Endothelin-1 Promotes Ca\(^{2+}\) Antagonist-Insensitive Coronary Smooth Muscle Contraction Via Activation of \(\varepsilon\)-Protein Kinase C

Lashonn L. McNair, Daisy A. Salamanca, Raouf A. Khalil

Abstract—Certain forms of coronary artery disease do not respond to treatment with Ca\(^{2+}\) channel blockers, and a role for endothelin-1 (ET-1) in Ca\(^{2+}\) antagonist-insensitive forms of coronary vasospasm has been suggested; however, the signaling mechanisms involved are unclear. We tested the hypothesis that a component of ET-1–induced coronary smooth muscle contraction is Ca\(^{2+}\) antagonist-insensitive and involves activation of protein kinase C (PKC). Cell contraction was measured in smooth muscle cells isolated from porcine coronary artery, \([\mathrm{Ca}^{2+}]_i\), was measured in fura-2 loaded cells, and the cytosolic and particulate fractions were examined for PKC activity and reactivity with isoform-specific PKC antibodies using Western blot analysis. In Hank’s solution (1 mmol/L Ca\(^{2+}\)), ET-1 (10\(^{-7}\) mol/L) caused a transient increase in \([\mathrm{Ca}^{2+}]_i\), (236±14 nmol/L) followed by a maintained increase in \([\mathrm{Ca}^{2+}]_i\), (184±8 nmol/L) and 35% cell contraction. The Ca\(^{2+}\) channel blockers verapamil and diltiazem (10\(^{-6}\) mol/L) abolished the maintained ET-1–induced \([\mathrm{Ca}^{2+}]_i\), but only partially inhibited ET-1–induced cell contraction to 18%. The verapamil-insensitive component of ET-1 contraction was inhibited by the PKC inhibitors calphostin C and \(\varepsilon\)-PKC\(_{\text{V1\_2}}\). ET-1 caused translocation of Ca\(^{2+}\)-dependent \(\alpha\)-PKC and Ca\(^{2+}\)-independent \(\varepsilon\)-PKC from the cytosolic to the particulate fraction that was inhibited by calphostin C. Verapamil abolished ET-1–induced translocation of \(\alpha\)-PKC, but not that of \(\varepsilon\)-PKC. Phorbol 12-myristate 13-acetate (10\(^{-6}\) mol/L), a direct activator of PKC, caused 22% cell contraction, with no increase in \([\mathrm{Ca}^{2+}]_i\), and translocation of \(\varepsilon\)-PKC that was inhibited by calphostin C, but not by verapamil. KCl (51 mmol/L), which stimulates Ca\(^{2+}\) influx, caused 35% cell contraction and increase in \([\mathrm{Ca}^{2+}]_i\), (291±11 nmol/L) that were inhibited by verapamil, but not by calphostin C, and did not cause translocation of \(\alpha\)- or \(\varepsilon\)-PKC. In Ca\(^{2+}\)-free (2 mmol/L EGTA) Hank’s solution, ET-1 caused 15% cell contraction, with no increase in \([\mathrm{Ca}^{2+}]_i\), and translocation of \(\varepsilon\)-PKC that were inhibited by \(\varepsilon\)-PKC\(_{\text{V1\_2}}\) inhibitory peptide. Thus, a significant component of ET-1–induced contraction of coronary smooth muscle is Ca\(^{2+}\) antagonist-insensitive and involves activation and translocation of Ca\(^{2+}\)-independent \(\varepsilon\)-PKC, and may represent a signaling mechanism of Ca\(^{2+}\) antagonist-resistant forms of coronary vasospasm. (Hypertension. 2004; 43:1-8.)

Key Words: endothelin ■ calcium ■ protein kinases

Coronary vasospasm is often associated with ischemic heart disease leading to different forms of angina and myocardial infarction, and excessive coronary vasoconstriction in response to endogenous vasoconstrictors has been suggested as one potential cause. Several potent endogenous vasoconstrictors have been described, including endothelin-1 (ET-1). Plasma levels of ET-1 are increased in patients with coronary artery disease. Also, coronary artery perfusion with ET-1 in dogs is associated with severe coronary vasoconstriction and vasospasm, suggesting a role of ET-1 in coronary vasospasm and coronary artery disease.

Ca\(^{2+}\) channel blockers such as verapamil and diltiazem are often recommended to decrease coronary vasospasm and reduce the incidence of angina and myocardial infarction. However, certain forms of angina and coronary artery disease do not respond adequately to treatment with Ca\(^{2+}\) channel blockers. The causes of Ca\(^{2+}\) antagonist-insensitive forms of coronary vasospasm are unclear, but could be due to decreased sensitivity of the coronary smooth muscle contraction induced by certain endogenous vasoconstrictors to Ca\(^{2+}\) channel blockers. For example, although ET-1 is a potent coronary vasoconstrictor, the sensitivity of the ET-1–stimulated mechanisms of coronary smooth muscle contraction to Ca\(^{2+}\) channel blockers is unclear.

A potential cause of Ca\(^{2+}\) antagonist-insensitive forms of coronary vasospasm is possible activation of a Ca\(^{2+}\)-sensitization, or perhaps Ca\(^{2+}\)-independent, mechanism of smooth muscle contraction. Vascular smooth muscle contraction is triggered by increases in intracellular free Ca\(^{2+}\).
([Ca$^{2+}$]) due to Ca$^{2+}$ release from the intracellular stores and Ca$^{2+}$ entry from the extracellular space. ET-1 has been shown to increase [Ca$^{2+}$], and to stimulate the Ca$^{2+}$ mobilization mechanisms of vascular smooth muscle contraction. In addition to the increase in [Ca$^{2+}$], the interaction of a vasoconstrictor agonist with its receptor is coupled to increased breakdown of plasma membrane phospholipids and increased production of diacylglycerol (DAG). DAG binds to and activates protein kinase C (PKC). PKC is a family of several Ca$^{2+}$-dependent and Ca$^{2+}$-independent isoforms that have different enzyme properties, subcellular distributions, substrates, and functions. PKC is mainly cytosolic under resting conditions and undergoes translocation to the particulate fraction when it is activated by endogenous DAG or exogenous phorbol esters. Also, direct activation of PKC by phorbol esters causes sustained contraction of vascular smooth muscle, suggesting a role for PKC in regulating smooth muscle contraction. However, the role of PKC as a potential signaling mechanism of the Ca$^{2+}$ antagonist-insensitive forms of coronary contraction, in general, and the ET-1 induced coronary smooth muscle, in particular, has not been fully investigated.

The purpose of this study was to test the hypothesis that a component of ET-1-induced coronary smooth muscle contraction is Ca$^{2+}$ antagonist-insensitive and involves activation of specific PKC isoforms. Since the PKC family includes both Ca$^{2+}$-dependent and Ca$^{2+}$-independent isoforms, any ET-1–induced changes in [Ca$^{2+}$], may determine which PKC isoform is activated. Therefore, experiments were designed to investigate the effects of ET-1 on coronary smooth muscle cell contraction, [Ca$^{2+}$], and PKC activity. The effects of ET-1 were compared with those of phorbol 12-myristate 13-acetate (PMA), a direct activator of PKC, and with membrane depolarization by high KCl solution, activator of Ca$^{2+}$ entry from the extracellular space. The sensitivity of coronary smooth muscle cell contraction, [Ca$^{2+}$], and PKC activity to two Ca$^{2+}$-channel antagonists, namely verapamil and diltiazem, and mechanistically distinct PKC inhibitors, namely calphostin C and selective ε-PKC$_{V1}$ inhibitory peptide, was also investigated.

**Methods**

**Tissue Preparation**

Castrated male Yorkshire pigs (30 kg) were anesthetized by inhalation of isoflurane. The heart was excised, and the left anterior descending coronary artery was dissected and sectioned into 2×2 mm strips. All procedures followed the guidelines of the Institutional Animal Care and Use Committee.

**Cell Isolation**

Coronary strips (50 mg) were placed in a digestion mixture containing collagenase (236 U/mg, Worthington), elastase (3.25 U/mg, Boehringer Mannheim), and trypsin inhibitor (10 000 U/mL, Sigma) in 7.5 mL Ca$^{2+}$- and Mg$^{2+}$-free Hank’s solution. The tissue preparation was placed in shaking water bath at 34°C in an atmosphere of 95% O$_2$-5% CO$_2$. Isolated cells were poured over glass coverslips, and Ca$^{2+}$ was gradually added back to avoid the “calcium paradox.”

**Contraction Studies**

Cells were placed on the stage of an inverted Nikon microscope and viewed using 100× objective. Only viable, healthy, and spindle-shaped cells ≥60 μm in length were selected. Viable cells adhered to glass coverslips and appeared bright and without visible nucleus. Cell viability was confirmed by their contraction in response to ET-1 and high KCl. Cell images were acquired using a PXL CCD camera and changes in cell length were measured after stimulation with ET-1, KCl, or the phorbol ester PMA. Cell contraction was expressed as the final cell length (L) as a fraction of the initial cell length (L$_0$). Contraction measurements were made at 37°C.

**Measurement of [Ca$^{2+}$]$_i$**

Cells were loaded with fura-2 for 30 minutes at 34°C and viewed through a 100× objective on an inverted microscope. The Ca$^{2+}$ indicator was excited alternately at 340 nm and 380 nm and the emitted light was collected at 510 nm. The ratio between 340 nm and 380 nm signals (R) was transformed to the corresponding [Ca$^{2+}$]$_i$.

$$[Ca^{2+}]_i = K_d(S_f/S_b)R/(R_{max} - R)$$

where $R_{min}$ and $R_{max}$ represent the minimal and maximal fluorescence ratios. $S_f/S_b$ is the ratio of the 380 signal in Ca$^{2+}$-free and Ca$^{2+}$-replete solutions, respectively. $K_d$ the dissociation constant of fura-2 for Ca$^{2+}$, was 224 nM. All experiments were performed at 37°C.

**Tissue Fractions**

Tissue strips (~80 mg) were transferred to homogenization buffer as previously described. The tissue was homogenized using a 2 mL tight-fitting homogenizer at 4°C, centrifuged at 100 000 rpm for 20 minutes, and the supernatant was used as the cytosolic fraction. The pellet was re-suspended in homogenization buffer and centrifuged at 100 000 rpm for 20 minutes. The supernatant was used as the particulate fraction. Protein concentrations in tissue fractions were determined using a protein assay kit (Bio-Rad).

**PKC Activity**

PKC activity was determined in cytosolic and particulate fractions by measuring the incorporation of $^3$P from [$^3$P]ATP (ICN) into histone or myelin basic protein (MBP). The assay mixture contained 25 mmol/L Tris.HCl (pH 7.5), 10 mmol/L MgCl$_2$, 200 μg/mL histone IIIIS or MBP, 80 μg/mL phosphatidylserine, 30 μg/mL diolein, [$^3$P]ATP (2×10$^5$ cpm/nmol), and 0.5 to 3 μg protein. After 5 minutes incubation at 30°C, the reaction was stopped by spotting 25 μL of the assay mixture onto phosphocellulose discs. The discs were washed with 5% trichloroacetic acid, placed in 4 mL Ecolite scintillation cocktail, and the radioactivity was measured in liquid scintillation counter.

**Immunoblotting**

Protein–matched samples of cytosolic and particulate fractions were subjected to electrophoresis on 8% SDS polyacrylamide gels then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated in 5% dried nonfat milk in PBS-Tween at 22°C for 1 hour then labeled with anti-PKC antibody at 4°C overnight. Polyclonal antibodies to α-, β-, γ-, δ-, ε-, and ζ-PKC (Gibco) were used. To maintain constant labeling conditions, we used the same antibody titer (1:500) and protein concentration (10 μg) in all tissue samples. The nitrocellulose membranes were washed in PBS, then incubated in horseradish peroxidase-conjugated anti-rabbit IgG for 1.5 hours. The blots were washed with PBS and visualized with enhanced chemiluminescence detection system (Amersham). The reactive bands were analyzed using GS-700 imaging densitometer (Bio-Rad).

**Drugs and Chemicals**

ET-1 (Sigma), verapamil, and diltiazem (Calbiochem) were dissolved in distilled water. Neomycin and U-73122 were purchased from Biomol. PMA (Alexis), calphostin C (Kamiya), and myristoyl-
tagged e-PKC<sub>V1,-2</sub> inhibitory peptide (Biomol) were dissolved in dimethylsulfoxide (DMSO). Final DMSO concentration in solution was ≤0.1%.

**Statistical Analysis**

Data from several cells of the same pig were averaged, and the data presented for each individual pig. The data from different pigs were compared using Student t test for unpaired data with P<0.05 considered significant.

**Results**

In cells incubated in Hank’s solution (1 mmol/L Ca<sup>2+</sup>) resting cell length was 73±6 μm and basal [Ca<sup>2+</sup>], was 81±2 nM. ET-1 (10<sup>-7</sup> mol/L) caused transient increase in [Ca<sup>2+</sup>], (236±14 nM) followed by maintained [Ca<sup>2+</sup>], (184±8 nM) and 34.1±2.3% cell contraction (Figure 1A). Pretreatment with the phospholipase C inhibitor neomycin (0.5 mmol/L) or U-73122 (10<sup>-5</sup> mol/L) for 10 minutes abolished ET-1-induced cell contraction and initial increase in [Ca<sup>2+</sup>]. The maintained ET-1–induced [Ca<sup>2+</sup>], was not significantly different from the basal levels in neomycin pretreated (92±8 nM) and U-73122 pretreated cells (89±11 nM).

Pretreatment with the Ca<sup>2+</sup> channel blocker verapamil (10<sup>-6</sup> mol/L) for 10 minutes reduced the initial ET-1–induced [Ca<sup>2+</sup>], to 123±14 nM, abolished the maintained ET-1–induced [Ca<sup>2+</sup>], and partially inhibited ET-1–induced cell contraction to 17.2±2.4% (Figure 1B). Pretreatment with another Ca<sup>2+</sup> channel blocker such as diltiazem (10<sup>-6</sup> mol/L) for 10 minutes also reduced the initial ET-1–induced [Ca<sup>2+</sup>], to 119±12 nM, abolished the maintained ET-1–induced [Ca<sup>2+</sup>], and partially inhibited ET-1–induced cell contraction to 16.8±2.2%. Pretreatment with the PKC inhibitor calphostin (10<sup>-6</sup> mol/L) for 10 minutes partially inhibited ET-1–induced cell contraction to 16.5±1.6% with no change in [Ca<sup>2+</sup>], (Figure 1C). Pretreatment with verapamil+calphostin for 10 minutes abolished ET-1–induced cell contraction and maintained [Ca<sup>2+</sup>], (Figure 1D). A small transient ET-1–induced increase in [Ca<sup>2+</sup>], to 121±13 nM could be observed in cells pretreated with verapamil+calphostin (Figure 1D), but was not sufficient to cause cell contraction.

Figure 1. Representative cell contraction (upper panels) and [Ca<sup>2+</sup>] (lower panels) in coronary smooth muscle cells activated with ET-1 (10<sup>-7</sup> mol/L) in absence (A) or presence of verapamil (10<sup>-6</sup> mol/L) (B), calphostin (10<sup>-6</sup> mol/L) (C), or verapamil+calphostin (D).

Treatment with PMA (10<sup>-6</sup> mol/L), direct activator of PKC, caused 20.7±2.1% cell contraction with no increase in [Ca<sup>2+</sup>], above basal levels (Figure 2A). Pretreatment with verapamil or diltiazem (10<sup>-6</sup> mol/L) did not affect PMA-induced contraction. Pretreatment of cells with calphostin (10<sup>-6</sup> mol/L) abolished PMA-induced contraction with no significant change in [Ca<sup>2+</sup>], (Figure 2B).

Membrane depolarization using high KCl (51 mmol/L) caused 35.8±2.9% cell contraction and increased [Ca<sup>2+</sup>], to 288±13.7 nM (Figure 2C). Pretreatment with verapamil (10<sup>-6</sup> mol/L) abolished KCl-induced contraction and [Ca<sup>2+</sup>], (Figure 2D). Similarly, pretreatment with diltiazem (10<sup>-6</sup> mol/L) abolished KCl-induced cell contraction and [Ca<sup>2+</sup>], (data not shown). Treatment of cells with calphostin (10<sup>-6</sup> mol/L) did not affect KCl-induced contraction or [Ca<sup>2+</sup>], (Figure 3).

The effect of specific PKC inhibitors on ET-1 and PMA-induced contraction was also tested. The membrane permeant selective e-PKC<sub>V1,-2</sub> inhibitory peptide (10<sup>-4</sup> mol/L) caused partial but significant inhibition of ET-1 contraction to 23.9±1.8% (P=0.003). The inhibition of ET-1 contraction by e-PKC<sub>V1,-2</sub> was smaller (P=0.041) compared with that of calphostin, which inhibited ET-1 contraction to 17.4±2.1%. Also, e-PKC<sub>V1,-2</sub> abolished the verapamil-insensitive component of ET-1 contraction and PMA contraction with no significant change in [Ca<sup>2+</sup>], (Figure 3). e-PKC<sub>V1,-2</sub> did not inhibit KCl-induced contraction or [Ca<sup>2+</sup>].

In resting tissues PKC activity was greater in the cytosolic than the particulate fraction, and the particulate/cytosolic
ratio was 0.5±0.1. ET-1 (10^{-7} \text{ mol/L}) caused an increase in PKC activity in the particulate fraction, a decrease in cytosolic fraction (Figure 4A), and increase in particulate/cytosolic ratio to a maximum of 2.1±0.2 (Figure 4B). In tissues pretreated with verapamil (10^{-6} \text{ mol/L}) ET-1–induced PKC activity was partially reduced (Figure 4B, 4C). Partial reduction of ET-1–induced particulate/cytosolic PKC ratio to 1.2±0.1 was also observed in tissues pretreated with diltiazem (10^{-6} \text{ mol/L}). Pretreating the tissues with calphostin (10^{-6} \text{ mol/L}) abolished ET-1–induced PKC activity (Figure 4B, 4C). Also, ε-PKC– inhibitory peptide (10^{-4} \text{ mol/L}) decreased the verapamil-insensitive ET-1–induced PKC activity ratio to 0.6±0.1, a level that was not significantly different from basal PKC activity ratio.

In comparison with ET-1, the phorbol ester PMA (10^{-6} \text{ mol/L}) caused significant increases in PKC activity that were inhibited by calphostin, but not by verapamil (Figure 4C). In tissues stimulated with KCl (51 \text{ mmol/L}), no increases in PKC activity could be observed, and pretreating the tissues with verapamil, diltiazem, or calphostin did not change PKC activity (Figure 4C).

Western blot analysis revealed significant amounts of ε-, δ-, and ζ-PKC. In resting tissues, α- and ε-PKC were mainly cytosolic (Figure 5), δ-PKC was slightly more in the particulate fraction, while ζ-PKC was equally distributed in the cytosolic and particulate fraction as previously described. ET-1 caused significant increase in the distribution of Ca^{2+}-dependent α-PKC (Figure 5A) and Ca^{2+}-independent ε-PKC (Figure 5B) in the particulate fraction compared with the cytosolic fraction. Pretreatment with verapamil inhibited ET-1–induced changes in the distribution of α-PKC (Figure 5A), but not ε-PKC (Figure 5B). Calphostin inhibited ET-1–induced changes in the distribution of both α-PKC (Figure 5A) and ε-PKC (Figure 5B). PMA did not change the distribution of α-PKC, but caused an increase in the distribution of ε-PKC in the particulate fraction that was inhibited in tissues pretreated with calphostin.

Experiments in Ca^{2+}-free (2mmol/L EGTA) solution indicated that significant ET-1–induced cell contraction, PKC activity, and increases in ε-PKC distribution in the particulate
ET-1-induced contraction, PKC activity, and absence or presence of verapamil (10^{-6} \text{ mol/L}) stimulates Ca^{2+} release from intracellular stores and diacylglycerol stimulates PKC.\textsuperscript{25,26} The inhibition of ET-1–induced cell contraction by the phospholipase C inhibitor neomycin and U-73122 provides evidence that ET-1 responses involve activation of phospholipase C and hydrolysis of plasma membrane phospholipids.

ET-1 (10^{-7} \text{ mol/L})–induced coronary smooth muscle contraction in Ca^{2+}–containing solution was associated with an initial followed by maintained elevation in [Ca^{2+}], suggesting that the increases in [Ca^{2+}], represent an important mechanism of ET-1 contraction. The agonist-induced transient increase in [Ca^{2+}], is mainly due to Ca^{2+} release from intracellular stores.\textsuperscript{21} The finding that the initial ET-1–induced [Ca^{2+}], was abolished in cells pretreated with neomycin and U-73122 suggests that it involves Ca^{2+} release from intracellular stores.

The main findings of the present study are: (1) ET-1 increases coronary smooth muscle contraction and [Ca^{2+}], and causes activation and translocation of Ca^{2+}–dependent \( \varepsilon \)-PKC and Ca^{2+}–independent \( \alpha \)-PKC. (2) Verapamil or diltiazem abolishes the maintained ET-1–induced [Ca^{2+}], and the activation and translocation of \( \alpha \)-PKC, but partially inhibits ET-1–induced contraction and does not inhibit the translocation of \( \varepsilon \)-PKC. (3) Calphostin and \( \varepsilon \)-PKC\textsubscript{VI,2} inhibitory peptide inhibit the verapamil-insensitive component of ET-1–induced contraction, PKC activity, and redistribution of \( \varepsilon \)-PKC. (4) ET-1–induced contraction, PKC activity, and \( \varepsilon \)-PKC redistribution in the absence of Ca^{2+} entry in Ca^{2+}–free solution are inhibited by \( \varepsilon \)-PKC\textsubscript{VI,2}.

The interaction of an agonist with its receptor activates phospholipase C and increases the hydrolysis of phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol.\textsuperscript{25,26,41} Inositol-1,4,5-trisphosphate stimulates Ca^{2+} release from intracellular stores and diacylglycerol stimulates PKC.\textsuperscript{25,26} The inhibition of ET-1–induced cell contraction by the phospholipase C inhibitors neomycin and U-73122 provides evidence that ET-1 responses involve activation of phospholipase C and hydrolysis of plasma membrane phospholipids.

**Discussion**

The main findings of the present study are: (1) ET-1 increases coronary smooth muscle contraction and [Ca^{2+}], and causes activation and translocation of Ca^{2+}–dependent \( \varepsilon \)-PKC and Ca^{2+}–independent \( \alpha \)-PKC. (2) Verapamil or diltiazem abolishes the maintained ET-1–induced [Ca^{2+}], and the activation and translocation of \( \alpha \)-PKC, but partially inhibits ET-1–induced contraction and does not inhibit the translocation of \( \varepsilon \)-PKC. (3) Calphostin and \( \varepsilon \)-PKC\textsubscript{VI,2} inhibitory peptide inhibit the verapamil-insensitive component of ET-1–induced contraction, PKC activity, and redistribution of \( \varepsilon \)-PKC. (4) ET-1–induced contraction, PKC activity, and \( \varepsilon \)-PKC redistribution in the absence of Ca^{2+} entry in Ca^{2+}–free solution are inhibited by \( \varepsilon \)-PKC\textsubscript{VI,2}.

The interaction of an agonist with its receptor activates phospholipase C and increases the hydrolysis of phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate stimulates Ca^{2+} release from intracellular stores and diacylglycerol stimulates PKC. The inhibition of ET-1–induced cell contraction by the phospholipase C inhibitors neomycin and U-73122 provides evidence that ET-1 responses involve activation of phospholipase C and hydrolysis of plasma membrane phospholipids.

ET-1 (10^{-7} \text{ mol/L})–induced coronary smooth muscle contraction in Ca^{2+}–containing solution was associated with an initial followed by maintained elevation in [Ca^{2+}], suggesting that the increases in [Ca^{2+}], represent an important mechanism of ET-1 contraction. The agonist-induced transient increase in [Ca^{2+}], is mainly due to Ca^{2+} release from intracellular stores. The finding that the initial ET-1–induced [Ca^{2+}], was abolished in cells pretreated with neomycin and U-73122 suggests that it involves Ca^{2+} release from intracellular stores.

**Figure 4.** PKC activity in cytosolic and particulate fraction (A) and particulate/cytosolic (Part/Cyt) PKC activity ratio (B) during coronary smooth muscle stimulation with ET-1 (10^{-7} \text{ mol/L}). The Part/Cyt PKC activity ratio after 30 minutes stimulation with ET-1, PMA (10^{-6} \text{ mol/L}), or KCl (51 mmol/L) was compared in absence or presence of verapamil (10^{-6} \text{ mol/L}), calphostin (10^{-6} \text{ mol/L}), or verapamil + calphostin (C). Data represent mean ± SEM of experiments on 18 to 24 tissue samples from 6 to 8 pigs. \*Measurements in presence of verapamil, calphostin, or verapamil + calphostin are significantly different (\( P < 0.05 \)) from control.

**Figure 5.** Distribution of \( \alpha \)-PKC (A) and \( \varepsilon \)-PKC (B) in cytosolic and particulate fraction of coronary smooth muscle at rest and after stimulation with ET-1 (10^{-7} \text{ mol/L}) for 30 minutes in absence or presence of verapamil (10^{-6} \text{ mol/L}) or calphostin (10^{-6} \text{ mol/L}). Data represent mean ± SEM of experiments on 6 to 8 pigs. \*Distribution of \( \alpha \)-PKC in particulate fraction of ET-1–treated tissues is significantly greater (\( P < 0.05 \)) than that in tissues at rest or treated with ET-1 + verapamil or ET-1 + calphostin. \#Distribution of \( \alpha \)-PKC in cytosolic fraction of ET-1–treated tissues is significantly less (\( P < 0.05 \)) than that in tissues at rest or treated with ET-1 + verapamil or ET-1 + calphostin. \$Distribution of \( \varepsilon \)-PKC in particulate fraction of tissues treated with ET-1 alone or ET-1 + verapamil is significantly greater (\( P < 0.05 \)) than that in tissues at rest or treated with ET-1 + calphostin. \$Distribution of \( \varepsilon \)-PKC in cytosolic fraction of tissues treated with ET-1 alone or ET-1 + verapamil is significantly less (\( P < 0.05 \)) than that in tissues at rest or treated with ET-1 + calphostin.
Evidence that verapamil and diltiazem mainly inhibit Ca\(^{2+}\) influx. The observation that verapamil or diltiazem, at concentrations that completely inhibit KCl-induced contraction and [Ca\(^{2+}\)]\(_i\), abolished the maintained ET-1-induced [Ca\(^{2+}\)]\(_i\), and significantly inhibited ET-1 contraction, support the contention that a component of ET-1 contraction is dependent on Ca\(^{2+}\) entry and is sensitive to Ca\(^{2+}\) antagonists. However, a component of ET-1 contraction in Ca\(^{2+}\)-containing solution was not inhibited by verapamil or diltiazem. Also, significant ET-1–induced contraction was observed in Ca\(^{2+}\)-free solution. These data suggest that a significant component of ET-1 contraction does not require Ca\(^{2+}\) entry and is thereby Ca\(^{2+}\)-antagonist-insensitive.

Although [Ca\(^{2+}\)]\(_i\) is a major determinant of smooth muscle contraction, other contraction pathways have been suggested, including activation of PKC.\(^{12,20}\) Direct activation of PKC by phorbol esters causes smooth muscle contraction with no change in [Ca\(^{2+}\)]\(_i\).\(^{29,30}\) Suggesting that PKC may regulate smooth muscle contraction by increasing the myofilament force sensitivity to [Ca\(^{2+}\)]. To investigate the role of PKC in the Ca\(^{2+}\)-antagonist-insensitive component of ET-1 contraction, we compared ET-1 response with that of the direct PKC activator PMA. PMA caused cell contraction that is most likely due to activation of PKC because: (1) PMA caused contraction in absence of changes in [Ca\(^{2+}\)]\(_i\), (2) PMA increased PKC activity, and (3) PMA-induced contraction and PKC activity were inhibited by calphostin, but not by verapamil. The observation that calphostin, at concentrations that completely inhibit PMA-induced contraction and PKC activity, caused significant inhibition of ET-1 contraction suggests that a significant component of ET-1 contraction involves activation of PKC. Also, combined pretreatment of the cells with calphostin and verapamil, both of which are partial inhibitors of ET-1 contraction, completely inhibited ET-1 contraction. These data suggest that calphostin inhibits the Ca\(^{2+}\)-antagonist-insensitive component of ET-1 contraction, and therefore, provide evidence that the Ca\(^{2+}\)-antagonist-insensitive component of ET-1 contraction involves activation of PKC. This is supported by the observation that ET-1 caused an increase in PKC activity that was inhibited by calphostin.

PKC is a family of Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent isoforms.\(^{25,26}\) The immunoblotting data suggest that in Ca\(^{2+}\)-containing solution ET-1 causes increases in the amount of Ca\(^{2+}\)-dependent \(\alpha\)-PKC and Ca\(^{2+}\)-independent \(\varepsilon\)-PKC in the particulate fraction compared with the cytosolic fraction, possibly due to PKC translocation from the cytosolic to particulate fraction. PKC translocation to the surface membrane allows the binding of cofactors such as membrane phospholipid and diacylglycerol to the PKC regulatory domain.\(^{25-28}\) That will lead to full activation of PKC, conformational change and unlocking of the ATP and substrate binding site in the PKC catalytic domain.\(^{25-28}\) These conformational changes would in turn allow PKC to phosphorylate the appropriate substrate and initiate a PKC-dependent response (eg, smooth muscle contraction).\(^{25-30}\) Thus, the immunoblotting data suggest that in Ca\(^{2+}\)-containing solution, ET-1 causes activation and translocation of Ca\(^{2+}\)-dependent \(\alpha\)-PKC and Ca\(^{2+}\)-independent \(\varepsilon\)-PKC.
The ET-1–induced translocation of α- and ε-PKC is inhibited by calphostin, confirming the specificity of the PKC translocation assay. Verapamil inhibited ET-1–induced translocation of α-PKC, but not that of ε-PKC. The inhibition of α-PKC translocation by verapamil is consistent with reports that α-PKC is Ca²⁺-dependent. The lack of inhibition of ε-PKC by verapamil is in accordance with reports that ε-PKC is Ca²⁺-independent and may therefore explain the Ca²⁺ antagonist-insensitive component of ET-1 contraction. The role of ε-PKC in Ca²⁺ antagonist-insensitive ET-1–induced contraction is supported by these findings: (1) Ca²⁺ antagonist-insensitive ET-1 contraction and PKC activity were inhibited by calphostin at concentrations that inhibit both Ca²⁺-dependent and Ca²⁺-independent PKC isoforms. (2) Ca²⁺ antagonist-insensitive ET-1 contraction and PKC activity were inhibited by calphostin at concentrations that inhibit both Ca²⁺-dependent and Ca²⁺-independent PKC isoforms.

**PMA, a PKC activator, caused contraction with no increase in [Ca²⁺], and induced activation and translocation of ε-PKC, consistent with a possible role for ε-PKC in Ca²⁺-insensitive smooth muscle contraction. The question arises as to why PMA did not activate α-PKC at basal levels of [Ca²⁺], while ET-1–caused activation of α-PKC when [Ca²⁺] was increased above basal levels. This could be related to the level of [Ca²⁺], required for activation of Ca²⁺-dependent PKC. This is consistent with reports that a threshold increase in [Ca²⁺] is required for activation of α-PKC in vascular smooth muscle cells. We should also note that calphostin inhibited ET-1–induced translocation of α- and ε-PKC and PMA–induced activation and translocation of ε-PKC. Calphostin competes with PMA or diacylglycerol for binding to the regulatory domain, and therefore inhibited the translocation of both α- and ε-PKC and abolished Ca²⁺-sensitive and Ca²⁺-independent PKC activity. On the other hand, ε-PKCv1-2-a 14 to 21 peptide sequence derived from the first variable region V1 of the regulatory domain of ε-PKC, and therefore specifically prevented ε-PKC translocation and inhibited Ca²⁺-insensitive PKC activity.

**Perspectives**

A significant component of ET-1–induced coronary smooth muscle contraction requires both Ca²⁺ entry and PKC activity and is inhibited by both Ca²⁺ channel blockers and PKC inhibitors; thus, it appears to involve Ca²⁺-dependent α-PKC isoform. An additional component of ET-1 contraction is Ca²⁺ antagonist-insensitive and appears to involve activation and translocation of Ca²⁺-independent ε-PKC. Ca²⁺-independent contraction of ET-1 may account for limited efficacy of Ca²⁺ channel blockers in some forms of cardiovascular dysfunction. Activation and translocation of Ca²⁺-independent ε-PKC may represent a signaling mechanism of Ca²⁺ antagonist-resistant forms of coronary vasospasm. The cellular mechanisms linking ET-1–induced activation and translocation of PKC and coronary contraction are not clear. PKC causes phosphorylation, either directly or indirectly, of actin-binding proteins such as calpontin or caldesmon, and thereby leads to increased force sensitivity of the contractile proteins to [Ca²⁺]. Whether ET-1 causes PKC-dependent phosphorylation of caldesmon and calponin in coronary artery smooth muscle should be investigated in future studies.

**Acknowledgments**

This work was supported by grants from the National Institutes of Health (HL–52696, HL–65998, HL–70659). R.A. Khalil is an Established Investigator of the American Heart Association.

**References**


17. Opie LH. Mechanisms whereby calcium channel antagonists may protect patients with coronary artery disease. 


19. Silvestry FE, Kimmel SE. Calcium-channel blockers in ischemic heart disease. 

   *Physiol Rev.* 1996;76:967–1003.


22. Rembold CM, Murphy RA. Myoplasmic [Ca^{2+}]i determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. 


26. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. 


30. Khalil RA, van Bremen C. Sustained contraction of vascular smooth muscle: calcium influx or C-kinase activation? 


32. Chen L, Wright LR, Chen CH, Oliver SF, Wender PA, Mochly-Rosen D. Molecular transporters for peptides: delivery of a cardioprotective epislonPKC agonist peptide into cells and intact ischemic heart using a glucose-pyruvate transporting system (RAT-4). 

33. Dubyak GR, Kerztes SB. Inhibition of GTP gamma S-dependent phospholipase D and Rho membrane association by calphostin is independent of protein kinase C catalytic activity. 

34. Kobayashi E, Nakano H, Morimoto M, Tamaoki T. Calphostin C (UCN-109C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. 

35. Khalil RA, Morgan KG. Phenylephrine-induced translocation of protein kinase C and shortening of two types of vascular cells of the ferret. 

36. Murphy JG, Khalil RA. Decreased [Ca^{2+}]i during inhibition of coronary smooth muscle contraction by 17B-estradiol, progesterone, and testosterone. 

37. Murphy JG, Khalil RA. Gender-specific reduction in contractility and [Ca^{2+}]i in vascular smooth muscle cells of female rat. 

38. Nayler WG, Perry SE, Elz JS, Daly MJ. Calcium, sodium, and the calcium paradox. 


40. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. 

41. Kanashiro CA, Khalil RA. Isoform-specific protein kinase C activity at variable Ca^{2+} entry during coronary artery contraction by vasoactive eicosanoids. 

42. Kanashiro CA, Altirkawi KA, Khalil RA. Preconditioning of coronary artery by endothelin-1 and prostaglandin F2alpha during repeated downregulation of nNOS. 
Endothelin-1 Promotes Ca^{2+} Antagonist-Insensitive Coronary Smooth Muscle Contraction Via Activation of \( \varepsilon \)-Protein Kinase C
Lashonn L. McNair, Daisy A. Salamanca and Raouf A. Khalil

Hypertension, published online February 23, 2004;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright \( \text{\textcopyright} \) 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2004/02/23/01.HYP.0000118520.92686.3b.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/