Phorbol Dibutyrate Stimulates the Release of Diffusible Endothelium-Derived Vasoconstrictor Factor(s) From Canine Femoral Arteries

Gabor M. Rubanyi, Allan Luisi, and Anthony Johns

The purpose of this study was to analyze the effect of the tumor-promoting phorbol ester 12,13-dibutyrate (PDBu) on the synthesis/release of nonprostanoid endothelium-derived vasoactive factors. In bioassay experiments (in the presence of 10^{-5} M indomethacin), infusion of PDBu (10^{-9}-10^{-7} M) through a femoral artery (donor) segment with endothelium evoked further, concentration-dependent contraction of superfused canine coronary artery bioassay rings without endothelium (already contracted with 10^{-7} M PDBu). Removal of the endothelium from the donor segment abolished further contractions of the bioassay ring to 10^{-9} M PDBu and significantly depressed the contractile responses to 10^{-8} and 10^{-7} M PDBu infused through the donor segment. The inactive phorbol ester 4a-phorbol 12,13-didecanoate had no effect on vascular preparations mounted in the bioassay system. Selective exposure of the bioassay tissue to 10^{-7} M PDBu completely inhibited its responsiveness to basally released endothelium-derived relaxing factor. These data indicate that PDBu stimulates the release of a diffusible and bioassayable vasoconstrictor mediator(s) from the endothelium of canine femoral arteries. (Circulation Research 1991;68:1527–1531)

The vascular endothelium produces nonprostanoid vasoactive substances that relax (endothelium-derived relaxing factor[s] [EDRF], see References 1 and 2) or contract (endothelium-derived contracting factor[s] [EDCF]) the underlying vascular smooth muscle. The intracellular mechanisms that control the biosynthesis of these mediators in endothelial cells is poorly understood. Although an increase in cytosolic concentration of free Ca^{2+} was reported to be a common signal for the synthesis of both EDRF and EDCF,1–4 several conditions (e.g., hypoxia, shear stress, and elevated transmural pressure) seem to modulate the biosynthesis of these two mediators in an opposite way.4,5,11

Recent studies indicate that many cell functions are controlled by the activity of protein kinase C.12 These indications were based on observations made largely with tumor-promoting phorbol esters whose only known receptor is protein kinase C.13 Several stimulants of the release of EDRF (e.g., acetylcholine, histamine, bradykinin, and 5-hydroxytryptamine) were reported to activate phosphatidylinositol hydrolysis (one product of which is diacylglycerol, a natural activator of protein kinase C). However, prolonged exposure to phorbol 12,13-dibutyrate (PDBu) inhibited endothelium-dependent relaxation evoked by histamine in the guinea pig pulmonary artery,14 by substance P in rabbit aorta,15 and by acetylcholine in canine femoral and coronary arteries.16 These results suggested that PDBu suppressed receptor-mediated processes linked to the synthesis/release of EDRF. It is not known, however, whether phorbol esters can trigger the synthesis or release of endothelium-derived vasoconstrictor factor(s).

The purpose of the present study was to analyze the effect of PDBu and the inactive phorbol ester 4α-phorbol 12,13-didecanoate (4α-PDD) on the synthesis or release of nonprostanoid endothelium-derived vasoactive factors. Experiments were designed using isolated canine femoral and coronary arteries mounted in a bioassay system, which allows separate analysis of the synthesis/release and action of endothelium-derived vasoactive factors.11,17

Materials and Methods

Experiments were performed on femoral and left circumflex coronary arteries isolated from mongrel dogs weighing 15–25 kg. The blood vessels were
cleaned of loose adipose and connective tissue and were studied in Krebs-Ringer bicarbonate solution with the following composition (mM): NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, edetate calcium disodium 0.026, and glucose 11.1 (control solution). All experiments were performed in the presence of 10^-5 M indomethacin to inhibit the synthesis of prostanoids in these blood vessels.10,18

**Bioassay Studies**

A bioassay technique described in detail earlier17 was used. Side branches of segments (3.0–3.5 cm long) of the left and right femoral artery were tied (donor segments). The segments were fixed to stainless steel cannulas (1.5 mm i.d.) and placed into an organ chamber maintained at 37°C and filled with 20 ml of aerated (95% O2–5% CO2) control solution. The segments were perfused at constant flow (2 ml/min) by means of a multichannel roller pump (Minipuls 2, Gilson Co., Inc., Worthington, Ohio) with control solution maintained at 37°C. A stainless steel tube was also placed in the organ chamber through which control solution was pumped at the same rate. A ring of coronary artery in which the endothelium had been removed (bioassay ring) was suspended directly below the organ chamber by two stainless steel stirrups passed through its lumen. One stirrup was connected to a force transducer (model FTO3D, Grass Instrument Co., Quincy, Mass.) for recording changes in isometric force. The assembly of bioassay ring, stirrups, and force transducer could be moved freely below the organ chamber, allowing the preparation to be superfused with the perfusate passing through the femoral artery segment or with the stainless steel tube (direct superfusion). The transit time between the donor segment and the bioassay ring was 1 second. Drugs were infused into the perfusate with infusion pumps (model 901, Harvard Apparatus, South Natick, Mass.) either upstream of the femoral artery (site 1, allowing contact with the donor segment) or below it (site 2, avoiding contact with the donor segment).

**Experimental Protocol**

Two femoral artery segments and two coronary artery bioassay rings were prepared from the same animal, mounted in two identical bioassay apparatuses, and studied in parallel. In one donor segment, the endothelium was removed by gentle mechanical rubbing of the intimal surface17 before mounting. The bioassay rings were superfused directly with control solution for 60 minutes. During this interval the rings were stretched in a stepwise manner until the basal tension reached approximately 8 g, the optimal tension for rings of isolated canine coronary arteries.19

To evoke sustained contraction of the bioassay rings (n=7), the endoperoxide analogue U46619 (5×10^-8 M) was added to the perfusate. After the contraction reached steady level (approximately 10–15 minutes), 10^-6 M acetylcholine was infused through the direct line to check the absence of functional endothelium on the rubbed bioassay ring. Then the bioassay ring was moved below the outlet from the donor segment, 10^-6 M acetylcholine was infused into the perfusate upstream of the perfused segment (at site 1), to determine the presence and absence of functional endothelium in the intact and rubbed donor segments, respectively. Sixty minutes after washout of U46619, the bioassay ring was contracted with 10^-7 M PDBu by infusing the drug downstream of the perfused segments (at site 2), and 30–45 minutes later, increasing concentrations (10^-8–10^-7 M) of PDBu were infused upstream of the donor segment (site 1) while the infusion of 10^-7 M PDBu was maintained at site 2. Control experiments were also performed using the same protocol but superfusing the bioassay ring through the stainless steel tube.

In separate experiments (n=3), the effect of selective exposure of the bioassay tissue to 10^-5 M PDBu on its reactivity to basally released EDRF was studied. First, the bioassay rings were contracted with 10^-8 M U46619, and the effect of basal EDRF was tested by moving the assay ring from the direct perfusion line to the outlet from a donor segment with endothelium. Thirty minutes after washout of U46619, the bioassay tissue was contracted by 10^-7 M PDBu infused at site 2, and the reactivity to basal EDRF was tested in a similar way to that described in the U46619-contracted bioassay rings.

**Drugs**

Acetylcholine hydrochloride, indomethacin, PDBu, and 4a-PDD were all purchased from Sigma Chemical Co., St. Louis. U46619 was purchased from The Upjohn Co., Kalamazoo, Mich. Drugs were dissolved in distilled water (acetylcholine and U46619), in 96% ethanol (PDBu and 4a-PDD), or in 1.5×10^-3 M Na2CO3 (indomethacin). Stock solutions were prepared daily and kept on ice during the experiments.

**Calculations and Statistical Analysis**

Changes in isometric force induced by drugs are expressed either as grams or as a percentage of the initial contractile responses. Data are shown as mean±SEM; n represents the number of dogs from which blood vessels were isolated.

Statistical comparisons were performed by Student’s t test for paired or unpaired observations. Significance was accepted at p≤0.05.

**Results**

Acetylcholine (10^-6 M) relaxed the bioassay rings contracted by U46619 when infused upstream of the donor femoral artery segment with endothelium (−85.4±5.1%; n=7) (Figure 1, lower left trace), but not when infused directly on the bioassay ring (−1.2±5.6%; n=8) or upstream of the donor segment without endothelium (2.6±3.2%; n=7) (Figure 1, upper left trace). Infusion of 10^-7 M PDBu downstream of the donor segment (at site 2, see “Materials and
Methods”) contracted the bioassay ring to a similar extent (5.0±0.3 g; n=7) than did 5×10⁻⁸ M U46619 (4.1±0.6 g; n=7). Infusion of increasing concentrations (10⁻⁶–10⁻⁷ M) of PDBu into the perfusate upstream of the donor segments (at site 1, see “Materials and Methods”) caused further, concentration-dependent increase in isometric force of the bioassay ring when the donor segment had endothelium (Figure 1, lower trace, and Figure 2). These responses started several minutes after the infusion of PDBu started, developed slowly, and were only partially reversible after the infusion of PDBu was stopped. Infusion of 10⁻⁶ M 4a-PDD had no effect (data not shown; n=3). Removal of the endothelium from the donor segment prevented the contractions evoked by 10⁻⁹ M PDBu and significantly depressed the contractions in response to infusion of 10⁻⁸ and 10⁻⁷ M PDBu (Figure 1, upper trace, and Figure 2). During direct superfusion (through the stainless steel tube), infusion of 10⁻⁹–10⁻⁷ M PDBu at site 1 caused no further contraction of the bioassay rings already contracted by 10⁻⁷ M PDBu (site 2) (10⁻⁸ M, 0%; 10⁻⁹ M, 2.3%; 10⁻⁷ M, −1.7%). Selective exposure of the bioassay tissue to PDBu (10⁻⁷ M) completely abolished the responsiveness of the bioassay vascular smooth muscle to basally released EDRF (Figure 3).

**Discussion**

The important and novel finding of the present bioassay study is that infusion of increasing concentrations of PDBu (10⁻⁹–10⁻⁷ M) through the donor femoral artery segments with endothelium evoked concentration-dependent increases of isometric force of superfused bioassay coronary artery rings already exposed to 10⁻⁷ M PDBu. The contractions of the bioassay ring induced by infusion of PDBu through

![Graph showing the effect of PDBu on isometric force](image1)

**Figure 1.** Tracings from an original bioassay experiment showing the change of isometric force in canine coronary artery rings without endothelium (bioassay tissue) superfused with the effluent from perfused donor canine femoral artery segments with (lower trace) or without (upper trace) endothelium. The absence (top) and presence (bottom) of functional endothelium in the donor segments was demonstrated by the absence and presence, respectively, of relaxations of the bioassay ring contracted by 5×10⁻⁸ M U46619 in response to infusion of 10⁻⁶ M acetylcholine (ACH) through the donor segment (left). Infusion of 10⁻⁷ M phorbol 12,13-dibutyrate (PDBu) downstream of the donor segment (at site 2) evoked slowly developing contractions in the bioassay rings. Infusion of increasing concentrations of PDBu (10⁻⁹–10⁻⁷ M) through the donor segment (at site 1) evoked further contraction of the superfused bioassay ring only if the endothelium was present in the donor artery.

![Graph showing the effect of PDBu on isometric force](image2)

**Figure 2.** Effect of infusion of increasing concentrations (10⁻⁹–10⁻⁷ M) of phorbol 12,13-dibutyrate (PDBu) through the donor femoral artery segment with (●) or without (○) endothelium on isometric force of bioassay coronary artery rings without endothelium contracted by 10⁻⁷ M PDBu (for experimental details see “Materials and Methods” and Figure 1). The change (increase) of isometric force of bioassay rings is expressed as grams (left) or percent of initial contraction (right) evoked by 10⁻⁷ M PDBu (100%: donor with endothelium, 5.0±0.3 g; donor without endothelium, 4.1±0.5 g). Data shown are mean±SEM of seven experiments. *p<0.05.
the donor segment must be due to the modulation of the release of vasoactive factor(s) from the endothelium, since removal of the endothelium from the donor segment significantly depressed the contractions. The experiments were carried out in the presence of indomethacin; thus the contribution of prostanoioids to the response can be ruled out.9

The observed phenomenon can be explained by two equally possible mechanisms: 1) PDBu inhibits the synthesis or release of EDRFs, and 2) PDBu facilitates (or triggers) the production of EDCF.s. Recent studies on the guinea pig pulmonary artery,14 rabbit aorta,15 and canine femoral and coronary arteries,16 showed that PDBu inhibited endothelium-dependent relaxations to a variety of agonists. Previous bioassay studies revealed that selective exposure of the donor tissue to PDBu depresses the production of EDRF released under basal conditions or in response to acetylcholine.16 Thus, inhibition of the release of EDRF from femoral arteries may explain the observed phenomenon.

However, inhibition of basal release of EDRF is an unlikely explanation for the contraction of the bioassay tissue in response to stimulation of the donor segment with 10⁻⁹ and 10⁻⁸ M PDBu (Figures 1 and 2) since responsiveness of the bioassay ring to basal EDRF was inhibited by its selective exposure to 10⁻⁸ M PDBu (Figure 3). Thus, the most likely explanation is that PDBu triggers the production of a diffusible and bioassayable endothelium-derived vasoconstrictor factor(s). Apparently this mechanism seems to be fully responsible for contractions evoked by low concentrations of PDBu (10⁻⁸ M). At higher PDBu concentrations (10⁻⁷ and 10⁻⁶ M), this mechanism facilitates the direct contractile effect of the phorbol ester on vascular smooth muscle.

Endothelin is a recently discovered potent endothelium-derived vasoconstrictor polypeptide24 whose gene was shown to have a phorbol ester-sensitive regulatory element (t-RA) in the 5’ flanking region.20 Indeed, phorbol esters stimulate the synthesis and release of endothelin in cultured endothelial cells.21 Although it is possible that PDBu stimulates the synthesis and release of endothelin from the endothelium of canine femoral artery, the present study was not designed to characterize the nature of the diffusible vasoconstrictror factor(s); it requires further investigations. PDBu is a tumor-promoting phorbol ester, which activates protein kinase C.13 Although non-specific phorbol effect cannot be ruled out completely, it is likely that the effects of PDBu are mediated by stimulation of protein kinase C. This conclusion is based on the finding that the inactive phorbol ester 4α-PDD (which does not activate protein kinase C13) was devoid of any contractile activity under the same experimental conditions. Protein kinase C is present in endothelial cells22,23 and is activated by PDBu and other active phorbol esters, but not by the inactive phorbol ester 4α-PDD.24,26

The present observations in combination with recent studies14-16 indicate that the phorbol ester PDBu depresses the production of EDRF and facilitates the synthesis/release of EDCF(s). The PDBu-induced disturbed balance between the production of relaxing and contracting endothelium-derived vasoactive factors mimic pathological conditions (e.g., hypoxia and hypertension) where a shift from EDRF to EDCF production were described.8,10,11,27 Thus,

![Figure 3. Effect of selective exposure of the bioassay ring to 10⁻⁸ M U46619 (top) or to 10⁻⁷ M phorbol 12,13-dibutylate (PDBu; bottom) on its responsiveness to basally released endothelium-derived relaxing factor. Left panel: Tracings from an original bioassay experiment. Right panel: Relaxation to basally released endothelium-derived relaxing factor of bioassay rings contracted by U46619 and its inhibition by contraction with PDBu. Data are expressed as percent inhibition of initial contraction (100%; U46619, 8.0±0.5 g; PDBu, 8.0±0.8 g) and shown as mean±SEM of three experiments. *The effect of PDBu was statistically significant (p<0.05).]
phorbol esters may be a useful tool for further characterization of the cellular events of endothelial dysfunction, which can lead to (or are the consequences of) various cardiovascular disorders.

Acknowledgments
The authors wish to thank Mrs. S. Packie for her excellent secretarial assistance. The Animal Facility at Berlex is accredited by the American Association for the Accreditation of Laboratory Animal Care.

References
22. Doctrow S, Folman J: Protein kinase C activators suppress stimulation of capillary endothelial cell growth by angiogenic endothelial mitogens, J Cell Biol 1987;104:679–687

Key Words • endothelium • endothelium-derived contracting factors • endothelium-derived relaxing factor • phorbol esters • vascular smooth muscle
Phorbol dibutyrate stimulates the release of diffusible endothelium-derived vasoconstrictor factor(s) from canine femoral arteries.

G M Rubanyi, A Luisi and A Johns

doi: 10.1161/01.RES.68.6.1527

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/68/6/1527

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* 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 *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/