A Role for Intracellular Histamine in Ultrastructural Changes Induced in Platelets by Phorbol Esters

Archibald McNicol, Satya P. Saxena, Lorne J. Brandes, and Jon M. Gerrard

In human platelets, phorbol esters, such as phorbol-12-myristate-13-acetate (PMA), induce morphological changes, including pseudopod formation and the swelling and fusion of intracellular granule membranes with those of the surface-connected canalicular system, effects which have been attributed to activation of protein kinase C. However, a novel intracellular histamine antagonist, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine.HCl (DPPE), previously has been shown to block PMA-induced aggregation independently of protein kinase C interaction, an effect reversible in permeabilized platelets by the addition of histamine. We now demonstrate that DPPE inhibits, in a concentration-dependent manner, the effects of PMA on human platelet ultrastructure. In permeabilized platelets, histamine reverses this inhibition, although it alone induces minimal effects on morphology. The results support a role for this amine to promote the labilization of platelet granules and pseudopod formation induced by PMA, presumably by acting in concert with additional PMA-activated pathways.


Phorbol esters, such as phorbol-12-myristate-13-acetate (PMA), have pronounced effects on a variety of cells.1–4 In platelets, PMA promotes the swelling and fusion of intracellular granules, the secretion of granular contents, the extension of small pseudopods, and aggregation,4 effects believed to be mediated by the activation of protein kinase C and the resultant protein phosphorylation.6 PMA also activates histidine decarboxylase (HDC), the enzyme responsible for histamine synthesis.6,7

Recently, we have shown that in human platelets, PMA-induced aggregation is accompanied by the formation of intracellular histamine. Both aggregation and histamine formation are attenuated in parallel by the HDC inhibitors, α-methylhistidine and α-fluoromethylhistidine.6 Furthermore, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine.HCl (DPPE), a histamine antagonist at a novel intracellular site,6 blocks PMA-induced platelet aggregation but not histamine production.8,10 In permeabilized, but not intact, platelets the inhibitory effects of DPPE and of HDC inhibitors are reversed by histamine at concentrations consistent with those synthesized internally,6 providing strong evidence that newly formed histamine modulates platelet activation.

While its precise function remains to be elucidated, recent reports that platelets from patients with peripheral vascular disease have elevated levels of intracellular histamine11 may suggest a novel mechanism for the enhanced responsiveness of platelets from such subjects. Thus, an improved understanding of the role of histamine in platelet physiology may be of great clinical importance.

In the present study, we have probed the mechanisms by which intracellular histamine acts in platelets by examining the effect of DPPE on PMA-induced ultrastructural changes, and those of histamine, subsequent to DPPE inhibition.

Methods

Materials

DPPE was synthesized as previously described, and its chemical structure was verified by nuclear magnetic resonance and mass spectroscopy.12 PMA, histamine, saponin, and inositol 1,4,5-trisphosphate were purchased from Sigma (St. Louis, MO). Electron microscope supplies were obtained from J.B.E.M. Supplies (St. Laurent, Montreal, Quebec). All other reagents were of the highest purity available.

Preparation of Platelets

Whole blood from human donors was collected, after obtaining informed consent, into citrate anticoagulant (38 mM citric acid, 75 mM trisodium citrate, 125 mM dextrose; 1.9 ml anticoagulant per 8.1 ml whole blood) and centrifuged at 800 g for 5 minutes at room temperature. Platelet-rich plasma (PRP) was collected, citrate anticoagulant (1.9 ml anticoagulant per 8.1 ml PRP) was added, and this was centrifuged at 800 g for 11 minutes at room temperature. The pellets were resuspended at a concentration of 2.5×10⁵ platelets per ml in a modified Hanks' balanced salt solution, as previously described.13

Preparation of Permeabilized Platelets

PRP was prepared from whole blood as above, and platelets were permeabilized using saponin as described by Authi and colleagues.14 The efficiency of permeabil-
HISTAMINE PROMOTES PLATELET CHANGES

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A

B

C

D

E

F
Electron Microscopy

Washed or permeabilized platelets were incubated with combinations of DPPE, PMA and histamine, or the relevant controls, for the times indicated. The platelets were fixed by the addition of an equal volume of 0.1% glutaraldehyde in White's saline, were subsequently postfixed by 3% glutaraldehyde and 1% osmium tetroxide, and were stained with 3% uranyl acetate as previously described. After dehydration by ethanol, the samples were embedded in L.R. White, hard grade, reagent. Thin sections were poststained with 0.03% lead acetate (P). Small pseudopods and the platelets had retained their discoid shape, extension of small pseudopods (P), and formation of swollen vacuoles (V) were still evident, x 10600. F. 100 /uM DPPE (30 seconds) failed to elicit any structural changes in human platelets. The addition of PMA to platelets produced a concentration-dependent extension of pseudopods and formation still occurred (data not shown). At higher concentrations of DPPE (50 and 75 /uM), there was a substantial granule swelling and fusion and pseudopod formation which occurred in response to PMA (Figure 1C) or granule size (Table 1). Although in some platelets, concentrations of DPPE (50 and 75 /uM), there was a substantial inhibition of vacuole formation and of pseudopod formation, although both were present to some degree (Figure 1E and Table 1). Concentrations of 100, 150, and 200 /uM DPPE virtually abolished the ultrastructural changes observed when platelets were incubated with PMA (Figure 1F and Table 1). The platelets had smooth exteriors without pseudopods; however, they were more rounded than those that had been exposed to neither DPPE nor PMA.

Results

Influence of DPPE on Platelet Ultrastructure

At concentrations ranging from 10 /uM to 100 /uM, DPPE failed to elicit any structural changes in human platelets (Figures 1A and 1B) or any alteration in the size of intracellular granules (Table 1). Although in some platelets, 200 /uM DPPE caused a modest centralization of cytosolic organelles, in most cases there was no change in the ultrastructure (Figure 1C) or granule size (Table 1).

Influence of Phorbol Acetate on Platelet Ultrastructure

The addition of PMA to platelets produced a concentration-dependent extension of pseudopods and the formation of vacuoles resulting from granule swelling and the fusion of granule membranes with those of the surface-connected canalicular system. A concentration of 30 nM PMA appeared optimal to cause ultrastructural changes (Figure 1D and Table 1) and was employed for subsequent studies on the influence of DPPE.

Influence of DPPE on Morphologic Changes Produced by Phorbol Myristate Acetate

DPPE pretreatment (30 seconds) produced a concentration-dependent inhibition of the granule fusion and swelling and the pseudopod formation seen in response to PMA. The PMA effects were only partially inhibited by preincubation with 10 or 25 /uM DPPE. In both cases, substantial granule swelling and fusion and pseudopod formation still occurred (data not shown). At higher concentrations of DPPE (50 and 75 /uM), there was a more substantial inhibition of vacuole formation and of pseudopod formation, although both were present to some degree (Figure 1E and Table 1). Concentrations of 100, 150, and 200 /uM DPPE virtually abolished the ultrastructural changes observed when platelets were incubated with PMA (Figure 1F and Table 1). The platelets had smooth exteriors without pseudopods; however, they were more rounded than those that had been exposed to neither DPPE nor PMA.

Reversal of Inhibitory Effect of DPPE by Histamine

Platelets are relatively impermeable to exogenously applied histamine. Therefore, to study the ability of histamine to reverse the inhibitory effects of DPPE on PMA-induced morphological changes, it was necessary to permeabilize the platelet membrane. The cytosol of permeabilized platelets was noticeably paler than that of intact ones; however, such platelets retained their intracellular organelles (Figure 2A) and functional viability. In permeabilized platelets, DPPE (125 /uM) per se had no effect on morphology (Figure 2B); however, preincubation with DPPE markedly attenuated the effects of 200 nM PMA (Figures 2C, 2D, and Table 2). When histamine (1 to 4 /uM) was co-added with PMA, the inhibitory effects of DPPE pretreatment were significantly

<table>
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<tr>
<th>Table 1. Influence of DPPE on PMA-Induced Formation of Large Vesicles in Intact Platelets</th>
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<tr>
<td>Percent of platelet constituted by surface-connected canalicular system and large vesicles</td>
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<tr>
<td>Control</td>
</tr>
<tr>
<td>30 nM PMA</td>
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<tr>
<td>50 /uM DPPE</td>
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<td>200 /uM DPPE</td>
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<td>50 /uM DPPE + 30 nM PMA</td>
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<td>100 /uM DPPE + 30 nM PMA</td>
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*p<0.001 with respect to 30 nM PMA. PMA=phorbol-12-myristate-13-acetate, DPPE=N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine.HCl. |
reversed (Figure 2E and Table 2). Large vacuoles were again evident. There was, however, very little effect of higher concentrations of histamine (40 or 400 μM).

Influence of Histamine on Platelet Morphology

Histamine (4 μM) elicited a small, but significant, effect on the morphology of permeabilized platelets (Figure 2F). There was some evidence for the fusion and/or swelling of intracellular granules (Table 2). However, the magnitude of these changes was so small that the significance is questionable.

Discussion

While the morphological effects of PMA on human platelets are mediated by protein kinase C, this study also indicates a critical role for histamine. DPPE inhibited the morphological changes induced by the action of PMA to both intact and permeabilized platelets, and this was reversed in the latter by histamine at a concentration similar to that produced by platelets in response to PMA.

The observation that high concentrations of DPPE (200 μM) caused modest granular centralization while abolishing PMA-induced aggregation suggests an additional effect on calcium, as this morphological effect is normally associated with the calcium-mediated activation of platelets. We have observed that high concentrations of DPPE do cause a rise in the cytosolic free calcium levels of platelets as monitored by Fura-2 (McNicol and Gerrard, unpublished data). However, the major anti-aggregatory effects of DPPE occur at significantly lower concentrations where no effect on cytoplasmic calcium levels is observed. In addition, previous studies have shown that agents that raise cytoplasmic calcium enhance, rather than inhibit, aggregation and secretion stimulated by PMA. Thus, the data are most consistent with DPPE acting to inhibit PMA-induced platelet aggregation and granule lysis by antagonizing intracellular histamine at some point distal to, or independent of, protein kinase C-mediated protein phosphorylation.

One possibility is that protein kinase C phosphorylates, and thereby activates, the HDC protein, resulting in an increased synthesis of intracellular histamine. However, as histamine alone is unable to mimic the effects of PMA, HDC activity (although essential) cannot be the only mediator of protein kinase C activity. It is also possible that HDC activation occurs independently of protein kinase C and that raised cytosol histamine then acts cooperatively with a protein kinase C-activated pathway to initiate platelet aggregation and granule fusion. In either case, it would appear that there are several parallel, intracellular events required for PMA-mediated platelet activation.

Finally, based on this and our previous reports, we wish to speculate that a potential explanation for the observed elevated platelet histamine levels in patients with peripheral vascular disease is a primary or secondary increase in its synthesis, or, perhaps, an aberrancy in its catabolism, resulting in a sensitizing action on platelets with consequent hyperaggregability.

Acknowledgments

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References


Index Terms: platelets • protein kinase C • histamine • granule fusion • morphology

Correction

The authors of the article, Increased Lipid Transfer Activities in Hyperlipidemic Rabbit Plasma by Son Y-CS and Zilversmit DB (Arteriosclerosis 6:345–351, May/June 1986) would like to correct the formula appearing on page 347. It should read as follows:

K, t = \ln \left( 1 - \frac{\text{HDL}}{\text{LDL}} \right) (1+c)/(1+c)

(5)
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