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N-ETHYL MALEIMIDE STIMULATES ARACHIDONIC ACID RELEASE THROUGH ACTIVATION OF THE SIGNAL-RESPONSIVE PHOSPHOLIPASE A_2 IN ENDOTHELIAL CELLS

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Abstract—Treatment of bovine endothelial cells with the alkylator *N*-ethyl maleimide results in arachidonic acid mobilization. *N*-ethyl maleimide-stimulated arachidonic acid release was dose and time dependent and maximum release was achieved after 10–15 min with 50 μM *N*-ethyl maleimide. *N*-ethyl maleimide-stimulated arachidonic acid release could be prevented by pretreating the cells with the phospholipase A_2 inhibitor quinacrine. Based on the finding that *N*-ethyl maleimide was not able to release oleic acid from oleic acid-preloaded cells, it was clear that the effect of *N*-ethyl maleimide was limited to an arachidonic acid-specific phospholipase. The effect of *N*-ethyl maleimide does not appear to be dependent on calcium, as shown by the observation that *N*-ethyl maleimide was not able to increase intracellular calcium concentration in FURA2-loaded cells. Pretreatment of the cells with staurosporine totally inhibited *N*-ethyl maleimide-stimulated arachidonic acid liberation. The tyrosine kinase inhibitor genistein was also able to significantly inhibit arachidonic acid release. It is concluded from the results obtained in this study that *N*-ethyl maleimide stimulates arachidonic acid release by stimulating the activity of a specific, signal-responsive phospholipase A_2. Furthermore this activation is not mediated by intracellular calcium fluxes but by a stimulation of intracellular kinase activity which eventually leads to the activation of this signal-responsive phospholipase A_2.

Key words: phospholipase A_2; *N*-ethyl maleimide; arachidonic acid; endothelium

The role of arachidonic acid metabolites in inflammatory diseases is well established and it is generally thought that the rate limiting step in the production of eicosanoids is the intracellular liberation of arachidonic acid from the *sn*-2 position of phospholipids [1]. The intracellular levels of arachidonate, and eventually the production of eicosanoids, are controlled by the PLA_2‡ system and the associated enzymes involved in the reacylation of free fatty acids: arachidonoyl-CoA:1-palmitoyl-*sn*-glycero-3-phosphocholine acyl transferase and the arachidonoyl-CoA synthetase [1].

PLA_2 (EC 3.1.1.4) can be classified into two different forms based on cellular localization: a low molecular mass (12–15 kDa) $sPLA_2$ and a high molecular mass 85 kDa $cPLA_2$ [2]. The $sPLA_2$ acts rather non-specifically at the *sn*-2 position of phospholipids, is responsive to calcium in the millimolar range and is almost inactive in reducing environments, such as that present in the cytoplasm [3, 4]. Recently it has been demonstrated that mammalian cells contain a PLA_2 that is responsive to physiologically relevant calcium concentrations [5–7] and G-protein activation [6, 8–10], one that is a substrate for PKC [11, 12] and mitogen-activated protein (MAP) kinase [12, 13]. $cPLA_2$ has also been found to be specific for arachidonic acid at the *sn*-2 position [5]. These findings strongly suggest that the release of arachidonic acid from cellular phospholipids is mediated by a signal-responsive PLA_2 ($cPLA_2$) and is under the control of cellular second messenger systems.

Much effort has been made to study oxidant-mediated arachidonic acid release from cellular systems and the possible mechanisms behind this release [14–21,§]. In addition, other thiol-reactive agents have been shown to induce arachidonic acid and eicosanoid release, such as methyl mercury [20, 22], cadmium [23] and NEM [24]. The mechanism by which these agents may cause arachidonic acid release is by an inhibition in the reacetylation pathway of free arachidonic acid, resulting in an imbalance in the acetylation/deacetylation cycle of arachidonic acid [20, 22, 24]. Recently, we have reported the activation of the signal-responsive PLA_2 by hydrogen peroxide,

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‡ Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; PKC, protein kinase C; NEM, *N*-ethyl maleimide; CPAE, bovine pulmonary endothelial cells; HHBSS, HEPES-buffered Hank's Buffered Salt Solution.

§ Boyer CS, Bannenberg GL, Neve EPA, Ryrfeldt Å and Moldéus P, Evidence for the activation of the signal-responsive phospholipase A_2 by exogenous hydrogen peroxide. *J. Applied Physiol*, submitted.

resulting in the release of arachidonic acid from bovine endothelial cells*. In this report we also observed that the thiol alkylator NEM was able to induce arachidonic acid release. The arachidonic acid release induced both by hydrogen peroxide and NEM was shown to be at least partly mediated by protein kinases. Recently, Ståhls reported a stimulatory effect of NEM on tyrosine kinase activity in the Jurkat T-cell line [25], which is in good agreement with our observations. In the present study we investigate the role of thiol modification in intracellular signalling in relation to arachidonic acid release pathways by using the thiol-alkylator NEM. In addition, the role of $cPLA_2$ in NEM-induced arachidonic acid release is presented.

MATERIALS AND METHODS

Materials. [^{3}H]Arachidonic acid (approx. 180–230 Ci/mmol) and [^{3}H]oleic acid (10 Ci/mmol) were obtained from Dupont-New England Nuclear (Wilmington, DE, U.S.A.) Genistein was obtained from Biomol (Hamburg, Germany). Fatty-acid-free BSA, HEPES, FURA2-AM and BAPTA-AM were obtained from Boehringer-Mannheim, (Mannheim, Germany). NEM, melittin, A23187, staurosporine and quinacrine were obtained from the Sigma Chemical Co. (Poole, U.K.). All other reagents were obtained from local sources and were of reagent grade or better.

Cell culture. Bovine pulmonary endothelial cells (CPAE) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in Dulbecco's Minimal Essential Medium (Gibco) supplemented with 10% foetal calf serum at 37° in an atmosphere of 95% air 5% CO_2. For arachidonic acid release experiments, cells were grown to confluence in 2 cm^2 wells on 24 well multiwell plates. For the measurement of intracellular calcium using FURA2, the cells were grown on glass coverslips as described below.

Arachidonic acid release. The cells were plated into 24 well plates and grown to confluency (approximately 5×10^4 cells/well). They were then loaded with 0.1 μCi [^{3}H]arachidonic acid, or in the case of oleic acid with 0.2 μCi [^{3}H]oleic acid, per well for 24 hr in growth medium. Over the 24 hr period, the cells incorporated an average of 70% of the added [^{3}H]arachidonic acid or 55% of the added [^{3}H]oleic acid. Prior to treatment, the cells were washed twice with HHBSS, pH 7.4 containing 1% fatty-acid-free BSA and subsequently washed once with BSA-free HHBSS. Cells were treated in a final volume of 500 μL of HHBSS. Pretreatments were carried out in the incubation medium and the arachidonic acid-releasing agents were added directly to the pretreatment medium. The various pretreatments were as follows: quinacrine, 250 μM for 10 min; staurosporine, 10 μM for 10 min and genistein, 10 μM for 30 min. For the intracellular chelation of calcium using BAPTA, BAPTA-AM (in 1 μL DMSO) was added to the growth medium to a final concentration of 20 μM 30 min prior to washing and treatment of the cells. The released radiolabel was determined by liquid scintillation counting of 400 μL aliquots of the extracellular medium. All treatments and pretreatments did not result in morphological changes of the cells or in an increase in LDH release as compared to untreated cells (data not shown). All radiolabel release results are given as per cent of the control for the particular experimental plate and as mean ± SEM of quadruplicate determinations.

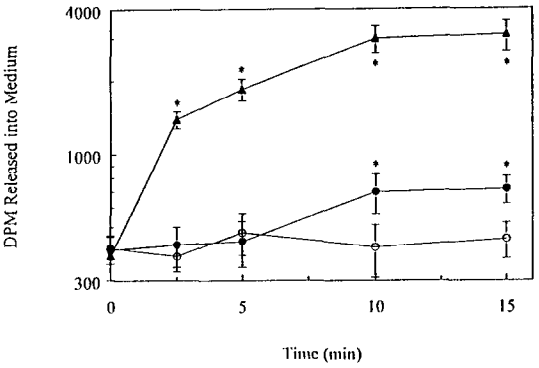

Fig. 1. Time course of arachidonic acid release from CPAE cells stimulated with 50 μM NEM (closed circles) or 4 μM calcium ionophore A23187 (closed triangles). Control release over the 15 min time course is also shown (open circles) and did not change significantly from the zero time point. Values represent the means ± SEM for two determinations done on separate CPAE cultures. Values that are significantly different ($P < 0.05$) from those of the controls at that particular time point are marked with an asterisk.

Measurement of intracellular calcium. The measurement of intracellular calcium fluxes using the fluorescent probe FURA2 was carried out essentially according to the methods of Wickham *et al.* [26]. After trypsinization, the cells were diluted to approx. 5×10^4 cells/mL and 0.1 mL was carefully applied to a glass slide (10 × 40 mm). The cells were allowed to attach to the glass for 4–8 hr, after which sufficient volume of growth medium was added to completely cover the glass slides. The cells were then allowed to grow to confluency. For FURA2 loading, each slide was immersed in 3.5 mL of growth medium containing 15 μM FURA2-AM for 30 min at 37°. The glass slide was then removed from the medium, washed four times in HHBSS and used immediately. The determination of the intracellular fluorescence of FURA2 was performed on a Sigma ZWS II spectrophotometer. The exposure medium in all experiments was HHBSS (1.3 mM Ca^{2+}).

Statistical analysis. Comparisons between treatment groups were performed by the use of one-way analysis of variance (ANOVA) followed by a *post hoc* Student's *t*-test. All values are presented as the means ± SEM.

RESULTS

The time course of arachidonic acid release from

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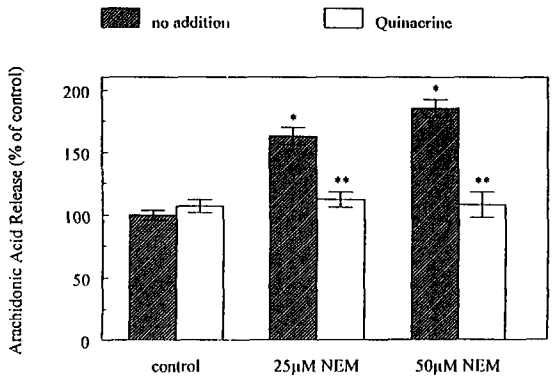

Fig. 2. Effect of the PLA_2 inhibitor quinacrine on NEM-stimulated arachidonic acid release from CPAE cells. [^{3}H]-Arachidonate loaded cells were washed and incubated for 10 min in the presence of 250 μM inhibitor prior to the addition of 25 or 50 μM NEM. Arachidonic acid release was determined at 15 min after addition of the stimulant. Values are expressed as the means ± SEM of the per cent control (N = 4). Control values were determined in quadruplicate for each 24 well plate and used as the control reference only for the treatment wells from that plate. Values that are significantly different ($P < 0.05$) from non-pretreated control values are marked with an asterisk and values that are significantly different from those within the particular treatment group are marked with a double asterisk.

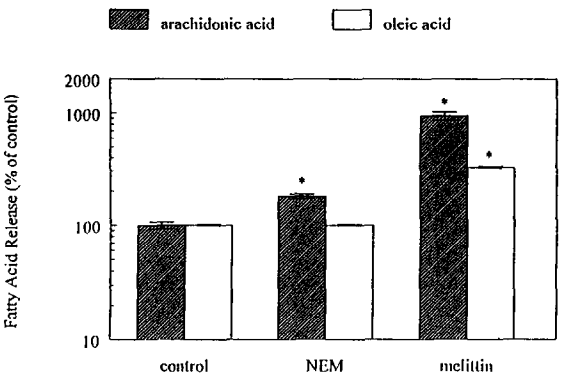

Fig. 3. Release of arachidonic acid or oleic acid from CPAE cells after NEM and melittin treatment. Arachidonic acid or oleic acid release was assessed at 15 min after addition of the stimulant, 50 μM NEM or 200 ng of melittin. Values are expressed as the means ± SEM of the per cent control (N = 4). Values that are significantly different from the control value for that particular fatty acid are marked with an asterisk. Further details are given in Fig. 2 and the Materials and Methods.

test CPAE cells is given in Fig. 1. From the data it is apparent that NEM increases extracellular arachidonic acid concentrations only after 5 min of exposure of the cells. After a 15 min incubation the amount of released arachidonic acid is increased approx. 2-fold as compared to the control incubation. The maximum release of arachidonic acid is achieved with the calcium ionophore A23187, which results in a more rapid release of arachidonate and reaches a plateau after approximately 10 min. Treatment of the cells with NEM resulted in a release of approx. 1% of the total incorporated arachidonate label, whereas treatment with A23187 resulted in a release of approx. 6% of the total incorporated label. These results are in close agreement with those recently obtained for the comparison of hydrogen peroxide- and A23187-induced release. NEM-induced arachidonic acid release appears to be concentration dependent (Fig. 2) as 25 μM NEM induced significant arachidonic acid release from CPAE cells, but less than that observed with 50 μM NEM. NEM-stimulated arachidonic acid release from the CPAE cells could be completely prevented by pretreating the cells with the PLA_2 inhibitor quinacrine (Fig. 2) [27]. The inhibition of NEM-stimulated release by the phospholipase inhibitor quinacrine indicates that the release is mediated by a PLA_2.

As described above, mammalian cells generally possess two forms of phospholipase A_2, $sPLA_2$ and $cPLA_2$. In order to establish the identity of the PLA_2 responsible for NEM-stimulated arachidonic acid release, the property of fatty acid specificity at the *sn*-2 position of phospholipids was utilized. Thus, $sPLA_2$ is rather non-specific for substituents at the *sn*-2 position of phospholipids, essentially cleaving any species of fatty acyl side chain. On the other hand, $cPLA_2$ is rather specific for arachidonic acid [3–5]. From the data presented in Fig. 3 it is clear that NEM is unable to release oleate from oleic acid preloaded CPAE cells, whilst the $sPLA_2$ stimulatory peptide melittin [28, 29] stimulates the release of either arachidonate or oleate. These results indicate that it is the stimulation of the activity of the $cPLA_2$ form which is responsible for NEM-mediated arachidonic acid mobilization. One of the possible mechanisms by which NEM can activate $cPLA_2$ is through interacting with a component of the signal transduction system that is responsible for the control of $cPLA_2$ activity [7, 12].

$cPLA_2$ has been shown to be responsive to physiologically-relevant calcium concentrations and the enhancement of intracellular calcium concentration by receptor-mediated pathways, or by ionophore treatment, results in the release of arachidonic acid [5, 30–32]. Therefore, a potential mechanism by which NEM could result in the stimulation of $cPLA_2$ activity is by enhancing intracellular calcium concentrations. Intracellular calcium levels in FURA2-loaded CPAE cells did not change during the 15 min period of incubation of the cells with 50 μM NEM (Fig. 4). However, when the cells were stimulated with the alkylator benzoquinone, which is also able to release arachidonate from CPAE cells to the same extent as NEM (data not shown), intracellular calcium levels increased immediately after addition and remained elevated compared to control levels. Furthermore, NEM-stimulated release of arachidonic acid was not affected by pre-loading the cells with the intracellular chelator BAPTA (data not shown). Thus, these results suggest that NEM does

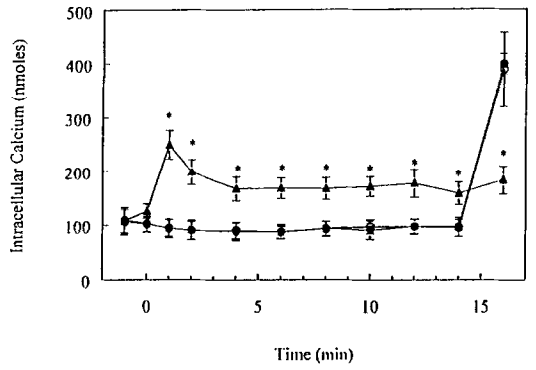

Fig. 4. Intracellular calcium levels in CPAE cells in the presence of NEM. The values shown are the means ± SEM of three separate experiments. After washing the glass slide was placed in a 3 mL quartz cuvette with constant stirring. At time 0 50 μM NEM (closed circles) or 25 μM benzoquinone (closed triangles) was added. The resting $[Ca^{2+}]_i$ was 105 ± 23 (N = 4) and did not change significantly over the 15 min time course in non-treated (open circles) and NEM-treated (closed circles) cells. Benzoquinone was used as a positive control. The mean $[Ca^{2+}]_i$ after the addition of bradykinin (1 μM) was 388 ± 68 (N = 5). Values that are significantly different ($P < 0.05$) from those of the controls at that particular time point are marked with an asterisk. Further details are given in the Materials and Methods.

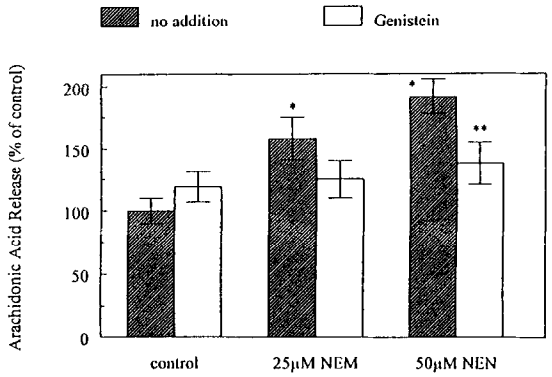

Fig. 6. Effect of the tyrosine kinase inhibitor genistein on NEM-stimulated arachidonic acid release. [^{3}H]Arachidonate loaded cells were washed and incubated for 30 min in the presence of 10 μM inhibitor prior to the addition of 25 or 50 μM NEM. Arachidonic acid release was determined at 15 min after addition of the stimulant. Values are expressed as the means ± SEM of the per cent control (N = 6). Values that are significantly different ($P < 0.05$) from non-pretreated control values are marked with an asterisk and values that are significantly different from those within the particular treatment group are marked with a double asterisk. Further details are given in Fig. 2.

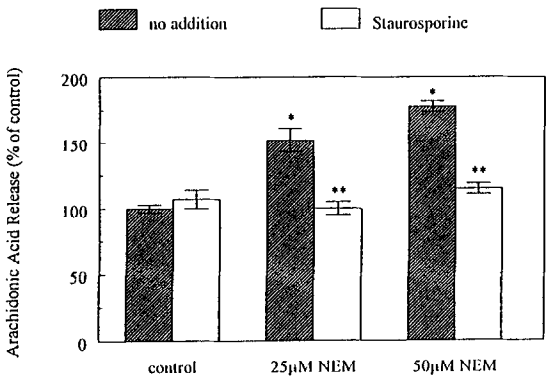

Fig. 5. Effect of the protein kinase C inhibitor staurosporine on NEM-stimulated arachidonic acid release from CPAE cells. [^{3}H]Arachidonate loaded cells were washed and incubated for 10 min in the presence of 10 μM inhibitor prior to the addition of 25 or 50 μM NEM. Arachidonic acid release was determined at 15 min after addition of the stimulant. Values are expressed as the means ± SEM of the per cent control (N = 4). Values that are significantly different ($P < 0.05$) from non-pretreated control values are marked with an asterisk and values that are significantly different from those within the particular treatment group are marked with a double asterisk. Further details are given in Fig. 2.

not stimulate the activity of $cPLA_2$ in CPAE cells by increasing intracellular calcium concentrations.

Activation of $cPLA_2$ can be achieved by phosphorylation of the protein, either by PKC or by MAP2 kinase. Oxidants have been shown to have stimulatory effects on the protein kinase C system [13, 21, 33, 34] and hydrogen peroxide in particular has been found to induce, at least partially, arachidonic acid release by a PKC-dependent mechanism*. The NEM-stimulated release of arachidonate was completely prevented by pretreatment of the cells with staurosporine as shown in Fig. 5. Also, pretreatment of the cells with the tyrosine kinase inhibitor genistein [35] resulted in a significant inhibition ($P < 0.05$) of NEM-stimulated arachidonate release (Fig. 6). This is in agreement with a recent report which describes the stimulatory effect of NEM on tyrosine kinase activity [25].

DISCUSSION

It is known from previous reports that thiol-reactive compounds, in particular NEM, are able to release arachidonic acid and arachidonic acid-derived products [36, 37]. It has been suggested that NEM induces the release of these products by inhibiting the incorporation of arachidonic acid into cellular phospholipids and not by stimulating the activity of PLA_2 [24]. In support of this suggestion is the observation that NEM is able to inhibit the reacetylation of arachidonic acid by inhibiting arachidonoyl-coenzyme A synthetase in a platelet membrane fraction [38]. However, these results were obtained using either high NEM-concentrations

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(millimolar range) or prolonged incubation times (several hours). In this report we show that NEM-induced arachidonic acid release occurs within minutes of addition of relatively low concentrations of NEM. No effect of NEM on arachidonic acid incorporation was observed during the 15 min incubation time, as compared to the incorporation into untreated cells (data not shown). Furthermore, NEM-induced release could be completely prevented by pretreatment of the cells with the PLA_2 inhibitor quinacrine (Fig. 2). These results strongly suggest that the activation of PLA_2 and not the inhibition of the acylation/deacylation cycle is responsible for arachidonic acid release. It is, however, possible that both mechanisms could be operative in NEM-stimulated cells; i.e. an early (first 15 min) stimulation of PLA_2 activity followed by a later inhibition of the reacylation pathways.

The identity of the particular form of PLA_2, $sPLA_2$ or $cPLA_2$ responsible for the liberation of arachidonic acid from CPAE cells can be clarified by using the fatty acid substrate specificity of the enzymes at the *sn*-2 position of the phospholipids. As is apparent from Fig. 3, NEM was unable to induce oleic acid release from oleic acid prelabelled cells, whilst the known $sPLA_2$ stimulator melittin was able to induce both oleic and arachidonic acid release. What is clear from these results is that NEM stimulates arachidonic acid release via an arachidonate-specific phospholipase; i.e. $cPLA_2$. Since $cPLA_2$ is signal-responsive in cells, we propose that NEM acts in this system as a signal: capable of precipitating a signal cascade that ultimately results in the activation of $cPLA_2$.

One possible mechanism by which NEM can stimulate $cPLA_2$ activity is by increasing intracellular calcium concentrations, since $cPLA_2$ can be activated by physiologically-relevant increases in intracellular calcium [5, 7] and alkylating agents have been shown to induce elevations in intracellular calcium [39]. From measurements of intracellular calcium fluxes using FURA2 it is clear that 50 μM NEM is unable to induce an elevation in this parameter in this cell type during the first 15 min after exposure. Additionally, the intracellular calcium chelator BAPTA had no effect on NEM-induced arachidonic acid release.

In addition to a Ca^{2+}-dependent form of $cPLA_2$ there is a Ca^{2+}-independent form of $cPLA_2$ present in cells. Recently, cytosolic forms of PLA_2 which do not require elevations in the intracellular calcium concentration for its catalytic activities have been isolated and characterized, indicating that increases in intracellular calcium are not necessary for arachidonic acid release by these forms of $cPLA_2$ [40, 41]. Furthermore, it has been reported that the phorbol ester phorbol 12-myristate 13-acetate was able to induce a stable modification of $cPLA_2$ which leads to an altered Ca^{2+}-dependency of this enzyme [42]. These observations together with the results obtained in the present study would indicate that an increase in intracellular calcium is not required for NEM-induced $cPLA_2$ activation and subsequent arachidonic acid release.

The activity of $cPLA_2$ is known to be regulated by a network of signalling systems. $cPLA_2$-activity has been shown to be regulated by phosphorylation, catalysed by either PKC or by MAP2 kinase [6, 11–13, 43]. The mechanisms behind the regulation of the kinases themselves are still unclear, but a number of pathways have been suggested, including phospholipase C [9], G-proteins [6, 8–10] and tyrosine kinases [44]. Stimulation of $cPLA_2$ activity and subsequent arachidonic acid release can also be realized by the use of the epidermal-growth-factor (EGF) [45, 46]. The tyrosine kinase activity of the EGF receptor seems to be essential for PLA_2 activation and arachidonic acid release [47]. Recently, Clark and Konstantopoulos [48] reported that NEM modulates the autophosphorylation of the EGF and insulin receptors. This, together with the observation that NEM induces hyperphosphorylation on tyrosine residues [25], indicates that NEM could modulate $cPLA_2$ activity by interacting with a receptor system.

In this study we have made an attempt to elucidate the mechanism by which NEM could induce arachidonic acid release. The serine–threonine kinase inhibitor staurosporine was able to prevent NEM-mediated arachidonic acid release, indicating that activation of one or more members of this class of kinases could play a role in the activation of $cPLA_2$ in endothelial cells. Also, the tyrosine kinase inhibitor genistein was able to inhibit NEM-mediated arachidonic acid release, which would imply that, in addition to serine–threonine kinases, tyrosine kinases are also involved in NEM-induced activation of $cPLA_2$. As the phosphorylation of $cPLA_2$ occurs only on serine residues [11], it seems unlikely that direct tyrosine phosphorylation is involved in the regulation of $cPLA_2$. It is possible that tyrosine phosphorylation could be involved in the regulation of other kinases, such as PKC or MAP2, which could in turn regulate $cPLA_2$ activity. One possibility is that NEM modifies a receptor system which possesses tyrosine phosphorylation capacity and that this ultimately leads to $cPLA_2$ activation. A cell-impermeable NEM analogue (NEM conjugated to hemocyanin) was able to release arachidonic acid from these cells to the same extent and in the same concentration range and time period as NEM itself (data not shown). This would indicate that NEM reacts with certain thiol groups located on or in the plasma membrane, possibly critical thiol groups of receptor systems. It has been shown that stimulation of a tyrosine kinase receptor, such as the EGF receptor [46], can result in arachidonic acid release from cells [45].

The present study has investigated the mechanism of NEM-stimulated arachidonic acid mobilization in endothelial cells. Based on the oleic acid versus arachidonic acid results, it appears that NEM stimulates the signal-responsive $cPLA_2$ rather than the low molecular mass secreted form of this enzyme. Elevation in the intracellular calcium concentration is not involved in the enhancement of $cPLA_2$ activity by NEM. Rather, NEM seems to stimulate $cPLA_2$ through the actions both of serine–threonine kinases and tyrosine kinase. The mechanism underlying NEM-stimulation of the signal transduction cascade, which eventually leads to the activation of $cPLA_2$, is not clear but there are some indications that NEM

could interact with thiol-containing components located on or in the plasma membrane. In this study we obtained evidence that NEM interacts with thiol groups of a cell surface receptor system. The exact signalling pathway by which NEM ultimately leads to activation of $cPLA_2$ and arachidonic acid release is not known but it is clear that in addition to a tyrosine kinase, a serine–threonine kinase, possibly PKC or MAP2 kinase, is involved. Thus it appears that NEM, and other thiol-reactive compounds in general, can cause the release of arachidonic acid through specifically stimulating $cPLA_2$ via the stimulation of intracellular kinase activity.

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