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Effects of Calcium Antagonists on the Erythrocyte Membrane

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Abstract—The effects of dihydropyridine compounds nimodipine, nicardipine and NB818 (isopropyl methyl-6-carbamoyloxymethyl-4-(2,3-dichlorophenyl)-1,4-dihydro-2-methyl-3,5-pyridine-dicarboxylate) on erythrocyte membranes have been studied. These compounds showed protective effects against hypotonic haemolysis, but not against heat-induced haemolysis. An increase in deformability of erythrocytes by these calcium antagonists was observed using a capillary tube centrifugal method. The erythrocytes showed slight stomatocytosis after 30 min of incubation with calcium antagonists, but did not show significant changes in mean corpuscular volume and ATP levels.

Dihydropyridine compounds such as nimodipine and nicardipine have been known to block calcium entry and to show pharmacological actions on the cardiovascular system (Henry et al 1978; Henry 1980; Kawai et al 1981; Brodsky et al 1982; Sherman & Liang 1983). Recently the protective effects of calcium antagonists against liver injury have been reported (Peck & Lefer 1981). At present neither potential-dependent calcium channels nor receptor-dependent calcium channels have been found in hepatocytes (Poggioli et al 1985; Putney 1986). Moreover it has been suggested that calcium antagonists show a protective effect on hepatocytes even after calcium ion influx into the cell (Iida et al 1989). Accordingly, the protective effects of calcium antagonists seem to be caused through cytoprotective effects such as membrane stabilization. However, the details of membrane changes caused by calcium antagonists remain to be clarified.

The present study has been undertaken as part of an investigation of the membrane changes induced by calcium antagonists.

Materials and Methods

Chemicals

Nimodipine, nicardipine and a new compound, NB818 (isopropyl methyl-6-carbamoyloxymethyl-4-(2,3-dichlorophenyl)-1,4-dihydro-2-methyl-3,5-pyridine-dicarboxylate) (Fig. 1) (Nagura et al 1986), were used as calcium channel blocking agents.

Measurement of haemolytic activity

Whole blood was collected from male Wistar rats, 200–250 g, under ether anaesthesia. Heparin was used to prevent clotting. The blood was washed three times with 0·9% NaCl (saline). The volume of erythrocytes was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution which contained in 1 L distilled water: $NaH_2PO_4.2H_2O$, 0·26 g; Na_2HPO_4, 1·15 g; NaCl, 9 g (10 mM sodium phosphate buffer). The pH was adjusted to 7·4. The erythrocyte suspension was incubated in a solution containing

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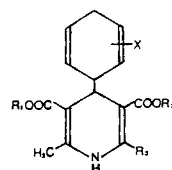

	X	R_1	R_2	R_3
Nimodipine	3-NO_2	$CH(CH_3)_2$	$CH_2CH_2OCH_3$	CH_3
Nicardipine	3-NO_2	CH_3	$CH_2CH_2N(CH_3)(CH_2—C_6H_5)$	CH_3
NB818	2,3-Cl_2	CH_3	$CH(CH_3)_2$	CH_2OCONH_2

FIG. 1. Chemical structures of calcium antagonists.

various concentrations of nicardipine, nimodipine and NB818 for 20 min at room temperature (20°C). The reaction mixture was centrifuged for 3 min at 1300 *g* and the absorbance of the supernatants was measured at 540 nm. The relative haemolysis was determined by comparison with a sample showing 100% haemolysis.

Assays for erythrocyte stabilization and lysis

The inhibition of heat-induced haemolysis was measured according to a modified method of Brown et al (1967). Portions (5 mL) of the isotonic buffer solution containing various concentrations of drugs, cooled to 0–5°C in an ice bath, were added to each of two duplicate sets of centrifuge tubes. The solution containing the drug was added to another tube in each of the sets as a control. Erythrocyte suspension (30 μL) was added to each tube and mixed gently by inversion. One pair of tubes was incubated for 20 min at 54°C in a water bath. The other pair was maintained at 0–5°C in an ice bath. The absorbance of the supernatant fluid was measured at 540 nm.

The extent of protection of erythrocytes in hypotonic solutions was measured according to Seeman & Weinstein (1966). The isotonic buffer solution was composed of 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7·4, and the hypotonic solution contained 56 mM NaCl in 10 mM sodium phosphate buffer (pH 7·4). The experiments were carried out in two duplicate pairs as in the experiment for heat-induced

haemolysis. Stock erythrocyte suspension (30 μL) was mixed with 5 mL of the hypotonic solution containing the drugs at a concentration between 10^{-7} and 10^{-3} M, while the control sample was mixed with the drug free solution. The mixtures were incubated for 10 min at room temperature, and centrifuged for 3 min at 1300 g. The absorbance of the supernatant was measured at 540 nm.

The percentage inhibition or acceleration of haemolysis was calculated according to the equation: %inhibition = 100 − 100 x (OD test sample heated or in hypotonic soln − OD test sample unheated or in isotonic soln)/(OD control sample heated or in hypotonic soln − OD control sample unheated or in isotonic soln).

Measurement of erythrocyte deformability

Erythrocyte deformability was evaluated with a slight modification of the Nagasawa method (Nagasawa et al 1980; Nagasawa 1981) using a microcapillary tube. Three solutions were layered by the discontinuous gradient method using a polyvinyl-pyrrolidine-coated colloidal silica matrix (Percoll; Pharmacia Fine Chemical AB, Uppsala, Sweden). Heparinized blood (5 μL) taken by venipuncture was suspended in 5 mL of Eagle MEM medium, and 50 μL of this suspension was layered onto the middle fraction consisting of an isotonic phosphate buffer solution. The capillary tube was centrifuged at 12 000 rev min^{-1} for 1 min at room temperature. The erythrocytes deformed by centrifugal force were fixed with glutaraldehyde. The resulting deformation was evaluated by measuring the elongation of erythrocytes using a phase-contrast microscope.

Measurement of mean corpuscular volume (MCV)

The MCV of erythrocytes was determined with a Sysmex Microcell Counter CC-150 (Toa Medical Electronics Ltd).

Scanning electron microscopy

The erythrocytes were dehydrated in a graded ethanol series after post-fixation, dried to the critical point with liquid CO_2 and then coated with gold. The samples were observed with a Hitachi S-450 at 20 kV.

Results

Haemolytic activity

The haemolytic activities of nimodipine, nicardipine and NB818 are shown in Fig. 2. None of these compounds showed haemolytic activity under 10^{-5} M. NB818 and nicardipine caused haemolysis at concentrations over 5×10^{-4} M, but nimodipine did not cause haemolysis even at a concentration of 10^{-4} M. NB818 showed the highest haemolytic activity in the comparison at 10^{-4} M.

Stabilization of erythrocyte membrane

In hypotonic haemolysis, nimodipine, nicardipine and NB818 showed strong stabilizing effects (Fig. 3), and the rates of inhibition of haemolysis were about 20, 40 and 48% at 10^{-5} M, respectively. No significant differences were seen between room temperature and 37°C. Concerning heat-induced haemolysis, none of the compounds effected protection, rather they caused acceleration (Fig. 4). The protective effect of nimodipine was lowest in hypotonic haemolysis and

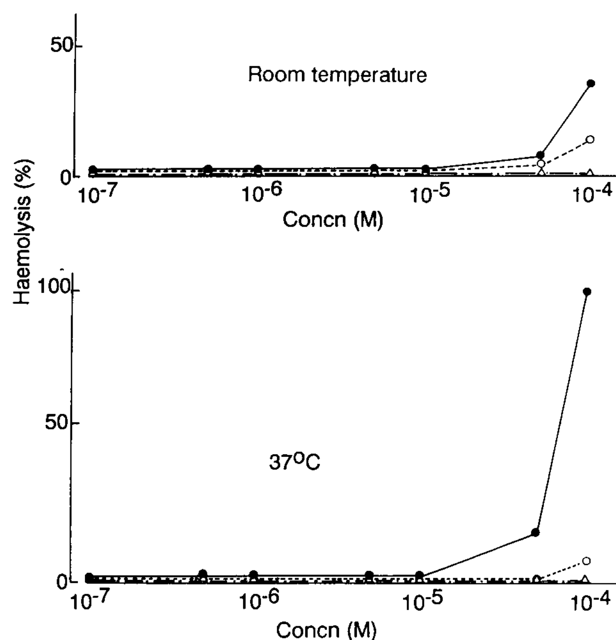

FIG. 2. The haemolytic activity of calcium antagonists. △; nimodipine, ○; nicardipine, ●; NB818.

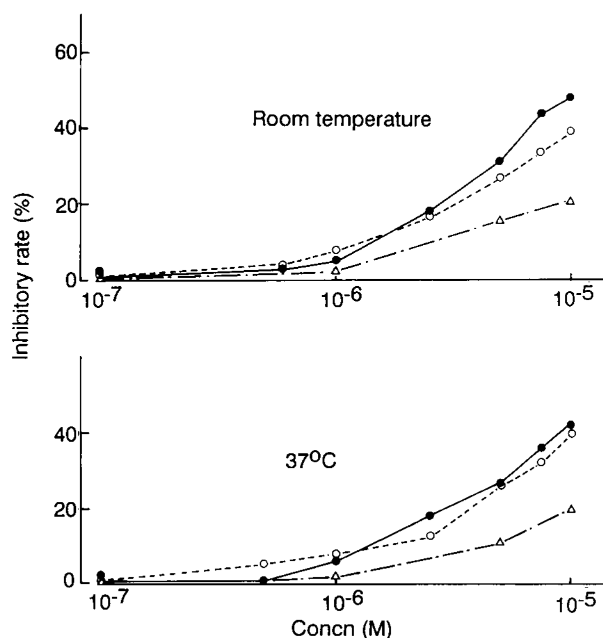

FIG. 3. The protective effects of calcium antagonists against hypotonic haemolysis. △; nimodipine, ○; nicardipine, ●; NB818.

the acceleration of heat-induced haemolysis was also lower than with the other compounds.

Erythrocyte deformability

The deformability of erythrocytes incubated with the drug-free solution did not significantly change until 30 min of incubation. All three compounds caused remarkable increases in deformability after 10 min of incubation. NB818 showed a significant effect at 10^{-6} and 5×10^{-6} M, but not at

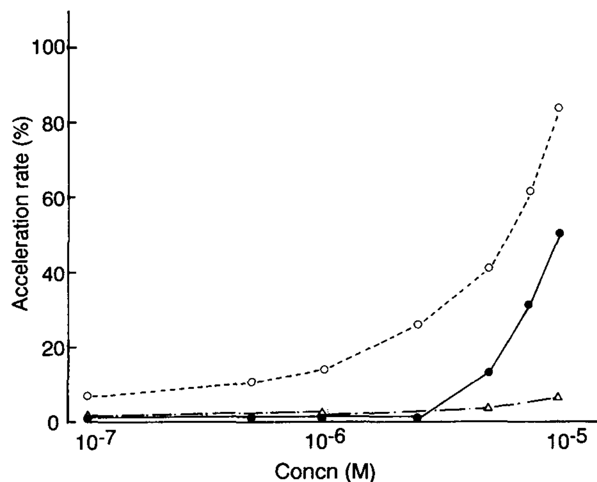

FIG. 4. The accelerated effects of calcium antagonists against heat-induced haemolysis. △; nimodipine, ○; nicardipine, ●; NB818.

10^{-5} M after 10 or 30 min of incubation. Nicardipine also caused an increase in deformability at 5×10^{-6} M, but did not show an effect at 10^{-6} M after 10 min of incubation (Table 1).

MCV

MCV was not significantly changed after incubation with any of the three drugs at the concentrations used (Table 1).

Electron microscopy

Fig. 5a, b, c, d shows scanning electron micrographs of control erythrocytes and erythrocytes treated with nimodipine, nicardipine and NB818, respectively. Fig. 5d, e, f, g show erythrocytes deformed by centrifugation in capillary tubes. Control erythrocytes revealed typical discoidal shapes even after 30 min of incubation. Most of the erythrocytes incubated with nicardipine, nimodipine and NB818 showed discoidal shapes but some exhibited as stomatocytosis. The control erythrocytes appeared handbag-shaped after centrifugation as did those treated with the compounds, but their longitudinal diameters were greater. The ratios of length against width in untreated erythrocytes and erythrocytes treated with nicardipine, nimodipine and NB818 were 1·329±0·044, 1·547±0·044, 1·447±0·031 and 1·528±0·052, respectively.

Discussion

Calcium antagonists protected erythrocytes against hypotonic haemolysis at concentrations showing haemolytic activity, but accelerated heat-induced haemolysis. A possible explanation for the inhibition of hypotonic haemolysis is an increase in the surface area/volume ratio of the cells which could be brought about by an expansion of the membrane, a shrinkage of the cell, or both. An increase in the surface area/volume ratio of red cells could make them osmotically less fragile. Moreover, Sheetz & Singer (1974) proposed that the intercalation of drugs into lipids in the exterior half of the membrane bilayer induces cells to crenate by expanding the exterior half-layer relative to the cytoplasmic half, while intercalation into the cytoplasmic half causes cells to form cup-shapes. Scanning electron microscopic observation of erythrocytes treated with calcium antagonists showed that those drugs cause slight cup-formations in some erythrocytes. Accordingly, calcium antagonists might be suggested to intercalate into the cytoplasmic half of the erythrocyte-bilayer, and to expand the interior half-layer, although no change in MCV was detected in the present study. On the other hand, heat-induced haemolysis is accelerated by calcium antagonists. Since protection against heat-induced haemolysis is thought to be caused by stabilization of proteins in the membrane, calcium antagonists seem to interact with membrane proteins but not to stabilize membrane proteins.

Calcium antagonists showed increasing effects in erythrocyte deformability when a capillary centrifugal method was used. A variety of techniques for investigating erythrocyte resistance to deformation is available, but most are not designed to examine deformability in individual cells. The techniques are influenced to some extent by interactions of the cells with solid surfaces. The technique used in the present experiment provides a means for examining rapidly deforming erythrocytes using a known force without requiring the cells to be in contact with solid surfaces. Erythrocyte deformation by the centrifugal method is influenced by three factors: internal viscosity, surface area/volume ratio and viscoelastic modules of the membrane. Calcium antagonists did not change the MCV, but the possibility of an increase in the area of the cytoplasmic bilayer is suggested. It has not been clarified whether the increase in area of the cytoplasmic bilayer significantly influences the measurement of deforma-

Table 1. Effects of calcium antagonists on erythrocytes.

Drugs	Dose (M)	Deformability (μm) 10 min	Deformability (μm) 30 min	MCV (μm³) 10 min	MCV (μm³) 30 min
Control		7·28±0·08	7·25±0·08	54·83±0·60	55·83±0·60
Nimodipine	5×10^{-4}	7·30±0·06	7·45±0·07*	55·17±0·76	55·67±0·93
	5×10^{-5}	8·04±0·08**	7·97±0·06**	55·33±0·84	55·83±0·60
	5×10^{-5}	7·83±0·07**	7·90±0·07**	55·33±0·84	56·17±0·68
Nicardipine	5×10^{-5}	7·49±0·06*	7·67±0·08**	55·00±0·62	55·50±0·61
	5×10^{-6}	7·83±0·07**	7·88±0·08**	54·83±0·68	55·83±0·60
	5×10^{-6}	7·39±0·06	7·65±0·06**	55·17±0·76	55·83±0·60
NB818	5×10^{-5}	7·43±0·06	7·37±0·06	54·83±0·60	55·67±0·61
	5×10^{-6}	7·51±0·07*	7·76±0·07**	54·83±0·60	55·83±0·60
	5×10^{-6}	7·52±0·06*	7·71±0·08**	55·33±0·73	55·83±0·60

*$P<0·05$, **$P<0·001$: vs control.

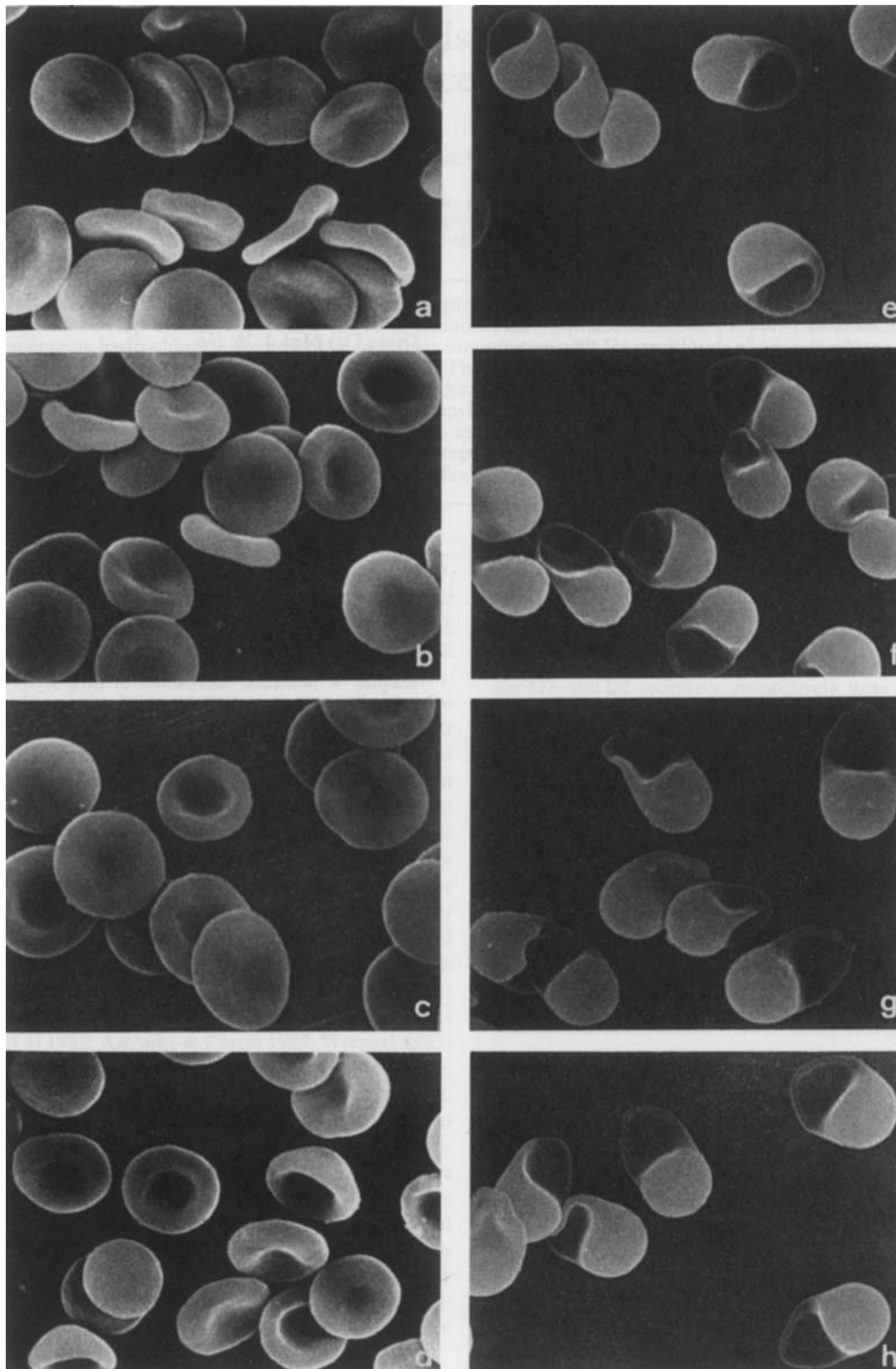

FIG. 5. Scanning electron micrographs of erythrocytes. a; untreated erythrocytes (control), b; erythrocytes treated with nimodipine (10^{-4} M), c; erythrocytes treated with nicardipine (10^{-5} M), d; erythrocytes treated with NB818 (10^{-5} M), e; erythrocytes centrifuged after incubation without drug for 30 min, f; erythrocytes centrifuged after incubation with nimodipine (10^{-4} M), g; erythrocytes centrifuged after incubation with nicardipine (10^{-5} M), h; erythrocytes centrifuged after incubation with NB818 (10^{-5} M).

bility by the present method. However, that an increase in deformability by calcium antagonists is not always proportional to their concentration, and that the ratio of width to length of erythrocytes deformed by centrifugation is also increased by calcium antagonists, suggests that calcium antagonists increase deformability through a decrease in internal viscosity or viscoelastic modules of the membrane. It has also been shown that the intracellular content of calcium in erythrocytes is closely related to cell volume, deformability, ATP level and life-span (Weed et al 1969; Shiga et al 1985). We examined the effects of nicardipine, nimodipine and NB818 on the intracellular calcium content of cultured cancer cells by the method using Fura 2 (Grynkiewicz et al 1985). NB818 showed the strongest inhibition in the increase of intracellular calcium content after 30 min of incubation under conditions for examining erythrocyte deformability and the inhibitory action was dose-dependent. The inhibitory actions of nimodipine and nicardipine were less in concentrations from 10^{-4} to 10^{-6} M compared with NB818, but their actions were dose-dependent (unpublished data). These results suggest the possibility that the changes in erythrocyte deformability caused by calcium antagonists can be attributed to the intracellular calcium content. However, changes in the erythrocyte membranes seem to play a role in the increase of deformability since the increasing rate of deformability is not dose-dependent and not always correlated with the strength of action inhibiting the increase of intracellular calcium content.

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