Biochimica et Biophysica Acta, 981 (1989) 36–42
Elsevier

BBA 74414

Distinction of two components of passive Ca^{2+} transport into human erythrocytes by Ca^{2+} entry blockers

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(Received 12 December 1988)

Key words: Calcium ion transport, passive; Calcium ion entry blocker; Vanadate, Quin 2; (Erythrocyte)

The nature of downhill Ca^{2+} net-transport into human erythrocytes was investigated using the experimental models of Ca^{2+} pump inhibition by vanadate and of intracellular chelation of Ca^{2+} by quin2. Ca^{2+} uptake by erythrocytes loaded with 0.5 mM vanadate and suspended in 145 mM Na^+–5 mM K^+ media was reduced by about 60% when medium K^+ was raised to 80 mM. Organic and inorganic Ca^{2+} entry blockers such as nifedipine (10^{-5} M), verapamil (10^{-4} M), diltiazem (10^{-4} M), Co^{2+} (1.5 mM) and Cu^{2+} (0.1 mM) as well as the K^+ channel blocker quinidine (1 mM) inhibited Ca^{2+} uptake in 145 mM Na^+–5 mM K^+ media by 60–75%. Flunarizine was less effective. In vanadate-loaded cells suspended in 70 mM Na^+–80 mM K^+ media, in contrast, flunarizine exerted a dose-dependent inhibition of Ca^{2+} uptake by up to 80% at 10^{-5} M, the other blockers being ineffective (except for verapamil at 10^{-4} M). A similar pattern of inhibition was seen in quin2-loaded erythrocytes. The different susceptibility towards inhibitors may indicate that passive Ca^{2+} uptake by vanadate-loaded erythrocytes suspended in 145 mM Na^+–5 mM K^+ media, on the one hand, and by vanadate-loaded erythrocytes suspended in 70 mM Na^+–80 mM K^+ media as well as by quin2-loaded erythrocytes, on the other hand, is mediated by two different transport components.

Introduction

The concentration of ionized Ca^{2+} within human erythrocytes is maintained low due to the action of the ATP-dependent Ca^{2+} pump which counterbalances passive Ca^{2+} downhill movements. Whereas the Ca^{2+} pump has been studied in detail [1], much less information is available concerning the mechanism of passive inward Ca^{2+} transport. A Na^+–Ca^{2+} exchange is apparently absent in human erythrocytes under physiological conditions [2]. Passive Ca^{2+} inward movements seem to be mediated mainly by a pathway that saturates at extracellular Ca^{2+} concentrations above 5 mM [2]. Recently, it has been claimed that passive Ca^{2+} uptake is mediated to a large extent by a 'Ca^{2+} channel' that is blocked by Ca^{2+} entry blockers [3,4] and bivalent cations such as Cu^{2+}, Zn^{2+}, Cd^{2+} and Co^{2+} [5].

The rate of passive Ca^{2+} uptake is low in human erythrocytes under physiological conditions (10–50 $\mu mol \cdot (litre\ cells)^{-1} \cdot h^{-1}$ [2]) as compared to other cells. Ca^{2+} uptake is enhanced in cells shrunken in hypertonic media [6], by hyperpolarization of the membrane potential [7], at elevated pH values [8], after replacement of saline by impermeant solutes such as sucrose [2], and in cells exposed to membrane-active drugs such as propranolol [9], salicylate [10] or the Ca^{2+} ionophore A23187 [11]. Replacement of medium Na^+ by K^+ lowers Ca^{2+} uptake [2].

The passive permeability to Ca^{2+} of human erythrocytes is usually assessed in cells with an arrested Ca^{2+} pump. Possibilities to reduce the Ca^{2+} pump activity are to deplete the cells of either ATP or of Mg^{2+} [12] or to lower the incubation temperature [10]. Recently, two alternative approaches have been proposed. Varecka and Carafoli [4] used preincubation with vanadate, a compound that inhibits the Ca^{2+} pump [13] and Na^+/K^+ pump [14] after permeation of the membrane through the anion exchange system [15]. Lew et al. [16] and McNamara and Wiley [17] lowered intracellular Ca^{2+} by loading Ca^{2+} chelators such as quin2 or benz2 into intact cells, using intracellular hydrolysis of the permeant acetoxymethyl esters [18].

In the methods based on inhibition of the Ca^{2+} pump the concentration of intracellular Ca^{2+} increases. At concentrations above 250 nM, intracellular Ca^{2+} activates a K^+ channel in human erythrocytes [7,19,20]

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(Gárdos-effect) without a concomitant change in the permeability to Na^+ and Cl^-. Due to the limited permeability of the Cl^- conductance pathway the cells hyperpolarize upon opening of the K^+ channel when intracellular K^+ is greater than extracellular K^+ by shifting the membrane potential to the K^+ equilibrium potential. Furthermore, the cells shrink progressively due to a loss of K^+, Cl^- and water. Both the inside more negative membrane potential and the cell shrinkage favour the entry of the positively charged Ca^{2+} ion [7,8,21]. At about 80 mM K^+ in the medium, cell hyperpolarization and shrinkage are prevented, and Ca^{2+} uptake is reduced (K^+-inhibitable component [4,16]). However, also other factors may contribute to the higher Ca^{2+} uptake of cells suspended in Na^+ media at low K^+ concentrations, since it has been shown that there exists a K^+-inhibitable component also in erythrocytes which apparently lack the Ca^{2+}-activated K^+ channel [22].

It was the aim of the present experiments to elucidate further the nature of the earlier reported inhibitory effects of organic [3,4] as well as inorganic [5] Ca^{2+} entry blockers on passive Ca^{2+} uptake. This was achieved by investigating the effects of Ca^{2+} entry blockers on Ca^{2+} entry into vanadate-treated red cells suspended either in 145 mM Na^+–5 mM K^+ or in 70 mM Na^+–80 mM K^+ media and on Ca^{2+} uptake by quin2 loaded erythrocytes. A preliminary report of this work has been published (Engelmann, B. and Duhm, J. (1988) Pflügers Arch. 411 Suppl. 1, R82).

Materials and Methods

Materials

Quin2 acetoxymethyl ester was from Sigma (Deisenhofen). Nifedipine was obtained from Bayer (Wuppertal), verapamil from Knoll (Ludwigshafen), diltiazem from Gödecke (Freiburg) and flunarizine from Janssen (Neuss). All other reagents were from Merck (Darmstadt). NaCl and KCl were suprapur.

Methods

Ca^{2+} uptake by vanadate-loaded erythrocytes. The method described by Varecka and Carafoli [4] was slightly modified. Freshly drawn venous blood (anticoagulated by 5 IU heparin/ml) was centrifuged for 2 min at 4500 × *g*. The buffy coat was removed and the cells were washed four times with 10 volumes of a 145 mM Na^+–5 mM K^+ buffer (5 mM glycylglycine, 5 mM glucose, pH 7.45 at room temperature), 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate (EGTA) being added in the first two washings. The washed erythrocytes were resuspended at a hematocrit of 10% in either 145 mM Na^+–5 mM K^+ or 70 mM Na^+–80 mM K^+ buffer (5 mM glycylglycine, 5 mM glucose, pH 7.45 at room temperature) containing 0.5 mM neutralized Na_3VO_4 and preincubated at 25 °C for 15 min. Subsequently, $CaCl_2$ was added at a final concentration of 1.25 mM. After further 60 min of incubation, the cells were sedimented by 1 min centrifugation at 4500 × *g* and washed five times in the incubation media containing 0.5 mM Na_3VO_4, no added $CaCl_2$, and 50 μM EGTA (room temperature).

Ca^{2+} uptake by quin2-loaded erythrocytes. The method described by McNamara and Wiley [17] was modified as follows. The erythrocytes were washed three times with a 10-fold excess of the 145 mM Na^+–5 mM K^+ buffer containing 1 mM EGTA (4 °C). The erythrocytes were loaded with quin2 by a 90 min preincubation (37 °C, hematocrit 1%) in the 145 mM Na^+–5 mM K^+ buffer (pH 7.45 at 37 °C) containing 1 mM EGTA and 1.45 μM quin2 acetoxymethyl ester (quin2/AM). This concentration of quin2/AM proved to be optimal with respect to the rate of Ca^{2+} uptake subsequently determined, and did not lower the red cell ATP content [23]. The quin2-loaded erythrocytes were washed three times in the 145 mM Na^+–5 mM K^+ buffer (4 °C). Then, Ca^{2+} uptake was initiated by resuspension of the cells in the 145 mM Na^+–5 mM K^+ buffer at 37 °C containing 1.25 mM $CaCl_2$ (hematocrit 10%). Ca^{2+} uptake proved to be linear over 30 min and, therefore, was usually terminated after 15 min of incubation by transferring the suspensions into an ice bath. Extracellular Ca^{2+} was removed subsequently by five washings with the 145 mM Na^+–5 mM K^+ buffer containing 1 mM EGTA (4 °C).

The intracellular Ca^{2+} content was determined before and after the incubations in the media containing 1.25 mM Ca^{2+} on perchloric acid extracts of the erythrocytes by atomic absorption spectrophotometry in a graphite furnace as described elsewhere [23]. The uptake rate was calculated from the difference of the two readings and normalized to 5.2 mmol hemoglobin tetramer, the hemoglobin content of 1 litre normal erythrocytes.

In some experiments the effect of Ca entry blockers (10^{-8}–10^{-4} M) on Ca^{2+} uptake was examined. Nifedipine was dissolved in ethanol, verapamil and diltiazem in saline, and flunarizine in a mixture of dimethylsulfoxide, 1 M HCl and H_2O (9 : 1 : 40, v/v). In control experiments it was verified that the solvents (63 μl ethanol and 11 μl dimethylsulfoxide in 7 ml of the red cell suspension) did not alter the rates of Ca^{2+} uptake. The experiments studying the effects of nifedipine were conducted in the dark. Nifedipine, diltiazem and verapamil were added to the suspension 5 min prior to the initiation of Ca^{2+} uptake. The preincubation time with flunarizine was 30 min. The Ca^{2+} entry blockers caused no hemolysis, except of flunarizine at concentrations greater than $5 \cdot 10^{-6}$ M with quin2-loaded cells and at concentrations greater than 10^{-5} M with vanadate-loaded erythrocytes.

Results

The concentration dependence of the vanadate effect on Ca^{2+} uptake by human erythrocytes and the concomitant cell volume changes are depicted in Fig. 1. Ca^{2+} uptake increased with rising vanadate concentration, the maximum effect being obtained at 3 mM vanadate (lower panel of Fig. 1). The Ca^{2+} uptake

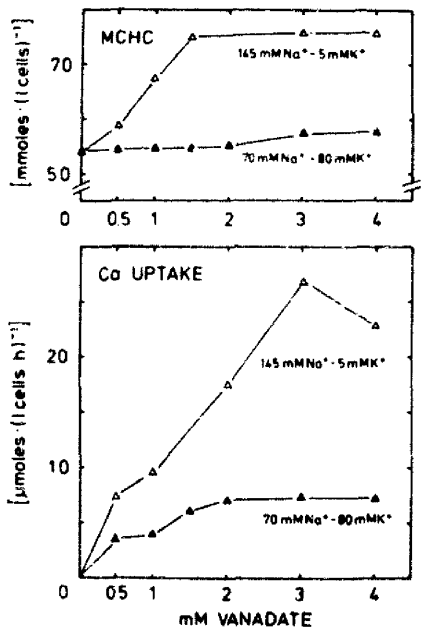

Fig. 1. Ca^{2+} uptake (bottom) and MCHC (mean cellular hemoglobin content in (mmol tetramer)·(litre cells)$^{-1}$, top) in Na^+ 145–K^+ 5 and Na^+ 70–K^+ 80 media as a function of vanadate concentration (25 °C, pH 7.45, 1.25 mM Ca^{2+}). 3–5 further experiments yielded similar results.

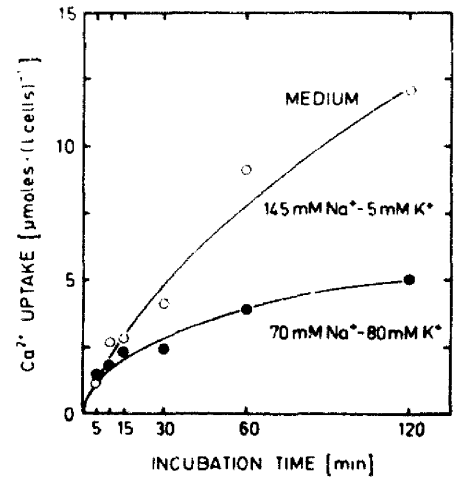

Fig. 2. Time course of Ca^{2+} uptake in cells treated with 0.5 mM vanadate and suspended either in Na^+ 145–K^+ 5 or Na^+ 70–K^+ 80 media (25 °C, pH 7.45).

induced by vanadate in 145 mM Na^+–5 K^+ media is paralleled by a cell shrinkage, as indicated by the increase in the mean cellular hemoglobin content shown in the upper panel of Fig. 1. The shrinkage resulted from a K^+ loss (data not shown) induced by the accumulation of intracellular Ca^{2+}. The cell shrinkage was prevented by raising the extracellular K^+ concentration to 80 mM, where K^+ is at electrochemical equilibrium and the hyperpolarization resulting from the opening of the Ca^{2+}-dependent K^+ channel is abolished. Simultaneously, the rate of Ca^{2+} uptake was reduced by about 60% (Fig. 1). A similar reduction of Ca^{2+} uptake by vanadate-loaded erythrocytes suspended in 145 mM Na^+–5 mM K^+ media was observed when the K^+

TABLE I

Ca^{2+} uptake [a] by human erythrocytes loaded with 0.5 mM vanadate and quin2

Effects of extracellular K^+ and cell shrinkage.

Ca^{2+} uptake					
vanadate method			quin2 method		
Na^+ 70–K^+ 80	Na^+ 105–K^+ 80	Na^+ 145–K^+ 5	Na^+ 145–K^+ 5	Na^+ 180–K^+ 5	Na^+ 145–K^+ 5 70 mM sucrose
300 mosmol/kg MCHC 5.2 [b]	370 mosmol/kg MCHC 5.7 [b]	300 mosmol/kg MCHC 5.9 [b]	300 mosmol/kg MCHC 5.2 [b]	370 mosmol/kg MCHC 5.9 [b]	370 mosmol/kg MCHC 5.9 [b]
	(n = 4) P < 0.05 [c] +23%			(n = 4) P < 0.05 [c] +18%	
3.2 ± 1.8	3.9 ± 1.3		30.3 ± 5.1	35.7 ± 4.2	37.7 ± 4.2
3.5 ± 1.2		8.2 ± 2.4			
	+134% P < 0.001 [c] (n = 11)			+24% P < 0.005 [c] (n = 4)	

[a] μmol·(litre cells)$^{-1}$·h^{-1}, mean values ± S.D. Ca^{2+} uptake was measured over 1 h at 25 °C and over 15 min at 37 °C in cells preloaded with vanadate and quin2, respectively.

[b] Mean cellular hemoglobin content in mmol hemoglobin tetramer per litre cells measured at the end of the Ca^{2+} uptake period.

[c] Paired t-test.

channel was blocked by 1 mM quinidine (data not shown).

The time course of Ca^{2+} uptake in cells treated with 0.5 mM vanadate and suspended in either 145 mM Na^+–5 mM K^+ or 70 mM Na^+–80 mM K^+ media is shown in Fig. 2. The rate of Ca^{2+} uptake steadily fell over the 120 min of incubation, both at 5 and 80 mM K^+. One possible explanation is that the Ca^{2+} pump was inhibited only in part at 0.5 mM vanadate (see Discussion), the rate of Ca^{2+} extrusion by the Ca^{2+} pump thus increasing with rising cellular Ca^{2+} content.

The contribution of the cell shrinkage to the accelerated Ca^{2+} uptake of cells treated with 0.5 mM vanadate and suspended in 145 mM Na^+–5 mM K^+ media can be evaluated from the data of Table I. Ca^{2+} uptake in vanadate-treated erythrocytes measured over 1 h was accelerated by about 130% upon lowering the extracellular K^+ concentration from 80 to 5 mM, concomitant with a cell shrinkage of about 12%. A similar cell shrinkage due to increasing the osmolality in the 70 mM Na^+–80 mM K^+ media by addition of NaCl enhanced Ca^{2+} uptake by about 18%. These results can be interpreted to indicate that the accelerated Ca^{2+} uptake seen in 145 mM Na^+–5 mM K^+ relative to 70 mM Na^+–80 mM K^+ media is mainly caused by the replacement of Na^+ by K^+ and the reduction of the transmembrane K^+ gradient, respectively. The relative small effect of cell shrinkage alone was confirmed in measurements of Ca^{2+} uptake using the quin2- method. Increasing the medium osmolality to 370 mosmol·(mg H_2O)$^{-1}$ by adding either 35 mM NaCl or 70 mM sucrose accelerated Ca^{2+} uptake by 24% (Table I).

Interindividual variability

The Ca^{2+} uptake rates determined in cells loaded either with vanadate or with quin2 varied up to 2-fold among different donors. In repeated determinations on the same donor the reproducibility of the results obtained was ±4.3% ($n = 5$) and ±6.3% ($n = 4$, ±S.D.) in erythrocytes loaded with quin2 and vanadate, respectively.

Ca^{2+} uptake determined over one hour in vanadate-treated erythrocytes suspended at 25°C in 70 mM Na^+–80 mM K^+ media showed a weak but significant correlation to Ca^{2+} uptake measured in quin2-loaded erythrocytes over 15 min at 37°C ($r = 0.69$, $2P < 0.05$, $n = 11$).

Effects of the inorganic Ca^{2+} entry blockers Co^{2+} and Cu^{2+}

The accelerated Ca^{2+} uptake seen in vanadate-loaded cells suspended in 145 mM Na^+–5 mM K^+ media was inhibited by 60 to 70% by Co^{2+} and Cu^{2+} at the concentrations indicated in Fig. 3. The residual Ca^{2+} uptake observed in the presence of Co^{2+} and Cu^{2+} resembled the rate of K^+-resistant Ca^{2+} uptake of

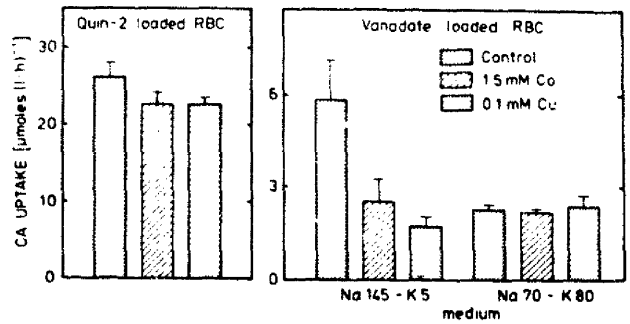

Fig. 3. Effects of 1.5 mM Co^{2+} and of 0.1 mM Cu^{2+} on Ca^{2+} uptake in erythrocytes loaded with quin2 ($n = 9$) and in red blood cells loaded with vanadate ($n = 3$) (mean values ± S.E.). The reductions by Co^{2+} and Cu^{2+} in cells loaded with quin2 were not significant.

vanadate-treated erythrocytes suspended in 70 mM Na^+–80 mM K^+ media. The two inorganic Ca^{2+} entry blockers only slightly reduced or did not affect Ca^{2+} uptake determined in quin2-loaded erythrocytes or in cells loaded with vanadate and suspended in 70 mM Na^+–80 mM K^+ media (Fig. 3).

Effects of organic Ca^{2+} entry blockers

As shown in Fig. 4, both the dihydropyridine derivative nifedipine and the phenylalkylamine verapamil blocked Ca^{2+} uptake in cells treated with vanadate and suspended in 145 mM Na^+–5 mM K^+ media in a concentration-dependent manner. Similar effects were exerted by the benzothiazepine derivative diltiazem and by the diphenylpiperazine derivative flunarizine (Fig. 4).

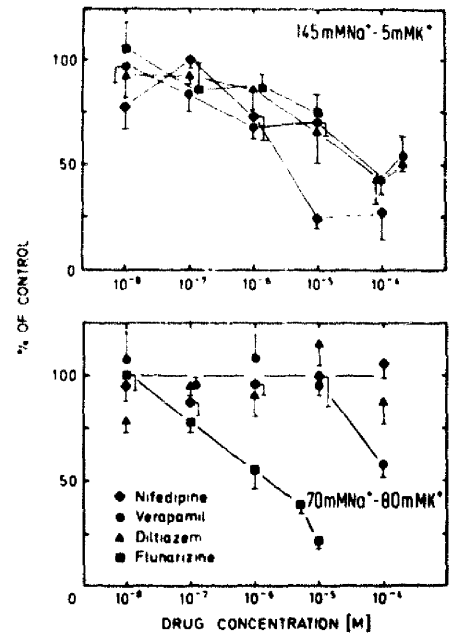

Fig. 4. Inhibition of Ca^{2+} uptake by Ca^{2+} entry blockers in Na^+ 145–K^+ 5 (top) and Na^+ 70–K^+ 80 media (bottom). Vanadate method ($n = 3$–7, bars indicate S.E.).

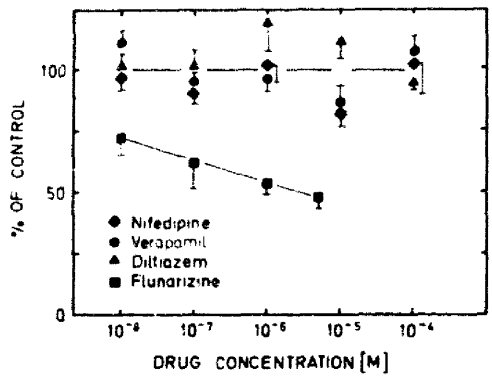

Fig. 5. Inhibition of Ca^{2+} uptake by Ca^{2+} entry blockers. Quin2 method (n = 3–6, S.E. is indicated by bars).

At 10^{-5} M, nifedipine was most effective, blocking about 75% of the Ca^{2+} uptake. The three other drugs reduced Ca^{2+} uptake only by 25–35% at 10^{-5} M, flunarizine being the least effective compound.

When vanadate-treated cells were incubated in 70 mM Na^+–80 mM K^+ media, nifedipine, verapamil and diltiazem did no longer block Ca^{2+} uptake at concentration below 10^{-4} (Fig. 4), the action of the drugs thus resembling that of Co^{2+} and Cu^{2+} (see Fig. 3). Flunarizine, in contrast, retained the property of inhibiting Ca^{2+} uptake at low concentrations, its action starting already at 10^{-7} M. Verapamil produced a 40% inhibition at 10^{-4} M (Fig. 4). Results similar to those of Fig. 4 were obtained in experiments using 2 mM vanadate (data not shown).

The effect of the organic Ca^{2+} uptake blockers on Ca^{2+} uptake by quin2-loaded erythrocytes is shown in Fig. 5. Flunarizine reduced Ca^{2+} uptake of quin2-loaded erythrocytes by up to 50%, the other entry blockers tested being ineffective.

Discussion

Both of the two methods applied in the present experiments to assess passive Ca^{2+} entry into human erythrocytes (inhibition of the Ca^{2+} pump by vanadate at 25°C and entrappment of intracellular Ca^{2+} by the Ca^{2+} chelator quin2) are subject to limitations. Vanadate exerts a multiplicity of biological effects [24–26]. Accordingly, a low vanadate concentration appears to be desirable to avoid possible side effects. On the other hand, vanadate concentrations of 0.5–1 mM such as applied by Varecka and Carafoli [4] and Varecka et al. [5] may be insufficient to block entirely Ca^{2+} extrusion by the Ca^{2+} pump. This view is supported by the data of Fig. 1 where the rate of Ca^{2+} uptake in vanadate-treated erythrocytes reached its maximum only at vanadate concentrations at or above 2 mM. Recently it has been suggested that the Ca^{2+} pump inhibition is incomplete in human erythrocytes even at 5 mM vanadate [27]. In view of these pros and cons of low and high vanadate concentrations, a concentration of 0.5 mM has been applied at 25°C in most experiments, as originally proposed by Varecka and Carafoli [4]. Similar results were obtained using 2 mM vanadate.

In addition, the alternative method of binding intracellular free Ca^{2+} by the entrapped Ca^{2+} chelator quin2 as described by McNamara and Wiley [17] was used to assess passive Ca^{2+} uptake at 37°C. With the quin2 method, Ca^{2+} uptake was linear over 30 min and then a steady-state was attained at about 11 μmol Ca^{2+}/litre cells, indicating an equilibrium between Ca^{2+} uptake and extrusion. It is important to note that at the concentrations of quin2 acetoxymethyl ester used in the present experiments red cell ATP remained within the normal range [23].

Identification of two components of passive Ca^{2+} entry into human erythrocytes

According to the data of Table I, the total Ca^{2+} uptake seen in erythrocytes treated with vanadate and suspended in 145 mM Na^+–5 mM K^+ media comprises a K^+-sensitive component (about 50%), a component induced by the cell shrinkage (about 10%) and a K^+-resistant component (about 40%). The Ca^{2+} uptake induced by cell shrinkage is rather small as compared to the other two components and, therefore, is neglected in the subsequent discussion. All of the six organic and inorganic Ca^{2+} entry blockers examined (nifedipine, verapamil, diltiazem, flunarizine, Co^{2+} and Cu^{2+}) inhibited Ca^{2+} uptake in vanadate-treated erythrocytes incubated in 145 mM Na^+–5 mM K^+ media by 25 to 75% (Figs. 3 and 4), thus confirming results reported by Varecka and Carafoli [4], Neyses et al. [3], and Varecka et al. [5]. Flunarizine was the only compound that inhibited both Ca^{2+} uptake in cells loaded with quin2 and K^+-resistant Ca^{2+} uptake in cells loaded with vanadate and suspended in 70 mM Na^+–80 mM K^+ media in a dose-dependent manner by up to 80% (Figs. 4 and 5). Nifedipine, verapamil, diltiazem, Co^{2+} and Cu^{2+} did not significantly affect Ca^{2+} uptake in cells loaded with quin2 (Figs. 3 and 5). Similarly, K^+-resistant Ca^{2+} uptake determined in vanadate-treated erythrocytes suspended in 70 mM Na^+–80 mM K^+ media was not affected by Co^{2+}, Cu^{2+}, nifedipine and diltiazem, and reduced by verapamil only at concentrations above 10^{-5} M (Figs. 3 and 4).

The simplest interpretation of these results is that there are two components for passive inward Ca^{2+} transport into human erythrocytes that can be identified by different inhibitors. (i) A Ca^{2+} uptake that is seen both in quin2-loaded erythrocytes and in cells treated with vanadate and suspended in 70 mM Na^+–80 mM K^+ media and that is inhibited by flunarizine but not by nifedipine, verapamil, diltiazem, Co^{2+} and Cu^{2+}. (ii) A Ca^{2+} uptake that is additionally present in vanadate-

loaded cells suspended in 145 mM Na^+–5 mM K^+ media. The latter component is inhibited by nifedipine, verapamil, diltiazem, Co^{2+} and Cu^{2+}, but probably not by flunarizine. On the basis of the present results it cannot be decided whether the two components of Ca^{2+} uptake by vanadate-loaded erythrocytes are mediated by one and the same Ca^{2+} transporter or by two distinct Ca transport pathways.

The second component is apparently also blocked by reduction of the K^+ gradient and in the presence of quinidine, in accordance with results of other authors [4]. One attractive explanation, first proposed by Gárdos et al. [7], would be that the two manoeuvres prevent membrane hyperpolarization resulting from Ca^{2+}-dependent opening of the K^+ channel in vanadate-treated cells suspended in 145 mM Na^+–5 mM K^+ media. Elevation of medium K^+ to 80 mM eliminates the electrochemical driving force for K^+, and quinine is known to block the K^+ channel, thereby preventing hyperpolarization.

This interpretation has been questioned by Jenkins and Lew [22] and by Ferreira and Lew [2] who reported that medium K^+ reduces Ca^{2+} uptake also in erythrocytes from species that apparently lack the Ca^{2+}-dependent K^+ channel. However, neither the effect of Ca^{2+} entry blockers nor the effect of medium K^+ on vanadate-treated erythrocytes has been studied in these species. Accordingly, further experiments are required to define the possible role of electric driving forces in the component of passive Ca^{2+} uptake that is blocked by extracellular K^+, quinidine as well as by nifedipine, verapamil, diltiazem, Co^{2+} and Cu^{2+} in human erythrocytes.

A second question concerns the possibility that Ca^{2+} uptake in quin2-loaded erythrocytes and the K^+-resistant component of Ca^{2+} uptake in vanadate-treated erythrocytes are mediated by the same transport mechanism. The inhibition of the two phenomena by flunarizine (but not by the other Ca^{2+} entry blockers) seemingly favours this possibility but gives no definite proof. The weak inhibitory effect of Co^{2+} exerted in the present experiments on Ca^{2+} entry into quin2-loaded cells (less than 15%, see Fig. 3) is in contrast to the 50% inhibition observed by Tiffert et al. in one experiment on erythrocytes loaded with benz2 [28].

The component of Ca^{2+} uptake blocked by flunarizine is possibly not identical to the component of Ca^{2+} entry into vanadate-loaded cells suspended in low K^+ media that is inhibited by the other entry blockers examined. The diphenylpiperazine derivative is a lipophilic drug that often is classified not to belong to the group of 'classic' Ca^{2+} entry blockers. It partitions easily into membranes and into cells. Accordingly, its inhibitory action may not be due to binding to extracellular receptors but rather related to interactions with the inner leaflet of the red cell membrane [29]. In accordance with this view is our observation that preincubation periods of at least 15 min were required for the inhibition of 'basal' Ca^{2+} uptake by flunarizine to develop. Although the evidence presented in this paper appears to be compelling, further work is needed to substantiate the notion that two different components of passive Ca^{2+} uptake by human erythrocytes can be distinguished by the use of Ca^{2+} entry blockers.

Acknowledgements

The precise and expert technical assistance of Renate Böhle is gratefully acknowledged. Thanks are also due to the valuable assistance provided by Jutta Kronauer in the initial phase of the experiments.

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