol-2-amine	ATP-binding site	PKCα		[564]
Perylenequinone Calphostin C (UCN-1028C)	1-[3,10-dihydroxy-12-[2-(4-hydroxyphenoxy)carbonyloxypropyl]-2,6,7,11-tetramethoxy-4,9-dioxoperylen-1-yl]propan-2-yl benzoate	DAG/phorbol ester-binding site	cPKCs, nPKCs	50 nM	[565]
Phenolic ketone Rottlerin (Mallotoxin)	5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene)	ATP-binding site	PKCδ Other nPKCs	PKCδ 5 μM Other PKCs 30 μM	[566]
Macrolactone Bryostatin 1 (NSC 339555)	(1S,3S,5Z,7R,8E,11S,12S,13E,15S,17R,21R,23R,25S)-25-(Acetyloxy)-1,11,21-trihydroxy-17-[(1R)-1-hydroxyethyl]-5,13-bis(2-methoxy-2-oxoethylidene)-10,10,26,26-tetramethyl-19-oxo-18,27,28,29-tetraoxatetracyclo[21.3.1.1^{3,7}.1^{11,15}]nonacos-8-en-12-yl (2*E*,4*E*)-2,4-octadienoate	C1 domain. DAG/phorbol ester-binding site	PKCε > PKCα, δ		[157, 269, 567]
Membrane lipids D-erythro-Sphingosine	2-Amino-4-octadecene-1,3-diol; trans-4-Sphingenine	Phosphatidyl-serine-binding site		2.8 μM	[568]
N, N-Dimethyl-D-erythro-sphingosine	(E,2S,3R)-2-(Dimethylamino)octadec-4-ene-1,3-dio	Phosphatidyl-serine-binding site		12 μM	[569]
Taxol Tamoxifen	2-[4-[(Z)-1,2-diphenylbut-1-enyl]phenoxy]-N, N-dimethylethanamine	Regulatory domain	cPKCs		[570]
Purine nucleoside Sangivamycin	4-amino-5-carboxamide-7-(D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine	ATP-binding site		10 μM	[571]
Other	α-tocopherol, adriamycin, aminoacridine, apigenin, cercosporin, chlorpromazine, dexniguldipine, polymixin B, trifluoperazine, UCN-02				[572]

Class/Inhibitor	Chemistry	Site of Action	Selectivity	K_d or IC_{50}	Ref
Carbonitrile	5-vinyl-3-pyridinecarbonitriles	Catalytic domain	PKCθ	4.7 nM	[573]
Pyrimidine	2,4-Diamino-5-nitropyrimidine	Catalytic domain	PKCθ		[574]
Sterols	Spheciosterol sulfate A	Catalytic domain	PKCζ	1.59 μM	[575]
	Spheciosterol sulfate B	Catalytic domain	PKCζ	0.53 μM	
	Spheciosterol sulfate C	Catalytic domain	PKCζ	0.11 μM	
Antisense oligonucleotides Isis3521 (CGP64128A, Aprinocarsen)	20-mer phosphorothioate oligodeoxynucleotide	Inhibits PKCα mRNA expression	PKCα	-	[576]
Isis9606	19-mer phophorothioate oligodeoxynucleotide	Inhibits PKCα mRNA	PKCα	-	[577]
Short peptides Myristoylated-pseudosubstrate peptide inhibitor	Peptide sequence: myr-FARKGALRQ	Substrate-binding site	cPKCs	-	[273]
αV5-3	Peptide sequence: QLVIAN	Site: aa 642–647	PKCα	-	[578]
βIV5-3	Peptide sequence: KLFIMN	Inhibits PKC translocation Site: aa 646–651	PKCβI	-	[579]
βIIV5-3	Peptide sequence: QEVIRN	Inhibits PKC translocation Site: aa 645–650	PKCβII	-	[580]
βC2–4	Peptide sequence: SLNPEWNET	Site: aa 218–226	All cPKCs	-	[188]
δV1-1 (KAI-9803, Delcasertib)	Peptide sequence: SFNSYELGSL	RACK-binding site Inhibits PKC translocation Site: aa 8–17	PKCδ	-	[581]
εV1-2 (KAI-1678)	Peptide sequence: EAVSLKPT	RACK-binding site Inhibits PKC translocation Site: aa 14–21	PKCε	-	[582]
KCe-12 and KCe-16		Substrate-binding site	PKCε	-	[583]
ZIP	Peptide sequence: SIYRRGARRWRKL	ζ–pseudo substrate	PKCζ and aPKCs	-	[584]
γV5-3	Peptide sequence: RLVLAS	Site: aa 659–664	PKCγ	-	[585]
Other PKC Inhibitors	α-tocopherol, adriamycin, aminoacridine, apigenin, cercosporin, chlorpromazine, dexniguldipine, polymixin B, trifluoperazine, UCN-02				[281, 282]

>, greater selectivity; aa, amino acid