Synthesis of intracellular histamine in platelets is associated with activation of protein kinase C, but not with mobilization of Ca^{2+}

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In previous reports, we have provided evidence indicating that newly formed histamine is an intracellular messenger in human platelets. The involvement of protein kinase C (PKC) and intracellular calcium (Ca^{2+}) in the synthesis of histamine was investigated. Human platelets were stimulated by phorbol 12-myristate 13-acetate (PMA), collagen and the Ca^{2+} ionophore A23187, with or without the PKC inhibitor staurosporine. Aggregation, histamine synthesis and phosphorylation of pleckstrin (47 kDa; P47) and myosin light chain (20 kDa; P20) proteins were monitored. Staurosporine inhibited PMA- and collagen-induced aggregation, histamine synthesis and phosphorylation of 47 kDa and 20 kDa proteins in a dose-dependent manner. For PMA, median inhibitory concentrations (IC_{50} values) for staurosporine inhibition of aggregation, histamine synthesis and phosphorylation were similar, suggesting that histamine synthesis induced by this agonist may be a consequence of PKC activation. Conversely, collagen-stimulated histamine synthesis was inhibited by staurosporine at concentrations significantly higher than those required to inhibit aggregation ($P < 0.005$) or pleckstrin phosphorylation ($P < 0.01$), indicating the possible involvement of non-PKC mechanism(s) in the synthesis of histamine induced by this agonist. A23187 failed to induce the synthesis of intracellular histamine in platelets, whereas staurosporine blocked A23187-induced aggregation and phosphorylation of the 20 kDa protein at significantly higher concentrations than those needed to inhibit PKC. When platelets were stimulated with a combination of A23187 and PMA, the increase in platelet histamine was less than that with PMA alone. The results provide evidence that the synthesis of intracellular histamine in platelets occurs as a consequence of PKC activation and may be down-regulated under conditions where there is a substantial rise in $[\text{Ca}^{2+}]_i$.

INTRODUCTION

The intracellular processes associated with platelet activation are complex, involving 1,2-diacylglycerol, cytosolic Ca^{2+} (Ca^{2+}_i), protein kinase C (PKC) and possibly intracellular histamine as messengers [for reviews see Siess (1989) and Brandes *et al.* (1990)]. One of the key events is the stimulation of PKC by diacylglycerol, an effect mimicked by phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (Castagna *et al.*, 1982). PKC phosphorylates a variety of proteins believed to be essential for platelet activation (Gerrard *et al.*, 1987a); however, their identity and function remain elusive (Haslam, 1987).

Recently, we have provided evidence that PMA- and collagen-induced platelet aggregation is associated with, and dependent on, the synthesis of intracellular histamine (Saxena *et al.*, 1989a,b, 1990), whose action occurs distal to, or independent of, PKC (Brandes *et al.*, 1988). Histamine, synthesized by the enzyme histidine decarboxylase (HDC; EC 4.1.1.22), is induced in skin by PMA (Watanabe *et al.*, 1981). In platelets, inhibitors of protein synthesis fail to affect PMA-induced histamine production (Saxena *et al.*, 1989a), indicating the non-genomic nature of HDC activation in these non-nucleated cells. Alternatively, the activity of HDC in platelets may be regulated by a PKC-mediated phosphorylation of the enzyme, or of an associated protein, as reported for the stimulation of ornithine decarboxylase by testosterone in rat kidneys (Koenig *et al.*, 1983).

In the present study we have used PMA and collagen to investigate the relationship between PKC activation and the synthesis of intracellular histamine in platelets. The Ca^{2+} ionophore A23187 was employed to examine the possible involvement of Ca^{2+}_i in histamine synthesis.

MATERIALS AND METHODS

Materials

PMA was purchased from LC Services Corp, Woburn, MA, U.S.A., and staurosporine and adenosine deaminase (ADA) were from Boehringer Mannheim, Dorval, Que., Canada. The Ca^{2+} ionophore A23187, collagen (type 1, from bovine achilles tendon), histamine, *o*-phthalaldehyde, 2-mercaptoethanol, acetylsalicylic acid, phosphocreatine (CP), creatine kinase (CPK) and apyrase were purchased from Sigma Chemical Co., St. Louis MO, U.S.A. $\text{H}_3^{32}\text{PO}_4$ was obtained from New England Nuclear, Mississauga, Ont., Canada. Electrophoresis supplies were from Bio-Rad, Mississauga, Ont., Canada. All other reagents and solvents were of analytical grade and were used without further purification. Glass-distilled water purified by the Milli-Q system was used throughout the study.

Preparation of platelet suspension

Washed platelets were prepared as described previously (Saxena *et al.*, 1990). Briefly, human venous blood was collected into acid-citrate-dextrose anticoagulant (3.8 mM-citric acid, 75 mM-trisodium citrate, 125 mM-dextrose; 1.9 ml of anticoagulant per 8.1 ml of whole blood). Platelet-rich plasma containing less than one leucocyte per 10^5 platelets was obtained 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 the appropriate buffer.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; CP, phosphocreatine; CPK, creatine kinase; DMSO, dimethyl sulphoxide; HDC, histidine decarboxylase; IC_{50} , median inhibitory concentration; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Aggregation studies

The platelet pellet was resuspended at a concentration of 4×10^8 /ml in histidine-free Hanks balanced salt solution modified by addition of 4.45 mM-dextrose, 3.35 mM- NaHCO_3 , 500 mM- MgCl_2 and 0.1% BSA, pH 7.5 (with NaOH). Aggregation was monitored photometrically at 37 °C with a Payton dual-channel aggregometer (Payton Associates, Scarborough, Ont., Canada). Samples (400–500 μl) of platelet suspension in silicone-treated glass cuvettes containing stirrer bars and 2.5 mM- CaCl_2 were equilibrated at 37 °C for 3–5 min. The PKC inhibitor staurosporine or vehicle control was added 30 s before the addition of agonist. In some experiments, platelets were first pretreated with the cyclo-oxygenase inhibitor aspirin (0.5 mM; 15 min, at room temperature), and later with the ADP-scavenging enzymes apyrase (20 units/ml) and ADA (4 units/ml) for 3 min at 37 °C, or with CP (5 mM) and CPK (40 units/ml) for 15 s at 37 °C, before the addition of agonist (Rittenhouse-Simmons, 1984). Aggregation was monitored in response to PMA (30 nM; 4 min), collagen (1.8 $\mu\text{g}/\text{ml}$; 3 min) and A23187 (0.5 μM , 1 μM and 5 μM ; 3 min).

In experiments where histamine was analysed, an alkaline-chloroform/butanol extraction was carried out on the entire sample as described by Keyzer *et al.* (1984).

Histamine analysis

Histamine was measured by h.p.l.c. (Saxena *et al.*, 1989a,b, 1990). Extracted histamine was converted into a fluorogenic derivative with *o*-phthalaldehyde (Shore *et al.*, 1959), and separated on a $\mu\text{Bondapak C}_{18}$ column by isocratic elution with a mobile phase containing 41.5% methanol/acetonitrile (83:17, v/v) and 58.5% 0.02 M-phosphate buffer, pH 6.0, at a flow rate of 1 ml/min. Fluorescence was monitored with a model 121 fluorimeter (Gilson, Middletown, WI, U.S.A.) with emission at 460 nm and excitation at 356 nm. Peaks were recorded and integrated with a model 745B Data Module (Waters Associates, Milford, MA, U.S.A.). The elution time of histamine was 24.5 min, and there were no interfering peaks. Histamine standards were run daily to ascertain consistency of the elution time and sensitivity of the assay. The detection limit of the assay was 200 pg of histamine, and day-to-day variation was less than 5%.

Protein phosphorylation studies

Platelets were resuspended in 1 ml of a phosphate-free buffer (150 mM- NaCl , 4 mM- KCl , 1 mM- MgCl_2 , 5 mM-Hepes, 10 mM-dextrose, pH 7.4, with NaOH) and preincubated with 1 mCi of $\text{H}_2^{32}\text{PO}_4$ for 1 h at 37 °C. After centrifugation at 800 *g* for 11 min at 20 °C, the platelets were resuspended at a concentration of 4×10^8 /ml in the modified Hanks solution and stirred in the aggregometer at 37 °C with or without inhibitor or agonist as described above. Samples (100 μl) were removed at 0 and 3 min when collagen and A23187 were used, or at 0 and 4 min when PMA was used, immediately added to 50 μl of denaturing solution (6% SDS, 6% 2-mercaptoethanol, 30% glycerol, 3 mM-EDTA, 12 mM-EGTA, 0.03% Bromophenol Blue, 450 mM-Tris, pH 6.8), heated at 100 °C for 3 min, and then frozen until electrophoresed.

Proteins were loaded on to a 4%-acrylamide stacking and a 12% running polyacrylamide/SDS vertical slab gel (Laemmli, 1970) and separated as described elsewhere (Gerrard *et al.*, 1987b). The gel was fixed, stained at 37 °C in 25% propan-2-ol/10% acetic acid/0.05% Coomassie Brilliant Blue R, destained in 10% methanol/10% acetic acid, dried under vacuum at 80 °C for 2 h, and exposed to Kodak BB-1 X-ray film. Phosphorylated protein bands were identified on the autoradiogram. Bands of molecular mass 47 kDa, corresponding to pleckstrin, and 20 kDa, corresponding to myosin light chain,

were cut from the gels, solubilized with 80% Protosol, and their radioactivity was determined in a Beckman LS 7800 liquid-scintillation counter.

Data analysis

The values are means $\pm$ S.E.M. unless otherwise indicated. Statistical differences between groups were determined by unpaired Student's *t* test, with *P* = 0.05 being considered a significant difference.

RESULTS

Effects of staurosporine on PMA-induced platelet activation

PMA (30 nM) induced aggregation, synthesis of intracellular histamine and phosphorylation of the platelet protein pleckstrin

Fig. 1. Inhibition of PMA-induced platelet aggregation (■), histamine synthesis (▲) and phosphorylation of pleckstrin (P47) (●) by staurosporine

Results are means $\pm$ S.E.M. for three to five experiments using platelets from different donors. Where error bars are not shown, the S.E.M. values lie within the dimensions of the symbols.

Fig. 2. Inhibition of collagen-induced platelet aggregation (■), histamine synthesis (▲) and phosphorylation of pleckstrin (P47) (●) by staurosporine

Results are means $\pm$ S.E.M. for three to five experiments using platelets from different donors. Where error bars are not shown, the S.E.M. values lie within the dimensions of the symbols.

Fig. 3. Effects of staurosporine on A23187-induced aggregation (■) and phosphorylation of myosin light chain (P20) (●)

Results are means $\pm$ S.E.M. for three to five experiments using platelets from different donors. Where error bars are not shown, S.E.M. values lie within the dimensions of the symbols.

(P47). Preincubation (30 s) with the PKC inhibitor staurosporine inhibited aggregation (IC_{50} 32 ± 3 nM), histamine synthesis (IC_{50} 39 ± 2 nM) and phosphorylation of P47 (IC_{50} 28 ± 6 nM) in a concentration-dependent manner (Fig. 1). These IC_{50} values were not significantly different. PMA also induced the phosphorylation of myosin light chain (P20), an effect inhibited in parallel with P47 phosphorylation by preincubation for 30 s with staurosporine.

Effects of staurosporine on collagen-induced platelet activation

Collagen ($1.8 \mu\text{g/ml}$) induced platelet aggregation, synthesis of intracellular histamine and phosphorylation of P47 and P20 proteins. Preincubation (30 s) with staurosporine inhibited collagen-induced aggregation (IC_{50} 35 ± 3 nM), histamine synthesis (IC_{50} 60 ± 8 nM) and phosphorylation of P47 protein (IC_{50} 24 ± 2 nM) (Fig. 2). The IC_{50} value for histamine synthesis was significantly higher than that for either aggregation ($P < 0.005$) or P47 phosphorylation ($P < 0.01$). The phosphorylation of P20 was also inhibited by staurosporine, but with a higher IC_{50} value, 119 ± 20 nM.

Effects of staurosporine on A23187-induced platelet activation (Fig. 3)

The Ca^{2+} ionophore A23187 ($1 \mu\text{M}$) stimulated platelet aggregation and phosphorylation of myosin light chain (P20) and,

Table 1. Histamine synthesis in platelets stimulated by PMA and A23187

Results are means $\pm$ S.E.M. for the numbers of samples in parentheses.

Additions	Histamine (pmol/ 10^9 platelets)
0.4 % DMSO	10.59 ± 0.23 (22)
30 nM-PMA	36.56 ± 0.76 (16)
0.5 μM -A23187	7.56 ± 1.67 (7)
1 μM -A23187	1.96 ± 0.99 (6)
0.5 μM -A23187 + 30 nM-PMA	36.38 ± 1.29 (4)
1.0 μM -A23187 + 30 nM-PMA	20.33 ± 0.84 (4)
5.0 μM -A23187 + 30 nM-PMA	13.00 ± 0.24 (4)

Table 2. Effects of inhibitors of cyclo-oxygenase and ADP on platelet histamine content

Results are means $\pm$ S.E.M. for four samples. Abbreviation: ADA, adenosine deaminase.

Additions	Histamine (pmol/ 10^9 platelets)
0.4 % DMSO	10.49 ± 0.46
Aspirin + 0.4 % DMSO	10.99 ± 1.74
Aspirin + 30 nM-PMA	37.38 ± 3.06
Aspirin + 0.5 μM -A23187	9.72 ± 0.79
Aspirin + apyrase + ADA + 0.4 % DMSO	6.42 ± 4.96
Aspirin + apyrase + ADA + 0.5 μM -A23187	10.46 ± 1.28
Aspirin + CP + CPK + 0.4 % DMSO	9.61 ± 1.15
Aspirin + CP + CPK + 0.5 μM -A23187	10.64 ± 0.30

to a lesser extent, of P47. Preincubation with staurosporine (30 s) inhibited A23187-induced aggregation (IC_{50} 188 ± 25 nM) and phosphorylation of P20 (IC_{50} 130 ± 40 nM) and P47 (IC_{50} 75 ± 12 nM).

Histamine synthesis in platelets stimulated by PMA and A23187

Platelets stimulated by PMA (30 nM; 4 min) showed an increase in intracellular histamine (Table 1), and this increase remained unaffected by pretreatment with aspirin (Table 2). In contrast, A23187 (0.5 μM ; 3 min) failed to cause any increase in histamine in the presence or absence of inhibitors of cyclo-oxygenase and ADP (Table 2). However, co-addition of PMA (30 nM) with 1 μM - or 5 μM -A23187 significantly decreased the PMA-induced histamine synthesis (Table 1).

DISCUSSION

The synthesis of intracellular histamine is important for platelet responses to agonists such as collagen, PMA, thrombin and platelet-activating factor (Saxena *et al.*, 1989a,b 1990; McNicol *et al.*, 1989). The present study associates PKC activation with this synthesis, since staurosporine, a potent PKC inhibitor (Tamaoki *et al.*, 1986; Erne *et al.*, 1987; Watson *et al.*, 1988), attenuated PMA-induced aggregation, P47 phosphorylation (an index of PKC activation) and histamine production with similar IC_{50} values (Fig. 1). The finding that myosin light chain was also phosphorylated in response to PMA, an effect inhibited by staurosporine at concentrations similar to those required to inhibit P47, is in agreement with previous reports which suggest that myosin light chain can serve as a substrate for PKC (Kawamoto *et al.*, 1989; Naka *et al.*, 1983). The close correlation between PKC activation and histamine synthesis suggests that the two are related events. As platelets are non-nucleated and protein-synthesis inhibitors fail to affect PMA-induced histamine production (Saxena *et al.*, 1989a), a non-genomic activation of the histamine-synthesizing enzyme HDC by PKC must occur. Since a variety of unidentified PKC substrates are phosphorylated during platelet activation (Haslam, 1987), HDC, or an associated regulatory protein, could be one. Alternatively, it is possible that PKC-mediated phosphorylation stimulated by PMA may activate a tyrosine kinase (Nakamura & Yamamura, 1989; Golden & Brugge, 1989), which in turn activates HDC or a regulatory protein. The latter possibility is unlikely, since thrombin is less potent to induce intracellular histamine synthesis in platelets than is PMA (Saxena *et al.*, 1989a), despite being more potent to initiate tyrosine phosphorylation.

Unlike that by PMA, collagen-induced platelet aggregation is not solely dependent on PKC activation (Kinlough-Rathbone *et al.*, 1977; Rink *et al.*, 1983; Pollock *et al.*, 1986). In the present study, a close correlation between activation of PKC and aggregation was observed, whereas histamine synthesis in response to collagen was less sensitive to inhibition by staurosporine (Fig. 2). These observations suggest that non-PKC (and therefore, less staurosporine-sensitive) pathway(s) could be involved in the collagen-induced synthesis of intracellular histamine. Since different type(s) of PKC isoforms may be activated by different agonists (Coussens *et al.*, 1986; Knopf *et al.*, 1986; Ohno *et al.*, 1987), staurosporine could preferentially inhibit certain of these PKC isoforms (Yamamoto *et al.*, 1989).

The Ca^{2+} -ionophore-A23187-induced platelet aggregation, which does not involve PKC activation (Rittenhouse-Simmons, 1984), was not associated with increased histamine, suggesting that a rise in $[\text{Ca}^{2+}]_i$ by itself is not sufficient to initiate histamine synthesis (Tables 1 and 2). Moreover, the inhibitory effects of staurosporine on A23187-induced aggregation and P20 phosphorylation occurred only at higher concentrations than those believed to be specific for PKC (Fig. 3).

The observation that A23187 did not increase platelet histamine is of potential interest, and was explored further in two ways. Firstly, we used conditions described by Rittenhouse-Simmons (1984) to block the production of thromboxane A_2 and remove ADP. These conditions would rule out the possibility of any PKC activation through A23187-induced and thromboxane- and ADP-mediated increase in diacylglycerol. Under such conditions, A23187 had no effect on the synthesis of intracellular histamine. Secondly, the possibility of down-regulation of histamine synthesis in the presence of a substantial increase in Ca^{2+} concentrations by A23187 was considered. Histamine synthesis was measured in platelets stimulated with both A23187 and PMA. As the concentration of A23187 was increased, there was a progressive decrease in PMA-induced histamine synthesis (Table 1). These findings suggest that an increase in $[\text{Ca}^{2+}]_i$ may decrease the PMA-induced increase in histamine, either by inhibiting its synthesis or by augmenting its metabolism. In addition, large increases in $[\text{Ca}^{2+}]_i$ may also over-ride the slight activation of PKC induced by A23187, resulting in no significant increase in histamine in these platelets. These results may also provide an explanation for our previous findings (Saxena *et al.*, 1989a,b, 1990) that platelets stimulated by collagen and thrombin, agonists which mobilize Ca^{2+} , as well as activate PKC, synthesize less intracellular histamine than those stimulated by PMA, an agonist known to act through direct activation of PKC.

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