Contrasting Effects of Phorbol Esters on Serotonin- and Vasopressin-Evoked Contractions in Rat Aorta and Small Mesenteric Artery

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Phorbol esters, which activate protein kinase C, modulate vasoconstrictor-induced tension in vascular smooth muscle. We examined the effects of phorbol esters (phorbol 12,13-dibutyrate [PDBu] and 12-O-tetradecanoylphorbol 13-acetate [TPA]) on receptor agonist (serotonin [5-HT] and arginine vasopressin [AVP])-induced high K+-, and caffeine-induced contractions in rings of rat aorta and a small (second-order) branch of the superior mesenteric artery (SMA). PDBu and TPA significantly augmented agonist-evoked contractions in aorta but diminished those in SMA. For example, 30 nM PDBu increased 5-HT- and AVP-evoked contractions 2.0–2.5-fold in aorta (p < 0.01) but decreased 5-HT- and AVP-induced contractions by 40–60% in SMA (p < 0.01). In contrast, PDBu and TPA amplified high K+-, and 10 mM caffeine-induced contractions in both aorta and SMA. Augmentation of agonist-induced contractions by PDBu was greater in endothelium-denuded aorta than in intact aorta. Two protein kinase C antagonists, H-7 and staurosporine, inhibited 5-HT-evoked contractions in the absence as well as in the presence of PDBu in both types of arteries. The augmentation of contractile responses to caffeine and K+ by phorbol esters in both types of arteries suggests that the phorbols increase the sensitivity of the contractile apparatus to Ca2+, probably by activating protein kinase C. However, the inhibitory effects of phorbols on 5-HT- and AVP-evoked responses in SMA suggest that under these conditions the dominant effect of the phorbols is a marked reduction in the availability of Ca2+ in the SMA but not in the aorta.

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Key Words • arginine vasopressin • H-7 • phorbol esters • protein kinase C • serotonin • vascular smooth muscle

Contraction in vascular smooth muscle (VSM) is triggered by a rise in the sarcoplasmic free calcium concentration ([Ca2+]i). This rise in [Ca2+]i activates the calmodulin–myosin light chain kinase cascade that is “necessary and sufficient” to initiate contraction. However, the relation between [Ca2+]i and tension is complicated. Recent evidence indicates that the sensitivity of the contractile apparatus to Ca2+ can be modulated; for example, it is increased by activators of protein kinase C (PKC) and inhibited by cyclic nucleotides.

Caffeine, potassium, and receptor agonists such as serotonin (5-HT) and arginine vasopressin (AVP) all activate VSM contraction by elevating [Ca2+]i; they raise [Ca2+]i in different ways, however. In large conduit vessels, such as the aorta, which has been routinely studied, the receptor agonists activate phospholipase C, thereby stimulating the production of inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG); the IP3, in turn, triggers Ca2+ release from stores in the sarcoplasmic reticulum (SR). The agonists also stimulate Ca2+ entry by opening receptor-operated Ca2+ channels in the sarcolemma. In contrast, caffeine directly releases Ca2+ from the SR, whereas K+ depolarizes VSM and thereby promotes Ca2+ entry via voltage-gated Ca2+ channels, although K+ also raises IP3 levels in some tissues. DAG activates PKC, but the role that DAG plays in receptor agonist–activated contractile responses is unknown. Phorbol esters, which selectively bind to PKC in place of DAG, raise resting tension in some VSMs, but they may do so without elevating [Ca2+]i. The influence of phorbol esters on vasoconstrictor-induced contractions is controversial, however; both augmentation and attenuation of contractions have been described, and one report has suggested that phorbol-augmented 5-HT responses are endothelium dependent. There is also some evidence that vasoconstrictor-induced increases in [Ca2+]i may be attenuated by phorbol esters, perhaps because receptor agonist–induced IP3 production is inhibited by phorbol esters.
The relative contributions to contractile activation of receptor agonist–stimulated Ca\(^{2+}\) release from the SR and Ca\(^{2+}\) entry from the extracellular fluid vary in different blood vessels.\(^{15,29}\) For example, in rat aorta, a conduit vessel, Ca\(^{2+}\) release from the SR apparently plays a prominent role, whereas in small muscular arteries such as branches of the rat mesenteric artery Ca\(^{2+}\) release from the SR appears to be much less important for the activation of contraction.\(^{30,31}\) Thus, IP\(_3\) (and perhaps DAG and PKC) may play very different roles in these two types of vessels; moreover, there is negligible information about activation of phospholipase C and production of IP\(_3\) and DAG in small peripheral vessels. With these differences in mind, we tested the effects of two phorbol esters on contractions activated by 5-HT and AVP and by caffeine and an

**Figure 1.** Effects of serotonin (5-HT), arginine vasopressin (AVP), phorbol 12,13-dibutyrate (PDBu), 4-α-PDBu, 12-O-tetradecanoylphorbol 13-acetate (TPA), and H-7 on isometric tension in rings of rat thoracic aorta. The rings were stimulated by brief exposures to 10 μM (panel A) or 20 μM (panels B and D) 5-HT or 60 nM AVP (panel C) at the times indicated by arrowheads. Panel A: After the initial control period, 25 nM and then 30 nM PDBu were added to the physiological salt solution (PSS) (open bars); 50 μM H-7 was also present during the period indicated by the thick black bar. Both the PDBu and H-7 were then washed out. Panels B and C: After the initial control period, 400 nM and then 600 nM TPA were added to the PSS (open bars); 50 μM H-7 was added later (thick black bar). Both the TPA and H-7 were then washed out. Panel D: After the initial control period, the ring was superfused for 30 minutes with PSS containing 3 μM 4-α-PDBu (hatched bar). The 4-α-PDBu was washed out for 30 minutes and 30 nM PDBu was then added (open bar). Finally, the PDBu was washed out for more than 2 hours. Temperature, 37°C; original “resting” tension, 500 mg; tissue wet weight, 2.3 mg (panel A), 2.1 mg (panel B), 2.7 mg (panel C), and 2.5 mg (panel D).

**Figure 2.** Effects of serotonin (5-HT) and cumulatively applied phorbol 12,13-dibutyrate (PDBu) on tension in rings of aorta (panel A) and small mesenteric artery (SMA, panel B) from the same rat. The rings were stimulated by brief exposures to 20 μM (panel A) and 0.5 μM (panel B) 5-HT at the times indicated by arrowheads. After the initial control period, PDBu was added to the physiological salt solution (PSS) (open bars). A much higher concentration of 5-HT was required to elicit contractions in the aorta than in the SMA. In contrast, the aorta was more sensitive to PDBu than was the SMA. Similar results were obtained in two other comparable experiments. Temperature, 37°C; “resting” tension, 500 mg (panel A) and 300 mg (panel B); tissue wet weight, 2.7 mg (panel A) and 0.12 mg (panel B).
FIGURE 3. Effects of Ca\(^{2+}\) removal from standard physiological salt solution (PSS, panel A) and 10 \(\mu\)M verapamil (panel B) on serotonin (5-HT)-evoked contractions in the presence of phorbol 12,13-dibutyrate (PDBu, large open bars) in rings of rat aorta. The rings were stimulated at the times indicated by arrowheads. Similar results were obtained in three other comparable experiments. Temperature, 37°C; "resting" tension, 500 mg; tissue wet weight, 2.4 mg (panel A) and 2.0 mg (panel B).

Elevated extracellular K\(^+\) concentration ([K\(^+\)]\(_{e}\)) in rings of rat aorta and a small (second-order) branch of the superior mesenteric artery (SMA).

Materials and Methods

Arterial Rings

Rings of rat thoracic aorta and SMA were used for these experiments. Normal Sprague-Dawley rats (200–250 g) were decapitated; the thoracic aorta as well as the mesenteric artery and surrounding tissues were rapidly removed and placed in physiological salt solution (PSS) at 37°C. Under a dissecting microscope, the thoracic aorta (1.0–1.5-mm diameter) and SMA (about 300-μm diameter) were dissected free of surrounding connective tissues and cut into 2-mm-long rings. The total dissection was usually accomplished within 45

FIGURE 4. Effects of phorbol 12,13-dibutyrate (PDBu, panel A) and 12-O-tetradecanoylphorbol 13-acetate (TPA, panel B) on serotonin (5-HT)- and arginine vasopressin (AVP)–evoked contractions in rings of rat aorta and small mesenteric artery (SMA). Control, 5-HT– or AVP–evoked contractions were determined during superfusion with standard physiological salt solution (aorta, 20–30 \(\mu\)M 5-HT or 60 nM AVP; SMA, 0.3–1.0 \(\mu\)M 5-HT or 10–20 nM AVP). After 30 minutes of superfusion with the phorbol esters (30 nM PDBu, panel A; 400 nM TPA, panel B), the arterial rings were again stimulated with 5-HT or AVP. Recovery, responses to the vasoconstrictors determined after 60 minutes of washout of phorbol ester. The data are normalized to the tension evoked by 5-HT and AVP before the introduction of phorbol ester (=100%). The data are mean±SEM and were statistically analyzed using Student’s unpaired t test. Panel A: n=7 (5-HT, aorta); n=5 (AVP, aorta); n=8 (5-HT, SMA); n=3 (AVP, SMA). Panel B: n=4 (5-HT, aorta); n=4 (AVP, aorta); n=3 (5-HT, SMA); n=4 (AVP, SMA). \(\ast p<0.05\), \(\ast\ast p<0.01\) vs. control; \(\dagger p<0.05\), \(\ddagger p<0.01\) vs. PDBu or TPA.
Figure 5. Effect of endothelium removal on the responses of rat aorta to serotonin (5-HT) and phorbol 12,13-dibutyrate (PDBu). Upper recordings (+E): Ring with endothelium intact; lower recordings (−E): adjacent ring with endothelium removed. 1: Contractions in response to 2.5 μM (+E) or 12.5 nM (−E) phenylephrine (PE). Acetylcholine (ACH, 10 μM) relaxed the +E ring but not the −E ring, indicating that the endothelium was completely destroyed in the −E ring. 2–6: Contractions in response to brief exposures to 0.6, 2.0, or 6.0 μM 5-HT (−E), or 2.0, 6.0, or 20 μM 5-HT (+E), as indicated (arrowheads) during superfusion with control physiological salt solution (PSS) or PSS containing 5 or 20 nM PDBu (open bars). Removal of the endothelium increased the response of aortic rings to PE by a factor of about 200, but it increased the responses to 5-HT and PDBu by factors of only about 3–4. Comparable results were obtained in two other similar experiments. Temperature, 37°C; “resting” tension, 500 mg; tissue wet weight, 2.6 mg (+E) and 2.0 mg (−E).

Reagents and Solutions

The standard PSS contained (millimolar) NaCl 138, KCl 4.7, CaCl2 1.8, MgSO4 1.2, NaH2PO4 1.2, glucose 10, and HEPES 5. The PSS was oxygenated with 100% O2. In high K+ solution (e.g., 30 mM K+ PSS), the NaCl in the standard PSS was replaced, mole for mole, by KCl. In Ca2+-free PSS, the CaCl2 was replaced by equimolar MgCl2. All solutions were adjusted to pH 7.4 with 2 M Tris.

Acetylcholine, caffeine, 5-HT, phenylephrine, phorbol 12,13-dibutyrate (PDBu), 12-O-tetradecanoylphorbol 13-acetate (TPA), staurosporine (a PKC inhibitor33), and 1-(5-isquinolinylsulfonyl)-2-methylpiperazone (H-7, also a PKC inhibitor44) were purchased from Sigma Chemical Co., St. Louis, Mo.; 4-α-PDBu was obtained from LC Services Corp., Woburn, Mass.; AVP was purchased from Peninsula Laboratories, Belmont, Calif.; and verapamil was a gift from Knoll Pharmaceuticals, Whippany, N.J. All drug solutions were prepared on the day of use. The 5-HT and AVP were made as concentrated stock solutions in distilled water; PDBu and 4-α-PDBu were made as 1 mM stock solutions in 10% dimethyl sulfoxide; TPA and staurosporine were made as 10 and 1 mM stock solutions, respectively, in 100% dimethyl sulfoxide; H-7 and phenylephrine were made as 10 mM stock solutions in distilled water; acetylcholine was prepared as a 1 M stock solution in distilled water; and verapamil and caffeine were dissolved directly in PSS. The final concentration of dimethyl sulfoxide in PSS was less than 0.006%.

Contraction Measurements

Two thin (0.3-mm diameter) stainless-steel hooks were inserted through the lumen of the aortic rings; tungsten wire hooks (0.1-mm diameter) were used for SMA rings. One hook was fixed to the bottom of the tissue chamber (volume, 0.75 ml), and the other hook was connected to an isometric force transducer (model 52-9529, Harvard Apparatus, South Natick, Mass.), which was mounted directly above the tissue chamber. Isometric tension was continuously monitored and recorded on a strip-chart recorder. The tissue was continuously superfused at a rate of 2 ml/min with well-oxygenated incubation fluid at 37°C. The arteries were equilibrated until the resting tension of aorta and SMA stabilized at 500 mg and 300 mg, respectively; this took 1.5–2.0 hours or more.

Most drugs and other reagents were added directly to the superfusion fluids and were therefore equilibrated in the tissue chamber. However, 5-HT and AVP were usually applied as small bolus injections (15 μl) in the superfusion line (see “Results”); the 5-HT and AVP were thus diluted 50-fold when they reached the tissue
chamber. Under these circumstances, the concentrations of 5-HT and AVP in the incubation chamber rose rapidly to a peak that lasted about 15–25 seconds and then declined with a half-time of about 15 seconds. Reproducible contractile responses to 5-HT and AVP were obtained with at least three repeated exposures to these agents before data collection was initiated (see "Results" and Reference 35).

Statistics

Student’s paired or unpaired t tests or analysis of variance was used to test for statistical significance when appropriate (see figure legends).

Results

Effects of PDBu and TPA on Resting Tension and Serotonin- and Arginine Vasopressin–Evoked Tension in Rat Aorta

Figure 1 shows representative data from rings of rat aorta on the effects of 5-HT- and AVP-evoked tension in the absence and presence of the phorbol esters PDBu and TPA. During superfusion with PSS (without phorbols), brief exposure to 5-HT (Figures 1A, 1B, and 1D) and AVP (Figure 1C) induced brief contractions followed by prompt relaxation back to the original “resting” tension as the 5-HT and AVP were washed out.

Effects of phorbol esters on resting tension. When 25 or 30 nM PDBu was added to the PSS, resting tension rose slowly toward a new steady level. This effect was dose dependent; as shown in Figures 1A and 2A, resting tension increased further when the PDBu concentration was raised from 25 to 30 nM or from 30 to 50 nM, respectively. TPA also slowly increased resting tension (Figures 1B and 1C), although a much higher concentration was required. On the average, 30 nM PDBu and 400 nM TPA increased aortic ring resting tension by 23.7±5.7 (n=12) and 20.1±4.9 (n=8) mg/mg wet wt, respectively. In agreement with others, this effect of phorbol esters on steady resting tension was dependent on external Ca2+ (Figure 3A) and was inhibited by the Ca2+ channel blocker verapamil (Figure 3B).

Effects of phorbol esters on vasoconstrictor-evoked contractions. Figures 1–3 also show that the tension responses evoked by submaximal concentrations of 5-HT and AVP in rat aorta were augmented in the presence of PDBu or TPA. Furthermore, when the receptor agonists were rapidly washed out of the tissue chamber, steady (resting) tension usually remained at a substantially elevated steady level. The rate of rise of tension, in response to 5-HT and AVP, was unaffected or only slightly increased by the phorbol esters.

Averaged data from a number of comparable experiments on the evoked responses of rat aortic rings to 5-HT (circles) and AVP (triangles) before, during (30-minute exposure), and after (60-minute washout) PDBu and TPA treatment are illustrated by the solid symbols in Figures 4A and 4B, respectively. PDBu (30 nM) increased 5-HT- and AVP-evoked contractions 1.99±0.09-fold (n=7, p<0.01) and 2.48±0.27-fold (n=5, p<0.01), respectively. TPA (400 nM) elevated 5-HT- and AVP-induced contractions 1.75±0.12-fold (n=4, p<0.05) and 1.50±0.16-fold (n=4, p<0.05), respectively.

When PDBu was washed out, it took at least 30 minutes before the steady tension returned to the original control resting tension level. Nevertheless, 5-HT usually continued to induce amplified tension responses for at least 60–90 minutes after PDBu and TPA were washed out (Figures 1D, 2A, and 4). On the other hand, after the phorbol esters were washed out for 60 minutes, AVP-evoked contractions became significantly smaller than the control AVP-evoked contractions (Figure 4; PDBu, 0.57±0.06-fold, n=5, p<0.01; TPA, 0.66±0.08-fold, n=4, p<0.05).

In contrast to the action of PDBu (25–30 nM), 4-α-PDBu (3 μM), a phorbol ester that does not activate PKC, had no effect on resting tension and even slightly decreased the contractile responses to 5-HT (Figure 1D).

Dependence on Ca2+. As shown in Figure 3, the contractile responses to 5-HT in the presence of PDBu were inhibited by the removal of extracellular Ca2+ (panel A) and by treatment with the Ca2+ channel blocker verapamil (panel B) in rings of aorta. These results suggest that Ca2+ influx from the extracellular fluid, perhaps via receptor-operated Ca2+ channels, is necessary for the PDBu-augmented contractile responses to 5-HT.

Effects of endothelium removal. Recently, Consigny reported that the amplification of the 5-HT response by TPA in rabbit aorta was dependent on the endothelium. We found that destruction of the endothelium (Figure 5, bottom panel) sensitized the rat aortic rings to both PDBu and 5-HT; the concentration of 5-HT required to increase tension was substantially reduced. Nevertheless, in contrast to Consigny’s report, PDBu still augmented the responses to 5-HT (Figure 5, segments 3 and 5) when the relaxation response to acetylcholine was completely abolished (segment 1).
Effects of PDBu and TPA on Resting Tension and Serotonin- and Arginine Vasopressin–Evoked Tension in Small Mesenteric Artery

The effects of PDBu on the resting and 5-HT–evoked responses in an aortic ring and an SMA ring from the same rat are compared in Figure 2. In both types of rings, PDBu induced a dose-dependent increase in resting tension. The absolute increases in resting tension in the SMA rings were small at low PDBu concentrations. When expressed on a wet weight basis, however, the increments in SMA resting tension were much larger than in the aortic rings: 169.8±51.4 (n=11) mg/mg wet wt for SMA and 23.7±5.7 (n=12) mg/mg wet wt for aorta with 30 nM PDBu. Furthermore, in control PSS, the SMA was more sensitive to 5-HT than was the aorta from the same rat (Figure 2; see References 37 and 38); i.e., the 5-HT dose–response curve for the SMA was located to the left of the comparable curve for the aorta (Figure 6). The SMA was also more sensitive to AVP than was the aorta.37,38

In contrast to the activating effects of the phorbols on vasoconstrictor-evoked contractions in aortic rings, both PDBu (Figures 2, 4, and 7) and TPA (Figures 4 and 7) significantly and reversibly inhibited the 5-HT– and AVP-evoked contractions in rings of SMA. As shown in Figure 6, PDBu shifted the 5-HT dose–response curve to the right and downward in SMA and to the left in aortic rings; in the aorta in the presence of PDBu, however, this 5-HT–evoked tension in the aorta was superimposed on a progressively rising baseline tension.

This marked difference between the aorta and SMA was not due to the brevity of exposure to the vasoconstrictor; PDBu also increased 5-HT–evoked tension in the aorta (Figure 8A) but reduced the active tension in the SMA (Figure 8B) when the rings were exposed to submaximal concentrations of the agonist for a more prolonged period. The inhibition of the 5-HT–evoked contraction by PDBu in the SMA was also observed in rings in which acetylcholine completely and reversibly relaxed the phenylephrine-evoked contraction (Figure

FIGURE 7. Effects of serotonin (5-HT), arginine vasopressin (AVP), phorbol 12,13-dibutyrate (PDBu), and 12-O-tetradecanoylphorbol acetate (TPA) on isometric tension in rings of rat small mesenteric artery. Panel D shows that the effect of PDBu on 5-HT–evoked responses in small mesenteric artery was not due to destruction of the endothelium; the phenylephrine (PE)-constricted ring relaxed completely in response to acetylcholine (ACh). The rings were stimulated by brief exposures to 0.5 μM (panel A) or 10 μM (panel D) 5-HT, or 10 nM (panel B) or 20 nM (panel C) AVP (at the arrowheads). After the initial period in physiological salt solution (PSS), 400 nM TPA (panels A and C) or 30 nM PDBu (panels B and D) was added. The phorbol esters were washed out at the end of the experiments. Temperature, 37°C; “resting” tension, 300 mg; tissue wet weight, 0.08 mg (panel A), 0.08 mg (panel B), 0.05 mg (panel C), and 0.06 mg (panel D).
7D) and thus could not be attributed to destruction of the endothelium.24

**Effects of Protein Kinase C Antagonists on Serotonin- and Arginine Vasopressin–Evoked Contractions in the Presence and Absence of Phorbols**

Activation of PKC, as may occur with tumor-promoting phorbol esters such as PDBu and TPA, apparently enhances VSM contraction39 by increasing the sensitivity of the contractile apparatus to Ca\(^{2+}\) (see Reference 3). We explored the possible role of PKC further by examining the effects of H-7, a PKC inhibitor,34 on aortic rings. As illustrated in Figures 1A–1C, H-7 relaxed aortic rings that were contracted by PDBu and TPA and markedly attenuated the contractile responses to 5-HT and AVP in the presence of PDBu and TPA; these effects of H-7 were reversible. Similar effects of H-7 on PDBu- and receptor agonist-activated contractions were also observed in rings of SMA (not shown).

Even in the absence of phorbol esters, however, H-7 reversibly inhibited the contractile responses evoked by 5-HT (Figure 9A). The 5-HT dose–response curves (Figures 9B and 9C) indicate that the inhibition by H-7 was noncompetitive, with an IC\(_{50}\) of 4.1±1.2 μM; H-7 reduced the maximal 5-HT–evoked contraction but did not significantly alter the apparent EC\(_{50}\) for 5-HT. This IC\(_{50}\) is very close to the \(K_i\) for inhibition of purified PKC, 6.0 μM.34 Because H-7 is a potent inhibitor of PKC but a poor inhibitor of myosin light chain kinase (\(K_i=97 \mu M\)),34,40 these actions of H-7 raise the possibility that PKC may modulate VSM contractility even in the absence of exogenous PKC activators.

This view is supported by studies with staurosporine, a PKC antagonist that acts via a different mechanism from that of H-7.41 Staurosporine also inhibited 5-HT–evoked contractions in the absence (Figure 10A) as well as in the presence (Figure 10B) of PDBu.

**Effects of Phorbol Esters on Caffeine- and High K\(^{+}\)–Evoked Contractions in Rat Aorta and Small Mesenteric Artery**

Phorbol esters inhibit the receptor agonist–stimulated formation of IP\(_3\),25–28 Therefore, it should be possible to evaluate the effects of PDBu on Ca\(^{2+}\) handling in rat aorta and SMA by comparing 5-HT– and AVP-evoked contractions to caffeine- and high K\(^{+}\)-induced contractions in the two types of arteries.

**Caffeine contractions.** PDBu augmented contractions induced by caffeine as well as the contractile responses to 5-HT in rat aorta (Figure 11A). PDBu also augmented caffeine-evoked contractions in SMA but, as noted above, diminished the contractile responses to 5-HT in the same rings (Figure 11B). Normal caffeine responses were restored in both aortic and SMA rings when the PDBu was washed out.

**K\(^{+}\)-evoked contractions.** Figure 12A shows the effects of PDBu on high K\(^{+}\)-evoked contractions in rat aorta. Increasing [K\(^{+}\)]\(_o\) from 4.7 to 10 or 15 mM did not alter tension in the absence of PDBu. In the presence of PDBu,
however, 10 mM K⁺ produced a small rise in tension and 15 mM K⁺ evoked a substantial contraction; moreover, the contractile response to 20 mM K⁺ was greatly augmented. These effects of PDBu were reversible.

PDBu also augmented K⁺-evoked contractions in rings of SMA. Figure 12B shows that PDBu increased the 30 mM K⁺-evoked contraction even though it attenuated the response to 5-HT in the same arterial ring. These effects of PDBu were completely reversible.

Data from a number of experiments similar to the ones illustrated in Figure 12 are summarized in Figure 13. While SMA rings were much more sensitive to 5-HT than aortic rings were (Figure 6), the aortic rings and SMA rings were equally sensitive to high [K⁺]o; that is, as shown in Figure 13 (left panel), the [K⁺]o versus relative evoked tension curves were nearly superimposable. Furthermore, as implied by the data in Figure 12, PDBu significantly shifted the [K⁺]o, versus tension curves for both aorta and SMA to the left along the [K⁺]o axis (Figure 13, right panel). Although not illustrated here, TPA also increased the contractility of the aorta and SMA in response to modest elevations of [K⁺]o.

Discussion

Effects of Phorbol Esters on Resting Tension in Vascular Smooth Muscle

Phorbol esters produce slowly developing and long-lasting contractions in unstimulated VSM. The mechanism underlying these contractions is uncertain, although data from permeabilized VSM preparations demonstrate that the sensitivity of the contractile apparatus to Ca²⁺ is increased (i.e., the relation between [Ca²⁺] and tension is shifted toward lower [Ca²⁺]) by phorbol esters. Nevertheless, the relation between the phorbol ester-induced contractions and [Ca²⁺] remains unresolved because of conflicting reports on the effects of these agents on Ca²⁺ movements and [Ca²⁺]: either an increase or no change or even a dual action with a low concentration of PDBu causing [Ca²⁺] to rise and a high concentration causing [Ca²⁺] to fall.

In a preliminary study in cultured A7r5 cells (derived from fetal rat aorta), 30–50 nM PDBu invariably induced a small, transient rise in [Ca²⁺] but, during maintained exposure to PDBu, [Ca²⁺] returned to the original resting level within 1 minute. This small PDBu-induced Ca²⁺ transient may be explained if the activation of PKC by
PDBu opens Ca\(^{2+}\) channels directly,\(^{47}\) thereby promoting rapid Ca\(^{2+}\) entry\(^{43}\) that is soon counterbalanced by enhanced Ca\(^{2+}\) extrusion,\(^{44}\) although release and resequestration of Ca\(^{2+}\) by the SR cannot be ruled out. Nevertheless, these data suggest that the contribution of Ca\(^{2+}\) influx to the phorbol ester–induced rise in steady tension may be relatively small. In agreement with previous reports,\(^{45,46,47}\) however, we observed inhibition of phorbol ester–induced contractions in unstimulated aortic rings on removal of extracellular Ca\(^{2+}\) or addition of the Ca\(^{2+}\) channel blocker verapamil (Figure 3). Therefore, these phorbol ester– induced contractions do seem to depend on Ca\(^{2+}\) influx from the extracellular fluid via voltage-regulated Ca\(^{2+}\) channels,\(^{47}\) but this does not necessarily imply that the sustained contractions require an elevated [Ca\(^{2+}\)].

**Phorbol Esters Increase the Sensitivity of the Contractile Apparatus to Ca\(^{2+}\)**

In both aortic and SMA rings, the contractile responses to caffeine and elevated [K\(^{+}\)], were significantly augmented by phorbol esters. Caffeine and K\(^{+}\) both

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**Figure 10.** Effects of staurosporine on serotonin (5-HT)-evoked contractions in rings of rat aorta. Panel A: Data obtained in the absence of phorbol 12,13-dibutyrate (PDBu). The ring was activated with 5 \(\mu\)M 5-HT for \(-20\) seconds (at the arrowheads). Staurosporine (10 nM and then 100 nM) was applied in physiological salt solution (PSS) for the periods indicated. Panel B: Data obtained in the presence of 35 nM PDBu. The ring was activated with 10 \(\mu\)M 5-HT for \(-20\) seconds (at the arrowheads). Staurosporine (100 nM) and PDBu (35 nM) were applied for the periods indicated. Temperature, 37°C; “resting” tension, 500 mg; tissue wet weight, 2.4 mg (panel A) and 2.3 mg (panel B).

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**Figure 11.** Effects of phorbol 12,13-dibutyrate (PDBu) on serotonin (5-HT)- and caffeine-evoked contractions in rings of rat aorta (panel A) and small mesenteric artery (panel B). The rings were stimulated by brief exposures to 20 \(\mu\)M (panel A) or 0.5 \(\mu\)M (panel B) 5-HT (at the arrowheads). The rings were also stimulated with 10 mM caffeine for 5 minutes (thick black bars). The rings were superfused with physiological salt solution (PSS) containing PDBu for 30 minutes before the second exposure to 5-HT and caffeine. The third exposure to these vasoconstrictors was carried out after 90 minutes of washout of PDBu. Similar results were obtained in one other aortic ring and two small mesenteric artery rings. Temperature, 37°C; “resting” tension, 500 mg (panel A) and 300 mg (panel B); tissue wet weight, 2.3 mg (panel A) and 0.05 mg (panel B).
FIGURE 12. Effect of phorbol 12,13-dibutyrate (PDBu) on K+-induced contractions in rat aorta (panel A) and small mesenteric artery (panel B). Panel A: Original recording shows responses to 10, 15, 20, and 30 mM K+ before, during, and after superfusion with physiological salt solution (PSS) containing 30 nM PDBu. The aortic ring was stimulated with each high K+ solution for 5 minutes (thick black bars). The data are representative of results obtained in four experiments. Panel B: The small mesenteric artery ring was stimulated by brief exposures to 0.6 μM serotonin (5-HT) (at arrowheads) and by 30 mM K+ for 5-minute periods (thick black bars). The data are representative of results obtained in five similar experiments. Temperature, 37°C; “resting” tension, 500 mg (panel A) and 300 mg (panel B); tissue wet weight, 2.2 mg (panel A) and 0.06 mg (panel B).

increase [Ca2+]i, albeit by different means: release from SR and entry via voltage-gated Ca2+ channels, respectively. The most parsimonious explanation for the augmented caffeine- and K+-evoked contractions, in the presence of the phorbol esters, is a PKC-induced increase in the sensitivity of the contractile apparatus to Ca2+ in both types of blood vessels.3,42 If this is the case, however, we must explain the seemingly paradoxical effects of the phorbol esters on the 5-HT- and AVP-activated contractions: augmentation in aortic rings and inhibition in SMA rings.

In the rat aorta, receptor agonists trigger the production of IP3,4 and release of Ca2+ from the SR, presumably mediated at least in part by IP3,5-7 contributes substantially to the rise in [Ca2+]i that activates contraction. Because phorbol esters inhibit the production of IP3,25-28 and may thereby attenuate agonist-evoked release of Ca2+ from the SR, phorbol esters might be expected to reduce agonist-evoked contractions in the aorta. The fact that these contractions are actually amplified therefore indicates that, in the aorta treated with a phorbol ester, the increased sensitivity of the contractile apparatus to Ca2+3,42 and/or enhanced (net) Ca2+ entry from the extracellular fluid plays the dominant role(s) in contractile activation. The endothelium does not mediate the augmenting effect of the phorbol esters (in contrast to the conclusion of Consigny24).

A very different explanation is required to account for the inhibitory effects of the phorbols on receptor agonist-evoked contractions in SMA. Release of Ca2+ from the caffeine-sensitive SR stores apparently contributes relatively little to the activation of contraction in SMA by receptor agonists under normal conditions31; indeed, a major role of the SR in these vessels may be to buffer the Ca2+ that enters from the extracellular fluid.31 Then, since phorbol esters apparently increase the sensitivity of the SMA contractile apparatus to Ca2+, we must conclude that the phorbol esters also markedly reduce agonist-activated Ca2+ entry and/or markedly enhance Ca2+ buffering, extrusion, and/or sequestration in the SR. In SMA, in contrast to aorta, the dominant effect of the phorbol esters may be a marked reduction of the agonist-induced rise in [Ca2+]i. Indeed, we have observed that 30–50 nM PDBu diminishes 5-HT-evoked Ca2+ transients in A549 cells.46

While this manuscript was in review, Boonen and De Mey48 reported that phorbol myristate acetate augments K+-evoked, but not norepinephrine-evoked, contractions in rat SMA, even after blockage or removal of the endothelium. Thus, it seems unlikely that the endothelium is responsible for the effects of phorbols that we observed in SMA.

The change in the effect of phorbols on the response to vasoconstrictors appears to be progressive along the arterial tree. In preliminary experiments on the main trunk of the superior mesenteric artery (not shown), PDBu had very little effect on the amplitude of 5-HT-evoked contractions.

Role of Protein Kinase C in Contractile Activation in Vascular Smooth Muscle

A straightforward explanation for the augmenting effects of phorbol esters on VSM contraction is that
these agents activate PKC by binding to this enzyme in place of DAG. If the PKC inhibitors H-7 and staurosporine blocked only the phorbol ester–induced changes in contraction, this might imply that PKC is not normally involved in VSM contraction. It is known, however, that 3–10 μM H-7 inhibits angiotensin II– and norepinephrine-evoked contractions of rat aorta in a noncompetitive manner in the absence of phorbols.49 Our data now show that staurosporine, as well as H-7, inhibits 5-HT-evoked contractions even in the absence of PDBu. Even though H-7 is not very selective, the low concentrations that we used (≤30 μM) reportedly inhibit PKC with little effect on myosin light chain kinase.34,40 Furthermore, the mode of action of staurosporine on PKC differs from that of H-7.41 Therefore, these data suggest that PKC may tonically modulate the Ca2+ sensitivity of contractile proteins under normal physiological condition.

The precise mechanism by which PKC increases the sensitivity of the contractile apparatus to Ca2+ and/or alters [Ca2+]c is not known. Phorbol esters can induce contractions without significantly increasing the phosphorylation of myosin light chains.50 Moreover, phosphorylation of myosin light chains catalyzed by PKC may actually promote relaxation.51 This suggests that another key Ca2+-regulated protein that helps to control contraction52,53 perhaps caldesmon,54,55 is modulated by PKC.

Conclusions

Augmentation of receptor agonist–induced contractions by PDBu in rat aorta cannot be attributed to enhanced receptor activation or to enhanced Ca2+ transients but rather primarily to the increased sensitivity of contractile elements to the available Ca2+ that apparently results from activation of PKC. The seemingly paradoxical inhibition of receptor agonist–activated contractions in SMA raises the possibility that phorbol esters may act preferentially to downregulate the receptor-activated rise in [Ca2+]c in these vessels. The apparent differences in the effects of phorbol esters on agonist-activated tension in different blood vessels7,13,15,17,48,56 (this report) may be explained by the complex interrelations involved in tension development that depend on 1) the fraction of receptors that are activated, 2) the availability of Ca2+ (from the extracellular fluid and the SR stores), and 3) the sensitivity of the contractile apparatus to that Ca2+. PKC may modulate all three of these processes, and the relative effects may be quantitatively different in different blood vessels. Therefore, activation of PKC may lead to augmented receptor agonist–activated contractions in some vessels and depressed receptor agonist–activated contractions in others. The depression of contraction seems to involve multiple agonist-specific receptors (e.g., 5-HT and AVP receptors, as in this study, and norepinephrine receptors, as in the study of Salaices and coworkers56). Therefore, it seems likely that a common factor is involved, perhaps the uncoupling of the receptors from G proteins, but this needs to be tested directly.

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\textit{Shima and Blaustein: Phorbol Esters Modulate 5-HT\textsubscript{2} and AVP-Evoked Tension}


Contrasting effects of phorbol esters on serotonin- and vasopressin-evoked contractions in rat aorta and small mesenteric artery.

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