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Effects of verapamil on calcium-induced rigidity and on filterability of red blood cells from healthy volunteers and patients with progressive systemic sclerosis

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1 The effects of verapamil on calcium-induced decrease deformability of red blood cells (RBCs) and on the filterability of RBCs from healthy volunteers and patients with progressive systemic sclerosis (PSS) were investigated *in vitro* using a gravity driven filtration technique.
2 The filterability of RBCs was increased by verapamil 1 μg/ml ($P < 0.01$) in healthy volunteers ($P < 0.05$) and in patients with PSS ($P < 0.05$).
3 A high concentration of verapamil (200 μg/ml) caused an 80% reduction ($P < 0.05$) in the filterability of RBCs from healthy volunteers.
4 The filterability of RBCs stored at 4°C for 24 h was increased by 1 μg/ml verapamil ($P < 0.05$).
5 Verapamil (1 μg/ml) prevented the decrease in deformability of RBCs due to an increase in either extracellular or intracellular calcium concentrations ($P < 0.05$).
6 By increasing red cell filterability verapamil may be useful in the treatment of PSS and other peripheral vascular diseases where decreased red cell deformability may play an important role in the pathogenesis.

Keywords verapamil red cell deformability progressive systemic sclerosis

Introduction

The maintenance of the normal shape and deformability of RBCs requires preservation of cellular ATP and a low content of calcium ions (Nakao *et al.*, 1961; Weed *et al.*, 1969; Lichtman & Weed, 1972; Palek *et al.*, 1974). Calcium ions are extruded by an adenosine triphosphate (ATP) dependent 'calcium pump' against a high concentration gradient (Schatzmann & Vincenzi, 1969; Lee & Shin, 1969; Olson & Cazort, 1969). ATP depletion and calcium uptake result in loss of cell deformability and are generally agreed to be mechanisms of physiologically important cell destruction. Weed *et al.* (1969) proposed that depletion of cellular ATP impaired the ability of cells to maintain their normal low calcium content, and that intracellular accumulation of calcium caused decreased deformability by inducing a sol-gel transformation of the cell membrane. This alteration of calcium metabolism would induce membrane stiffening which may underlie the reduced deformability of both ATP depleted cells and cells loaded with calcium without prior ATP depletion. The reduced deformability of irreversibly sickled cells (Palek, 1979; Eaton *et al.*, 1979) and of RBCs stored in plasma for an extended period (Weed *et al.*, 1969) has also been explained on this basis. The RBCs of patients with progressive systemic sclerosis (PSS) have decreased red cell deformability (Kovacs *et al.*, 1983), the exact mechanism of which is not known. The role of calcium entry blockers, such as verapamil, on calcium-induced

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red cell rigidity has not been well established. Calcium entry blockers have been used and proven to be beneficial in the treatment of PSS and Raynaud's phenomenon (Kahan, 1981; Winston *et al.*, 1983). The role of calcium entry blockers on the deformability of normal or rigidified RBCs has not been well established. We have therefore studied the effects of verapamil on the calcium-induced rigidity of red cells and on the filterability of normal and abnormal RBCs.

Methods

Blood samples were obtained from healthy volunteers aged between 28–65 years and from patients with PSS aged between 28–64 years. All PSS patients fulfilled the preliminary diagnostic criteria set out by the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980).

Methodology

RBCs were separated from heparinised venous blood (heparin sodium without preservatives, 20 u/ml, Weddel Pharmaceuticals, UK). The buffy coat was carefully removed and the red cells washed three times in Dulbecco's phosphate buffered saline (PBS-A), pH 7.4 (Oxoid Ltd, UK) containing 0.25% (v/v) human albumin (Immuno Ltd, Sevenoaks, Kent, UK). Packed cell volume was determined from a concentrated suspension (about 50%) with a microhaematocrit centrifuge (Hawksley, UK) and then adjusted to 5% (v/v) with PBS-A (prefiltered through a 3 μm pore diameter filter). The white cell con-

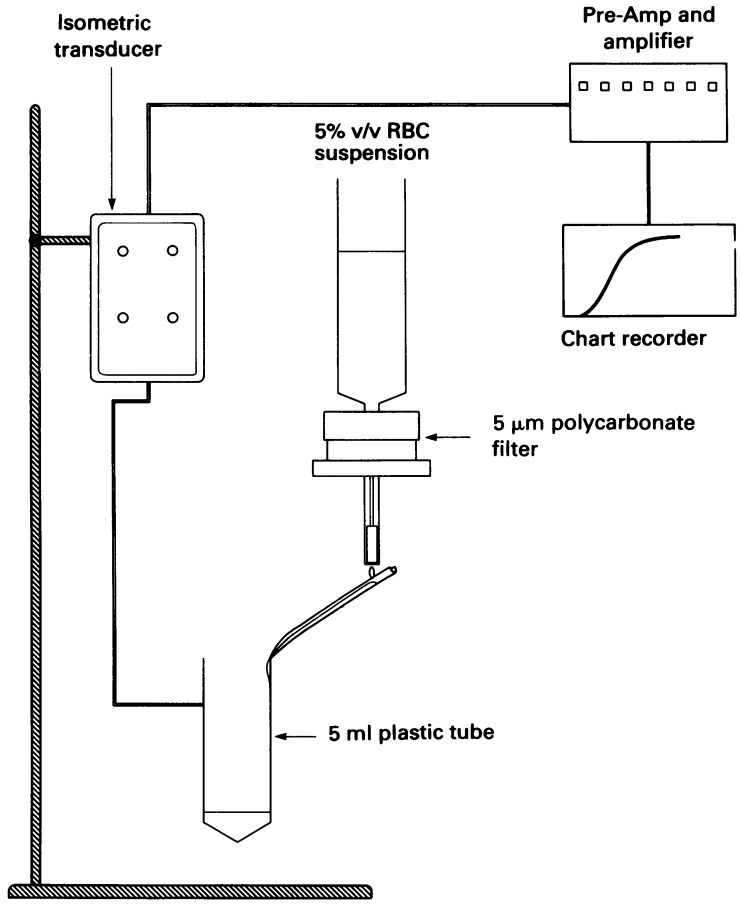

Figure 1 Schematic representation of the various components of the filtration apparatus. This improved filtration system which is based on a decreasing head pressure, produced by decreasing volume of cell suspension filtering by gravity, has the advantage that a physiological range of shear stresses is applied.

tamination in the final suspension was less than 0.1×10^9/l. A schematic diagram of the equipment used is shown in Figure 1. For each filterability test, a new polycarbonate membrane filter (13 mm diameter pore diameter of 5 μm, Nuclepore Corporation, Pleasanton, California, USA) was inserted into a 'Pop Top' filter holder. The outlet of the filter holder was connected to a short piece of siliconised rubber tube (a three way tap may be used) which was closed with an arterial clip. The filter chamber was filled through the inlet with 400 μl of PBS-A. Care was taken during this operation to avoid the introduction of air bubbles into the filter chamber. A 5 ml disposable syringe (without the plunger) was inserted into the inlet of the filter holder, and then filled from above with PBS-A or cell suspension until the level of the uppermost marking on the barrel was reached. The flow rate of the buffer was first determined in order to verify proper functioning of the system. The measurement started when the arterial clip was released and 5 ml of buffer was allowed to flow across the filter into a collecting tube. The tube was connected to an isometric transducer, the output of which was registered on a chart recorder. The linear part of the filtration curve obtained was used to calculate the slope, i.e. the filtration rate. At the end of each test, the syringe barrel was removed and replaced with a clean and dry new one. The buffer content was then replaced with 5% (v/v) RBC suspension and the filtration procedure repeated.

Filterability tests were performed on the following samples:

i. 5% (v/v) normal and PSS RBC suspensions (in PBS-A as suspending medium) pre-incubated with either PBS-A or verapamil (0.50, 1.0, 25.0 and 200 μg/ml final concentrations) at 37°C for 30 min.
ii. 5% (v/v) normal RBC suspension (in plasma prefiltered through 3 μm pore diameter filter) pre-incubated with either PBS-A or verapamil (1.0 and 25.0 μg/ml final concentrations) at 37°C for 30 min.
iii. Whole blood was pre-incubated with 1 and 25 μg/ml of verapamil (final concentrations) at 37°C for 30 min. At the end of the incubation period, 5% (v/v) RBC suspension was prepared and used for filterability studies.

Filterability measurements were made in triplicate on each sample and the mean calculated. The results were expressed as relative filterability (RF):

$$RF = \frac{\text{Rate of flow of RBC suspension}}{\text{Rate of flow of the suspending medium}}$$

i.e. =

$$\frac{\text{Height of filtration curve of RBC suspension at 10 s}}{\text{Height of filtration curve of suspending medium at 10 s}}$$

The coefficient of variation of 10 replicate measurements using this technique was 2.98%.

Calcium loading (extracellularly)

Freshly washed RBCs were added to isotonic saline (0.9% sodium chloride, Travenol Laboratories Ltd UK) or saline containing verapamil (25 μg/ml final concentration) to make a 50% (v/v) RBC suspension. The RBC suspensions were incubated at 37°C for 15 min. At the end of the incubation the haematocrit was readjusted to 5% (v/v) with isotonic saline containing varying amounts of calcium (1, 2, 4, 6, 8, 16 mM final concentrations), and the cells were then incubated for a further 15 min before the filterability test.

Calcium loading (intracellularly)

5% (v/v) RBC suspensions were pre-incubated with saline/verapamil (25 μg/ml) at 37°C for 15 min prior to the addition of 5 μM calcium ionophore and 0.25 mM $CaCl_2$. The RBCs were then incubated at 37°C for a further 15 min before the filterability test at 25°C. A stock solution of calcium ionophore (A23187) of 5×10^{-3} M was prepared in dimethylsulphoxide (DMSO) and the final concentration was adjusted to 5 μM with saline in the red cell suspension. Concentrations of DMSO identical to those present with A23187 were added without ionophore to control cell suspensions.

Storage of RBCs

Blood samples from healthy volunteers were stored at 4°C with PBS-A or PBS-A containing verapamil (1 and 25 μg/ml final concentrations) for 24 h. At the end of 24 h storage, the blood samples were placed in a water bath at 37°C for 30 min. The RBCs were then separated and washed three times in PBS-A as described earlier. The cells were then resuspended in PBS-A and the haematocrit readjusted to 5% (v/v) with PBS-A. The RBC suspensions were then filtered at 25°C using the gravity-driven filtration technique described earlier.

Measurement of haematological parameters

Mean cell volume (MCV) and mean haemoglobin concentration (MCHC) were measured in all the red cell suspensions treated with or without verapamil (0.50, 1, 25, 200 μg/ml), calcium chloride (1, 2, 4, 8, 16 mM) and calcium ionophore (5 μM) plus 0.25 mM $CaCl_2$, using a Coulter cell analyser (Model S plus IV with 3 population differential, Coulter Electronics Ltd, England).

Statistical analysis of results

Differences were analysed with Wilcoxon matched-pairs signed rank test (a non parametric test). Statistical procedures for calculation of regression lines and comparison of slopes are fully described by Sokal & Rohlf (1969).

Results

Effect of verapamil on filterability of RBCs

The effect of different concentrations of verapamil on filterability of RBCs from healthy volunteers and patients with PSS is shown in Table 1. Pre-incubation with verapamil resulted in a concentration-dependent increase in RBC filterability which was maximal at 25 μg/ml. A higher concentration of verapamil (200 μg/ml) significantly ($P < 0.05$) decreased the filterability of RBCs. The increase in filterability was significant ($P < 0.01$) for both 1 and 25 μg/ml verapamil in healthy volunteers and ($P < 0.05$) in PSS patients. The dose-response effects of verapamil on RBCs from patients with PSS and controls did not differ in parallelism.

The improvement in RBC filterability was still present when the RBCs were suspended in the volunteer's own plasma. This was also significant ($P < 0.05$) for both 1 and 25 μg/ml verapamil.

Most drug trials involving filterability tests include oral or intravenous administration of the active agent, at the end of which blood samples are taken for filterability studies when the plasma concentration of the drug is at its peak level. In order to simulate *in vivo* conditions, whole blood samples were incubated with verapamil (1 and 25 μg/ml final concentration). The maximum plasma level of the drug in treated patients was found to be 1 μg/ml (Woodcock *et al.*, 1980). When RBCs were separated and washed (three times), the filterability of cells which had been incubated with verapamil was significantly increased ($P < 0.05$).

Effect of verapamil on stored blood

RBCs stored at 4°C for 24 h showed a significant decrease ($P < 0.05$) in filterability (Table 1). This decrease was significantly ($P < 0.05$) prevented by incubation with verapamil (25 μg/ml) (Table 1).

Table 1 The effect of different concentrations of verapamil on filterability of RBCs from healthy volunteers and patients with progressive systemic sclerosis

Experimental conditions	*Verapamil (μg/ml)*	*Mean relative filterability (RF) ± s.e. mean*	n
Normal RBCs resuspended in PBS-A	—	0.637 ± 0.014	16
	0.50	0.653 ± 0.13	16
	1	0.677 ± 0.012**	16
	25	0.704 ± 0.008**	16
	200	0.078 ± 0.004*	6
Normal RBCs resuspended in prefiltered plasma	—	0.408 ± 0.36	7
	0.50	0.390 ± 0.030	7
	1	0.441 ± 0.038*	7
	25	0.529 ± 0.033*	7
Normal whole blood pre-incubated with verapamil (see method)	—	0.517 ± 0.036	6
	1	0.596 ± 0.011*	6
	25	0.664 ± 0.018*	6
RBCs from patients with progressive systemic sclerosis resuspended in PBS-A	—	0.410 ± 0.040	7
	1	0.493 ± 0.037*	7
	25	0.580 ± 0.036*	7
Normal RBCs filtered within 2 h of venepuncture	—	0.678 ± 0.019	6
24 h stored RBCs	—	0.549 ± 0.051†	6
24 h stored RBCs	1	0.586 ± 0.040‡	6
24 h stored RBCs	25	0.621 ± 0.033‡	6

* $P < 0.05$; compared with saline
** $P < 0.01$; compared with saline
† $P < 0.05$; compared with normal RBCs filtered within 2 h of venepuncture
‡ $P < 0.05$; compared with untreated 24 h stored RBCs

Table 2 Effects of verapamil on rigidity of RBCs induced by increased intracellular calcium

Number of samples	*Verapamil (μg/ml)*	*Calcium ions (mM)*	*Calcium ionophore (μM)*	*Relative filterability (RF)*
6	—	—	—	0.524 ± 0.074
6	—	0.25	5	0.224 ± 0.035*
6	25	0.25	5	0.320 ± 0.051†

*$P < 0.05$ compared with normal RBCs (not loaded with calcium)
†$P < 0.05$ compared with calcium loaded RBCs

Effect of calcium loading on RBC filterability

Normal RBCs pre-incubated with 5 μM calcium ionophore (A23187) and 0.25 mM calcium showed a significant ($P < 0.05$) decrease in filterability (Table 2). This decrease was partially prevented by 25 μg/ml verapamil ($P < 0.05$) (Table 2).

Effect of increased extracellular calcium ion concentrations on filterability

An increase in the extracellular concentration of calcium ions caused a concentration-dependent decrease in RBC filterability (Figure 2). The percentage decrease ranged from 11 to 89% at the lowest and highest concentrations respectively. The decrease in RBC filterability was significant ($P < 0.02$) for 2 and 4 mM Ca^{2+} and ($P < 0.01$) for 8 mM. This effect was partially prevented by pre-incubation with verapamil, the percentage blockade being unrelated to the extracellular calcium ion concentration (Figure 2). This effect of verapamil (25 μg/ml) on calcium-induced rigidity of RBCs was significant at $P < 0.02$ and $P < 0.05$ for 2 and 4 mM Ca^{2+} respectively and ($P < 0.01$) for 8 mM Ca^{2+}.

Effects of calcium ions and verapamil on haematological parameters of RBCs

Neither increased concentrations of extracellular calcium ions nor verapamil caused any significant change in MCV or MCHC. Calcium loading (intracellularly) of RBCs caused a significant decrease in MCV and an increase in MCHC which were partially reversed by pre-incubation with 25 μg/ml verapamil ($P < 0.05$).

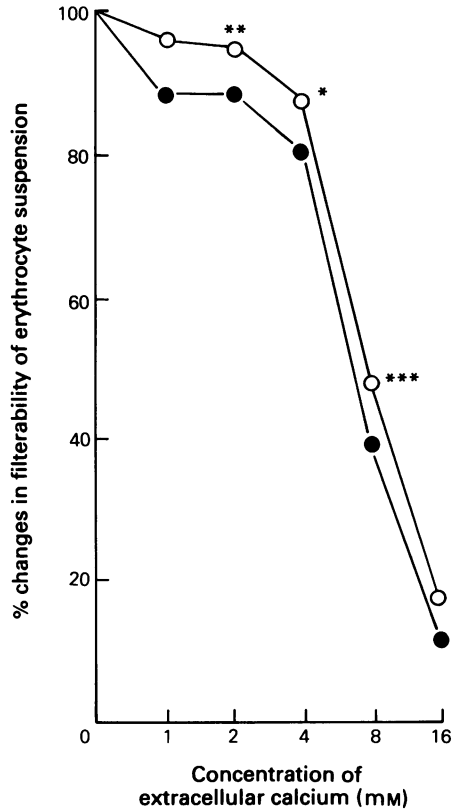

Figure 2 Percentage relative filterability of erythrocyte suspensions with increasing concentrations of extracellular calcium ions in the presence (○) or absence (●) of 25 μg/ml of verapamil. Each point at (2, 4 and 8 mM Ca^{++}) represents the mean of 10 separate readings, and at 1 mM Ca^{++}, $n = 4$ and 16 mM Ca^{++}, $n = 5$. *$P < 0.05$; **$P < 0.02$; ***$P < 0.01$ (significance of difference from control).

Discussion

The introduction of small amounts of calcium into the calcium-poor interior of normal RBCs has deleterious effects upon the shape, viscoelastic properties and metabolism of the cell (Weed *et al.*, 1969).

Fortier *et al.* (1977) showed that an increase in the extracellular concentration of calcium ions could induce a small but significant increase

in the rate of exchange between extracellular calcium ions and the exchangeable red cell calcium. Under these conditions the net calcium content remained unchanged because of the low permeability of the red cell membrane to calcium (Ferreira & Lew, 1975) and also because of a powerful calcium extrusion pump (Schatzmann & Vincenzi, 1969). This increased rate of calcium fluxes, coupled with an increased permeability of the cell membrane to potassium ions (Hoffman, 1966; Gardos, 1959) initiates a series of changes in the cell membrane which are similar to that seen in calcium loaded RBCs, namely (1) cellular shrinkage (elevated MCHC), (2) loss of water and potassium, (3) rapid and near total hydrolysis of intracellular ATP, (4) conversion of cell shape from a biconcave disc to an echinocyte and spheroechinocyte, (5) greatly diminished cellular deformability and elasticity, (6) the accumulation of 1:2 diacylglycerol (DAG), (Burris *et al.*, 1980). Our results show that RBC filterability decreases when either extracellular or intracellular calcium ion concentration is increased, and that verapamil added to the whole blood or to the separated RBCs can inhibit that induced decreased filterability of cells. Storage of RBCs for 24 h or longer can result in metabolic depletion, conversion of the cells to echinocytes and an increase in the cellular content of calcium (Weed *et al.*, 1969). Our results show that incubation of the red cells with verapamil partially reversed these effects. This partial recovery may be associated with a reduction in the cellular levels of calcium ions, but full recovery may require the presence of ATP. Verapamil had a biphasic effect on RBC filterability. In low concentrations (1 and 25 μg/ml) it significantly increased red cell filterability in healthy volunteers and in patients with PSS, while a high concentration (200 μg/ml) reduced the filterability of red cells from healthy volunteers.

In order to explain the effect of verapamil on calcium-induced cell rigidity, one can assume that the drug inhibited Ca^{2+} influx, or that it increased the efficiency of the calcium pump efflux, or that it had a combined effect. It is also possible that verapamil affected the filterability of RBCs in a way not directly related to Ca^{2+} movements, possibly by interfering with ATP production of the cell (Spearman & Butcher, 1983). It is also known that the binding of Ca^{2+} to cell membranes may lead to conformational changes of the external membrane proteins or of the lipid/protein bilayer itself. Verapamil may also interfere with the binding of external Ca^{2+} to the membrane proteins.

It has been shown that a high concentration of verapamil not only inhibits calcium influx but also prevents intracellular Ca^{2+} mobilisation (Han *et al.*, 1983), some of which might be needed for the activation of the contractile proteins that are involved in the maintenance of red cell shape and deformability. High concentrations of verapamil also caused a slight decrease in the MCV, and this may have led to an increase in the internal viscosity and therefore to decreased deformability of RBCs (Chien, 1981).

The percentage increase in RBC filterability after incubation with verapamil is considerably greater in PSS RBCs than that seen in normal RBCs. The slopes of the lines for the drug effect on PSS RBCs did not differ from the slope of the effect of drug on normal RBCs. This difference in response between PSS and normal RBCs to verapamil may be related not only to the initial rigidity of PSS RBCs but also to the sensitivity of the RBC membranes to extracellular calcium ions. The above observation raises the interesting possibility that the membranes of PSS RBCs may be either excessively permeable to calcium ions or possess a defective ATPase-dependent calcium extrusion pump, similar to that which has recently been observed in the red cells of patients with sickle cell anaemia (Bookchin *et al.*, 1984). Small amounts of calcium ions in the extracellular fluid or blood may therefore lead to accumulation within the PSS, RBCs, resulting in reduced filterability. The minimum concentration of verapamil which significantly increased RBC filterability in our studies is equivalent to the maximum plasma level seen in some patients on long term therapy with the drug (Woodcock *et al.*, 1980).

We can assume that by improving haemorheological conditions verapamil treatment might be beneficial in diseases where decreased deformability of RBCs is involved in the pathogenesis.

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