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Characterization of maleimide-activated Ca^{2+} entry in neutrophils

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Abstract

N-Ethylmaleimide (NEM), a thio-alkylating agent, concentration-dependently stimulated the elevation of $[Ca^{2+}]_i$ in rat neutrophils in the presence of external Ca^{2+}. This effect was not observed in Ca^{2+}-free medium and was abrogated by dithiothreitol pretreatment. The application of NEM after cyclopiazonic acid (CPA) stimulated the store-emptying activation of Ca^{2+} entry. Unlike CPA-induced cation entry, NEM showed poor uptake of Ba^{2+} and Sr^{2+} and did not induce Mn^{2+} influx. NEM diminished CPA-induced Mn^{2+} influx, an effect that was blocked by dithiothreitol. Both Ni^{2+} and La^{3+} attenuated the elevation of $[Ca^{2+}]_i$ in response to NEM; however, greater resistance was observed to Ni^{2+} inhibition of NEM-induced Ca^{2+} influx than inhibition of store-operated Ca^{2+} entry. Both *cis-N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A) and 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole (SKF-96365), Ca^{2+} channel blockers, and calyculin A, an inhibitor of protein serine/threonine phosphatases 1/2, diminished the NEM-induced Ca^{2+} entry. Treatment of cells with genistein, a general tyrosine kinase inhibitor, or with wortmannin and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), phosphatidylinositol 3-kinase inhibitors, had no appreciable inhibitory effects on the action of NEM. However, 2-aminoethyldiphenyl borate, an inositol trisphosphate receptor antagonist, enhanced rather than inhibited the $[Ca^{2+}]_i$ change in response to NEM. These results indicate that NEM stimulates Ca^{2+} entry and regulates Ca^{2+} signaling through direct thiol oxidation, bypassing the cellular signal transduction pathway. The NEM-regulated Ca^{2+} signal demonstrates characteristics that distinguish it from the store-emptying operation in neutrophils, and therefore represents two distinct modes of Ca^{2+} regulation.

Keywords: *N*-Ethylmaleimide; Cation entry; Intracellular free Ca^{2+}; Cyclopiazonic acid; Neutrophils

1. Introduction

Neutrophils play a pivotal role in inflammatory reactions and constitute the first line of host defense. Ca^{2+} signals have been implicated in many cellular functions of neutrophils [1,2]. The induction of receptor-mediated cytosolic Ca^{2+} signals involves two closely coupled events: a rapid and transient release of stored Ca^{2+}, followed by slow entry of extracellular Ca^{2+} [3]. 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 generate the second messenger, inositol trisphosphate (IP_3), which in turn interacts with the IP_3 receptor in releasing calcium from internal stores [4]. 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 intracellular Ca^{2+} stores induces entry of Ca^{2+} across the plasma membrane, referred to as store-operated Ca^{2+} entry (capacitative Ca^{2+} entry) [5]. Although several hypotheses have been proposed regarding direct or indirect coupling mechanisms of the internal stores and the plasma membrane (reviewed recently in Ref. [6]), little is known about the intracellular signals governing store-operated Ca^{2+} entry or the Ca^{2+} channels mediating this particular form of Ca^{2+} entry. The transient receptor potential (TRP) channel, which was discovered through genetic studies of the *Drosophila* visual transduction mutation, has been reported to have characteristics similar to store-operated Ca^{2+} entry channels (for a recent review see Ref. [7]).

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Abbreviations: 2-APB, 2-aminoethyldiphenyl borate; CPA, cyclopiazonic acid; DTT, dithiothreitol; HBSS, Hanks' balanced salt solution; IP_3, inositol trisphosphate; NEM, *N*-ethylmaleimide; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C.

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Although such store-operated entry of Ca^{2+} can be demonstrated clearly in a wide variety of different cells, it is far from certain that this mechanism is the only one involved in the increase in Ca^{2+} entry in non-excitable cells. A non-store-operated Ca^{2+} entry mechanism that involves protein kinase C has been reported in human platelets [8]. A phosphatidylinositol 3,4,5-trisphosphate-sensitive Ca^{2+} entry that is independent of the filling state of internal Ca^{2+} stores was observed in FcεRI-stimulated mast cells [9]. In addition, arachidonic acid activates non-store-operated Ca^{2+} entry into smooth muscle cells [10]. There is growing evidence supporting thiol modification of a number of important membrane proteins or channels for the regulation of $[Ca^{2+}]_i$, including microsomal Ca^{2+}-ATPase [11], ryanodine receptor Ca^{2+} release [12], Na^+-Ca^{2+} exchanger [13], N-type Ca^{2+} channel [14], and high K^+-induced Ca^{2+} influx [15]. Here, we show that NEM, a thiol-alkylating agent, induces Ca^{2+} entry and regulates the Ca^{2+} signal in rat neutrophils with characteristics that distinguish it from the better known store-operated mechanism.

2. Materials and methods

2.1. Materials

Dextran T-500 was purchased from Pharmacia, and Hanks' balanced salt solution (HBSS) was obtained from Gibco. Fluo-3/AM and fura-2/AM were purchased from Molecular Probes. *cis-N*-(2-Phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A) was obtained from Alexis. Calyculin A, wortmannin, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole (SKF-96365), and 2-aminoethyldiphenyl borate (2-APB) were obtained from Calbiochem. 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) was obtained from Biomol Research. All other reagents and chemicals were purchased from Sigma. The final volume of DMSO in the reaction mixture was ≤0.5% (v/v).

2.2. Preparation of rat neutrophils

Neutrophils were isolated from Sprague–Dawley rats as described previously [16]. Briefly, fresh whole blood was obtained from the abdominal aorta and immediately mixed with EDTA. The neutrophils were purified by dextran sedimentation followed by centrifugation through Ficoll-Hypaque and hypotonic lysis of the erythrocytes. Purified neutrophils of >95% viability were suspended in HBSS containing 10 mM HEPES (pH 7.4) and 4 mM $NaHCO_3$, and kept in an ice bath before use.

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

Neutrophils (5×10^7 cells/mL) were loaded with 5 μM fluo-3/AM at 37° for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 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: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$, where F is the observed fluorescence intensity [17]. F_{max} and F_{min} values were obtained at the end of the experiments by the sequential addition of 0.33% Triton X-100 and 50 mM EGTA. The K_d was taken as 400 nM. Some data are expressed as means ± SEM.

2.4. Measurement of cation permeability

Neutrophils (5×10^7 cells/mL) were loaded with 5 μM fura-2/AM at 37° for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/mL [16]. Fura-2-loaded cells were stimulated in a Ca^{2+}-free medium followed by the addition of Ca^{2+}, Ba^{2+}, or Sr^{2+} to the medium. Fluorescence was monitored with a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) at 510 nm with excitation at 340 and 380 nm in the ratio mode. The entry of Mn^{2+} into the cells was measured by a fura-2 fluorescence quenching technique [18]. Fluorescence changes were monitored at 510 nm with excitation at 360 nm, the isosbestic point where fura-2 is insensitive to changing $[Ca^{2+}]_i$; fluorescence intensity declined as Mn^{2+} was added into the Ca^{2+} (1 mM)-containing medium. Diethylenetriamine pentaacetic acid (2 mM) was added at the end of the experiments, and indicated that <5% of the total fluorescence quenched by Mn^{2+} was due to leakage of fura-2.

2.5. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance. $P < 0.05$ was considered to be a significant difference.

3. Results and discussion

3.1. Maleimide-mediated Ca^{2+} entry

The NEM concentration-dependent increase in $[Ca^{2+}]_i$ was preceded by a pronounced lag in rat neutrophils (Fig. 1A). NEM has been reported to inhibit Ca^{2+}-ATPase activity and to promote Ca^{2+} release from Ca^{2+}-loaded nuclei, microsomes, and sarcoplasmic reticulum [11,12]. In the absence of external Ca^{2+}, NEM induced no changes in $[Ca^{2+}]_i$ (Fig. 1B), which clearly reflects a Ca^{2+} entry mechanism rather than release from internal stores. It is conceivable that NEM, a membrane-permeant alkylating agent, reacts rapidly with the reactive thiol groups of proteins. The fact that NEM-activated Ca^{2+} entry was prevented by dithiothreitol (DTT) (Fig. 1C), which maintains protein thiol groups in a reduced state, implicates

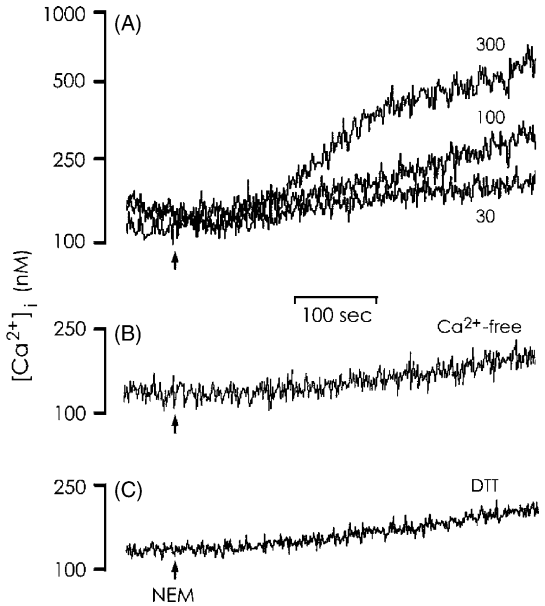

Fig. 1. Stimulatory effect of NEM on $[Ca^{2+}]_i$. (A) Cells were stimulated with the indicated concentrations (μM) of NEM (arrow) in Ca^{2+} (1 mM)-containing medium. In some experiments, cells were stimulated with 300 μM NEM (arrow) (B) in a Ca^{2+}-free medium or (C) were preincubated with 1 mM DTT in the presence of Ca^{2+} for 1 min before NEM stimulation. Similar results were obtained from three independent experiments.

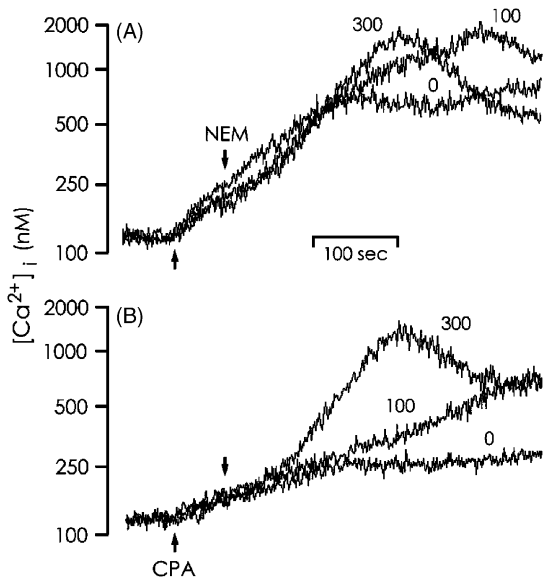

Fig. 2. Effect of NEM on CPA-induced $[Ca^{2+}]_i$ change. Cells were stimulated with (A) 5 μM or (B) 1 μM CPA (upward arrow) for 1 min in a Ca^{2+} (1 mM)-containing medium before the addition or without the addition of various concentrations (μM) of NEM (downward arrow) into the medium. Similar results were obtained from three independent experiments.

protein thiol oxidation. Thus, the NEM-induced $[Ca^{2+}]_i$ elevation is believed to reflect a Ca^{2+} entry mechanism activated through the direct oxidation of reactive thiols of the entry channel or an associated protein that might produce a conformational change governing the influx of extracellular Ca^{2+}.

The transport activity of the Na^+-Ca^{2+} exchanger expressed in HEK 293 cells has been reported to be sensitive to thiol modification [13]. To address this possibility, neutrophils were suspended in HEPES buffer (124 mM NaCl, 4 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 10 mM HEPES, pH 7.4, 5.56 mM dextrose, 15.2 mM $NaHCO_3$, and 1 mM $CaCl_2$) or in Na^+-deprived HEPES buffer (the sodium salts were replaced by 124 mM *N*-methyl-D-glucamine/gluconic acid, 0.64 mM K_2HPO_4, and 15.2 mM $KHCO_3$). Similar responses of NEM-activated Ca^{2+} entry were obtained in normal as well as in Na^+-deprived HEPES buffer (data not shown), which indicates the independence of the Na^+-Ca^{2+} exchange.

3.2. Effect of store emptying on maleimide-induced Ca^{2+} entry

Previous studies have revealed that the NEM-induced increase in $[Ca^{2+}]_i$ is enhanced substantially by store emptying in DDT_1MF-2 smooth muscle cells [19] but not in HEK 293 cells [20]. Store-operated Ca^{2+} entry differs significantly between cells. This phenomenon has been proposed to represent a family of distinct channel proteins and/or different associations with regulatory proteins [21,22]. We investigated the mutual influence on Ca^{2+} entry in rat neutrophils of the NEM-induced process and the store-emptying operation, using CPA. CPA selectively inhibits the Ca^{2+} pump of internal stores [23], allowing the stores to be emptied independently of receptor activation, thereby activating the store-operated Ca^{2+} entry mechanism. Application of NEM following treatment with 1 or 5 μM CPA showed the concentration dependence of the enhancement of $[Ca^{2+}]_i$ elevation (Fig. 2A and B). The NEM-induced stimulatory effect was more pronounced in cells treated with a low concentration of CPA. This was probably because a near maximal Ca^{2+} influx response was obtained with 5 μM CPA. A similar enhancement of $[Ca^{2+}]_i$ elevation was also observed by treatment with NEM followed by CPA, and the stimulation of $[Ca^{2+}]_i$ elevation was prevented by 2-mercaptoethanol (data not shown), which maintains protein thiol groups in the reduced state. Favre *et al*. [19] have proposed that store emptying allows the Ca^{2+} entry channel itself or associated proteins to alter its configuration so as to expose a key thiol group that is important in enhancing channel activity.

3.3. Characteristics of maleimide-mediated cation permeability

Store-emptying stimulation of Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mn^{2+} entry has been reported in human neutrophils [24]. In the absence of external Ca^{2+}, CPA induced only a slight change in $[Ca^{2+}]_i$. A large and rapid Ca^{2+} entry, representing the overshoot response for the store-operated Ca^{2+} channel, commenced immediately upon the re-addition of Ca^{2+}. Removal of external Ca^{2+} followed by the addition of Ba^{2+} resulted in Ba^{2+} entry, which, as for Sr^{2+}, was also

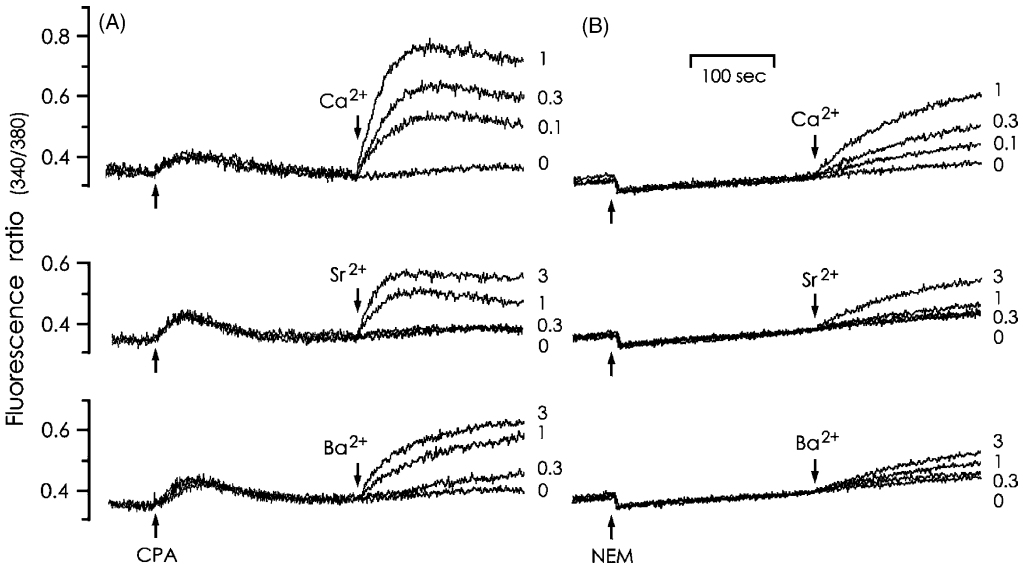

Fig. 3. Comparison of cation permeability in response to CPA and NEM. Fura-2-loaded cells were stimulated (upward arrow) with (A) 10 μM CPA or (B) 300 μM NEM for 4 min in a Ca^{2+}-free medium before the addition (downward arrow) of the indicated concentrations (mM) of Ca^{2+}, Sr^{2+}, or Ba^{2+} into the medium. The changes in fluorescence were monitored by fura-2 ratio-fluorimetry. Similar results were obtained from three independent experiments.

detectable by fura-2 ratio-fluorimetry (Fig. 3A). Entry of the divalent cations was concentration-dependent with permeability in the following order: $Ca^{2+} > Ba^{2+} \geq Sr^{2+}$, which is consistent with data reported in the literature [25]. A concentration dependence of externally applied Ca^{2+} entry activated by NEM was observed under the same conditions. However, appreciable entry of Ba^{2+} and Sr^{2+} occurred only at higher external cation concentrations (Fig. 3B). The results with Ba^{2+} and Sr^{2+} reveal a difference in the apparent selectivity for passage of cations activated by store emptying as opposed to maleimide alkylation in neutrophils.

Activation of the store-operated entry mechanism induces an increased rate of Mn^{2+} influx [25]. Mn^{2+} has been shown to permeate through the neutrophils *via* a Ca^{2+} influx pathway activated by CPA [18] and subsequent quenching of the fluorescence signal by its high-affinity binding activity to fura-2. Mn^{2+} is not a substrate for the Ca^{2+} pump and, hence, a surrogate of Ca^{2+} influx. In rat neutrophils, CPA induced Mn^{2+} influx, a response that was abolished in the presence of La^{3+}, a Ca^{2+} channel blocker. Surprisingly, NEM did not stimulate Mn^{2+} influx in rat neutrophils (Fig. 4A). This result suggests a fundamental difference between the function of store-operated Ca^{2+} entry and the NEM-induced entry mechanism. Moreover, the Mn^{2+} influx in response to CPA was almost eliminated in the presence of NEM, and this inhibitory effect was prevented by pretreatment of the cells with DTT (Fig. 4B). The finding that NEM enhanced Ca^{2+} entry but prevented Mn^{2+} influx in store emptying implies that there is a different regulation mechanism governing store-operated Ca^{2+} entry and Mn^{2+} influx.

The effectiveness of specific Ca^{2+} channel blockers, La^{3+} and Ni^{2+}, in blocking the passage of Ca^{2+} has been widely used as a criterion for defining differences between putative entry mechanisms. Various concentrations of La^{3+} or Ni^{2+} were added after the addition of NEM. La^{3+} (0.1 mM) eliminated the NEM-induced Ca^{2+} entry, while up to 1 mM Ni^{2+} showed about 50% inhibition (Fig. 5B). Under the same experimental conditions, the CPA-induced Ca^{2+} entry was abolished by 0.1 mM La^{3+} and 1 mM Ni^{2+} (half-maximal inhibition was observed with approximately 0.1 mM Ni^{2+}) (Fig. 5A). The greater resistance to inhibition by Ni^{2+} of NEM-induced vs. store-operated Ca^{2+} entry re-affirmed the difference between the two modes of Ca^{2+} entry.

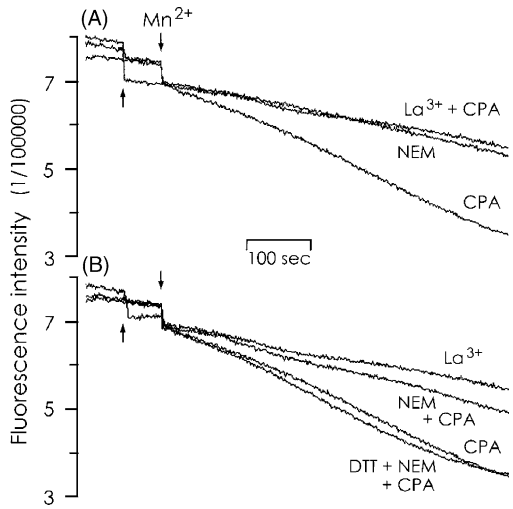

Fig. 4. Effect of NEM on Mn^{2+} influx. Fura-2-loaded neutrophils were preincubated (A) with or without 5 mM La^{3+} followed by 300 μM NEM or 10 μM CPA (upward arrow), and were subsequently exposed to 0.5 mM Mn^{2+} (downward arrow) in Ca^{2+} (1 mM)-containing medium, or (B) with 5 mM La^{3+} or 10 μM CPA, with or without 1 mM DTT for 1 min preceding 300 μM NEM for an additional 1 min followed by CPA (upward arrow), and then were exposed to 0.5 mM Mn^{2+} (downward arrow) in a Ca^{2+} (1 mM)-containing medium. Similar results were obtained from three independent experiments.

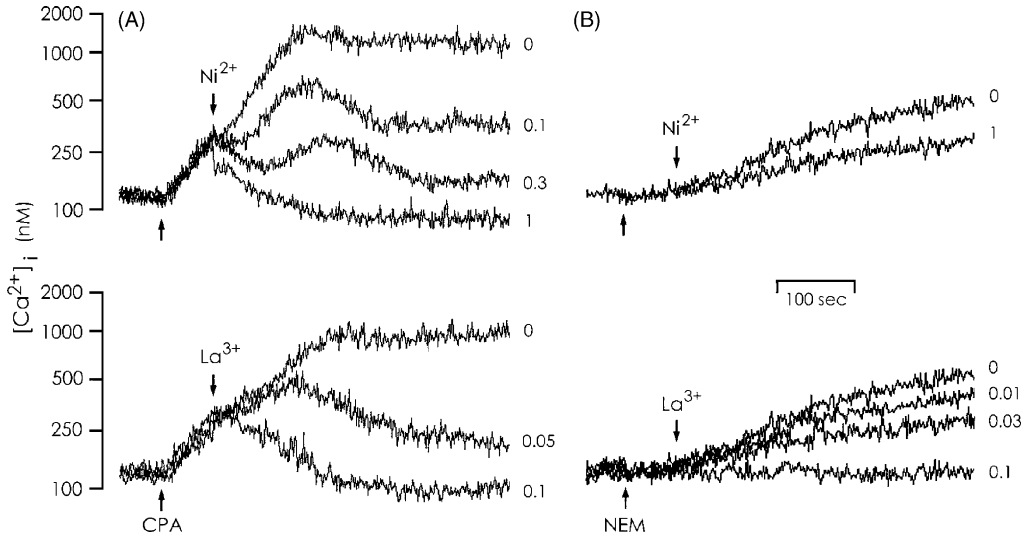

Fig. 5. Inhibitory effects of Ni^{2+} and La^{3+} on $[Ca^{2+}]_i$ change. Cells were stimulated with (A) 5 μM CPA (upward arrow) or (B) 300 μM NEM (upward arrow) for 1 min in a Ca^{2+} (1 mM)-containing medium before the addition of the indicated concentrations (mM) of Ni^{2+} or La^{3+} (downward arrow) into the medium. Similar results were obtained from three independent experiments.

3.4. Effects of Ca^{2+} signal blockers on maleimide-mediated Ca^{2+} entry

A useful tool in elucidating the coupling mechanism for store-operated channel activation has been the cell-permeant IP_3 receptor blocker 2-APB, which blocks receptor-induced Ca^{2+} release from internal stores [26]. Pretreatment of neutrophils with 2-APB diminished the CPA-induced $[Ca^{2+}]_i$ changes (Fig. 6B), but enhanced the NEM-induced response (352 ± 24 nM for control vs. 1074 ± 52 nM, $P < 0.01$) (Fig. 6A). This result suggests the independence of the IP_3 receptor in NEM-mediated Ca^{2+} entry. A recent report indicated that a non-capacitative Ca^{2+} entry caused by arachidonic acid is unaffected by

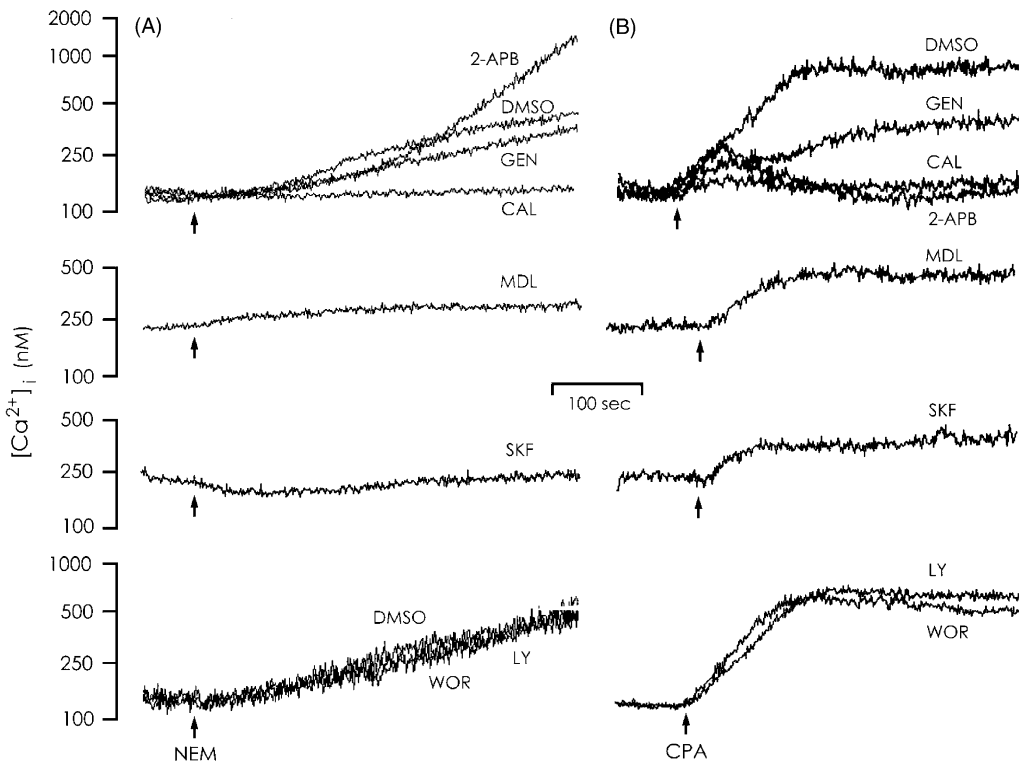

Fig. 6. Effects of 2-APB, calyculin A, genistein, MDL-12,330A, wortmannin, and LY294002 on $[Ca^{2+}]_i$ change. Cells were preincubated with DMSO (as control), a 100 μM concentration of each 2-APB or genistein (GEN) for 1 min, 50 μM SKF-96365 (SKF) for 3 min, 0.1 μM calyculin A (CAL) or 100 μM MDL-12,330A (MDL) for 10 min, or DMSO (as control), 10 μM wortmannin (WOR) or 50 μM LY294002 (LY) for 1 min before stimulation with (A) 300 μM NEM (upward arrow) or (B) 5 μM CPA (upward arrow) in a Ca^{2+} (1 mM)-containing medium. Similar results were obtained from three independent experiments.

2-APB [27]. At present, we have no evidence to explain the 2-APB enhancement of the NEM-induced response. MDL-12,330A blocked Ca^{2+} entry, independent of adenylyl cyclase, following store emptying in a number of different cell types [28]. In addition, SKF-96365, a receptor-operated and voltage-gated Ca^{2+} channel inhibitor, blocked the formyl peptide-activated Ca^{2+} entry in neutrophils [24]. Pretreatment of cells with MDL-12,330A and SKF-96365 prevented maleimide-mediated Ca^{2+} entry in neutrophils (34 ± 17 nM and 28 ± 14 nM, respectively, both $P < 0.01$) (Fig. 6A) and also greatly reduced the CPA-induced response (Fig. 6B).

There are a number of theories about store-operated Ca^{2+} entry. One of these hypotheses is a secretion-like mechanism involving close but reversible interactions between the internal stores and the plasma membrane [29]. Calyculin A, an inhibitor of protein serine/threonine phosphatases 1/2, activates the translocation of existing F-actin to the cell periphery, which prevents store-operated Ca^{2+} entry activation by blocking the association of internal stores with the plasma membrane [30]. It has been shown that treatment with calyculin A greatly attenuates the CPA-induced Ca^{2+} entry in human neutrophils [24], and the present study demonstrated that it also diminished the action of NEM (24 ± 15 nM, $P < 0.01$) (Fig. 6A). This finding suggests that stabilization of the cortical F-actin network acts as a physical barrier to prevent Ca^{2+} entry in the store-emptying operation as well as in a maleimide-mediated process.

The PLC-γ isoforms are activated by receptor and non-receptor protein tyrosine kinases [31,32]. Genistein, a general tyrosine kinase inhibitor, has been demonstrated to inhibit store-operated Ca^{2+} entry in human neutrophils [33], but in our study it only slightly affected the NEM-induced response (335 ± 23 nM, $P > 0.05$) (Fig. 6A). Thus, under these conditions, protein tyrosine phosphorylation plays a minor role.

The involvement of phosphatidylinositol 3-kinase (PI3K) and its product, phosphatidylinositol 3,4,5-trisphosphate, which mediates recruitment of PLC-γ to the lipid bilayer, in the full activation of PLC-γ, has been reported in many different cell types [34–36]. Thus, PI3K is at the crossroads of a tyrosine kinase-mediated Ca^{2+} signaling pathway. In RBL-2H3 mast cells, phosphatidylinositol 3,4,5-trisphosphate directly stimulates a Ca^{2+} transport system in the plasma membrane that is independent of PLC activity [9]. Two distinct inhibitors of PI3K, wortmannin and LY294002 [37,38], have been useful tools for examining the role of PI3K. These inhibitors have been demonstrated to block PLC-γ activation and/or Ca^{2+} signals in a wide variety of different cells [9,36,39], including Fcγ receptor-mediated Ca^{2+} release and influx in human neutrophils [40]. The observations that both wortmannin and LY294002 failed to inhibit the maleimide-mediated $[Ca^{2+}]_i$ change (384 ± 28 nM for control vs. 372 ± 27 nM and 397 ± 32 nM for inhibitors, respectively, $P > 0.05$) (Fig. 6A) and only slightly affected the CPA-induced response (Fig. 6B) preclude the involvement of the PI3K pathway. Taken together, the different mode of actions between the store-emptying operation and that of NEM is further strengthened by a comparison of the actions of these signal transduction blockers.

In conclusion, NEM induced Ca^{2+} entry and regulated the Ca^{2+} signal in rat neutrophils through direct protein thiol oxidation, bypassing the cellular signaling pathways. Analysis of cation permeability and the actions of Ca^{2+} blockers suggests a fundamental difference between the function of the store-emptying operation and the maleimide-mediated mechanism.

Acknowledgments

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