The Effect of Calcium Antagonists on the Activation of Guinea Pig Neutrophils

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Abstract—The role of calcium ions in the activation of guinea pig neutrophil functions was examined by evaluating the effects of calcium antagonists. The data presented here show that calcium antagonists inhibit the activation of guinea pig neutrophil functions elicited by N-formyl-methionyl-leucyl-phenylalanine (FMLP) such as chemotaxis, superoxide anion generation and granule enzyme release in a concentration-dependent manner. The concentrations of calcium antagonists demonstrating the inhibition of neutrophil functions may be somewhat different for each function and higher than that of smooth muscle cells. Calcium ionophore A23187 (A23187) caused superoxide anion generation and granule enzyme release of the neutrophils. A23187 also potentiated the FMLP-induced superoxide anion generation and granule enzyme release of the neutrophils. On the contrary, A23187 neither elicited neutrophil chemotaxis nor affected FMLP-induced neutrophil chemotaxis. These results indicate the possibility that the inhibitory effect of calcium antagonists on the activation of neutrophil functions is probably not simply mediated by inhibition of calcium uptake but also by inhibition of a calcium-dependent intracellular target.

In the vital-defense mechanisms, the phagocytic leukocytes are known to play an important role against invading pathogens. They find their targets by chemotaxis and migrate toward their source. During phagocytosis, the phagocytic leukocytes release lysosomal enzymes and free radicals of oxygen. Several workers have shown that calcium ions are needed for chemotaxis (1–5), superoxide anion generation (6–11) and granule enzyme release (12–17) in response to several kinds of stimulation. The nature of these mechanisms, however, remains to be clarified. In this regard, calcium has been demonstrated to play a central role in these cellular functions. Therefore, drugs which modify the cellular handling of calcium ions might be expected to profoundly influence the vital-defense mechanisms. The purpose of this study is to clarify the role of calcium ions in the activation of guinea pig neutrophil functions such as chemotaxis, superoxide anion generation and granule enzyme release by evaluating the effects of various calcium antagonists (18).

Materials and Methods

Drugs and reagents: $^{45}$CaCl$_2$ (23.29 mCi/mg) was purchased from New England Nuclear; shellfish glycogen, bovine serum albumin (BSA), N-formyl-methionyl-leucyl-phenylalanine (FMLP), ferricytochrome C, cytochalasin B, bovine superoxide dismutase (SOD) and p-nitrophenyl-N-acetyl-$eta$-D-glucosaminide from Sigma; N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) from Dojin Chemicals; Diff-Quick from Kokusai Shiyaku; and calcium ionophore A23187 (A23187) from Calbiochem. We are grateful for the generous gifts of each of the following agents: prenylamine (Hoechst), verapamil (Eisai), nifedipine
Preparation of guinea pig peritoneal neutrophils: Unless otherwise stated, adult male Hartley guinea pigs (350 to 450 g) purchased from a local supplier were used. Guinea pig peritoneal exudate cells were collected 16 hr after intraperitoneal injection of a 1% shellfish glycogen solution in saline (10 ml/100 g body weight). The cells were suspended in lysing buffer (19) consisting of 150 mM NH₄Cl, 1 mM KHCO₃ and 1 mM EDTA, pH 7.2, to lyse contaminating red cells and were washed twice with 20 mM phosphate-buffered saline, pH 7.2. The washed cells were resuspended in Gey’s balanced salt solution containing 2% BSA and 20 mM HEPES, pH 7.2. These cells were 96% neutrophils as identified by the Diff-Quick stain. Viability averaged 98% as assessed by trypan blue exclusion.

Neutrophil chemotaxis assay: Cell migration was measured with the membrane filter method using a 48 well microchemotaxis chamber (Neuro Probe) (20, 21). The two compartments of the chamber were separated by a polyvinylpyrrolidone-free polycarbonate membrane filter (pore size, 3 μm; Nucleopore). As a chemoattractant, FMLP (22), 10 nM, was used. Fifty μl of neutrophils (1 X 10⁶/ml) were placed in the upper compartment of the chamber with or without test compounds. After a 60 min incubation at 37°C in a humidified incubator, the filter was removed from the chamber, stained with Diff-Quick and quantitated. Data represent the mean±1 S.E. of neutrophils per high power field (20 fields counted) for triplicate filters.

Assay of calcium influx: Calcium influx was measured as the uptake of ⁴⁵Ca²⁺ into neutrophils according to the method of Otani et al. (23). The cells (10×10⁶) were suspended in 1 ml of CaCl₂-free Eagle’s minimum essential medium containing 20% dialyzed calf fetal serum. ⁴⁵CaCl₂ (1 μCi) was added to the cell suspension immediately after the addition of FMLP, and incubation was continued at 37°C with aeration using 5% CO₂ in air. At the indicated times, the uptake of ⁴⁵Ca²⁺ was stopped by the addition of 0.2 ml of the cell suspension to 1 ml of La³⁺ washing medium which contained 5 mM Tris, 139 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂ and 2 mM LaCl₃, pH 7.2. After centrifuging the cells at 2,000×g for 1 min, the supernatant was decanted. The cells were suspended in 1.4 ml of cold fresh La³⁺ washing medium, collected on a Millipore filter (0.45 μm) and subsequently washed 3 times with 2 ml of La³⁺ washing medium. The filter was dried and placed in a counting vial containing 5 ml of a toluene scintillator (0.4% PPO and 0.01% POPOP), and the radioactivity was measured using a liquid scintillation counter (LSC-903SP, Aloka).

Assay of superoxide anion generation: Superoxide anion generation was measured by the reduction of ferricytochrome C according to the method of Goldstein et al. (24). All experiments were performed in triplicate in a volume of 0.6 ml in plastic tubes. Serially added were 0.15 ml of Hanks’ balanced salt solution supplemented with 1 mg/ml glucose and 1 mg/ml BSA, pH 7.4, or test compounds, 0.15 ml of neutrophils (12 to 20×10⁶/ml), 0.15 ml of ferricytochrome C solution (4 mg/ml) and 0.15 ml of FMLP (160 nM). Tubes were then placed in a 37°C water bath and periodically agitated. After 5 min, the tubes were removed, the reactions were stopped by pelleting the cells by centrifugation at 8,000×g for 30 sec, and the supernatants were decanted. The amount of decreased cytochrome C was assayed by a spectrophotometer (UV-200, Shimadzu). The addition of 10 μg/ml SOD to the reaction mixture resulted in at least 90% inhibition of stimuli-induced ferricytochrome C reduction, which indicates that the reduction was specific for superoxide anion generation. Appropriate control tubes also demonstrated no spontaneous reduction of ferricytochrome C by FMLP or by any of the test compounds in the absence of neutrophils.

Assay of degranulation: Lysosomal enzyme secretion was measured by the activity of secreted N-acetyl-β-D-glucosaminidase according to the method of Fanton et al. (25). After neutrophils (20×10⁶/ml) were treated with 5 μg/ml of cytochalasin B for 10 min at 37°C, the reaction was started by the addition of the indicated...
time. Cells were quickly chilled to 0°C on ice and centrifuged at 500 x g for 10 min to stop the reaction. Fifty μl of the supernatant was incubated with 4 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in 50 mM sodium citrate buffer, pH 4.5, in a total volume of 500 μl for 30 min at 37°C. Reaction was terminated by the addition of 400 mM glycine-NaOH buffer, pH 10.5, and released p-nitrophenol absorbance was measured at 410 nm. Total enzyme activities were determined by adding 0.2% Triton X-100 to lyse the cells. Results are expressed as the percentage of total activity.

The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was monitored in order to determine if the test compounds have any damaging effects. The release of LDH was assayed by measuring the conversion of NADH to NAD⁺ during the reaction of pyruvate to lactate. The enzyme activities were expressed as a percentage of the total enzyme activity. These were determined after disruption of the cells with 0.2% Triton X-100 (26).

**Statistics:** Statistical analyses were made by Student’s t-test.

**Results**

**Effect of calcium antagonists on neutrophil chemotaxis:** A concentration gradient of chemotactic peptide FMLP causes chemotaxis in guinea pig neutrophils. As can be seen in Fig. 1, all calcium antagonists tested inhibited the neutrophil migration to FMLP in a concentration-dependent manner. However, the concentrations required for inhibition of the neutrophil chemotaxis were mostly one or two orders of magnitude higher than those required for inhibition of Ca²⁺ translocation across smooth muscle cell membrane. Because calcium antagonists in high concentrations could damage the cells, we tested the membrane damaging effect of

![Fig. 1. Effect of calcium antagonists on neutrophil chemotaxis. Data represent the mean±1 S.E. of triplicate filters (20 oil immersion fields/filter).](image-url)
these drugs on neutrophils. The drug concentrations used in these experiments elicited no significant LDH release from neutrophils, except prenylamine which caused the discharge of approximately 10% of the available LDH at 100 μM (data not shown). At the concentrations employed in this study, none of the agents tested affected neutrophil viability as assessed by trypan blue exclusion (data not shown).

Effect of calcium antagonists on Ca\(^{2+}\)-influx: \(^{45}\)Ca\(^{2+}\)-influx of neutrophils induced by 10 nM FMLP was inhibited by the calcium antagonists in a concentration-dependent manner. The ID50s for nifedipine, prenylamine, bepridil, verapamil, nicardipine and diltiazem were 5.2, 29.8, 88.9, 105.3, 123.7 and 189.7 μM, respectively. These concentrations were also higher than those for smooth muscle cell membranes.

Effect of calcium antagonists on FMLP-induced superoxide anion generation in neutrophils: Figure 2 shows the effect of calcium antagonists on superoxide anion generation of neutrophils induced by 40 nM FMLP. As shown, calcium antagonists demonstrated concentration-dependent inhibition. The ID50s for nifedipine, prenylamine, verapamil, bepridil, nicardipine and diltiazem were 1.8, 15.7, 39.5, 52.2, 68.8 and 127.5 μM, respectively. These concentrations were about one fourth of the inhibitory dose for neutrophil chemotaxis.

Effect of calcium antagonists on degranulation: Guinea pig neutrophils pretreated with cytochalasin B released 65.4±5.2% of the total N-acetyl-β-D-glucosaminidase in the presence of 200 nM FMLP for 5 min. No significant release of LDH was detected during the incubation. As shown in Fig. 3, calcium antagonists inhibited FMLP-induced selective release of N-acetyl-β-D-glucosa-

Fig. 2. Effect of calcium antagonists on FMLP-induced superoxide anion generation in neutrophils. Incubations were performed at 37°C for 5 min in the presence of 40 nM FMLP. Results are expressed as the mean±1 S.E. of three separate experiments, each performed in triplicate.
minidase from guinea pig neutrophils in a concentration-dependent manner. These inhibitory concentrations were of an intermediate level between those for neutrophil chemotaxis and those for neutrophil superoxide anion generation.

Effect of A23187 on neutrophil functions: A23187 caused calcium-influx, superoxide anion generation and granule enzyme release in guinea pig neutrophils. A23187 also activated the FMLP-induced calcium-influx, superoxide anion generation and granule enzyme release.

Fig. 3. Effect of calcium antagonists on FMLP-induced granule enzyme release in neutrophils. The cells were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°C. They were then incubated with 10 nM FMLP for 5 min at 37°C. Cell-free supernatants were analyzed for secreted N-acetyl-β-D-glucosaminidase. Total enzyme activities were determined by adding 0.2% Triton X-100 to lyse the cells. Results are expressed as the percentage of the total activity. Each value is the mean±1 S.E. of three separate experiments.

Table 1. Effect of FMLP and A23187 on superoxide anion generation and granule enzyme release of guinea pig neutrophils

| Compound          | Superoxide anion generation (%) | Granule enzyme release (%) |
|-------------------|----------------------------------|----------------------------|
| FMLP (40 nM)      | 100.0a                           | 65.4b                      |
| A23187 (1 μM)     | 82.7                             | 48.2                       |
| FMLP+A23187       | 175.5                            | 89.5                       |

a FMLP (40 nM)-induced superoxide anion generation of the neutrophils expressed as 100%. b Guinea pig neutrophils were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°C. The cells were then incubated with FMLP for 5 min at 37°C.
enzyme release of the neutrophils. By contrast, A23187 neither elicited neutrophil chemotaxis nor affected FMLP-induced neutrophil chemotaxis (Tables 1 and 2).

**Discussion**

There have been numerous reports on the participation of calcium ions on the activation of phagocytic cells such as chemotaxis, superoxide anion generation and granule enzyme release. Boucek and Snyderman (3) have shown that calcium ions are needed for chemotaxis and that stimulation of leukocytes by various chemotactic factors causes a rapid influx of calcium ions from the extracellular medium. The respiratory burst which follows chemotactic stimulation of leukocytes is probably dependent upon an influx of extracellular calcium ions (8-10) or the release of calcium ions from intracellular binding sites (6, 7), and this results in the production of superoxide anions (11). Calcium ions are also required for the release of lysosomal enzymes from leukocytes in response to chemotactic stimulation (12, 15-17). However, the precise mechanisms of the regulatory role of calcium ions on the activation of leukocytes remains to be clarified. Thus the role of calcium ions in the activation of guinea pig neutrophils was examined in this study by evaluating the effects of various calcium antagonists (18). The organic calcium antagonists are a chemically heterogenous class of drugs with similar pharmacological properties. Though their mechanism of action is not completely clear, it is evident that they interfere with the availability of calcium for many calcium-dependent cell functions (27-30).

The results show that all six calcium antagonists, prenylamine, verapamil, nifedipine, nicardipine, diltiazem and bepridil, inhibited chemotaxis by guinea pig peritoneal neutrophils to FMLP in a concentration-dependent manner. Though several nonspecific effects of these compounds have recently been described (31), all six calcium entry blockers exerted similar patterns of inhibitory activities on the chemotaxis of guinea pig neutrophils. These data suggest a common mode of action for these agents, i.e., interference with the calcium-dependent step in the chemotaxis of neutrophils. As compared with the effect of the calcium antagonists on calcium ion transportation across cardiac and smooth muscle cell membranes (32-34), inhibition of the neutrophil chemotaxis occurs at very high concentrations. The concentrations of calcium antagonists in these experiments, however, did not induce LDH release or affect neutrophil viability. It is therefore not likely that inhibition of neutrophil chemotaxis is due to the cytotoxic effects of calcium antagonists. Calcium-influx of neutrophils induced by FMLP was inhibited by calcium antagonists, and the inhibitory dose for calcium-influx was also higher than those for smooth muscle cells. This fact indicates that a difference of cell type may be one of the reasons why calcium antagonists inhibit the neutrophil chemotaxis at very high concentrations.

FMLP-induced superoxide anion generation of neutrophils was also inhibited by calcium antagonists in a concentration-dependent manner. The ID50 of calcium antagonists for superoxide anion generation in neutrophils was about one fourth of the inhibitory dose for neutrophil chemotaxis. One possible explanation of this difference would be that chemotaxis is one of the whole

| Experimental condition | Neutrophil chemotaxis (cells/μl immersion field±1 S.E.) |
|------------------------|--------------------------------------------------------|
| Control                | 3±1                                                    |
| FMLP (10 nM)           | 235±10**                                               |
| A23187 (10 μM)         | 5±2                                                   |
| FMLP+A23187            | 241±6**                                                |

Significant differences from the control were calculated by Student's t-test. **P<0.01.
cell functions of neutrophils, while superoxide anion generation is one of the membrane associated functions of neutrophils.

Incubation of guinea pig neutrophils with calcium antagonists in the present studies inhibited the release of N-acetyl-β-D-glucosaminidase induced by cytochalasin B and FMLP in a concentration-dependent manner. The inhibitory dose of calcium antagonists for granule enzyme release from neutrophils was about half of that for neutrophil chemotaxis.

The data presented here show that calcium antagonists inhibit the activation of guinea pig neutrophil functions elicited by FMLP such as chemotaxis, superoxide anion generation and granule enzyme release. Among the six calcium antagonists, nifedipine is known to be the most potent one in the cardiovascular system both in vivo and in vitro (35), and it also exhibits the most potent inhibition of neutrophil activation in this study. The concentrations of calcium antagonists demonstrating the inhibition of neutrophil enzymes may be somewhat different for each function and higher than those of smooth muscle cells. Though the neutrophil activating mechanisms of FMLP are not clear, it has been reported that FMLP activates phospholipase C and enhances phosphatidylinositol breakdown to diacylglycerol, which is further degraded to monoaecylglycerol and non-esterified fatty acid or phosphorylated to phosphatidic acid in leukocytes (36). The accumulated phosphatic acid is thought to act as an ionophore and cause an increase of intracellular free calcium ions, superoxide anion generation and granule enzyme release. The mechanism of activation of neutrophil chemotaxis by FMLP differs from that of other functions of neutrophils, but further experimental work is required to clarify this aspect.

A23187, which is known to stimulate calcium influx into various cells, caused superoxide anion generation and granule enzyme release of guinea pig neutrophils. A23187 also potentiated the FMLP-induced superoxide anion generation and granule enzyme release of guinea pig neutrophils. These actions of A23187 were also inhibited by calcium antagonists in a concentration-dependent manner (data not shown). On the contrary, A23187 neither elicited neutrophil chemotaxis nor affected FMLP-induced neutrophil chemotaxis. These facts suggest that activation of neutrophil functions causes the increase of calcium uptake from extracellular medium, but increase of calcium uptake does not necessarily cause the activation of neutrophil functions such as neutrophil chemotaxis.

These results indicate that the inhibitory effect of calcium antagonists cannot be ascribed to their ability as calcium channel blockers. The lipophilic characteristics of these drugs permit an easy penetration into the cells. It has also been shown that calcium antagonists acting within the cells generally require higher concentrations than are required for inhibition of calcium translocation across the plasma membranes of cardiac cells (37–39). Some of the calcium antagonists are also calmodulin inhibitors (40, 41). We have recently shown that calmodulin inhibitors strongly interfere with the activation of neutrophil functions elicited by FMLP (42).

These facts indicate some possibility that the inhibitory effect of calcium antagonists on the activation of neutrophil functions is probably not simply mediated by inhibition of calcium uptake but also by inhibition of a calcium-dependent intracellular target. However, additional studies will be required to clarify the effect of calcium antagonists on the activation of neutrophil functions at the intracellular level.

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