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GEA3162 stimulates Ca^{2+} entry in neutrophils

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Abstract

We showed that 5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium (GEA3162), a lipophilic nitric oxide (NO)-releasing agent, induced Ca^{2+} entry into rat neutrophils in a concentration-dependent manner, whereas the guanylyl cyclase inhibitors, 6-anilino-5,8-quinolinequinone (LY83583) and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), had no effect on GEA3162-induced response. The GEA3162-induced Ca^{2+} entry was not observed in a Ca^{2+}-free medium. GEA3162 did not potentiate but reduced the store-emptying activated Ca^{2+} entry caused by cyclopiazonic acid. Stimulation of cells with GEA3162 in the absence of extracellular Ca^{2+} followed by addition of cations showed that only Ca^{2+} but not Ba^{2+} and Sr^{2+} entry occurs. Store-operated Ca^{2+} entry was sensitive to La^{3+} and Ni^{2+} inhibition, whereas the GEA3162-induced Ca^{2+} entry was sensitive to La^{3+} but resistant to Ni^{2+}. *cis*-*N*-(2-Phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A) and calyculin A diminished the Ca^{2+} entry activated by cyclopiazonic acid as well as by GEA3162. In contrast, 2-aminoethyldiphenyl borate (2-APB) diminished cyclopiazonic acid–but enhanced GEA3162-induced $[Ca^{2+}]_i$ change. Genistein effectively attenuated the cyclopiazonic acid–but slightly inhibited GEA3162-induced $[Ca^{2+}]_i$ change. Application of neomycin and high extracellular Ca^{2+} concentration did not induce $[Ca^{2+}]_i$ rise. These data suggest that GEA3162 induced Ca^{2+} entry and regulated Ca^{2+} signal, through direct protein thiol oxidation. The action of GEA3162 demonstrates characteristics that distinguish it from the store-operated mechanism in neutrophils and therefore is likely to represent an entirely distinct pathway. Extracellular Ca^{2+}-sensing receptor is not existing in neutrophils.

Keywords: GEA3162; *S*-Nitrosylation; Cation entry; Ca^{2+}, intracellular, free; Cyclopiazonic acid; Neutrophil

1. Introduction

In neutrophils, Ca^{2+} signals have been implicated in many cellular functions (Korchak et al., 1984; O'Flaherty et al., 1991). Receptor-induced cytosolic Ca^{2+} signals involve two closely coupled components: rapid and transient release of Ca^{2+} from internal stores, followed by slowly developing extracellular Ca^{2+} entry (Von Tscharner et al., 1986). It is conceivable that the initial Ca^{2+} spike is mediated by the phosphoinositide cascade through the activation of phospholipase C to generate the second messenger, inositol trisphosphate (IP_3), which interacts with the IP_3 receptor to release Ca^{2+} from the internal stores (Berridge and Irvine, 1989). However, the nature of the Ca^{2+} entry pathway and its regulation is far from clear. Receptor-enhanced entry of Ca^{2+} in nonexcitable cells is generally ascribed to a store-operated mechanism (capacitative Ca^{2+} entry) in which the activation of the entry pathway is specifically dependent on the emptying of agonist-sensitive internal Ca^{2+} stores. The resulting depletion of Ca^{2+} store serves as the primary trigger for a message, which is returned to the plasma membrane resulting in the slow activation of store-operated Ca^{2+} entry (Putney, 1990). This Ca^{2+} entry phase of Ca^{2+} signaling serves to mediate longer term cytosolic Ca^{2+} elevations and provides a means to replenish internal stores (Putney and Bird, 1993). The mechanism for coupling internal store depletion with Ca^{2+} entry is not yet clear. It may involve the release and/or generation of a diffusible signaling molecule within the cell that activates the plasma membrane channels responsible for Ca^{2+} entry (Randriamampita and Tsien, 1993; Graier et al., 1995). Alternatively, a more direct molecular coupling may occur between the stores and the plasma membrane channels (Somasundaram et al., 1995). Transient receptor potential proteins have been described, which display characteristics similar to store-operated Ca^{2+} entry channels, as reviewed recently (Putney and McKay, 1999).

Although such store-operated entry of Ca^{2+} can be clearly demonstrated in a wide variety of different cells, it

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is far from certain that such mechanism is the only one involved in the increase in Ca^{2+} entry in nonexcitable cells. A nonstore-operated Ca^{2+} entry mechanism which involves protein kinase C has been reported in human platelets (Rosado and Sage, 2000). 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 (Ching et al., 2001). In addition, arachidonic acid activates the nonstore-operated Ca^{2+} entry in smooth muscle cells (Broad et al., 1999). Studies have revealed that a number of important plasma membrane and intracellular channels for Ca^{2+} are regulated by nitric oxide (NO)-mediated thiol nitrosylation (Lipton et al., 1993; Campbell et al., 1996; Xu et al., 1998). Here, we show that 5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium (GEA3162), a lipophilic NO-releasing agent (Kankaanranta et al., 1996), induced Ca^{2+} entry and regulated Ca^{2+} signal with characteristics distinct from that of the store-operated mechanism in rat neutrophils.

2. Materials and methods

2.1. Materials

Dextran T-500 was purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Hanks' balanced salt solution was obtained from Gibco Life Technologies (Gaithersburg, MD, USA). Fluo-3 acetoxymethyl ester (fluo-3/AM) and fura-2/AM were purchased from Molecular Probes (Eugene, OR, USA). 5-Amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium (GEA3162) and *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12,330A) were obtained from Alexis (San Diego, CA, USA). Calyculin A and 2-aminoethyldiphenyl borate (2-APB) were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). 6-Anilino-5,8-quinolinequinone (LY83583) was purchased from Research Biochemicals International (Natick, MA, USA). 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All other reagents and chemicals were purchased from Sigma (St. Louis, MO, USA). The final volume of dimethyl sulfoxide (DMSO) in the reaction mixture was $\leq 0.5\%$ (v/v).

2.2. Preparation of rat neutrophils

Neutrophils were isolated from Sprague–Dawley rats as described previously (Wang et al., 1995). Briefly, fresh whole blood was obtained from the abdominal aorta and immediately mixed with ethylenediamine tetraacetate. The neutrophils were purified by dextran sedimentation followed by centrifugation through Ficoll-Hypaque and hypotonic lysis of erythrocytes. Purified neutrophils of >95% viability were suspended in Hanks' balanced salt solution (HBSS) containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (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 for 45 min at 37 °C. 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 (Merritt et al., 1990). 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. In some experiments, data are expressed as means ± S.D.

2.4. Measurement of cation permeability

Neutrophils (5×10^7 cells/ml) were loaded with 5 μM fura-2/AM at 37 °C for 45 min. After being washed, the cells were resuspended in HBSS to 5×10^6 cells/ml (Wang et al., 1995). Fura-2-loaded cells were stimulated in a Ca^{2+}-free medium followed by 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.

3. Results and discussion

3.1. S-Nitrosylation-mediated Ca^{2+} entry

Application of the lipophilic NO donor, GEA3162, revealed an increase in $[Ca^{2+}]_i$ in rat neutrophils in a concentration-dependent manner. Unlike the chemotactic peptide-induced strong and rapid response, the $[Ca^{2+}]_i$ change caused by GEA3162 was weak and preceded by a concentration-dependent lag (Fig. 1A). This GEA3162-induced $[Ca^{2+}]_i$ change was abolished in a Ca^{2+}-free medium, reflecting a Ca^{2+} entry mechanism rather than release from internal stores (Fig. 1B). Several studies have shown that NO donor-induced *S*-nitrosylation results in activation of Ca^{2+} entry, which is strikingly similar to entry of Ca^{2+} induced by cell-permeable alkylators (Broillet and Firestein, 1997; Favre et al., 1998; Xu et al., 1998). The observation that GEA3162 activated Ca^{2+} entry was prevented by the disulphide-reducing agent 2-mercaptoethnaol (data not shown) suggests the involvement of protein thiol oxidation. GEA3162 might selectively donate NO at the surface of the membrane in the vicinity of reactive thiols of the entry channel or an associated protein, as has been proposed in DDT_1MF-2 smooth muscle cells (Favre et al.,

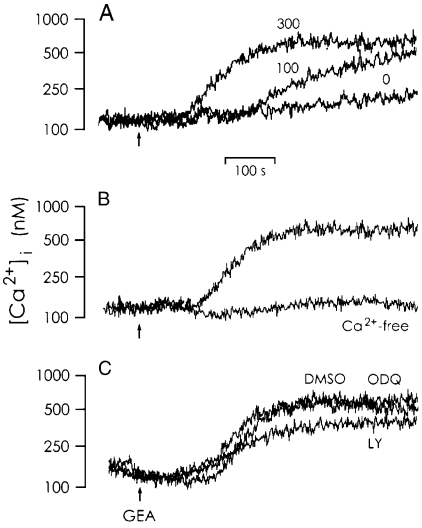

Fig. 1. GEA3162 stimulated $[Ca^{2+}]_i$ changes in rat neutrophils. (A) Concentration dependence of GEA3162 (0–300 μM) stimulated (arrow) the increase in $[Ca^{2+}]_i$ in Ca^{2+} (1 mM)-containing medium. (B) Stimulation of cells with 300 μM GEA3162 (arrow) in the presence or absence of external Ca^{2+}. (C) Cells were preincubated with 20 μM LY83583 (LY) or 20 μM ODQ for 3 min before stimulation with 300 μM GEA3162 (GEA, arrow) in a Ca^{2+}-containing medium. Similar results were obtained from three independent experiments.

1998). It is conceivable that NO is a strong soluble guanylyl cyclase activator, and the NO donor activates soluble guanylyl cyclase in neutrophils (Feelisch and Noack, 1987). However, GEA3162 activated Ca^{2+} entry independent of guanylyl cyclase because no appreciable changes in $[Ca^{2+}]_i$ could be observed with the application of LY83583 and ODQ (403.3 ± 32.1 nM for control vs. 355.1 ± 34.2 and 412.6 ± 31.5 nM, respectively, both $P>0.05$) (Fig. 1C), inhibitors of guanylyl cyclase (Garthwaite et al., 1995; Schmidt et al., 1985). This result is in line with the previous report in DDT_1MF-2 cells (Favre et al., 1998). In addition, our previous report indicated that cGMP does not play an important role in the regulation of $[Ca^{2+}]_i$ in rat neutrophils (Wang et al., 2001). Thus, GEA3162-induced $[Ca^{2+}]_i$ elevation is believed to reflect a Ca^{2+} entry mechanism, independent of guanylyl cyclase, activated through the direct *S*-nitrosylation of protein thiols that might produce conformational change governing the influx of extracellular Ca^{2+}.

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

Previous research has revealed that the *S*-nitrosylation-dependent Ca^{2+} entry mechanism activated by GEA3162 is potentiated by store emptying in DDT_1MF-2 cells but not in human embryonic kidney (HEK) 293 cells (Ma et al., 1999). Since store-operated Ca^{2+} entry differs significantly between cells, it has been proposed that there is a family of distinct channel proteins and/or different association with regulatory proteins (Putney and Bird, 1993; Parekh and Penner, 1997).

In order to evaluate the influence of the store emptying on the effects of GEA3162, we studied the effect of cyclopiazonic acid on GEA3162-activated Ca^{2+} entry. Cyclopiazonic acid selectively inhibits the Ca^{2+} pump of internal stores (Seidler et al., 1989), allowing the stores to be emptied independently of receptor activation and thereby activating the store-operated Ca^{2+} entry mechanism. Under these conditions, emptying of internal Ca^{2+} stores with 1 or 5 μM cyclopiazonic acid did not enhance the GEA3162-induced Ca^{2+} influx (Fig. 2A, B). In contrast, treatment with GEA3162 reduced the cyclopiazonic acid-induced $[Ca^{2+}]_i$ change, suggesting a different entry mechanism.

3.3. Characteristics of cation permeability in S-nitrosylation

Store emptying stimulates entry of Ca^{2+}, Ba^{2+}, Sr^{2+} and Mn^{2+} into human neutrophils (Wenzel-Seifert et al., 1996). Removal followed by the readdition of 1 mM Ca^{2+} in cyclopiazonic acid treatment rat neutrophils caused a large and rapid Ca^{2+} entry representing the overshoot response for store-operated Ca^{2+} channel. Further removal of Ca^{2+} followed by the addition instead of Ba^{2+} resulted in Ba^{2+} entry, which, as for Sr^{2+}, was also detectable by fura-2 ratio-fluorimetry (Fig. 3A). The concentration dependence of externally applied Ca^{2+}, Ba^{2+} or Sr^{2+} (each 0–3 mM) entry activated by store emptying was observed (data not shown). The cation permeability of store-operated entry mechanism followed the order $Ca^{2+} > Ba^{2+} \geq Sr^{2+}$, in accordance with the literature (Fasolato et al., 1994). Under the same conditions, upon readdition of external Ca^{2+} (1 mM) to the GEA3162-treated cells, a slow but prominent Ca^{2+} entry was observed. However, with externally applied Ba^{2+} or Sr^{2+}, only a very small amount of Sr^{2+} entered (Fig. 3B). Up to 3 mM each of Ba^{2+} or Sr^{2+} did not result in any appreciable increase in cation entry (data not shown).

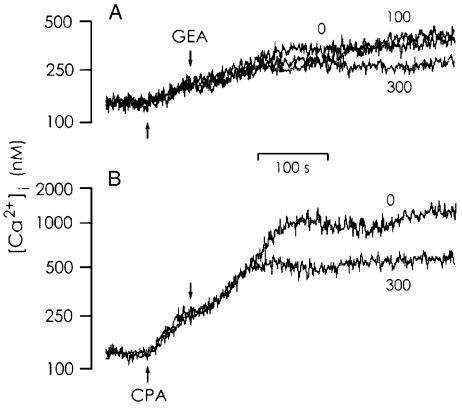

Fig. 2. Effect of cyclopiazonic acid on GEA3162-induced $[Ca^{2+}]_i$ changes in rat neutrophils. Cells were stimulated with (A) 1 μM or (B) 5 μM cyclopiazonic acid (CPA, first arrow) for 1 min in a Ca^{2+}(1 mM)-containing medium before addition or without addition of GEA3162 (GEA, second arrow) into the medium. Similar results were obtained from three independent experiments.

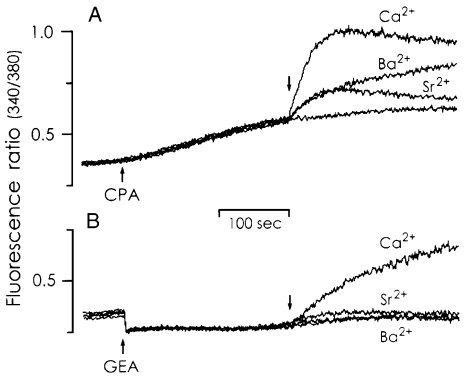

Fig. 3. Comparison of the entry of Ca^{2+}, Ba^{2+} and Sr^{2+} into rat neutrophils in response to cyclopiazonic acid and GEA3162. Fura-2-loaded cells were stimulated (first arrow) with (A) 10 μM cyclopiazonic acid (CPA) or (B) 300 μM GEA3162 (GEA) for 4 min in a Ca^{2+}-free medium before addition (second arrow) or without addition of 1 mM each of Ca^{2+}, Ba^{2+} or Sr^{2+} into the medium. The fluorescence changes were monitored by fura-2 ratio-fluorimetry. Similar results were obtained from three independent experiments.

The results with Ba^{2+} and Sr^{2+} indicated a significant difference in the apparent selectivity for passage of cations activated by store emptying as opposed to GEA3162 stimulation in neutrophils.

Our results are in contradiction with a previous report that GEA3162-activated entry mechanism allowed passage of Ba^{2+}, with one order of magnitude upper the rate of store-operated Ba^{2+} entry, and Sr^{2+} in DDT_1MF-2 cells (Ma et al., 1999). The discrepant results imply the characteristics of cation entry in response to GEA3162 differ significantly between DDT_1MF-2 cells and neutrophils. Mn^{2+} is not a substrate for the Ca^{2+} pump and hence a surrogate of Ca^{2+} influx. Activation of the store-operated entry mechanism induces an increased rate of Mn^{2+} influx (Fasolato et al., 1994). The Mn^{2+} influx experiment was not performed because GEA3162 (300 μM) alone greatly reduced the Fluorescence intensity of fura-2 at 360/510 nm, obscuring the effect of Mn^{2+}.

In other studies on Ca^{2+} entry, criteria for defining differences between putative entry mechanisms have rested on the effectiveness of La^{3+} and Ni^{2+}, the specific Ca^{2+} channel blockers, in blocking passage of Ca^{2+}. Varying concentrations of La^{3+} or Ni^{2+} were added either after store emptying or following GEA3162 addition. Analysis of the La^{3+} dependence of blocking Ca^{2+} entry revealed a significant difference between the two modes of activation. La^{3+} (0.03 mM) showed only slight inhibition of cyclopiazonic acid-activated Ca^{2+} entry, but almost eliminated the GEA3162-induced response (Fig. 4A). In contrast, Ni^{2+} (0.3 mM) greatly attenuated the Ca^{2+} entry in pool emptying, but had little effect on this response to *S*-nitrosylation (Fig. 4B). These results reaffirmed the difference between the two modes of Ca^{2+} entry.

3.4. Effects of Ca^{2+} signal blockers on S-nitrosylation- and store emptying-activated Ca^{2+} entry

2-Aminoethyldiphenyl borate (2-APB), the cell-permeable antagonist of IP_3 receptor (Maruyama et al., 1997), blocks receptor-induced Ca^{2+} release from internal stores and has been a useful tool in elucidating the coupling mechanism for store-operated cation entry. Pretreatment of neutrophils with 2-APB abolished the cyclopiazonic acid-induced $[Ca^{2+}]_i$ change, but enhanced rather than inhibited GEA3162-induced response (Fig. 5). The failure of inhibition of *S*-nitrosylation-activated Ca^{2+} entry by 2-APB is in agreement with the previous report in DDT_1MF-2 cells (Van Rossum et al., 2000), suggesting the independence of the IP_3 receptor mechanism. A recent report indicated that a non-capacitative Ca^{2+} entry caused by arachidonic acid was also unaffected by 2-APB (Luo et al., 2001). MDL-12,330A blocked the Ca^{2+} entry, independent of adenylyl cyclase,

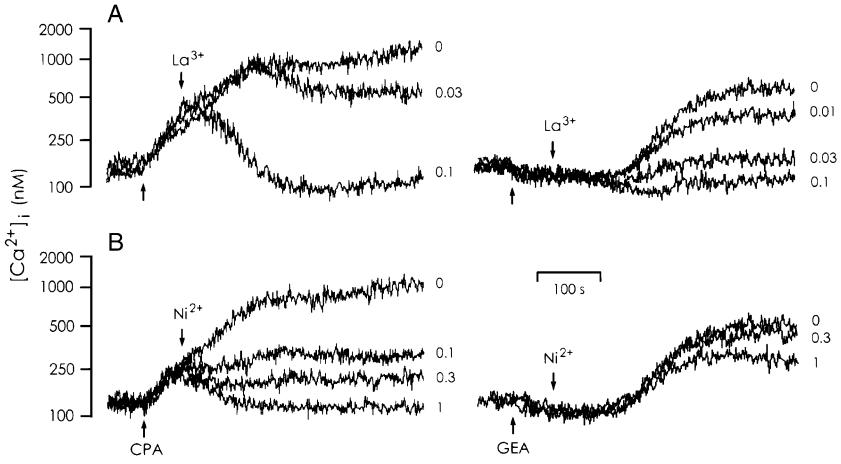

Fig. 4. Comparison of the effects of La^{3+} and Ni^{2+} on cyclopiazonic acid- and GEA3162-stimulated $[Ca^{2+}]_i$ changes in rat neutrophils. Cells were stimulated (first arrow) with 5 μM cyclopiazonic acid (CPA, left panel) or 300 μM GEA3162 (GEA, right panel) for 1 min in a Ca^{2+} (1 mM)-containing medium before addition (second arrow) of indicated concentrations of (A) La^{3+} or (B) Ni^{2+} into the medium. Similar results were obtained from three independent experiments.

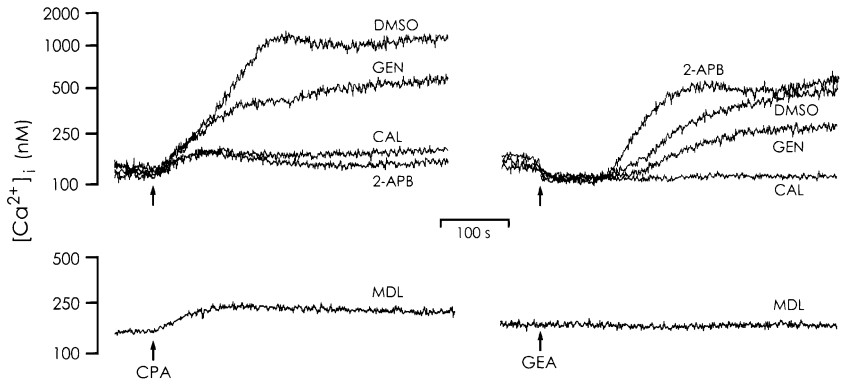

Fig. 5. Effects of 2-APB, calyculin A, genistein and MDL-12,330A on cyclopiazonic acid- and GEA3162-stimulated $[Ca^{2+}]_i$ changes in rat neutrophils. Cells were preincubated with DMSO (as control), 100 μM each of 2-APB or genistein (GEN) for 1 min, 0.1 μM calyculin A (CAL) or 30 μM MDL-12,330A (MDL) for 10 min before stimulation (first arrow) with 5 μM cyclopiazonic acid (CPA, left panel) or 300 μM GEA3162 (GEA, right panel) in a Ca^{2+}(1 mM)-containing medium. Similar results were obtained from three independent experiments.

following store emptying in a number of different cell types (Van Rossum et al., 2000). Thus, the different mode of actions between the operation of store-operated and *S*-Nitrosylation is further strengthened by comparison of the actions of 2-APB. The results that MDL-12,330A prevented store-operated and *S*-nitrosylation-mediated Ca^{2+} entry in neutrophils (Fig. 5) are consistent with previous report in DDT_1MF-2 cells (Van Rossum et al., 2000).

The operation of store-operated Ca^{2+} entry is proposed to be mediated by a physical secretion-like mechanism involving close but reversible interactions between the internal stores and the plasma membrane (Patterson et al., 1999). Calyculin A, an inhibitor of protein phosphatases 1/2A, activates translocation of existing F-actin to the cell peripheral, which prevents store-operated Ca^{2+} entry activation via blockade of the association of internal stores with the plasma membrane (Patterson et al., 1999). Treatment with calyculin A diminished the cyclopiazonic acid-induced Ca^{2+} entry in human neutrophils (Wenzel-Seifert et al., 1996) as well as in rat neutrophils (Fig. 5). Calyculin A failure to inhibit GEA3162-induced response was observed in DDT_1MF-2 cells (Ma et al., 1999), but prevented the action of GEA3162 in neutrophils. It is plausible that the cortical actin filament network acts as a physical barrier to prevent Ca^{2+} entry in store-emptying operation as well as in *S*-nitrosylation-induced process.

Protein tyrosine phosphorylation has been reported to play a role in regulating $[Ca^{2+}]_i$ (Jayaraman et al., 1996; Rhee and Bae, 1997). Genistein, a general tyrosine kinase inhibitor, inhibits the plateau phase of the chemotactic peptide- and Ca^{2+} pump inhibitor-induced Ca^{2+} response, while leaving the transient phase intact in human neutrophils (Waddell et al., 1995). Loss of the plateau phase by genistein was due to blockage of Ca^{2+} entry. Pretreatment of cells with genistein for 1 min reduced the cyclopiazonic acid-induced $[Ca^{2+}]_i$ change. Under the same conditions, genistein slightly, however significantly, affected the GEA3162-induced response (355.7 ± 34.2 nM for control vs. 265.2 ± 31.3 nM, $P<0.05$) (Fig. 5). Thus the tyrosine phosphorylation probably plays a role in *S*-nitrosylation-induced Ca^{2+} entry. Prolonging the preincubation time with genistein for 30 min did not enhanced its inhibitory activity (372.5 ± 29.8 nM for control vs. 254.6 ± 28.7 nM). The mechanism of genistein action besides the inhibition of tyrosine phosphorylation has not been excluded.

3.5. Examination of the Ca^{2+}-sensing receptor

Extracellular Ca^{2+}-sensing receptor plays a key role in mineral ion homeostasis by sensing small perturbations in the level of $[Ca^{2+}]_0$ and modulating the functions of parathyroid and kidney (Brown et al., 1993; Zhang et al., 2001). Recently reports demonstrated the expression of Ca^{2+}-sensing receptor in bone marrow derived cells of

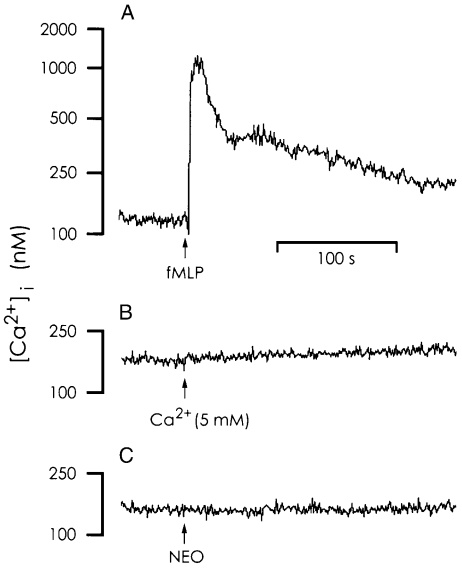

Fig. 6. Effects of neomycin and high extracellular Ca^{2+} concentration on $[Ca^{2+}]_i$ in rat neutrophils. Cells were treated (arrow) with (A) 0.3 μM fMLP, (B) 4 mM Ca^{2+} or (C) 2 mM neomycin (NEO) in a Ca^{2+}(1 mM)-containing medium. Similar results were obtained from three independent experiments.

the macrophage/monocyte lineage and in human peripheral blood monocytes (Yamaguchi et al., 1998). The Ca^{2+}-sensing receptor, which forms disulfide-linked dimers, is known to bind its agonists and produce a G protein-dependent activation of phospholipase C, leading to an elevation in the cellular levels of IP_3, thereby releasing Ca^{2+} from its internal stores and producing increases in $[Ca^{2+}]_i$ (Godwin and Soltoff, 1997). The possibility of direct activation of Ca^{2+}-sensing receptor by *S*-nitrosylation, bypassing the cellular signal process, was examined. In rat neutrophils, the normal receptor-induced $[Ca^{2+}]_i$ changes caused by formyl-Met-Leu-Phe (fMLP) were observed (Fig. 6A). The inability of neomycin and high $[Ca^{2+}]_o$, both Ca^{2+}-sensing receptor agonists (Brown et al., 1993), to elicit increases in $[Ca^{2+}]_i$ (Fig. 6B, C) argue against the existence of Ca^{2+}-sensing receptor in neutrophils.

In conclusion, the NO-releasing agent GEA3162 activated Ca^{2+} entry and regulated Ca^{2+} signal in rat neutrophils involving protein thiol oxidation. The present results suggested a fundamental difference in the regulation of cellular Ca^{2+} signal between the store-emptying operation and the *S*-nitrosylation-induced process.

Acknowledgements

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