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Calcium-independent phospholipase A₂ participates in KCl-induced calcium sensitization of vascular smooth muscle

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Abstract

In vascular smooth muscle, KCl elevates intracellular free Ca²⁺ ([Ca²⁺]_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 Ca²⁺ sensitization (increased T/[Ca²⁺]_i ratio). Precisely how KCl causes ROCK-dependent Ca²⁺ sensitization remains to be determined. Using fura-2-loaded isometric rings of rabbit artery, we found that the Ca²⁺-independent phospholipase A₂ (iPLA₂) inhibitor, bromoenol lactone (BEL), reduced the KCl-induced tonic but not early phasic phase of T and potentiated [Ca²⁺]_i, reducing Ca²⁺ sensitization. The PKC inhibitor, GF-109203X (≥ 3 μM) and the pseudosubstrate 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 iPLA₂ links KCl stimulation with ROCK activation. The cPLA₂ inhibitor, pyrrolidine-1, inhibited KCl-induced tonic increases in [Ca²⁺]_i but not T, whereas the inhibitor of 20-HETE production, HET0016, acted like the ROCK inhibitor H-1152 by causing Ca²⁺ desensitization. These data support a model in which iPLA₂ activity regulates Ca²⁺ sensitivity.

Keywords

vascular smooth muscle; rabbit artery; contraction; K⁺-depolarization; Ca²⁺ signaling; fura-2; signal transduction; Ca²⁺ sensitization; Rho-kinase; PLA₂; BEL; ETYA; NDGA; HET0016; H-1152; HA-1077; Y-27632; 20-HETE; pyrrolidine-1; GF-109203X; PKC pseudosubstrate inhibitor

1. Introduction

In vascular smooth muscle, contractile stimuli generally cause elevations in [Ca²⁺]_i that increases the activity of Ca²⁺ 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 [Ca²⁺]_i. This mechanism, termed “Ca²⁺

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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 $[\text{Ca}^{2+}]_i$, 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;7;8;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 greater increases in T for a given increase in $[\text{Ca}^{2+}]_i$ compared to KCl [10;11;12;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;18;19;20] support the notion that KCl-induced $[\text{Ca}^{2+}]_i$ 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 lysophospholipids [23]. An elevation in $[\text{Ca}^{2+}]_i$ 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 PKC ζ [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, MgSO₄ 1.2, CaCl₂ 1.6, NaHPO₄ 1.2, morpholino-propanesulfonic acid (MOPS) 2.0 (adjusted to pH 7.4), Na₂ethylenediamine 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 K₂SO₄ 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, [Ca²⁺]_i and the degree of Ca²⁺ sensitization (T/[Ca²⁺]_i), tissues were stimulated twice with KCl to produce two responses, termed T₁ (1st contractile response) Ca₁ (1st Ca response), T₂ (2nd contractile response) and Ca₂ (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 T₁ response produced during a first contraction (T/T_{1(10')}). Otherwise, contractions were calculated as T/T₀, where T₀ was the maximum T produced at the optimum muscle length (L₀) [32;33]. Arteries contracted with KCl were incubated with 1μM phentolamine to block potential α-adrenergic receptor activation caused by release of norepinephrine from peri-arterial nerves.

2.2. [Ca²⁺]_i

[Ca²⁺]_i was measured in artery rings at L₀ as previously described [34]. Tissues were loaded for 2 hours 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 MnCl₂ plus 20 μM ionomycin, was subtracted prior to calculating the fluorescence ratios. [Ca²⁺]_i was normalized by dividing the fluorescence ratio by the difference between the 10 min [Ca²⁺]_i response produced during the 1st KCl-induced contraction (Ca₁) and the basal response produced just prior to the 1st KCl contraction (Ca/Ca_{1(10')}). The degree of Ca²⁺ sensitization was calculated as (T₂/Ca₂). 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 TrisHCl (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-amidino-phenyl)-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 PKC ζ -pseudosubstrate inhibitor (PKC ζ -PI) were from EMD Biosciences (formerly Calbiochem), San Diego, CA. Ionomycin was from VWR, Bridgeport, NJ. Pyrrolidine-1 was kindly provided by Dr. Suzanne Barbour. Fura 2-PE3 (AM) and Pluronic F-127 were from Tef Labs, Austin, TX. Ionomycin was dissolved in ethanol; BEL, HET0016, 17-ODYA, NDGA, pyrrolidine-1 and GF-109203X were dissolved in dimethylsulfoxide (DMSO); all other inhibitors were dissolved in distilled water. Ethanol and DMSO were added at a final concentration no greater than 0.1%, a concentration that had no effect on KCl-induced increases in T or $[Ca^{2+}]_i$.

2.5. Statistics

The null hypothesis was examined using Students' t-test (when 2 groups were compared) or using a one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test to assess whether each "test" group was different than the control group. In all cases, the null hypothesis was rejected at $P < 0.05$. For each study described, the n value was equal to the number of rabbits from which arteries were taken.

3. Results

3.1. General T & $[Ca^{2+}]_i$ protocol

Arteries loaded with fura-2 were twice stimulated with KCl for ~15 minutes, and the T and $[Ca^{2+}]_i$ responses produced during the 1st and 2nd contractions were labeled, respectively, T₁ and T₂ (Fig 1A), and Ca₁ and Ca₂ (Fig 1B). The time-dependent isometric T and fluorescence $[Ca^{2+}]_i$ responses resulting from the two sequential KCl stimulation episodes were plotted as normalized to the 10 min values produced during the 1st contraction (Figs 1A and 1B). Tissues were washed 3-times with PSS for ~30–60 min between the two stimulation episodes. For some control tissues, 0.1% DMSO or 0.1% ethanol, the vehicles for some drugs used in this study, was added 30 min before the second stimulation with KCl. Other control tissues were not exposed to drug vehicle. DMSO and ethanol had no effect on T or $[Ca^{2+}]_i$ (not shown), and therefore, vehicle-control and no-vehicle-control data were pooled and termed "control".

The time-loop for the 2nd KCl-induced contraction in which T was plotted as a function of $[Ca^{2+}]_i$ displayed counter clockwise hysteresis (Fig 1C) because the increase in $[Ca^{2+}]_i$ was much greater than the increase in T for about the 1st 3 sec and because T increased while $[Ca^{2+}]_i$ decreased from 15 sec to 3 min (Fig 1C and Figs 1D and 1E). An inflection in the T tracing occurred at about the peak of the $[Ca^{2+}]_i$ increase (Figs 1D and 1E; vertical dotted lines at 15 sec identifies the peak $[Ca^{2+}]_i$ response), and thereafter, $[Ca^{2+}]_i$ declined whereas T continued to increase slowly until ~3 min (Figs 1C–1E). After 3 min, T and $[Ca^{2+}]_i$ declined very slowly together for at least 10 min (Figs 1A–1C). Because of this slow decline in both parameters, tissues were considered to have reached a pseudo-steady-state by 3 min. Interestingly, because the decrease in $[Ca^{2+}]_i$ from 15 sec to 3 min was approximately equivalent to the increase from 3 sec to 15 sec, the $[Ca^{2+}]_i$ value at 3 min was not significantly different than at 3 sec (Fig 1C). Thus, Ca^{2+} sensitization of T appears to occur from ~3" to 3'. That is, most T development occurs from 3 sec to 3 min, despite no net increase in $[Ca^{2+}]_i$ over this time period.

The 1st and 2nd KCl-induced contractions were similar in strength over a 3 min period (Fig 1D, T₁ and T₂, respectively). Thus, the T₂/T₁ ratio taken at 3 min was not different than unity

(Fig 1F). However, the 2nd KCl stimulation produced an ~15% stronger increase in $[Ca^{2+}]_i$ compared to the 1st stimulation at 3 min (Fig 1E, Ca_2 and Ca_1 , respectively). Thus, Ca_2/Ca_1 at 3 min was, on average, ~1.15-fold greater than unity (Fig 1F). The T_2/Ca_2 ratio produced during the tonic phase of the 2nd KCl-induced contraction (Fig 1F) was assessed as a measure of the degree of Ca^{2+} sensitization induced by KCl. These data suggest that mechanisms regulating $[Ca^{2+}]_i$ and Ca^{2+} sensitivity were not identical during two sequential KCl-induced contractions. In particular, the data suggest that, compared to the 1st contraction, the 2nd contraction displayed increased Ca^{2+} mobilization not associated with increased T (Fig 1F, Ca_2/Ca_1), and therefore, Ca^{2+} desensitization (Fig 1F, T_2/Ca_2).

To assess the effects of various inhibitors on the ability of KCl to induce increases in $[Ca^{2+}]_i$ and T, each agent was added to muscle rings ~15–30 min (3 hrs for PKC ζ -PI) before the second KCl stimulation, and the relative changes in $[Ca^{2+}]_i$ and T, as well as Ca^{2+} sensitivity as assessed by the T_2/Ca_2 ratio, were compared to the control responses produced at 3 min of a KCl-induced contraction (as shown in Figs 1D–1F, and reproduced for comparison in Figs 2A–2C).

3.2. Effect of ROCK inhibitors on KCl-induced T, $[Ca^{2+}]_i$ and Ca^{2+} sensitivity

The ROCK inhibitor H-1152 at a relatively low concentration (0.1 μ M) produced a strong inhibition of KCl-induced T (Fig 1G, T_2 H-11) while producing no inhibition of KCl-induced increases in $[Ca^{2+}]_i$ (Fig 1H, Ca_2 , compare to Ca_2 of Fig 1E). Thus, H-1152 caused a significant reduction in the 3 min KCl-induced T_2/Ca_2 value compared to control (Fig 1I). A higher concentration of the structurally similar compound, HA-1077 (10 μ M), produced a stronger reduction in KCl-induced tonic T (Fig 1G) and had no effect on KCl-induced $[Ca^{2+}]_i$ (Fig 1H, the Ca_2 values produced in the presence of 0.1 μ M H-1152 and 10 μ M HA-1077 were nearly super-imposable). The 3 min KCl-induced T_2/Ca_2 value produced in the presence of 10 μ M HA-1077 was 65% that of control (HA-1077: 0.30 ± 0.06 , $n=3$ versus control: 0.86 ± 0.04 , $n=9$, $P<0.05$). The structurally dissimilar ROCK inhibitor, Y-27632 (3 μ M), reduced 3 min KCl-induced T and not 3 min KCl-induced $[Ca^{2+}]_i$, in a manner similar to 10 μ M HA-1077, yielding a significant reduction in the 3 min KCl-induced T_2/Ca_2 value (0.34 ± 0.04) compared to control. Interestingly, 3 μ M Y-27632 attenuated the early (< 3 min) KCl-induced increase in $[Ca^{2+}]_i$ (data not shown). The Ca^{2+} desensitization of the tonic-phase (3 min) of the KCl-induced contraction induced by ROCK inhibition supports the hypothesis that ROCK participates in causing Ca^{2+} sensitization during the tonic phase of a KCl-induced contraction.

3.3. Effect of pyrrolidine-1, BEL, PKC ζ -PI and ETYA on KCl-induced T, $[Ca^{2+}]_i$, and Ca^{2+} sensitivity

In smooth muscle KCl can activate CamKII [37;38] and ERK [35,] and these kinases can activate cPLA₂ [39] which generates arachidonic acid, a stimulus of ROCK ~3-fold stronger than rhoA [25]. To determine whether cPLA₂ contributed to KCl-induced Ca^{2+} sensitization, tissues were treated with 10 μ M of the cPLA₂ inhibitor, pyrrolidine-1 [40] and contracted with KCl. Pyrrolidine-1 had no effect on the tonic phase of KCl-induced T (Fig 2A). Thus, the 3 min KCl-induced T_2/T_1 produced in the presence of pyrrolidine-1 was identical to that produced by control tissues (Fig 2C). In the presence of pyrrolidine-1, the KCl-induced average increase in $[Ca^{2+}]_i$ (Ca_2 , Fig 2B) was equal to or less than that produced during a 1st stimulation with KCl (Ca_1 , Fig 2B), so the 3 min Ca_2/Ca_1 ratio was less than that produced by control tissues (Fig 2C). The resulting apparent increase in T_2/Ca_2 was not significant (Fig 2C). These data suggest that cPLA₂ may play a role in regulation of Ca^{2+} homeostasis but not in causing Ca^{2+} sensitization. Moreover, these data support the notion that KCl can cause an increase in $[Ca^{2+}]_i$ that may not participate directly in T development.

KCl can mobilize Ca^{2+} from sarcoplasmic reticular (SR) stores [21;22], and Ca^{2+} store depletion can generate a Ca^{2+} influx factor that activates iPLA_2 by displacing calmodulin [23]. To test whether iPLA_2 links K^+ -depolarization with tonic contraction, we examined the ability of the suicide iPLA_2 inhibitor, BEL [41], to inhibit a KCl-induced contraction [28]. BEL inhibited T while potentiating the KCl-induced increase in $[\text{Ca}^{2+}]_i$, resulting in a reduction in KCl-induced Ca^{2+} sensitization (Figs 2D–2F). These data suggest that iPLA_2 plays a role in regulation of $[\text{Ca}^{2+}]_i$ and T in smooth muscle. Arachidonic acid can activate the atypical PKC isoform, $\text{PKC}\zeta/\lambda$ [30]. Moreover, this PKC isoform is present in rabbit femoral artery, and inhibition of $\text{PKC}\zeta/\lambda$ using a selective pseudosubstrate peptide inhibitor ($\text{PKC}\zeta\text{-PI}$) attenuates phenylephrine- and KCl-induced Ca^{2+} sensitization [19;42]. We re-investigated this effect and confirmed that $10\mu\text{M}$ $\text{PKC}\zeta\text{-PI}$ caused a reduction in T and potentiation of $[\text{Ca}^{2+}]_i$ induced by KCl, resulting in a reduction in KCl-induced Ca^{2+} sensitivity (Figs 2G–I) not unlike that seen with exposure to BEL. These data suggest that iPLA_2 and $\text{PKC}\zeta$ play a role in causing KCl-induced Ca^{2+} sensitization.

3.4. Effect of arachidonic acid inhibitors on KCl-induced contraction

Arachidonic acid metabolites can activate plasma membrane G protein-coupled receptors causing smooth muscle contraction. Thus, it is possible that KCl-induced Ca^{2+} sensitization reflects, in part, G protein-coupled receptor-induced Ca^{2+} sensitization because of the generation by PLA_2 of one or more arachidonic acid metabolites that act as autocrine or paracrine agents. We tested this hypothesis by using the general inhibitor of arachidonic acid metabolism, ETYA. At $30\mu\text{M}$, ETYA inhibited T (Fig 2J) and $[\text{Ca}^{2+}]_i$ (Fig 2K) induced by KCl, with the greatest effect on $[\text{Ca}^{2+}]_i$ occurring during the early portion of the response (Fig 2K, dotted line). The concomitant reductions in 3 min T and $[\text{Ca}^{2+}]_i$ resulted in no change in KCl-induced Ca^{2+} sensitivity (Fig 2L). These data alone suggest that an arachidonic acid metabolite may play a role in regulation of Ca^{2+} homeostasis during a KCl-induced contraction, but these data do not support the hypothesis that KCl-induced Ca^{2+} sensitization was caused by production of an arachidonic acid metabolite acting on G protein-coupled receptors.

We examined further the ability of more selective inhibitors of arachidonic acid metabolism to attenuate a KCl-induced contraction. Indomethacin ($10\mu\text{M}$) did not affect KCl-induced contraction (data not shown), suggesting that cyclooxygenase products did not participate in causing KCl-induced T. As with the broad-spectrum arachidonic acid metabolism inhibitor, ETYA, the lipoygenase inhibitor NDGA ($10\mu\text{M}$) reduced the average values of KCl-induced increases in $[\text{Ca}^{2+}]_i$ and T, with greater inhibition occurring during the early portion (within the first 15 sec) of contraction (Figs 3A and 3B). The net result was that NDGA did not reduce KCl-induced Ca^{2+} sensitivity (see Fig 7F). These data suggest that a lipoxygenase product may participate in regulating Ca^{2+} mobilization but not Ca^{2+} sensitization during a KCl-induced contraction. 17-ODYA is used to block both cytochrome P450 epoxygenase and ω -hydroxylase activities, and HET0016 is used to selectively block cytochrome P450 ω -hydroxylase activity [43]. Whereas $30\mu\text{M}$ 17-ODYA exerted no effect on a KCl-induced contraction, $1\mu\text{M}$ HET0016 reduced KCl-induced early (at 15 sec) and pseudo-steady-state (3 min) T by ~25% (Fig 3C). In tissues loaded with fura-2 to measure T and $[\text{Ca}^{2+}]_i$ simultaneously, the HET0016-induced reduction in 3 min KCl-induced T did not correspond with a reduction in $[\text{Ca}^{2+}]_i$ compared to control (Fig 3D). Thus, like H-1152 (a ROCK inhibitor), HET0016 (an inhibitor of the production of 20-HETE) caused a reduction in KCl-induced Ca^{2+} sensitization (Fig 3D, T_2/Ca_2).

3.5. Effect of BEL on KCl-induced and phenylephrine-induced T, and on basal, KCl-induced and EGTA-induced MYPT1-pT853

Tissues treated with $15\mu\text{M}$ BEL and contracted with KCl were frozen at 3 min and processed to measure ROCK substrate (MYPT1) phosphorylation at threonine 853 (MYPT1-pT853). As

shown previously, KCl produced a rapid increase in T that peaked at ~15 sec followed by a slow further increase in T during the next ~3 min (Fig 4A, solid line). BEL caused a slowing in the rate of the initial T rise induced by KCl and attenuated 3 min T (Fig 4A, dash-dotted line). At 3 min of a KCl-induced contraction, MYPT1-pT853 was elevated ~2-fold above the basal level (Figs 4B, “KCl”, and 4C, “3’ KCl”, open bar). BEL inhibited basal MYPT1-pT853 by ~50% (Figs 4B, “Basal”, and 4C, “Basal”, hatched bar) and abolished the ability of KCl to increase MYPT1-pT853 (Figs 4B, “KCl+BEL”, and 4C, “2’ KCl”, hatched bar). Substitution of KCl for NaCl can cause cell swelling within about 10 min when added to rabbit aorta, whereas substitution of K₂SO₄ for NaCl does not [44]. BEL (Fig 4D) and H-1152 (data not shown) inhibited the tonic and not the phasic phase of a K₂SO₄-induced contraction in a manner similar to the inhibition of a KCl-induced contraction. Likewise, BEL (Fig 4D) and H-1152 (data not shown) inhibited the 3 min K₂SO₄-induced increase in MYPT1-pT853. These data suggest that the effects of BEL and H-1152 were not due to cell swelling induced by KCl.

EGTA can cause strong activation of iPLA₂ in arterial smooth muscle [45]. Incubation of arterial rings in a Ca²⁺-free solution containing EGTA reduces [Ca²⁺]_i and can deplete SR calcium [46], and SR Ca²⁺ depletion is necessary and sufficient to activate iPLA₂ in smooth muscle [47]. KCl-induced contraction was abolished in tissues that were incubated in a Ca²⁺-free solution containing 1mM EGTA to chelate extracellular Ca²⁺ (not shown) and EGTA, like KCl, *increased* the degree of MYPT1-pT853 by ~2-fold (Fig 4B, “EGTA”, open bar). Also like that seen in the response to KCl, both BEL and H-1152 abolished the ability of EGTA to cause an increase in MYPT1-pT853 (Fig 4B, “EGTA”, hatched bars). Interestingly, BEL (Fig 4B) and H-1152 [19] inhibited basal MYPT1-pT853 by more than 50%. For a comparison, and in confirmation with a recent publication [28], BEL greatly inhibited the ability of the α-adrenergic receptor agonist, phenylephrine, to cause a strong contraction in rabbit arterial muscle (Figs 4C and 4D).

These data together suggest that a KCl-induced contraction is dependent on iPLA₂-dependent ROCK activity. In support of this contention, H-1152, a potent and selective ROCK inhibitor, produced only a weak inhibition of the tonic phase of a contraction induced by KCl in tissues that had been pretreated with 15 μM BEL, while producing a strong inhibition in the absence of BEL (Fig 5). That is, H-1152 was a less effective relaxant agent in tissues that had been pretreated with BEL than in control tissues, suggesting that iPLA₂ is an upstream regulator of ROCK-dependent KCl-induced Ca²⁺ sensitization and tonic tension maintenance.

3.6. T-[Ca²⁺]_i time-loops

As shown in the T-[Ca²⁺]_i time-loops of Fig 6A, the ROCK inhibitor H-1152 (0.1 μM) prevented the KCl-induced elevation in T from 15 sec to 3 min compared to control. HA-1077 (10μM) produced a similar effect but actually caused T to decline from 15 sec to 3 min (data not shown). Importantly, KCl-induced T also did not increase from 15 sec to 3 min in the presence of the structurally distinct ROCK inhibitor, Y-27632 (data not shown). These data support the contention that ROCK participates only modestly in the early portion of KCl-induced T development, and that T maintenance is nearly completely dependent on ROCK activity.

The inhibitors of arachidonic acid metabolism, ETYA and NDGA (Fig 6B), and the cPLA₂ inhibitor, pyrrolidine-1 (Fig 6C), caused a leftward shift and depression of the KCl-induced T-[Ca²⁺]_i time-loop. Importantly, in tissues exposed to ETYA and NDGA, the KCl-induced 3 min value fell on the diagonal dotted line linking the zero and 3 min control values (Fig 6B). The diagonal dotted line reflects proportionate reductions in T and [Ca²⁺]_i, and thus, the 3 min T₂/Ca₂ values produced by tissues exposed to ETYA and NDGA were not different than the control value (see Fig 2L for example). However, in tissues exposed to pyrrolidine-1, the KCl-induced 3 min value fell to the *left* of the diagonal dotted line (Fig 6C), indicating that

pyrrolidine-1 caused Ca^{2+} sensitization rather than Ca^{2+} desensitization. The KCl-induced T- $[\text{Ca}^{2+}]_i$ time-loop induced in the presence of the cytochrome P450 ω -hydroxylase inhibitor, HET0016, was similar to that induced in the presence of H-1152 (compare Figs 6D and 6A). That is, neither H-1152 nor HET0016 reduced KCl-induced increases in $[\text{Ca}^{2+}]_i$ but both caused Ca^{2+} desensitization by reducing tonic T maintenance. These data suggest that 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) may participate in regulation of KCl-induced Ca^{2+} sensitization because HET0016 is a selective inhibitor for the production of this eicosanoid [43;48].

The iPLA₂ inhibitor, BEL, like H-1152 and HET0016, caused a reduction in the KCl-induced 3 min T value (Fig 6E). However, unlike the effect induced by the ROCK inhibitors, T development from 15 sec to 3 min was not greatly reduced. Notably, unlike the inhibitors of ROCK and arachidonic acid metabolism, BEL caused a rightward shift in the KCl-induced T- $[\text{Ca}^{2+}]_i$ time-loop while suppressing KCl-induced T (Fig 6E). That is, BEL potentiated KCl-induced increases in $[\text{Ca}^{2+}]_i$. Like BEL, the PKC inhibitors, GF-109203X and PKC ζ -PI caused a rightward shift in the KCl-induced T- $[\text{Ca}^{2+}]_i$ time loop while suppressing KCl-induced T (Fig 6F).

In summary, in the presence of H-1152, HET0016, BEL and PKC inhibitors, $[\text{Ca}^{2+}]_i$ induced by KCl at 3 min was the same as or greater than that induced in control tissues (Figs 6A, 6D, 6E & 6F, note that 3 min data are equal to or to the right of the vertical dotted line identifying the 3 min control KCl-induced $[\text{Ca}^{2+}]_i$ level). However, T at 3 min was below the control level (the horizontal dotted line identifying the 3 min control KCl-induced $[\text{Ca}^{2+}]_i$ level). These data together suggest that iPLA₂, 20-HETE, ROCK and PKC ζ / λ participate in regulating KCl-induced Ca^{2+} sensitization.

4. Discussion

This study presents evidence that constitutive iPLA₂ activity plays an essential role in KCl-induced Ca^{2+} sensitization and T maintenance. Our study confirms the finding by Guo et al [28] that the strength of the early, phasic phase of a KCl-induced contraction is not affected by BEL (see Figs 4A and 4D for example), and broadens their model by introducing the proposal that the tonic phase of a KCl-induced contraction, which clearly depends on ROCK activity (reviewed by [16]), does require iPLA₂ activity. Moreover, our data support the contention that in addition to iPLA₂ and ROCK, 20-HETE and PKC ζ / λ also participate in causing KCl-induced Ca^{2+} sensitization in rabbit tonic artery. The precise mechanism(s) by which iPLA₂ becomes constitutively activated was not pursued in this study. However, based on the literature, one speculative model is that the high level of basal Ca^{2+} entry through the plasma membrane could generate sufficient calcium influx factor to activate iPLA₂ in resting (i.e., not activated) muscle [23;49;50].

KCl appeared to activate cPLA₂ because 10 μM pyrrolidine-1, a potent inhibitor of cPLA₂ that has little effect on iPLA₂ [40], inhibited KCl-induced increases in $[\text{Ca}^{2+}]_i$ by ~25%. The surprising aspect of these data was that KCl-induced T was largely unaffected by pyrrolidine-1 (see Figs 3A and 6C). It is well-established that Ca^{2+} entry to replenish SR Ca^{2+} stores in smooth muscle can occur without the development of T [51;52]. We therefore speculate that the “extra” increase in $[\text{Ca}^{2+}]_i$ that was dissociated from T upon stimulation with KCl revealed in the present study by inhibition of cPLA₂ may contribute to refilling the SR Ca^{2+} stores.

Contraction produced upon stimulation with KCl reached a pseudo steady-state by 3 min, and the KCl-induced 3 min T/ $[\text{Ca}^{2+}]_i$ ratio was considered a measure of the degree of Ca^{2+} sensitivity developed by KCl. The ROCK inhibitors H-1152 and HA-1077 reduced KCl-induced 3 min T without inhibiting $[\text{Ca}^{2+}]_i$, and this effect resulted in a reduction in the 3 min

T/[Ca²⁺]_i ratio. Notably, these compounds did not alter the rapid phase of T development that occurred prior to the inflection in T development at ~15 sec (see especially Fig 6A). Thus, these data suggest that the slow-phase, and not the rapid-phase of T development induced by KCl, was dependent on ROCK.

To our surprise, BEL had a dual effect on a KCl-induced contraction. Like the ROCK inhibitors, BEL inhibited KCl-induced 3 min T without reducing [Ca²⁺]_i, and like inhibitors of PKC, BEL potentiated the KCl-induced increase in [Ca²⁺]_i. BEL reduced basal MYPT1-pT853 by ~50% and abolished the KCl-induced 3 min increase in MYPT1-pT853. Neither 1 μM nifedipine nor 10 μM GF-109203X inhibits basal MYPT1-pT853, but both abolish the KCl-induced 2 min increase in MYPT1-pT853 [19]. These data together suggest that a PKC isotype and ROCK participate in causing Ca²⁺ sensitization during a KCl-induced contraction, and that the former may be regulated by Ca²⁺ entry and activated iPLA₂ whereas the latter may be dependent on constitutive iPLA₂ activity. Moreover, the notion that constitutive iPLA₂ and ROCK activities can participate in pseudo steady-state T is supported by the finding that the ROCK inhibitor H-1152 caused almost no relaxation of a KCl-induced tonic contraction in the presence of BEL (see Fig 5).

ETYA, a general inhibitor of arachidonic acid metabolism inhibited KCl-induced increases in T and [Ca²⁺]_i equally, suggesting that an eicosanoid plays a role in KCl-induced contraction. Because indomethacin did not inhibit a KCl-induced contraction, a cyclooxygenase product may be excluded from further consideration. However, lipoxygenase and cytochrome P450 metabolites may participate because NDGA and HET0016 altered KCl-induced contraction. The finding that HET0016 caused inhibition was surprising because another structurally distinct cytochrome P450 ω-hydroxylase inhibitor, 17-ODYA [53], did not inhibit a KCl-induced contraction. Thus, the action of HET0016 may have been non-specific. However, 17-ODYA is an equally effective inhibitor of EET and 20-HETE production [53]. EETs produce vasodilatation, so inhibition of EET production may counteract the concomitant inhibition of 20-HETE. HET0016 is selective for ω-hydroxylase, and therefore would specifically inhibit production of 20-HETE [53;54]. Like H-1152, 1 μM HET0016 inhibited T without inhibiting [Ca²⁺]_i. Vascular smooth muscle contractions induced by 20-HETE are attributed, in part, to activation of PKC and ERK [55;56], and we have shown in this and other studies [19;35] that KCl can activate PKC and ERK. Moreover, PKC has been shown to be an upstream activator of rhoA and ROCK [57]. Thus, data from the present study are at least consistent with the notion that sufficient 20-HETE may be produced constitutively and/or upon KCl-stimulation to participate in activation of these enzymes. Because the IC₅₀ of HET0016 for inhibition of ω-hydroxylase activity is 25 nM [53], 1 μM HET0016 would be expected to abolish 20-HETE production, and this concentration reduced the 3 min KCl-induced T by no more than 23%. However, the IC₅₀ of H-1152 for ROCK inhibition is likewise quite low (12 nM [58]), and 0.1 μM H-1152 reduced the 3 min KCl-induced T by ~31%. Moreover, the IC₅₀ value of HA-1077 for ROCK inhibition is ~10-fold higher than H-1152 [58] and 10 μM HA-1077 reduced the 3 min KCl-induced T equally by no more than ~63%.

In summary, these data suggest that 20-HETE participates in, but is likely not the sole regulator of, KCl-induced Ca²⁺ sensitization. In summary, results from this study support a model of KCl-induced vascular smooth muscle contraction in which constitutive (basal) and stimulated iPLA₂ and ROCK activities, as well as 20-HETE and PKCζ/λ, participate in causing KCl-induced Ca²⁺ sensitization.

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Figure 1.

Examples of tension (T) versus time (A) and $[Ca^{2+}]_i$ versus time (B) tracings showing two sequential KCl-induced control contractile responses (respectively, T_1 and T_2 in A, and Ca_1 and Ca_2 in B) and the resulting average T- $[Ca^{2+}]_i$ time loop (C, $n=9$). For clarity, SE bars were included only for data taken at 0 sec (0"), 3 sec (3"), 15 sec (15"), 3 min (3') and 10 min (10'). The first 3 min of the average values for first (solid lines) and second (dotted lines) of two sequential control contractile responses ($n=9$) were graphed together starting at time = 0 so that the two T (D) and $[Ca^{2+}]_i$ (E) responses could be compared. The vertical dotted line in D and E identifies the time at which $[Ca^{2+}]_i$ achieved a peak value. A plot of the average ratios of the second over the first T and $[Ca^{2+}]_i$ responses, and of the second T over the second

$[Ca^{2+}]_i$ Ca response (measure of Ca^{2+} sensitivity) taken at 3 min of a KCl-induced contraction (open symbols in F and I) indicating that the second T response was identical to the first, and that the $[Ca^{2+}]_i$ signal had increased by ~15% ($n=9$). KCl-induced increases in T (G) and $[Ca^{2+}]_i$ (H), produced in the presence of the ROCK inhibitors, H-1152 (H-11, 0.1 μ M) and H-1077 (H-10, 10 μ M). The 3 min ratios of the second over the first T and $[Ca^{2+}]_i$ responses and the second T over the second $[Ca^{2+}]_i$ response for H-1152 (I, bars). Horizontal dotted line (F and I) indicates unity. w = wash. * = $P < 0.05$ comparing data points indicated in C, comparing Ca_1 and Ca_2 at 3 min in E, and comparing control to H-1152 in I. Data in C–I are means, and in C, F and I, control data are shown as open symbols. Data depicted by open circles and bars are means $\pm$ SE, $n = 9$ and 4 respectively.

Figure 2.

KCl-induced increases in T (A, D, G, J), $[Ca^{2+}]_i$ (B, E, H, K), and 3 min ratios of the second over the first T and $[Ca^{2+}]_i$ responses and the second T over the second $[Ca^{2+}]_i$ response (C, F, I, L) produced in presence of the cPLA $_2$ inhibitor, pyrrolidine-1 (PYRR-1, 1 μ M, A–C), the iPLA $_2$ inhibitor, bromoenol lactone (BEL, 15 μ M, D–F), the pseudosubstrate inhibitor of PKC ζ , (PKC ζ -PI, 10 μ M, G–I), and the general inhibitor of arachidonic acid metabolism, ETYA (30 μ M, J–L). Data are means, and in C, F, I and L, control data are shown as open symbols, effects of drugs are shown as bars, and data are means $\pm$ SE. $n = 3-9$. * = $P < 0.05$ compared to control.

Figure 3.

KCl-induced increases in T (A, B), $[Ca^{2+}]_i$ (C), and 3 min ratios of the second over the first T and $[Ca^{2+}]_i$ responses and the second T over the second $[Ca^{2+}]_i$ response (F) produced in the presence of the general lipooxygenase inhibitor, NDGA (10 μ M, A, B), the general cytochrome P450 inhibitor, 17-ODYA, (30 μ M, C), and the inhibitor of 20-HETE production, HET0016 (1 μ M, C, D). Data are means, and in D, control data are shown as open symbols, effects of HET0016 are shown as bars, and data are means $\pm$ SE. $n = 3-9$. * = $P < 0.05$ comparing HET0016 to control (in C, data are compared at 15 sec and 3 min).

Figure 4.

Increases in T (A, D, E and F) and MYPT1-pT853 (B, C and D) induced by KCl (A–C), a Ca^{2+} -free solution (EGTA, C), K_2SO_4 (D) and phenylephrine- (PE, E and F) under control conditions and after pretreatment with 15 μ M BEL for 2 hr followed by a 30 min BEL washout period before addition of KCl, EGTA, K_2SO_4 and PE, and during incubation with the ROCK inhibitor, H-1152 (0.3 μ M) and EGTA (C). Data are means $\pm$ SE, $n = 3$ –11 (except D is $n=2$). * = $P < 0.05$, compared to control (A), and greater than 1 (C). ψ = $P < 0.05$ less than 1 (C). Dotted lines in A, E and F are $\pm$ SE values.

Figure 5.

Examples (A) and summary data (B) of contractions produced by KCl in tissues not treated (Control) and pretreated with 15 μ M BEL for 2 hr followed by a 30 min BEL washout period before addition of KCl, and then relaxed by addition of the ROCK inhibitor, H-1152. Data in "B" were taken at the times indicated by thick arrows in "A", and are means $\pm$ SE, $n=7$. * = $P < 0.05$ comparing "Post-H-1152" to "Pre-H-1152". The degree of relaxation induced by H-1152 was not different when comparing control tissues to tissues that had been pretreated with BEL (B, "NS").

Figure 6.

Average T - $[Ca^{2+}]_i$ time loops for KCl-induced contractions recorded from 0 to 3 min in control tissues (open symbols and connecting dashed line, A–F) and tissues incubated with inhibitors (grey symbols and connecting solid lines A–F), including the ROCK inhibitor H-1152 (0.1 μM, A), the general inhibitor of eicosanoid production (30 μM ETYA, B) the general lipoxygenase inhibitor NDGA (10 μM, B), the cPLA₂ inhibitor pyrrolidine-1 (1 μM PYRR, C), an inhibitor of 20-HETE production (1 μM HET0016, B), an inhibitor of iPLA₂ (15 μM BEL, E) and inhibitors of PKC (F, GF-109203X at 3 μM and 10 μM and the pseudosubstrate inhibitor of PKCζ, PKCζ-PI, 10 μM). For clarity, SE bars were included only for data taken at 0 sec (0"), 3 sec (3"), 15 sec (15") and 3 min (3') as shown in "A" near the control values. Horizontal

and vertical dotted lines connect the 3 min control value to the axes, which acts as an aid to visualize which inhibitors reduced the KCl-induced 3 min T and not 3 min $[Ca^{2+}]_i$ (for example, ROCK inhibitors, BEL, HET0016 and PKC inhibitors). An agent that caused the three min KCl-induced increases in T and $[Ca^{2+}]_i$ to fall on the diagonal dotted line connecting control 0 and 3 min values reduced equally the KCl-induced pseudo steady-state increases in T and $[Ca^{2+}]_i$ (for example, NDGA and ETYA).