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Inhibition of erythrocyte membrane Ca^{2+}-pumping ATPase of hypertensive humans by nifedipine, a calcium entry blocker

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Summary

Nifedipine, a 1,4-dihydropyridine antihypertensive drug, inhibited the basal activity of erythrocyte Ca^{2+},Mg^{2+}-ATPase of hypertensive individuals in a concentration-dependent manner. About 50% inhibition was obtained at nifedipine concentrations ≥ 300 μM. The extent of inhibition of the ATPase action was increased in the presence of calmodulin. Maximal inhibition at 400 μM was 76%. Furthermore, the activity of the partially trypsinized enzyme was inhibited by about 50% by 300 μM nifedipine. Similar results were obtained with membranes from normotensive individuals.

These findings suggest that nifedipine could prevent Ca^{2+}-pumping by the erythrocyte Ca^{2+},Mg^{2+}-ATPase.

Résumé

Nifedipine, un médicament antihypertensif de 1,4-dihydropyridine a inhibé l'activité basal de l'erythrocyte Ca^{2+},Mg^{2+}-ATPase des personnes hypertensives d'une façon entraînant une dépendence de concentration. On a obtenu à peu près 50% de l'inhibition utilisant des concentrations de nifedipine de ≥ 300 μM. Avec la présence de calmodulin, le degré de l'inhibition de l'ATPase a augmenté. L'inhibition maximale à 400 μM était de 76%. En outre, l'activité de l'enzyme partiellement trypsinée a été inhibée par à peu près 50% à 300 μM de nifedipine. On a obtenu des résultats semblables avec les membranes des personnes normotensives.

Ces résultats donnent l'impression que nifedipine pourrait empêcher Ca^{2+} d'être pompé par l'erythrocyte de Ca^{2+},Mg^{2+}-ATPase.

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Introduction

Nifedipine, a 1,4-dihydropyridine calcium entry blocker, is widely used in the management of systemic hypertension [1,2]. Although there is abundant evidence that Ca^{2+} entry blockers interfere with the influx of Ca^{2+} through slow Ca^{2+} channels in the plasma membrane [3–6], it seems probable that these blockers may interact with other calcium-binding proteins and calcium-dependent enzymes. For instance, the interaction of felodipine, another 1,4-dihydropyridine antihypertensive drug, with calmodulin has been shown to result in an alteration in the Ca^{2+}-binding properties of the protein [7]. Furthermore, nifedipine and related substances are now known to antagonize calmodulin and to inhibit calmodulin-stimulated and basal activity of cyclic AMP phosphodiesterase [8,9]. We have recently shown that nifedipine inhibits the basal and calmodulin-stimulated activity of erythrocyte Ca^{2+}-pumping ATPase [10], an integral plasma membrane protein which moves Ca^{2+} ions across the plasma membrane against a 10,000-fold chemical concentration gradient with energy derived from the hydrolysis of ATP [11].

In view of the occurrence of an elevated

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intracellular free Ca^{2+} concentration in essential hypertension [12] and of the finding that erythrocyte membrane Ca^{2+},Mg^{2+}-ATPase of hypertensive humans [13] and of spontaneously hypertensive rats [14] exhibits a reduced responsiveness to calmodulin, it was thought desirable to examine the behaviour of this pump protein in the presence of nifedipine. In this paper, we present evidence that nifedipine antagonizes calmodulin and inhibits the Ca^{2+}-pumping ATPase of the erythrocyte membrane of hypertensive humans. The calcium entry blocker also inhibits the activity of the partially trypsinized membrane-bound enzyme.

Materials

Ethyleneglycol - bis - (aminoethylether) N,N,N′,N′-tetra acetic acid (EGTA), adenosine 5′-triphosphate (vanadium free), 4-(2-hydroxyethyl) - 1 - piperazine-ethanesulphonic acid (HEPES), bovine serum albumin (fatty acid-free) and phenylmethylsulphonylfluoride (PMSF) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were of the highest purity available and were purchased from Fluka AG, Zurich, Switzerland and British Drug Houses, U.K.

Patients and methods

Isolation of calmodulin-free erythrocyte ghost membrane

Blood samples were collected in acid-citrate-dextrose buffer from individuals who were newly identified as having essential hypertension and who did not receive any medication or dietary therapy prior to the time blood was collected. Calmodulin-deficient erythrocyte ghost membranes were prepared after haemolysis in 1 mM EGTA, essentially as described by Dodge *et al.* [15] and as previously reported [16]. Ghost membranes were stored in 130 mM KCl, 20 mM Hepes, pH 7.4, 500 μM $MgCl_2$ and 50 μM $CaCl_2$ at −40°C. All solutions contained 0.1 mM PMSF.

Determination of erythrocyte ghost membrane protein

Membrane protein was determined by the method of Lowry *et al.* [17], as modified by Markwell *et al.* [18] following precipitation by treatment of whole membrane preparations with 0.05% (w/v) deoxycholic acid and 10% TCA. Fatty acid-free serum albumin was used as a standard.

Assay of Ca^{2+},Mg^{2+}-ATPase activity

Ca^{2+},Mg^{2+}-ATPase activity was determined by following the rate of liberation of inorganic phosphate from the γ-position of ATP, as previously described [16]. Reaction medium contained in final concentrations: 120 mM KCl, 50 mM Hepes pH 7.4, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM EDTA and 50–100 μg membrane protein in a total volume of 900 μl. Aliquots of nifedipine were added where necessary. The reaction was started by the addition of 2 mM ATP. The assay was run in duplicate with or without calmodulin (120 nM). At the end of 30 min, 10% sodium dodecylsulphate was used to terminate the reaction. The inorganic phosphate liberated was estimated colorimetrically. Ca^{2+},Mg^{2+}-ATPase activity was obtained by subtracting Mg^{2+}-ATPase activity from total activity in the presence of calcium.

Limited proteolysis of membranes by trypsin

Plasma membranes (75 μg protein) were incubated with trypsin (8.5×10^{-7} M) in an incubation medium containing 50 mM K-Hepes (pH 7.4), 120 mM KCl, 0.1 mM ouabain, 5 mM $MgCl_2$, 2 mM $CaCl_2$ and 5 mM EDTA. Proteolysis was started by adding the protease and incubating at 20°C for 60 min.

Tryptic digestion was stopped by the addition of a 10-fold concentration of soybean trypsin inhibitor, and the Ca^{2+},Mg^{2+}-ATPase activity of the membranes was assayed in the presence of aliquots of nifedipine. In some experiments Ca^{2+},Mg^{2+}-ATPase was assayed in the presence of calmodulin (120 nm).

Results

The data presented in Table 1 show that the basal activity of erythrocyte membrane Ca^{2+}, Mg^{2+}-ATPase of hypertensive humans was inhibited by nifedipine in a concentration-

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Table 1. Influence of nifedipine on erythrocyte plasma membrane Ca^{2+},Mg^{2+}-ATPase of hypertensive humans

Nifedipine (μM)	Ca^{2+},Mg^{2+}-ATPase activity (μmol Pi/mg protein/h)	
	Basal	Calmodulin-stimulated
0	0.59 ± 0.06	1.62 ± 0.09
50	0.56 ± 0.05	1.11 ± 0.09
100	0.49 ± 0.06	0.71 ± 0.08
200	0.37 ± 0.05	0.39 ± 0.03
300	0.30 ± 0.03	0.35 ± 0.03
400	0.30 ± 0.03	0.33 ± 0.02

Each value is a mean of six determinations ± standard deviation.

dependent manner. Significant inhibition (≥ 37%) of this ATPase action was obtained at nifedipine concentrations greater than or equal to 200 μM, while maximal inhibition (49%) was attained at 300 μM nifedipine. The results show further that the extent of inhibition of the enzyme was higher in the presence of calmodulin than in its absence. In this instance, maximal inhibition of about 76% was obtained at 400 μM nifedipine. Interestingly, nifedipine (50 μM) significantly inhibited (by 30%) this ATPase action. Taken together, these results indicate that, although the basal activity of the pump is only partially inhibited by nifedipine, the calmodulin-stimulated enzyme is more susceptible to the effect of the antihypertensive drug. These results are in agreement with previous findings in our laboratory on the effects of nifedipine on the Ca^{2+}-ATPase of the membranes of normal and healthy individuals [10].

In order to ascertain whether nifedipine interacts directly with the ATPase or prevents calmodulin from binding to the enzyme, we investigated the effect of the drug on partially trypsinized erythrocyte membrane Ca^{2+},Mg^{2+}-ATPase which was no longer stimulable by calmodulin. Our findings, summarized in Table 2, indicate that the enzyme from normotensive or hypertensive individuals was stimulated by mild treatment with trypsin. Additions of varying amounts of nifedipine to the reaction medium revealed that the activity of the trypsinized enzyme of the membranes of hypertensive and normal individuals was inhibited by nifedipine. The lowest (16–20%), and highest (51–53%) values of inhibition were, respectively, obtained at 50 and 500 μM nifedipine in normal and hypertensive subjects.

Table 2. Effect of nifedipine on the activity of partially trypsinized erythrocyte membrane Ca^{2+},Mg^{2+}-ATPase of normotensive (NTN) and hypertensive (HTN) humans

Nifedipine (μM)	Ca^{2+},Mg^{2+}-ATPase activity (μmol Pi/mg protein/h)	
	NTN	HTN
0	4.34 ± 0.38	3.98 ± 0.43
50	3.51 ± 0.29	3.36 ± 0.31
100	2.63 ± 0.21	2.42 ± 0.22
200	2.20 ± 0.22	1.99 ± 0.21
300	2.16 ± 0.20	1.88 ± 0.23
400	2.14 ± 0.15	1.87 ± 0.19

Each value is a mean of six determinations ± standard deviation.

Discussion

Although the use of calcium entry blockers in the treatment of cardiovascular disease has its roots in the ability of these drugs to prevent contraction of cardiac and smooth muscle by interfering with the entry of calcium through

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the slow calcium channels of excitable cells, the concept of a specific channel which allows the passage of Ca^{2+} has been extended to non-excitable cells such as the erythrocytes [19]. The present results show that nifedipine inhibits the erythrocyte membrane Ca^{2+},Mg^{2+}-ATPase of hypertensive subjects, whether or not calmodulin is present (Table 1). These results suggest, in particular, that nifedipine could interact with the erythrocyte Ca^{2+}-pumping ATPase of hypertensive humans, such that few Ca^{2+} ions are transported out of the cell even in the presence of calmodulin.

It appears, therefore, from these findings that the interaction of nifedipine with erythrocyte Ca^{2+},Mg^{2+}-ATPase and calmodulin could produce an effect that is opposed to the ability of nifedipine to reduce the level of intracellular free Ca^{2+} in essential hypertension. Even though the experiments reported here were not carried out under the same conditions that prevail in essential hypertension, the involvement of other Ca^{2+} transport systems in the regulation of intracellular free Ca^{2+} concentration during exposure to nifedipine may not be ruled out. The active participation of such a system will be inevitable if the level of intracellular free Ca^{2+} must remain very low. Alternatively, the ability of nifedipine to complex Ca^{2+} should reduce the level of intracellular free Ca^{2+}, especially at very high doses of the drug.

Apart from calmodulin, acidic phospholipids and unsaturated long chain fatty acids, limited proteolysis by trypsin [11] or calpain [20], an endogenous Ca^{2+}-dependent neutral protease, will activate the erythrocyte membrane Ca^{2+}, Mg^{2+}-ATPase. The finding that the activity of the partially trypsinized enzyme was sensitive to nifedipine (Table 2) provides additional evidence that the 1,4-dihydropyridine interacts directly with the ATPase. Furthermore, the observation that limited proteolysis by trypsin raised the activity of the enzyme of hypertensive subjects to about the same level (or stimulation fold by calmodulin) as that of normal membranes supports the suggestion by Wang *et al.* [20] that calpain could irreversibly stimulate Ca^{2+},Mg^{2+}-ATPase during periods of prolonged or uncontrolled increase in intracellular free Ca^{2+} concentration in certain pathological states.

Finally, further research on the effect of calpain on the Ca^{2+}-pumping ATPase in essential hypertension should enhance our understanding of the mechanism of calcium homeostasis in this disease state and during nifedipine therapy.

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