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Calmodulin antagonism and inhibition of erythrocyte plasma membrane Ca^{2+}-pump by nifedipine, a calcium channel blocker

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Summary

Nifedipine, a 1,4-dihydropyridine Ca^{2+}-channel blocker inhibits calmodulin-stimulated activity of erythrocyte plasma membrane (Ca^{2+}+ Mg^{2+}) -ATPase. Its inhibition of the basal activity of the enzyme increases on pre-incubation with membranes. The maximum velocity, affinity for ATP and Ca^{2+}-sensitivity of pump are reduced by micromolar amounts of the antihypertensive drug. These results suggest that 1,4-dihydropyridines may interact with calcium-binding proteins and calcium-dependent enzymes at their calcium-binding sites.

Résumé

Nifedipine, medicament de 1,4-dihydropyridine qui bloque le canal Ca^{2+} + Mg^{2+})-ATPase du plasma de l'erythrocyte stimulée par calmodulin. Son inhibition de l'activité basale de l'enzyme croit aprés préincubation avec les membranes. La vélocité et laffinité maximales pour la sensibilité d'ATP et de Ca^{2+} de la pompe diminuent utilisant des quantités micromolaires du médicament antihypertensif. Ces résultants donnent l'impression qu'une action réciproque des 1,4-dihydropyridines a lieu avec les protéines reliant le calcium et avec les enzymes dépendant du calcium.

Introduction

The plasma membrane Ca^{2+}-pump actively removes Ca^{2+} from all eukaryotic cells investigated to date[1,2]. The enzyme, an ATPase of the P-type because it forms an acylphosphate intermediate during its catalytic cycle[3,4], is the major mechanism responsible for the extrusion of Ca^{2+} from erythrocytes[4,5]. In other cells, particularly those of excitable tissues, the Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger are involved in the regulation of intracellular free Ca^{2+} concentration[2]. The ATPase, M_r 136[6] and representing less than 0.1% of total membrane proteins effectively maintains erythrocyte intracellular free calcium concentration in the rate of 10^{-7} to 10^{-8}M by transporting Ca^{2+} ions against a 10,000-fold electrochemical gradient with energy derived from ATP[1]. A major feature of the protein is the regulation of its calcium sensitivity by calmodulin[7], a ubiquitous calcium binding protein which also requires Ca^{2+} ions for potency[8].

Although a significant body of evidence suggests that Ca^{2+}-entry blockers interfere with the influx of Ca^{2+} through Ca^{2+} channels by binding to specific sites on the proteinaceous channel in the plasma membrane[9,11], there are indications that these substances may act on intra-cellular or membrane-bound calcium-binding proteins[12]. In particular, Minocherhomjee and Roufogalis[13] have shown that nifedipine and related calcium entry blockers antagonise calmodulin and phosphodiesterase. The observation that carboxylate groups are involved in the binding of Ca^{2+} to the outer surface of the channel[14] suggests that these groups could be directly or indirectly masked by the Ca^{2+} entry blockers. It seems pertinent to us to study the effect of nifedipine on the Ca^{2+}-pumping ATPase in the absence and presence of calmodulin because the ATPase, like its endogeneous regulator, possesses a number of carboxylate groups for Ca^{2+} binding at its Ca^{2+} binding domains[6,15].

Materials

Phenylmethylsulfonyl fluoride (PMSF), adenosine 5^1-triphosphate (vanadium-free), Ethyleneglycolbis (2-aminoethylether), N,N,N^1,N^1-tetraacetic acid (EGTA), 4-(20-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) and fatty acid free bovine serum albumin were obtained from Sigma Chemical Co. (London). Bovine brain calmodulin was obtained from Calbiochem La Jolla, (California). All other chemicals were of the highest purity grade available. Nifedipine was a generous gift of Dr. S. O. Lawal, Department of Medicine, University of Ibadan.

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Methods

Isolation of calmodulin-deficient erythrocyte ghost membrane

Calmodulin-free erythrocyte plasma membranes were isolated from whole blood collected in acid-citrate-dextrose buffer by the procedure of Jarret and Penniston[16] using the principle of hypotonic lysis developed by Dodge *et al* [17]. Fresh blood was centrifuged at 5,800*g* for 10 min at 4°C. The plasma and buffy layers were carefully aspirated to obtain packed red cells which were washed thrice in 5 vols of 130mM KCl, 20mM Tris-HCl pH 7.4 by spining the cells suspension at 5,800*g* for 10 min. Washed cells were gently resuspended in 5 vols of 1mM EGTA, 10mM HEPES (pH 7.4). The suspension was spun at 20,000*g* for half an hour. The step was repeated five times to ensure complete haemolysis. Membranes were washed with 10mM HEPES (pH 7.4) until they became white. Finally, the white ghost membranes were resuspended in 130mM KCl, 20mM HEPES, pH 7.4, 500 µM $MgCl_2$, 50µM $CaCl_2$, and stored at -40°C. The membranes were used within two weeks after isolation. All buffers contained 0.1mM PMSF.

Protein estimation

Membrane proteins were estimated by a modification of the procedure of Lowry *et al* [10] as described by Markwell *et al* [19]. The proteins were first precipitated with 0.05% (w/w) deoxycholic acid and 10% (w/w) trichloroacetic acid in order to prevent interference by HEPES and phospholipids. Fatty acid-free bovine serum albumin was used as standard.

Assay of Ca^{2+}-ATPase activity

Ca^{2+}-pump activity was determined by following the rate of release of inorganic phosphate from the γ-position of ATP as previously described[6]. Assay medium consisted of 120mM KCl, 50mM HEPES, 3mM $MgCl_2$, 0.2mM $CaCl_2$, 0.1mM EGTA and erythrocyte ghost membrane (50-100µg membrane protein) in a total volume of 950µl. The mixture was pre-incubated for 5 min at 37°C with constant shaking prior to the initiation of reaction by addition of 50µl of 40mM ATP. The assay was run for 30 mins in duplicates with or without 120mM calmodulin. Inorganic phosphate was measured by a modification of the method of Fiske and Subbarow[20] following termination of reaction with 1ml of 10% sodium dodecylsulphate. The intensity of the blue colour that was developed on addition of ammonium molydate in H_2SO_4 and ascorbate respectively, was measured at 820nm in a Pye unicam SP 600 spectrophotometer. Blanks were run to correct for non-enzymic hydrolysis of ATP. Ca^{2+}-ATPase activity was obtained by subtracting Mg^{2+}-ATPase activity (activity in the presence 5mM EGTA instead of 0.2mM $CaCl_2$) from total ATPase activity in the presence of calcium. Activity is expressed as µmol Pi released per mg protein per hour.

Results

Figure 1 shows the pattern of inhibition of calmodulin-activated erythrocyte plasma membrane Ca^{2+}-ATPase by nifedipine.

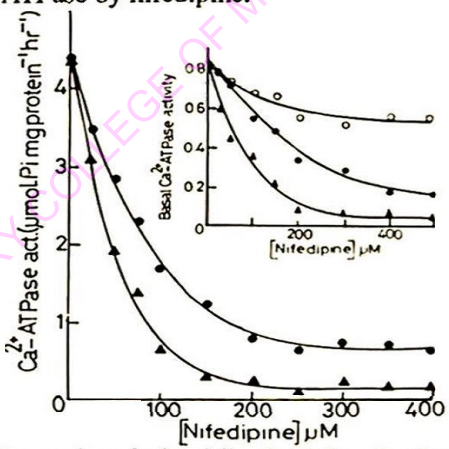

Fig 1. Antagonism of calmodulin stimulation of erythrocyte membrane (Ca^{2+}+ Mg^{2+})-ATPase by nifedipine on pre-incubation with membranes. Zero minute (●) and 30 mins (▲). Insert: Effect of nifedipine on basal (Ca^{2+}+ Mg^{2+})-ATPase activity without preincubation (0), on preincubation for 30 min (●) and 1 hour (▲). Preincubation was done at 4°C.

It is clear from the figure that nifedipine antagonised calmodulin-stimulation of the ATPase in a concentration-dependent manner whether or not the membranes were pre-incubated with the Ca^{2+}-entry blocker. Half maximal inhibition (IC_{50}) was obtained at 40µM and 70µM nifedipine, respectively, with and without pre-incubation with membranes. The results show further that the inhibition of the Ca^{2+}-ATPase was at least 70% when the channel blocker (200µM) was added to the membranes just before commencement of reaction, whereas inhibition was almost total at nifedipine concentrations greater than 200µM on pre-incubation with membranes for at least half an hour. In addition, the results presented in Fig. 2 show that

both the V_{max} and the K_m values of the enzyme were modified by 100μM nifedipine.

A study of the dependence of the inhibition by nifedipine on calmodulin concentration (Fig.3), indicates that the extent of inhibition by nifedipine is reduced by increasing calmodulin concentration in the reaction medium. These results suggest that nifedipine antagonizes the interaction of calmodulin with the Ca^{2+}-ATPase.

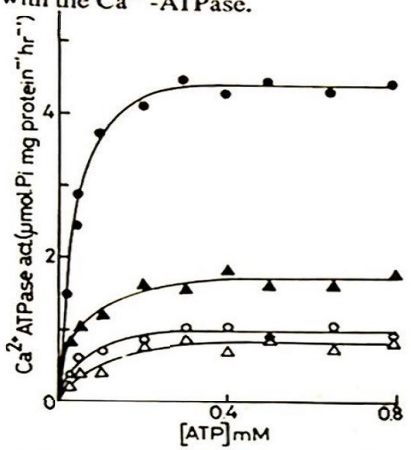

Fig 2. ATP-dependence of erythrocyte membrane (Ca^{2+}+ Mg^{2+})- ATPase in the absence (circles) and presence (triangles) of 100μM nifedipine. Assay was run with (filled symbols) or without (open symbols) 120nM calmodulin.

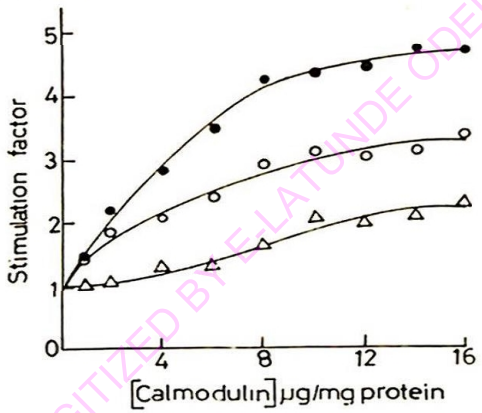

Fig. 3: Calmodulin-dependence of erythrocyte membrane (Ca^{2+}+ Mg^{2+})-ATPase. Assay was run in the absence of nifedipine (●); in the presence of 100μM (0) and 200μM nifedipine (△).

Furthermore, an assessment of the effect of nifedipine on the Ca^{2+} sensitivity of the ATPase reveals that the Ca^{2+} channel blocker antagonizes calmodulin as the V_{max} and the Ca^{2+} sensitivity of the enzyme were not significantly altered by calmodulin (Fig.4). For instance, calmodulin could not increase the V_{max} of the enzyme by two-fold in the presence of 100μM nifedipine. In addition, calmodulin stimulation was inhibited by nifedipine (50μM and 100μM) at all the concentrations of free Ca^{2+} tested.

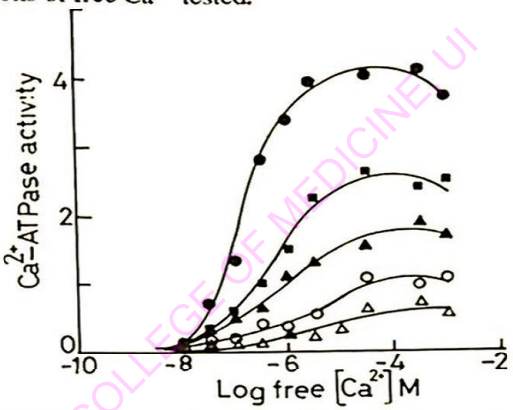

Fig. 4. Effect of nifedipine on the Ca^{2+} sensitivity of erythrocyte membrane (Ca^{2+}+ Mg^{2+})-ATPase in the presence (filled symbols) or absence (open symbols) of calmodulin (120nM). No nifedipine (circles), 100μM nifedipine (triangles) and 50μM nifedipine (squares).

Figure 1 (insert) shows the effect of varying concentrations of nifedipine on the basal activity of the Ca^{2+}-ATPase. The activity of the pump was only inhibited by about 25 percent at very high concentrations of the drug (≥200μM) when added to the membranes in the reaction medium just before initiation of the ATPase reaction. The results show, also that the extent of inhibition increased with increasing nifedipine concentration on pre-incubation with membranes for at least 30 mins. In this instance, activity of the pump was almost totally inhibited when membranes were pre-incubated with nifedipine (≥200μM) for at least 1 hour. The ATP-dependence of the basal activity of the pump did not change with increasing ATP concentrations. An assay of the basal activity of the Ca^{2+}-ATPase over a range of free Ca^{2+} concentration reveals that nifedipine reduced the sensitivity of the enzyme to Ca^{2+} (Fig.4). The enzyme was inhibited by nifedipine at all the concentrations of free Ca^{2+} examined. Figure 5 shows the effect of varying pre-incubation times on the inhibition by nifedipine (10-100μM).

The results indicate that over 20 percent inhibition of ATPase activity was obtained after 25 minutes of pre-incubation of the plasma membranes with 50μM and 100μM nifedipine. About

50 and 60 percents inhibition was observed after 1hr of pre-incubation with 50μM and 100μM nifedipine, respectively. The extent of inhibition was increased with increasing time of exposure of membranes to the drug. For instance, about 75 percent of the basal activity of the ATPase was inhibited on pre-incubation of membranes with 100μM nifedipine for approximately 2hr.

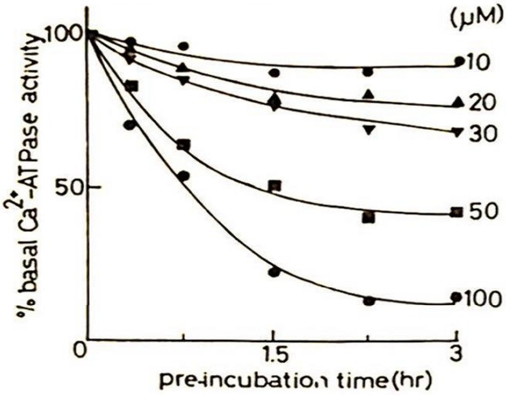

Fig. 5. Basal activity of erythrocyte membrane (Ca^{2+}+ Mg^{2+})- ATPase following pre-incubation for varying periods with different amounts of nifedipine. Pre-incubation was carried out on ice (4°C).

Furthermore, the degree of inhibition of the enzyme was lower than 60 percent when membranes were pre-incubated with 50μM nifedipine for 2 to 3 hours. Lower concentrations of nifedipine (20-30μM) inhibited about 25 percent of the ATPase action only after pre-incubation for 2 hours. These results indicate that the basal activity of the pump is only partially inhibited by low concentrations of nifedipine except if the membranes were exposed to the drug for at least half an hour prior to assay of the ATPase action.

Discussion

The affinity and selectivity of certain proteins for Ca^{2+} is made possible by the ability of these proteins to arrange six to eight carboxylate oxygen atoms, each carrying a partial or fully negative charge in a helix-loop-helix structure of E-F hand for binding Ca^{2+} [21]. Calmodulin, the erythrocyte Ca^{2+}-ATPase and certain Ca^{2+}-binding proteins or Ca^{2+}-dependent enzymes have been shown to possess this structure in their Ca^{2+} binding domains[15,21]. Unlike most Ca^{2+}-binding proteins, very little is known about the nature of the Ca^{2+}-binding ligand of the proteinaceous Ca^{2+}-channel. Although one carboxylate group has been implicated in the binding of penetrating Ca^{2+} at the outer opening of the channel, the use of labelled dihydropyridines which block Ca^{2+} entry, to tag Ca^{2+} channel components during isolation and purification procedures suggest that these substances could form a covalent linkage with the proteinaceous slow channels[14].

In this study we present evidence to show that calmodulin stimulation of erythrocyte Ca^{2+}-ATPase is antagonized by micromolar amounts of nifedipine; the effect of the Ca^{2+} entry blocker being more pronounced on pre-incubation with the ghost membranes (Figs. 1 and 5). Although, it is not known whether the Ca^{2+} channel blocker binds directly to calmodulin and/or to the Ca^{2+}-ATPase, it appears that the drug could bind to calmodulin in the same manner as felodipine, another dihydropyridine derivative[22]. Specifically, studies on the effect of felodipine on the NMR spectrum of ^{113}Cd in $(Cd)_4$-calmodulin complex have shown that the binding of the antihypertensive drug to calmodulin modifies the conformation of the protein, at least at one Ca^{2+}-binding site[22]. Furthermore, potentiometric titrations of 4 mols of felodipine per mol of protein show that the ability of calmodulin to bind Ca^{2+} was reduced to 2 mol Ca^{2+} [22]. These findings did not show that felodipine interacts with calmodulin at the Ca^{2+}-binding domain or at some other sites different from the domain. However, more recent findings on the deduction of the amino acid sequence of the plasma membrane Ca^{2+}-ATPase show the occurrence of calmodulin-like domains and E-F hands, which resemble those in calmodulin, in the structure of this pump[15]. Based on these facts, it appears reasonable to surmise that nifedipine could interact with the Ca^{2+}-ATPase at those sites which have resemblance to calmodulin, only if those sites are accessible to nifedipine. Interestingly, our results show that the effect of nifedipine on the basal activity of the Ca^{2+}-pump increases with an increase in the concentration of the drug and the time of pre-incubation; thus indicating that the sites to which the drug binds are not readily accessible (Fig.1).

It is not known if nifedipine actually plugs the Ca^{2+} passage in the (Ca^{2+}+ Mg^{2+})-ATPase or if it merely binds to Ca^{2+}-binding domains on the enzyme and thus modulates its catalytic activity. However, nifedipine interaction with Ca^{2+}-binding proteins or Ca^{2+}-dependent enzymes will be specific if the drug recognises the Ca^{2+} binding E-F hands of these proteins. Clearly, the finding

that labelled dihydropyridines are used to tag Ca^{2+} channel components in purification procedures[23] rules out the possibility of a lowering of free Ca^{2+} by complexation by nifedipine, and offers a strong support for the possibility of a direct interaction between the Ca^{2+} binding domains of calmodulin or the Ca^{2+}-pumping ATPase and dihydropyridines. Such interactions could modify the catalytic properties of the enzyme such as K_m, V_{max} (Fig. 2) and Ca^{2+} sensitivity (Fig. 4). Consequently, the observation that an increase in calmodulin concentration diminishes the effect of nifedipine may be interpreted to mean that calmodulin probably binds more of the drug and thus reduces the concentration of free drug available for modifying the Ca^{2+}-binding domain of the ATPase and of free calmodulin (Fig. 3).

Although this effect of nifedipine has no relationship whatsoever with the pharmacological action of dihydropyridine calcium channel blockers[13], it is pertinent to investigate in future studies, the exact site and mechanism of interaction of these substances with the Ca^{2+}-binding E-F hands of calcium-binding proteins/enzymes.

References

1. Schatzmann HJ, ATP-dependent Ca^{2+}-extrusion from human red cells. Experentia (Basel) 1966; 22; 22: 364-368.
2. Carafoli E, Intracellular calcium homeostasis. Ann. Rev. Biochem. 1987; 56: 395-433.
3. Penniston JT, Plasma membrane Ca^{2+}-ATPases as active pumps. In: Cheung WYed. Calcium and cell function. Academic Press. London, New York 1983.
4. Schatzman HJ, The plasma membrane calcium pump of erythrocytes and other animal cells. In: Carafoli E, ed. Membrane transport of calcium. Academic Press, London, New York 1982.
5. Roufogalis BD, Regulation of Calcium translocation across the red blood cell membrane. Can. J. Physiol. Pharmacol. 1979; 57: 1331-1349.
6. Olorunsogo O.O., Villalobo A, Wang KKW, Roufogalis BD, The effect of calmodulin on the interaction of carbodimides with the purified human erythrocyte (Ca^{2+}+ Mg2+)-ATPase. Biochem. Biophys. Acta 1988; 945: 33-40.
7. Scharff O, Foder B, Reversible shift between two states of Ca^{2+}-ATPase in human erythrocytes mediated by Ca^{2+} and a membrane bound activator. Biochem. Biophys. Acta 1978; 509: 67-74.
8. Cheung WY, Calmodulin plays a pivotal role in cellular regulation. Science 1980; 207: 19-27.
9. Fleckenstein A, Specific pharmacology of calcium in myocardium, cardiac pacemakers and vascular smooth muscle. Ann. Rev. Pharmacol. Toxicol. 1977; 17: 149-166.
10. Nayler WG, Grinwald P, Calcium entry blockers and myocardial function Fed. Proc. 1982; 40: 2855-2861.
11. Godfraind T, Mechanisms of action of calcium entry blockers. Fed. Proc. 1981; 40: 2866-2871.
12. Epstein PM, Fiss K, Hachisu R, Andrenyak DM, Interaction of calcium antagonists with cyclic AMP phosphodiesterase and calmodulin. Biochem. Biophys. Res. Commum. 1982; 105: 1142-1149.
13. Minocherhomjee AM, Roufogalis BD, Antagonism of calmodulin and phosphodiesterase by nifedipine and related calcium entry blockers. Cell Calcium. 1984; 5: 57-63.
14. Flockerz V, Oeker HJ, Hofmann F, Pelzer D, Cavalie A, Trantwein W, Purified dihydropyridine-binding site from skeletal muscle tubule is a functional calcium channel. Nature (London), 1986; 323: 66-68.
15. Verma AK, Filoteo AG, Fischer R, *et al.* Complete primary structure of a human plasma membrane Ca^{2+}-pump. J. Biol. Chem. 1988; 263: 14152-14159.
16. Jarret HW, Penniston JT, Purification of the Ca^{2+}-stimulated ATPase activator from human erythrocytes: its membership in the class of Ca^{2+}-binding modulator proteins. J. Biol. Chem. 1978; 253: 4076-4682.
17. Dodge JT, Mitchell C, Hanahan DJ, The preparation and chemical characteristics of haemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 1963; 100: 119-126.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.
19. Markwell MK, Haas SM, Tolbert NE, Bioerber LL, Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol. 1981; 72: 296-303.
20. Fiske CH, Subbarow T, The colorimetric determination of phosphorus. J. Biol. Chem. 1925; 66: 375-400.
21. Kretsinger RH, Calcium-binding proteins. Ann. Rev. Biochem. 1976; 45: 239-266.
22. Bostrom SL, Ljung B, Mardh S, Forsen S, Thulin E, Interaction of the antihypertensive drug felodipine with calmodulin. Nature (London) 1981; 292: 777-778.
23. Curtis BM, Catteral WA, Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. Biochemistry 1984; 23: 2113-2118.

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