Phorbol Ester, Vascular Relaxation, and Cyclic Guanosine 3',5'-Monophosphate

WARRILOCKETTE, BRUCE BRENNAMAN, AND YUJI OTSUKA

SUMMARY The relaxation of phenylephrine-contracted blood vessels by acetylcholine, nitroprusside, or atrial natriuretic factor has been linked to elevations in cyclic guanosine 3',5'-monophosphate (cGMP). Also, 8-bromo-cGMP can induce vascular relaxation in isolated vascular smooth muscle contracted with phenylephrine. We determined whether these cGMP-dependent vasodilators could relax isolated rat aortas contracted with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. cGMP was measured by radioimmunoassay. Acetylcholine, nitroprusside, and atrial natriuretic factor induced relaxation in vascular smooth muscle contracted by 12-O-tetradecanoylphorbol-13-acetate. These relaxation responses were accompanied by elevations of cGMP. However, the sensitivity to these vasodilators was markedly decreased in phorbol ester–contracted vessels compared to phenylephrine-contracted vessels. Nifedipine and superoxide dismutase induced small but significant relaxations in phorbol ester–contracted vessels; however, blood vessels contracted with phenylephrine and phorbol ester relaxed completely with papaverine. There was a marked decrease in sensitivity to 8-bromo-cGMP in phorbol ester–treated vessels compared to phenylephrine-contracted vessels. Contractions induced by phorbol ester were not inhibited by amiloride or chlorpromazine. Also, following incubation in potassium-free salt solution, vessels incubated with phenylephrine or phenylephrine and phorbol ester underwent similar relaxations when exposed to potassium chloride. The contractile state induced by phorbol ester has decreased sensitivity to cGMP-dependent vasodilators. This may be due to nonspecific effects of the phorbol ester or to the mechanism by which protein kinase C activation maintains vascular tone. (Hypertension 9 [Suppl III]: III-91-III-95, 1987)

KEY WORDS • protein kinase C • rats • vascular resistance • blood vessels

VASCULAR smooth muscle contraction is regulated by calcium and calmodulin-activated myosin light chain kinase. The degree of myosin light chain phosphorylation rises during isometric contraction; however, the level of phosphorylation may decline while the isometric stress is maintained. This phenomenon is known as the latch state. It has been suggested that this latch state stress is maintained by the activation of protein kinase C by hydrolysis products of phosphatidylinositol.1-3 The relaxation of vascular smooth muscle by agents that stimulate the release of endothelium-derived relaxing factors such as acetylcholine, by nitrososvadis-
MA, USA) that weighed from 250 to 350 g. They were cut helically, attached to isometric force transducers, and bathed with a physiological salt solution of the following composition (in mM): NaCl, 130; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄·7H₂O, 1.17; CaCl₂·2H₂O, 1.6; NaHCO₃, 14.9; dextrose, 5.5; and CaNa₂EDTA, 0.03. The strips were aerated with 95% O₂-5% CO₂, and the bath was maintained at 37°C. A dose-response curve to phenylephrine was constructed for each vessel and, following numerous rinses, the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma), was added in a concentration of either 3.2 × 10⁻⁸ M or 3.2 × 10⁻⁷ M. The vessels developed tension very slowly, and when the contractile force reached a magnitude equivalent to 30% of the maximum phenylephrine-induced contraction (the ED₃₀ value for phenylephrine), either acetylcholine (Miochol, Cooper Vision), sodium nitroprusside (Nipride, Abbott), ANF (rat auriculin B, Peninsula Labs), or 8-Br-cGMP (Sigma) was added in cumulative doses. Since baseline tone could not again be achieved once the phorbol ester was added, only one drug was used with each particular vessel. Relaxation (−), or further tension development (+), was expressed as a percent change in tone from the ED₃₀ value of the average maximal contractile force generated by phenylephrine HCl (Neo-Synephrine, Winthrop).

Similarly treated vessels were also frozen in liquid nitrogen, placed in 6% trichloroacetic acid and extracted. Then cGMP was measured with a commercially available radioimmunoassay.

To elucidate the mechanisms of changes in vascular responsiveness to cGMP-dependent vasodilators in the presence of TPA, nifedipine (10⁻⁷ M), superoxide dismutase (200 units/ml, Sigma), amiloride (10⁻⁶ M, Merck), or chlorpromazine (10⁻⁶ M–10⁻⁴ M, Sigma) was added prior to the addition of TPA or when a TPA-induced contraction developed tone equivalent to the ED₃₀ for phenylephrine.

To determine the role of membrane depolarization induced by inhibition of vascular Na⁺,K⁺-ATPase, vessels were placed in potassium-free physiological salt solution for 15 minutes. A contraction was induced with the addition of either 3.2 × 10⁻⁷ M TPA or 3.2 × 10⁻⁷ M TPA + 10⁻⁶ M phenylephrine. Potassium chloride was then added in cumulative fashion, and the subsequent relaxation response was expressed as a percent of the contractile force generated in response to TPA or TPA plus phenylephrine.

To examine relaxation responses to a non-cGMP-dependent vasodilator, papaverine (Lily) was added cumulatively to vessels contracted to equal magnitudes with either TPA or phenylephrine.

Statistical analysis to compare dose-response curves was done using the Mann-Whitney nonparametric procedure to examine the two groups (phenylephrine vs TPA) for differences in responses at the various dose levels. The Bonferroni method was used for multiple testing; this correction allowed for significance of overall testing at the level of p < 0.05.

Results
At a dose of 3.2 × 10⁻¹ M, TPA induced a maximal contractile force of 920 ± 42 mg (n = 17). Phenylephrine (10⁻⁶ M) induced a maximal contractile force of 893 ± 52 (n = 20, p > 0.05).

Acetylcholine, sodium nitroprusside, and ANF were able to induce dose-dependent relaxations of TPA-contracted vessels, as demonstrated in Figure 1.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Acetylcholine (Ach; A), sodium nitroprusside (SNP; B), and atrial natriuretic factor (ANF; C) were able to induce relaxation in vascular smooth muscle contracted with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). ANF and SNP were significantly more potent than Ach in inducing relaxation in TPA-contracted vessels (n = 5). The changes in tone (+) or (−) are expressed relative to the ED₃₀ value of the maximal contractile response to TPA.
These relaxation responses were accompanied by significant elevations in cGMP (Figure 2).

The relaxation responses to 8-Br-cGMP were markedly attenuated in vessels similarly contracted with TPA or phenylephrine. Although 8-Br-cGMP was eventually able to induce complete relaxation following either treatment, the sensitivity to this agent was significantly shifted to the right in the TPA-treated vessels (Figure 3).

As demonstrated in Table 1, amiloride ($10^{-6}$ M) and chlorpromazine ($10^{-6}$ M) did not cause significant relaxation responses of TPA-contracted vessels. Superoxide dismutase (200 U/ml) did induce a significant relaxation response, and this relaxation was accompanied by a significant increase in cGMP.

Unlike the cGMP-dependent vasodilators, papaverine induced complete relaxation in the TPA- and phenylephrine-treated vessels. There were no differences in sensitivity to papaverine (Figure 4).

To determine whether the TPA inhibited relaxation responses by inhibiting Na⁺,K⁺-ATPase activity, we depolarized isolated blood vessels by placing them in a potassium-free salt solution for 15 minutes. There were no differences in the relaxation response to the subsequent addition of potassium chloride between the phenylephrine-contracted vessels and phenylephrine-contracted vessels that were treated with phorbol ester (Figure 5).

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Acetylcholine (Ach, $10^{-5}$ M), sodium nitroprusside (SNP, $10^{-7}$ M), and atrial natriuretic factor (ANF, $10^{-7}$ M) markedly stimulated cGMP levels in the presence of the phorbol ester TPA.

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Vascular relaxations in response to 8-Br-cGMP were markedly attenuated in vessels treated with the phorbol ester TPA compared to vessels having a comparable level of tone induced with phenylephrine (PE).

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Papaverine caused a dose-dependent decrease in equivalent tone induced by either the phorbol ester TPA or phenylephrine (PE). There were no significant differences between the papaverine-induced relaxation responses of the TPA- and PE-induced contractions.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** Potassium chloride-induced relaxations in phorbol ester (TPA)- and phenylephrine (PE)-treated vessels. The strips were placed in potassium-free salt solution for 15 minutes. Equivalent tone was then generated with PE ($10^{-6}$ M) or PE + TPA ($3.2 \times 10^{-7}$ M). There were no differences in the potassium-induced relaxations, an indicator of Na⁺,K⁺-ATPase activity, between vessels treated with TPA alone or TPA + PE.

**Table 1.** Relaxation Responses and cGMP Levels in TPA-Treated Vessels

<table>
<thead>
<tr>
<th>Agent</th>
<th>Relaxation (%)</th>
<th>cGMP (pmol/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA ($3.2 \times 10^{-7}$ M)</td>
<td>0.0</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>TPA + amiloride</td>
<td>0.0</td>
<td>97 ± 7</td>
</tr>
<tr>
<td>TPA + nifedipine</td>
<td>27.2*</td>
<td>95 ± 6</td>
</tr>
<tr>
<td>TPA + superoxide dismutase</td>
<td>34.7*</td>
<td>242 ± 20*</td>
</tr>
<tr>
<td>TPA + chlorpromazine</td>
<td>0.0</td>
<td>104 ± 10</td>
</tr>
</tbody>
</table>

cGMP values are means ± SE. TPA = 12-O-tetradecanoylphorbol-13-acetate.

Relaxation was induced in TPA-contracted vessels with either amiloride ($10^{-6}$ M), nifedipine ($10^{-7}$ M), superoxide dismutase (200 U/ml), or chlorpromazine ($10^{-6}$ M).

*p < 0.05, n ≥ 5; results analyzed by paired t-test.
Discussion

The phorbol ester TPA is able to induce sustained contractions of isolated rat aortas, and cGMP-dependent vasodilators are able to induce vascular relaxation of these vessels. However, these relaxation responses were considerably less than the relaxation responses to acetylcholine, sodium nitroprusside, and ANF of isolated vessels contracted with phenylephrine. Sodium nitroprusside and ANF (unpublished observations) induce complete relaxation at a concentration of $10^{-7}$ M, and a $10^{-5}$ M concentration of acetylcholine induces nearly an 80% relaxation of vessels contracted to a much greater degree with phenylephrine (i.e., contraction with an ED$_{50}$ dose of phenylephrine). The cGMP generated in response to these vasodilators was significantly less than the cGMP generated in response to the same concentration of vasodilators in phenylephrine-contracted vessels (unpublished observations.) Vascular relaxation responses of the TPA-contracted vessels were much less than relaxation responses of the phenylephrine-treated vessels when similar levels of cGMP were induced by increasing the concentration of the vasodilators.

The mechanism by which cGMP induced vascular relaxation is not known. It has been suggested that cGMP-dependent vasodilators may alter calcium flux and that this may result in dephosphorylation of proteins involved in vascular smooth muscle contraction. It has also been suggested that cGMP may interfere with the hydrolysis of phosphatidylinositol following activation of $\alpha$-adrenergic receptors. Our data suggest that cGMP may induce relaxation at a step in the contraction cascade prior to the activation of protein kinase C.

Nifedipine caused only a slight relaxation in TPA-treated vessels; when added prior to TPA, the contractile responses to TPA were attenuated. This observation was surprising, since TPA did not appear to alter relaxation responses to potassium chloride following incubation in potassium-free salt solution. This procedure has been shown to inhibit Na$^+$-K$^+$-ATPase, and inhibition of this enzyme may result in depolarization of vascular smooth muscle. It has been previously reported that calcium channel antagonists may inhibit phorbol ester-induced vascular contractions. It is possible that TPA results in phosphorylation of potential-operated calcium channels, and this may result in calcium channel activation without a membrane depolarization. Alternatively, it is possible that phenylephrine, in the concentration used, maximally activates protein kinase C, and the addition of an exogenous protein kinase C activator may not be able to induce any further depolarization.

Superoxide dismutase induced a significant relaxation of TPA-contracted vessels. TPA has been shown to produce superoxide anion in endothelial cells, and superoxide anion has been demonstrated to inactivate endothelium-derived relaxing factors. It is possible that scavenging of superoxide anion by superoxide dismutase results in the protection of the basal production of endothelium-derived vascular relaxing factor.

Alternatively, superoxide dismutase has also been shown to stimulate guanylate cyclase directly. Amiloride had no consistent effect on TPA-induced contractions. TPA has previously been demonstrated to stimulate Na$^+$-H$^+$ exchange in cultured vascular smooth muscle cells. This suggests that the TPA-induced contractions are not directly dependent on the Na$^+$-H$^+$ exchange; the exact role for this exchange mechanism in the control of vascular tone remains to be determined.

To determine the role of calmodulin-dependent mechanisms in the contractile response of TPA, relaxation responses to chlorpromazine at a concentration known to inhibit calmodulin were studied; no significant relaxation was induced by $10^{-6}$ M of chlorpromazine. The relative inability of chlorpromazine to inhibit the TPA-induced contractions suggests that TPA-induced contractions may be calmodulin-independent.

The role for cGMP in vascular relaxation remains obscure; however, it appears that cGMP is less able to induce relaxation in phorbol ester--treated vessels than in blood vessels treated with other vasoconstricting agents. We were also unable to induce relaxation with acetylcholine in vessels contracted with mezerein, $10^{-7}$ M, another activator of protein kinase C ($n = 4$, data not shown). It has been demonstrated that different activators of protein kinase C have different effects on isolated blood vessels. For example, it is reported that TPA and phorbol dibutyrate differ in their requirement for extracellular calcium, and different vascular beds may respond differently to the same phorbol ester activators of protein kinase C.

The TPA-treated blood vessels do not appear to be incapable of relaxation; papaverine-induced relaxations did not differ between the phorbol ester--treated vessels and the phenylephrine-contracted vessels. It has also been reported previously that forskolin, an agent that raises cyclic adenosine 3',5'-monophosphate levels, is also able to relax phorbol ester--treated blood vessels. It is also possible that acetylcholine, sodium nitroprusside, ANF, and superoxide dismutase are able to induce vascular relaxation through non-cGMP-dependent pathways. For example, we have found that sodium nitroprusside is able to relax phenylephrine-contracted rat aortas when guanylate cyclase is inhibited with methylene blue (unpublished observations). It remains to be determined what role the non-cGMP-dependent mechanisms of vascular relaxation induced by sodium nitroprusside and ANF play in attenuating TPA-induced contraction.

Since not all vasodilators are able to induce relaxation of vessels contracted with activators of protein kinase C, it is significant that vasodilators linked to elevations of cGMP are able to induce relaxation of isolated rat aortas contracted with TPA. The mechanisms by which vascular relaxation induced by agents that have been linked to elevations in cGMP are attenuated in TPA-induced contractions in isolated rat aorta is unknown. Perhaps this is due to the potency of TPA, which exceeds that of endogenous, physiological protein kinase activators in vascular smooth muscle.
Another possibility is that cGMP may be more important in regulating other cellular elements of vascular contraction, such as calcium flux or calmodulin activity. An elucidation of these pathways may play a role in clinical situations marked by the refractory contraction of blood vessels and increased vascular resistance, such as hypertension or cerebral vasospasm following subarachnoid hemorrhage.

References

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