use of beads as markers, this low-amplitude shear oscillation should be measurable in the absence of bending in fragments of demembranated flagella examined under conditions appropriate for reactivating the bending of intact flagella.

These applications will be facilitated by development of more automatic and higher precision methods for photographic image analysis to obtain measurements of bead separation in both straight and curved regions of a flagellum. Other recent work has demonstrated the ability of digital image-processing methods to extract positional information from light microscopic images at nanometer dimensions, well below the resolving distance of light microscopy (*10, 21*).

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4. Spermatozoa were demembranated with Triton X-100, activated by incubation for 90 s with cyclic adenosine monophosphate and ATP, and treated for 30 s with 2 m*M* Ca^{2+} to extract calmodulin (*5*). A 100-μl aliquot was then added to 1 ml of reactivation solution lacking polyethylene glycol or methyl cellulose, containing 100 μl of a suspension of 40-nm gold beads (Janssen Life Science Products, Piscataway, NJ). After 2 min, a 100-μl portion of this mixture was diluted with 1 ml of reactivation solution containing, in addition to standard components (*5*), 1 to 10 m*M* lithium acetate to increase bend angles (*6*) and 0.1 to 0.2% methyl cellulose to decrease the bending wavelengths (7). Spermatozoa swimming next to a water-oil interface were observed in thin hanging-drop preparations with the use of a well slide filled with mineral oil (*8*). All procedures and observations were carried out at 18°C.
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11. Selected portions of the photographic negatives were digitized at 2048 by 768 pixels (21.5 pixel/μm), 256 gray levels, with an Eikonix 850 camera (Eikonix Corporation, Bedford, MA) and transferred to a Hewlett-Packard 320 microcomputer. A portion of the image was displayed, and a line parallel to the head axis was entered manually. A second line was entered, parallel to the flagellum and transecting the images of two beads selected for measurement. The angle between these two lines was used as a measure of shear angle at the location of the beads. Pixel intensity along the line through the beads was calculated for a band of pixels, usually 11 pixels in width, centered on the line, and displayed. By visual inspection, a threshold was selected to retain only the peaks corresponding to the two beads. The position of the centroid of each peak was then computed from intensity values above the threshold and used to obtain the distance between the two beads. This is a simplified, one-dimensional version of the procedure described by Gelles *et al.* (*21*).
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13. The values are the 81 values of 61 nm times the absolute value of the function $\cos[0.698\ (n-1)] - \cos[0.698\ (m-1)]$ evaluated for all values of n,m, from 1 to 9. In this calculation it is assumed that the axonemal cross section is circular (or elliptical but symmetric around the bending plane), that the bending plane passes through doublet 1 and the 5-6 bridge, and that the axonemal diameter, measured at the center of the doublets, is 132 nm in the bending plane. This distribution is appropriate either for the case where each bead attaches to just one doublet or for the case where each bead attaches to two adjacent doublets. If both types of attachment occur, then there are 4×81 possible combinations, and the distribution of "doublet separation" is smoother.
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Histamine Is an Intracellular Messenger Mediating Platelet Aggregation

SATYA P. SAXENA, LORNE J. BRANDES, ALLAN B. BECKER, KEITH J. SIMONS, FRANK S. LABELLA, JON M. GERRARD*

Inhibition of human platelet aggregation by *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine-HCl (DPPE), a novel antagonist of histamine binding, suggested that histamine might serve a critical role in cell function. Phorbol-12-myristate-13-acetate (PMA) or collagen was found to increase platelet histamine content in parallel with promotion of aggregation. Inhibitors of histidine decarboxylase (HDC) suppressed both aggregation and the elevation of histamine content, whereas DPPE inhibited aggregation only. In saponin-permeabilized platelets, added histamine reversed the inhibition by DPPE or HDC inhibitors on aggregation induced by PMA or collagen. The results indicate a role for histamine as an intracellular messenger, which in platelets promotes aggregation.

HISTAMINE IS A WELL-ESTABLISHED extracellular messenger in numerous physiological and pathophysiological conditions, including allergies, inflammation, gastric acid secretion, neurotransmission, cardiac dysfunction, and uterine contraction, and is implicated in others such as immunoregulation (*1*). Almost 30 years ago, Kahlson *et al.* (*2*) suggested that newly formed histamine might be an important mediator of cell growth. Histidine decarboxylase (HDC) activity, the ability of cells and tissues to form histamine, has been correlated with cell multiplication and growth (*3*). Studies of DPPE, an antiproliferative agent that antagonizes histamine binding at a novel (non-H_1, non-H_2, non-H_3) affinity site that binds histamine in micromolar amounts (*4*), showed that DPPE inhibits platelet aggregation stimulated by PMA, a tumor promoter usually considered to act primarily through activation of protein kinase C (*5*). However, DPPE did not inhibit protein kinase C–induced phosphorylation of a platelet M_r 47,000 protein (p47) (*6*). Thus, the antiaggregatory effect of DPPE is not due to antagonism of protein kinase C. Since PMA in-

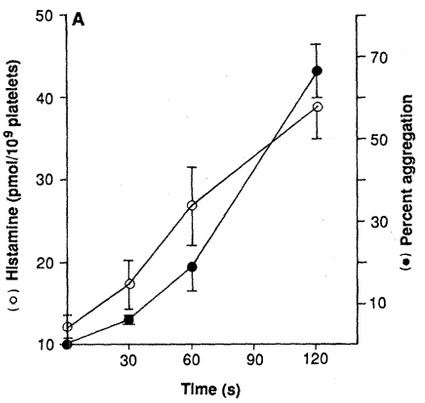
S. P. Saxena and J. M. Gerrard, Manitoba Institute of Cell Biology and Department of Pediatrics, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0V9.
L. J. Brandes, Manitoba Institute of Cell Biology and Departments of Medicine and Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0V9.
A. B. Becker, Department of Pediatrics, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0V9.
K. J. Simons, Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.
F. S. LaBella, Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0V9.

*To whom correspondence should be addressed.

duces histidine decarboxylase (7), we evaluated the possibility that PMA-induced platelet aggregation, antagonized by DPPE, might be mediated in part by stimulation of intracellular histamine formation.

We used high-performance liquid chromatography (HPLC) to measure intracellular histamine production in PMA-treated human platelets. The histamine found in unstimulated platelets (12.2 ± 1.5 pmol per 10^9 cells) agrees well with previously reported values (*8*). In platelets activated by PMA (480 n*M*), histamine production increased in parallel with the extent of aggregation (Fig. 1A), reaching a level of 38.7 ± 3.8 pmol per 10^9 platelets by 2 min, an increment of 27 pmol of histamine per 10^9 cells. A lower dose of PMA (15 n*M*) also stimulated platelet aggregation and histamine formation (to 37.5 ± 1.3 pmol per 10^9 cells at 4 min) as did 1 U of thrombin per milliliter (to 22.8 ± 5.1 pmol per 10^9 platelets by 2 min). When the platelet suspension was cooled by the addition of cold buffer and then centrifuged at 800*g* for 5 min, 87% ± 5% (n = 5) of the histamine in the PMA-treated platelets was accounted for in the pellet. When platelets were permeabilized with saponin and treated with DPPE to displace histamine binding, most (75%) of the histamine was now in the supernatant (*9*), suggesting that histamine formed in response to PMA is cytoplasmic. If a mean platelet volume of 7.3 μm^3 is assumed (*10*), 27 pmol of histamine per 10^9 platelets yields a rise in cytoplasmic histamine of 3.7 μ*M* in response to PMA.

The formation of histamine from histidine is accomplished by a single enzyme, HDC (E.C. 4.1.1.22). Since phorbol esters increase HDC in other cells and tissues (*7, 11*), the PMA-stimulated increase in platelet histamine may reflect increased HDC activity. The medium used in our experiments was histidine-free, but platelets contain sufficient endogenous histidine (~300 μ*M*) (*12*) to allow the measured production of histamine. α-Methyl histidine (α-MH), a reversible inhibitor, or (*S*)-α-fluoromethylhistidine (α-FMH), an irreversible inhibitor of HDC, inhibited histamine formation



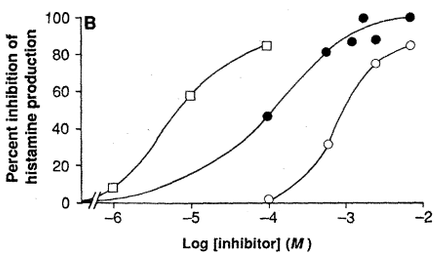


Fig. 1. (**A**) Platelet aggregation and histamine production in response to PMA as a function of time. (**B**) Inhibition of PMA-induced histamine production by α-MH (●) and α-FMH (○, □). Aggregation of washed platelets in histidine-free medium (*27*) was recorded 2 min after PMA addition, with a Payton dual-channel aggregometer. Histamine content was assessed by HPLC. Washed platelets (2.5×10^8 to 9×10^8 per milliliter) were extracted with alkaline chloroform and butanol (4:1, v/v) as described by Keyzer *et al.* (*8*). Histamine was derivatized to a fluorogenic compound with *o*-phthalaldehyde (*28*) and was injected onto a μBondapak C_{18} column. Isocratic elution was carried out with a mobile phase containing 41.5% methanol:acetonitrile (83:17) and 58.5% 0.02*M* P buffer, *p*H 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 of histamine. Incubation of an extracted platelet sample with histaminase (0.75 mg/ml) (Sigma) selectively removed the histamine peak, confirming its identity. Further confirmation was obtained through the use of an RIA for histamine (Immunotech), which gave identical results to those shown in (A) for platelet histamine production in response to PMA (*29*). Day-to-day variation was less than 5%. Where used, inhibitors of HDC were ordinarily added 30 s before PMA (480 n*M*) (○, ●). For the 2-hour incubation experiment, α-FMH was added to whole blood, and each subsequent buffer and the platelets were stimulated with PMA (15 n*M*) for 4 min (□). The number of independent replicates in (A) was 18, 7, 6, and 18 for the 0, 30-s, 1-min, and 2-min time points, respectively, and in (B) was 2 to 4 for each time point. Comparison of data sets with more than one time point show that they represent a set of parallel curves.

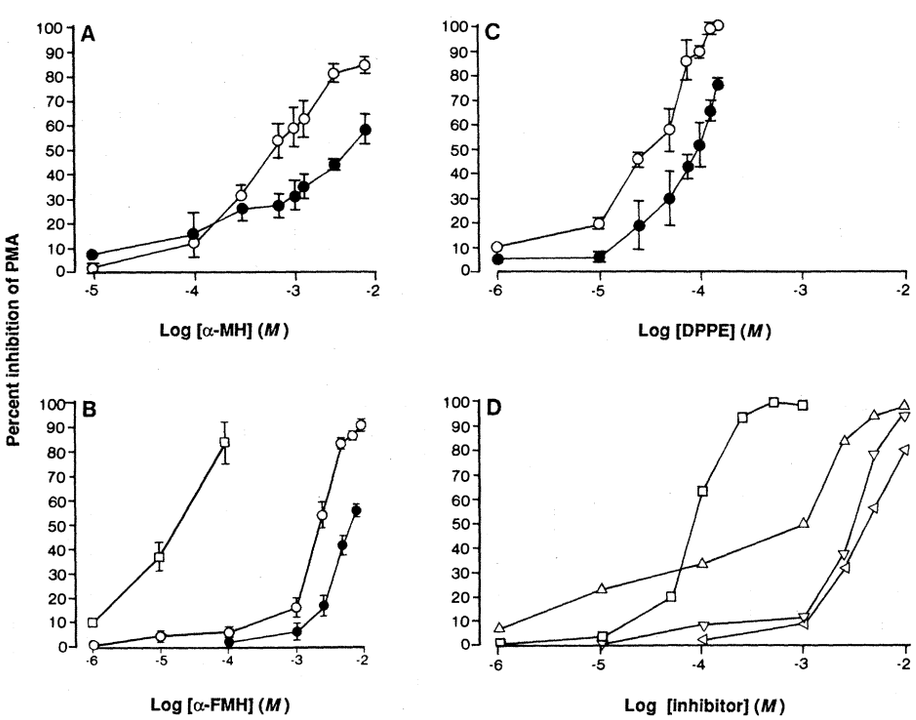


Fig. 2. Inhibitory effects of various antagonists on PMA-induced aggregation in washed (open symbols) and saponin-permeabilized (closed symbols) platelets. Platelets were prepared by a modification of previously described methods (*23*). Various antagonists including (**A**) α-MH (○, ●); (**B**) α-FMH (○, □, ●); (**C**) DPPE (○, ●); (**D**) phenyltoloxamine (□), pyrilamine (△), ranitidine (▽), and cimetidine (◁), or vehicle controls were added, after a 2-min exposure to saponin (10 to 13 μg/ml), and then PMA (480 n*M*) was added 30 s later. In (B), the effect of a 2-hour preincubation with α-FMH (see Fig. 1 legend for details) is also shown (□). Platelet aggregation was recorded on a Payton dual-channel aggregometer for 3 min after addition of PMA. Each point shown is the mean and standard error of up to nine independent replicates. The results, for each inhibitor, came out as a set of parallel curves.

(Fig. 1B) and platelet aggregation in parallel (Fig. 2, A and B). When added 30 s before PMA, α-MH was more potent [median inhibitory concentration (IC_{50}), 600 μ*M*] than α-FMH (IC_{50}, 2.4 m*M*). The slope of the regression, for individual data points, between the extent of aggregation and the measured platelet histamine content was 2.2 ± 0.5 for α-FMH ($P = 0.0007$) and 1.2 ± 0.3 for α-MH ($P = 0.0008$). The IC_{50} observed for α-MH was similar to that reported to inhibit HDC in other tissues (*13*), whereas for α-FMH, the IC_{50} was much higher (*14*). Since α-FMH produces a time-dependent inactivation of HDC (*14*), we evaluated histamine production in platelets incubated with α-FMH for 2 hours. Under these conditions, α-FMH again inhibited platelet aggregation and histamine production in parallel, but at much lower concentrations (IC_{50}, 10 μ*M*) than that found for the shorter incubation period (*15*), and one that agrees with the findings of others (*14*). In contrast to α-MH and α-FMH, 100 μ*M* DPPE inhibited PMA-induced platelet aggregation by >90%, but did not influence the production of histamine (35.5 ± 4.2 pmol of histamine per 10^9 platelets), evidence that DPPE is not an HDC inhibitor, but consistent with its antagonism of intracellular histamine.

The rapid rise (30 s) in histamine levels in response to PMA suggests nongenomic activation of HDC, especially as platelets are nonnucleated. In keeping with this, inhibitors of protein synthesis (cycloheximide, 5 μg/ml) or of RNA synthesis (actinomycin D, 5 μg/ml) failed to affect PMA-induced histamine synthesis (*16*). Thus, as in the case of rapid (30 s) stimulation of ornithine decarboxylase in kidney cells by testosterone (*17*), activation of HDC in platelets may involve a receptor-mediated posttranslational event, possibly a reversible phosphorylation of the enzyme or associated protein.

We next tested DPPE and traditional H_1 and H_2 antagonists on PMA-induced platelet aggregation in order to evaluate the nature of the putative platelet histamine receptor. DPPE was the most potent inhibitor (IC_{50}, 32 μ*M*)(Fig. 2C). The H_1 antagonist and ortho-isomer of DPPE, phenyltoloxamine was significantly weaker (IC_{50}, 85 μ*M*), whereas the H_1 antagonist pyrilamine and the H_2 antagonists cimetidine and ranitidine were very weak, with IC_{50} values in the millimolar range (Fig. 2D). The rank order of antiaggregative potency for the various agents correlated with that for antagonizing [^{3}H]histamine binding at the lower affinity site in rat cortex membranes (*4, 18*).

If newly formed histamine has a role in PMA-induced platelet function, the antiaggregative effects of HDC inhibitors and DPPE should be reversed by exogenous histamine. In intact platelets, the addition of histamine, which does not readily cross the plasma membrane (*19*), was without effect. However, in platelets permeabilized with saponin, histamine reversed (by about 40%) the effect of the HDC inhibitors and DPPE (Fig. 3, A to C). The dose-response curve for histamine reversal was bell-shaped with an optimal effect at $10^{-6}M$ and lesser, though still significant, effects at 10^{-5} and $10^{-4}M$ (*20*).

The optimal concentration of histamine (1 to 10 μ*M*) to reverse the effects of DPPE and HDC inhibitors corresponded to the measured increment in intracellular histamine (3.7 μ*M*) produced in response to PMA. The fact that full reversal was not achieved may relate to the high concentrations of HDC inhibitors and DPPE required to inhibit PMA-induced platelet aggregation in saponin-permeabilized platelets (Fig. 2, A to C). Alternatively, the permeabilized platelets may be less responsive to agonists because saponin may cause leakage of small molecular weight chemicals from the cell. In contrast to the findings for histamine, the polyamines spermine, spermidine, and putrescine, and the polyamine precursor ornithine had no significant effect on inhibition by α-MH (Fig. 3A).

Collagen (1.8 μg/ml), a more physiologic mediator of platelet function, promoted platelet aggregation (Fig. 4) and stimulated

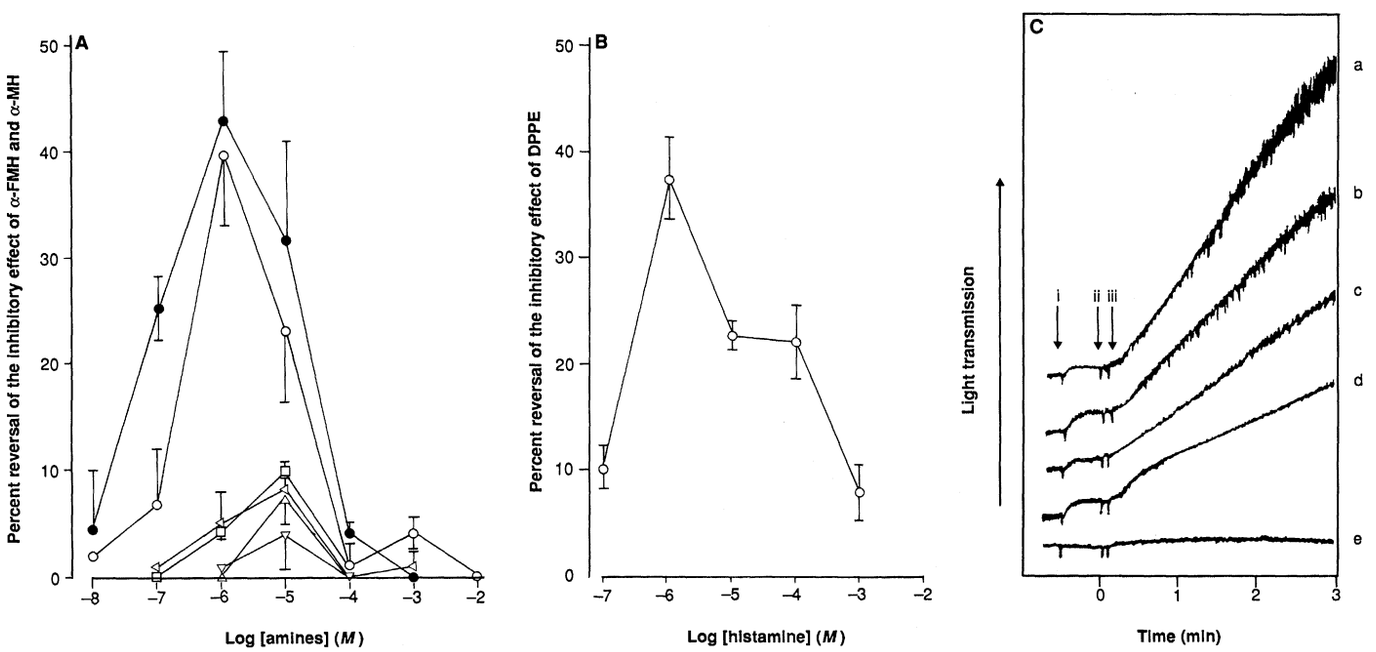


Fig. 3. Histamine, but not other amines, was able to reverse the inhibition of PMA-induced platelet aggregation produced by prior incubation with (**A**) the HDC inhibitors α-MH (open symbols) and α-FMH (●), and (**B**) DPPE (○). Amines were added 5 s after PMA (480 n*M*) to saponin-permeabilized platelets pretreated with HDC inhibitors or DPPE, and percent aggregation was recorded 3 min later. Amines studied included histamine (○, ●), spermine (□), spermidine (△), putrescine (▽), and ornithine (◁). Each point shown is the mean and standard error of up to 12 independent replicates. For each inhibitor, the results of histamine reversal studies were a set of parallel lines. (**C**) Results of a typical experiment are shown with platelets stirred with saponin for 2 min, inhibitor or buffer added at (i) PMA or buffer added at (ii) and histamine or buffer added at (iii). The upper curve (a) shows the platelet response to PMA alone. DPPE (100 μ*M*) inhibited PMA-induced aggregation (d) and such inhibition was significantly reversed by $10^{-6}M$ histamine (b) or $10^{-5}M$ histamine (c). Histamine alone at $10^{-6}M$ is shown in tracing e.

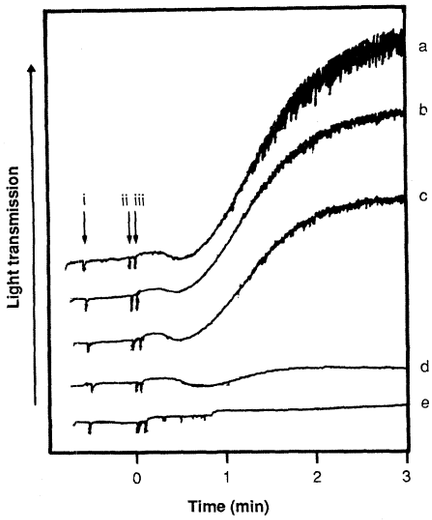


Fig. 4. Aggregation of permeabilized platelets in response to 1.8 μg/ml collagen (a), was markedly inhibited by 10 μ*M* DPPE (d). Histamine at 10^{-7}*M* (b) or 10^{-6}*M* (c) reversed the inhibition by DPPE. Histamine alone at 10^{-6}*M* is shown in (e). Reagents were added at (i), (ii), and (iii) as in Fig. 3C.

histamine formation (to 33.6 pmol per 10^9 platelets by 2 min). In permeabilized platelets, DPPE inhibited collagen-stimulated aggregation (IC_{50}, 6 ± 1.2 μ*M*); histamine reversed this inhibition at an optimal concentration of 10^{-7}*M*. Compared to PMA, the lower effective concentrations of both histamine and DPPE are consistent with studies of cytoplasmic calcium levels in relation to platelet function. Higher levels of cytoplasmic calcium are necessary for responses to ionomycin, which acts primarily by modulating calcium flux, than for collagen or thrombin, which utilize multiple second messenger systems (*21, 22*). Similarly, end responses to PMA may rely to a larger extent upon the contribution of newly formed histamine than those to collagen, so that higher cytoplasmic concentrations of the amine are required.

Histamine, unlike inositol trisphosphate (IP_3) (*23*), did not by itself aggregate saponin-permeabilized platelets at any concentration tested (Figs. 3C and 4). Thus, although histamine formation is critical for aggregation, the full platelet response to PMA and collagen must need the simultaneous activation of more than one intracellular process.

Our results show that intracellular histamine formation is necessary for platelet responses to external stimuli and may explain induction of platelet aggregation evoked by the HDC stimulator thapsigargin (*24*) as well as lend relevance to the finding of increased histamine levels in platelets from patients who have peripheral vascular disease (*25*). In addition, the activation of HDC and the increased histamine present during the proliferation of cultured lymphocytes, various cancer cells, and many rapidly growing fetal tissues, suggest a broader role for histamine as an intracellular messenger (*2, 3, 26*).

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16. In this experiment, 480 n*M* PMA increased platelet histamine from 11.2 ± 0.87 to 38.5 ± 2.7 pmol per 10^9 platelets. Platelets pretreated with cycloheximide or actinomycin D before PMA contained 37.5 ± 1.6 and 37.9 ± 1.4 pmol of histamine per 10^9 platelets, respectively.
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27. Citrate anticoagulant (final concentration, 0.7 m*M* citric acid, 9.3 m*M* sodium citrate, and 17.6 m*M* dextrose, *p*H 6.5) was added to blood drawn from normal human donors, and platelet-rich plasma, with less than one leukocyte per 10^5 platelets, was collected by centrifugation at 800*g* for 5 min at 20°C. Platelets were sedimented by centrifugation at 800*g* for 11 min, and the platelet pellet was resuspended in Hanks balanced salt solution modified by addition of 4.45 m*M* dextrose, 3.35 m*M* $NaHCO_3$, 500 μ*M* $MgCl_2$, 0.1% bovine serum albumin, *p*H 7.5 (with NaOH), in experiments with washed platelets and in 90 m*M* NaCl, 5 m*M* KCl, 36 m*M* citric acid, 10 m*M* EDTA, and 5 m*M* dextrose, *p*H 6.5 (with KOH), for the preparation of platelets to be permeabilized. Platelets were further sedimented after the addition of citrate anticoagulant and finally resuspended at 2.5×10^8 cells per milliliter in the modified Hanks balanced salt solution for washed platelets or in a Ca^{2+}-free medium resembling the cytosolic ionic environment (1 m*M* dextrose; 1 m*M* $MgCl_2$, 0.42 m*M* NaH_2PO_4, 11.9 m*M* $NaHCO_3$, 140 m*M* KCl, and 5 m*M* Hepes, *p*H 7.35) for the study of permeabilized cells. The concentration of saponin used to permeabilize the platelets was carefully titrated for each experiment. Aggregation by IP_3 (10 μ*M*) after 2 min of initial saponin (10 to 13 μg/ml) exposure reflected successful permeabilization.
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29. Comparison of HPLC and radioimmunoassay (RIA) in three experiments gave the following values: (nanograms per sample). HPLC values are given first and RIA determinations second. (i) Control (12.2, 21.5); 480 n*M* PMA × 2 min (52.6, 67.4); (ii) control (19.3, 21.0); 1.8 μg of collagen per milliliter × 2 min (54.4, 40.1); (iii) control (21.2, 20.8); 15 n*M* PMA × 4 min (59.1, 63.0); 100 μ*M* α-FMH × 2 hours + 15 n*M* PMA × 4 min (13.6, 7.8).

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