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N-Ethylmaleimide Inhibition of Thrombin-Induced Platelet Aggregation

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ABSTRACT. The data presented in this report show that *N*-ethylmaleimide (NEM) is a powerful inhibitor of thrombin-induced platelet aggregation. NEM increased guanosine 3′, 5′-cyclic monophosphate (cGMP) and adenosine 3′, 5′-cyclic monophosphate (cAMP) levels in intact cells. The inhibition of cAMP high-affinity phosphodiesterase and cGMP phosphodiesterase was implicated in the elevation of the cyclic nucleotides. NEM dose dependently blocked the thrombin-stimulated, but not the phorbol myristate acetate-dependent phosphorylation of the protein kinase C substrate pleckstrin. Myosin light chain phosphorylation was also inhibited by NEM. In addition, the sulphydryl reagent inhibited Ca^{2+} mobilisation induced by thrombin. The data indicate that phospholipase C activation by thrombin is interrupted by NEM at the level of receptor-mediated signal transduction. BIOCHEM PHARMACOL **58**;8:1293–1299, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. NEM; aggregation; thrombin; cyclic nucleotides; human platelets

It is known that various intracellular functions are modified by an imbalance between oxidants and antioxidants. The intracellular redox state may influence the activity of a variety of enzymes. GSH is the major intracellular redox buffer in almost cell types, and lowering intracellular GSH selectively increases the stimulation of tyrosine phosphorylation by inflammatory cytokines in lymphocytes [1]. Moreover, oxidants that react with thiols, such as phenylarsine oxide, diamide, and NEM†, increase tyrosine phosphorylation in the Jurkat T-cell line [1, 2]. The ligand–receptor interaction and specific steps in signal transduction may be influenced by modifications in the cellular redox state. Insulin and epidermal growth factor give different responses to NEM and iodoacetamide depending on whether they are in the basal or the activated state [3]. The thiol-reactive agent NEM is able to induce arachidonic acid release in endothelial cells [4] and human platelets [5]. NEM is also involved in the modulation of noradrenaline release from rabbit hippocampus synaptosomes [6].

Thrombin is a potent and efficient inhibitor of adenylate cyclase in human platelet membranes [7]. Thrombin-induced inhibition of adenylate cyclase is accompanied by an increased GTP hydrolysis by stimulation of high-affinity GTPase in the G_{Si} [8]. NEM at rather low concentrations abolishes thrombin-induced adenylate cyclase inhibition and partially blocks GTPase stimulation [8] through the alkylation of specific cysteine residues present in the $G_{o\alpha}$ subunit and involved in the functions of the signal-coupling proteins [9].

In this paper we show that NEM specifically inhibits thrombin-induced platelet aggregation. Increases in cGMP and cAMP intracellular levels are likely to be involved in platelet inhibition by NEM. In addition, the sulphydryl reagent affects the phospholipase C pathway by blocking Ca^{2+} elevation as well as pleckstrin and myosin light chain phosphorylation produced by thrombin.

MATERIALS AND METHODS

Chemicals

[^{3}H]cAMP, [^{3}H]cGMP, cAMP and cGMP radioimmunoassays, [^{32}P]phosphoric acid, and HyperfilmMP were from Amersham International. Thrombin, PMA, ADP, 5′-nucleotidase from *Crotalus atrox* venom (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), NEM, EGTA, leupeptin, Dowex 1 × 8-400, and forskolin from Sigma Chemical Co. Fura-2acetoxymethyl ester and PAF-C18 were from Calbiochem-Novabiochem International. Pico-Fluor™ 40 was from Packard Instruments Co. and collagen from Mascia Brunelli SpA. The stable PGI_2 analogue iloprost was a gift from Schering AG. PMA and PAF-C18 stock ethanol solutions were further diluted in saline.

Human Platelet Preparation and Aggregation

Human blood obtained from healthy volunteers was collected in 130 mM Na-citrate (9:1), and PRP and WP were prepared as previously described [10]. Aggregation, performed on an Aggrecoder PA-3210 Menarini Diagnostics aggregometer, was measured according to the turbidimetric

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† *Abbreviations:* cAMP, adenosine 3′, 5′-cyclic monophosphate; cGMP, guanosine 3′, 5′-cyclic monophosphate; IP_3, inositol 1,4,5-trisphosphate; NEM, *N*-ethylmaleimide; PAF-C18, platelet-activating factor-C18; PGI_2, prostacyclin; PKC, protein kinase C; PMA, phorbol myristate acetate; PRP, platelet-rich plasma; and WP, washed platelets.

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method of Born [11] and quantified by the light transmission reached within 3 min. PRP and/or WP were preincubated for 2 min at 37° with saline or NEM before agonist addition.

cAMP and cGMP Measurement

Cyclic nucleotide formation was measured as previously described [12]. WP (1.0×10^9 platelets/mL) were preincubated for 5 min at 30° with saline or NEM, then iloprost, forskolin, or thrombin was added where required. The reaction was stopped after 5 min by the addition of cold 2.0 M perchloric acid. The mixtures were centrifuged for 3 min at 12,000 *g*, and the supernatants neutralised with 2.0 M NaOH were analysed for cAMP or cGMP content by radioimmunoassay kits.

Phosphodiesterase Assay

Phosphodiesterase activity was measured in a soluble fraction obtained as follows. WP (2.0×10^9 platelets/mL) containing 50 μg/mL leupeptin were sonicated twice for 15 sec. The disrupted platelet suspension was centrifuged at 1000 *g* for 20 min and the recovered supernatant was centrifuged at 100,000 *g* for 60 min. Phosphodiesterase activity was assayed after 10 min of preincubation of the soluble fraction with saline or NEM at room temperature. A further 10-min incubation at 30° was started by the addition of 1.0 μM [^{3}H]cAMP or 1.0 μM [^{3}H]cGMP and terminated by boiling. Then, each mixture was added to 1.25 U/mL of 5′-nucleotidase and incubated for a further 10 min at 30°. [8-^{3}H] Adenosine was eluted with methanol from a 1.5-mL column of Dowex 1×8-400 and counted for ^{3}H in Pico-Fluor™ 40 reagent.

Protein Phosphorylation

Platelets, resuspended at 2.5×10^9 platelets/mL in pH 7.4 HEPES buffer containing 1.0 mM EGTA and 5% platelet-poor plasma, were incubated for 60 min at 37° with 250 μCi of [^{32}P]phosphoric acid/mL under gentle shaking, washed once, and finally resuspended to 2.0×10^8 platelets/mL in the same buffer containing 2.0 mM $CaCl_2$. Samples were then incubated for 30 min at 37° with saline or NEM as indicated. In the experiments in which the effect of NEM on phosphorylation induced by thrombin or PMA was studied, platelets were preincubated for 10 min at 37° with saline or NEM and then stimulated for 6 min with thrombin or PMA. A suitable aliquot of 2X Laemmli SDS reducing gel sample buffer containing 10% β-mercaptoethanol stopped incubation. Samples were boiled for 2 min and proteins separated by 13% SDS-PAGE. Running was performed in the presence of molecular weight markers. Gels were dried and [^{32}P]-phosphorylated bands were observed by autoradiography by exposure to Amersham HyperfilmMP.

TABLE 1. Effect of NEM on platelet aggregation

Agonists	IC_{50} (μM) PRP	WP
ADP	157 ± 35	ND
Collagen	185 ± 50	ND
PAF	315 ± 54	ND
Thrombin	ND	18 ± 1
PMA	335 ± 33	ND

PRP or WP (2.0×10^8 platelets/mL) were incubated with saline (control) or NEM for 2 min at 37°, after which 5.0 μM ADP, 5.0 μg/mL collagen, 1.0 μM PAF, 1.0 μM PMA, or 0.1 U/mL thrombin was added. Values are means ± SD of at least six separate determinations. ND: not determined.

Measurements of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured according to Rotondo *et al.* [13].

Statistical Analysis

Student's *t*-test was used to determine the significance of differences between two groups.

RESULTS

The NEM effect on platelet aggregation induced by agonists able to produce irreversible aggregation of PRP was studied. To avoid any clotting problems, thrombin was tested in WP. In PRP, the alkylating agent poorly inhibited platelet aggregation, showing the highest potency in the presence of ADP (IC_{50} = 157 ± 35 μM) and the lowest power in platelets challenged by PMA (IC_{50} = 335 ± 33 μM). Nevertheless, NEM was much more active in WP stimulated with thrombin, the IC_{50} value being 18 ± 1 μM (Table 1). The inhibiting effect of NEM on thrombin-induced aggregation was dose dependently potentiated by the PGI_2 analogue iloprost. In the presence of 10 or 20 nM iloprost, concentrations that by themselves produce a very poor inhibition of aggregation, the NEM IC_{50} value decreased from 17.2 μM to 12.8 or 5.4 μM, respectively (Fig. 1). Iloprost inhibited aggregation induced by thrombin with an IC_{50} of about 40 nM (inset).

In order to clarify the action mechanism of NEM, we wanted to test the effect of the sulphydryl reagent on cGMP and cAMP platelet intracellular formation. The results showed that NEM increased cGMP both in resting and in thrombin-stimulated platelets but produced the maximal effect in stimulated platelets, where the basal level rose from 1.2 ± 0.2 to 2.0 ± 0.3 pmoL/10^8 platelets (Fig. 2). Thus, cGMP elevation may enhance the cAMP level and response through the inhibition of cAMP high-affinity phosphodiesterase [14]. As expected, NEM was able to significantly increase the platelet cAMP basal level (Fig. 3, inset) and to potentiate adenylate cyclase activators such as iloprost and forskolin by producing the maximal cooperation at 50 μM. Higher concentrations than this rapidly decreased cAMP in platelets treated with iloprost and had

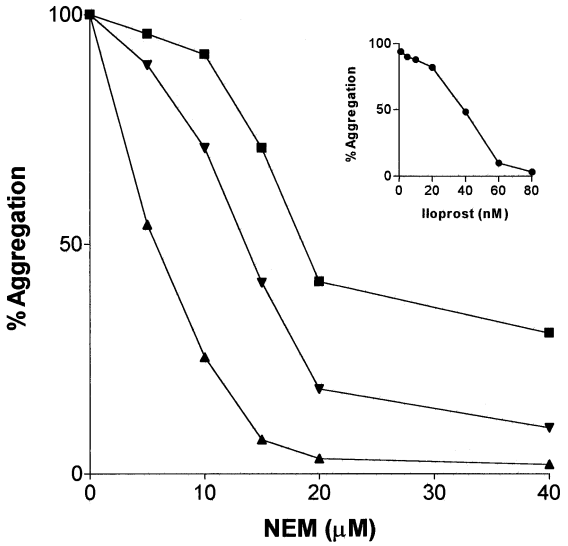

FIG. 1. Inhibition of thrombin-induced platelet aggregation by NEM and/or iloprost. WP (2.0×10^8 platelets/mL) preincubated with NEM and/or iloprost for 2 min at 37° were stimulated with 0.1 U/mL thrombin. Data, expressed as the percentage of the maximum platelet aggregation obtained in the control sample, are from a single measurement, but representative of three confirmatory experiments. (■) NEM; (▼) NEM + 10 nM iloprost; (▲) NEM + 20 nM iloprost.

a smaller effect in platelets incubated with forskolin (Fig. 3).

Thrombin was able to significantly reduce cAMP intracellular levels both in control and in platelets treated with iloprost. NEM counteracted the thrombin effect and cooperated with iloprost by producing a significant increase in cAMP levels (Fig. 4). The cAMP elevation could be

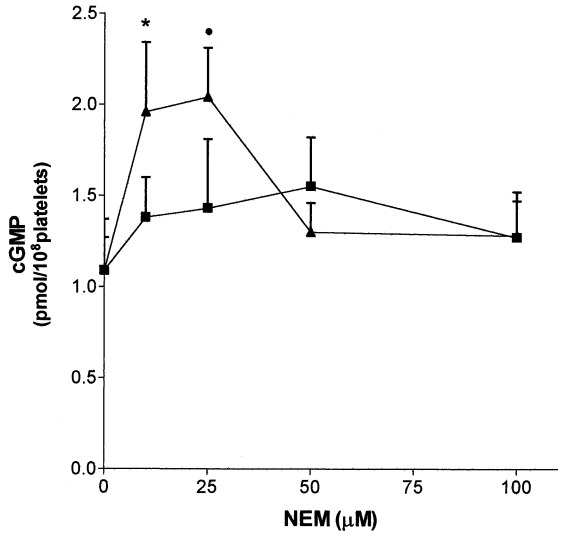

FIG. 2. Effect of NEM on cGMP formation. WP (1.0×10^9 platelets/mL) were preincubated with increasing NEM concentrations for 5 min at 30°, challenged by thrombin (0.2 U/mL) where required, and incubated for 5 min at 30°. cGMP was measured by an RIA kit. Data are means ± SD of four experiments. (■) None; (▲) Thrombin. *$P < 0.025$; ●$P < 0.05$ versus none.

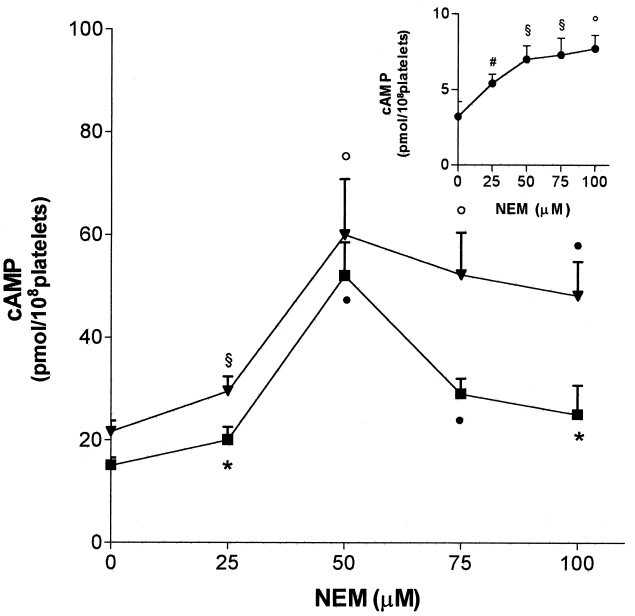

FIG. 3. Effect of NEM on cAMP elevation. WP (1.0×10^9 platelets/mL) were preincubated with NEM for 5 min at 30°, then saline (●), 50 nM iloprost (■), or 50 μM forskolin (▼) was added. After 5 min at 30°, the reaction was stopped and cAMP measured by an RIA kit. Each point represents the mean ± SD of four determinations. ●$P < 0.0005$; ○$P < 0.0025$; *$P < 0.025$; §$P < 0.01$; #$P < 0.05$ versus control without NEM.

dependent on the stimulation of adenylate cyclase and/or the inhibition of cAMP phosphodiesterase. Since NEM (10–50 μM) does not modify adenylate cyclase activity in membranes of human platelets [8 and our data, not shown], the involvement of cAMP phosphodiesterase was likely. cAMP breakdown could be inhibited by a light elevation of cGMP intracellular concentration [14] and/or by a direct effect at the level of one or more specific residues involved in the catalytic activity of the enzyme. From three series of experiments in which NEM was tested at six increasing concentrations, a percentage inhibition–concentration curve was derived from which the IC_{50} value was calculated. As shown in Fig. 5, NEM is a strong inhibitor of cAMP high-affinity phosphodiesterase, the IC_{50} being 16 ± 2 μM. Moreover, low concentrations of NEM were also able to inhibit cGMP phosphodiesterase (IC_{50} = 20 ± 6 μM). The inhibition of cAMP and cGMP phosphodiesterase could be consistent with the modification of one or more essential cysteine residues.

It is known that a minute increase in cAMP blocks platelet function and that many different steps are involved [15], such as receptor-mediated phosphoinositide hydrolysis by phospholipase C activation [16]. As thrombin activates the phospholipase C pathway, Ca^{2+} elevation and phosphorylation of pleckstrin (47 kDa) and myosin light chain (20 kDa) can be considered indicative of phospholipase C activation. Therefore, we attempted to investigate the NEM effect both on protein phosphorylation and Ca^{2+} elevation. Preincubation of platelets with NEM strongly

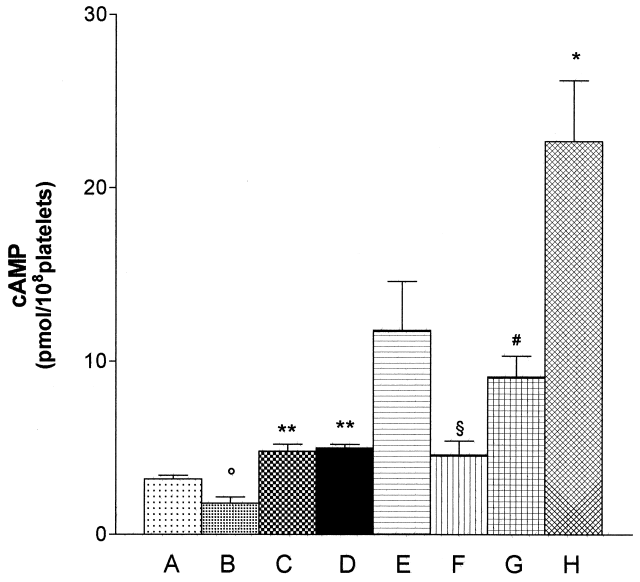

FIG. 4. Effect of NEM and/or iloprost on cAMP elevation in thrombin-stimulated platelets. WP (1.0×10^9 platelets/mL) were preincubated with saline or NEM for 5 min at 30°, then 50 nM iloprost and/or thrombin (0.2 U/mL) was added when required. Values are means ± SD of three experiments carried out in duplicate. (A) none; (B) thrombin; (C) thrombin + 25 μM NEM; (D) thrombin + 50 μM NEM; (E) iloprost; (F) thrombin + iloprost; (G) thrombin + iloprost + 25 μM NEM; (H) thrombin + iloprost + 50 μM NEM. ○$P < 0.0025$ versus none; **$P < 0.0005$ versus thrombin; §$P < 0.005$ versus iloprost; *$P < 0.0005$; and #$P < 0.0025$ versus thrombin + iloprost.

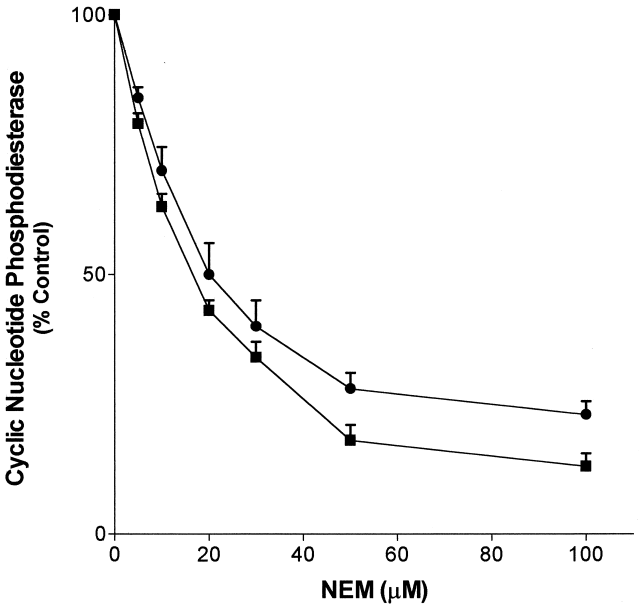

FIG. 5. Inhibition of platelet cyclic nucleotide phosphodiesterases by NEM. Cytosolic human cAMP high-affinity phosphodiesterase (■) and cGMP phosphodiesterase (●) activities were determined in the presence or absence of increasing concentrations of NEM. Data are presented as percentage of maximal activity (mean ± SD).

inhibited the thrombin-induced phosphorylation of pleckstrin and myosin light chain. At 25 μM NEM, phosphorylation of both proteins was abolished. To investigate which step in the activation by thrombin was sensitive to NEM, the experiments were repeated during direct stimulation of PKC by PMA. No inhibition in PMA-challenged platelets was observed. (Fig. 6A). Unstimulated platelets showed no significant change in [^{32}P] radioactivity of pleckstrin or myosin light chain during platelet treatment with NEM. Nevertheless, two proteins with apparent molecular masses of 50 and 68 kDa appeared to be phosphorylated by the alkylating agent (Fig. 6B). Platelet treatment with thrombin caused a dramatic increase in cytosolic Ca^{2+} levels. NEM was able to inhibit the thrombin-induced Ca^{2+} elevation in a time- and dose-dependent manner. One hundred micromoles/L NEM produced a rapid drop in the intracellular Ca^{2+} level attained, while 25 and 50 μM NEM had a slower effect (Fig. 7A). To define the sources of the increased cytoplasmatic Ca^{2+} inhibited by NEM, the experiments were performed in the presence of 2.5 mM EGTA. Under these conditions, NEM decreased the peak of intracellular Ca^{2+} produced by thrombin and accelerated its removal, suggesting that the sulphydryl reagent is able to block both the intracellular signal IP_3 formation for Ca^{2+} mobilisation and the Ca^{2+} extracellular flux (Fig. 7B).

DISCUSSION

NEM is a potent inhibitor of platelet aggregation induced by thrombin and cooperates with the PGI_2 analogue iloprost in this effect. Low concentrations of NEM increase cGMP intracellular levels both in resting and in thrombin-stimulated platelets. The cGMP elevation can be consistent with the strong inhibition exerted by NEM on cGMP phosphodiesterase. In addition, NEM elevates cAMP in human platelets and markedly potentiates the effect of known adenylate cyclase activators such as iloprost and forskolin. It is known that cGMP and cAMP both act as inhibitors of platelet activation. The cyclic nucleotides are regulated by various phosphodiesterases. In platelets, the majority of cAMP phosphodiesterase activity is localised in the cytosolic fraction and is inhibited by low cGMP concentrations. Thus, it is likely that as platelet cGMP levels begin to rise in response to NEM, cAMP phosphodiesterase is inhibited [14]. This inhibition leads to an increase in cAMP concentration, resulting in both nucleotides contributing to platelet inhibition. Moreover, other potential sites of interaction of these cyclic nucleotides are present in platelets, such as the activation of the cyclic nucleotide-dependent protein kinases [17] and the phosphorylation of the same substrate in response to an increase in the concentration of either cAMP or cGMP [18].

Thrombin is a potent platelet activator which stimulates aggregation and secretion and induces phospholipase C activation, arachidonate release, PKC activation, and Ca^{2+} mobilisation [16]. Moreover, thrombin inhibits adenylate cyclase activity in both membrane preparations and intact

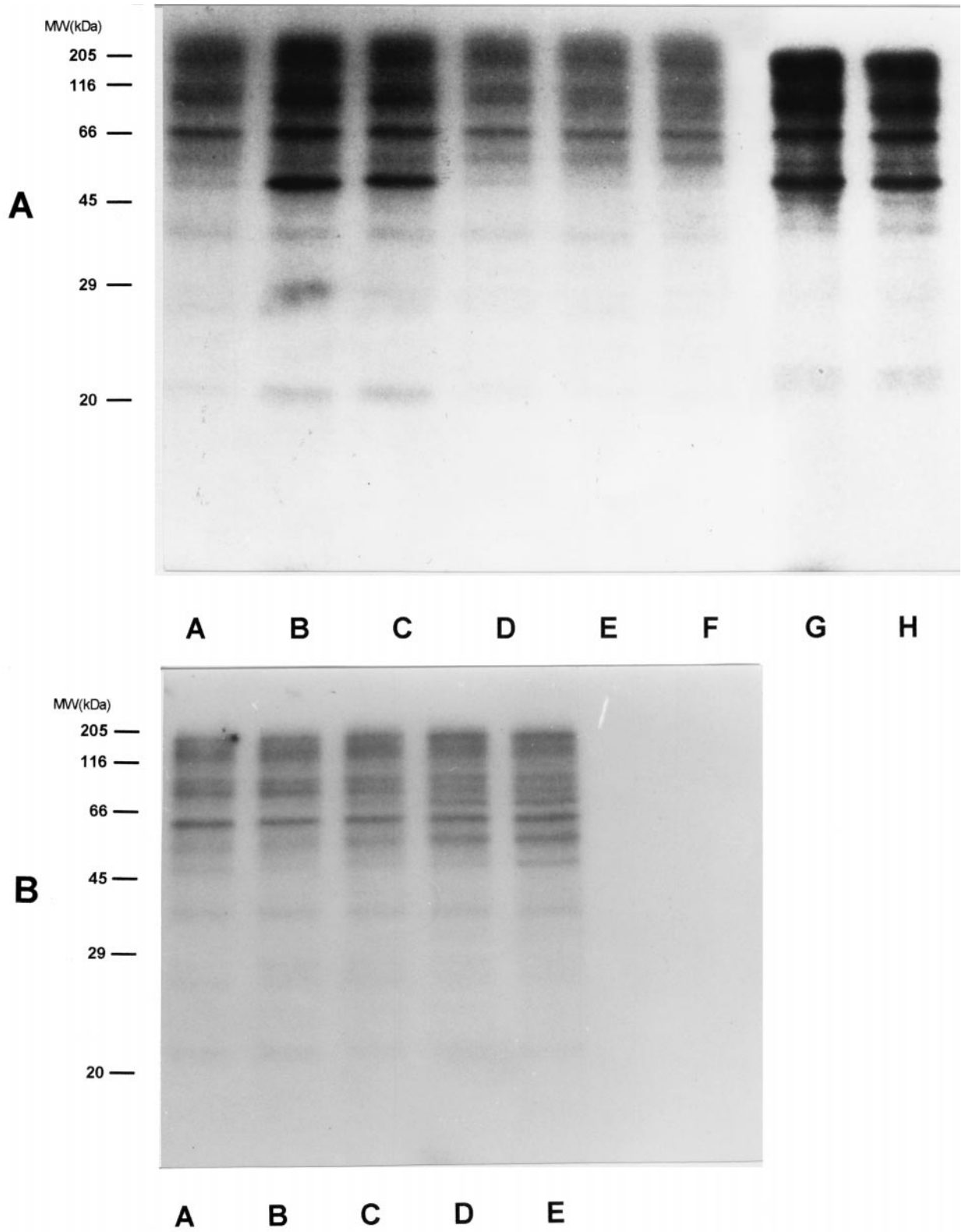

FIG. 6. Effect of NEM on platelet phosphorylation. WP (2.0×10^8 platelets/mL), labelled with [^{32}P], were preincubated for 10 min at 37° with saline or NEM and stimulated with 0.1 U/mL thrombin or 1 μM PMA (A). In (B), WP were incubated for 30 min at 37° with saline or increasing concentrations of NEM. In both types of experiments, incubation was stopped by boiling samples for 2 min. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography. The figure shows the autoradiography patterns of 13% SDS-PAGE representative of three separate experiments. (A) Lane A: none; lane B: thrombin; lane C: thrombin + 10 μM NEM; lane D: thrombin + 25 μM NEM; lane E: thrombin + 50 μM NEM; lane F: thrombin + 100 μM NEM; lane G: PMA; lane H: PMA + 100 μM NEM. (B) Lane A: none; lane B: 10 μM NEM; lane C: 25 μM NEM; lane D: 50 μM NEM; lane E: 100 μM NEM.

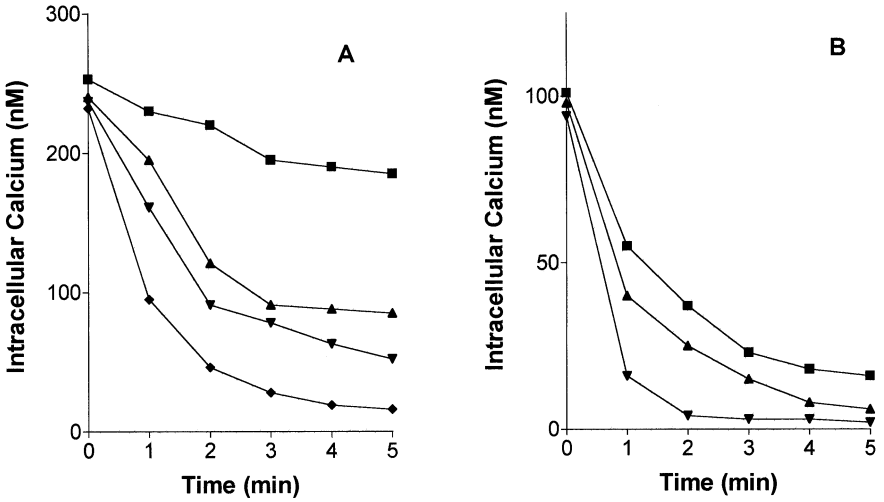

FIG. 7. Effect of NEM on thrombin-induced intracellular Ca^{2+} mobilisation. Fura-2-loaded platelets, resuspended at 2.0×10^8 platelets/mL in pH 7.4 HEPES buffer containing 1.0 mM $CaCl_2$ (A) or 2.5 mM EGTA (B), were preincubated for 2 min at 37° with saline (■), 10 μM (▲) NEM, 25 μM (▼) NEM, or 50 μM (◆) NEM, prior to the addition of 0.1 U/mL thrombin. The data are representative from experiments performed in triplicate with similar results.

human platelets, thus reducing intracellular cAMP levels [7, 19]. A pertussis toxin-sensitive G_i protein is involved in this inhibition [7, 8]. Since thrombin stimulation of phospholipase C activity in platelets is sensitive to pretreatment with pertussis toxin, it is tempting to suggest a role for $G\beta\gamma$ subunits derived from G_i in stimulating phospholipase C activity [20].

After NEM treatment of platelet membranes, thrombin-induced adenylate cyclase inhibition is partially reduced [7] and the intracellular cAMP levels subsequently increased. The cAMP elevation prevents agonist-induced activation of phosphoinositide-specific phospholipase C [16] and inhibits IP_3-induced Ca^{2+} release [21]. In addition, a small rise in cGMP intracellular concentration inhibits phospholipase C activation, IP_3 generation, and Ca^{2+} mobilisation [22]. cGMP inhibitory effects are mediated by a cGMP-dependent protein kinase [23]. Recently, it has been shown that thromboxane A_2-receptors are substrates for the cGMP-dependent protein kinase [24]. NEM inhibits the thrombin-induced phosphorylation of 20 and 47 kDa proteins. Since these proteins of platelets are phosphorylated during thrombin stimulation by activated myosin light chain kinase or PKC, respectively, the results shown in Fig. 6A suggest that not only the cAMP-dependent protein kinase, but also the cGMP-dependent protein kinase inhibit the activation pathways for both PKC and myosin light chain kinase. On the other hand, NEM has no effect in platelets challenged with PMA, which bypasses receptors and activates PKC, suggesting that the NEM effect is directed to one or more specific steps in the very early activation of G-protein-coupled receptors. NEM also inhibits the phosphorylation of myosin light chain (20 kDa). Since the phosphorylation of this light chain occurs when intracellular Ca^{2+} concentrations rise and the calmodulin-dependent myosin light chain kinase is activated [25], the inhibitory action of NEM may result from its ability to prevent the thrombin-induced increase in cytoplasmatic Ca^{2+} concentrations (Fig. 7A). On the other hand, NEM by itself is able to induce protein phosphorylation. In particular, the 50 kDa and 68 kDa proteins appeared clearly phosphorylated upon NEM treatment. The cAMP-dependent protein kinase is involved in the 68 kDa phosphorylation [26], and the 50 kDa phosphorylation "*in vitro*" was shown to be mediated by the cAMP- and cGMP-dependent protein kinases [21, 27, 28].

In conclusion, the data reported in the present paper suggest that NEM inhibits platelet aggregation induced by thrombin through a significant intracellular elevation of both cGMP and cAMP.

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