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HISTAMINE FORMED IN STIMULATED HUMAN PLATELETS IS CYTOPLASMIC

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SUMMARY: The localization of histamine formed by human platelets in response to agonists was evaluated. 87 ± 5% of the histamine in a suspension of platelets exposed to phorbol-12-myristate-13-acetate (PMA) was associated with the platelet pellet. Incubation of saponin-permeabilized platelets with the intracellular histamine antagonist, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine·HCl (DPPE), released 75 ± 3.9% of the histamine into the supernatant. Under conditions where 90% of platelet serotonin was secreted into the supernatant, the majority (80%) of platelet histamine remained associated with the pellet. The results suggest that histamine synthesized in response to agonists is largely cytoplasmic.

Histamine has been found in platelets in a number of studies (1-7). Platelets from certain species, particularly rabbits and pigs, contain considerable histamine, primarily localized in dense granules (1-2). In contrast, resting human platelets contain relatively small amounts of histamine (4-7), but they synthesize additional histamine in response to agonists, including PMA, collagen, thrombin and platelet activating factor (PAF)(7,8). Since this newly synthesized histamine promotes platelet aggregation (7,8) the exact location of this amine in human platelets is of considerable interest. In the present study, we have investigated the location of the histamine formed in human platelets in response to agonists.

MATERIALS AND METHODS

Materials: Stock solutions of reagents were prepared fresh daily. N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine.HCl (DPPE) was synthesized as described previously (9). Phorbol-12-myristate-13-acetate (PMA) was purchased from LC Services Corp., Worburn, MA; thrombin, saponin, histamine, o-phthaldehyde, 2-mercaptoethanol and D-myo-inositol trisphosphate from Sigma Chemical Co., St. Louis, MO; and platelet activating factor (PAF) from Calbiochem, San Diego, CA; and U46619 (a thromboxane mimetic) from Calman, Ann Arbor, MI. ^{14}C-serotonin was purchased from New England Nuclear. All other reagents and solvents were of analytical grade

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and were used without further purification. Glass distilled water purified with Milli-Q system was used throughout the study.

Preparation of Platelets: Blood obtained from healthy male and female donors was collected into plastic syringes containing citrate anti-coagulant (final concentration 0.7 mM citric acid, 9.3 mM sodium citrate, 13.6 mM dextrose, pH 6.5). Platelet-rich plasma was collected by centrifugation at 800 xg for 5 min at 20°C. Platelets were sedimented by centrifugation at 800 xg for 11 min and the platelet pellet resuspended in either Hank's balanced salt solution modified by the addition of 4.45 mM dextrose, 3.35 mM $NaHCO_3$, 500 μM $MgCl_2$ and 0.1% bovine serum albumin, pH 7.5 for experiments with washed platelets, or in a buffer containing 90 mM NaCl, 5 mM KCl, 5 mM dextrose, 10 mM EDTA and 36 mM citric acid, pH 6.5 for the preparation of permeabilized platelets. Platelets were further sedimented after the addition of citrate anti-coagulant and resuspended at 2.5 x 10^8 cells/ml in either modified Hank's balanced salt solution for washed platelets or in buffer containing 140 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$, 0.42 M $NaHPO_4$, 11.9 mM $NaHCO_3$, pH 7.35 (10) for platelets to be permeabilized. Saponin was used to permeabilize the platelets and its concentration carefully titrated (10-13 μg/ml) and tested using IP_3 to ensure permeabilization (10,11).

Platelet Aggregation: Platelet aggregation was recorded on a Payton dual channel aggregometer (Payton Assoc, Scarborough, Ontario) at 37°C. 100 μM DPPE, where used, was added 30 sec before PMA. After the addition of PMA, thrombin, PAF or U46619, aggregation was recorded for two minutes. Aliquots of 500 μl platelet suspension were cooled by the addition of an equal amount of cold (4°C) buffer, and centrifuged at 800 xg for 5 min to collect the platelet pellet and supernatant.

Histamine Analysis: Histamine was extracted separately from the pellet and the supernatant using alkaline chloroform:butanol (4:1) as described by Keyzer et al (5) and analyzed by high performance liquid chromatography after derivitization to a fluorogenic compound using o-phthaldehyde (12). Using a μ-Bondpak C18 column, isocratic elution was carried out with a mobile phase containing 41.5% methanol:acetonitrile (83:17) and 58.5% 0.02 M phosphate buffer pH 6.0, at a flow rate of 1 ml/min. Fluorescence was monitored (emission/ excitation ratios 460:356). The elution time for histamine was 24.5 min and there were no interfering peaks. The limit of detection of the method is 200 pg histamine. The identity of the histamine was previously confirmed using histaminase (incubation of an extracted platelet sample with 0.75 mg/ml histaminase selectively removed the histamine peak) as well as by a radioimmunoassay for histamine (Immunotech, S.A. Harseville, FL) which gave similar results (r=0.984) (8).

Release of Serotonin from Platelet Dense Granules: Secretion of serotonin was assessed in platelets following incubation of the platelet-rich plasma for 15 min. at 37°C with [^{3}H]-serotonin (1 μCi/ml), then washing and resuspending the platelets as described above. Aggregation was carried out as above. Two minutes after the addition of agonist (or vehicle control), 1 ml of 0.1% glutaraldehyde in White's saline was added to stop further secretion. Platelet samples were then pelleted at 500 x g for 10 min to separate platelets and supernatant. Serotonin secretion was expressed as the radioactivity of the supernatant as a percentage of the total radio-activity in the supernatant plus the pellet (13).

RESULTS AND DISCUSSION

After stimulation by PMA, there was a significant increase in the total platelet content of histamine (Table 1), consistent with our previous

TABLE 1: The Influence of the Intracellular Histamine Antagonist DPPE and of Saponin-Permeabilization on the Distribution of Platelet Histamine

Experiments	Histamine pmoles/10^9 cells Pellet	Supernatant	% Histamine in Pellet	n
A. NON PERMEABILIZED				
Platelets + vehicle control	14.7 ± 1.3	0.3 ± 0.9	97.8 ± 2.3	8
Platelets + PMA (480 nM)	36.0 ± 3.5	5.6 ± 2.3	89.0 ± 4.5	5
Platelets + DPPE (100 μM) + PMA (480 nM)	33.1 ± 3.3	11.5 ± 1.6	74.3 ± 1.5	3
B. PERMEABILIZED				
Platelets + vehicle control	12.2 ± 0.2	1.7 ± 0.3	88.0 ± 1.5	3
Platelets + PMA (480 nM)	28.4 ± 1.8	12.3 ± 0.6	69.6 ± 1.5	3
Platelets + DPPE (100 μM) + PMA (480 nM)	8.7 ± 0.9	26.5 ± 2.6	25.1 ± 3.9	3

findings (8). This histamine was largely associated with the platelet pellet, rather than released into the supernatant (Table 1). To test whether the histamine present in the platelet suspension after stimulation with PMA might have been secreted and then bound extracellularly, non-permeabilized platelets were incubated with the histamine receptor antagonist, DPPE (9, 14, 15), which inhibits histamine binding to a novel intracellular μM affinity site. Treatment with DPPE increased only slightly, the histamine in the supernatant. However, when the platelets were permeabilized with saponin, a detergent which has some preferential ability to permeabilize the plasma membrane as compared to intracellular membranes, and then treated with DPPE, the majority of platelet histamine was now released into the supernatant, providing further evidence that platelet histamine is largely cytoplasmic (Table 1).

However, it remained necessary to confirm that the histamine was not located in the dense granules, since stimulation by PMA resulted in the secretion of only approximately 30% of the platelet serotonin. Using thrombin, PAF, or the combination of PMA and U46619, the platelets released up to 90% of the granule serotonin; however, more than 80% of the histamine remained within the platelet pellet (Table 2), providing strong evidence that in human platelets, histamine formed in response to agonists does not arise from dense granules, but rather is cytoplasmic in nature; a considerable proportion of it may be bound.

TABLE 2: **Synthesis and Distribution of Histamine in Response to Various Agonists**

Experiments	Histamine pmoles/10^9 cells		% Histamine	% Serotonin
	Pellet	Supernatant	in Pellet	Secretion
Platelets + vehicle control	14.7 ± 1.3 (8)*	0.3 ± 0.9 (8)	97.8 ± 2.2 (8)	0.00 (6)
Platelets + Thrombin (1U/ml)	24.3 ± 0.1 (3)	4.3 ± 0.9 (3)	85.0 ± 1.5 (3)	90.4 ± 1.2 (6)
Platelets + PAF (1 μM)	23.7 ± 0.8 (6)	5.2 ± 0.8 (6)	82.0 ± 0.9 (6)	83.7 ± 1.9 (6)
Platelets + PMA (480 nM) + U46619 (1 μM)	37.4 ± 1.5 (3)	4.8 ± 0.9 (3)	88.6 ± 2.3 (3)	56.5 ± 1.8 (6)
Platelets + PMA (480 nM)	36.0 ± 3.5 (5)	5.6 ± 2.3 (5)	89.0 ± 4.5 (5)	32.3 ± 2.0 (6)

* Figures in parentheses indicate the number of samples.

These findings are in keeping with an intracellular second messenger role for histamine, mediating aggregation in response to various agonists which bind to receptors on the external membrane.

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