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N-Ethylmaleimide-Stimulated Arachidonic Acid Release in Human Platelets

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ABSTRACT. Treatment of human platelets with the alkylating agent *N*-ethylmaleimide (NEM) induces arachidonic acid release. The effect was time- and dose-dependent. NEM-stimulated arachidonic acid mobilisation could be prevented by pretreating platelets with the cytosolic phospholipase A_2 ($cPLA_2$)-specific inhibitor arachidonyltrifluoromethyl ketone. Moreover, the tyrosine kinase inhibitor genistein was able to significantly inhibit arachidonic acid mobilisation. NEM-stimulated release of arachidonic acid appears to be a Ca^{2+}-dependent mechanism, as shown by the observation that arachidonic acid mobilisation was significantly reduced by platelet treatment with EGTA and abolished by preloading platelets with the intracellular chelator 1,2-*bis* (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA/AM). In Fura-2-loaded platelets, NEM was able to significantly increase the intracellular Ca^{2+} level. The Ca^{2+} elevation was significantly reduced in the presence of EGTA and suppressed by cell treatment with BAPTA/AM. Arachidonic acid released by NEM produced a significant increase in reactive oxygen species (ROS) intracellular levels, which was partially inhibited by diphenyleneiodonium and almost completely suppressed by 5,8,11,14-eicosatetraynoic acid. In conclusion, the results in this study demonstrate that NEM stimulates arachidonic acid release by $cPLA_2$ activation through intracellular Ca^{2+} elevation. In addition, tyrosine specific protein kinases seem to be involved in arachidonic acid release. ROS was also shown to be formed during arachidonic acid metabolisation. BIOCHEM PHARMACOL **57**;7:785–791, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. human platelets; *N*-ethylmaleimide; cytosolic phospholipase A_2; arachidonic acid; calcium; reactive oxygen species

PLA_2† is a class of enzymes which catalyses the hydrolysis of arachidonic acid and other unsatured fatty acids from the sn-2 position of phospholipids. Two PLA_2 isoforms have been described in human platelets: type II 14kDa secreted enzyme ($sPLA_2$) and type IV 85kDa enzyme ($cPLA_2$). The 14kDa isoform is thought to play a role in extracellular fluids after its release from secretory granules, where it is stored [1]. Whether $sPLA_2$ is involved in the liberation of arachidonic acid in stimulated platelets is still unclear. On the contrary, the involvement of $cPLA_2$ in the receptor-coupled release of arachidonic acid has been well documented [2, 3]. This enzyme has a cytosolic localisation, is highly specific for arachidonyl residues [2], and requires a submicromolar concentration of Ca^{2+} and phosphorylation for full activation [3]. Arachidonic acid released from membrane phospholipids can subsequently be metabolised to various bioactive lipid mediators such as prostaglandins, thromboxane, and leukotrienes [4, 5]. In addition, arachidonic acid, during its metabolisation both by the cyclooxygenase and lipooxygenase pathways [6, 7], can be a source of important mediators for processes such as inflammation, immune response, and NADPH oxidase activation. Many authors have reported that oxygen free radicals activate PLA_2 through stimulation of several signal pathways [8, 9]. Oxidant-mediated arachidonic acid release from various cellular systems is well described [10–12]. In addition, thiol-reactive agents such as methyl mercury [13], cadmium [14], and NEM [15] have been shown to induce arachidonic acid and eicosanoid release.

In the present study, we investigated the effect of the sulphydryl reagent NEM in intracellular signalling in relation to arachidonic acid release, intracellular Ca^{2+} concentration, and ROS formation.

MATERIALS AND METHODS

Chemicals

NEM, thrombin, genistein, PMA, EGTA, DPI, ETYA, quinacrine, aristolochic acid, *N*-acetyl-L-cysteine, and fat-

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† *Abbreviations:* PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; NEM, *N*-ethylmaleimide; PMA, phorbol myristate acetate; DPI, diphenyleneiodonium; ETYA, 5,8,11,14-eicosatetraynoic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; D609, tricyclodecan-9-yl-xanthogenate; RHC-80267, 1,6-*bis*(cyclohexyloximinocarbonylamino) hexane; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine; Ro-31-8220, 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide methane sulfonate; BAPTA/AM, 1,2-*bis*(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid tetra(acetoxymethyl)ester; fura-2/AM, fura-2 acetoxymethyl ester; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; ROS, reactive oxygen species; PKC, protein kinase C; and MAP kinase, mitogen-activated protein kinase.

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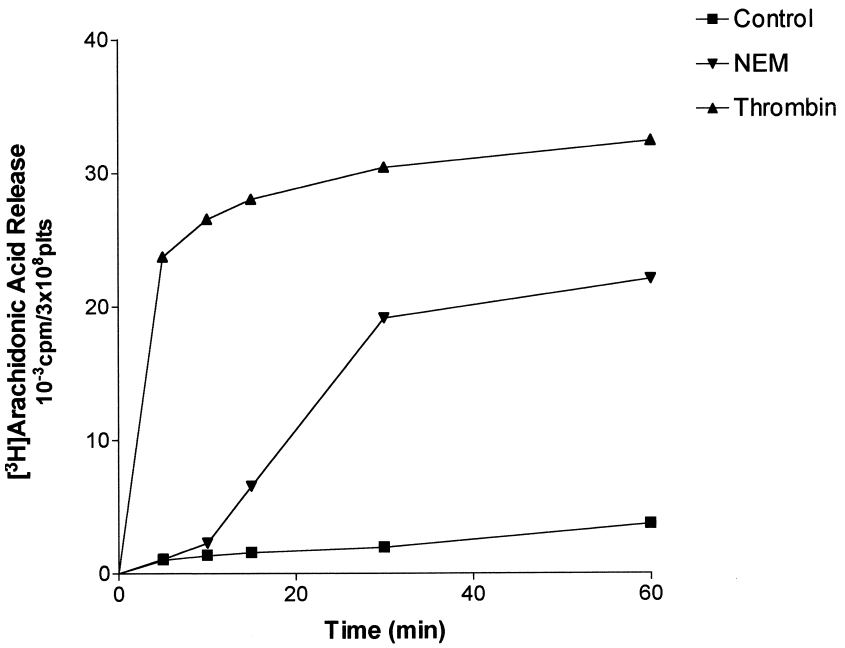

FIG. 1. Time–course of [^{3}H]arachidonic acid release from human platelets stimulated with 0.5 U/mL thrombin or 100 μM NEM. At the indicated time, suitable aliquots of the mixture were withdrawn, stopped by adding cold block mix, and counted by liquid scintillation. Values are duplicate determinations from one representative experiment.

ty-acid-free BSA were from Sigma Chemical Co. AA-$COCF_3$, D609, RHC-80267, ET-18-OCH_3, Ro-31-8220, BAPTA/AM, and fura-2/AM were from Calbiochem-Novabiochem Corp. [^{3}H]Arachidonic acid was from NEN™ Life Science Laboratories. Collagen was from Mascia Brunelli SpA. Prostaglandin E_1 was a gift from Wellcome. DCF was purchased from Fluka Chemie AG. DCFH-DA was prepared from DCF according to Brandt and Keston [16]. DCFH-DA was dissolved in DMSO, kept in the dark at room temperature at a concentration of 0.1 M, and further diluted in buffer. PMA stock ethanol solution was further diluted in saline.

Statistics

Statistically significant differences were determined by using the Student's two-tailed unpaired *t*-test.

Platelet Preparation

Blood from healthy volunteers was collected in 130 mM Na Citrate (9:1). Platelet-rich plasma was obtained by centrifuging blood at 100 *g* for 20 min. Washed platelets, prepared as previously described [17], were resuspended in pH 7.4 Tyrode's-HEPES buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$-$6H_2O$, 5.6 mM glucose, 10 mM HEPES), if not otherwise indicated.

Arachidonic Acid Release

Blood was collected in ACD (acid-citrate-dextrose)-Lagarde (9:1). Washed platelets, resuspended in pH 7.4 Tyrode's-HEPES buffer with 0.2% BSA (fatty-acid-free), were incubated with [^{3}H]arachidonic acid (1 μCi/mL) for 60 min at 37°. Over this time, platelets incorporated an average of 80% of the added [^{3}H]arachidonic acid. Prior to treatment, labelled platelets were washed twice with pH 7.4 Tyrode's-HEPES buffer and finally resuspended to 3.0×10^8/mL in the same buffer containing 0.2% fatty-acid-free BSA. All the experiments were carried out in the presence of 1.0 mM $CaCl_2$. Ten μM ETYA was added to prevent arachidonic acid metabolism. For the intracellular chelation of calcium, 100 μM BAPTA/AM was added after loading platelets with [^{3}H]arachidonic acid. Labelled platelets, preincubated for 10 min at 37°, were treated in a final volume of 500 μL with the indicated agents. Incubation, started by adding NEM, was prolonged for a further 30 min and then stopped in ice by adding 50 μL of cold block mix containing 5 mM EGTA, 5 mM theophylline, and 0.2 μg/mL prostaglandin E_1. [^{3}H]Arachidonic acid released was determined by liquid scintillation counting of 450 μL aliquots.

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured according to Rotondo *et al.* [18].

Fluorimetric Assay of ROS

ROS were measured by the fluorescent, intracellulary produced compound DCF as previously published [19].

RESULTS

Effect of NEM on [^{3}H]Arachidonic Acid Release

The time–course of arachidonic acid release in human platelets is shown in Fig. 1. The amount of arachidonic acid released by NEM was increased as compared to the control, with the difference being particularly relevant after 15 min

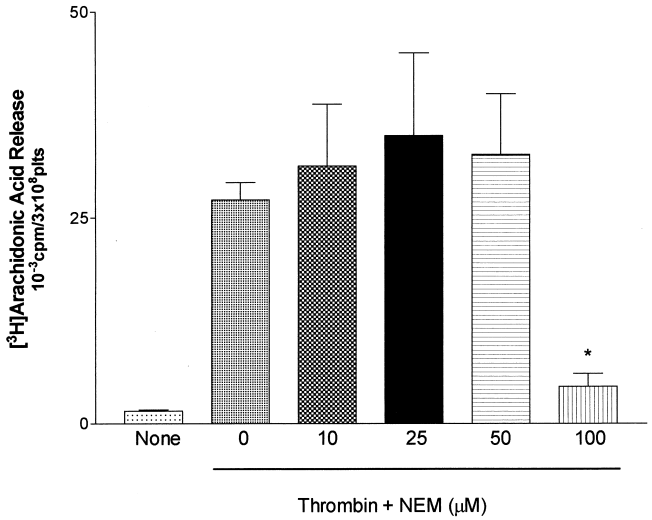

FIG. 2. The NEM effect on [^{3}H]arachidonic acid release induced by thrombin. Labelled platelets (3.0 × 10^8 platelets/mL) were preincubated for 10 min at 37° with saline or NEM and then stimulated with 0.5 U/mL thrombin. The incubation was prolonged for a further 6 min. Data are the mean values ± SD of four experiments carried out in duplicate. *$P < 0.0005$ versus thrombin.

or prolonged incubation times. Thrombin appeared to be more rapid and more effective than NEM. Treatment of platelets with thrombin resulted in a release of 7% of the total incorporated label, whereas treatment of platelet with NEM released about 4.5% of the incorporated arachidonic acid. Nevertheless, the alkylating reagent was more active than collagen or PMA. The [^{3}H]arachidonic acid released by NEM decreased from 19132 ± 867 cpm/3.0 × 10^8 platelets to 9379 ± 915 and 6146 ± 108 cpm/3.0 × 10^8 platelets in the presence of collagen and PMA, respectively. The NEM effect on arachidonic acid release induced by thrombin was also studied. NEM at low concentrations (10–50 μM) potentiated arachidonic acid release by thrombin, the maximum effect being achieved by 25 μM NEM. On the contrary, 100 μM NEM significantly ($n = 4$, $P < 0.0005$) inhibited arachidonic acid release by thrombin (Fig. 2). The platelet release of arachidonic acid was mainly catalysed by PLA_2 [3] and by the sequential actions of phospholipase C and diacylglycerol lipase [20]. To determine which pathway could be involved in arachidonic acid liberation induced by NEM, we treated platelets with $AACOCF_3$, the trifluoromethylketone analogue of arachidonic acid, previously shown to block almost all the arachidonate specifically released by $cPLA_2$ [21]. As shown in Fig. 3, the NEM-stimulated arachidonic acid release was significantly reduced by $AACOCF_3$ to 30% of control ($N = 4$, $P < 0.0005$). Moreover, genistein, an inhibitor of protein tyrosine kinases, significantly diminished arachidonic acid release ($N = 4$, $P < 0.0005$). On the contrary, both ET-18-OCH_3 and D609, which are selective inhibitors of phosphatidylinositol-specific phospholipase C or phosphatidylcholine-specific phospholipase C respectively, as well as RHC-80267, a diacylglycerol lipase inhibitor [22], did not produce inhibition of arachidonic acid release (data not shown). In addition, platelet preincubation with the potent and selective PKC inhibitor Ro-31-8220 slightly increased arachidonic acid release, suggesting that PKC is not involved in $cPLA_2$ activation, as previously observed [23, 24]. NEM-stimulated arachidonic acid release could be a Ca^{2+} mediated mechanism. To elucidate the relationship between NEM-induced Ca^{2+} influx and arachidonic acid release, labelled platelets were stimulated under conditions in which Ca^{2+} influx was blocked. The results reported in Table 1 show that the preincubation of platelets with the external Ca^{2+} chelator EGTA significantly decreased, but did not abolish, arachidonic acid release elicited by NEM.

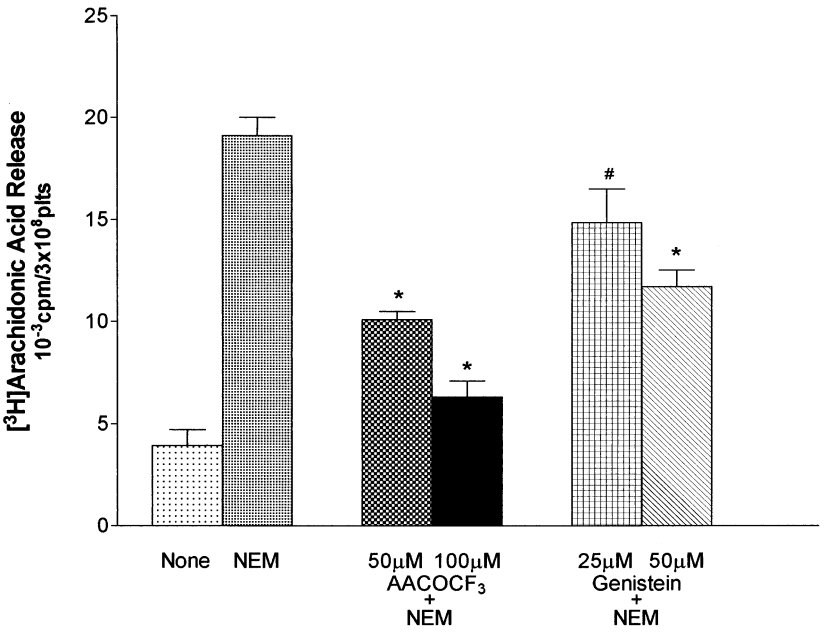

FIG. 3. Effect of $AACOCF_3$ and genistein on NEM-induced [^{3}H]arachidonic acid release. Labelled platelets (3.0 × 10^8 platelets/mL), preincubated for 10 min at 37° with saline, $AACOCF_3$, or genistein at the indicated concentrations, were treated with 100 μM NEM and incubated for a further 30 min. Data are means ± SD of four experiments carried out in duplicate. *$P < 0.0005$; #$P < 0.0025$ versus NEM.

TABLE 1. Effect of free Ca^{2+} on [^{3}H]arachidonic acid release induced by NEM

Treatment	[^{3}H]Arachidonic acid release (cpm/3.0 × 10^8 plts)	
	Ca^{2+}	EGTA
Control	4176 ± 330	4090 ± 500
NEM (50 μM)	6254 ± 717	4021 ± 950
NEM (100 μM)	20632 ± 2280	7544 ± 1077

Labelled platelets (3.0 × 10^8 plts/mL), resuspended in pH 7.4 Tyrode's-HEPES buffer containing 0.2% BSA (fatty-acid-free), were incubated with NEM for 30 min at 37° in the presence of 1.0 mM $CaCl_2$ or 2.5 mM EGTA, respectively. Results are given as means ± SD of three independent experiments carried out in triplicate.

Furthermore, arachidonic acid release was suppressed by preloading platelets with the intracellular chelator BAPTA/AM (data not shown).

Effect of NEM on Intracellular Ca^{2+} Concentration

A potential mechanism by which NEM could induce arachidonic acid mobilisation is through enhancing intracellular Ca^{2+} concentrations. In fura-2-loaded platelets, NEM, in a dose-dependent manner, produced a significant increase in the intracellular Ca^{2+} concentration. One hundred μM NEM elevated platelet resting calcium from 66 to 147 nM (Fig. 4A). In the presence of the extracellular Ca^{2+} chelator EGTA, the dose-dependent effect of NEM was greatly reduced but not suppressed (Fig. 4B). On the contrary, treatment of platelets with BAPTA/AM completely inhibited the Ca^{2+} increase induced by NEM (data not shown). It is likely that the relatively small increase in Ca^{2+} concentrations induced by NEM during the 15-min incubation is involved in the observed arachidonic acid mobilisation (Table 1).

The NEM Effect on ROS Formation

Arachidonic acid release is the rate-limiting step of eicosanoid formation. Arachidonic acid metabolism and ROS production are closely connected. The fluorescent signal generated by DCF trapped inside the platelets is considered indicative of ROS formation [19]. NEM was able to accumulate DCF more effectively than thrombin. Moreover, NEM strongly cooperated with thrombin by significantly elevating intracellular DCF. In the presence of extracellular Ca^{2+}, DCF formation induced by NEM and/or thrombin was significantly potentiated. Chelation of extracellular Ca^{2+} decreased DCFH oxidation to the control levels both in basal and in thrombin-stimulated platelets. On the contrary, in NEM-treated platelets EGTA did not abolish DCF accumulation (Fig. 5). *N*-Acetyl-L-cysteine, a potent thiol-containing antioxidant, suppressed DCFH oxidation produced by NEM and/or thrombin (data not shown).

In order to establish if ROS were produced in the PLA_2 cascade, we incubated platelets with the $cPLA_2$ specific inhibitor $AACOCF_3$ or with other less specific PLA_2 inhibitors such as aristolochic acid and quinacrine. Unfortunately, all these compounds produced aspecific DCFH oxidation, so that we were unable to measure DCF generated by NEM and/or thrombin precisely. Therefore, we carried out experiments in platelets treated with ETYA, which blocks arachidonic acid metabolisation [25]. ETYA reduced the intracellular DCF formation by NEM to 25%, but was less active in the presence of thrombin or in platelets pretreated with both agents. In addition, DPI, inhibitor of neutrophil NADPH oxidase [26] and of macrophage nitric oxide synthase [27], decreased DCF accumulation by NEM by about 40%, but was more effective (about 50% of inhibition) in platelets treated with thrombin or with both agents (Table 2). The DCF produced by NEM was not abolished by D609 or ET-18-OCH_3, confirming that the NEM oxidative mechanism does not involve the phospholipase C pathway.

DISCUSSION

The results in this report demonstrate that NEM induces arachidonic acid release in human platelets by the activation of $cPLA_2$. Since arachidonic acid release could be

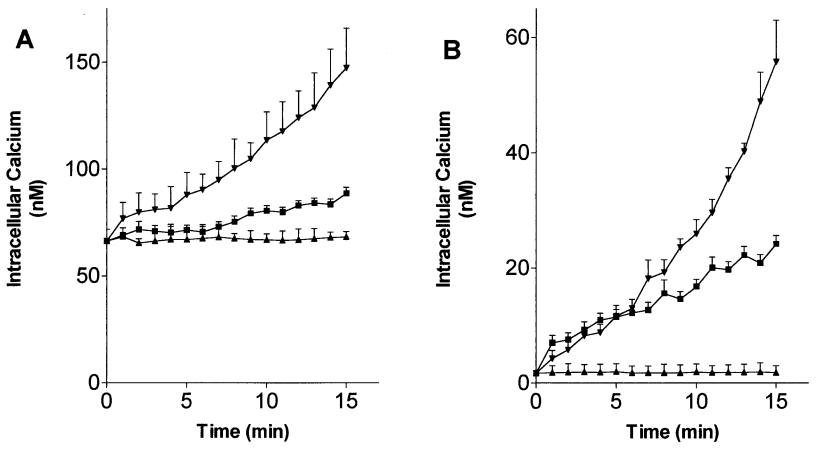

FIG. 4. Time–course of intracellular Ca^{2+} elevation induced by NEM. Fura-2-loaded platelets, resuspended at 2.0 × 10^8 platelets/mL in pH 7.4 Tyrode's-HEPES buffer containing 1.0 mM $CaCl_2$ (A) or 2.5 mM EGTA (B), were preincubated for 2 min at 37° prior to the addition of 50 μM (■), 100 μM (▼) NEM, or an equivalent volume of saline (▲). Thrombin was used as a positive control. The mean Ca^{2+} concentration after the addition of 0.1 U/mL thrombin was 509 ± 120 nM. Platelet fluorescence was measured in a Perkin–Elmer luminescence spectrometer, model LS50B. Data are the means ± SD of three experiments carried out in duplicate.

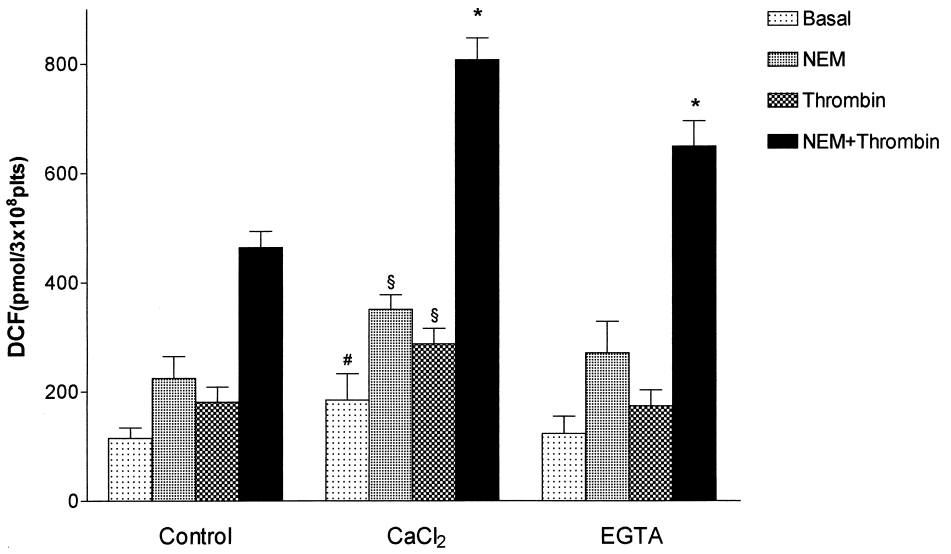

FIG. 5. DCFH oxidation after platelet treatment with NEM and/or thrombin. Washed platelets (5.0×10^7 platelets/mL), resuspended in pH 7.4 HEPES buffer with 1.0 mM $CaCl_2$ or 2.5 mM EGTA where required, were incubated for 15 min at 37° with 5 μM DCFH-DA in the presence of 5 μM NEM and then stimulated with 0.1 U/mL thrombin. The incubation, prolonged for a further 15 min, was stopped by putting samples in ice. Data are means ± SD of five experiments carried out in triplicate. #$P < 0.025$; §$P < 0.0025$; *$P < 0.0005$ versus the corresponding control without Ca^{2+}.

almost completely prevented by platelet treatment with the specific $cPLA_2$ inhibitor $AACOCF_3$, the involvement of this enzyme was likely. NEM-induced arachidonic acid release occurred within minutes of the addition of relatively low concentrations of the alkylating agent and appears to be a Ca^{2+}-mediated mechanism. Similar data were reported by Neve *et al.* [15] in endothelial cells, but there the NEM-mediated arachidonic acid release did not depend on Ca^{2+} levels. Moreover, staurosporine significantly reduced the NEM-stimulated arachidonic acid release in endothelial cells, suggesting the involvement of PKC in $cPLA_2$ activation. On the contrary, the specific PKC inhibitor Ro-31-8220 did not inhibit arachidonic acid release by NEM in platelets. In addition, tyrosine phosphorylation seems to be involved in the NEM effect to a different extent: genistein was much more effective in endothelial cells than in platelets. It is likely that different mechanisms are implicated in NEM-induced arachidonic acid release in endothelial cells and human platelets.

TABLE 2. Effect of DPI and ETYA on DCFH oxidation in human platelets

DCF (inhibition %)				
	DPI		ETYA	
Treatments	1.0	5.0	1.0	5.0
	(μM)			
NEM	27	37	70	75
Thrombin	39	52	41	53
NEM + Thrombin	40	54	47	60

Washed platelets (5.0×10^7/mL), resuspended in pH 7.4 HEPES buffer, were preincubated for 15 min at 37° with 5 μM DCFH-DA in the presence of DPI or ETYA and/or 5 μM NEM, and then stimulated with 0.1 U/mL thrombin where required. Incubation was prolonged for a further 15 min. Each value is the mean of four experiments in triplicate and is expressed as percentage inhibition of the DCF measured in control experiments carried out in the absence of DPI or ETYA.

In platelets, NEM produced a significant elevation in Ca^{2+} concentration (Fig. 4A) that was abolished by BAPTA/AM and partially inhibited by EGTA (Fig. 4B), suggesting that NEM could stimulate fluxes of extracellular Ca^{2+} and/or produce mobilisation of intracellular Ca^{2+} from storage sites.

$cPLA_2$ is regulated posttraslationally both by phosphorylation and by the level of intracellular Ca^{2+}. Calcium plays a role by promoting binding of $cPLA_2$ to membrane, which is mediated by a Ca^{2+}-phospholipid-binding domain at the amino terminals of this enzyme [28]. Although many different studies have suggested the involvement of the intracellular Ca^{2+} level in regulating arachidonic acid release, alternative mechanisms have been proposed by Qiu *et al.* [29] in mouse peritoneal macrophages where okadaic acid induces arachidonic acid release without increasing Ca^{2+} levels. Treatment of a variety of cell types with physiological agonists induces phosphorylation of $cPLA_2$, resulting in an increase in its activity [3, 30–32]. $cPLA_2$ is a substrate for PKC, cyclic AMP-dependent kinase, and MAP kinase "*in vitro*", but only phosphorylation by MAP kinase induces a consistent increase in $cPLA_2$ activity [33–35]. MAP kinase is phosphorylated and activated by a protein tyrosine kinase-dependent pathway [34]. Phosphorylation of protein tyrosine kinases has been shown to play a crucial role in the induction of cellular responses to extracellular stimuli. Many agonists induce tyrosine phosphorylation in platelets, indicating potential roles in platelet activation processes [36]. Tyrosine phosphorylation may be induced by intracellular Ca^{2+} elevation [37]. Moreover, protein tyrosine inhibitors such as genistein have been

shown to block several platelet events, including activation and thrombin-induced Ca^{2+} mobilisation, by affecting polyphosphoinositide turnover [38]. The NEM-induced arachidonic acid release was shown to be significantly inhibited by genistein, which would imply that tyrosine kinases are involved in NEM-induced activation of $cPLA_2$. As the phosphorylation of $cPLA_2$ occurs only on serine residues [35], it seems unlikely that direct tyrosine phosphorylation can be involved in the regulation of $cPLA_2$. It is likely that NEM, reacting with thiol groups located on or in the plasma membrane, modifies a receptor system which possesses tyrosine phosphorylation activity leading to $cPLA_2$ activation. Indeed, protein tyrosine kinase is sensitive to both exogenous and endogenous oxidising agents and antioxidants [39].

In this report, we have shown that NEM is able to increase ROS basal concentrations by strongly cooperating with thrombin. In the presence of extracellular Ca^{2+}, ROS formation is increased and the cooperation between the sulphydryl agent and thrombin is significantly potentiated. Chelation of extracellular Ca^{2+} with EGTA decreases ROS to the basal levels in control samples and in platelets stimulated by thrombin, but not in NEM-treated platelets (Fig. 5). It is likely that the Ca^{2+} released by NEM from storage sites (Fig. 4B) may be sufficient to activate arachidonic acid mobilisation (Table 1), leading to ROS formation (Fig. 5). Moreover, ETYA, which blocks arachidonic acid metabolism, almost completely inhibits ROS accumulation, suggesting that in NEM-treated platelets ROS are in large part generated during arachidonic acid metabolisation. Alternative mechanisms could be involved in platelets challenged by thrombin, such as the direct or indirect activation of NADP(H) oxidase, to which free arachidonic acid could contribute [40]. The involvement of NADP(H) oxidase in ROS formation during platelet treatment with NEM and/or thrombin is suggested by results obtained in the presence of DPI that show that it partially inhibits ROS formation.

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References

1. Kramer RM, Hession C, Johansen B, Hayes G, McGray P, Chow EP, Tizard R and Pepinsky RB, Structure and properties of a human non-pancreatic phospholipase A_2. *J Biol Chem* **264:** 5768–5775, 1989.
2. Takayama K, Kudo I, Kim DK, Nagata K, Nozawa Y and Inoue K, Purification and characterization of human platelet phospholipase A_2 which preferentially hydrolyzes arachidonoyl residue. *FEBS Lett* **282:** 326–330, 1991.
3. Kramer RM, Roberts EF, Manetta JV, Hylsop PA and Jakubowski JA, Thrombin-induced phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. *J Biol Chem* **268:** 26796–26804, 1993.
4. Smith JB and Willis AL, Selective inhibition of synthesis of prostaglandin in human platelets. *Nature New Biol* **230:** 235–237, 1971.
5. Samuelsson B, Dahlén SE, Lindgren JA, Rouzer CA and Serhan CN, Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* **237:** 1171–1176, 1987.
6. Egan RW, Gale PH, Baptista EM, Kennicott KL, Vanden-Heuvel WJA, Walker RW, Fagerness PE and Kuehl Jr FA, Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase. *J Biol Chem* **256:** 7352–7361, 1981.
7. Kulmacz RJ, Tsai AL and Palmer G, Heme spin states and peroxide-induced radical species in prostaglandin H synthase. *J Biol Chem* **262:** 10524–10531, 1987.
8. Iuliano L, Praticò D, Bonavita MS and Violi F, Involvement of phospholipase A_2 in H_2O_2-dependent platelet activation. *Platelets* **2:** 87–90, 1992.
9. Iuliano L, Pedersen JZ, Praticò D, Rotilio G and Violi F, Role of hydroxyl radicals in the activation of human platelets. *Eur J Biochem* **221:** 695–704, 1994.
10. Hashizume T, Yamaguchi H, Kawamoto A, Tamura A, Sato T and Fujii T, Lipid peroxide makes rabbit platelet hyperaggregable to agonists through phospholipase A_2 activation. *Arch Biochem Biophys* **289:** 47–52, 1991.
11. Goldman R, Ferber E and Zort U, Reactive oxygen species are involved in the activation of cellular phospholipase A_2. *FEBS Lett* **309:** 190–192, 1992.
12. Chakraborti S, Michael JR, Gurtner GH, Ghosh SS, Dutta G and Merker A, Role of a membrane-enocieted serine esterose in the oxidant activation of phopholipose A_2 by t-butylhydroperoxide. *Biochem J* **292:** 585–589, 1993.
13. Hornberger W and Patscheke H, Primary stimuli of icosanoid release inhibit arachidonoyl-CoA synthetase and lysophospholipid acyltransferase. *Eur J Biochem* **187:** 175–181, 1990.
14. Nelson JM, Duane PG, Rice KL and Niewoehner DE, Cadmium ion-induced alterations of phospholipid metabolism in endothelial cells. *Am J Respir Cell Mol Biol* **5:** 328–336, 1991.
15. Neve EPA, Boyer CS and Moldeus P, *N*-ethylmaleimide stimulates arachidonic acid release through activation of the signal-responsive phospholipase A_2 in endothelial cells. *Biochem Pharmacol* **49:** 57–63, 1995.
16. Brandt R and Keston AS, Synthesis of diacetyldichlorofluorescin: a stable reagent for fluorimetric analysis. *Anal Biochem* **11:** 6–9, 1965.
17. Leoncini G, Maresca M, Buzzi E, Piana A and Armani U, Platelets of patients affected with essential thrombocythemia are abnormal in plasma membrane and adenine nucleotide content. *Eur J Haematol* **44:** 115–119, 1990.
18. Rotondo S, Evangelista V, Manarini S, De Gaetano G and Cerletti C, Different requirement of intracellular calcium and protein kinase C for arachidonic acid release and serotonin secretion in cathepsin G-activated platelets. *Thromb Haemost* **78:** 919–925, 1997.
19. Leoncini G, Maresca M and Colao C, Oxidative metabolism of human platelets. *Biochem Int* **25:** 647–655, 1991.
20. Bell RL, Kennerly DA, Stanford N and Majerus PW, Diglyceride lipase: A pathway for arachidonate release from human platelets. *Proc Natl Acad Sci* USA **76:** 3238–3241, 1979.
21. Bartoli F, Lin H-K, Ghomashchi F, Gelb MH, Jain MK and Apitz-Castro R, Tight binding inhibitors of 85-kDa phospholipase A_2 but not 14-kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. *J Biol Chem* **269:** 15625–15630, 1994.
22. Bross TE, Prescott SM and Majerus PW, RHC 80267 does not inhibit the diglyceride lipase pathway in intact platelets. *Biochem Biophys Res Comm* **116:** 68–74, 1983.
23. Börsch-Haubold AG, Kramer RM and Watson SP, Cytosolic phospholipase A_2 is phosphorylated in collagen and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. *J Biol Chem* **270:** 25885–25892, 1995.

24. Iorio P, Gresele P, Stasi M, Nucciarelli F, Vezza R, Nenci GG and Goracci G, Protein kinase C inhibitors enhance G-protein-induced phospholipase A_2 activation in intact human platelets. *FEBS Lett* **381:** 244–248, 1996.
25. Kühn H, Holzhütter HG, Schewe T, Hiebsch C and Rapoport SM, The mechanism of inactivation of lipoxygenases by acetylenic fatty acids. *Eur J Biochem* **139:** 577–583, 1984.
26. O'Donnell VB, Tew DG, Jones OTG and England PJ, Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* **290:** 41–49, 1993.
27. Stuehr DJ, Fasehum OA, Kwon NS, Gross SS, Gonzales JA, Levi R and Nathan CF, Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J* **5:** 98–103, 1991.
28. Nalefski EA, Sultzman LA, Martin DM, Kriz RW, Towler PS, Knopf JL and Clark JD, Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. *J Biol Chem* **269:** 18239–18249, 1994.
29. Qiu ZH and Leslie CC, Protein kinase C-dependent and -independent pathways of mitogen-activated protein kinase activation in macrophages by stimuli that activate phospholipase A_2. *J Biol Chem* **269:** 19480–19487, 1994.
30. Qiu ZH, de Carvalho MS and Leslie CC, Regulation of phospholipase A_2 activation by phosphorylation in mouse peritoneal macrophages. *J Biol Chem* **268:** 24506–24513, 1993.
31. Doerfler ME, Weiss J, Clark JD and Elsbach P, Bacterial lipopolysaccharide primes human neutrophils for enhanced release of arachidonic acid and causes phosphorylation of an 85-kD cytosolic phospholipase A_2. *J Clin Invest* **93:** 1583–1519, 1994.
32. Gronich J, Konieczkowski M, Gelb MH, Nemenoff RA and Sedor JR, Interleukin-1α causes rapid activation of cytosolic phospholipase A_2 by phosphorylation in rat mesangial cells. *J Clin Invest* **93:** 1224–1233, 1994.
33. Nemenoff RA, Winitiz S, Qian NX, Van Putten V, Johnson GL and Heasley LE, Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J Biol Chem* **268:** 1960–1964, 1993.
34. Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A and Davis RJ, $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72:** 269–278, 1993.
35. Lin LL, Lin AY and Knopf JL, Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc Natl Acad Sci* USA **89:** 6147–6151, 1992.
36. Golden A, Brugge JS and Shattil SJ, Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of specific platelet proteins. *J Cell Biol* **111:** 3117–3127, 1990.
37. Vostal JG, Jackson WL and Shulman NR, Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *J Biol Chem* **266:** 16911–16916, 1991.
38. Ozaki Y, Yatomi Y, Jinnai Y and Kume S, Effects of genistein, a tyrosine kinase inhibitor, on platelet functions. Genistein attenuates thrombin-induced Ca^{2+} mobilisation in human platelets by affecting polyphosphoinositide turnover. *Biochem Pharmacol* **46:** 395–403, 1993.
39. Fialkow L, Chan CK, Grinstein S and Downey GP, Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase. Role of reactive oxygen intermediates. *J Biol Chem* **268:** 17131–17137, 1993.
40. Maridonneau-Parini I and Tauber AI, Activation of NADPH-oxidase by arachidonic acid involves phospholipase A_2 in intact human neutrophils but not in the cell-free system. *Biochem Biophys Res Comm* **138:** 1099–1105, 1986.