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. (Arteriosclerosis 9:684–689, September/October 1989)

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

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. Furthermore, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine.HCl (DPPE), a histamine antagonist at a novel intracellular site, blocks PMA-induced platelet aggregation but not histamine production. 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, 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 histamine 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. 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 by histamine at concentrations consistent with those synthesized internally, 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 histamine 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.

**Preparation of Permeabilized Platelets**

PRP was prepared from whole blood as above, and platelets were permeabilized using saponin as described by Authi and colleagues. The efficiency of permeabil-
zation was determined by monitoring the ability of inositol 1,4,5-trisphosphate to initiate platelet aggregation in a stirred sample.\(^1\),\(^2\)

**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.\(^3\) After dehydration by ethanol, the samples were embedded in L.R. White, hard grade, reagent. Thin sections were poststained with 0.03% lead acetate as previously described. Thin sections were poststained with 0.03% lead acetate and examined with a Philips EM400 electron microscope. The percentage of platelet cross-sections constituted by swollen and/or fused granules was determined by computer-aided analysis using a Kontron Image Analysis system.

**Results**

**Influence of DPPE on Platelet Ultrastructure**

At concentrations ranging from 10 \(\mu\)M to 100 \(\mu\)M, 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 \(\mu\)M 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.\(^4\),\(^5\) 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 \(\mu\)M 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 \(\mu\)M), there was a more substantial inhibition of vacuole formation and pseudopod formation, although both were present to some degree (Figure 1E and Table 1). Concentrations of 100, 150, and 200 \(\mu\)M 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.\(^6\) 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 \(\mu\)M) 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 \(\mu\)M) was co-added with PMA, the inhibitory effects of DPPE pretreatment were significantly

---

**Table 1. Influence of DPPE on PMA-Induced Formation of Large Vesicles In Intact Platelets**

| Condition                        | Percent of platelet constituted by surface-connected canalicular system and large vesicles |
|----------------------------------|------------------------------------------------------------------------------------------|
| Control                          | 1.72±0.28                                                                                 |
| 30 nM PMA                        | 29.30±1.92                                                                                 |
| 50 \(\mu\)M DPPE                 | 3.66±0.85                                                                                 |
| 200 \(\mu\)M DPPE                | 1.67±0.65                                                                                 |
| 50 \(\mu\)M DPPE + 30 nM PMA      | 11.88±1.93*                                                                               |
| 100 \(\mu\)M DPPE + 30 nM PMA     | 3.20±0.77*                                                                                |
| 200 \(\mu\)M DPPE + 30 nM PMA     | 3.55±0.72*                                                                                |

\(^*p<0.001\) with respect to 30 nM PMA.

PMA = phorbol-12-myristate-13-acetate, DPPE = N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine.HCl.

---

\(^1\) In some platelets, centralization of intracellular organelles, in most cases there was no change in the ultrastructure (Figure 1C) or granule size (Table 1).

\(^2\) After dehydration by ethanol, the samples were embedded in L.R. White, hard grade, reagent. Thin sections were poststained with 0.03% lead acetate and examined with a Philips EM400 electron microscope. The percentage of platelet cross-sections constituted by swollen and/or fused granules was determined by computer-aided analysis using a Kontron Image Analysis system.

\(^3\) The PMA effects were only partially inhibited by preincubation with 10 or 25 \(\mu\)M 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 \(\mu\)M), there was a more substantial inhibition of vacuole formation and pseudopod formation, although both were present to some degree (Figure 1E and Table 1). Concentrations of 100, 150, and 200 \(\mu\)M 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.

\(^4\) The 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 \(\mu\)M) 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 \(\mu\)M) was co-added with PMA, the inhibitory effects of DPPE pretreatment were significantly

---

\(^5\) In some platelets, centralization of intracellular organelles, in most cases there was no change in the ultrastructure (Figure 1C) or granule size (Table 1).

\(^6\) The PMA effects were only partially inhibited by preincubation with 10 or 25 \(\mu\)M 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 \(\mu\)M), there was a more substantial inhibition of vacuole formation and pseudopod formation, although both were present to some degree (Figure 1E and Table 1). Concentrations of 100, 150, and 200 \(\mu\)M 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.
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 (McNicoll 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 labilization 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 phosphorlylates, 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**

The authors gratefully thank Catherine Robertson for technical assistance, Susan Snusher for typing this manuscript, and Frank Baldwin, University of Manitoba, Department of Pathology, for the use of the Image Analysis System.

**Table 2. Influence of DPPE and Histamine on PMA-induced Formation of Large Vesicles in Permeabilized Platelets**

| Condition                          | Percent of platelet constituted by surface-connected canicular system and large vesicles |
|------------------------------------|------------------------------------------------------------------------------------------|
|                                    | Means±SEM | n       |
| Control                           | 2.30±0.60 | 26      |
| 200 nM PMA                        | 24.19±3.94| 19      |
| 125 μM DPPE + 200 nM PMA          | 3.28±0.70 | 32      |
| 125 μM DPPE + 200 nM PMA + 1 μM histamine | 12.19±1.64 | 22      |
| 125 μM DPPE + 200 nM PMA + 4 μM histamine | 11.13±1.83 | 28      |
| 4 μM histamine                    | 4.51±0.76 | 29      |

PMA=phorbole-12-myristate-13-acetate, DPPE=N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine.HCl. *p<0.001 with respect to 200 nM PMA. †p<0.001 with respect to 125 μM DPPE + 200 nM PMA. §0.02<Δ<0.05 with respect to control.
HISTAMINE PROMOTES PLATELET CHANGES

McNicol et al. 689

References

1. Hacker E. Isolation and characterization of the carcinogenic principles from croton oil. Methods Cancer Res 1967; 6:439–484

2. Silvsk A, van Duuren BL. Cellular interactions of phorbol myristate acetate in tumor promotion. Chem Biol Interact 1971; 3:401–411

3. White JG, Repine JE. Fine structural alterations induced in erythrocytes by phorbol myristate acetate. Am J Pathol 1978; 91:571–580

4. Ettensen RD, White JG. Ultrastructural features of the platelet response to phorbol myristate acetate. Am J Pathol 1974; 74:441–452

5. Nishizuka Y. Studies and perspectives of protein kinase C. Science 1986; 233:305–312

6. Watanabe T, Tazuchl Y, Sasaki K, Tsuyama K, Kltamura Y. Increase in histidine decarboxylase activity in mouse skin after application of the tumor promotor tetradecanoylphorbol acetate. Biochem Biophys Res Commun 1981; 100:427–432

7. Taguchi Y, Tsuyama K, Watanabe T, Wada H, Kltamura Y. Increase in histamine decarboxylase activity in skin of genetically mast-cell-deficient W/Wv mice after application of phorbol-12-myristate-13-acetate: Evidence for the presence of histamine producing cells without basophilic granules. Proc Natl Acad Sci USA 1982; 79:6837–6841

8. Saxena SP, Brandes LJ, Becker AB, Simons KJ, LaBella FS, Gerrard JM. Histamine is an intracellular messenger mediating platelet aggregation. Science 1989; 243:1596–1599

9. Brandes LJ, Bogdanovic RP, Cawker MD, LaBella FS. Histamine and growth: Interaction of antiestrogen binding site ligands with a novel histamine site that may be associated with calcium channels. Cancer Res 1987; 47:4025–4031

10. Brandes LJ, Gerrard JM, Bogdanovic RP, Limt DW, Reid RE, LaBella FS. Correlation of the antiproliferative action of diphenylmethane-derivative antiestrogen binding site ligands with antagonism of histamine binding but not of protein kinase C-mediated phosphorylation. Cancer Res 1988; 48:3954–3958

11. Gill DS, Barradas MA, Tonesca VA, Gracey L, Dandonia P. Increased histamine content in leukocytes and platelets of patients with peripheral vascular disease. Am J Clin Pathol 1988; 89:622–626

12. Brandes LJ, Hermontat MW. A diphenylmethane derivative specific for the antiestrogen binding site found in rat liver microsomes. Biochem Biophys Res Commun 1984; 123:724–728

13. Friesen LL, Gerrard JM. The effect of 1-octyl-2-acetylglycerol on platelet protein phosphorylation and platelet ultrastructure. Am J Pathol 1985; 121:79–87

14. Authi KS, Evenden BJ, Crawford N. Metabolic and functional consequences of introducing inositol-1,4,5-trisphosphate into saponin-permeabilized human platelets. Biochem J 1986; 233:709–718

15. Israels SJ, Robinson P, Docherty JC, Gerrard JM. Activation of permeabilized platelets by inositol-1,4,5-trisphosphate. Thromb Res 1985; 40:499–509

16. Hoell E, Hoell L. Autoradiographic localization of binding sites for [3H] histamine and H1- and H2-antagonists on cultured neurons and glial cells. Neuroscience 1984; 13:663–870

17. Beattie LL, Park J, Israels SJ, Gerrard JM. A role for protein kinase C (PKC) in the membrane fusion necessary for granule secretion (abstract). Blood 1986; 68(suppl):1130

18. Cohen I, Gerrard JM, Bergman RM, White JG. The role of contractile filaments in platelet activation. In: Proteins of the biological fluids. Elmsford, NY: Pergamon Press, 1979; 555

19. Yamanishi J, Takai Y, Kalbuchi K, Sano K, Castagna M, Nishizuka Y. Synergistic functions of phorbol ester and calcium in serotonin release from human platelets. Biochem Biophys Res Commun 1983; 12:778–786

20. Halenda SP, Zavolco GB, Feinstein MB. Phorbol esters and oleoyl acetylgluceroic enhances release of arachidonic acid in platelets stimulated by Ca2+ ionophore A23187. J Biol Chem 1985; 260:12484–12491

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:

$$K_{i,t} = -\ln(1 - (HDL^*_t/\text{LDL}^*_a)) \frac{(1+c)}{(1+c)}$$

(5)
A role for intracellular histamine in ultrastructural changes induced in platelets by phorbol esters.
A McNicol, S P Saxena, L J Brandes and J M Gerrard

Arterioscler Thromb Vasc Biol. 1989;9:684-689
doi: 10.1161/01.ATV.9.5.684

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/9/5/684

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/