Abstract—Endothelin-1 (ET-1), a potent vasoconstrictor, has been implicated in the pathogenesis of coronary vasospasm by enhancing coronary vasoconstriction to vasoactive eicosanoids; however, the cellular mechanisms involved are unclear. We investigated whether physiological concentrations of ET-1 enhance coronary smooth muscle contraction to vasoactive eicosanoids by activating specific protein kinase C (PKC) isoforms. Cell contraction was measured in single smooth muscle cells isolated from porcine coronary arteries, intracellular free Ca$^{2+}$ ([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 anti-PKC antibodies using Western blots. In Hanks’ solution (1 mmol/L Ca$^{2+}$), ET-1 (10 pmol/L) did not increase basal [Ca$^{2+}]_i$, (81 ± 2 nmol/L), but it did cause cell contraction (9%) that was inhibited by GF109203X (10$^{-6}$ mol/L), an inhibitor of Ca$^{2+}$-dependent and Ca$^{2+}$-independent PKC isoforms. The vasoactive eicosanoid prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; 10$^{-7}$ mol/L) caused increases in cell contraction (11%) and [Ca$^{2+}]_i$ (108 ± 7 nmol/L) that were inhibited by the Ca$^{2+}$ channel blocker diltiazem (10$^{-6}$ mol/L). Pretreatment with ET-1 (10 pmol/L) for 10 minutes enhanced cell contraction to PGF$_{2\alpha}$ (35%) with no additional increase in [Ca$^{2+}]_i$ (112 ± 8 nmol/L). Direct activation of PKC by phorbol 12,13-dibutyrate (PDBu, 10$^{-7}$ mol/L) caused cell contraction (10%) and enhanced PGF$_{2\alpha}$ contraction (33%) with no additional increase in [Ca$^{2+}]_i$ (115 ± 7 nmol/L). The ET-1–induced enhancement of PGF$_{2\alpha}$ contraction was inhibited by Gö6976 (10$^{-6}$ mol/L), an inhibitor of Ca$^{2+}$-dependent PKC isoforms. Both ET-1 and PDBu caused an increase in PKC activity in the particulate fraction and a decrease in the cytosolic fraction and increased the particulate/cytosolic PKC activity ratio. Western blots revealed the Ca$^{2+}$-dependent α-PKC and the Ca$^{2+}$-independent δ-, ε-, and ζ-PKC isoforms. In resting tissues, α- and ε-PKC were mainly cytosolic, δ-PKC was mainly in the particulate fraction, and ζ-PKC was equally distributed in the cytosolic and particulate fraction. ET-1 (10 pmol/L) alone or PDBu (10$^{-7}$ mol/L) alone caused translocation of ε-PKC from the cytosolic to the particulate fraction, localized δ-PKC more in the particulate fraction, but did not change the distribution of ζ-PKC. PGF$_{2\alpha}$ (10$^{-7}$ mol/L) alone did not change PKC activity. In tissues pretreated with ET-1 or PDBu, PGF$_{2\alpha}$ caused additional increases in α-PKC activity. Thus, the enhancement of PGF$_{2\alpha}$-induced coronary smooth muscle contraction by physiological concentrations of ET-1 involves activation and translocation of α-PKC in addition to δ- and ε-PKC isoforms, and this may represent one possible cellular mechanism by which ET-1 could enhance coronary vasoconstriction to vasoactive eicosanoids in coronary vasospasm. (Hypertension. 2001; 37[part 2]:497-504.)

Key Words: endothelin ■ prostaglandin ■ calcium ■ muscle, smooth, vascular ■ myocardial contraction

Coronary vasospasm is often associated with ischemic heart disease leading to different forms of angina or myocardial infarction, and excessive coronary vasoconstriction in response to endogenous vasoconstrictors has been suggested as one potential cause of ischemic heart disease. Some have suggested that endothelin-1 (ET-1), one of the most potent vasoconstrictors described, may act as an endogenous modulator of coronary vascular tone and may play a role in the setting of coronary vasospasm. In addition, local release of vasoactive eicosanoids such as prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) in response to cardiac tissue injury causes significant coronary vasoconstriction that may dangerously interfere with adequate coronary blood flow.

Although previous studies have shown that both ET-1 and PGF$_{2\alpha}$ are potent coronary vasoconstrictors, the effects of ET-1 on smooth muscle contraction have often been evaluated separately from the effects of PGF$_{2\alpha}$. Also, in most mechanistic studies, high unphysiological concentrations of...
ET-1 and PGF$_{2\alpha}$ have often been used to activate maximally the possible mechanisms of smooth muscle contraction. This is in sharp contrast to the in vivo conditions, where the coronary artery is usually exposed to more than one vasoconstrictor at the same time, and the increases in the concentration of vasoconstrictor agonists are usually within the physiological range. Although high concentrations of ET-1 alone or PGF$_{2\alpha}$ alone are predicted to and have been shown to cause significant coronary contraction, it is not clear whether physiological concentrations of ET-1 enhance coronary vasoconstriction to small concentrations of PGF$_{2\alpha}$. In addition, although high concentrations of ET-1 alone or PGF$_{2\alpha}$ alone are predicted to activate maximally one or more mechanisms of smooth muscle contraction, the cellular mechanisms involved in the possible ET-1 induced enhancement of coronary vasoconstriction to the vasoactive eicosanoid PGF$_{2\alpha}$ are unclear.

It is widely accepted that vascular smooth muscle contraction is triggered by increases in intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) due to Ca$^{2+}$ release from the intracellular stores and Ca$^{2+}$ entry from the extracellular space. Other studies have also shown that the interaction of a vasoconstrictor agonist with its specific receptor is coupled to increased breakdown of plasma membrane phospholipids and increased production of diacylglycerol. Diacylglycerol binds to and activates protein kinase C (PKC). PKC is a family of several isoforms that have different enzyme properties, substrates, and functions and exhibit different subcellular distributions. PKC is mainly cytosolic under resting conditions and undergoes translocation to the particulate fraction when it is activated by endogenous diacylglycerol or exogenous phorbol esters. Also, direct activation of PKC by phorbol esters such as phorbol 12,13-dibutyrate (PDBu) causes sustained contraction of vascular smooth muscle with no significant change in [Ca$^{2+}$]. This suggests that PKC may have a role in regulating smooth muscle contraction, at least in part, by increasing the myofilament force sensitivity to [Ca$^{2+}$].

The purpose of the present study was to test the hypothesis that physiological concentrations of ET-1 enhance coronary smooth muscle contraction to the vasoactive eicosanoid PGF$_{2\alpha}$ by increasing the activity of specific PKC isoforms. Because the PKC family includes both Ca$^{2+}$-dependent and Ca$^{2+}$-independent isoforms, any ET-1 or PGF$_{2\alpha}$ induced changes in coronary smooth muscle [Ca$^{2+}$] may determine which PKC isofrom would be activated. Therefore, experiments were designed to investigate (1) whether physiological concentrations of ET-1 enhance PGF$_{2\alpha}$-induced contraction in coronary smooth muscle, (2) whether the ET-1 enhancement of PGF$_{2\alpha}$-induced coronary smooth muscle contraction is associated with increases in [Ca$^{2+}$], and (3) whether the ET-1 enhancement of PGF$_{2\alpha}$-induced contraction is associated with increases in the activity of specific PKC isoforms in coronary smooth muscle. The effects of ET-1 were compared with those of the phorbol ester PDBu, a direct activator of PKC, and sensitivity to the effects of the Ca$^{2+}$ channel antagonist diltiazem and the PKC inhibitors GF109203X and G6976 was also investigated.

**Methods**

**Tissue Preparation**

Castrated male Yorkshire pigs (30 kg) from a local breeder were anesthetized by isoflurane inhalation. The heart was rapidly excised and placed in Krebs’ solution. The left anterior descending coronary artery was dissected, cleaned of connective and adipose tissue, and opened by cutting along its longitudinal axis. The endothelium was removed by rubbing the vessel interior with wet filter paper. The tunica media was carefully dissected from the tunica adventitia under microscopic visualization using sharp-tipped forceps and then sectioned into 2×2-mm strips. All procedures followed the guidelines of the Institutional Animal Care and Use Committee.

**Single Cell Isolation**

Coronary artery strips (50 mg) were placed in a tissue digestion mixture containing collagenase type II (236 U/mg protein, Worthington), elastase grade II (3.25 U/mg protein, Boehringer Mannheim), and trypsin inhibitor type II (10 000 U/mL, Sigma) in 7.5 mL of Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ solution supplemented with 30% bovine serum albumin (Sigma). The tissue was incubated 3 times in the tissue digestion mixture to yield 3 batches of cells. For the first batch, the tissue was incubated with 5 mg of collagenase, 4 mg of elastase, and 147 µL of trypsin inhibitor for 60 minutes. For batches 2 and 3, the collagenase was reduced to 2.5 mg, the trypsin inhibitor was reduced to 122 µL, and the incubation period was reduced to 30 minutes. The tissue preparation was placed in a shaking water bath at 34°C in an atmosphere of 95% O$_2$ and 5% CO$_2$. The preparation was rinsed with 12.5 mL of Hanks’ solution, poured over glass coverslips placed in wells, and cooled to 2°C. The cells were allowed to settle by gravity and adhere to the glass coverslips. Ca$^{2+}$ was gradually added back to the preparation to avoid the "calcium paradox." 21

**Contraction Studies**

Coverslips with the attached cells were placed on the stage of an inverted Nikon microscope and viewed using a Nikon 100× oil immersion objective. The cell isolation procedure yielded smooth muscle cells of variable lengths. Only viable, healthy, spindle-shaped cells ≥60 µm in length were selected. Viable, healthy cells adhered to the glass coverslips and appeared bright, with a halo along the periphery and without a visible nucleus when viewed with phase-contrast optics. The viability of the smooth muscle cells was confirmed by their consistent and significant contraction in response to ET-1 and PGF$_{2\alpha}$. The cells were further characterized and consistently showed significant immunofluorescence signal when fixed and labeled with anti-smooth muscle myosin antibody. Cell images were acquired using a FXL CCD camera and displayed on a computer using PMIS image analysis software (Photometrics). The number of pixels corresponding to the cell length in the cell image was transformed into microns using a calibration bar. The magnitude of cell contraction was expressed as the final cell length as a fraction of the initial cell length. All contraction measurements were made at 37°C. The changes in cell contraction in response to ET-1, PGF$_{2\alpha}$, and the phorbol ester PDBu were measured.

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

Single coronary smooth muscle cells were loaded with the Ca$^{2+}$ indicator fura-2 for 30 minutes at 34°C. The fura-2 loading solution was made of normal Hanks’ solution, 1 µmol/L of the cell permeant fura-2 acetoxymethyl ester (Molecular Probes), and 0.01% Pluronic F-127 (Sigma). The fura-2–loaded cells were washed twice and further incubated in Hanks’ solution for at least 30 minutes to allow complete de-esterification of the fura-2 acetoxymethyl ester. Precautions were taken throughout the procedure to avoid excessive photobleaching of fura-2.
The fura-2–loaded cells were viewed through a Nikon Fluor 100× oil-immersion objective (NA 1.3) on an inverted Nikon (Diaphot-300) microscope. The Ca²⁺ indicator was excited alternately at 340±2.5 nm and 380±2.6 nm using a filter wheel that alternates at a frequency of 0.5 Hz. The emitted light was collected at 510 nm to a photomultiplier tube R928 (Ludl Electronic Products) through a pinhole aperture 1 μm in diameter positioned 1 μm from the plasma membrane and 1 μm from the nucleus. The fluorescent signal was digitized using a module (Mac 2000, Ludi) and analyzed on a computer using data analysis software. The fluorescent signal was background-subtracted. Spectral shifts that result from the binding of Ca²⁺ to fura-2 make it possible to use the ratio method, thus rendering the measurements of [Ca²⁺], less sensitive to changes in cell thickness or the extent of dye loading and photobleaching. The ratio between the fluorescence intensity at 340 nm and 380 nm (R) was transformed to the corresponding levels of [Ca²⁺], as described by Grynkiewicz et al as follows:

\[
\frac{[Ca^{2+}]}{[Ca^{2+}]} = K_0 \times \frac{S_f}{S_b} \times \frac{(R - R_{min})}{(R_{max} - R)}
\]

where \(R_{min}\) and \(R_{max}\) represent the minimal and maximal fluorescence ratios; they were measured by adding fura-2 pentapotassium salt (50 μmol/L) to Ca²⁺–free (10 mmol/L EGTA) and Ca²⁺–replete (2 mmol/L) solutions, respectively. \(S_f/\) is the ratio of the dissociation constant of fura-2 for Ca²⁺ and was established at 224 nmol/L under these experimental conditions. All experiments were performed at 37°C. The changes in [Ca²⁺], in response to ET-1, PGF₂α, and the phorbol ester PDBu were measured.

**Tissue Fractions**

Tissue strips (~80 mg) at rest or stimulated with ET-1, PGF₂α, or PDBu for 30 minutes were rapidly transferred to iced-cold equilibrating buffer A containing (in mmol/L): Tris-HCl 25 (pH 7.5), EGTA 5, leupeptin 0.02, phenylmethylsulfonylfluoride 0.2, and dithiothreitol 1. To measure PKC activity, the tissue was transferred to homogenization buffer B, which has the same composition as buffer A plus 250 mmol/L sucrose. For Western blots, the tissue was transferred to a homogenization buffer containing 20 mmol/L 3-[N-morpholino]propane sulfonic acid, 4% sodium dodecylsulfate, 10% glycerol, 2.3 mg of dithiothreitol, 1.2 mmol/L ethylenediaminetetraacetic acid, 0.02% bovine serum albumin, 5.5 μmol/L Leupeptin, 5.5 μmol/L pepstatin, 2.15 μmol/L aprotinin, and 20 μmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride. The tissue was homogenized using a 2 mL tight-fitting homogenizer ( Kontes Glass) at 4°C and centrifuged at 100,000 rpm for 20 minutes at 4°C (Utra-Centrifuge TL-100, Beckman). The supernatant was used as the cytosolic fraction. The pellet was resuspended in a homogenization buffer containing 1% Triton X-100 for 20 minutes, diluted with homogenization buffer to a final concentration of 0.2% Triton, and centrifuged at 100,000 rpm for 20 minutes at 4°C. The supernatant was used as the particulate fraction. Protein concentrations in tissue fractions were determined using a protein assay kit (Bio-Rad).

**PKC Activity**

The cytosolic and particulate fractions were applied to diethylaminoethyl-cellulose columns (0.8×4.0 cm; Bio-Rad). The columns were washed with buffer A, and the protein was eluted with 0.1 mol/L NaCl. PKC activity in the aliquots was determined by measuring the incorporation of ³²P from [γ³²P]ATP (ICN) into histone HII.²³-²⁴ The assay mixture contained 25 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl₂, 200 μg/mL histone HII, 80 μg/mL phosphatidylserine, 30 μg/mL diolein, [γ³²P]ATP (1 to 3×10⁵ cpm/nmol), and 0.5 to 3 μg of protein. After 5 minutes of incubation at 30°C, the reaction was stopped by spotting 25 μL of the assay mixture onto phosphocellulose discs. The discs were washed 3×5 minutes with 5% trichloroacetic acid and placed in a 4-mL Ecolite scintillation cocktail, and radioactivity was measured in a liquid scintillation counter.

**Immunoblotting**

Protein-matched samples of the cytosolic and particulate fractions were subjected to electrophoresis on 8% sodium dodecylsulfate–polyacrylamide gels and then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated in 5% dried milk in PBS-Tween at 22°C for 1 hour, washed with PBS-Tween for 3×5 minutes, and then incubated in the primary anti-PKC antibody solution at 4°C overnight. Polyclonal antibodies to α-, β-, γ-, δ-, ε-, and ζ-PKC (Gibco) were used. These antibodies have been shown to react with the specific PKC isoforms in porcine aortic endothelial cells and in airway and coronary smooth muscle cells.²³-²⁶ The specificity of the antibodies was confirmed by the observation that the peptide controls were successful only with the peptide to which the antibodies were raised and not with other sequences of the PKC molecule. To maintain constant labeling conditions, we used the same anti-PKC antibody titer (1:500) and protein concentration (10 μg) in all tissue samples. These antibody titer and protein concentrations gave optimal immunoreactive signals while remaining on the linear portion of the titration curve. The nitrocellulose membranes were washed 5×15 minutes in PBS-Tween and then incubated in horseradish peroxidase–conjugated anti-rabbit secondary antibody for 1.5 hours. The blots were washed with PBS-Tween for 5×15 minutes and visualized with enhanced chemiluminescence detection system (Amersham). PBS-Tween contained the following (in mmol/L): NaH₂PO₄, 80, NaH₂PO₄ 20, and NaCl 100 plus 0.05% Tween. The reactive bands corresponding to PKC isoforms were analyzed quantitatively by optical densitometry using a GS-700 imaging densitometer (Bio-Rad).

**Solutions**

Krebs solution contained (in mmol/L): NaCl 120, KCl 5.0, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.2, and dextrose 11.5 at pH 7.4. Hanks’ solution contained (in mol/L): NaCl 137, KCl 5.4, KH₂PO₄ 0.44, NaH₂PO₄ 0.42, NaHCO₃ 4.17, dextrose 5.55, and HEPES 10 at pH 7.4. For Ca²⁺– and Mg²⁺–containing Hanks’ solution, 1 mmol/L CaCl₂ and 1.2 mmol/L MgCl₂ were added, respectively.

**Drugs and Chemicals**

ET-1, PGF₂α (Sigma), and diltiazem (Calbiochem) were dissolved in distilled water. PDBu (Alexis Laboratory.), GF109203X, and Gö6976 (Kamiya) were dissolved in DMSO. The final concentration of DMSO in solution was ≤0.1%. All other chemicals were of reagent grade or better.

**Statistical Analysis**

The data were analyzed and presented as the mean±SEM and compared using Student’s t test for unpaired data. P<0.05 was considered significant.

**Results**

The cell isolation procedure produced relatively long cells (Figure 1). In cells incubated in Hanks’ solution (1 mmol/L Ca²⁺), the resting cell length was 73±6 μm and the basal [Ca²⁺]i was 81±2 mmol/L. High concentrations of ET-1 (10⁻⁷ mol/L) caused significant cell contraction (74.9±2.8%) and an initial increase in [Ca²⁺], to 496±37 mmol/L, followed by a maintained increase to 167±7 mmol/L (Figure 1A). High concentrations of PGF₂α (10⁻⁴ mol/L) also caused significant cell contraction (73±2.3%) and a transient increase in [Ca²⁺], to 423±29 mmol/L and a maintained increase to 181±5 mmol/L (Figure 1B).

Small concentrations of ET-1 (10⁻¹¹ mol/L) caused a small but significant cell contraction (9.2±2.3%), with no significant increases in [Ca²⁺], (Figure 2C). However, small concentrations of PGF₂α (10⁻⁷ mol/L) caused small increases in both contraction (11.3±2.2%) and [Ca²⁺], (108±7 mmol/L;
In cells pretreated with ET-1 (10⁻¹¹ mol/L) for 10 minutes, PGF₂α (10⁻⁷ mol/L) caused a large contraction (34.9 ± 3.2%), with no additional increases in [Ca²⁺]ᵢ (112 ± 7 nmol/L; Figure 1E).

Cumulative data from different cells stimulated with increasing concentrations of ET-1 or PGF₂α were used to construct concentration-response curves (Figure 2). ET-1 alone caused concentration-dependent increases in cell contraction and [Ca²⁺]ᵢ. PGF₂α alone caused concentration-dependent increases in contraction and [Ca²⁺]ᵢ, although PGF₂α was less potent than ET-1. In cells pretreated with ET-1 (10⁻¹¹ mol/L) for 10 minutes, the PGF₂α concentration-contraction curve was significantly enhanced (Figure 2A), with no additional increases in [Ca²⁺]ᵢ (Figure 2B). Direct activation of PKC by PDBu (10⁻⁷ mol/L) caused cell contraction (10%) with no significant change in [Ca²⁺]ᵢ (115 ± 7 nmol/L). In cells pretreated with PDBu (10⁻⁷ mol/L) for 10 minutes, PGF₂α contraction was enhanced to levels similar to those observed in cells pretreated with ET-1 and then stimulated with PGF₂α (Figure 2A), with no additional increase in [Ca²⁺]ᵢ (Figure 2B).

The effects of the Ca²⁺ channel blocker diltiazem on the responses of ET-1, PGF₂α, and ET-1 plus PGF₂α were investigated. Pretreatment with diltiazem (10⁻⁶ mol/L) for 10 minutes did not affect the cell contraction (Figure 3A) or [Ca²⁺]ᵢ (Figure 3B) induced by ET-1 (10⁻¹¹ mol/L) alone, suggesting that Ca²⁺ entry from the extracellular space is not involved. Diltiazem completely abolished the contraction (Figure 3A) and [Ca²⁺]ᵢ (Figure 3B) induced by PGF₂α (10⁻⁷ mol/L) alone, suggesting that the PGF₂α responses involve Ca²⁺ entry. Diltiazem also significantly inhibited the ET-1 induced enhancement of PGF₂α contraction (Figure 3A), suggesting that the enhanced response is dependent on Ca²⁺ entry.

The effects of PKC inhibitors on the responses of ET-1, PGF₂α, and ET-1 plus PGF₂α were also investigated. Pretreatment with GF109203X (10⁻⁶ mol/L), an inhibitor of both Ca²⁺-dependent and Ca²⁺-independent PKC isoforms, for 10 minutes significantly inhibited the cell contraction induced by ET-1 (10⁻¹¹ mol/L) alone (Figure 3A), with no significant change in [Ca²⁺]ᵢ (Figure 3B), suggesting the involvement of PKC. In cells stimulated with PGF₂α (10⁻⁷ mol/L) alone, GF109203X did not affect contraction (Figure 3A) or [Ca²⁺]ᵢ (Figure 3B), suggesting that PKC is not activated during stimulation by PGF₂α (10⁻⁷ mol/L) alone. In cells pretreated...
with ET-1, GF109203X inhibited the enhancement of PGF$_{2\alpha}$ contraction (Figure 3A), with no significant change in [Ca$^{2+}$]$_i$ (Figure 3B), suggesting the involvement of PKC. Pretreatment with Gö6976 (10$^{-6}$ mol/L), an inhibitor of Ca$^{2+}$-dependent PKC isoforms, for 10 minutes did not affect the cell contraction (Figure 3A) or [Ca$^{2+}$]$_i$ (Figure 3B) induced by ET-1 (10$^{-11}$ mol/L) alone or PGF$_{2\alpha}$ (10$^{-7}$ mol/L) alone. However, in cells stimulated with ET-1 (10$^{-11}$ mol/L) plus PGF$_{2\alpha}$ (10$^{-7}$ mol/L), Gö6976 caused a significant reduction in contraction (Figure 3A), with no significant change in [Ca$^{2+}$]$_i$ (Figure 3B), suggesting the involvement of a Ca$^{2+}$-dependent PKC isoform.

PKC activity was measured in tissue fractions of coronary smooth muscle. In resting tissues, PKC activity was greater in the cytosolic fraction than the particulate fraction (Figure 4A), with a particulate/cytosolic PKC activity of 0.46$\pm$0.05 (Figure 4B). ET-1 (10$^{-11}$ mol/L) caused time-dependent increases in PKC activity in the particulate fraction, a decrease in the cytosolic fraction (Figure 4A), and an increase in the particulate/cytosolic PKC activity ratio (Figure 4B). PGF$_{2\alpha}$ (10$^{-7}$ mol/L) alone did not cause any significant increase in PKC activity (Figure 4A and 4B). In tissues pretreated with ET-1 (10$^{-11}$ mol/L), PGF$_{2\alpha}$, (10$^{-7}$ mol/L) caused additional increases in PKC activity (Figure 4A and 4B). Direct activation of PKC by PDBu (10$^{-7}$ mol/L) caused a significant increase in PKC activity that was roughly similar to that in tissues stimulated with ET-1 (10$^{-11}$ mol/L) alone (Figure 4C). In tissues pretreated with PDBu (10$^{-7}$ mol/L), PGF$_{2\alpha}$ (10$^{-7}$ mol/L) caused additional increases in PKC activity (Figure 4C). Gö6976 (10$^{-6}$ mol/L), an inhibitor of Ca$^{2+}$-dependent PKC isoforms, did not affect the PKC activity induced by ET-1 alone or PDBu alone, but it abolished the additional increase in PKC activity caused by PGF$_{2\alpha}$ in tissues pretreated with ET-1 or PDBu (Figure 4C).

Western blots in tissue fractions revealed the Ca$^{2+}$-dependent $\alpha$-PKC and the Ca$^{2+}$-independent $\delta$, $\epsilon$, and $\zeta$-PKC isoforms. In resting tissues, $\alpha$-PKC was mainly cytosolic (Figure 5A), $\delta$-PKC seemed to appear slightly more in the particulate fraction (Figure 5B), $\epsilon$-PKC was mainly cytosolic (Figure 5C), and $\zeta$-PKC was equally distributed in the cytosolic and particulate fractions (Figure 5D). In the presence of ET-1 (10$^{-11}$ mol/L) alone, the distribution of $\alpha$-PKC did not change (Figure 5A), $\delta$-PKC was more...
localized in the particulate fraction (Figure 5B), \( \alpha \)-PKC showed significant translocation from the cytosolic to the particulate fraction (Figure 5C), and the distribution of \( \zeta \)-PKC was unchanged (Figure 5D). PGF\(_{2\alpha}\) (10\(^{-7}\) mol/L) alone did not significantly change the distribution of PKC isoforms. In tissues pretreated with ET-1 (10\(^{-11}\) mol/L), PGF\(_{2\alpha}\) (10\(^{-7}\) mol/L) caused additional translocation of \( \alpha \)-PKC from the cytosolic to the particulate fraction (Figure 5A). Direct activation of PKC by PDBu (10\(^{-7}\) mol/L) alone caused changes in the distribution of \( \delta \)- and \( \epsilon \)-PKC that were similar to those observed in tissues treated with ET-1 (10\(^{-11}\) mol/L) alone. In tissues pretreated with PDBu (10\(^{-7}\) mol/L), PGF\(_{2\alpha}\) (10\(^{-7}\) mol/L) caused additional translocation of \( \alpha \)-PKC from the cytosolic to the particulate fraction in a manner similar to that observed in tissues stimulated with ET-1 plus PGF\(_{2\alpha}\) (data not shown).

**Discussion**

The present study showed that ET-1 at concentrations \( \approx 10^{-10} \) mol/L causes significant contraction of coronary smooth muscle cells. At these high concentrations, ET-1-induced contraction is associated with a significant increase in \([Ca^{2+}]\). These results are consistent with previous reports that vascular smooth muscle contraction in response to ET-1 is triggered by increases in \([Ca^{2+}]\), due to \( Ca^{2+} \) release from the intracellular stores and \( Ca^{2+} \) entry from the extracellular space.\(^{27,30}\) ET-1 at very small and physiological concentrations (10\(^{-11}\) mol/L) still caused a significant contraction of coronary smooth muscle cells that was not associated with any increases in \([Ca^{2+}]\), suggesting activation of other mechanisms of smooth muscle contraction that may increase the myofilament force sensitivity even to basal levels of \([Ca^{2+}]\). Although the ET-1 contraction at physiological concentrations seemed to be relatively small, this contraction could be of considerable significance because only minimal circumferential coronary vasoconstriction is often necessary to critically reduce the luminal cross-sectional area in the setting of significant coronary vasospasm.\(^{31}\) Also, if ET-1 coronary contraction is mediated by a \( Ca^{2+} \)-sensitizing pathway rather than by increasing \([Ca^{2+}]\), vasodilators functioning solely by lowering \([Ca^{2+}]\), would be ineffective in overcoming this form of coronary vasospasm. Furthermore, if the ET-1-stimulated \( Ca^{2+} \) sensitizing pathway is combined with another agonist, which by itself causes only small increases in coronary smooth muscle contraction and \([Ca^{2+}]\), the resulting synergistic effect could dangerously enhance coronary vasoconstriction and lead to severe coronary vasospasm.

Several studies have shown that direct activation of PKC by phorbol esters causes significant and sustained contraction of smooth muscle with no significant change in \([Ca^{2+}]\),\(^{16,17}\) suggesting a role for PKC in regulating smooth muscle contraction, at least in part, by increasing the myofilament force sensitivity to \([Ca^{2+}]\). The present results suggest that ET-1, at small and physiological concentrations, increases the myofilament sensitivity to \([Ca^{2+}]\), by activating PKC because (1) ET-1 contraction was not associated with any significant increase in \([Ca^{2+}]\); (2) ET-1 contraction was not inhibited by the \( Ca^{2+} \) channel blocker diltiazem; (3) direct activation of PKC by phorbol ester caused a contraction similar to that of ET-1, with no significant change in \([Ca^{2+}]\); (4) ET-1 contraction was completely inhibited by GF109203X, an inhibitor of both \( Ca^{2+} \)-dependent and \( Ca^{2+} \)-independent PKC isoforms; and (5) ET-1 caused an increase in PKC activity that was completely inhibited by GF109203X. Thus, the contraction induced by small concentrations of ET-1 alone does not require significant increases in \([Ca^{2+}]\), but it seems to involve the activation of PKC. The observations that the contraction and PKC activation induced by ET-1 were completely inhibited by GF109203X, an inhibitor of both \( Ca^{2+} \)-dependent and \( Ca^{2+} \)-independent PKC isoforms, but not by Gö6976, a relatively specific inhibitor of the \( Ca^{2+} \)-dependent isoforms, raises the possibility that the responses induced by ET-1 alone involve a \( Ca^{2+} \)-independent PKC isoform. This is consistent with the observation that ET-1 alone caused a
translocation of the Ca$$^{2+}$$-independent ε-PKC and, to a lesser extent, δ-PKC but not the Ca$$^{2+}$$-dependent α-PKC.

Small concentrations of PGF$$\_2$$α alone caused a cell contraction that was associated with a significant increase in [Ca$$^{2+}$$]i. PGF$$\_2$$α-induced contraction and [Ca$$^{2+}$$]i seem to be mainly due to Ca$$^{2+}$$ entry from the extracellular space because they were inhibited by the Ca$$^{2+}$$ channel blocker diltiazem and were not associated with any significant change in PKC activity or the distribution of PKC isoforms.

In cells pretreated with ET-1, the PGF$$\_2$$α contraction was significantly enhanced, with no additional increases in [Ca$$^{2+}$$]i. These results are in agreement with reports that ET-1 enhances vascular smooth muscle contraction to other agonists such as 5-hydroxytryptamine. Although the enhanced PGF$$\_2$$α contraction in cells pretreated with ET-1 did not involve additional increases in [Ca$$^{2+}$$]i, it seems to require extracellular Ca$$^{2+}$$ because it was completely inhibited by diltiazem. In addition, the enhancement of PGF$$\_2$$α contraction by ET-1 seems to involve PKC because (1) direct activation of PKC by PDBu caused similar enhancement of PGF$$\_2$$α contraction, (2) the enhancement of PGF$$\_2$$α contraction by ET-1 was associated with an ≈2-fold increase in PKC activity, and (3) the enhancement of PGF$$\_2$$α-induced contraction and PKC activity by ET-1 were inhibited by PKC inhibitors. Thus, the enhanced PGF$$\_2$$α-induced contraction and PKC activity in tissues pretreated with ET-1 seem to require both Ca$$^{2+}$$ and PKC, which raises the possibility that the contraction involves a Ca$$^{2+}$$-dependent PKC isoform. This is consistent with the observation that the enhancement of PGF$$\_2$$α contraction by ET-1 was associated with the activation and translocation of the Ca$$^{2+}$$-dependent α-PKC.

The question arises regarding why ET-1 did not activate α-PKC at basal levels of [Ca$$^{2+}$$]i, while causing significant activation of α-PKC when [Ca$$^{2+}$$]i was slightly increased above basal levels. This could be related, at least in part, to the level of [Ca$$^{2+}$$]i required for the activation of Ca$$^{2+}$$-dependent PKC isoforms. This is consistent with previous reports that a threshold increase in [Ca$$^{2+}$$]i is required for the activation of α-PKC in vascular smooth muscle cells from the ferret and the pig.

In summary, physiological concentrations of ET-1 cause coronary smooth muscle contraction, with no significant increase in [Ca$$^{2+}$$]i, but with significant increases in the activity of the Ca$$^{2+}$$-independent δ- and ε-PKC. Small concentrations of PGF$$\_2$$α cause coronary smooth muscle contraction that is associated with a significant increase in [Ca$$^{2+}$$]i but not in PKC activity. Physiological concentrations of ET-1 significantly enhance coronary smooth muscle contraction to PGF$$\_2$$α with no additional increases in [Ca$$^{2+}$$]i, and are associated with an increase in α-PKC activity. Thus, the enhancement of PGF$$\_2$$α-induced coronary smooth muscle contraction by physiological concentrations of ET-1 involves activation and translocation of α-PKC in addition to δ- and ε-PKC isoforms. The additional activation of α-PKC may represent one possible cellular mechanism by which ET-1 may cause exaggerated coronary vasoconstriction to vasoactive eicosanoids in the setting of coronary vasospasm.

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References


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