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

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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^{-5}–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 4α-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).^{12} 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
Bioassay Studies

A bioassay technique described in detail earlier 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 surface 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.

To evoke sustained contraction of the bioassay rings (n=7), the endothelium 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 (10⁻⁷–10⁻⁶ 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 10⁻⁷ 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.

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

The bioassay rings were 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 (10⁻⁷–10⁻⁶ 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.

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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 (10⁻⁹–10⁻⁷ 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 4α-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...
the donor segment must be due to the modulation of
the release of vasoactive factor(s) from the endothe-
lum, since removal of the endothelium from the
donor segment significantly depressed the con-
tractions. The experiments were carried out in the
presence of indomethacin; thus the contribution of pros-
tanoids 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 EDCFs.
Recent studies on the guinea pig pulmonary artery,14
rabbit aorta,15 and canine femoral and coronary
arteries16 showed that PDBu inhibited endothelium-
dependent relaxations to a variety of agonists. Previ-
ous bioassay studies revealed that selective exposure
of the donor tissue to PDBu depresses the produc-
tion 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 bio-
assay 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^{-7}
M PDBu (Figure 3). Thus, the most likely explana-
tion is that PDBu triggers the production of a diffus-
able and bioassayable endothelium-derived va-
soconstrictor 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^{-8} and 10^{-7} M), this me-
chanism facilitates the direct contractile effect of the
phorbol ester on vascular smooth muscle.

Endothelin is a recently discovered potent endo-
thelium-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 endo-
thelium of canine femoral artery, the present study
was not designed to characterize the nature of the
diffusible vasoconstrictor 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
4a-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 4a-PDD.24,26

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

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