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THE EFFECT OF VERAPAMIL AND OTHER CALCIUM ANTAGONISTS ON CHEMOTAXIS OF POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—The calcium antagonists verapamil, D600, prenylamine, diltiazem and perhexiline inhibit chemotaxis and (stimulated) locomotion by rabbit polymorphonuclear leukocytes (PMN's). Inhibition occurs at relatively high concentrations (5.10^{-5}–10^{-3} M). Inhibition was most pronounced in the absence of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+} there was also inhibition of chemotaxis, but to a lesser degree than in the absence of Ca^{2+}, thus extracellular Ca^{2+} partly reverses the inhibition of chemotaxis by calcium antagonists.

The results suggest that the mechanism of action of these drugs in PMN chemotaxis differs from that in smooth muscle cell contraction, where much lower concentrations of these drugs are active. The data obtained suggest that calcium antagonists inhibit PMN chemotaxis and locomotion by interference with a Ca^{2+}-dependent intracellular target.

The organic calcium antagonists are a chemically heterogeneous class of drugs with similar pharmacological properties. Important representatives of this class of drugs are verapamil, D600, nifedipine, diltiazem, perhexiline and prenylamine. These substances have been investigated thoroughly in the past years. A large number of laboratory and clinical studies suggest, that they have a remarkable therapeutic effect in coronary artery diseases [1–5]. They are used now in the treatment of angina pectoris, arrhythmias, hypertension and obstructive cardiomyopathies [4, 7–9].

Though their mechanism of action is not completely clear it is evident that they interfere with the availability of calcium for the contractile machinery [1, 6]. This interference can be antagonized by an increase of the calcium concentration in the medium, hence their name. They are thought to act as calcium channel blockers; their interference with translocation of calcium ions across the cardiac and smooth muscle cell membrane is a plausible base for their beneficial effects [1, 4, 6]. However, some authors have suggested that calcium antagonists might also interfere with other calcium dependent targets, which are located intracellularly.

Apart from their effect on the contractile properties of cardiac and smooth muscle cells, calcium antagonists also interfere with other calcium-requiring processes, such as secretion of vasopressin in isolated rat neurohypophysis [15], the release of hormones from rat pituitary tissue [16], insulin release from isolated rat islets [17, 18], exocytosis of histamine by mast cells [19] and the aggregation of blood platelets [20, 21].

Recently it has been shown that verapamil and nifedipine interfere with granulocyte functions, such as phagocytosis, degranulation [22], superoxide-production [23, 24] and aggregation [25]. The effects could be partially reversed by an increase of the extracellular Ca^{2+} concentration, hence it could be considered as a true calcium antagonistic effect. The concentrations required for inhibition of granulocyte functions was mostly one or two orders of magnitude higher than those required for inhibition of Ca^{2+}-translocation across smooth muscle cell membranes.

Chemotaxis by polymorphonuclear leukocytes (PMN's) is an important function which enables the body to respond rapidly to invasion of microorganisms. Because divalent cations are supposed to play an important but little understood role in PMN chemotaxis [26–30], we have studied the effect of verapamil and a number of other calcium antagonists on this process.

MATERIALS AND METHODS

Rabbit peritoneal PMN's were obtained as described previously [31]. The cells were washed with 1 mM EDTA to remove adherent divalent cations and then suspended in a medium consisting of 128 mM NaCl, 10 mM KCl, 9 mM KH_2PO_4, 3 mM K_2HPO_4, 4.5 mM Na_2HPO_4, 0.5% bovine serum albumin and 30 mM HEPES, pH 7.3. The final cell suspension during the experiments contained 2.10^6 PMN's per ml.

Cell migration was measured with the Boyden chamber technique, as described by Boyden [32]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter (pore size 3 μm). As a chemotactic agent formylmethionylleucylphenylalanine (FMLP), 10^{-9} M, was used. PMN's were preincubated with or without calcium antagonist for 20 min at room temperature, after which they were placed in the upper compartment of the Boyden chamber. The Boyden chambers were incubated for 60 min at 37°, after which the filters with PMN's were fixed in ethanol and stained with Mayer's hemalum solution. Cell migration was

determined microscopically according to the leading front technique [33]. The assays were carried out in triplicate and the migration distance of the PMN's was determined at three different filter sites.

Three types of experiments were carried out: locomotion (no FMLP); stimulated locomotion (FMLP in both upper and lower compartment of Boyden chamber); and chemotaxis (FMLP in lower compartment of the Boyden chamber). In the migration distance given for chemotaxis no correction has been made for (stimulated) locomotion. When chemotaxis or (stimulated) locomotion were determined in the absence of extracellular divalent cations, the divalent cation complexing agent EDTA was included in the medium to eliminate Ca^{2+} which is adherent to or liberated by the PMN.

The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was determined in order to establish a possible damaging effect of the calcium antagonists. PMN's were incubated with calcium antagonists for 60 min at 37°, after which the cells were centrifuged for 5 min at 500 rpm. The release of LDH was determined as described previously [34].

The calcium antagonists were obtained as a gift from the manufacturing firms. Verapamil and D600 were obtained from Knoll A.G. (Ludwigshafen); nifedipine was obtained from Bayer Nederland B.V. (Mijdrecht); prenylamine was from Hoechst Holland N.V. (Amsterdam), diltiazem was from Synthélabo (Paris) and perhexiline was obtained from Merrell Dow Pharmaceuticals Inc. (Cincinnati, OH). The drugs were dissolved in dimethylsulfoxide just before the experiment to give a concentrated stock solution from which μl quantities were added to the mixture; the final concentration of dimethyl sulfoxide did not exceed 0.3%.

The chemotactic peptide *N*-formyl methionylleucylphenylalanine (FMLP) and other reagents were obtained from Sigma Chemical Co.

RESULTS

A concentration gradient of the chemotactic peptide FMLP causes chemotaxis in rabbit PMN's. However, in the presence of FMLP there is also an increase in non-directional motility: stimulated locomotion. Whereas the locomotion (in the absence of FMLP) is small, the stimulated locomotion is considerable (Fig. 1). Because stimulated locomotion may play a certain, but difficult to estimate role in the measurement of chemotaxis, we also determined the effect of calcium antagonists on stimulated locomotion.

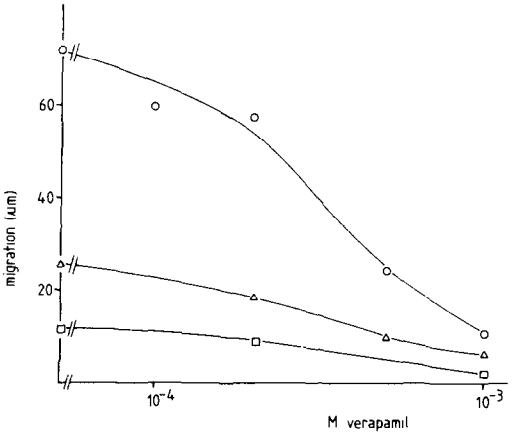

Fig. 1. Effect of verapamil on locomotion —□—; on stimulated locomotion —△—; and on chemotaxis —○—, in rabbit peritoneal PMN's. The values given are the mean values of three experiments.

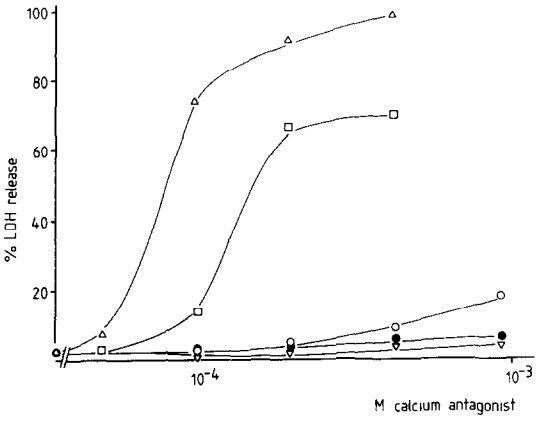

Fig. 2. Effect of calcium antagonists on cell integrity. Damage to PMN's was measured as LDH release. —○— verapamil; —●— D600; —▽— diltiazem; —□— prenylamine; —△— perhexiline. The values given are the mean values of two experiments.

As can be seen in Fig. 1, the calcium-antagonistic drug verapamil inhibits chemotaxis, locomotion and stimulated locomotion. The concentration range in which the drug is effective, is 10^{-4}–10^{-3} M. There is no inhibiting effect of verapamil or the other calcium antagonists in the range 0.1–10 μM, where these drugs are effective in inhibiting cardiac and smooth muscle cell contraction.

Because verapamil and the other calcium antagonists might damage cells in high concentrations, we have tested the membrane damaging effect of these drugs on granulocytes. In Fig. 2 the release of the cytoplasmic enzyme LDH as a function of drug concentration is depicted; the release of LDH is a measure for membrane damage. Especially prenylamine and perhexiline are lytic at relatively low concentrations. The drug concentrations, used in our experiments, were chosen so that lysis did not play a significant role.

In the system we used, chemotaxis occurs in the presence of the divalent cations Ca^{2+} and Mg^{2+}, but also in its absence. As a general rule chemotaxis in the absence of divalent cations was somewhat less than in their presence (see legends of Tables). All calcium antagonists tested strongly inhibit chemotaxis and stimulated locomotion in the absence of divalent cations. The inhibiting drug concentration was relatively high (0.5 mM); prenylamine and perhexiline were active at lower concentrations (Tables 1–3). The calcium antagonist nifedipine was also tested, but because this drug strongly binds to albumine—which is an essential constituent of our test system—no results were obtained with this drug.

Table 1. Effect of organic calcium antagonists on chemotaxis in the presence or absence of extracellular Ca^{2+}

Calcium antagonist	(%) Inhibition* in the presence of 1 mM EDTA	2 mM Ca^{2+}
0.5 mM verapamil	60 ± 1	33 ± 3
0.5 mM D600	53 ± 2	35 ± 3
0.5 mM diltiazem	50 ± 6	31 ± 5
0.1 mM prenylamine	78 ± 5	44 ± 5
0.05 mM perhexiline	69 ± 10	21 ± 12

* The migration of PMN's in the absence of drug was 50 μm in the presence of 1 mM EDTA, and 81 μm in the presence of 2 mM Ca^{2+}. The difference between migration in the absence and the presence of drug represented the inhibition, which was expressed as a percentage of migration in the absence of drug.

Values given are the mean of three experiments ± S.D.

Table 2. Effect of organic calcium antagonists on chemotaxis in the presence or absence of extracellular Ca^{2+} and Mg^{2+}

Calcium antagonists	(%) Inhibition* in the presence of 1 mM EDTA	1 mM Ca^{2+} + 1 mM Mg^{2+}
0.5 mM verapamil	77 ± 3	37 ± 10
0.5 mM D600	44 ± 5	15 ± 10
0.5 mM diltiazem	40 ± 11	18 ± 10
0.1 mM prenylamine	72 ± 6	23 ± 6
0.05 mM perhexiline	65 ± 7	15 ± 6

* The migration of PMN's in the absence of drug was 57 μm in the presence of 1 mM EDTA and 80 μm in the presence of 1 mM Ca + 1 mM Mg^{2+}.

Table 3. Effect of organic calcium antagonists on stimulated locomotion in the presence or absence of extracellular Ca^{2+}

Calcium antagonist	(%) Inhibition* in the presence of 1 mM EDTA	2 mM Ca^{2+}
0.5 mM verapamil	76 ± 4	63 ± 6
0.5 mM D600	58 ± 6	38 ± 9
0.5 mM diltiazem	64 ± 4	26 ± 5
0.1 mM prenylamine	79 ± 4	63 ± 6
0.05 mM perhexiline	74 ± 4	51 ± 6

* The migration of PMN's in the absence of drug was 31 μm in the presence of EDTA and 37 μm in the presence of 2 mM Ca^{2+}.

In the presence of divalent cations (2 mM Ca^{2+} or 1 mM Ca^{2+} + 1 mM Mg^{2+}) the calcium antagonists are also inhibiting but to a lesser degree than in the absence of divalent cations; apparently the presence of extracellular Ca^{2+} (or Ca^{2+} + Mg^{2+}) causes a partial reversal of the inhibiting effect. Both the inhibition of calcium antagonists, and the partial reversal of inhibition by divalent cations apply to chemotaxis (Tables 1 and 2) and stimulated locomotion (Table 3).

Because the absolute value of migration in chemotaxis, as well as the inhibiting effect of the calcium antagonists, strongly vary with experimental conditions, i.e. different batches of cells, inhibition of calcium antagonists in the presence and absence of divalent cations was determined on PMN's of the same batch of cells, under identical circumstances.

In order to find out whether only Ca^{2+}, or also Mg^{2+} was able to reverse the inhibitory effect of the calcium antagonist, we tested the inhibitory effect of verapamil in the presence of 1 mM EDTA; 2 mM Ca^{2+}; 2 mM Mg^{2+} or 1 mM Ca^{2+} + 1 mM Mg^{2+}. The latter condition was included because both Ca^{2+} and Mg^{2+} are generally present in media for granulocytes. The results are shown in Table 4. Whereas there is a reversal of inhibition with 2 mM Ca^{2+}, or 1 mM Ca^{2+} + 1 mM Mg^{2+}, the inhibition in the presence of 2 mM Mg^{2+} is little affected.

We also tested the effect of some inorganic calcium channel blockers [35] on chemotaxis, under the conditions of our experiments (Table 5). We found that chemotaxis is little affected by the inorganic calcium channel blockers Co^{2+}, Ni^{2+} and Mn^{2+}. In the absence of extracellular Ca^{2+} a slight stimulation was observed which was not significant. In the presence of Ca^{2+} some ions were slightly inhibitory. In accord-

Table 4. Reversal of verapamil-induced inhibition of chemotaxis by divalent cations

	Distance travelled in the presence of 0.5 mM verapamil		
	(μm)	(μm)	(%) Inhibition
1 mM EDTA	80 ± 8	31 ± 4	61
2 mM Ca^{2+}	81 ± 6	56 ± 8	31
1 mM Ca^{2+} + 1 mM Mg^{2+}	92 ± 8	66 ± 7	28
2 mM Mg^{2+}	100 ± 6	44 ± 13	57

Chemotaxis is expressed as migrated distance in μm. Values given are the mean values of three experiments ± S.D.

ance with the results of Boucek and Snyderman [36] we found that La^{3+} was strongly inhibitory at 1 mM, both in the presence and absence of extracellular Ca^{2+}.

DISCUSSION

The results show that verapamil inhibits locomotion, stimulated locomotion and chemotaxis by rabbit peritoneal PMN's, in the concentration range of 0.1–1 mM. Whereas prenylamine and perhexiline inhibit at somewhat lower concentrations, diltiazem and D600 require the same inhibiting concentration as verapamil. As compared with the effect of verapamil and the other calcium antagonists on Ca^{2+} transportation across cardiac and smooth muscle cell membranes [1, 4, 6], where inhibition occurs in the micromolar concentration range, inhibition of chemotaxis of PMN's occurs at very high concentrations. This is a first indication that the mechanism of inhibition in PMN chemotaxis might be different from that in other systems. There are some other processes, such as phagocytosis, exocytosis, aggregation and superoxide production by PMN's [22–25], and serotonin release from platelets [37] which require a high inhibitory concentration of calcium antagonists.

Two of the compounds tested are toxic in high concentrations, which can be deduced from the leakage of the cytoplasmic enzyme LDH. However, the concentrations used in our experiments did not induce LDH release. Furthermore, the inhibiting effect of prenylamine and perhexiline can be modulated by Ca^{2+} as for the other non-toxic calcium antagonists. It is therefore not likely that inhibition of chemotaxis by prenylamine and perhexiline is due to the membrane-damaging effects of these compounds.

Table 5. Effect of inorganic Ca^{2+}-channel blockers on chemotaxis in the presence or absence of extracellular Ca^{2+}

Me^{2+}	Distance travelled in the presence of 2 mM Ca^{2+}	
	(μm)	(μm)
None	66 ± 8	83 ± 5
1 mM Co^{2+}	65 ± 8	70 ± 6
1 mM Ni^{2+}	73 ± 3	68 ± 3
1 mM Mn^{2+}	70 ± 8	70 ± 13
0.1 mM La^{3+}	69 ± 8	73 ± 12
1 mM La^{3+}	24 ± 5	31 ± 12

Chemotaxis is expressed as migrated distance in μm. Values given are the mean values of three experiments ± S.D. In the absence of Ca^{2+} no EDTA was added, to avoid complexation with the metal ions.

Under the condition of our experiments chemotaxis occurs also in the absence of extracellular divalent cations. This does not mean that Ca^{2+} (and possible Mg^{2+}) is not required for chemotaxis. It has been shown that PMN's can perform a number of Ca^{2+}-dependent cell functions by mobilizing Ca^{2+} from intracellular stores [38, 39]. Verapamil and the other calcium antagonists strongly inhibit chemotaxis and (stimulated) locomotion in the absence of extracellular Ca^{2+}. This indicates that the inhibiting effect of calcium antagonists cannot be ascribed to their ability to block Ca^{2+}-channels and therewith the translocation of Ca^{2+} across the membrane. It seems therefore likely that the calcium antagonists have an intracellular site of action. This view is supported by the fact that the lipophilic characteristics of these drugs permit an easy penetration into the cells. Furthermore it has been shown that calcium antagonists acting on intracellular structures [6, 10–14, 40, 41] generally require higher concentrations as compared with inhibition of Ca^{2+} translocation across the plasma membranes of cardiac cells. The relative unimportance of Ca^{2+}-channels in the effect of calcium antagonists on PMN chemotaxis is underlined by the lack of inhibition by the inorganic Ca^{2+}-channel blockers Co^{2+}, Mn^{2+} and Ni^{2+}.

The inhibiting effect of verapamil and the other calcium antagonists on PMN chemotaxis and stimulated locomotion can be partially reversed by an increase in the extracellular Ca^{2+} concentration. Though the action of calcium antagonists on chemotaxis and other PMN functions occurs at much higher concentrations as compared with the effect on cardiac cells, they remain true "calcium antagonists" in the sense that Ca^{2+} ions antagonize their inhibiting effect. It strengthens the view that the calcium antagonists interfere with an (intercellular) Ca^{2+}-dependent target. The properties of the chemotactic peptide FMLP, which is present in those conditions where extracellular Ca^{2+} causes a reversal of inhibition, explain why extracellular Ca^{2+} interferes with an intracellular action of calcium antagonists. FMLP, in concentrations as low as 10^{-10} M, causes a change in permeability for Ca^{2+} of the membrane [42, 43]. Using $^{45}Ca^{2+}$, Petroski *et al.* [44] have shown that FMLP causes an increase in the steady state level of cell-associated $^{45}Ca^{2+}$; the

increase is dependent on the extracellular Ca^{2+} concentration. This indicates that an increase of extracellular Ca^{2+} causes a (transient) higher intracellular Ca^{2+} concentration, which enables the reversal of the inhibition by calcium antagonists of a Ca^{2+}-dependent structure.

The nature of the intracellular structure on which the calcium antagonists act, is hitherto unknown. There are a number of potential targets. One of the possibilities is that calcium antagonists interfere with Ca^{2+} translocation across membranes of intracellular organelles. This possibility has been suggested for some cell types where Ca^{2+} has been identified in specific intracellular organelles [6, 10]. For the PMN however, such membrane-bounded stores of Ca^{2+} are not known; most authors believe that the PMN stores its intracellular Ca^{2+} at the inside of the plasma membrane [45]. It is thus unlikely that the calcium antagonists interfere with Ca^{2+} translocation across membranes but they may interfere with Ca^{2+} release from the plasma membrane.

Another possibility with which calcium antagonists may interfere, is calmodulin. The action of this molecule is Ca^{2+} dependent and this fits in with the Ca^{2+} dependent reversal of antagonist inhibition. Another support for this view is that prenylamine, one of the calcium antagonists tested, is known as a calmodulin inhibitor [46]. The calmodulin dependent contraction of skinned smooth muscle is inhibited by prenylamine (200 μM); this inhibition can be overcome by an increase of the Ca^{2+} concentration [47]. We have recently shown that calmodulin inhibitors strongly interfere with chemotaxis of PMN's [48], thus making it likely that calmodulin is involved in PMN chemotaxis.

The experimental basis for these hypotheses is rather weak; other possibilities are not excluded and further experimental work is required to clarify this aspect.

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