Phorbol ester effects on neurotransmission: Interaction with neurotransmitters and calcium in smooth muscle

(acetylcholine/bradykinin/histamine/protein kinase C/serotonin)

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ABSTRACT Stimulation of the phosphatidylinositol cycle by neurotransmitters generates diacylglycerol, an activator of protein kinase C, which may regulate some forms of neurotransmission. Phorbol esters, potent inflammatory and tumor-promoting compounds, also activate protein kinase C. We demonstrate potent and selective effects of phorbol esters on smooth muscle, indicating a role for protein kinase C in neurotransmission. In rat vas deferens and dog basilar artery, phorbol esters synergize with calcium to mimic the contractile effects of neurotransmitters that act through the phosphatidylinositol cycle. In guinea pig ileum and rat uterus, phorbol esters block contractions produced by these neurotransmitters.

Stimulation by many neurotransmitters of the phosphatidylinositol (PtdIns) cycle may mediate their influence on cellular activity (1, 2). One of the products of the PtdIns cycle is diacylglycerol, which stimulates protein kinase C (3, 4), a calcium- and phospholipid-dependent phosphorylating enzyme (5, 6). Thus, protein kinase C may mediate physiological effects of the neurotransmitters that stimulate PtdIns turnover. However, no direct evidence has yet been provided for a role of protein kinase C in neurotransmission.

Phorbol esters, a class of very potent inflammatory and tumor-promoting compounds, bind to receptors (7) in numerous tissues (8) and activate protein kinase C (9–13). Since phorbol esters and diacylglycerol stimulate protein kinase C by acting at the same site (14), we have used phorbol esters to mimic endogenously produced diacylglycerol in isolated smooth muscle preparations to investigate the role of protein kinase C in neurotransmission. In some smooth muscles, phorbol esters mimic the contractile action of neurotransmitters that stimulate PtdIns turnover, whereas in others they potently and selectively block the actions of these neurotransmitters.

MATERIALS AND METHODS

Two- to 3-cm strips of guinea pig ileum, rat vas deferens, or rat uterus were isolated, as described (15). They were suspended with a resting tension of ≈0.5 g in aerated Tyrode's buffer maintained at 32°C. Muscle tension was recorded isometrically. For some experiments with ileum and vas deferens, a depolarizing Tyrode's buffer was used, which contained 80 mM KCl and 75 mM NaCl.

Female rats were pretreated with 25 μg of estradiol benzoate (Progynon, Schering) subcutaneously on the day prior to sacrifice. For uterine preparations, a modified Tyrode's buffer containing 0.5 mM $CaCl_2$ was used to avoid spontaneous contractions. Segments of dog basilar artery were mounted on parallel prongs in Mops [3-(N-morpholino)propanesulfonic acid] buffer as described (16). In some experiments where indicated, $CaCl_2$ was deleted from the standard Mops buffer.

The K_i values for phorbol ester analogues in the guinea pig ileum were obtained by testing a contractile agent before and 2–3 min after bath application of the phorbol ester. The dose ratio was estimated from these responses and the K_i was calculated from the relationship $K_i =$ [phorbol ester]/(dose ratio -1). ED_{50} values for a series of phorbol ester analogues in the rat vas deferens were determined from cumulative dose–response curves obtained in the presence of depolarizing Tyrode's buffer following bath application of 1 mM $CaCl_2$.

Specific binding of [³H]phorbol 12,13-dibutyrate (PBt_2) in rat vas deferens was determined by a modification of the method of Driedger and Blumberg (7). Vasa deferentia were dissected from rats and stored at $-20°C$ until assayed. Tissue was homogenized with a Brinkmann Polytron in 100 vol of buffer containing 50 mM Tris·HCl at pH 7.7 and 1 mM $CaCl_2$ and then centrifuged at $50,000 \times g$ for 10 min. This procedure was repeated with the pellet and then membranes were diluted to a concentration of 2–3 mg/ml. Incubations were carried out at room temperature for 30 min in a volume of 0.55 ml containing 1–1.5 mg of tissue and 6–8 nM [³H]PBt_2. Nonspecific binding was assessed in the presence of 5 μM PBt_2. Incubations were stopped by rapid filtration over Schleicher & Schuell no. 32 glass fiber filters presoaked in 0.5% polyethyleneimine. Membranes were washed with 15 ml of ice-cold 50 mM NaCl and radioactivity retained by the filters was measured.

Phorbol ester analogues were obtained from LC Services (Woburn, MA), and [³H]PBt_2 was from New England Nuclear. Other drugs were obtained from standard commercial sources.

RESULTS

Guinea Pig Ileum. Oxotremorine, a muscarinic cholinergic agonist that stimulates PtdIns turnover (1), contracts the longitudinal muscle of the guinea pig ileum (17). A potent phorbol ester, PBt_2 (20–100 nM), did not induce ileal contractions but rapidly and reversibly blocked oxotremorine-induced contractions (Fig. 1) with a K_i of 31 nM (Table 1). 12-Deoxyphorbol 13-isobutyrate (DPB), another phorbol ester, which has slightly less affinity than PBt_2 for phorbol ester receptors (18), also blocked the effects of oxotremorine (Fig. 1). To determine whether these effects involve pharmacologically relevant phorbol ester receptors, we examined a series of phorbol esters (Table 1). The relative potencies of six

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Abbreviations: PtdIns, phosphatidylinositol; PBt_2, phorbol 12,13-dibutyrate; DPB, 12-deoxyphorbol 13-isobutyrate; PAc_2, phorbol 12,13-diacetate.
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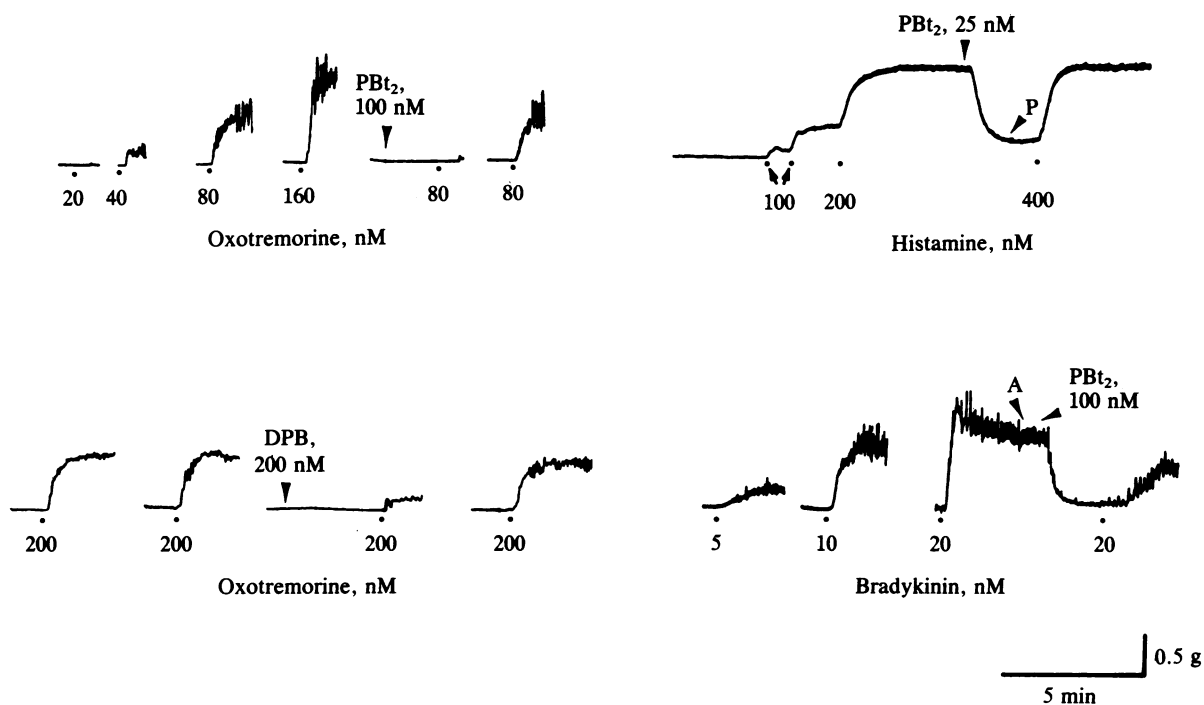‖To whom reprint requests should be addressed.

FIG. 1. Phorbol ester blockade of neurotransmitter-induced contractions of guinea pig ileum. In the top left panel, PBt$_2$ (100 nM) did not contract the guinea pig ileum strip. However, within 2 min of bath application it completely blocked contractile effects of oxotremorine (80 nM). Following a brief wash this action of PBt$_2$ was completely reversed. The bottom left panel illustrates a similar effect of DPB (200 nM). DPB rapidly and reversibly blocked oxotremorine's action. In the top right panel, a contraction was elicited by increasing doses of histamine. PBt$_2$ potently reversed this contraction. Propranolol (2 μM) (P) did not alter PBt$_2$'s inhibition, but addition of another 400 nM of histamine overcame PBt$_2$'s blockade. The bottom right panel illustrates the contractile action of bradykinin. Atropine (1 μM) (A) did not block bradykinin's effect but PBt$_2$ (100 nM) exerted a powerful inhibition that was partially reversed by 20 nM bradykinin. Data depicted in Figs. 1–3 are from typical experiments that were replicated at least three times.

phorbol derivatives closely parallel their affinity for receptors measured by the binding of [^{3}H]PBt$_2$.

Histamine and bradykinin, which contract ileal smooth muscle by a direct action (17), also stimulate PtdIns turnover (1, 19). PBt$_2$ blocked their contractile effects much as it affected oxotremorine contractions (Fig. 1). The prevention of oxotremorine, histamine, and bradykinin contractions by PBt$_2$ was completely reversible with washing or the addition of more of the contractile agent. Serotonin contracts ileal smooth muscle both directly and indirectly via release of acetylcholine from intrinsic nerves (20). PBt$_2$ also blocked serotonin-induced contractions in the presence of 1 μM atro-

pine, which would prevent serotonin actions involving secondary release of acetylcholine.

To ascertain whether the phorbol esters block these four contractile substances via the same mechanism, we examined the potency of PBt$_2$ in influencing contractions induced by oxotremorine, histamine, serotonin, and bradykinin. The K_i for PBt$_2$'s effect is similar for all four agents (Table 2).

We wondered whether the influence of phorbol esters is exerted directly upon receptor-linked events or whether it acts at voltage-dependent ion channels, the contractile machinery of the muscle itself, or other sites. Accordingly, we examined ileal contractions elicited by potassium depolarization (Table 2). PBt$_2$ at concentrations up to 200 nM had no effect upon KCl-induced contractions.

Is the blockade exerted by phorbol esters selective for those contractile agents that stimulate PtdIns turnover? We

Table 1. Comparison of phorbol ester analogues in smooth muscle systems and receptor binding

Drug	K_i for inhibition of guinea pig ileum contractions, nM	ED$_{50}$ for contraction of rat vas deferens, nM	K_i for inhibition of [^{3}H]PBt$_2$ binding in rat vas deferens, nM
PBt$_2$	31 ± 4	16 ± 5	36 ± 6
DPB	78 ± 19	25 ± 6	180 ± 20
PAc$_2$	470 ± 160	140 ± 40	1,100 ± 100
P 13-Ac	20,000 ± 4000	6,000 ± 2100	22,000 ± 3000
P 13,20-Ac$_2$	>25,000	>25,000	>25,000
Phorbol	>25,000	>25,000	>25,000

K_i values in guinea pig ileum were determined for blockade of oxotremorine-induced contractions. ED$_{50}$ values in the rat vas deferens were obtained from strips immersed in depolarizing Tyrode's buffer following addition of 1 mM CaCl$_2$. P 13-Ac, phorbol 13-acetate; P 13,20-Ac$_2$, phorbol 13,20-diacetate. Data are mean values of three to six determinations with standard errors of the mean.

Table 2. Selectivity of PBt$_2$ blockade in guinea pig ileum

Stimulus	K_i for inhibitory potency of PBt$_2$, nM
Oxotremorine	31 ± 4
Histamine	23 ± 5
Serotonin	25 ± 5
Bradykinin	29 ± 5
KCl	>200
Isoproterenol	>200

KCl stimulation of guinea pig ileum was performed by addition of increasing doses of CaCl$_2$ to strips bathed in depolarizing buffer. Isoproterenol (1–5 μM) partially relaxed strips of guinea pig ileum stimulated by 1 mM CaCl$_2$ in depolarizing Tyrode's solution. PBt$_2$ (200 nM) did not alter the responses to stimulation by KCl or isoproterenol. Data are mean values of three to six determinations with standard errors of the mean.

are not aware of any transmitter substances that directly contract ileal muscle and do not also stimulate PtdIns turnover. However, catecholamines relax the guinea pig ileum though adenylate cyclase systems rather than the PtdIns cycle (17). PBt_2, up to 200 nM, failed to affect isoproterenol (1–5 μM)-induced relaxation of the guinea pig ileum (Table 2).

The apparent blockade by phorbol esters of contractions might reflect relaxing effects caused by release of endogenous catecholamines acting through β-adrenergic receptors. Accordingly, we examined the effect of the β-adrenergic antagonist propranolol upon the action of PBt_2. The blockade by PBt_2 of oxotremorine or histamine contractions (Fig. 1) was not affected by propranolol (2 μM).

Rat Uterus. To determine if the phorbol ester effects observed in guinea pig ileum reflect some idiosyncratic property of that tissue, we evaluated contractile responses of the estrous rat uterus (Fig. 2). Uterine muscle contracts in response to oxotremorine, bradykinin, and serotonin (17). The contractile actions of all three agents were reversibly blocked by PBt_2 (25–100 nM) and another phorbol ester, phorbol 12,13-diacetate (PAc_2) (1–2 μM). As had been found in the guinea pig ileum, PBt_2 (100 nM) failed to alter contractions produced by potassium depolarization (Fig. 2).

Rat Vas Deferens. In the rat vas deferens, phorbol esters facilitated contractile activity. Alone, PBt_2 (200 nM) or PAc_2 (2 μM) did not elicit contraction in physiological buffer. However, they enhanced nonrepinephrine-induced contractions (Fig. 3). To determine if this action was selective for transmitter-induced stimulation, we examined phorbol ester effects on contractions elicited by KCl depolarization. In vas deferens strips exposed to depolarizing Tyrode's buffer without added calcium, PBt_2 and PAc_2 were inactive. However, in the presence of a low concentration of calcium (1 mM), which produces only a partial contraction of the depolarized vas deferens, PBt_2 and PAc_2 (Fig. 3) elicited further contractions.

The relative potencies of various phorbol esters in enhancing depolarization-induced contractions closely paralleled their affinity for phorbol ester receptor binding sites in the vas deferens (Table 1). Moreover, the absolute potencies in contracting the vas deferens were even greater than their potencies in the guinea pig ileum.

Dog Basilar Artery. The pattern of phorbol ester effects in the dog basilar artery differed from that seen in the vas deferens, ileum, or uterus. In the absence of added extracellular calcium in a nondepolarizing buffer, PBt_2 contracted the
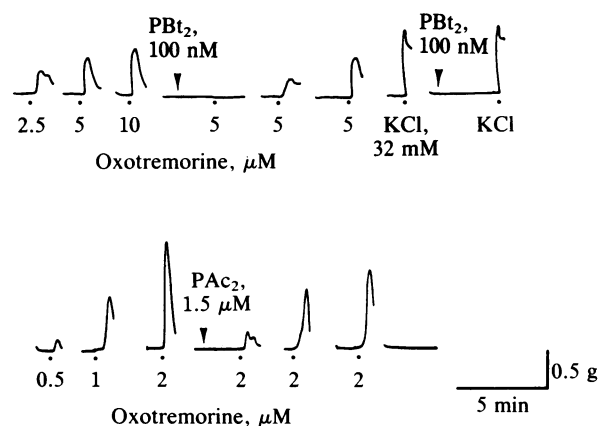

FIG. 2. Inhibition of oxotremorine-induced contraction of rat uterus. In the record presented in the top panel, PBt_2 (100 nM) did not alter baseline muscle tension but completely prevented the effect of oxotremorine (5 μM). In contrast, the same dose of PBt_2 did not influence uterine contractions initiated by bath application of 32 mM KCl. Similar effects of another phorbol ester, PAc_2, are illustrated in the lower trace.
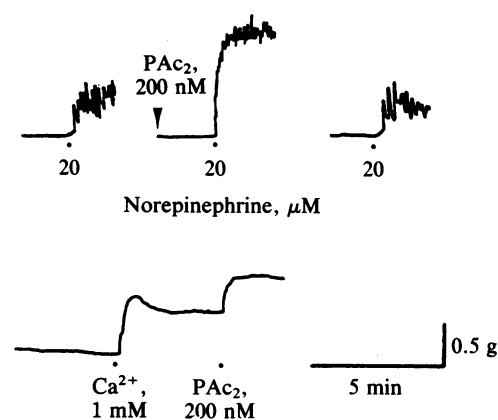

FIG. 3. Facilitation of contractile action by phorbol ester in rat vas deferens. In the top trace, PAc_2 (200 nM) was without effect when administered alone. However, it facilitated the contractile response to norepinephrine (20 μM). In the bottom trace, PAc_2 enhanced the contraction induced by 1 mM $CaCl_2$ in tissue bathed in depolarizing Tyrode's buffer

basilar artery in 2–4 min with an ED_{50} of 24 nM. When added to tissue bathed in standard calcium-containing buffer, PBt_2 produced two to three times the contraction observed in the absence of added extracellular calcium.

DISCUSSION

Our results establish that protein kinase C is involved in neurotransmission in smooth muscle. The most direct evidence comes from studies in the ileum and uterus where contractile effects of several neurotransmitters are blocked by phorbol esters. The close correlation between the potencies of phorbol esters in blocking neurotransmitter action and their affinity for phorbol ester receptors strongly supports the conclusion that these effects of phorbol esters are mediated by specific phorbol ester receptors—i.e., protein kinase C. Recently, Dunn and Blumberg (18) differentiated between two phorbol ester receptor binding sites, one associated with tumor promotion and the other with inflammatory effects of the phorbol esters. The relative potencies of phorbol esters in both guinea pig ileum and rat vas deferens correlate better with their affinity at the "inflammatory" phorbol ester receptor site.

The contractile effects of phorbol esters in vas deferens and basilar artery suggest that protein kinase C also mediates physiological responses in these tissues. The enhancement by phorbol esters of calcium-induced contractions in the vas deferens fits with observations of Nishizuka and colleagues that phorbol esters increase the affinity of calcium for protein kinase C (3, 4, 21). Since norepinephrine's action in the vas deferens involves an increase in intracellular calcium (22), the facilitation of norepinephrine effects by phorbol esters may reflect a synergistic interaction of phorbol esters and calcium on protein kinase C activation. The phorbol ester-elicited contractions of dog basilar artery are also consistent with a synergism between phorbol esters and calcium on protein kinase C activation. According to this model, the interaction of phorbol esters with available intracellular calcium leads to protein kinase C activation, which produces the observed contraction.

Augmented PtdIns turnover elicited by neurotransmitters generates diacylglycerol, which, in turn, activates protein kinase C. If phorbol esters mimic diacylglycerol, one would expect them to produce similar effects to those elicited by contractile agents, as observed in the basilar artery and vas deferens. In contrast, phorbol esters block the contractile effects of neurotransmitters in the ileum and uterus. What might account for this discrepancy? Activation of the PtdIns

cycle by neurotransmitters stimulates the breakdown of phosphatidylinositol-4,5-bisphosphate (PtdIns-P_2) leading to the formation of diacylglycerol and inositol triphosphate (2). Inositol triphosphate releases calcium from intracellular stores (23, 24), which may also mediate the action of neurotransmitters that stimulate PtdIns turnover. In several tissues, diacylglycerol and inositol triphosphate appear to act in a complementary fashion to produce cellular responses (2). Recent evidence indicates that a feedback mechanism regulates the PtdIns cycle (25). Activation of protein kinase C inhibits the formation of PtdIns-P_2 (26–28). Through such a feedback system, stimulation of protein kinase C by phorbol esters may inhibit the synthesis of PtdIns-P_2. Conceivably, in the ileum and uterus phorbol esters act preferentially via this feedback mechanism to block the action of neurotransmitters, whereas in the basilar artery and vas deferens they synergize with calcium in producing the physiological response.

The central nervous system contains extremely high concentrations of protein kinase C (14). Recently, the neurophysiological effects of phorbol esters have been examined in the hippocampal splice preparation. Intracellular recordings from hippocampal pyramidal neurons have demonstrated that phorbol esters potently affect neuronal excitability (unpublished data). These findings suggest that protein kinase C is directly involved in the regulation of neuronal activity.

Phorbol esters may be valuable probes for exploring the involvement of protein kinase C and the PtdIns cycle in a wide variety of physiological systems. The use of smooth muscle systems may facilitate a search for antagonists of phorbol ester receptors.

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