Increased Ca$^{2+}$ sensitivity of contractile elements via protein kinase C in α-toxin permeabilized SMA from young spontaneously hypertensive rats

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Abstract

Objective: The purpose of the present investigation was to examine the Ca$^{2+}$ sensitivity of the contractile elements via protein kinase C (PKC) in superior mesenteric artery (SMA) from young (5–6 weeks old) spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY).

Methods: Staphylococcal aureus α-toxin, which produces pores in the plasma membrane too small to allow passage of proteins such as PKC, was used to investigate the signal transduction system in vascular smooth muscle cells. We investigated the Ca$^{2+}$ sensitivity of the contractile apparatus via PKC in intact and α-toxin skinned SMA from young SHR and WKY.

Results: In intact SMA, high K$^+$ responses were not different between SHR and WKY. However, phorbol 12,13-dibutyrate (PDBu, a PKC activator) augmented high K$^+$-evoked contractions and PKC inhibitors, such as 1-(5-isoquinolinesulfonyl)-2-methylpiperezine (H-7) and calphostin C, suppressed them more in SHR as compared with WKY. In α-toxin skinned SMA, the [Ca$^{2+}$]$_c$-force relationship curve was not significantly different between SHR and WKY. However, PDBu augmented [Ca$^{2+}$]$_c$-evoked contractions and PKC inhibitors suppressed them more in SHR than in WKY.

Conclusion: These results suggest that the Ca$^{2+}$ sensitivity of the contractile elements via PKC is significantly greater in prehypertensive SHR than in age-matched WKY. This abnormality in small muscular arteries may be involved in the pathogenesis of hypertension in SHR.

Keywords: Protein kinase C; Vascular smooth muscle; Spontaneously hypertensive rat; Wistar-Kyoto rat; Calcium sensitivity; Contractile elements

1. Introduction

It is generally accepted that intracellular calcium concentration ([Ca$^{2+}$]$_c$) is the primary regulator of the contractile state of smooth muscle. Elevation of [Ca$^{2+}$]$_c$ induces Ca$^{2+}$-calmodulin (CaM) complex formation and activates myosin light chain kinase (MLCK), thereby increasing the phosphorylation of myosin light chain (MLC) and initiating tension development [1].

Recent evidence indicates that the sensitivity of the contractile apparatus to Ca$^{2+}$ can be modulated; for example, it is increased by activators of protein kinase C (PKC) and inhibited by cyclic nucleotides [2]. It was reported that 1,2-diacylglycerol (DAG) activates PKC [3]. Phorbol esters, which selectively bind to PKC in place of DAG [4], raise resting tension in some vascular smooth muscle [5–8], but they may do so without elevating [Ca$^{2+}$]$_c$ [9]. In contrast, 1-(5-isoquinolinesulfonyl)-2-methylpiperezine (H-7) selectively inhibits the PKC-mediated reaction without inhibiting Ca$^{2+}$-CaM-dependent enzymes such as MLCK [10] and suppresses the arterial contraction.

The alterations of PKC-mediated responses in rings of superior mesenteric artery (SMA) between young spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) may be considered as one factor in the pathogenesis of hypertension, but they have not been examined in detail. Recently, it was reported that staphylococcal α-toxin skinned fibers retain intact receptors and signal transduction system [11,12]. α-Toxin produces small pores in the plasma membrane (2–3 nm) [13] by forming hexamers.
These pores allow passage of agents whose molecular weights are less than about 1000, such as inorganic ions, ATP, and EGTA [14], and prevent the passage of proteins such as α-toxin itself, protein kinase A (PKA), protein kinase G (PKG), PKC, CaM, adenylate cyclase, guanylate cyclase, MLCK, and phosphodiesterase into or out of the cell. Therefore, intracellular Ca\(^{2+}\) can be clamped at specified values (10\(^{-3.5}\)–10\(^{-4}\) M) using EGTA-Ca\(^{2+}\) buffer. We used this new method and examined the Ca\(^{2+}\) sensitivity of the contractile apparatus through PKC in rings of a small branch of SMA of young SHR and age-matched normotensive WKY.

2. Methods

2.1. Arterial rings

Systolic blood pressure was measured by the tail-cuff method. Male 5–6 weeks old SHR rats and WKY rats were decapitated under anesthesia; SMA and surrounding tissues were rapidly removed and placed in physiological salt solution (PSS) at 37°C. Under a dissecting microscope, SMA (about 200 μm diameter) were dissected free of surrounding connective tissues and cut into 2 mm long rings. The total dissection was usually accomplished within 45 min.

2.2. Reagents and solutions

The standard PSS contained (mM) NaCl 138, KCl 4.7, CaCl\(_2\) 1.8, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 1.2, glucose 10, and HEPES 5. The PSS was oxygenated with 100% O\(_2\). In high K\(^+\) solution (e.g., 30 mM K\(^+\) PSS), the NaCl in the standard PSS was replaced, mole for mole, by KCl. In Ca\(^{2+}\)-free PSS, the CaCl\(_2\) was replaced by equimolar MgCl\(_2\). All solutions were adjusted to pH 7.4 with 2 M Tris. Permeabilization was accomplished by exposing the arterial rings to staphylococcal α-toxin (50 μg protein/ml) for 15 min in cytoplasmic substitution solution (CSS). This experimental CSS solution contained (mM) EGTA 2, K propionate 130, MgCl\(_2\) 4, Na\(_2\)ATP 4, creatine phosphate 22, HEPES 5. The PSS was oxygenated with 100% O\(_2\). In high K\(^+\) solution, the NaCl in the standard PSS was replaced, mole for mole, by KCl. In the presence of PDBu more in SHR than in WKY. Two tungsten wire hooks (75 μm diameter) were passed through the lumen of SMA rings. One hook was fixed to the bottom of the tissue chamber (volume, 750 μl) and the other hook was connected to an isometric force transducer (Type TB-68417, Nihon Kohden, Tokyo, Japan), which was mounted directly above the tissue chamber. Isometric tension was continuously monitored and recorded on a strip-chart recorder. The tissue was continuously superfused at a rate of 2 ml/min with oxygenated incubation fluid at 37°C. The arteries were equilibrated until the resting tension at SMA stabilized at 300 mg, respectively; this took 1.5–2.0 h or more. Most drugs and other reagents were superfused with 30 mM K\(^+\)-PSS containing 15 nM PDBu for 10 min. Moreover, the rings were superfused with 30 mM K\(^+\)-PSS containing 15 nM PDBu and 40 μM H-7 for 10 min. Temperature 37°C; resting tension 300 mg (A: upper panel). High K\(^+\) responses were not different between SHR and WKY. However, PDBu augmented high K\(^+\)-evoked contraction more in SHR than in WKY (B: lower-left panel). In contrast, H-7 suppressed contractile response to high K\(^+\) in the presence of PDBu more in SHR than in WKY (C: lower-right panel).

Fig. 1. Effects of phorbol 12,13-dibutyrate (PDBu) and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on high potassium (K\(^+\))-induced contraction in intact small mesenteric artery from SHR and WKY. The arterial rings from SHR and WKY were stimulated with 30 mM K\(^+\)-PSS. The rings were augmented by 30 mM K\(^+\)-PSS containing 15 nM PDBu for 10 min. Moreover, the rings were superfused with 30 mM K\(^+\)-PSS containing 15 nM PDBu and 40 μM H-7 for 10 min. Temperature 37°C; resting tension 300 mg (A: upper panel). High K\(^+\) responses were not different between SHR and WKY. However, PDBu augmented high K\(^+\)-evoked contraction more in SHR than in WKY (B: lower-left panel). In contrast, H-7 suppressed contractile response to high K\(^+\) in the presence of PDBu more in SHR than in WKY (C: lower-right panel).
were added directly to the superfusion fluids and were therefore equilibrated in the tissue chamber.

2.4. Statistics

Student’s t-tests or analysis of variance (the experiment of Ca²⁺–force relationship) was used to test for statistical significance when appropriate.

3. Results

3.1. Systolic blood pressure and body weight in SHR and WKY

Systolic blood pressure were 130 ± 6 mmHg in SHR (n = 19) and 128 ± 5 mmHg in WKY (n = 19). Body weight was 122 ± 5 g in SHR and 126 ± 6 g in WKY. There were no significant differences between SHR and WKY.

3.2. Effects of PDBu and PKC inhibitors on high K⁺-evoked contractions in rat intact mesenteric artery

High K⁺ responses were not different between SHR and WKY. However, 15 nM PDBu augmented high K⁺-evoked contraction more in SHR than in WKY (Fig. 1A,B). In contrast, 40 μM H-7 suppressed contractile response to high K⁺ in the presence (Fig. 1A,C) or absence (Fig. 2) of PDBu more in SHR than in WKY. 0.1 μM calphostin C (another PKC inhibitor) also attenuated high K⁺-induced contraction in the presence of PDBu more in SHR than in WKY (Fig. 3A).

3.3. Effects of calphostin C on high potassium (K⁺)-induced tension in intact superior mesenteric artery (SMA) and Ca²⁺-evoked contraction in α-toxin permeabilized SMA from SHR and WKY.

Calphostin C suppressed contractile response to high K⁺ in the presence of PDBu more in SHR than in WKY in intact SMA (A: left panel). Calphostin C also diminished [Ca²⁺]i-evoked contraction in the absence of PDBu more in SHR than in WKY in α-toxin permeabilized SMA (B: right panel).
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Fig. 4. Ca\(^{2+}\)-force relationship in the absence and presence of 200 nM PDBu in α-toxin permeabilized SMA rings from SHR and WKY. Values are expressed as percentage of maximum contraction. The [Ca\(^{2+}\)]_e-force relationship curve was not significantly different between SHR and WKY in the absence of PDBu in CSS. However, analysis of variance indicated that PDBu augmented [Ca\(^{2+}\)]_e-evoked contraction more in SHR than in WKY (P < 0.01). Student’s unpaired t-test was also used to compare Ca\(^{2+}\)-induced tension in the presence of PDBu in SHR with one in WKY: *P < 0.05, **P < 0.01.

3.3. Effects of PDBu and PKC inhibitors on Ca\(^{2+}\)-induced contractions in rat α-toxin permeabilized small mesenteric artery

We observed [Ca\(^{2+}\)]_e-induced contraction in the absence or presence of 200 nM PDBu in SHR and WKY. Data from a number of experiments are summarized in Fig. 4. The [Ca\(^{2+}\)]_e-force relationship curve was not significantly different between SHR and WKY in the absence of PDBu in CSS. However, PDBu augmented [Ca\(^{2+}\)]_e-evoked contraction more in SHR than in WKY (Fig. 4). In contrast, 40 μM H-7 suppressed [Ca\(^{2+}\)]_e-evoked contraction in the absence (Fig. 5) or presence (data not shown) of PDBu more in SHR than in WKY. 0.1 μM calphostin C also diminished [Ca\(^{2+}\)]_e-induced tension more in SHR than in WKY (Fig. 3B).

4. Discussion

Skinning techniques have been used to investigate the intracellular signal transduction system and to change intracellular circumstances directly. However, most skinned fibers have lost some protein kinases and receptor function [16–18]. In contrast, α-toxin could be successfully used to permeabilize small preparations of smooth muscle tissue which retain functional receptors and receptor-coupled transduction systems [11]. Recently, in a preliminary study, we have demonstrated that Ca\(^{2+}\)-induced contractions were dose-dependently increased in α-toxin permeabilized SMA in Wistar rats. Moreover, PDBu significantly shifted the [Ca\(^{2+}\)]_e-force relationship curve to the left [19]. These findings are consistent with Nishimura’s report [2]. Therefore, α-toxin skinned fibers appear to be useful, in that [Ca\(^{2+}\)]_i is able to be clamped at specified values (10\(^{-8.5}\)–10\(^{-4}\) M) using EGTA-Ca\(^{2+}\) buffer.

Mrwa et al. [20] have reported that calcium requirement for activation of Triton-X-100 permeabilized tail artery were not significantly different between 7-month-old SHR (stroke-prone strain) and WKY. However, this explanation may not be complete, because Triton-X-100 skinned fibers have lost CaM, some protein kinases, and SR function [16]. In order to elucidate clearly the role of PKC in vascular smooth muscle contraction in hypertensive rats, we examined whether PKC activation influences the relationship between [Ca\(^{2+}\)]_i and tension in two kinds of small arterial rings from prehypertensive SHR and normotensive WKY; one ring was intact SMA, and the other ring was α-toxin permeabilized SMA.

We observed in this study that, in intact SMA (α-toxin non-treated artery), high K\(^+\) responses were not different

Fig. 5. Effects of 1-(5-isooquinolinesulfonyl)-2-methylpiperazine H-7 on Ca\(^{2+}\)-induced contraction in α-toxin permeabilized small mesenteric artery from SHR and WKY. The α-toxin skinned SMA from SHR and WKY were stimulated with pCa 7 (10\(^{-7}\) M Ca\(^{2+}\)-cytoplasmic substitution solution (CSS)). The rings were then additionally exposed to 40 μM H-7 for 10 min. Temperature 37°C; resting tension 300 mg (A: left panel). Ca\(^{2+}\)-induced contractions were not different between SHR and WKY. However, H-7 suppressed [Ca\(^{2+}\)]_e-evoked contraction in the absence of PDBu more in SHR than in WKY (B: right panel).
between young SHR and WKY. These findings may be explained by the evidence that Ca\(^{2+}\) influx via voltage-dependent Ca\(^{2+}\) channels was the same in young SHR and WKY [21]. PDBu augmented high K\(^+\)-evoked contraction more in SHR than in WKY, and PKC inhibitors, such as H-7 and calphostin C, suppressed contractile response to high K\(^+\) in the absence or presence of PDBu more in SHR than in WKY. These findings indicate that Ca\(^{2+}\) sensitivity of arterial contractile apparatus via PKC may be greater in SHR than in WKY. In order to determine these assumptions, we tested the effects of PKC on [Ca\(^{2+}\)]\(_i\)-induced tension using a more direct approach: α-toxin permeabilized artery in young SHR and WKY. The [Ca\(^{2+}\)]\(_i\)-force relationship curve was not significantly different between SHR and WKY. This finding is consistent with the report that basal PKC activity in aorta was not significantly different between young SHR and WKY [22]. However, PDBu augmented [Ca\(^{2+}\)]\(_i\)-evoked contraction more in SHR than in WKY. In contrast, PKC inhibitors suppressed [Ca\(^{2+}\)]\(_i\)-induced tension in the absence or presence of PDBu more in SHR than in WKY. These results are explained by the idea that the Ca\(^{2+}\) sensitivity of vascular smooth muscle contractile elements through PKC is greater in SHR than in WKY in the prehypertensive phase.

Previously, we reported that norepinephrine (NE) overflow by electrical stimulation in mesenteric artery was significantly increased in 7-week-old SHR as compared with WKY [23]. Moreover, it was reported that NE-induced contractions in mesenteric artery were significantly increased in young SHR as compared with WKY [24,25]. This finding may be explained by the evidence that Ca\(^{2+}\) influx via receptor-operated Ca\(^{2+}\) channels is significantly increased in SHR as compared with WKY [26]. Our report might include the key points to explain increased NE responses in young SHR. This result may be due to increased Ca\(^{2+}\) sensitivity of contractile elements via PKC. It was reported that basal PKC activity in aorta is almost the same in young SHR and WKY [22]. Because [Ca\(^{2+}\)]\(_i\) stays at the resting level and PKC is not activated under the usual conditions (non-excited resting phase), blood pressure might be not high in young SHR. But sometimes PKC in vascular smooth muscle cell may be activated by some receptor agonists, so increased Ca\(^{2+}\) sensitivity of contractile elements via PKC might be involved in the pathogenesis of hypertension.

In contrast, in adult rats, Soloviev reported that the Ca\(^{2+}\)-force relationship curve was significantly shifted to the left and PKC inhibitors significantly decreased Ca\(^{2+}\)-evoked tension in SHR as compared with WKY in saponin skinned fibers [18]. These findings are consistent with our data [27]. However, the results may be due to increased basal PKC activity in adult SHR as compared with age-matched WKY [22].

Our results in this study suggest that increased Ca\(^{2+}\) sensitivity of contractile elements via PKC in α-toxin permeabilized SMA from young prehypertensive SHR may be involved in the pathogenesis of hypertension. Research into PKC isoforms [28,29] is ongoing in an effort to learn what their roles in the signal transduction system are and how they regulate Ca\(^{2+}\) sensitivity of the contractile elements and arterial contractile state.

References


