Relationship between Intracellular Ca\(^{2+}\) Store and Protein Kinase C in Agonist-Induced Contraction of Hypertensive Rat Aortae

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Abstract

The roles of intracellular Ca\(^{2+}\) store and protein kinase C (PKC) in vascular contractile responses independent of Ca\(^{2+}\) influx were studied using aortic rings from spontaneously hypertensive rat (SHR) and Wistar-Kyoto rat (WKY). The functional sizes of agonist-sensitive intracellular Ca\(^{2+}\) store were estimated as the peak response to agonist after PKC inhibition with calphostin C (Cal-C), a PKC inhibitor. The participation of PKC in 5-hydroxytryptamine-, phenylephrine-, and endothelin-1 (ET-1)-induced contractions in aortae of SHR was equal to, or greater than that in WKY. In contrast, compared with WKY, SHR aortae possessed a greater size of endothelin-1-sensitive Ca\(^{2+}\) store, a similar size of 5-hydroxytryptamine-sensitive Ca\(^{2+}\) store, and a smaller size of phenylephrine-sensitive Ca\(^{2+}\) store. Based on these data, both PKC activation and functional size of intracellular Ca\(^{2+}\) store differ between SHR and WKY and these differences are selective among agonists.

Key words: spontaneously hypertensive rat, intracellular Ca\(^{2+}\) store, protein kinase C, smooth muscle (vascular)

Introduction

It is well established that there are differences in the contractile reactivity of vascular smooth muscles from spontaneously hypertensive rats (SHR) compared to their normotensive counterparts (Wistar-Kyoto rats (WKY)). These differences may be due to an altered effectiveness of protein kinase (PKC), since vascular smooth muscles from SHR demonstrated...
an increased sensitivity to contractile phorbol esters, which directly activate PKC (Turla and Webb, 1987; Mackay and Cheung, 1987). Alteration of intracellular Ca\(^{2+}\) handling may also be responsible for the different contractility in SHR. There is substantial evidence for increases in Ca\(^{2+}\) permeability of sarcolemma in vascular smooth muscle of hypertensives (Winquist et al., 1982), for the Ca\(^{2+}\)-accumulating properties of intracellular structures (Postnov, 1990), and for a decrease in plasma membrane Ca\(^{2+}\) pump activity (Kwan, 1985) in vascular smooth muscle of hypertensives. All these abnormalities may lead to increased intracellular Ca\(^{2+}\) levels. Although the free Ca\(^{2+}\) concentration in cytoplasm is an important factor determining the contractile force of smooth muscle, the smooth muscle may contract without an increase in the cytosolic free Ca\(^{2+}\) concentration, as a result of the increase in Ca\(^{2+}\) sensitivity (Rasmussen and Barrett, 1984). Indeed, direct measurements of basal or agonist elevated cytosolic free Ca\(^{2+}\) level in vascular smooth muscle cells from SHR and WKY have shown inconsistent results (Erne and Hermsmeyer, 1989; Sugiyama et al., 1990).

This study compares the role of intracellular mechanisms of aortic smooth muscle contraction in Ca\(^{2+}\)-free condition between SHR and WKY. The availability of functional Ca\(^{2+}\) stores was investigated after incubating aortic strips in Ca\(^{2+}\)-free conditions so that mechanisms of activation which are not dependent on Ca\(^{2+}\) entry through the sarcolemma could be studied. Furthermore, the participation of PKC in vascular contraction was also determined under Ca\(^{2+}\)-free condition using calphostin C, a PKC inhibitor (Shimamoto, et al., 1993).

**Methods**

**Animals and blood pressure**

Male spontaneously hypertensive rats (SHR) of about 10 weeks and age-matched Wistar-Kyoto normotensive rats (WKY) (170–190 g, body weight) were used. Systolic blood pressures were measured in the conscious state by a tail-cuff method (pneumatic transducer). Systolic blood pressures of SHR (185±12 mmHg, n=48) were significantly higher than those of WKY (112±11 mmHg, n=52) (p<0.01).

**Tissue isolation and handling**

Rats were killed by cervical dislocation. This procedure followed the guideline of the University Animal Care Committee. The descending aortae were excised rapidly and cleaned of excess fat and connective tissue and subsequently cut into 3-mm wide rings. The vascular endothelium was denuded by gently rubbing the intimal surface of the vessel with a wooden stick. The functional removal of the endothelium was tested by applying acetylcholine to precontracted aortic rings (Shimamoto et al., 1992). The vascular rings were suspended in 3- or 9-ml organ bath containing Krebs-bicarbonate solution (Ca\(^{2+}\)-containing medium) of the following composition (10\(^{-3}\) M): NaCl, 115.0; KCl, 4.6; CaCl\(_2\), 2.5; MgNO\(_4\), 1.2; NaH\(_2\)PO\(_4\), 1.2; NaHCO\(_3\), 21.9; and glucose, 11.0 (pH 7.4–7.5). Tissue baths were maintained at 37°C and aerated with 95% O\(_2\)/5% CO\(_2\). Resting tensions were maintained at 2 g. Vascular rings were allowed to equilibrate for 120 minutes before starting the experiments. Isometric tensions were recorded on a Gould 2800 Recorder or Beckman 611.
All experiments were performed under ordinary fluorescent lighting because of the photoactivatable properties of Cal-C (Bruns et al., 1991).

Validation of $10^{-6}$ M calphostin C (Cal-C) as a selective PKC inhibitor

Since we define the action of PKC with pharmacological tool, Cal-C, in this work. Its selectivity on PKC modulated contractile responses were studies in preliminary experiments to validate its use as a selective inhibitor of PKC. In rat aortae isolated from both SHR and WKY, contractile responses to $10^{-6}$ M 12-0-tetradecanoylphorbol-13-acetate (TPA) or $6 \times 10^{-5}$ M KCl were obtained in the absence and presence of $3 \times 10^{-7}$ M, $10^{-6}$ M and $3 \times 10^{-6}$ M Cal-C under Ca$^{2+}$-containing condition. Cal-C ($3 \times 10^{-7}$ M) inhibited the maximal tension of $10^{-6}$ M TPA-induced contraction by $54.5 \pm 11.2\%$ ($p<0.01$) in WKY and $52.9 \pm 10.8\%$ ($p<0.01$) in SHR. Contractile responses to $10^{-6}$ M TPA were completely inhibited by $10^{-6}$ M Cal-C (both $p<0.01$ in WKY and SHR), whereas $10^{-6}$ M Cal-C had no effect on $6 \times 10^{-2}$ M KCl-induced contraction in either SHR and WKY. Cal-C ($3 \times 10^{-6}$ M) abolished $10^{-6}$ M TPA-induced contraction in SHR and WKY (both $p<0.01$), but it also reduced contractile responses to $6 \times 10^{-2}$ M KCl in WKY and SHR (both $p<0.05$). Because PKC apparently does not participate in KCl-induced contraction (Haller et al., 1990; Takuwa et al., 1988), $10^{-6}$ M Cal-C appeared to be the appropriate concentration under Ca$^{2+}$-containing condition.

We also confirmed that in Ca$^{2+}$-free medium, $10^{-6}$ M TPA-induced contraction was completely inhibited by $10^{-6}$ M Cal-C in SHR and WKY. In this study, therefore, we regarded Cal-C as a selective PKC inhibitor when used at $10^{-6}$ M.

Inhibitory effects of $10^{-6}$ M Cal-C on agonist-induced contraction in Ca$^{2+}$-free conditions

On the assumption that Ca$^{2+}$ mobilization from intracellular Ca$^{2+}$ store and activation of PKC may account for agonist-induced contractile responses in Ca$^{2+}$-free condition, we examined the effect of $10^{-6}$ M Cal-C on agonist-induced responses in Ca$^{2+}$-free condition and assessed the participation of PKC and the functional sizes of agonist-sensitive Ca$^{2+}$ stores.

TPA- and ET-1-induced contractions

Tissues were incubated in Ca$^{2+}$-free medium + $10^{-3}$ M EGTA, which was prepared by omitting CaCl$_2$ and adding $10^{-3}$ M EGTA in order to completely eliminate contaminating Ca$^{2+}$ from both fluid and superficially membrane-bound Ca$^{2+}$ (Daniel et al., 1983; Guan et al., 1988). Tissues were incubated in Ca$^{2+}$-free medium + $10^{-3}$ M EGTA for 15 minutes before addition of either $10^{-8}$ M ET-1 and $10^{-6}$ M TPA. In separate paired preparations, the same procedure was performed following 45-minute preincubation with $10^{-6}$ M Cal-C. In some experiments, tissues were exposed to $3 \times 10^{-5}$ M cyclopiazonic acid (CPA) at least 15 minutes prior to addition of $10^{-8}$ M ET-1 or $10^{-6}$ M TPA in Ca$^{2+}$-free medium + $10^{-3}$ M EGTA in order to deplete intracellular Ca$^{2+}$ store by selectively inhibiting the sarcoqlasmic reticulum (SR) Ca$^{2+}$ pump activity (Deng and Kwan, 1991; Seidler et al., 1989).

PE- and 5-hydroxytryptamine(5-HT)-induced contractions

In both SHR and WKY, $10^{-5}$ M PE or $10^{-4}$ M 5-HT in Ca$^{2+}$-free medium + $5 \times 10^{-5}$ M
EGTA elicited a peak phasic contraction, which then subsequently declined toward a suprabasal level. After washout, readministration of either agonist failed to elicit a second peak phasic contraction, reflecting complete depletion of intracellular PE- or 5-HT-sensitive Ca\(^{2+}\) store.

PE- or 5-HT-sensitive intracellular Ca\(^{2+}\) is readily influenced by the extracellular Ca\(^{2+}\) concentration; i.e., these Ca\(^{2+}\) stores are easily depleted in Ca\(^{2+}\)-free medium + 10\(^{-3}\) M EGTA (Guan et al., 1988). Conversely, these stores are, with time, repleted when they are exposed to Ca\(^{2+}\)-containing medium. Therefore, in this study on 5-HT- and PE-sensitive intracellular Ca\(^{2+}\) stores, 5 \times 10^{-5} M EGTA was added to nominally Ca\(^{2+}\)-free medium to minimize the decrease in Ca\(^{2+}\) concentration of these stores. This concentration of EGTA is also sufficient to chelate contaminating Ca\(^{2+}\) (Guan et al., 1988).

Either 10\(^{-5}\) M PE or 10\(^{-4}\) M 5-HT in Ca\(^{2+}\)-free medium + 5 \times 10^{-5} M EGTA (S\(_1\) stimulation) elicited a peak phasic increase in force, which then subsequently declined toward a suprabasal level (Fig. 1A). Peak tension (S\(_1\)) after an addition of either agonist in Ca\(^{2+}\)-free medium was measured in its phasic response. After the peak phasic contraction had declined toward a suprabasal level, the agonist was washed out and tissues were exposed to 2.5 \times 10^{-3} M Ca\(^{2+}\) for 45 minutes, prior to a second administration of either 10\(^{-5}\) M PE or 10\(^{-4}\) M 5-HT in Ca\(^{2+}\)-free medium + 5 \times 10^{-5} M EGTA (S\(_2\) stimulation) (Fig. 1A). Peak tension (S\(_2\)) after the second addition of either agonist in Ca\(^{2+}\)-free medium + 5 \times 10^{-5} M EGTA was measured in its phasic response.

**Fig. 1.** Typical tracings of restoring the phenylephrine (PE)-induced contraction in SHR. Rat aortic rings were challenged with 10\(^{-5}\) M PE in Ca\(^{2+}\)-free medium + 5 \times 10^{-5} M EGTA (S\(_1\) stimulation), resulting a peak phasic increase in force which then subsequently declined toward a suprabasal level. Then tissues were washed with Ca\(^{2+}\)-free medium + 5 \times 10^{-5} M EGTA (W). Tissues were exposed to 2.5 \times 10^{-3} M Ca\(^{2+}\) for 45 minutes. Ca\(^{2+}\)-containing medium was switched to Ca\(^{2+}\)-free medium + 5 \times 10^{-5} M EGTA (W), and aortic rings were stimulated again with 10\(^{-5}\) M PE (S\(_2\) stimulation). Open boxes indicate the presence of agents.

(A) Control. After the first phenylephrine-induced contraction (S\(_1\) stimulation), the PE-sensitive Ca\(^{2+}\) stores were emptied. Reintroduction of Ca\(^{2+}\) resulted in the entry of Ca\(^{2+}\), which was rapidly taken up by the PE-sensitive Ca\(^{2+}\) pool without eliciting a contractile response. There was no significant difference between S\(_1\) and S\(_2\) stimulations, suggesting an effective repletion of the PE-sensitive Ca\(^{2+}\) pool.

(B) Following complete refilling of Ca\(^{2+}\) store by 45 minutes, calphostin C (Cal-C, 10\(^{-8}\) M) was added to the medium for another 45 minutes. Calphostin C (10\(^{-8}\) M) decreased the maximal tension of 10\(^{-5}\) M PE-induced peak phasic contraction (S\(_2\) stimulation) and also inhibited the following small, but sustained contraction.
response. In our preliminary study, since the ratio of $S_2/S_1$ in the PE- or 5-HT-induced contraction were not significantly different from unity, a 45-minute incubation time was considered to be enough exposure to Ca$^{2+}$ to completely restore both PE- and 5-HT-sensitive Ca$^{2+}$ stores. In order to evaluate the participation of PKC in PE- and 5-HT-induced contractions in Ca$^{2+}$-free medium $+5 \times 10^{-5}$ M EGTA, $10^{-6}$ M Cal-C was added following complete refilling of Ca$^{2+}$ store by 45-minute exposure to Ca$^{2+}$ (Fig.1B). Cal-C ($10^{-6}$ M) was added for another 45 minutes. Tissues were then washed with Ca$^{2+}$-free medium $+5 \times 10^{-5}$ M EGTA $+10^{-4}$ M Cal-C prior to a second administration of an agonist. Percentages of inhibition of $S_2$ by $10^{-6}$ M Cal-C [$100 \times (S_2-S_1)/S_1$] were calculated (Fig.1B). In each experiment, we confirmed that $S_2/S_1$ was not different from unity in control preparations.

In some experiments, tissues were exposed to $3 \times 10^{-5}$ M CPA for at least 15 minutes prior to administration of Ca$^{2+}$ so that refilling of the Ca$^{2+}$ store might be fully prevented (Fig.2A).

**Determination of the functional Ca$^{2+}$ store**

Since PKC activation may initiate contraction without an elevation of cytosolic Ca$^{2+}$ levels, after blocking PKC activation by calphostin C the agonist-induced contraction in Ca$^{2+}$-free medium would reflect the response solely to the elevation of intracellular Ca$^{2+}$. KCl elicited the same contractile response in SHR and WKY in Ca$^{2+}$-free medium, showing the same
sensitivity and responsiveness of contractile elements to $\text{Ca}^{2+}$ between SHR and WKY. Therefore, we have defined the peak response to agonists after PKC inhibition by calphostin C in $\text{Ca}^{2+}$-free medium as the measure of the "functional $\text{Ca}^{2+}$ store".

**Statistical analysis**

All values are presented as the mean±S.D. Significance of difference between the two means was determined by either Wilcoxon matched-pairs, signed rank test or nonparametric Wilcoxon U test. Differences between mean values with $p<0.05$ were considered significant.

**Drugs**

Drugs used in this study were phenylephrine (Sigma Chemical Company, St. Louis, MO, USA), 5-hydroxytryptamine (Sigma), cyclopiazonic acid (Sigma), endothelin-1 (Bachem Inc, Torrance, CA, USA), TPA (Sigma), calphostin C (Kamiya Biomedical Co. CA, USA) and EGTA; ethylene glycol-bis(beta-aminoethyl ether)-N, N, N, N-tetracetic acid (Sigma). Cal-C was used immediately after being dissolved.

**Results**

**Effects of calphostin C on TPA- and ET-1-induced contractions and their functional size of intracellular $\text{Ca}^{2+}$ store**

In $\text{Ca}^{2+}$-free medium + $10^{-3}$ M EGTA, $10^{-6}$ M TPA elicited a higher sustained contraction in SHR than that in WKY ($p<0.05$). Calphostin C ($10^{-6}$ M) abolished $10^{-6}$ M TPA-induced contraction in both WKY and SHR in $\text{Ca}^{2+}$-free medium + $10^{-3}$ M EGTA. Furthermore, $3\times10^{-5}$ M CPA had no effect on TPA-induced contraction in SHR and WKY.

In $\text{Ca}^{2+}$-free medium + $10^{-3}$ M EGTA, $10^{-8}$ M ET-1 induced a slowly-developing and sustained contraction in both SHR and WKY. The maximal tension in SHR was significantly greater than that in WKY ($p<0.05$). Calphostin C ($10^{-6}$ M) inhibited $10^{-8}$ M ET-1-induced contraction to the same extent in SHR and WKY. The functional size of ET-1-sensitive intracellular $\text{Ca}^{2+}$ store, estimated as the maximal tension in $\text{Ca}^{2+}$-free medium after treatment with $10^{-6}$ M calphostin C, was also greater in SHR than that in WKY ($0.43\pm0.13$ g in WKY vs $0.56\pm0.15$ g in SHR, $p<0.05$).

In the presence of $3\times10^{-5}$ M CPA, there was no difference in contractions induced by $10^{-8}$ M ET-1 between WKY and SHR derived aortae ($0.14\pm0.08$ g in WKY, $n=6$; $0.17\pm0.12$ g in SHR, $n=6$). Fig. 3 demonstrated the effect of CPA on ET-1-induced contraction in $\text{Ca}^{2+}$-free medium + $10^{-3}$ M EGTA. In the ET-1 precontracted tissue, $3\times10^{-5}$ M CPA elicited a transient contraction and then markedly reduced the vascular tone, but not to the resting level. This observation suggests that $\text{Ca}^{2+}$ uptake by sarcoplasmic reticulum was blocked by CPA, thus redirecting $\text{Ca}^{2+}$ released from ET-1-sensitive $\text{Ca}^{2+}$ store to contractile elements for contraction, and that the residual tension may be due to PKC activation. CPA ($3\times10^{-5}$ M) exerted a greater inhibitory effect on ET-1-induced contraction in SHR than in WKY ($0.54\pm0.12$ g in SHR, $n=6$; $0.39\pm0.10$ g in WKY; $n=7$, $p<0.05$) resulting in a similar contractile response to ET-1 in $\text{Ca}^{2+}$-free medium + $10^{-3}$ M EGTA + $3\times10^{-5}$ M CPA in both strains. This suggested
that ET-1-induced PKC activation was similar in the two tissues. Furthermore, in Ca²⁺-free medium + 10⁻³ M EGTA containing both CPA and calphostin C, ET-1 failed to induce appreciable responses in either strain of rat, which confirmed the assumption that ET-1-induced contraction in Ca²⁺-free medium + 10⁻³ M EGTA may be caused by PKC activation and Ca²⁺ release from an ET-1-sensitive Ca²⁺ store.

Effects of Cal–C on PE- and 5-HT-induced contraction and their functional sizes of intracellular Ca²⁺ stores

The maximal tension of the peak phasic contraction elicited by 10⁻⁵ M PE was smaller in SHR than that in WKY (p<0.05). Cal–C (10⁻⁶ M) decreased the maximal tension of 10⁻⁵ M PE-induced peak phasic contraction and also inhibited the following small sustained contraction in both SHR and WKY (Fig. 1B). Since there was no significant difference in degree of inhibition of peak phasic contraction by 10⁻⁶ M Cal–C between SHR and WKY, the PE-induced maximal tension in the presence of 10⁻⁶ M Cal–C, defined as the functional size of PE-sensitive Ca²⁺ pool, was still greater in WKY than that in SHR (0.93±0.18 g in WKY vs 0.78±0.15 g in SHR, p<0.05).

The maximal tension of 10⁻⁴ M 5-HT-induced contraction was greater in SHR than that in WKY (p<0.05) in Ca²⁺-free medium + 5×10⁻⁵ M EGTA. However, 10⁻⁶ M Cal–C inhibited 5-HT-induced contraction in this medium more effectively in SHR than that in WKY (p<0.01) (Table 1). As a consequence, in Ca²⁺-free medium + 5×10⁻⁵ M EGTA + 10⁻⁶ M Cal–C, there was no significant difference in the maximal tension of 5-HT-induced phasic contraction between SHR and WKY, suggesting a similar functional size of the 5-HT-sensitive intracellular Ca²⁺ store.

The effects of 3×10⁻⁵ M CPA on PE- or 5-HT-induced peak phasic contraction in Ca²⁺-free medium + 5×10⁻⁵ M EGTA was investigated in order to examine the functional size of 5-HT- or PE-sensitive Ca²⁺ store (Fig. 2A). On washout of 10⁻⁵ M PE or 10⁻⁴ M 5-HT after this agonist-sensitive Ca²⁺ store has been depleted, reintroduction of Ca²⁺ elicited a prominent contraction presumably because, in the presence of CPA, extracellular Ca²⁺ reaches the cytoplasm and contractile elements without refilling PE- or 5-HT-sensitive Ca²⁺ stores. Pretreatment with 3×10⁻⁵ M CPA almost abolished the phasic components of the PE- or 5-
Table 1. Comparison of the maximal tension in Ca\(^{2+}\)-free medium between WKY and SHR, and inhibitory effects of 10\(^{-6}\) M calphostin C on the maximal tension in Ca\(^{2+}\)-free conditions.

| Agonist | Maximal tension in Ca\(^{2+}\)-free conditions | % Inhibition of maximal tension by 10\(^{-6}\) M calphostin C | Functional size of agonist-sensitive Ca\(^{2+}\) store |
|---------|-----------------------------------------------|---------------------------------------------------------------|-----------------------------------------------|
| 10\(^{-5}\) M PE | WKY 1.60±0.22 g (14) | 19.5±7.6% (14) | 0.93±0.18 g (14) |
|         | SHR 0.97±0.19 g (14)* | 21.1±8.8% (14) | 0.78±0.15 g (14)* |
| 10\(^{-4}\) M 5-HT | WKY 1.01±0.23 g (12) | 10.1±4.3% (12) | 0.89±0.20 g (12) |
|         | SHR 1.31±0.20 g (12)* | 25.5±8.3% (12)*d | 0.97±0.18 g (12) |
| 10\(^{-8}\) M ET-1 | WKY 0.56±0.15 g (12) | 21.5±15.5% (12)* | 0.43±0.13 g (12) |
|         | SHR 0.73±0.17 g (12)* | 26.5±18.5% (12)* | 0.56±0.15 g (12)* |
| 10\(^{-8}\) M TPA | WKY 0.79±0.21 g (12) | 100% (12)* | 0 g (12) |
|         | SHR 0.99±0.22 g (12)* | 100% (12)* | 0 g (12) |

Values are means±S.D. for the number of rats given in parentheses. \(\%\) Ratio—the percent ratio of maximal tension in Ca\(^{2+}\)-free conditions to that in Ca\(^{2+}\)-containing medium. Inhibitory effects are expressed as percent decreases in the maximal tension to agonists in Ca\(^{2+}\)-free conditions in the presence of 10\(^{-6}\) M calphostin C. TPA=12-O-tetradecanoylphorbol-13-acetate.

\*p<0.05, \#p<0.01, significantly different compared to corresponding WKY. \(\dagger p<0.05, \ddagger p<0.01, \) statistical significance of percent difference in the absence and presence of 10\(^{-6}\) M calphostin C.

Discussion

In this study, the functional sizes of the intracellular Ca\(^{2+}\) stores responsible for agonist-induced contractions were not always greater in SHR than in WKY. Compared with WKY, SHR aortae possessed a greater functional size of ET-1-sensitive Ca\(^{2+}\) store, a similar size of 5-HT-sensitive Ca\(^{2+}\) store, and a smaller size of PE-sensitive Ca\(^{2+}\) store. On the other hand, the participation of PKC in agonist-induced contractions in SHR was equal to or greater than that in WKY under Ca\(^{2+}\)-free condition, estimated from the degree of inhibition of agonist-induced contractions by 10\(^{-6}\) M Cal-C. Thus, both activation of PKC and functional size of intracellular Ca\(^{2+}\) stores differ between SHR and WKY and these differences are selective among agonists. The experimental evidence and the rationale for these conclusions are discussed below.

**Contractile mechanisms in agonist-induced contraction under Ca\(^{2+}\)-free condition**

As to the time course in 5-HT- and PE-induced contractions in Ca\(^{2+}\)-free condition, phasic contractions were elicited within a few seconds, followed by small sustained contractions, as shown in this study. We assumed that PKC activation is involved all throughout the time.
course of 5-HT- or PE-induced phasic contraction. Therefore, PKC participation in these phasic contractions was estimated from the extent of inhibition by calphostin C.

In rat aorta, 5-HT and PE activate phospholipase C via 5-HT_2 receptors and alpha_1-adrenoceptors, respectively (Roth et al., 1986), resulting in the release of two second messengers, water soluble inositol 1, 4, 5-triphosphate (IP_3) and membrane-associated diacylglycerol. IP_3 liberates Ca^{2+} from intracellular stores, whereas diacylglycerol activates PKC (Berridge, 1984).

If both IP_3 and diacylglycerol initiated signal transduction cascades are responsible for this contraction, receptor-mediated turnover of phosphoinositides should occur within a few seconds after the agonist activates its receptor. In cultured rat aortic smooth muscle cells angiotensin II has been shown to induce accumulation of IP_3 within 5 seconds (Griendling et al., 1987). Huzoor-Akbar and Anwer (1988) have shown that thrombin induces detectable hydrolysis of PIP_2 in washed rat platelets within 3 seconds and maximal hydrolysis of PIP_2 within 15 seconds. On the other hand, diacylglycerol is initially produced as a result of hydrolysis of inositol phospholipids, which is normally transient and temporally corresponds to the formation of IP_3. Frequently it is followed by a more sustained increase in the amount of diacylglycerol (Nishizuka, 1992), and this second phase of diacylglycerol formation probably results from hydrolysis of phosphatidylcholine (Nishizuka, 1992). Therefore, PKC can also be activated within a few seconds in response to these agonists, concomitantly with IP_3. Furthermore, as illustrated in this study (Fig. 2A), PE-induced, small, sustained contractions, developed rapidly in the presence of CPA. As mentioned above, because PKC dependent contraction has already been superimposed in the agonist-induced phasic contractions in Ca^{2+}-free condition, it seems reasonable to estimate the component of contraction dependent on PKC participation and the functional size of agonist-sensitive Ca^{2+} stores, independent of PKC activation, from the extent of inhibition of these phasic contractions by calphostin C.

When ET-1 induced a slowly-developing and sustained contraction, concentration- and time-dependent formation of IP_3 and release of intracellular Ca^{2+} have been demonstrated (Marsden et al., 1989; Resink et al., 1988). Moreover, ET-1 generates diacylglycerol (Resink et al., 1988), which can subsequently activate PKC. In this study, ET-1-induced contractions were composed of two components; one component is dependent on CPA- or ET-1-sensitive intracellular Ca^{2+}, and the other is sensitive to calphostin C and requires activation of PKC.

There were no significant differences in TPA-induced responses in the absence and presence of CPA. Thus TPA seems to act independently of Ca^{2+}-mobilization from the CPA-sensitive Ca^{2+} store, or alternately TPA-sensitive intracellular Ca^{2+} store seems negligible. These results were supported by the observations that TPA did not initiate a redistribution of intracellular Ca^{2+} from intracellular Ca^{2+} stores into the cytoplasm, or changes in cytosolic free Ca^{2+} concentration (Neusser et al., 1993). Since calphostin C abolished TPA-induced contraction, TPA elicited a sustained contraction dependent on activation of PKC.

**Functional sizes of 5-HT-, PE-, and ET-1-sensitive intracellular Ca^{2+} stores**

Regarding resting cytosolic free Ca^{2+} concentration, Neusser et al. (1993) and Erne and Hermsmeyer (1989) both demonstrated that there is no significant difference of resting cytosolic
free Ca\textsuperscript{2+} concentration between SHR and WKY. Responses of contractile elements to raised cytosolic Ca\textsuperscript{2+} concentrations are regarded as identical between SHR and WKY, because similar KCl-induced contractions were seen between these strains in the absence and presence of calphostin C. Therefore, when the functional size of agonist-sensitive Ca\textsuperscript{2+} store is defined as the ability of the SR to take up, store, and release Ca\textsuperscript{2+} (independent of PKC activation), SHR aorta possesses a greater ET-1-sensitive Ca\textsuperscript{2+} store, an equal size of 5-HT-sensitive Ca\textsuperscript{2+} store, and a smaller PE-sensitive Ca\textsuperscript{2+} store, as compared to WKY aorta. In Ca\textsuperscript{2+}-free condition, ET-1-induced contraction is mainly (70%) caused by Ca\textsuperscript{2+} release from this agonist-sensitive Ca\textsuperscript{2+} store, which is also sensitive to CPA. The contraction dependent on Ca\textsuperscript{2+} release from ET-1-sensitive Ca\textsuperscript{2+} store was slowly developing and sustained, presumably because Ca\textsuperscript{2+} release was slow. In contrast, 5-HT- and PE-sensitive Ca\textsuperscript{2+} stores appeared to release Ca\textsuperscript{2+} rapidly but transiently as indicated by the time course of their contractions. The mechanism involved in agonist-induced sustained contractions may be an important factor in the development of hypertension. Then the functional size of ET-1-sensitive Ca\textsuperscript{2+} store influenced by factors such as Ca\textsuperscript{2+} pump activity, leakiness of sarcoplasmic reticulum and different release channels, may partly explain the genesis of hypertension.

**Role of PKC in agonist-induced contractions**

In this study, we demonstrated the increased responsiveness of SHR aortae to TPA in Ca\textsuperscript{2+}-free condition, which is consistent with previous reports (Bruschi et al., 1988; MacKay and Cheung, 1987; Silver et al., 1988, 1992; Soloviev and Bershtein, 1992; Turla and Webb, 1987). This finding supports our conclusion that the participation of PKC in 5-HT-, PE-, and ET-1-induced contractions in SHR is equal to or greater than that in WKY, when estimated by the inhibitory effect of calphostin C on each agonist-induced contraction. PKC activation is another major determinant eliciting a long-lasting contraction with or without extracellular Ca\textsuperscript{2+}. Therefore, an increased responsiveness to TPA and a greater or equal participation of PKC in agonist-induced contraction may enhance the contractile properties of the vascular smooth muscle in SHR. Our conclusion has not taken account the presence of multi-isoforms of PKC and future studies on the possible involvement of PKC isoforms in the contractile properties of the vascular smooth muscle in hypertension are warranted.
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