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FELODIPINE-INDUCED INHIBITION OF POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS

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Abstract—Felodipine inhibits fMet-Leu-Phe or ionophore A23187-induced exocytosis in rabbit peritoneal polymorphonuclear leukocytes (PMNs), in the concentration range 1–50 μM. Activation of the metabolic burst, and migration of PMNs towards fMet-Leu-Phe are equally inhibited by felodipine in the same concentration range. The effect is not due to blocking of calcium channels in the plasma membrane, because the degree of inhibition remains the same when Ca^{2+} is omitted from the medium. Felodipine interferes with ionophore A23187-induced association of ^{45}Ca with the PMN but this interference occurs at lower concentrations than the inhibition of exocytosis. Hypotonic hemolysis of erythrocytes is inhibited by felodipine; maximal protection against hemolysis occurs at a concentration of 50 μM felodipine. It is suggested that at least a part of the inhibiting effect on PMN functions might be due to an anesthetic-like membrane effect of felodipine.

Felodipine, a dihydropyridine derivative, is a calcium-antagonistic drug. Its ability to inhibit the Ca^{2+} entry through calcium channels is thought to be the basis of a number of clinically interesting properties. It is a potent vasodilator and could be quite useful in the treatment of hypertension [1–4].

Besides the inhibiting effect on calcium channels felodipine has been shown to interact with calmodulin [5–7]. This interaction is the basis of inhibition of myosin P-light chain phosphorylation, and of actin–myosin interactions. Silver *et al.* [8] demonstrated that this effect occurred at higher concentrations of felodipine than the effect on force development in smooth muscle, which was supposed to be due to a blockade of Ca^{2+} entry channels.

Calcium fluxes and calmodulin play an, as yet, incompletely defined role in polymorphonuclear leukocyte (PMN) function [9–17]. An impairment of PMN functions by felodipine through an interference with Ca^{2+} fluxes or with calmodulin might affect the defence system of the body [18] during treatment with this drug. On the other hand, the known properties of felodipine might contribute to insight in the role of calcium channels and calmodulin in PMN functions. For this reason we studied the effect of felodipine on exocytosis, chemotaxis and metabolic burst in PMNs.

MATERIALS AND METHODS

Polymorphonuclear leukocytes. Rabbit peritoneal PMNs were obtained as described previously [19]. The cells were suspended in a medium consisting of 140 mM NaCl, 5 mM KCl, 10 mM glucose and 20 mM Hepes pH 7.3. The final cell suspension was supplemented with 1 mM Ca^{2+}, unless otherwise indicated, and contained 3×10^6 PMNs per ml.

Exocytosis. Exocytosis was measured as the release of the granule-associated enzymes lysozyme and glucuronidase, under conditions that there was no significant release of the cytoplasmic marker enzyme lactate dehydrogenase (LDH). Lysozyme was measured by estimating the rate of lysis of *Micrococcus lysodeikticus*. Glucuronidase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucuronide. The release of LDH was determined as a measurement of cell damage; it was estimated by measuring the conversion of NADH into NAD^+ during the LDH catalyzed conversion of pyruvate into lactate. Enzyme release is expressed as a percentage of a maximum value, obtained by treating the cells with 0.05% Triton X100.

Locomotion. PMN migration towards the chemotactic peptide fMet-Leu-Phe was measured with the Boyden chamber technique as described previously [20]. The experiment was carried out in the absence of albumin unless otherwise indicated, because albumin interferes with felodipine inhibition.

Metabolic burst. The metabolic burst was measured as an increased nitroblue tetrazolium (NBT) reduction [21]. NBT reduction was measured by including 0.4 mM NBT into the mixture of 3×10^6 cells per ml. After preincubation with or without felodipine for 10 min at 37° the activator was added followed by incubation for 15 min at 37°. The reaction was terminated by adding 5 ml 0.5 M HCl. After centrifugation the residue was dissolved in 2 ml pyridine by warming for 10 min in a boiling water-bath. After cooling to room temperature the absorbance of the pyridine solution was measured at 510 nm. The results are expressed as nmoles NBT reduced per 3×10^6 PMNs per 15 min.

^{45}Ca uptake. The association of ^{45}Ca with the PMN was estimated according to Korchak *et al.* [13], with minor modifications. Cells were preincubated with or without felodipine for 10 min at 37°. Then, together with ionophore A23187 (0.5 μM), 1 μCi ^{45}Ca was added to the cells to a final Ca^{2+} concentration of 1 mM. After incubation for 5 min at

37° the cells were rapidly filtered through Schleicher and Schuell BA85 filters with a pore width of 0.45 μm. The filters were washed and dried, and subsequently the radioactivity associated with the cells was measured. In a parallel experiment, carried out in the same way but without radioactivity, the lysozyme release was measured.

Erythrocytes. Heparinized human blood was centrifuged, and the buffy coat was removed by aspiration. Then the blood cells were washed three times with a buffer (290 mOsM NaCl, 20 mOsM Tris–HCl pH 7.3). After the final washing the erythrocytes were diluted to give a stock suspension containing 2×10^9 erythrocytes per ml. Erythrocytes were added to buffer with or without felodipine, and incubated for 30 min at 37°. Then the cells were centrifuged and the absorbance of the supernatant was measured (after dilution) at 540 nm. The final erythrocyte concentration during the experiment was 2×10^8 cells per ml.

Materials. Felodipine was obtained from Hässle, Sweden. It was dissolved in DMSO before the experiment, and the resulting solution was added to the medium in quantities of 2 μl per ml suspension. ^{45}Ca was from Amersham Nederland. Ionophore A23187 was obtained from Boehringer Mannheim. The other chemicals were obtained from Sigma Chemical Co.

RESULTS

Lysozyme release, induced by fMet-Leu-Phe in the presence of cytochalasin B, is inhibited by felodipine in the concentration range of 5–100 μM (Fig. 1). Lysozyme release appears to be a direct measure for exocytosis because there is little or no LDH release. Inhibition of lysozyme release is maximal at a concentration of 50 μM felodipine. Higher concentrations of felodipine cause an increase of lysozyme release. Though there is a slight increase of LDH release at these felodipine concentrations, this is much less than the percentage of lysozyme release. In a separate experiment we found that in the absence of an activator the highest concentration of felodipine (200 μM) gave a LDH release of 5 ± 1%, and a lysozyme release of 16 ± 3%. Lower concentrations of felodipine did not induce significant enzyme release. Ionophore A23187-induced lysozyme release is equally inhibited by felodipine, at slightly lower concentrations than fMet-Leu-Phe-induced lysozyme release. Here, too, an optimum in the inhibitory concentrations can be observed at 50 μM felodipine (Fig. 1).

Activation of the metabolic burst in cytochalasin B treated PMNs can be measured as an increase of NBT reduction. FMet-Leu-Phe-induced NBT reduction is inhibited by felodipine at the same concentrations as exocytosis (Fig. 2). Activation of the metabolic burst in rabbit PMNs by fMet-Leu-Phe not only occurs in the presence of extracellular Ca^{2+}, but also in its absence. In the presence of EDTA, which is added to remove adherent Ca^{2+}, activation of the metabolic burst by fMet-Leu-Phe is only slightly less than in the presence of Ca^{2+}. The inhibiting effect of felodipine in the absence of extracellular Ca^{2+} is not significantly different from the inhibition in the presence of Ca^{2+} (Fig. 2).

Felodipine inhibits fMet-Leu-Phe-induced migration of PMNs, in the absence of albumin, in

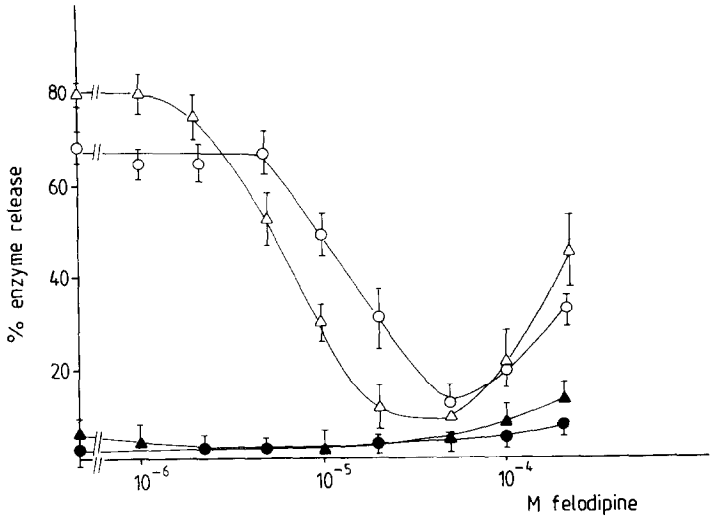

Fig. 1. Effect of felodipine on enzyme release during exocytosis. Cells were exposed to the given concentration of felodipine for 10 min at 37°. Then activator was added, followed by incubation for 20 min at 37°. Subsequently the mixture was centrifuged, and enzyme release in the supernatant was measured: —○—, lysozyme release, activator: fMet-Leu-Phe (10^{-8} M) + cytochalasin B (5×10^{-6} M); —●—, LDH release, activator: fMet-Leu-Phe (10^{-8} M) + cytochalasin B (5×10^{-6} M); —△—, lysozyme release, activator: ionophore A23187 (5×10^{-7} M); —▲—, LDH release, activator: ionophore A23187 (5×10^{-7} M). Values given are the means of four experiments ± SD.

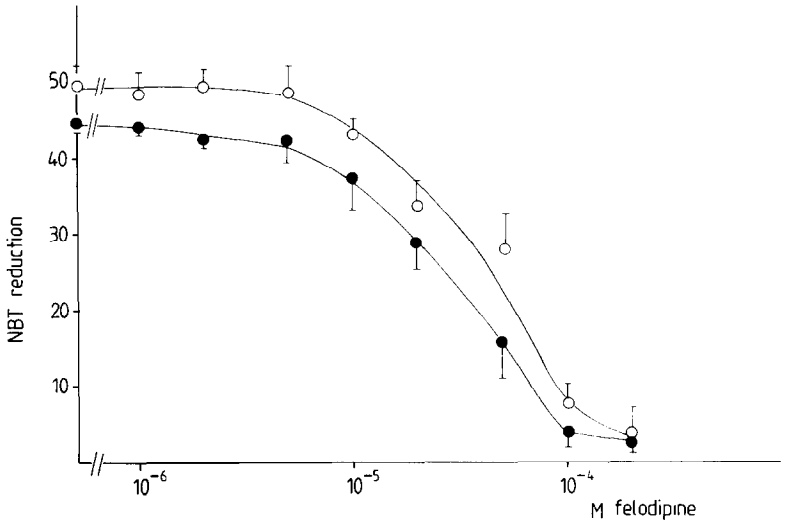

Fig. 2. Inhibition of fMet-Leu-Phe activated NBT reduction by felodipine in the presence or absence of extracellular Ca^{2+}. Cells were exposed to the given concentration of felodipine for 10 min at 37°, in the presence of 0.4 mM NBT. Subsequently activator (fMet-Leu-Phe (10^{-8} M) + cytochalasin B 5×10^{-6} M) was added, followed by incubation for 15 min at 37°. Then the reaction was terminated and NBT reduction by the cells was established. NBT reduction is expressed as nmoles NBT reduced per 3×10^6 PMNs per 15 min. —○—, 1 mM Ca^{2+} present; —●—, 1 mM EDTA present. Values given are the means of three experiments ± SD.

the concentration range of 10–100 μM (Fig. 3). Migration of PMNs is much higher in the presence of albumin than in its absence. Albumin, however, strongly interferes with inhibition by felodipine (Table 2). Inhibition of PMN functions may be due to an interaction with sulfhydryl groups [19]. Because a modulating effect of albumin in such a case could be due to a reaction with the sulfhydryl group in the albumin molecule, we considered the effect of the sulfhydryl compound dithiothreitol. This compound, however, has the opposite effect: inhibition by felodipine is slightly potentiated (Table 1). Inhibition of exocytosis by felodipine is modulated in the same way by albumin and dithiothreitol as is migration. Though lysozyme release is strongly reduced in the presence of felodipine and dithiothreitol, there is a small but significant LDH release.

In order to examine a possible involvement of calmodulin in felodipine-induced inhibition of PMN functions, we considered the effect of a combination of suboptimal concentrations of felodipine and another inhibitor. These inhibitors were either interfering with calmodulin (W7, trifluoperazine [22,23]) or with another target such as sulfhydryl groups

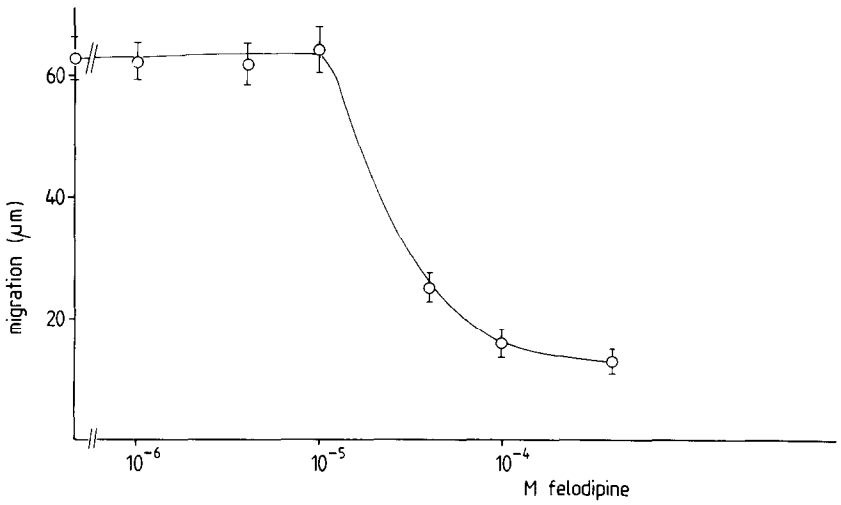

Fig. 3. Interference of felodipine with fMet-Leu-Phe induced migration of PMNs. Cells were preincubated with the given concentration of felodipine for 10 min at 37°, and were subsequently placed in the upper compartment of a Boyden chamber on a filter with a pore width of 3 μm. In the lower compartment 10^{-9} M fMet-Leu-Phe was present. The cells were allowed to migrate (in the absence of albumin) for 90 min at 37°. Then the filters were fixed and stained, and migration in μm was established according to the leading front technique. Values given are the means of three experiments ± SD.

Table 1. Effect of albumin and dithiothreitol on felodipine-induced inhibition of exocytosis and migration

A. Exocytosis

	Enzyme release (%) (in the presence of)			
	0		40 μM felodipine	
	LDH	lys	LDH	lys
—	2 ± 1	58 ± 4	5 ± 2	26 ± 2
Albumin, 0.5 mg/ml	1 ± 1	78 ± 6	0 ± 1	73 ± 4
Dithiothreitol, 0.5 mM	2 ± 0	74 ± 3	11 ± 2	23 ± 4

B. Migration

	Migration (μm) (in the presence of)	
	0	40 μM felodipine
—	68 ± 3	43 ± 2
Albumin, 0.5 mg/ml	99 ± 3	95 ± 4
Dithiothreitol, 0.5 mM	51 ± 3	26 ± 2

PMNs were preincubated with or without felodipine in the presence of the reagents indicated, for 10 min at 37°. For exocytosis activator (10^{-8} M fMet-Leu-Phe + 5 × 10^{-6} M cytochalasin B) was added, followed by incubation for 20 min. For migration the cell suspension was placed in the upper compartment of a Boyden chamber. In the lower compartment 10^{-9} M fMet-Leu-Phe and reagent (albumin, dithiothreitol) was present. The cells were allowed to migrate for 90 min (control, dithiothreitol) or 60 min (albumin). Albumin: rabbit serum albumin; lys: lysozyme. Values given are the means of four (exocytosis) or three (migration) experiments ± SD.

(ethacrynic acid, triphenyltinchloride [19, 24]) and metabolism (2-deoxy-glucose) [25, 26]. The inhibitory effect of felodipine is additive with the inhibitory effect of the calmodulin inhibitors W7 and trifluoperazine, but also with the effect of the other inhibitors (Table 2).

In a separate experiment the combined effect of felodipine and prenylamine was considered (Table 4), because the latter compound has been shown to enhance strongly the binding of felodipine to calmodulin [7]. Prenylamine itself inhibits PMN functions at about 40 μM, and is lytic at still higher concentrations, as we have shown previously [27, 28]. For this reason two concentrations of prenylamine were taken which are below or at the beginning of the inhibitory concentration range. There is a slight enhancement of inhibition with 10 μM felodipine, but this enhancement is absent with 20 μM felodipine. The combination of 20 μM felodipine and 20 μM prenylamine results in an increase of LDH release and an absence of inhibition of lysozyme release (Table 3).

Table 2. Inhibition of fMet-Leu-Phe induced exocytosis by a combination of felodipine and another inhibitor

	Enzyme release (%) (in the presence of)			
	0		20 μM felodipine	
	lys	gluc	lys	gluc
—	75 ± 5	67 ± 5	53 ± 3	48 ± 5
10 μM Trifluoperazine	68 ± 1	57 ± 5	44 ± 3	32 ± 6
40 μM W7	73 ± 2	62 ± 6	50 ± 4	48 ± 2
50 μM Ethacrynic acid	44 ± 2	49 ± 6	29 ± 4	26 ± 7
0.5 μM Triphenyltin chloride	58 ± 6	47 ± 2	39 ± 6	36 ± 4
10 mM 2-Deoxyglucose	26 ± 5	16 ± 5	8 ± 4	4 ± 1

Cells, in medium devoid of glucose, were preincubated without or with felodipine, and the indicated concentration of other inhibitor, for 10 min at 37°. Then activator (10^{-8} M fMet-Leu-Phe + 5 × 10^{-6} M cytochalasin B) was added, followed by incubation for 20 min at 37°.

W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; lys, lysozyme; gluc, glucuronidase.

Values given are the means of four experiments ± SD.

Table 3. Inhibition of fMet-Leu-Phe induced enzyme release by a combination of felodipine and prenylamine

	Enzyme release (%) (in the presence of)					
	0		10		20 μM felodipine	
	LDH	lys	LDH	lys	LDH	lys
—	0 ± 1	66 ± 4	2 ± 1	59 ± 1	1 ± 0	30 ± 3
5 μM Prenylamine	1 ± 0	60 ± 5	1 ± 0	47 ± 4	3 ± 0	32 ± 4
20 μM Prenylamine	2 ± 1	61 ± 6	5 ± 1	43 ± 2	17 ± 3	69 ± 6

Cells were preincubated in the presence of the given concentrations of felodipine and prenylamine for 10 min at 37°. Then activator (10^{-8} M fMet-Leu-Phe + 5×10^{-6} M cytochalasin B) was added, followed by incubation for 20 min at 37°. Lys, lysozyme.

Values given are the means of four experiments ± SD.

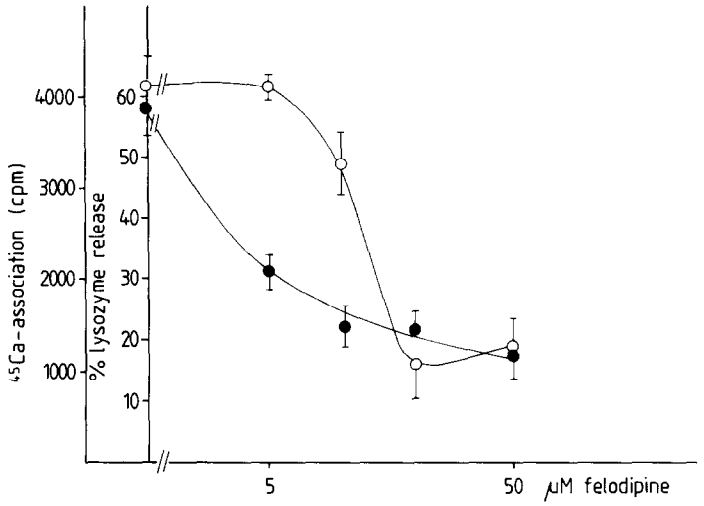

Fig. 4. Effect of felodipine on ionophore A23187 induced association of ^{45}Ca with the PMN, and on A23187 induced lysozyme release. PMNs were incubated for 10 min at 37° with the given concentration of felodipine, in two series of experiments. In the first series of experiments subsequently ^{45}Ca was added in a total Ca^{2+} concentration of 1 mM, in the second series 1 mM Ca^{2+} was added. In both series of experiments ionophore A23187 (5×10^{-7} M) was added, followed by incubation for 5 min at 37°. In the first series of experiments ^{45}Ca association was established. In the second series lysozyme release was measured: —●—, association of ^{45}Ca with the PMN; —○—, lysozyme release.

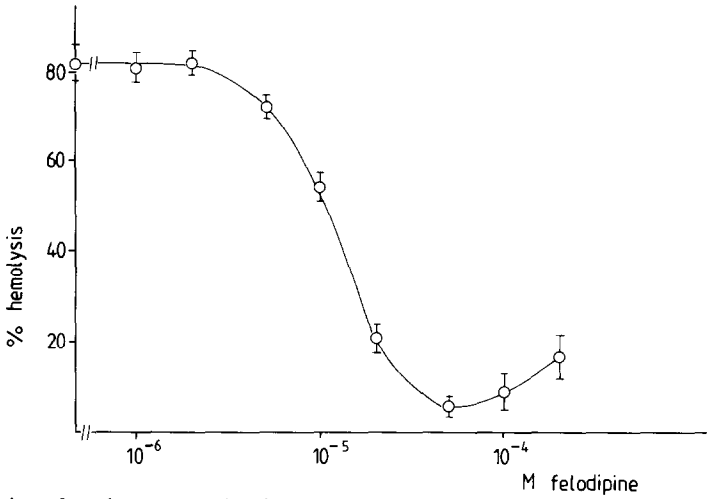

Fig. 5. Protection of erythrocytes against hypotonic hemolysis by felodipine. Human erythrocytes were placed in a hypotonic salt solution (138 mOsM NaCl, mOsM Tris–HCl pH 7.3), in the presence of the given concentration of felodipine. After incubation for 30 min at 37° the mixture was centrifuged and the degree of hemolysis was established. Hemolysis is expressed as a percentage of complete hemolysis. The values given are the means of three experiments ± SD.

During A23187 activated exocytosis a strong association of ^{45}Ca with the PMN occurs. At relatively low concentrations this association of ^{45}Ca with the cell is inhibited by felodipine. Under the same experimental conditions higher concentrations of felodipine are required for inhibition of A23187-induced lysozyme release than for inhibition of ^{45}Ca-association (Fig. 4).

Hypotonic hemolysis of erythrocytes is reduced if felodipine is present in the medium. Under the conditions of this experiment maximal protection against hypotonic hemolysis occurs at a concentration of 50 μM felodipine (Fig. 5).

DISCUSSION

Felodipine has an inhibitory effect on all PMN functions studied *in vitro*. Inhibiton of chemotaxis, exocytosis and metabolic burst occurs in the same concentration range. This suggests that the target of felodipine is of importance for all functions studied.

It is unlikely that the inhibitory effect of felodipine on the PMN occurs with the clinically effective concentrations unless the drug is accumulated into the PMN. Felodipine concentrations which are required to treat hypertension [1–4] are considerably lower than the 5–100 μM which are inhibitory for PMN functions. The effective inhibitory concentrations *in vivo* are still higher because of the presence of albumin, which eliminated part of the inhibition.

Felodipine is a calcium channel blocker, but a blockade of calcium channels in the plasma membrane is probably not the basis for the inhibition of PMN functions. The calcium channel blocking action, which is supposed to be associated with the clinically beneficial effects of felodipine, occurs in other cell types at lower concentrations than required for inhibition of PMN functions [1–4].

Another argument against the involvement of calcium channels is the observation that fMet-Leu-Phe-induced activation of the metabolic burst in the absence of extracellular Ca^{2+} is inhibited by felodipine. Translocation of Ca^{2+} across the plasma membrane by ionophore A23187 is inhibited by felodipine, but there is no relation between the inhibitory concentration for Ca^{2+} translocation and that for exocytosis. The nature of the inhibition of Ca^{2+} translocation by felodipine is not known; it might have the same basis as inhibition of Ca^{2+} channels, or may be a membrane effect.

Previously we found that a number of calcium antagonistic drugs, such as verapamil, nifedipine, diltiazem, prenylamine and perhexiline, inhibited PMN functions in a way that did not involve a calcium channel blocking effect, but that nevertheless was calcium antagonistic [27–29]. We postulated that these drugs interfered with an intracellular target via a Ca^{2+} dependent mechanism. Though felodipine is a related drug, its effect on the PMN is not antagonized by an increase of extracellular Ca^{2+}, and that indicates another mechanism of action.

Felodipine binds to calmodulin, which has two binding sites for this drug [6, 7]. Inhibitors of calmodulin inhibit PMN functions, suggesting that calmodulin is required for PMN functions [14–17]. An interaction between felodipine and calmodulin might thus result in an inhibition of PMN functions. Though such an interference cannot be excluded, there is on the other hand, no clear experimental evidence for it. The combined action of calmodulin inhibitors and felodipine is not different from the combined action of other types of inhibitors and felodipine. Prenylamine has been shown to bind to calmodulin, resulting in a binding of felodipine to the remaining site with greatly enhanced affinity; both felodipine binding and its potentiation by prenylamine were dependent on Ca^{2+} [7]. The combined presence of prenylamine and felodipine, however, does not result in a strongly enhanced inhibition of PMN functions.

Felodipine stabilizes the membrane of erythrocytes, suggesting that it is a membrane-active drug. Anesthetic-like compounds show a biphasic effect on erythrocytes: a membrane-stabilizing effect at low concentrations, and lysis at higher concentrations [30]. These compounds have been shown to inhibit all PMN functions [31–33]. Because felodipine shows a biphasic effect, both with regard to its action on erythrocytes and on PMNs, it is tempting to speculate that at least part of the inhibitory effect of felodipine on PMN functions is due to an interaction with the plasma membrane according to an anesthetic-like mechanism.

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