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Inhibition by divalent cations and sulphydryl reagents of the passive Ca^{2+} transport in human red blood cells observed in the presence of vanadate

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The uptake of $^{45}Ca^{2+}$ by human red blood cells induced by vanadate was found to be inhibited by a number of divalent cations. The following order of potencies was determined (in parentheses, IC_{50} in mmol/l): Cu^{2+} (0.006), Zn^{2+} (0.014), Cd^{2+} (0.030), Co^{2+} (0.20), Ni^{2+} (0.25), Mn^{2+} (8.0), Ba^{2+} (9.0), Sr^{2+} (14.0). The effects of Cu^{2+}, Zn^{2+} and Cd^{2+} were biphasic — over a critical concentration their inhibitory potencies decreased, and finally, were lost. Besides Ca^{2+}, Sr^{2+}, Ba^{2+} and Mn^{2+} were also taken up, but only Ca^{2+} and Sr^{2+} were capable of eliciting the Gárdos effect. Ni^{2+} was not taken up. Several HS reagents also inhibited $^{45}Ca^{2+}$ uptake. The following order of potencies was determined (in parentheses, IC_{50} in mmol/l): mersalyl (0.0025), 5,5′-dithiobis(2,2′-dinitrobenzoic acid) (0.011), *p*-chloromercuric acid (0.042), *N*-ethylmaleimide (2.0). The effects of all HS reagents except *N*-ethylmaleimide were biphasic. The biphasicity of the actions of the indicated agents was caused by the opening of a new pathway for $^{45}Ca^{2+}$ entry which is different from that observed in the presence of vanadate alone, and is inhibited by low concentrations of these agents. The modified form of the anion channel seems to be identical with the former pathway. The last one is mediated by a transport protein which has an ionic specificity similar to Ca^{2+} channels in excitable tissues, and contains an HS group which is essential for the transport function.

Introduction

It has been found in several types of cell that stopping active Ca^{2+} transport leads to the accumulation of Ca^{2+} in the cell cytoplasm [1–5]. This suggests that there is a stationary cycling of calcium ions across the cytoplasmic membrane. Its existence implies that there are transport systems for Ca^{2+} in cell membranes that catalyse the passive transport of Ca^{2+} in the resting state (i.e., without externally added activators) of the cells, also. The consequence of this is that these putative transport systems operate without the intervention of receptors and/or voltage changes.

The most suitable experimental model for testing this hypothesis is probably the mammalian red blood cell. It has preserved no internal membranes, and no apparent receptor-operated functions. The value, and the origin, of its membrane potential does not favour the presence of excitable mechanisms in its membranes [6]. Moreover, several methods have been developed which induce the uptake of $^{45}Ca^{2+}$ by human red blood cells. Some of them, such as ATP depletion [3] and inhibition of Ca^{2+}-ATPase by La^{3+} [4] and vanadate [5], seem to unmask the physiological turnover of Ca^{2+} cycling, a fact which has been confirmed by a method not based on the inhibition of Ca^{2+} pumping [7]. Although the mechanisms of the active Ca^{2+} transport in human red blood cells have been elucidated up to molecular details (see Refs. 9, 10, for review), only scanty

data are available about the mechanism(s) of the inward (passive) Ca^{2+} transport [3,5,14].

In the present work we have used vanadate for induction of $^{45}Ca^{2+}$ uptake. Previously published results using this method showed that the uptake possessed some characteristics of the carrier-mediated transport, such as saturability and inhibition by Co^{2+} and verapamil [5], and that the uptake was inhibited and activated by dihydropyridine Ca^{2+} antagonists and agonists, respectively [8]. In this paper we add further findings supporting the existence of the carrier protein in the human red blood cell membrane catalyzing the passive Ca^{2+} transport, and characterizing its properties.

Methods

Red blood cells

Fresh citrate-treated human blood was obtained from healthy volunteers using the facilities of Haematology and Transfusion Clinics in Bratislava. It was stored under sterile conditions at 0–4°C, and used within 2 weeks. Pig citrate-treated blood was obtained from a local slaughterhouse, and was used for experiments the same day.

Red blood cells were prepared immediately before the experiments. Blood was centrifuged at $2500 \times g$ for 5 min. The plasma and the buffy coat were sucked off, and the red blood cells were suspended in a medium (henceforth referred to as 'medium') comprising (mmol/l) 20 Tris-HCl, 130 NaCl, 5 KCl, 10 glucose (pH 7.3). The suspension was centrifuged as above, and the washing procedure was repeated four times. The washed cells were resuspended in the same medium at a concentration of 30% (microhaematocrit).

$^{45}Ca^{2+}$ uptake experiments

The procedure of Varečka and Carafoli [5] was used modified as follows. (A) $NaVO_3$ was used instead of Na_3VO_4: We have found that both compounds were equally effective in inducing the $^{45}Ca^{2+}$ uptake (not shown). (B) Cells were suspended in the medium described above. (C) The extracellular radioactivity was washed out with a solution comprising (mmol/l) 20 Tris-HCl, 105 KCl, 30 NaCl, 10 glucose, 1 EDTA, 1 $NaVO_3$. In control test tubes, vanadate was omitted.

$^{45}Ca^{2+}$ efflux and K^+ efflux experiments

Experiments were performed according to Varečka and Carafoli [5].

Divalent cation uptake

The uptake of Ca^{2+}, Mn^{2+}, Ni^{2+} was measured by atomic absorption spectrophotometry, the uptake of Sr^{2+} and Ba^{2+} by atomic emission spectrophotometry. The experimental protocol was as in radionuclide uptake experiments except that a 5-fold volume of the suspension was used per assay, and that $LaCl_3$ was omitted from the deproteinization solution.

Chemicals

$NaVO_3$ was obtained from Reachim, Moscow, U.S.S.R.; $^{45}CaCl_2$ from the Radiochemical Institute of the Hungarian Academy of Sciences, Budapest, Hungary; Valinomycin; *N*-ethylmaleimide, and ionophore A23187 from Calbiochem, Luzern, Switzerland; Dithiothreitol, and Mersalyl from Sigma, St. Louis, MO, U.S.A.; 5-5′-dithiobis(2,2′-dinitrobenzoic acid) from Merck, Darmstadt, F.R.G., and trichloroacetic acid from Reanal, Budapest, Hungary. All other chemicals of reagent grade purity were purchased from Lachema, Brno, Czechoslovakia.

Results

Effects of divalent cations (M^{2+})

In order to obtain further information about the vanadate-induced $^{45}Ca^{2+}$ uptake we have studied the effects of several divalent cations on this process. Experiments were performed at constant concentrations of $NaVO_3$ (1 mmol/l) and $^{45}Ca^{2+}$ (2.5 mmol/l). The incubation with $^{45}Ca^{2+}$ was 1 h. Under these conditions the uptake had an almost linear time-course. The results are summarized in Fig. 1. Divalent cations inhibited the vanadate-induced $^{45}Ca^{2+}$ uptake with varying efficiency. Of all M^{2+} tested, only Mg^{2+} exerted no inhibitory effect up to 10 mmol/l. The inhibiting M^{2+} could be divided into three groups according to their inhibitory potency and other typical characteristics. (a) Weak inhibitors (Sr^{2+}, Ba^{2+}, Mn^{2+}), which had an IC_{50} in the 10^{-3}–10^{-2} mol/l range. When Mn^{2+} was used at lower (e.g., 0.5–2.0 mmol/l) concentrations, it had a small

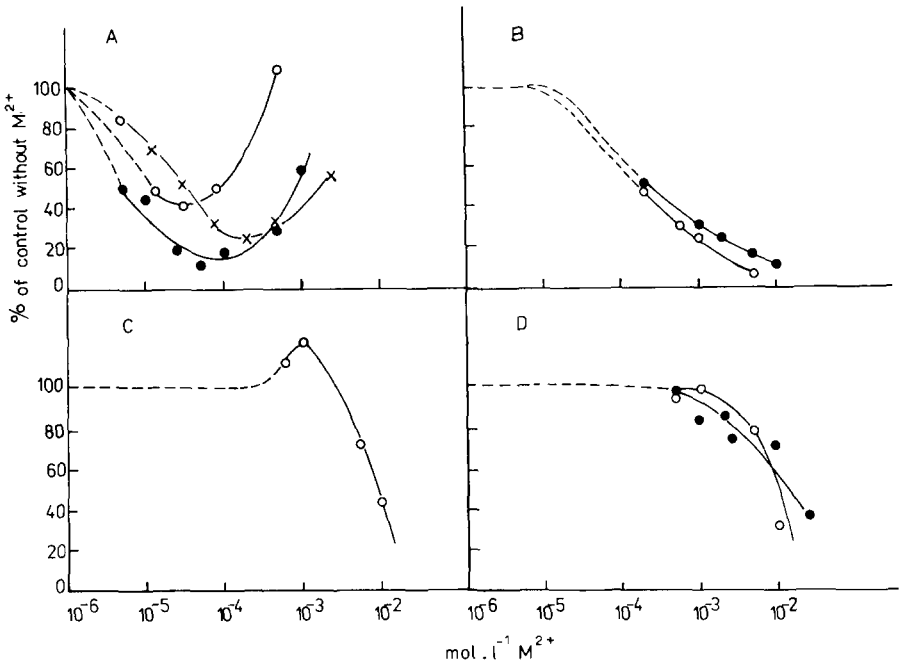

Fig. 1. The inhibition by divalent cations of $^{45}Ca^{2+}$ uptake induced by the presence of vanadate. Each dose-effect curve is typical of 2–4 independent experiments as described in Methods. The values of the uptake were corrected for the residual uptake in the absence of vanadate, and are expressed as percentage of control without M^{2+}. The curves are plotted in a semi-logarithmic plot. Symbols: (A) ●, Cu^{2+}; ○, Zn^{2+}; ×, Cd^{2+}. (B) ●, Ni^{2+}; ○, Co^{2+}. (C) ○, Mn^{2+}. (D) ●, Sr^{2+}; ○, Ba^{2+}.

TABLE I

EFFECTS OF Co^{2+}, Cu^{2+}, MERSALYL AND DTNB ON THE ^{40}Ca-INDUCED $^{45}Ca^{2+}$ EFFLUX OBSERVED IN THE PRESENCE OF VANADATE

The initial rate of the efflux was calculated from the difference of the radioactivity of samples at time zero and at 6 min, and is expressed in μmol/l cells per h. The results of three independent experiments are shown. Procedures as in Methods.

Experiment	Additions	Efflux ($\mu mol \cdot l^{-1} \cdot h^{-1}$)
1	none	34
	1.5 mmol/l $CoCl_2$	34
2	none	33
	20 μmol/l $CuCl_2$	28
	100 μmol/l $CuCl_2$	20
	1 000 μmol/l $CuCl_2$	28
3	none	17.7
	12.5 μmol/l DTNB	16.1
	78.1 μmol/l DTNB	16.7
	250 μmol/l DTNB	15.4
	none	18.2, 17.1
	2.5 μmol/l mersalyl	12.4
	10.0 μmol/l mersalyl	15.7
	100.0 μmol/l mersalyl	14.9

stimulatory effect. The latter was observed in the presence of vanadate only. (b) Medium inhibitors (Ni^{2+}, Co^{2+}), which had an IC_{50} in the 10^{-4} mol/l range. They exhibited monophasic inhibition up to 10 mmol/l. In a pilot experiment we have found that Co^{2+} competitively inhibits the $^{45}Ca^{2+}$ uptake (not shown). (c) Strong inhibitors (Cu^{2+}, Zn^{2+}, Cd^{2+}) had an IC_{50} in the 10^{-6}–10^{-5} mol/l range. Their effects were biphasic – if used in higher concentrations (e.g., 10^{-4}–10^{-3} mol/l) their inhibitory potency decreased and, finally, they increased the uptake over control values. These effects were usually observed in the presence of vanadate only. Only Cu^{2+}, if present in concentrations over 1 mmol/l, stimulated the uptake also in controls without vanadate (Fig. 6). Experiments were performed which exclude the possibility that the inhibition of $^{45}Ca^{2+}$ uptake by M^{2+} was due to the reactivation of the Ca^{2+} pumping rate set at a low level by the presence of vanadate. These experiments were based on the effects of selected M^{2+} on the $^{40}Ca^{2+}$-induced $^{45}Ca^{2+}$ efflux from red blood cells loaded previously with $^{45}Ca^{2+}$ by means of vanadate under

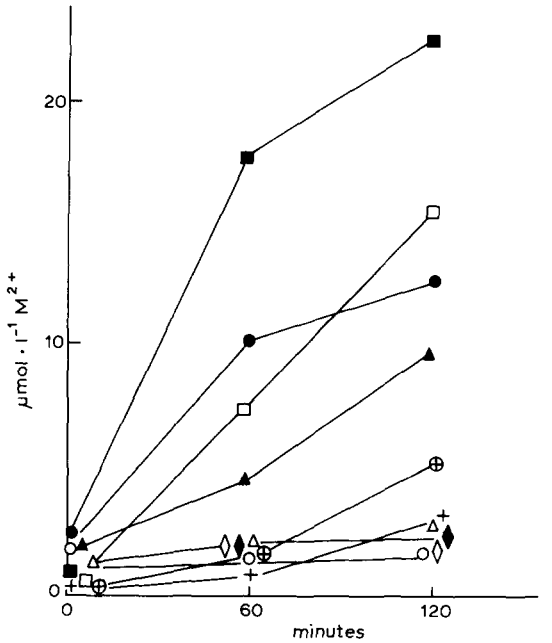

Fig. 2. The uptake of divalent ions by human red blood cells in the presence and absence of vanadate. The time-course of the uptake was measured at times indicated according to the procedure described in Methods, except that vanadate was present at 2 mmol/l concentration. Symbols: ●, ○, Ca^{2+}; ▲, △, Sr^{2+}; ■, □, Ba^{2+}; ◆, ◇, Ni^{2+}; closed symbols – vanadate present, open symbols – no vanadate; ⊕, Mn^{2+} plus vanadate; ×, Mn^{2+} without vanadate. Representative of two or three independent experiments. The uptake in each experiment was measured in duplicate.

conditions identical to those in $^{45}Ca^{2+}$ uptake experiments. As expected, these experiments, summarized in Table I, gave no indication of the activation of the Ca^{2+} pumping rate by M^{2+}.

In order to obtain information about the transport specificity of the vanadate-induced $^{45}Ca^{2+}$ uptake, transport of several M^{2+} was measured by means of atomic absorption (Ca^{2+}, Mn^{2+}, Ni^{2+}) or atomic emission (Sr^{2+}, Ba^{2+}) spectrophotometry under the conditions used in the experiments with a radioactive tracer. All of these ions except Ni^{2+} were found to enter red blood cells (Fig. 2). The uptake of Ca^{2+}, Sr^{2+} and Mn^{2+} was inhibited by DTNB (not shown). This effect will be described below. Ca^{2+} and Sr^{2+} entered red blood cells in the presence of vanadate only, whereas Ba^{2+} and Mn^{2+} did so both in the presence and in the absence of vanadate (slightly less in its absence). The uptake of Ni^{2+} was less than 2 μmol/l cells. The uptake of Ba^{2+} and Mn^{2+} in the absence of vanadate could be explained if one took into account the fact that red blood cell Ca^{2+}-ATPase transports Ca^{2+} and Sr^{2+} only [9]. In the next experiment we tested the effects of several selected ions on the Gárdos effect induced by vanadate and Ca^{2+}. It is shown in Fig. 3 that these ions also inhibited the Gárdos effect. Cu^{2+}, Mn^{2+} and Co^{2+} inhibited the Gárdos effect as strongly as they did the $^{45}Ca^{2+}$ uptake, whereas Cd^{2+} inhibited the Gárdos effect less potently than the uptake. We have no explanation for this discrepancy. It must be mentioned that the inhibition of the Gárdos effect by Cu^{2+} and Cd^{2+} was not biphasic, in contrast to their effect on the uptake. A possible explanation is that these ions at higher concentrations enter red blood cells together with Ca^{2+} and effectively compete with the latter for the Ca^{2+}-binding site of the Ca^{2+}-activated K^+ channel. This suggestion is supported by the findings of Rabenstein et al. [11], who reported NMR mea-

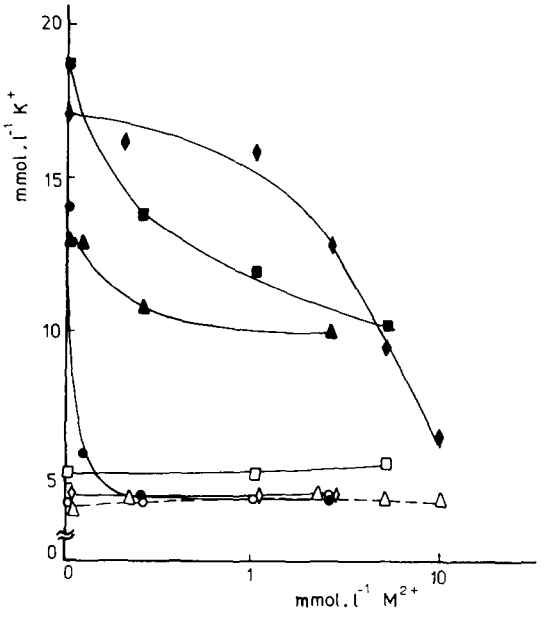

Fig. 3. Inhibition of the vanadate-induced Gárdos effect by divalent cations. The Gárdos effect was elicited by 1 mmol/l vanadate and 2.5 mmol/l Ca^{2+} in the presence of indicated concentrations of the following cations: ●, ○, Cu^{2+}; ■, □, Cd^{2+}; ▲, △, Co^{2+}, ◆, ◇, Mn^{2+}. The ordinate represents the extracellular K^+ concentration after 1 h incubation with Ca^{2+}. Open symbols – controls without vanadate. All experiments were independent. Results shown are typical of at least two experiments.

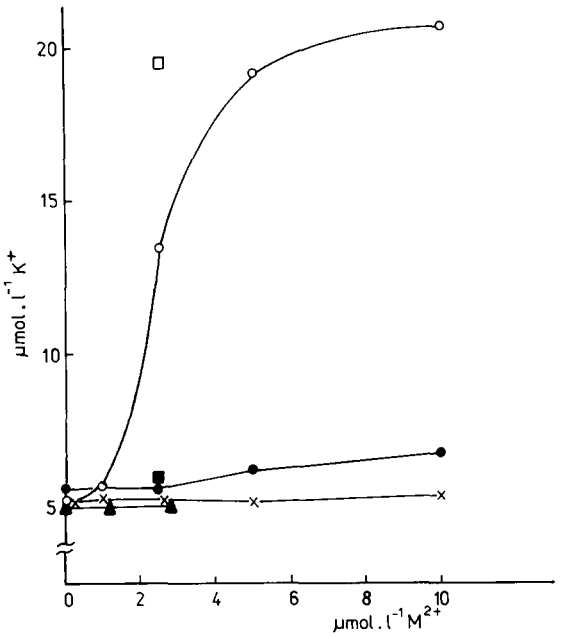

Fig. 4. The activation of the Gárdos effect by various cations in the presence of vanadate. The Gárdos effect was elicited in red blood cells pretreated with 1 mmol/l vanadate by indicated concentrations of divalent cations. Symbols: □, Ca^{2+}; ■, Ca^{2+} + 50 μmol/l DTNB; ○, Sr^{2+}; ●, Sr^{2+} + 50 μmol/l DTNB; ×, Mn^{2+}; ▲, Ba^{2+}. On the ordinate are plotted extracellular K^+ concentrations after 1 h incubation with M^{2+}.

surements which suggest that Cd^{2+} applied in mmol/l concentrations permeate the red blood cell membrane and bind to haemoglobin.

The next experiment was designed to find out which of the divalent cations that enter red blood cells is capable of activating Ca^{2+}-activated K^+ channel in the presence of vanadate, and in the absence of Ca^{2+}. We have found that, besides Ca^{2+}, only Sr^{2+} was able to do this, Ba^{2+} and Mn^{2+} being ineffective (Fig. 4). The efflux of K^+ induced by Sr^{2+} in vanadate-treated cells was saturated with Sr^{2+} at about 5 mmol/l. This concentration was higher than the corresponding one for Ca^{2+} (about 2.5 mmol/l). The Gárdos effect induced by Sr^{2+} and vanadate was sensitive to DTNB (Fig. 4) (see below). This experiment, together with that shown in Fig. 2, indicates that the specificity of the Ca^{2+} gate of the Ca^{2+}-activated K^+ channel is higher than that of the passive transport of Ca^{2+} observed in the presence of vanadate.

The effects of sulphydryl reagents

The capability of exerting a biphasic effect on $^{45}Ca^{2+}$ uptake was shared by Cu^{2+}, Cd^{2+} and Zn^{2+} (Fig. 1). One of common properties of these M^{2+} is their ability to react with HS groups, and create mercaptide bonds [13]. This led us to propose that the strong potency in the $^{45}Ca^{2+}$ uptake inhibition of these ions as compared with other M^{2+} is due to their interaction with an essential HS group in the molecule of the putative transport protein that catalyzes the passive transport of Ca^{2+}. The following experiments support this proposal.

It is shown in Fig. 5 that HS-group reagents inhibit vanadate-induced $^{45}Ca^{2+}$ uptake. The most

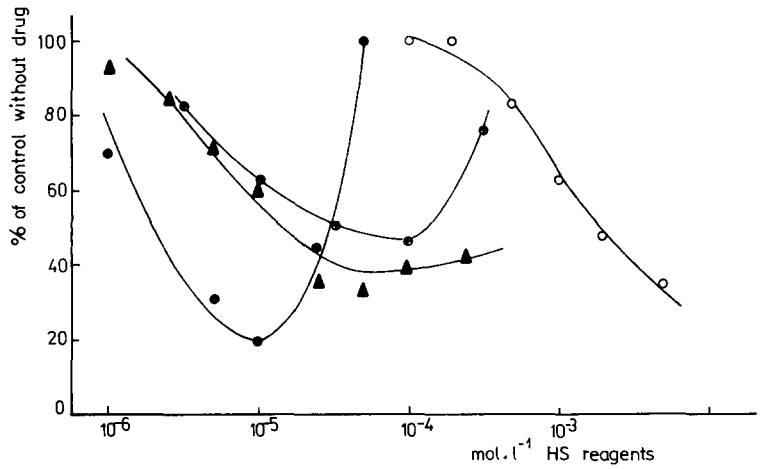

Fig. 5. Effects of HS reagents on $^{45}Ca^{2+}$ uptake induced by vanadate. $^{45}Ca^{2+}$ uptake was measured as described in Methods in the presence of the indicated concentrations of HS reagents. The values were corrected for uptake without vanadate. ●, mersalyl; ▲, DTNB; ⊕, pCMB; ○, *N*-ethylmaleimide. Each curve is representative of at least two independent experiments.

TABLE II

THIOLS REVERSE THE INHIBITION OF THE GÁRDOS EFFECT BY MERSALYL

The results of a pilot experiment are presented which was performed according to the procedure described in Methods. The order of additions was following (in parentheses, time of incubation in minutes at 25°C): Experiment 1: 1 mmol/l vanadate (15), 100 μmol/l mersalyl (2), 2 mmol/l dithiothreitol (2), 2.5 mmol/l $CaCl_2$ (60). Experiment 2: 1 mmol/l vanadate (15), 10 μmol/l mersalyl, (2), 2.0 mmol/l thioglycolate, sodium salt (2), 2.5 mmol/l $CaCl_2$ (60). DTT, dithiothreitol.

Experiment	Additions	$[K^+]$ ($mmol \cdot l^{-1}$)
1	vanadate + $CaCl_2$ + mersalyl	7.2
	vanadate + $CaCl_2$ + mersalyl + DTT	18.5
	vanadate + $CaCl_2$ + DTT	18.5
	vanadate + DTT	5.4
	vanadate + mersalyl + DTT	5.4
	none	5.4
2	vanadate + $CaCl_2$	19.8
	vanadate + $CaCl_2$ + mersalyl	11.1
	vanadate + $CaCl_2$ + mersalyl + thioglycolate	19.0
	vanadate + mersalyl	5.4
	vanadate + thioglycolate	5.4

potent inhibitor was mersalyl, which inhibited the uptake with an IC_{50} of 2.5 μmol/l, followed by DTNB (11 μmol/l), pCMB (42 μmol/l), and *N*-ethylmaleimide, which had an IC_{50} of 2 mmol/l. Mersalyl, DTNB and pCMB exerted biphasic effects on the $^{45}Ca^{2+}$ uptake, similarly to Cu^{2+}, Zn^{2+} and Cd^{2+}. *N*-Ethylmaleimide, at the concentrations used, had a monophasic inhibitory effect. In the control experiments without vanadate, the HS reagents, except for *N*-ethylmaleimide (tested up to 250 μmol/l), themselves induced the $^{45}Ca^{2+}$ uptake. The extent of this was always less than that induced by the simultaneous presence of vanadate and HS reagents. We did not test whether the inhibition of $^{45}Ca^{2+}$ uptake by HS reagents was reversed by thiols. However, this is very probable, because the inhibition by mersalyl of the vanadate and Ca^{2+}-induced Gárdos effect was completely reversed by dithiothreitol (Table II). The latter by itself stimulated the vanadate-induced $^{45}Ca^{2+}$ uptake in most experiments (Table III). In experiments not presented here we have found that the inhibition of the $^{45}Ca^{2+}$ uptake by Cu^{2+} was completely reversed by 1 mmol/l dithiothreitol. This effect, however, could be due to the chelation of Cu^{2+} from the medium, lowering its analytical concentration. Therefore, this experiment cannot support our thesis that Cu^{2+} (and also Zn^{2+} and Cd^{2+}) inhibits the $^{45}Ca^{2+}$ uptake by attacking an essential HS group. Despite this, a comparison of the data presented in Figs. 1 and 5 strongly supports this thesis. In the experiments shown in Table I and described in detail for M^{2+} in the previous section, we have excluded the possibility that the inhibition by HS reagent is caused by the unblocking of the vanadate inhibition of the Ca^{2+}-ATPase.

TABLE III

STIMULATION OF $^{45}Ca^{2+}$ UPTAKE INDUCED BY VANADATE BY DITHIOTHREITOL

The effect of dithiothreitol (DTT) was measured as described in Methods in the suspensions obtained from seven healthy donors in independent experiments. No effect of dithiothreitol was observed without vanadate. The data were not corrected for the uptake in the absence of vanadate. The data in experiments 4–7 were obtained in duplicate; both values are shown.

Expt. No.	$^{45}Ca^{2+}$ uptake (μmol/l cells per h)	
	control	+1 mmol/l DTT
1	6.7	13.6
2	23.5	40.5
3	5.3	9.9
4	61.0, 62.3	61.2, 59.9
5	9.2, 9.9	18.3, 19.5
6	5.73, 7.7	4.8, 4.7
7	16.0, 15.6	17.4, 15.4

The relationship between the $^{45}Ca^{2+}$ uptake induced by vanadate, and by HS reagents

If one proposes, as we do, that the inhibitory effects of Cu^{2+}, Zn^{2+} and Cd^{2+}, or mersalyl, DTNB and pCMB on the vanadate-induced $^{45}Ca^{2+}$ uptake are caused by their interaction with the molecule of the putative transport system, then the decrease or their inhibitory effect upon increasing their concentration (i.e., ascending parts of the dose-effect curves in Fig. 1 and 5) cannot be explained other than by assuming the opening of a new route for the $^{45}Ca^{2+}$ uptake. We have obtained evidence that this really is the case in our experimental system.

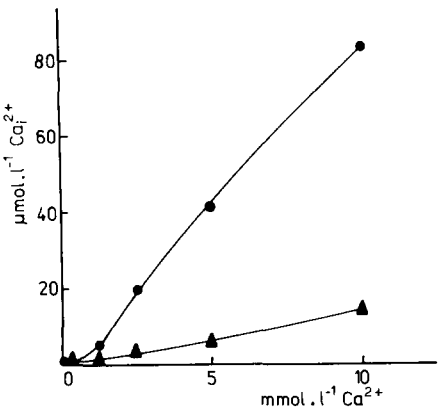

Fig. 6. The dependence of the $^{45}Ca^{2+}$ uptake induced by Cu^{2+} and mersalyl on the external $^{45}Ca^{2+}$ concentration. Aliquots of a red blood cell suspension were preincubated 15 min at 25°C with 0.1 mmol/l mersalyl (●), or 5.0 mmol/l $CuCl_2$ (▲) $^{45}Ca^{2+}$ was added to the final concentration indicated in the figure, and the uptake was measured as described in Methods. One of two independent experiments.

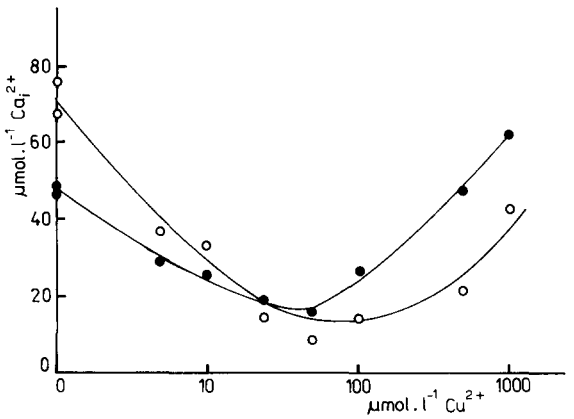

Fig. 7. The dose-effect curve of Cu^{2+} on the $^{45}Ca^{2+}$ uptake induced by vanadate in the absence and presence of valinomycin. The uptake of $^{45}Ca^{2+}$ was measured in the presence of 1 mmol/l vanadate and the Cu^{2+} concentrations indicated in the figure, in the presence (●) and absence (○) of 1 μg/ml valinomycin according to the procedure described in Methods. In control test tubes, an equal volume of solvent (0.3% (v/v) methanol) was present. One of two independent experiments.

In Fig. 6 is shown the dependence of the $^{45}Ca^{2+}$ uptake induced by 5 mmol/l Cu^{2+} and 100 μmol/l mersalyl on the extracellular $^{45}Ca^{2+}$ concentration. There is no saturation of the uptake induced by these two agents up to 10 mmol/l $^{45}Ca^{2+}$, in contrast to the vanadate-induced $^{45}Ca^{2+}$ uptake [5], which we confirmed also under the conditions of the experiment in Fig. 6 (not shown). This suggests either a substantial difference in the affinity for Ca^{2+} of both Cu^{2+}- and mersalyl-induced $^{45}Ca^{2+}$ uptake as compared with the vanadate-induced one, or the absence of the carrier-mediated transport induced by HS reagents. So far we have carried out no experiment which could distinguish between these two possibilities.

Vanadate- and HS reagent-induced $^{45}Ca^{2+}$ uptake respond differently to the membrane potential imposed by the presence of the K^+-specific ionophore valinomycin in a medium containing 5 mmol/l K^+. This is shown in Fig. 7 for Cu^{2+}. If the $^{45}Ca^{2+}$ uptake was blocked by Cu^{2+}, the Gárdos effect was inhibited in parallel (Fig. 3). Unlike the $^{45}Ca^{2+}$ uptake, the Gárdos effect was not stimulated when the Cu^{2+} concentration was increased (Figs. 1, 3). In the presence of valinomycin, which mimics the potential change elicited by the Gárdos effect, the uptake is altered. In the descending part of the dose-effect curve (Fig. 7), valinomycin additionally inhibited the uptake. In other similar experiments we observed no inhibition of the uptake by valinomycin under the same conditions. The source of this variability is not known to us at present. On the other hand, the uptake of $^{45}Ca^{2+}$ shown in the ascending part of the dose-effect curve was always stimulated by valinomycin. Similar results were obtained with Cd^{2+}, Zn^{2+} and mersalyl (not shown). The inability of valinomycin to release the inhibition of the uptake in the descending part of the dose-effect curve does not favour the role of the membrane potential created by the opening of the Ca^{2+}-activated K^+ channel in the vanadate-induced $^{45}Ca^{2+}$ uptake. This is in accord with the data of Gárdos et al. [14], Szász et al. [4] and Varečka and Carafoli [5]. These experiments exclude the uptake observed after the inhibition of the Ca^{2+}-ATPase being due to a membrane leak which is reinforced by the Gárdos-effect-induced hyperpolarization of the membrane. Unfortunately, these experiments do not allow one to determine the charge-compensation properties of the Ca^{2+}-transporting system that operates in the descending part of the dose-effect curve. The Ca^{2+}-transport observed in the ascending part of

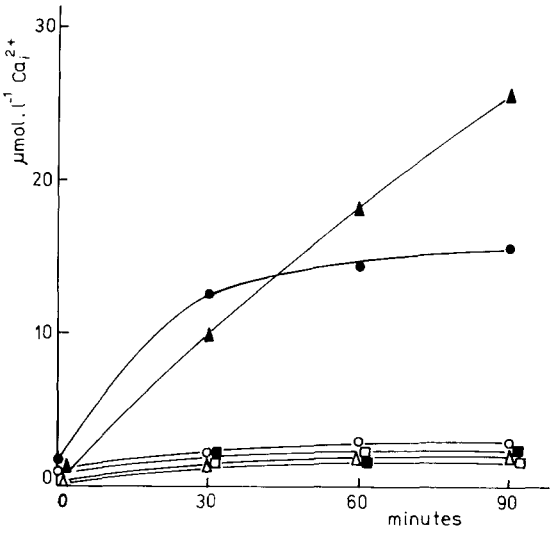

Fig. 8. Comparison of the $^{45}Ca^{2+}$ uptake induced by vanadate and La^{3+} in human and pig red blood cells. The uptake was induced by 1 mmol/l vanadate (▲, △), or 0.2 mmol/l La^{3+} (●, ○), and measured as described in Methods. Control samples without additions (■, □) were treated in parallel. Closed symbols, human red blood cells; open symbols, pig red blood cells. One of two independent experiments.

the dose-effect curve seems to be of a uniport (i.e., channel-like) type according to its membrane-potential dependence.

The following experiments were performed with pig red blood cells. We have found that pig red blood cells do not take up $^{45}Ca^{2+}$ in the presence of vanadate (Fig. 8). Control experiments (Fig. 8) showed that fail to take up $^{45}Ca^{2+}$, even in the presence of 0.2 mmol/l La^{3+}. According to Szász et al. [4] La^{3+} does not enter red blood cells, and apparently inhibits Ca^{2+}-ATPase from the outer side of the membrane. This experiment shows that the lack of the effect of vanadate is not due to its inability to cross the membrane in order to reach its target, and suggests that the transport protein which conveys Ca^{2+} across the human red blood cell membrane is absent, or non-functional, in the pig red blood cell membrane. For comparison, typical experiments with human red blood cells are shown in Fig. 8 which illustrate both vanadate- and La^{3+}-induced $^{45}Ca^{2+}$. As is shown in Fig. 9, both mersalyl and Cu^{2+} were still able to induce the uptake of $^{45}Ca^{2+}$ in pig red blood cells. It is further shown in Fig. 9 that vanadate potentiates the effects of both mersalyl and Cu^{2+}. This indicates that vanadate enters the pig red blood cell membrane and inhibits the Ca^{2+}-ATPase in a similar manner as in human red blood cells. This inhibition may occur simultaneously with that by HS reagents [16]. In experiments not detailed here we have confirmed earlier data [27] that the Gárdos effect can be elicited in pig red blood cells by the ionophore A23187 and Ca^{2+}. This experiment ex-

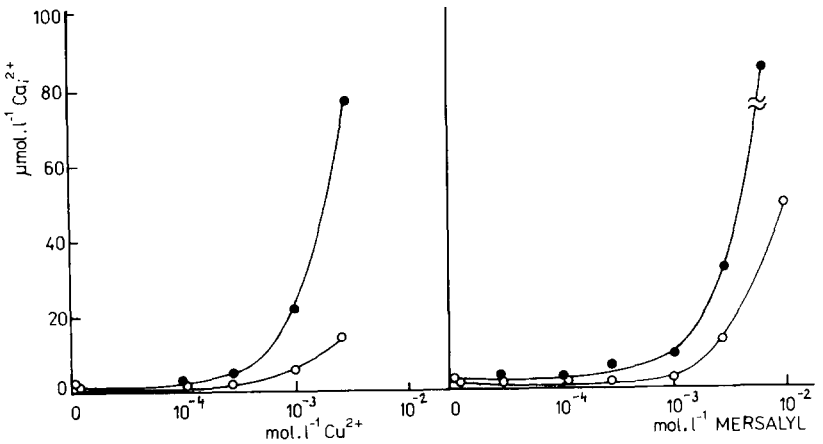

Fig. 9. The induction by Cu^{2+} and mersalyl of $^{45}Ca^{2+}$ uptake by pig red blood cells; effect of vanadate. The suspension of pig red blood cells was preincubated 15 min with 1 mmol/l vanadate (closed symbols), and 2 min with indicated concentrations of Cu^{2+} (left plot), or mersalyl (right plot). The $^{45}Ca^{2+}$ uptake was measured as described in Methods. Control samples were treated as above but without vanadate (open symbols). One of two experiments.

cludes the possibility that the transport of Ca^{2+} induced by vanadate or La^{3+} in human red blood cells is associated with a moiety of the Ca^{2+}-activated K^+ channel, unless we accept a less probable possibility that there are substantial interspecies differences in the structure of this protein. A similar argument can be applied for the anion channel, which must be present in both human and pig red blood cell membranes. The anion channel is a likely candidate for a site of the action of high concentrations of HS reagents. Its modification by HS reagents, and an analysis of their action, has been recently described [17].

Discussion

It seems to be fairly well established by experiments published previously [5,8], and is shown above, that the passive Ca^{2+} transport observed in the presence of vanadate is a carrier-mediated process. This can be documented by its saturability [5], substrate specificity (Fig. 2), inhibition by substrate analogues (Fig. 1), inhibition by other inhibitors (Refs. 5, 8; Fig. 5). The low inhibitory potency of $^{45}Ca^{2+}$ transport by Ca^{2+}-like cations (Sr^{2+}, Ba^{2+}), which cross the membrane via the same pathway, suggests that the putative Ca^{2+} carrier is strongly selective for Ca^{2+}. Its transport specificity is similar to that of Ca^{2+} channels of excitable tissues [18–21] and is different from the Ca^{2+}-gating specificity of the Ca^{2+}-activated K^+ channel (Ref. 22, Fig. 4). It is clear from our results (Figs. 6–9) that Cu^{2+}, Zn^{2+} and Cd^{2+} cannot be tested for their ability to be transported by the putative Ca^{2+} carrier, because when applied in concentrations corresponding to $K_{m(Ca)}$ of the vanadate-induced $^{45}Ca^{2+}$ transport, they created a new pathway for the Ca^{2+} entry. A possibility remains open that they also enter red blood cells, if present in μmol/l concentrations, as was recently shown by Simons [12] for Pb^{2+} transport. Therefore, the question of the transport specificity of the transport system described above is still not answered definitively.

The role of HS groups in vanadate-induced Ca^{2+}-transport is suggested on the basis of its susceptibility to HS reagents (Fig. 5), and its stimulation by dithiothreitol (Table III). The Ca^{2+} transport is more (about two orders of magnitude) sensitive to HS reagents than is the anion transport [35] or the Gárdos effect as measured in red blood cells previously loaded with Ca^{2+} in inhibitor-free conditions [23]. This, together with other evidence (Figs. 8, 9), proves that the putative Ca^{2+} carrier is distinct from both the anion channel and the Ca^{2+}-activated K^+ channel. The absence of vanadate-induced $^{45}Ca^{2+}$ transport in pig red blood cells excludes also the conceivable explanation that a modified form of the Ca^{2+}-ATPase could mediate the passive transport of Ca^{2+} in vanadate-treated red blood cells.

Although positive identification of the putative Ca^{2+} carrier is not possible on the basis of our results, some data published by others could be mentioned which might be pertinent to this problem. Brown et al. [24], who studied the inhibition of the water transport across the red blood cell membrane by DTNB, found that in *N*-ethylmaleimide-treated membranes DTNB was bound to a single protein band only, which, according to its molecular weight, corresponded to band 3. This does not ultimately contradict our results, which exclude the identity of the anion channel and the putative Ca^{2+} carrier. It has been repeatedly shown that band 3 consists of several proteins [25,26].

Our results (Fig. 5) have shown the high potency of carboxylic compounds over *N*-ethylmaleimide. Regarding the results of Motais [31], the difference in the potencies of these compounds cannot be explained by their different ability to permeate the red blood cell membrane. It is more probable that the causative factor is the presence of the carboxylic groups in their structures. This implies the existence of a cationic group in the proximity of the target site of HS reagents. It must be mentioned that similar structure-effect relationships were observed in the inhibition of Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase by HS reagents (see Ref. 30, for review).

The high sensitivity of the inward-directed Ca^{2+} transport to heavy metals does not seem to be restricted to red blood cells. Recently it has been shown by Suszkiw et al. [32] that the inward transport of Ca^{2+} in synaptosomes is inhibited by Cd^{2+} and Pb^{2+} at micromolar concentrations of these ions. The same has been shown by Cooper and Manakis [33] for a Ca^{2+} current in a neuromuscular junction. Furthermore, Ca^{2+} uptake by a

neuronal cell line was inhibited by Cd^{2+}, Co^{2+} and Sr^{2+} in concentrations similar to those used in our observations [34]. However, all these authors failed to observe any biphasicity in the dose-effect curves. The latter may be linked to the properties of the anion channel [17], and, therefore, specific for the red blood cell membrane.

The nature of the defect in the vanadate-induced $^{45}Ca^{2+}$ transport in pig red blood cells has so far not been investigated. It should be mentioned at this point that pig red blood cells are defective also in the enzymatic mechanism involved in the metabolism of inositol phospholipids [27], which is known to participate in the process of cell activation [28], and they have an extremely low glucose metabolism as compared to red blood cells from other mammalian species [29]. Therefore, pig red blood cells may be useful as a 'mutant', for the investigation not only of the molecular mechanism of the passive Ca^{2+} transport in red blood cells, but also of the significance of the Ca^{2+} cycling across the red blood cell membrane.

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References

1 Cittadini, A.M. and Van Rossum, G.V.D. (1978) J. Physiol. (Lond.) 281, 29–43
2 Hinnen, R., Miyamoto, H. and Racker, E. (1979) J. Membrane Biol. 49, 309–324
3 Ferreira, H.G. and Lew, V.L. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 53–91, Academic press, New York
4 Szász, I., Sarkádi, B., Schubert, A. and Gárdos, G. (1978) Biochim. Biophys. Acta 512, 331–340
5 Varečka, Ľ. and Carafoli, E. (1982) J. Biol. Chem. 257, 7714–7721
6 Hoffman, J.F. and Laris, P.C. (1974) J. Physiol. (London) 239, 519–552
7 Lew, V.L., Tsien, R.Y., Miner, C. and Bookchin, R.M. (1982) Nature 298, 478–481
8 Stimpel, M., Neyses, L., Locher, R., Groth, H. and Vetter, V. (1984) J. Hypertension 2, Suppl. 3, 577–580
9 Schatzmann, H.J. (1982) in Membrane Transport of Calcium (Carafoli, E., ed.), pp. 41–108, Academic Press, London
10 Carafoli, E. and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279–301
11 Rabenstein, D.L., Isab, A.A., Kadima, W. and Mahanakrishnan, P. (1983) Biochim. Biophys. Acta 762, 531–554
12 Simons, T.J.B. (1984) FEBS Lett. 172, 250–254
13 Torchinskii, Yu.M. (1971) in Sulfhydryl and Disulfidic Groups in Proteins, Nauka, Moscow (in Russian)
14 Gárdos, G., Szász, I., Sarkádi, B. and Szebeni, J. (1980) in Membrane Transport in Erythrocytes (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 163–177, Munksgaard, Copenhagen
15 Lew, V.L. and Ferreira, H.G. (1978) Curr. Top. Membranes Transp. 10, 218–279
16 Sarkádi, B. and Tosteson, D.C. (1979) in Membrane Transport in Biology (Giebisch, G., Tosteson, D.C. and Ussing, H.H., eds.), Vol. II, pp. 117–160, Springer, Berlin
17 Lukacovic, M.F., Toon, M.R. and Solomon, A.K. (1984) Biochim. Biophys. Acta 772, 313–320
18 Kostyuk, P.G. (1982) in Membrane Transport of Calcium (Carafoli, E., ed.), pp. 1–39, Academic Press, London
19 McDonald, T.F. (1982) Annu. Rev. Physiol. 44, 425–434
20 Ochi, R. (1976) J. Physiol. (London) 263, 139–156
21 Anderson, M. (1979) J. Exp. Biol. 82, 227–238
22 Simons, T.J.B. (1976) J. Physiol. (London) 256, 227–244
23 Szász, I., Sarkádi, B. and Gárdos, G. (1978) Acta Biochim. Biophys. Acad. Sci. Hung. 13, 133–141
24 Brown, P.A., Feinstein, M.B. and Sha'afi, R.I. (1975) Nature 254, 523–525
25 Anselstetter, V. and Horstmann, H.-J. (1975) Eur. J. Biochem. 56, 259–269
26 Ideguchi, H., Matsuyama, H. and Hamasaki, N. (1982) Eur. J. Biochem. 125, 665–671
27 Allan, D., and Michell, R.H. (1977) Biochem. J. 166, 495–499
28 Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–147
29 Kim, M.D., McManus, T.J. and Bartlett, G.R. (1973) in Erythrocytes, Thrombocytes, Leucocytes (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds.), pp. 146–148, Georg Thieme Verlag, Stuttgart
30 Schuurmans Stekhoven, F. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76
31 Motais, R. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 197–220, Academic Press, New York
32 Suszkiw, J., Toth, G., Muawsky, M. and Cooper, G.P. (1984) Brain Res. 323, 31–46
33 Cooper, G.P. and Manakis, R.S. (1984) Eur. J. Pharmacol. 99, 251–256
34 Freedman, S.B., Dawson, G., Vilereal, M.L. and Miller, R.J. (1984) J. Neurosci. 4, 1453–1467
35 Reithmeier, R.A.F. (1983) Biochim. Biophys. Acta 732, 122–125