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# Distinct effects of *N*-ethylmaleimide on formyl peptide- and cyclopiazonic acid-induced $Ca^{2+}$ signals through thiol modification in neutrophils

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**Abstract**

In this study, we demonstrate that *N*-ethylmaleimide (NEM), a cell permeable thiol-alkylating agent, enhanced the $[Ca^{2+}]_i$ rise caused by stimulation with cyclopiazonic acid (CPA), a sarcoplasmic-endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor, in rat neutrophils. In addition, NEM attenuated the formyl-Met-Leu-Phe (fMLP)-induced $[Ca^{2+}]_i$ rise whether NEM was added to cells prior to or after fMLP stimulation. Moreover, application of NEM after fMLP activation in the absence of external $Ca^{2+}$ inhibited the $Ca^{2+}$ signal upon addition of $Ca^{2+}$ to the medium. Similar patterns were also obtained by using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), a cell impermeable dithiol-oxidizing agent, which replaced NEM in the CPA- and fMLP-induced $[Ca^{2+}]_i$ rise experiments. Treatment with dithiothreitol (DTT), a cell permeable dithiol-reducing agent, *N*-acetyl-L-cysteine (NAC), a cell permeable monothiol-reducing agent, and tris-(2-carboxyethyl)phosphine (TCEP), a cell impermeable reductant without a thiol group, all rescued the fMLP-induced $Ca^{2+}$ signal from NEM. Rat neutrophils express the mRNA encoding for transient receptor potential (TRP) C6, inositol trisphosphate receptor ($IP_3R$) 2 and $IP_3R3$. NEM had no effect on the mitochondrial membrane potential. NEM could restore the polarization and F-actin accumulation of fMLP-treated cells to those of the control. In the absence of external $Ca^{2+}$, NEM rendered the CPA-induced $[Ca^{2+}]_i$ elevation persistently but inhibited the fMLP-induced $Ca^{2+}$ spike, which was reversed by tris-(2-cyanoethyl)phosphine (TCP), a cell permeable reductant without a thiol group. DTNB did not affect the $Ca^{2+}$ spike caused by fMLP. These results indicate that through protein thiol oxidation, NEM affects the receptor-activated and the store depletion-derived $Ca^{2+}$ signals in an opposing manner.


*Keywords:* *N*-Ethylmaleimide; $Ca^{2+}$ entry; $Ca^{2+}$ release; Cyclopiazonic acid; fMLP; Neutrophils

## 1. Introduction

Neutrophils play a crucial role in first-line defense against invading microorganisms. $Ca^{2+}$ signals have been implicated in many cellular functions of neutrophils. The induction of receptor-activated cytosolic $Ca^{2+}$ signals results in two closely coupled events: a rapid and transient release of stored $Ca^{2+}$, followed by slowly developing extracellular $Ca^{2+}$ entry. It is well established that the initial $Ca^{2+}$ spike is mediated by the activation of phosphoinositide-specific phospholipase C (PLC) which hydrolyzes membrane phosphatidylinositol 4,5-bisphosphate to

*Abbreviations:* CPA, cyclopiazonic acid; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; fMLP, formyl-Met-Leu-Phe; FPR, formyl peptide receptor; HBSS, Hanks' balanced salt solution; $IP_3$, inositol trisphosphate; $IP_3R$, inositol trisphosphate receptor; NAC, *N*-acetyl-L-cysteine; NEM, *N*-ethylmaleimide; PLC, phospholipase C; ROCE, receptor-operated $Ca^{2+}$ entry; RT-PCR, reverse transcription-polymerase chain reaction; SERCA, sarcoplasmic-endoplasmic reticulum $Ca^{2+}$-ATPase; SOCE, store-operated $Ca^{2+}$ entry; TCEP, tris-(2-carboxyethyl)phosphine; TCP, tris-(2-cyanoethyl)phosphine; TRP, transient receptor potential

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generate the second messenger, inositol trisphosphate ($IP_3$), which interacts with $IP_3$ receptor ($IP_3R$) on the internal stores causing the release of $Ca^{2+}$ [1]. However, the mechanism regulating $Ca^{2+}$ influx across the plasma membrane, which accounts for the sustained increase in $[Ca^{2+}]_i$, is still unclear. In non-excitable cells, including neutrophils, depletion of the intracellular $Ca^{2+}$ stores induces entry of $Ca^{2+}$ across the plasma membrane, referred to as store-operated $Ca^{2+}$ entry (SOCE) [2], thereby replenishing the depleted intracellular storage compartments and prolonging the initial agonist-induced $Ca^{2+}$ signal. Homologues of the *Drosophila* transient receptor potential (*trp*) channel gene are widely distributed in mammalian tissues. TRP proteins are assumed to play a part in SOCE and/or receptor-operated $Ca^{2+}$ entry (ROCE), however this proposition remains a controversial issue. Recently, Roos et al. [3] have suggested that stromal interaction molecule 1 (STIM1), a ubiquitously expressed protein, may be a common component of SOCE.

Thiols play a principal role in maintaining the appropriate oxidation-reduction state of proteins. The susceptibility of thiols to oxidation can lead to the formation of disulfides and higher oxidation products. Evidence has demonstrated that thiol modification of a number of important plasma membrane and intracellular channels has an influence on the regulation of $[Ca^{2+}]_i$ [4–6]. Our previous report indicated that *N*-ethylmaleimide (NEM), a cell permeable thiol-alkylating agent, stimulated $Ca^{2+}$ entry in neutrophils through a non-SOCE mechanism probably via direct protein thiol oxidation [7]. To further explore the effect of NEM on $Ca^{2+}$ entry in neutrophils, the present study demonstrates that NEM affects formyl-Met-Leu-Phe (fMLP)- and cyclopiazonic acid (CPA)-induced $Ca^{2+}$ signals, via direct protein thiol oxidation, with very distinct features.

## 2. Materials and methods

### 2.1. Materials

Dextran T-500 was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Hanks' balanced salt solution (HBSS) was obtained from Invitrogen. Fluo-3/AM, tris-(2-carboxyethyl)phosphine (TCEP) and fluorescein phalloidin were purchased from Molecular Probes. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Calbiochem-Novabiochem. All other reagents and chemicals were purchased from Sigma-Aldrich. The final volume of dimethyl sulfoxide (DMSO) in the reaction mixture was $\leq$0.5% (v/v).

### 2.2. Preparation of rat neutrophils

Blood was collected from the abdominal aorta of male Sprague–Dawley rats (3–4 months old) and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll–Paque, and hypotonic lysis of erythrocytes [7]. Purified neutrophils containing >95% viable cells were normally resuspended in HBSS supplemented with 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice-bath until used. All experiments in the present study were performed under the guidelines of the Institutional Experimental Laboratory Animal Committee and were in strict accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

### 2.3. Measurement of intracellular free $Ca^{2+}$

Neutrophils ($5 \times 10^7$ cells/ml) were incubated with 5 μM fluo-3/AM for 45 min at 37 °C. After being washed, the cells were resuspended in HBSS to $5 \times 10^6$ cells/ml. Fluorescence was monitored with a fluorescence spectrophotometer at 535 nm with excitation at 488 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where $F$ is the observed fluorescence intensity [8]. The values $F_{max}$ and $F_{min}$ were obtained at the end of experiments by the sequential addition of 0.33% Triton X-100 and 50 mM EGTA. The $K_d$ was taken as 400 nM. The superimposed $[Ca^{2+}]_i$ response was obtained from the same batch of neutrophil preparation.

### 2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and electrophoresis of products

Total RNA was prepared and the PCR amplification was performed as previously described [9]. The sequences of the primer pairs used along with the predicted size of their expected fragments (shown in parentheses in base pairs) are as follows: 5′-acagatgttacaagattttggg-3′ and 5′-aacttccattctttatcctcatg-3′ (TRPC1, 393 bp), 5′-cctgagcgaagtcacactcccac-3′ and 5′-ccactctacatcactgtcatcc-3′ (TRPC3, 529 bp), 5′-gcctacacctttcaatgtcatccc-3′ and 5′-cttaggttatgtctctcggaggc-3′ (TRPC4, 492 bp), 5′-ctatgagaccagagctattgatg-3′ and 5′-ctaccagggagatgacgttgtatg-3′ (TRPC5, 221 bp), and 5′-gtgccaagtccaaagtccctgc-3′ and 5′-ctgggcctgcagtacgtatc-3′ (TRPC6, 315 bp) [10]; 5′-cagtttcacccgattggcgtat-3′ and 5′-ctttggggatggcaggatgtta-3′ (TRPC2, 487 bp) [11]; 5′-gtggaggtttcatctgcaagc-3′ and 5′-gctttcgtggaatactcggtc-3′ ($IP_3R1$, 524 bp), and 5′-gctcttgtccctgacattg-3′ and 5′-cccatgtctccattctcatagc-3′ ($IP_3R2$, 362 bp) [12]; 5′-catctgcttctccattgcc-3′ and 5′-tgacattaaacagcgtctcc-3′ ($IP_3R3$, 338 bp) (L06096). The total RNA (5 μg) and oligo$(dT)_{15}$ were used for the first strand cDNA synthesis. PCR amplification was performed with initial heating for 5 min at 95 °C, followed by 28 cycles of 45 s denaturation at 94 °C, annealing for 30–45 s at 60–63 °C, extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR products were resolved using a 2% agarose gel, and the sequences of these products were

confirmed using a CEQ 2000 capillary sequencer (Beckman Coulter) with Dye Terminator Cycle Sequencing kit.

### 2.5. Measurement of mitochondrial membrane potential

Neutrophils ($5 \times 10^7$ cells/ml) were incubated at room temperature with 5 μM JC-1 for 10 min. After being washed, the cells were resuspended in HBSS to $5 \times 10^6$ cells/ml. Fluorescence changes were monitored with a double-wavelength fluorescence spectrophotometer alternatively at 528 nm with excitation at 485 and at 633 nm with excitation at 575 in a ratio mode [13]. JC-1 fluorescence has two emission peaks, with red fluorescence of J-aggregates indicating hyperpolarized mitochondria and green fluorescence (JC-1 monomers) indicating low mitochondria membrane potential.

### 2.6. Confocal microscopy

Neutrophils were fixed for 10 min with 3% (v/v) paraformaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$, pH 7.5) at room temperature, and then plated onto poly-L-lysine-coated coverslips. Cells were twice rinsed thoroughly and permeabilized with 0.2% (w/v) saponin. After being rinsed, cells were incubated with 1% (w/v) BSA followed by incubation with fluorescein phalloidin for 10 min. Cells were then washed twice and mounted with 50% (v/v) glycerol. Fluorescence images were viewed with an oil objective (100×, 1.40 NA) using an upright Leica confocal laser scanning microscope system (TCS SP2). F-actin was identified by its green emission at LP515 nm with excitation at 488 nm. To study the distribution and localization of intracellular fluorescence, cells were scanned with *XY*-sections in *Z* direction throughout the central portion of cells. The fluorescence density of F-actin of a total of 150 cells for each test was quantified using Multi Gauge (Fuji) software and expressed as quantum level (QL) per cell. Samples were also examined in differential interference contrast (DIC).

### 2.7. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison test and the Student's *t*-test method for a comparison of two groups; $P < 0.05$ was considered statistically significant. Values are expressed as means ± S.D.

## 3. Results

### 3.1. Effect of NEM on CPA- and fMLP-induced $[Ca^{2+}]_i$ elevation

CPA, a sarcoplasmic-endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibitor, selectively inhibits the $Ca^{2+}$ pump of internal $Ca^{2+}$ stores, allowing store depletion by resting $IP_3$ levels independent of activation of receptors on the plasma membrane, and stimulates SOCE. As shown in Fig. 1A, NEM concentration-dependently enhanced the

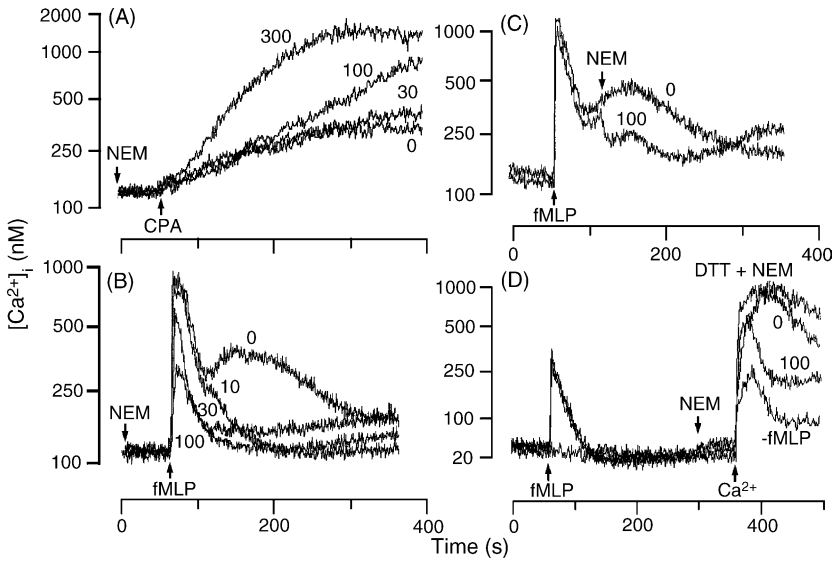


Fig. 1. Effect of NEM on fMLP- and CPA-induced $[Ca^{2+}]_i$ changes in neutrophils. Cells were treated with the indicated concentrations (μM) of NEM for 1 min before stimulation with (A) 1 μM CPA or (B) 0.3 μM fMLP in a $Ca^{2+}$ (1 mM)-containing medium. (C) Cells were stimulated with fMLP followed by addition or no addition of NEM in a $Ca^{2+}$ (1 mM)-containing medium. (D) Cells were stimulated or not stimulated with fMLP followed by addition or no addition of NEM, or addition of 1 mM DTT 1 min prior to NEM addition in a $Ca^{2+}$-free medium, then readdition of 1 mM $Ca^{2+}$ into medium. Similar results were obtained in three to four independent experiments.

$Ca^{2+}$ signal caused by subsequent stimulation with CPA. This result reaffirms our earlier report of a similar effect caused by NEM, via thiol oxidation, following stimulation with CPA [7]. Interestingly, NEM concentration-dependently inhibited the $[Ca^{2+}]_i$ rise in response to fMLP (Fig. 1B), which activates a G-protein-coupled formyl peptide receptor (FPR) on the plasma membrane. The receptor-activated $Ca^{2+}$ signaling causes an initial $Ca^{2+}$ release phase followed by external $Ca^{2+}$ entry through the plasma membrane channels. One might assume that the inhibition by NEM is largely attributed to the blockade of external $Ca^{2+}$ entry in that NEM mainly affects the late phase of fMLP-induced $Ca^{2+}$ signal at low concentrations ($\leq$30 μM), whereas inhibition of the initial $Ca^{2+}$ release phase was more prominent at higher concentrations ($38.5 \pm 7.2\%$ inhibition of maximal $[Ca^{2+}]_i$ at 30 μM NEM, $P < 0.05$; $71.3 \pm 6.4\%$ inhibition of maximal $[Ca^{2+}]_i$ at 100 μM NEM, $P < 0.01$). To address this hypothesis, we assessed its effects in the following two treatment paradigms, bypassing the $Ca^{2+}$ release component. In the first treatment, following the initial $Ca^{2+}$ spike after the fMLP stimulation, the external $Ca^{2+}$ influx was preceded by delayed onset of the activated $Ca^{2+}$ entry [14]. Addition of 100 μM NEM after the initial spike immediately inhibited the $Ca^{2+}$ entry (Fig. 1C). In the second treatment, after stimulation with fMLP in a $Ca^{2+}$-free medium, the subsequent addition of $Ca^{2+}$ to the medium elicited an instantaneous $Ca^{2+}$ spike in cells, representing the overshoot response of $Ca^{2+}$ entry through the plasma membrane channel. Addition of 100 μM NEM after fMLP activation inhibited the $Ca^{2+}$ signal (Fig. 1D). These results further confirm the above notion of NEM inhibition of fMLP-activated $Ca^{2+}$ entry. This inhibitory effect appears to be linked to thiol oxidation because of the prevention by a cell permeable dithiol-reducing agent dithiothreitol (DTT) (Fig. 1D), which maintains protein thiol groups in reduced state.

### 3.2. TRP expression in neutrophils

The family of TRPC proteins (composed of TRPC1–TRPC7) is believed to assemble into a wide variety of ion channels [15]. Previous reports demonstrated the presence of mRNA of TRPC6 using RT-PCR in human neutrophils [15,16]. Recently, TRPC1, TRPC3, TRPC4, and TRPC6 were shown to express as mRNA as well as membrane proteins in human neutrophils [17]. In the present study, RT-PCR was performed using six specific primer pairs to screen for *TRPC1–TRPC6* gene products in rat neutrophils, and while no assay was performed for *TRPC7*, we have confirmed the existence of TRPC6 mRNA in neutrophils (Fig. 2), whereas rat brain expresses the mRNA of all six TRPCs [10]. Comparison of sequences obtained with the GenBank database (data not shown) demonstrated 99% identity between PCR product and the published rat TRPC6.

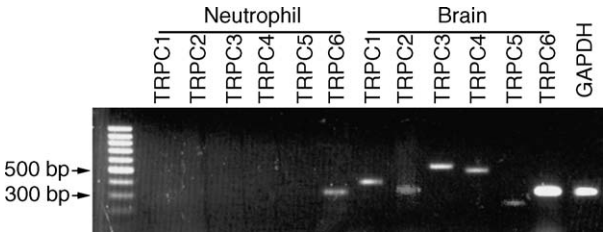


Fig. 2. Identification of neutrophil TRP isoforms using RT-PCR. Total RNA was extracted from rat neutrophils and brain, and RT-PCR was performed with primers for TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, TRPC6, and GAPDH. Similar results were obtained in three independent experiments.

### 3.3. Effect of DTNB on CPA- and fMLP-induced $[Ca^{2+}]_i$ elevation

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), which has been widely used for modification and quantitative estimation of thiol groups in proteins, enhanced the CPA-induced $[Ca^{2+}]_i$ rise (Fig. 3A) but inhibited the fMLP-evoked late phase of $Ca^{2+}$ signaling (Fig. 3B and C). DTNB (3 mM) alone had no effect on the basal $[Ca^{2+}]_i$ (Fig. 3D). As DTNB readily forms a mixed disulfide with thiols, liberating the intense yellow chromophore 5-mercapto-2-nitrobenzoic acid, it makes experimentation with DTT prevention impractical.

### 3.4. Preventive effect of NAC and TCEP on NEM inhibition of fMLP-induced $[Ca^{2+}]_i$ elevation

In attempt to clarify the role of thiol in NEM inhibition of fMLP-induced $Ca^{2+}$ response, we also examined the effects of other reductants with or without thiol group, besides DTT, on the inhibition by NEM. Pretreatment of cells with 1 mM *N*-acetyl-L-cysteine (NAC), a cell permeable monothiol-reducing agent, for 10 min prevented the NEM inhibition of fMLP-induced $[Ca^{2+}]_i$ rise (Fig. 4A). In addition, cells pretreated with 0.3 mM TCEP, a cell impermeable reductant without a thiol group, for 10 min prior to NEM also prevented the inhibition by NEM (Fig. 4B). This result excludes the possibility that NEM targets the thiol group of reductants.

### 3.5. Effect of NEM on mitochondrial membrane potential

Mitochondria are well known participants in the regulation of $[Ca^{2+}]_i$ homeostasis, capable of modulating cytosolic $Ca^{2+}$ signals. Functional mitochondria appear to maintain SOCE by effective sequestration of subplasmalemmal $Ca^{2+}$ and by the consequential attenuation of $Ca^{2+}$-induced inactivation of SOC channels [18]. Hyperpolarization increases the driving force for mitochondrial $Ca^{2+}$ uptake. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, dissipates mitochon-

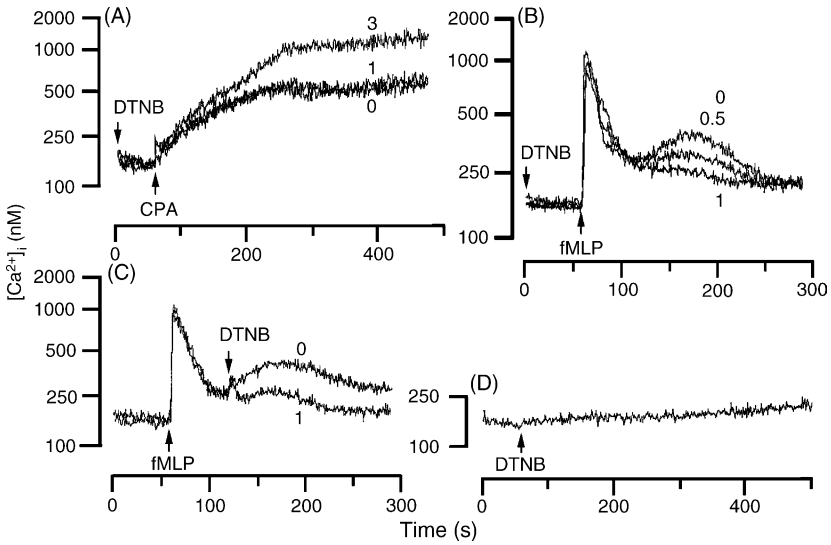


Fig. 3. Effect of DTNB on fMLP- and CPA- induced $[Ca^{2+}]_i$ changes in neutrophils in a $Ca^{2+}$ (1 mM)-containing medium. Cells were treated with the indicated concentrations (mM) of DTNB for 1 min before stimulation with (A) 1 μM CPA or (B) 0.3 μM fMLP. (C) Cells were stimulated with fMLP followed by addition or no addition of DTNB. (D) Cells were treated with 3 mM DTNB. Similar results were obtained in three to four independent experiments.

drial $H^+$ gradients, thus reducing the mitochondrial membrane potential and inactivating SOCE channels [19]. The fluorescence remained relatively unchanged after addition of 300 μM NEM to JC-1-loaded cells (Fig. 5) indicates that NEM did not affect mitochondrial membrane potential.

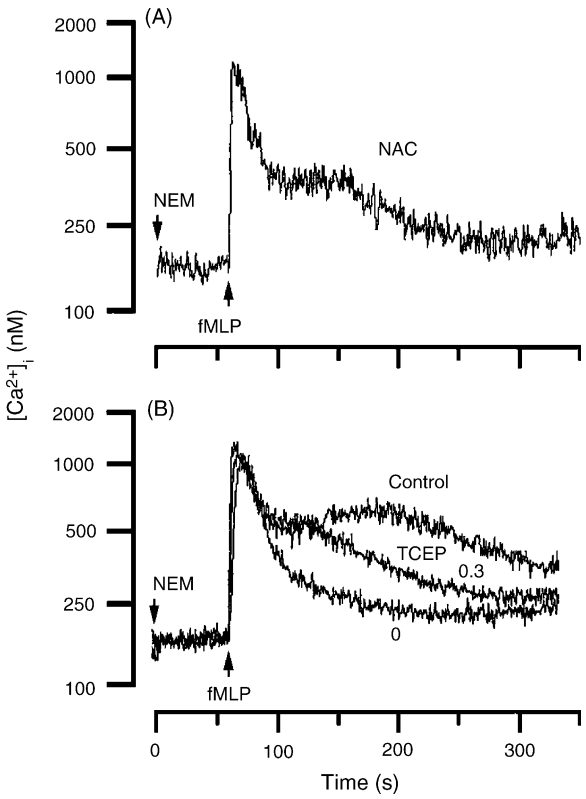


Fig. 4. Effect of NAC and TCEP on NEM inhibition of fMLP-induced $[Ca^{2+}]_i$ changes in neutrophils in a $Ca^{2+}$ (1 mM)-containing medium. (A) Cells were treated with 1 mM NAC for 10 min followed by addition of 100 μM NEM before stimulation with 0.3 μM fMLP. (B) Cells were treated with or without 0.3 mM TCEP for 10 min followed by addition or no addition of NEM before stimulation with fMLP. Similar results were obtained in three to four independent experiments.

### 3.6. Effect of NEM on the organization of actin filament

Reorganization of the actin has been implicated in the activation of SOCE [20]. The actin cytoskeleton network is organized in two major structures: a cytosolic actin filament network and a membrane-associated cytoskeleton. Unstimulated neutrophils show a diffuse cytoplasmic distribution of F-actin. Upon cell activation, neutrophils show a transformation of G-actin to F-actin and a shift of F-actin to the cell outline [21]. Stimulation of neutrophils with 0.3 μM fMLP resulted in morphologic changes, polarization of front-tail shaped cells, and promoted actin filament accumulation ($19715 \pm 462$ versus $40531 \pm 654$ QL, $P < 0.01$) in the anterior lamellipodium and tail (Fig. 6).

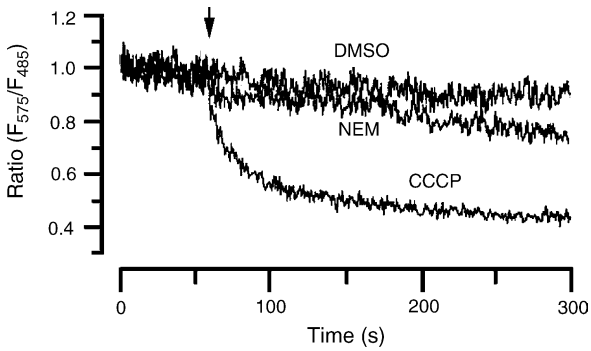


Fig. 5. Effect of NEM on mitochondrial membrane potential. DMSO, 300 μM NEM or 1 μM CCCP was added to JC-1-loaded neutrophils in a $Ca^{2+}$ (1 mM)-containing medium. Fluorescence changes were measured in a ratio mode. Similar results were obtained in three independent experiments.

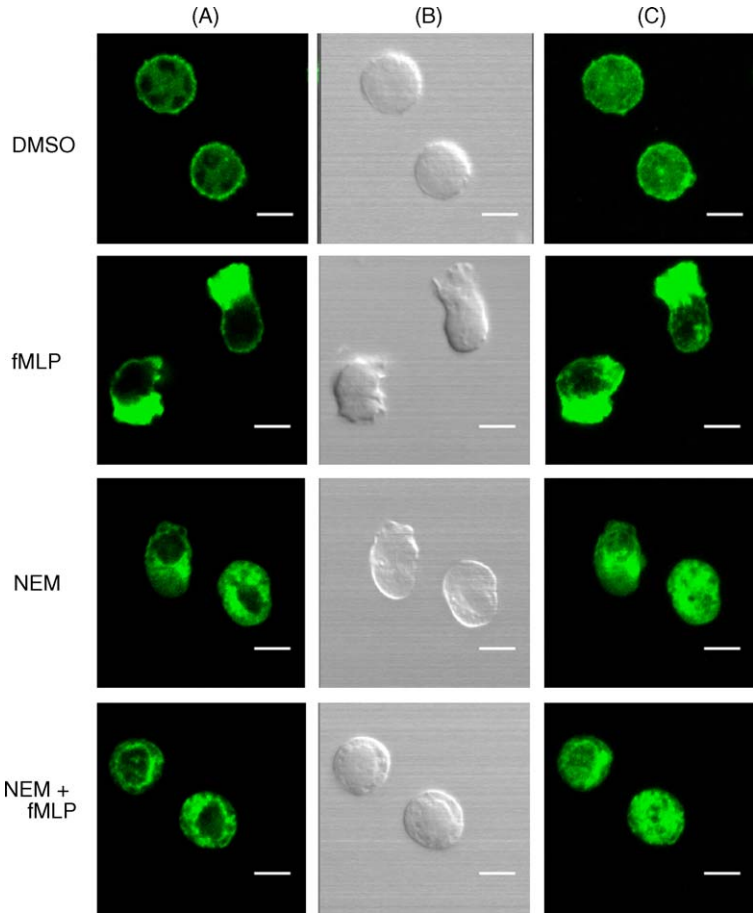


Fig. 6. Effect of NEM on the organization of actin filament in neutrophils in response to fMLP. Cells were preincubated with DMSO (as control) or 100 μM NEM for 1 min before addition of DMSO or 0.3 μM fMLP for 3 min in a $Ca^{2+}$ (1 mM)-containing medium. After fixation, cells were then stained with fluorescein phalloidin. Samples are shown in (A) *XY* sections, (B) DIC images, and (C) *XY* projections. Scale bar is 8 μm. Similar results were obtained in three to four independent experiments.

Treatment of cells with 300 μM NEM did not change either the morphology or the level of actin filaments in cells (20991 ± 442 QL, $P > 0.05$), but showed diffuse cytoplasmic distribution of F-actin. Sequential addition of NEM and fMLP prevented both the fMLP-induced morphologic changes and F-actin accumulation (19023 ± 665 QL, $P < 0.01$).

### 3.7. Effect of NEM on internal $Ca^{2+}$ release

We next examined the $[Ca^{2+}]_i$ changes in the absence of extracellular $Ca^{2+}$, uncomplicated by the effect of NEM on $Ca^{2+}$ entry. fMLP evoked a transient $Ca^{2+}$ spike without a subsequent $Ca^{2+}$ entry phase in the absence of external $Ca^{2+}$. NEM concentration-dependently attenuated the $Ca^{2+}$ spike (Fig. 7A); significant inhibition was observed at concentrations of NEM ≥30 μM (43.7 ± 7.6 and 78.6 ± 7.1% inhibition of maximal $[Ca^{2+}]_i$ at 30 and 100 μM NEM, respectively, both $P < 0.01$). A slow $[Ca^{2+}]_i$ rise, reached a maximal level at 1 min and then gradually declined, this was observed upon stimulation of cells with CPA in the absence of external $Ca^{2+}$. In contrast to the inhibition of fMLP-induced response, NEM sustained the CPA-induced $[Ca^{2+}]_i$ rise (Fig. 7B). Tris-(2-cyanoethyl)phosphine (TCP), a cell permeable reductant without a thiol group, prevented the NEM inhibition of fMLP-induced $[Ca^{2+}]_i$ rise (Fig. 7C). Pretreatment with DTNB had no effect on fMLP-induced $Ca^{2+}$ spike (Fig. 7D).

### 3.8. Expression of $IP_3R$ receptors

$IP_3R$ is a pivotal molecule for cytosolic $Ca^{2+}$ mobilization in neutrophils. G-protein-coupled receptor agonist-induced $Ca^{2+}$ entry requires recognition of $IP_3$ by the $IP_3R$, the subsequent conformation changes in the $IP_3R$ gate endogenous $Ca^{2+}$ entry channels. There are at least three $IP_3R$ isoforms ($IP_3R1$–$IP_3R3$) [22], which are distributed differently in tissue and are anticipated to have different regulatory properties. To determine the $IP_3R$ expressed in rat neutrophils, RT-PCR was performed with primers designed to amplify the cDNA of $IP_3R$ isoforms. Of the three putative $IP_3Rs$, two pairs of primers, namely those complementary to $IP_3R2$ and

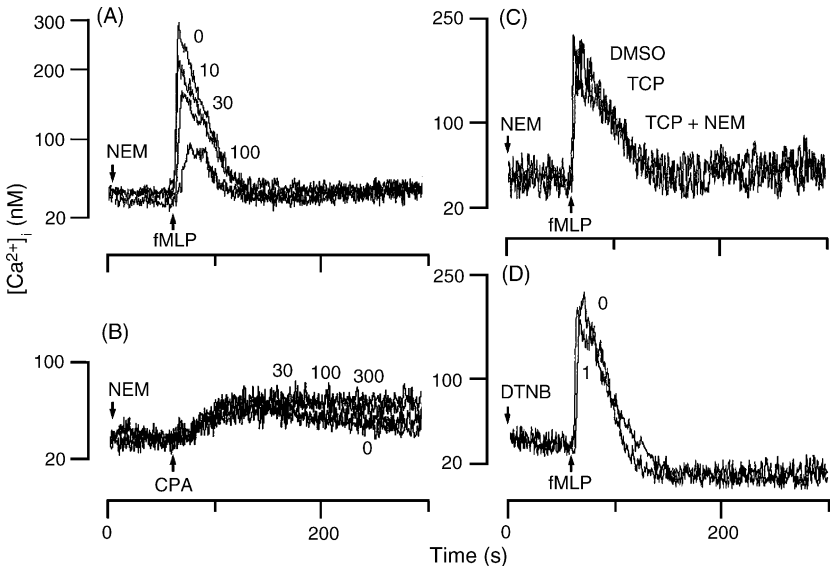


Fig. 7. Effect of NEM on fMLP- and CPA-induced $[Ca^{2+}]_i$ changes in neutrophils in a $Ca^{2+}$-free medium. Cells were treated with the indicated concentrations (μM) of NEM for 1 min before stimulation with (A) 0.3 μM fMLP or (B) 10 μM CPA. (C) Cells were treated with DMSO or 1 mM TCP for 10 min, or with TCP for 10 min followed by addition of 100 μM NEM before stimulation with fMLP. (D) Cells were treated with or without 1 mM DTNB for 1 min before stimulation with fMLP. Similar results were obtained in three to four independent experiments.

$IP_3R3$ produced a single PCR product band of the expected size of each product in agarose electrophoresis (Fig. 8). The clear band was detected for $IP_3R3$ but seen less extensively in the $IP_3R2$. No evidence was found of *$IP_3R1$* gene product, whereas this product could be amplified in rat cerebellum [12]. Comparison of sequences obtained with the GenBank database (data not shown) demonstrated 100 and 97% identity between PCR products and the published rat $IP_3R2$ and $IP_3R3$, respectively. The finding of *$IP_3R2$* and *$IP_3R3$* gene products by RT-PCR suggested the presence of $IP_3R2$ and $IP_3R3$ proteins in neutrophils and presumably their involvement in $Ca^{2+}$ signaling.

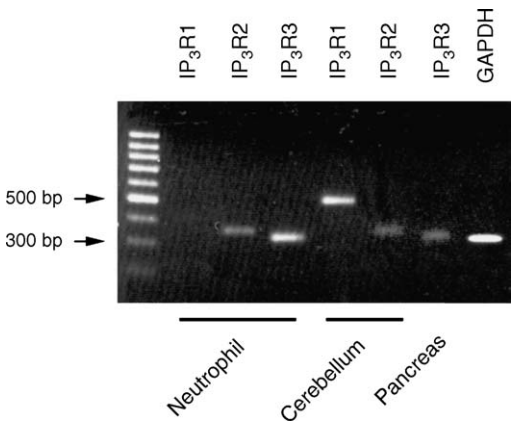


Fig. 8. Identification of neutrophil $IP_3R$ isoforms using RT-PCR. Total RNA was extracted from rat neutrophils, cerebellum and pancreas, and RT-PCR was performed with primers for $IP_3R1$, $IP_3R2$, $IP_3R3$, and GAPDH. Similar results were obtained in three independent experiments.

## 4. Discussion

Our previous report demonstrated that NEM stimulated a slow $[Ca^{2+}]_i$ rise, significant response was observed at concentrations of NEM ≥100 μM, after a concentration-dependent lag in rat neutrophils [7]. This $Ca^{2+}$ signal was attributed to the $Ca^{2+}$ entry through the non-SOCE pathway, via direct protein thiols oxidation, but not to the $Ca^{2+}$ release from the internal stores. The great increase in CPA-induced $[Ca^{2+}]_i$ rise might result from additional $Ca^{2+}$ entry through the non-SOCE pathway activated by NEM because these both occur within similar concentration ranges. However, our previous reports demonstrate that the activation of non-SOCE might attenuate CPA-induced $Ca^{2+}$ entry [23–25]. Alternatively, Favre et al. [26] have proposed that store emptying allows either the $Ca^{2+}$ entry channel itself or associated proteins to alter its configuration thereby exposing a key thiol group for modification in enhancing channel activity. However, thiol oxidation by NO donor inhibited SOCE in neutrophils [23]. The reasons for these disparate results are unclear but might be attributed to modification of either the different protein thiols by these two stimulants or the same critical thiols through alkylation and *S*-nitrosylation, respectively, by NEM and NO donor, which results in distinct configurational changes.

In contrast with the results obtained in experiments with CPA, NEM inhibited the fMLP-induced $Ca^{2+}$ response. Similar discrepant results were also obtained by using a dithiol-oxidizing agent DTNB replaced NEM in the experiments. In addition to DTT, both NAC and TCEP prevented the NEM inhibition of fMLP-induced $Ca^{2+}$

signal strengthens the notion that NEM targets the protein thiol. The membrane impermeable property of DTNB and TCEP implies that the protein thiol in the outer plasma membrane is linked to the regulation of $Ca^{2+}$ entry. The discrepancy between the effects of NEM on $Ca^{2+}$ signals in response to fMLP and CPA may be due to the different roles of protein thiols in the regulation of $Ca^{2+}$ signals with different stimulants. A number of differences between the characteristics of SERCA inhibitor- and formyl peptide-induced $Ca^{2+}$ signal has been reported in neutrophils, which suggests that these two stimulants-activated $Ca^{2+}$ entry pathways have differing underlying regulation [27]. Besides the SOCE pathway, fMLP is able to activate the ROCE pathway in HL60 cells [28]. Several different channel molecules are likely to be involved in receptor-activated $Ca^{2+}$ signaling. It has been reported that intact thiol groups are required for FPR ligand-binding in neutrophils [29,30]. However, the possibility that the blockade of fMLP binding to FPR mediated the NEM inhibition of fMLP-induced $Ca^{2+}$ signal could be obviated as our results are incompatible with the previous observations [29] that NEM, but not the cell impermeable thiol-oxidizing agents, caused rapid suppression of FPR ligand-binding. Alternatively, PLC-γ has been implicated, as a permissive adaptor without necessarily requiring release of $Ca^{2+}$ from stores, in ROCE but not SOCE in PC12 or A7r5 cells [31]. Whether PLC-γ plays an adaptor role in fMLP-induced $Ca^{2+}$ entry in neutrophils is still unclear.

The diverse TRP superfamily of channels has been divided into TRPC, TRPV, and TRPM subfamilies based on structural motifs. There is more literary evidence to suggest that the TRPCs form ROC channels than the other two TRP channel subgroups [15]. However, the expression systems in cell lines cannot prove the native ROCE depends on TRPCs, thus the evidence that TRP proteins are functionally important in wild-type cells remains indirect. Although TRPC6 mRNA exists in rat neutrophils as determined by RT-PCR, it is not known whether the TRPC6 protein is expressed to any significant extent because the antibodies for specific rat TRPC6 are not available yet. Thus whether TRPC6 mediates NEM inhibition of external $Ca^{2+}$ entry awaits further investigation. It has been reported that TRPC6 is directly activated by diacylglycerol, independent of PKC [32]. The lack of $[Ca^{2+}]_i$ rise in human neutrophils in response to 1-oleoyl-2-acetyl-*sn*-glycerol [16,33] makes the role of TRPC6 uncertain in the functional regulation of $Ca^{2+}$ entry in neutrophils.

Besides the blockade of external $Ca^{2+}$ entry, promoting the removal of the cytosolic free $Ca^{2+}$ may also account for the decrease in $[Ca^{2+}]_i$. After cell activation, removal of $Ca^{2+}$ from cytosol occurs via extrusion by the plasma membrane $Ca^{2+}$-ATPase (PMCA) and $Na^+/Ca^{2+}$ exchanger as well as sequestration into internal stores via the SERCA. It has been reported that NEM inhibits the transport activity of the $Na^+/Ca^{2+}$ exchanger expressed in HEK 293 cells [34]. However, our previous report suggested that NEM has no effect on the $Na^+/Ca^{2+}$ exchanger activity in neutrophils [7]. It is unlikely that the promotion of PMCA and SERCA activities may explain the apparent discrepancy between the effects of NEM on CPA- and fMLP-induced $Ca^{2+}$ responses. In addition, liver microsomal $Ca^{2+}$-ATPase activity is decreased by NEM [5]. Mitochondrial permeability transition is accompanied by a significant decrease in the total membrane protein thiol content [13,35], thereby depolarizing mitochondria and decreases the driving force for mitochondrial sequestration of subplasmalemmal $Ca^{2+}$. However, mitochondria might play a minor role in NEM inhibition of $Ca^{2+}$ entry as evidenced from the lack of mitochondrial depolarization by NEM. This result is consistent with the lack of inhibition of CPA-induced SOCE by NEM. The nature of the precise molecular target for regulation of $Ca^{2+}$ entry in response to thiol oxidation in neutrophils remains to be seen.

Based on the secretion-like coupling model, reorganization of the actin cytoskeleton plays a key role in the activation of SOCE [20]. However, the role played by the actin cytoskeleton in regulation of $Ca^{2+}$ entry through the ROCE pathway is less well defined. Although the receptor-activated and store depletion-derived $Ca^{2+}$ signals are regarded as distinct sometimes, in reality they display many similar characteristics. It has been reported that calyculin A, a protein phosphatase 1/2A inhibitor, reorganizes actin filaments into a tight cortical layer and inhibits CPA-induced SOCE [7] as well as fMLP-induced polarization response and $Ca^{2+}$ entry in neutrophils [21,36]. Moreover, TRPC proteins are initially localized at the cell surface and their internalization by calyculin A was observed in expression systems [37] as well as in human neutrophils [17]. The result that NEM prevented both the fMLP-induced morphologic changes and F-actin accumulation raise the possibility of a role for actin reorganization in the NEM inhibition of fMLP-induced $Ca^{2+}$ entry in neutrophils, despite the distinct patterns of cytoskeletal reorganization for NEM and calyculin A. Rho family has been implicated in actin filament reorganization [38] and RhoA possesses vicinal cysteines within the guanine nucleotide-binding region and the phosphohydrolase activity site. Phenylarsine oxide, which complexes with vicinal sulfhydryl groups of proteins to form stable dithioarsine ring structures, inhibit RhoA, reduce and reorganize the actin cytoskeleton in Caco-2 cells [39]. It is plausible that the inactivation of RhoA by NEM could be responsible for reorganization of the cytoskeleton. Further study will be required to clarify the relationship between the cytoskeletal reorganization and the regulation of ROCE.

In the absence of extracellular $Ca^{2+}$, the discrepant results that NEM inhibited fMLP-evoked $Ca^{2+}$ spike but sustained the CPA-induced $[Ca^{2+}]_i$ rise are consistent with those obtained in the presence of external $Ca^{2+}$. The

prevention of NEM inhibition by TCP together with the lack of inhibition by DTNB of fMLP-induced $Ca^{2+}$ spike indicate that the intracellular protein thiol is linked to the regulation of $Ca^{2+}$ release. A previous study demonstrated that thiol oxidation by thimerosal alone is able to release $Ca^{2+}$ from internal stores and causes a persistent accumulation of $Ca^{2+}$ within human neutrophils after stimulation by fMLP [40]. Thus the distinct features of $Ca^{2+}$ signal for NEM and thimerosal is probably mediated by the modification of different thiols.

$IP_3R$ is a pivotal intracellular $Ca^{2+}$ release channel for cytosolic $Ca^{2+}$ mobilization in neutrophils. A previous report indicated that human polymorphonuclear leukocytes predominantly express $IP_3R2$ [41]. The RT-PCR experiment showed the presence of $IP_3R2$ and $IP_3R3$ mRNA in rat neutrophils. However, it is not known whether the $IP_3R2$ and $IP_3R3$ proteins are expressed to any significant extent because the antibodies for specific $IP_3R$ isoforms are not available yet. Phosphorylation of $IP_3R$ increases the potency of $IP_3$ in stimulating $Ca^{2+}$ flux from microsomes [42]. Calcineurin (or protein phosphatase 2B) is physiologically associated with the $IP_3R$. The calcineurin inhibition of $Ca^{2+}$ flux reflects dephosphorylation of $IP_3R$. However, the report that NEM inhibition of calcineurin activity [43] makes it unlikely to be involved in the NEM inhibition of fMLP-induced $Ca^{2+}$ release in neutrophils. Tyrosine phosphorylation of the $IP_3R$ may increase their open probability and may play a role in regulating $[Ca^{2+}]_i$ [44]. Protein tyrosine phosphatase contains redox-sensitive cysteines at the active site, and oxidation of the thiol group inactivates enzyme activity [45]. Whether NEM inhibition of protein tyrosine phosphatase therefore increasing the tyrosine phosphorylation of $IP_3R$ occurs in neutrophils is unclear. Since $IP_3$ mediates both fMLP- and CPA-induced $Ca^{2+}$ release, the opposite $Ca^{2+}$ response to NEM between two stimulants obviate $IP_3/IP_3R$ as possible sites of action. NEM elicits $Ca^{2+}$ release through the activation of ryanodine receptor in the sarcoplasmic reticulum vesicles of skeletal muscle [6]. Human neutrophils contain ryanodine-sensitive $Ca^{2+}$ stores, which may play a minor role in the modulation of $[Ca^{2+}]_i$ accumulation mediated by a SERCA inhibitor [46]. Whether the sustained CPA-induced $Ca^{2+}$ signal by NEM is attributed to the activation of ryanodine-sensitive $Ca^{2+}$ stores awaits further investigation. Further studies are necessary to clarify the nature of the precise intracellular molecular target for regulation of $Ca^{2+}$ release in response to thiol modification in neutrophils.

In conclusion, NEM enhanced the CPA-induced $[Ca^{2+}]_i$ rise, but inhibited the fMLP-induced $Ca^{2+}$ signal, in the presence or absence of external $Ca^{2+}$ in rat neutrophils through direct protein thiol oxidation. Further studies are required to identify the membrane-associated and the internal protein thiols, which mediate the regulation of receptor-activated and store depletion-derived $Ca^{2+}$ signals.

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