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Calcium-independent phospholipase A_2 participates in KCl-induced calcium sensitization of vascular smooth muscle

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ABSTRACT

In vascular smooth muscle, KCl not only elevates intracellular free Ca^{2+} ($[Ca^{2+}]_i$), myosin light chain kinase activity and tension (*T*), but also can inhibit myosin light chain phosphatase activity by activation of rhoA kinase (ROCK), resulting in Ca^{2+} sensitization (increased $T/[Ca^{2+}]_i$ ratio). Precisely how KCl causes ROCK-dependent Ca^{2+} sensitization remains to be determined. Using Fura-2-loaded isometric rings of rabbit artery, we found that the Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) inhibitor, bromoenol lactone (BEL), reduced the KCl-induced tonic but not early phasic phase of *T* and *potentiated* $[Ca^{2+}]_i$, reducing Ca^{2+} sensitization. The PKC inhibitor, GF-109203X (≥3 μM) and the pseudo-substrate inhibitor of PKCζ produced a response similar to BEL. BEL reduced basal and KCl-stimulated myosin phosphatase phosphorylation. Whereas BEL and H-1152 produced strong inhibition of KCl-induced tonic *T* (~50%), H-1152 did not induce additional inhibition of tissues already inhibited by BEL, suggesting that $iPLA_2$ links KCl stimulation with ROCK activation. The $cPLA_2$ inhibitor, pyrrolidine-1, inhibited KCl-induced tonic increases in $[Ca^{2+}]_i$ but not *T*, whereas the inhibitor of 20-HETE production, HET0016, acted like the ROCK inhibitor H-1152 by causing Ca^{2+} desensitization. These data support a model in which $iPLA_2$ activity regulates Ca^{2+} sensitivity.

1. Introduction

In vascular smooth muscle, contractile stimuli generally cause elevations in $[Ca^{2+}]_i$ that increase the activity of Ca^{2+} and calmodulin-dependent myosin light chain kinase [1], causing elevations in myosin light chain phosphorylation, actomyosin crossbridge cycling, muscle shortening, and *T* development [2]. Certain contractile stimuli may also activate signaling systems causing inhibition of myosin light chain phosphatase activity to elevate contractile *T* independently of further increases in $[Ca^{2+}]_i$. This mechanism, termed "Ca^{2+} sensitization" [3], is caused by activation of G protein-coupled receptors linked to G(q) and G(12/13) that activate PKC and ROCK.

An assumption that remains a central theme for smooth muscle biologists is that high KCl concentrations bypass plasma membrane receptor activation, causing contraction solely by elevating Ca^{2+} entry and $[Ca^{2+}]_i$, and activating myosin light chain kinase [4,5]. As such, KCl has been used for decades as a surrogate for membrane depolarization (electromechanical coupling) in cell signaling studies as a comparison to receptor-mediated (pharmacomechanical coupling) smooth muscle activation [6–9]. For example, the notion that G protein-coupled receptor stimuli can cause Ca^{2+} sensitization of smooth muscle was strengthened by seminal work showing that G protein-coupled receptor stimuli can produce

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greater increases in T for a given increase in $[Ca^{2+}]_i$ compared to KCl [10–13].

However, several studies challenge the assumption that KCl is a stimulus that acts solely by causing activation of myosin light chain kinase. A study by Yanagisawa and Okada provided compelling evidence that KCl can increase Ca^{2+} sensitivity in coronary artery [14]. Moreover, Ratz [15] showed that KCl-induced contraction can be desensitized, implying that KCl, like G protein-coupled receptor stimuli, can induce Ca^{2+} sensitization. Finally, a series of studies published several years ago independently showed that KCl can cause Ca^{2+} sensitization by activation of ROCK [16]. Notably, Sakurada et al. [17] were the first to record an elevation in active rhoA upon stimulation of vascular smooth muscle with KCl, and to suggest that KCl-induced Ca^{2+} sensitization reflects Ca^{2+}-dependent rhoA stimulation. However, the precise mechanisms linking K^+-depolarization with elevated Ca^{2+} sensitivity of cross bridges remains elusive.

There is evidence that membrane depolarization alone can cause KCl-induced Ca^{2+} sensitization [14], while other studies [17–20] support the notion that KCl-induced $[Ca^{2+}]_i$ sensitization depends on Ca^{2+} entry through dihydropyridine-sensitive voltage-operated Ca^{2+} channels. However, KCl can cause Ca^{2+}-release from intracellular stores [21,22], and Ca^{2+} store-depletion could activate "Ca^{2+}-independent" phospholipase A_2 ($iPLA_2$) to generate arachidonic acid and lysophosphospholipids [23]. An elevation in $[Ca^{2+}]_i$ could also activate Ca^{2+}-dependent PLA_2 ($cPLA_2$) to generate arachidonic acid [24]. Arachidonic acid and certain lysophospholipids are stronger activators of ROCK than is rhoA [25], and several arachidonic acid metabolites are known modulators of vascular contractile activity, so PLA_2-generated eicosanoids resulting from K^+-depolarization could act as autocrine and paracrine agents to stimulate certain G protein-coupled receptors to cause Ca^{2+} sensitization. Importantly, arachidonic acid causes Ca^{2+} sensitization [26] that is diminished by the ROCK inhibitor, Y-27632 [27]. Notably, the study by Guo et al. [28] using BEL and rabbit venous smooth muscle was the first to reveal that constitutive $iPLA_2$ activity plays a significant role in establishing basal arachidonic acid production necessary for α-adrenergic receptor activation-induced, but not for KCl-induced, contraction and Ca^{2+} sensitization. However, only the early, phasic phase of a KCl-induced contraction was examined in this study, and it is the tonic phase that is attenuated by inhibition of ROCK [29]. In addition to activation of ROCK, arachidonic acid can potentially activate PKCζ [30]. Thus, there is sufficient reason to suspect that KCl can lead to more complex cell signaling events than simply activation of voltage-operated Ca^{2+} channels leading to increased myosin light chain kinase activity. The focus of the present study was to determine whether PLA_2 participates in causing KCl-induced Ca^{2+} sensitization in rabbit vascular smooth muscle.

2. Methods

2.1. Tissue preparation and isometric tension (T)

Each endothelium-denuded 3–4 mm femoral and renal artery ring isolated from adult New Zealand white rabbits was prepared as previously described [31] and secured in a myograph tissue chamber filled with aerated physiological salt solution (PSS) maintained at 37 °C. The PSS composition was, in mM, NaCl 140, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.6, $NaHPO_4$ 1.2, morpholino-propanesulfonic acid (MOPS) 2.0 (adjusted to pH 7.4), Na_2ethylenediamine tetraacetic acid (EDTA, to chelate heavy metals) 0.02, and D-glucose 5.6. For all studies except that shown in Fig. 4D, KCl (110 mM) was substituted isosmotically for NaCl to produce K^+-depolarization. In the study shown in Fig. 4D, 72.75 mM K_2SO_4 was used instead of 110 mM KCl. Contractile T was measured as previously described [31]. In the protocol used to assess the affect of certain selective pharmacological agents on T, $[Ca^{2+}]_i$ and the degree of Ca^{2+} sensitization ($T/[Ca^{2+}]_i$), tissues were stimulated twice with KCl to produce two responses, termed T_1 (1st contractile response) Ca_1 (1st Ca response), T_2 (2nd contractile response) and Ca_2 (2nd Ca response). Tissues were washed 3 times with PSS after the 1st KCl stimulation to cause complete relaxation, and a pharmacological agent or no drug (control) was added for ~30 min prior to and during the 2nd stimulation with KCl. T was normalized by dividing the contractile response by the 10 min T_1 response produced during a first contraction ($T/T_{1(10')}$). Otherwise, contractions were calculated as T/T_o, where T_o was the maximum T produced at the optimum muscle length (L_o) [32,33]. Arteries contracted with KCl were incubated with 1 μM phentolamine to block potential α-adrenergic receptor activation caused by release of norepinephrine from peri-arterial nerves.

2.2. $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in artery rings at L_o as previously described [34]. Tissues were loaded for 2 h with 7.5 μM Fura-2-PE3 (AM) and 0.01% (wt/vol) Pluronic F-127 (TefLabs, Austin, TX) to enhance solubility. Fluorescence emission intensities at 510 nm collected by a photomultiplier tube were expressed as excitation ratios (340 nm/380 nm, DeltaRam V, Photon Technologies Inc., Lawrenceville, NJ) using Felix software (Photon Technology International). Background fluorescence, determined by incubating tissues in 4 mM $MnCl_2$ plus 20 μM ionomycin, was subtracted prior to calculating the fluorescence ratios. $[Ca^{2+}]_i$ was normalized by dividing the fluorescence ratio by the difference between the 10 min $[Ca^{2+}]_i$ response produced during the 1st KCl-induced contraction (Ca_1) and the basal response produced just prior to the 1st KCl contraction ($Ca/Ca_{1(10')}$). The degree of Ca^{2+} sensitization was calculated as (T_2/Ca_2). Pyrrolidine-1, ETYA, NDGA, 17-ODYA and HET0016 had no effect on the fluorescence ratio.

2.3. Western blot

Phosphorylation of MYPT1 was measured by Western blot analysis of artery ring homogenates using phospho-specific antibodies as described previously [35,36]. Tissues were homogenized in 1% SDS, 10% glycerol, 20 mM dithiothreitol, 25 mM Tris–HCl (pH 6.8), 5 mM EGTA, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 20 mg/ml leupeptin, 2 mg/ml aprotinin, and 20 mg/ml (4-amidinophenyl)-methanesulfonyl fluoride, heated, clarified, and stored at −70 °C. Thawed homogenates were assayed for protein concentration, loaded into gel wells, and proteins were separated by 1-dimensional SDS PAGE on 7.5% gels followed by Western blotting. Phosphorylated MYPT1 was identified using anti-MYPT1–p853 antibody (Upstate) and horseradish peroxidase-labeled secondary antibody (Santa Cruz), enhanced chemiluminescence (ECL) film (Amersham), and digital image analysis (Scion Image software). Total MYPT1 (BD Transduction) was assessed to quantify loading accuracy. Band intensities were reported as the degree of change from the control basal level.

2.4. Drugs

Phentolamine and phenylephrine were from Sigma, St. Louis, MO. Bromoenol lactone (BEL), *N*-Hydroxy-*N*′-(4-butyl-2-methylphenyl) formamidine (HET0016), 17-octadecynoic acid (17-ODYA) and nordihydroguaiaretic acid (NDGA) were from Cayman Chemical, Ann Arbor, MI. H-1152, HA-1077, Y-27632, GF-109203X and the cell permeable (myristoylated) form of the PKCζ-pseudo-substrate inhibitor (PKCζ-PI) were from EMD