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THE MECHANISM OF VANADIUM ACTION ON SELECTIVE K^+-PERMEABILITY IN HUMAN ERYTHROCYTES

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Low concentrations of chelating agents such as EDTA prevent the air oxidation of vanadyl (VO^{2+}, +4 oxidation state) to vanadate (VO_3^-, +5 oxidation state). Under these conditions, the ionophore A23187 mediates the rapid entry of vanadyl into human erythrocytes. In the presence of A23187, vanadyl at concentrations in excess of EDTA gives rise to a dramatic increase in K^+ permeability, which is very similar to the Gardos Ca^{2+}-induced K^+ permeability increase with respect to ion selectivity, response to inhibitors, effects of pH, and stimulation by external K^+. In ultrapure media with very low Ca^{2+}, however, vanadyl has no effect on K^+ permeability. These experiments suggest that Ca^{2+} is displaced from EDTA by vanadyl and then enters the cell via A23187 where it triggers the increase in K^+ permeability. This hypothesis is confirmed by experiments demonstrating that vanadyl does displace Ca^{2+} from EDTA. Vanadate, an inhibitor of Ca^{2+}-ATPase, causes a selective increase in K^+ permeability in metabolically depleted cells, but the increase is abolished by low concentrations of EDTA, indicating that this effect is also due to entry of extracellular Ca^{2+}. Earlier observations of effects of vanadyl and vanadate on erythrocyte K^+ permeability can thus be explained on the basis of inhibition of the Ca^{2+} pump by vanadium, leading to an increase in intracellular Ca^{2+} concentration.

Introduction

In the last few years vanadium compounds have gained increasing attention because of their ubiquity in mammalian systems and selective biochemical actions (reviewed in Refs. 1–4). The oxy-cation vanadyl (VO^{2+}, +4 oxidation state) is one of the most stable ions in the first row of transition elements; it forms strong anionic, cationic, and neutral complexes with various types of ligands [4]. From this behaviour an interaction with a variety of biological functional groups can be predicted. However, vanadyl is most unstable at physiological pH and it is oxidized rapidly by air oxygen to form vanadate (+5 oxidation state). When vanadate enters the cell interior it is converted back to vanadyl by metabolism [3,5]. Although NADH and NADPH in connection with oxidoreductases are able to convert vanadate to vanadyl [5–7], most of the reduction in vivo seems to be accomplished by glutathione [8].

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Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

Recently Siemon et al. [9] reported that vanadium increased selective K^+ permeability in human erythrocytes and the effect was similar to that produced by calcium ions [10–12]. In human erythrocytes that had been ATP-depleted by incubation with iodoacetate and adenosine, vanadate induced a 10–15-fold increase of K^+ permeability. The selective change of K^+ permeability was brought about regardless of whether vanadate anion or vanadyl cation was added. In both cases, the presence of EDTA prevented the permeability change. Blocking of the anion-transport system by H_2DIDS was used to discriminate between the effects of vanadate anion and vanadyl cation in producing the K^+ loss. Since blocking of the anion-transport for vanadate anion had little if any effect on the efficiency and in view of the fact [5] that vanadate appears mostly as vanadyl in the cell interior, the suggestion was made that, like Ca^{2+} and Pb^{2+} [10–12], vanadyl (VO^{2+}) can open the 'potassium channel' in the erythrocyte membrane, possibly by binding to the same site which normally binds calcium.

An alternate mechanism for the effects of vanadium on potassium permeability, suggested by Rega (personal communication), would involve a direct action of calcium. Since vanadate is a potent inhibitor of the calcium pump [13,14] and since vanadyl might also compete with calcium for the Ca^{2+}-ATPase, it is possible that the inhibition of active calcium transport out of the cell leads to a buildup of calcium inside which then triggers the selective increase in potassium permeability. This might seem unlikely, since the cells used in the experiments of Siemon et al. [9] were metabolically depleted, so the level of calcium pump activity would be low. Nevertheless, since the calcium sensitivity of the K^+ permeability increase is enhanced in metabolically depleted cells [15], even a small change in intracellular calcium concentration might have a large effect. In addition, there is the possibility that the vanadyl cation, produced in the cell by reduction of vanadate, might bind to intracellular calcium-binding sites (amounting to about 20 μmol/l cells [11]) and thereby displace calcium, causing an increase in intracellular free calcium and thus increasing the potassium permeability.

To further investigate these possible mechanisms, we have made use of a new system to assess the effects of vanadyl on membrane transport. One problem in studying the effects of the vanadyl cation as opposed to effects of the vanadate anions is that vanadyl is readily oxidized to vanadate in solutions containing atmospheric oxygen. We have found that addition of small amounts of EDTA to the solutions prevents or greatly reduces this oxidative effect. To facilitate entry of vanadyl into the cell interior, we have made use of the ionophore A23187, which is capable not only of transporting ions such as calcium and magnesium, but also other divalent and trivalent ions [16] including, as we demonstrate below, vanadyl. An alternate method for getting vanadyl into the cell involves exposure of the cells to vanadate, which is rapidly taken up via the anion exchange system, and which is then reduced intracellularly to vanadyl. Data obtained using these techniques suggest that the apparent effects of vanadyl on potassium permeability are in fact the result of traces of calcium in the medium.

Methods

Materials. Valinomycin, oligomycin, and A23187 were obtained from Calbiochem, Inc., San Diego, CA. For the low calcium experiments, the acid form of A23187 was used. Quinidine was obtained from Sigma Chemical Co., St. Louis, MO, and H_2DIDS was from Molecular Probes, Inc., Junction City, OR. Vanadyl sulfate and sodium orthovanadate were obtained from Fisher Chemical Co., Pittsburgh, PA. For both vanadate and vanadyl, stock solutions of 15 mM were prepared in either 150 mM NaCl or 150 mM $NaNO_3$.

Cell preparation and flux measurements. Human red blood cells freshly drawn with heparin as anticoagulant were washed two times in 150 mM NaCl/1 mM EDTA, pH 7.6. For most experiments the cells were then washed two times in 150 mM $NaNO_3$/1 mM KNO_3/20 mM Hepes, pH 7.6 at room temperature. For the 'Gardos type' experiments, the cells were washed instead with 150 mM NaCl and 20 mM Hepes, pH 7.6. The cells were made up to a hematocrit of 50 percent and experiments were started by addition of cells to pre-warmed media, except for experiments with A23187 or valinomycin, when the experiment was

started by addition of ionophore. At various times, samples of the cell suspension were added to ice-cold 113 mM $MgCl_2$ containing 100 μM quinidine and the cells were washed three times with this solution. Na^+ and K^+ were determined by flame photometry and hemoglobin was measured as cyanomethemoglobin [17]. Net potassium efflux in the presence of valinomycin was determined by light-scattering as previously described [18].

Experiments in low calcium media. For these experiments, cells were washed two additional times in medium containing 1 mM KNO_3 and 160 mM $NaNO_3$ (Suprapur, E. Merck, Darmstadt, F.R.G.), and fluxes were measured in the same medium. For these experiments, freshly prepared 15 mM vanadyl sulfate (Fluka AG, Buchs, Switzerland) dissolved in distilled water was used. Ca^{2+} was determined with an Atomic Absorption Spectrophotometer Model 372 (Perkin-Elmer) equipped with a graphite furnace H6A-76B. The calcium concentration in the flux medium was 0.24 μM.

Absorbance spectra. The spectra were recorded on a Cary Model 210 spectrophotometer (Fig. 1) or on an Aminco DW_2 double beam spectrophotometer (Fig. 10).

Electron spin resonance measurements. Electron spin resonance (ESR) spectra were taken on a Varian E-9 instrument equipped with a TM 100 cavity. The samples were contained in 50 μl glass capillaries inside a standard ESR quartz tube. The frequency of measurement was about 9.24 GHz, with a modulation amplitude of 8 gauss. For measurements of red cells, the cells were first centrifuged at 12800 × *g* in an Eppendorf micro-centrifuge and the pellet was then drawn up into a 50 μl capillary tube. Data were normalized to the number of cells in the tube by measuring the hemoglobin content. This was done by rinsing the contents of the tube into Drabkin's solution and measuring the absorbance at 540 nm.

Results

Stabilization of vanadyl in the presence of EDTA

Vanadyl ion exhibits a characteristic absorbance at 750 nm, which is not shown by the vanadate ion, so the absorbance at this wavelength can be used to monitor changes in the amount of vanadyl in solution [4]. Vanadyl is stable at acid pH, but is unstable in neutral solutions in the presence of oxygen. As shown in Fig. 1, the absorbance at 750 nm of a solution of vanadyl at neutral pH first increases, and then decreases to near zero. The decrease in absorbance at 750 nm is accompanied by an increase in absorption around 260 nm, characteristic of vanadate. The initial increase in absorbance at 750 nm probably is due to the formation of vanadyl complexes [19].

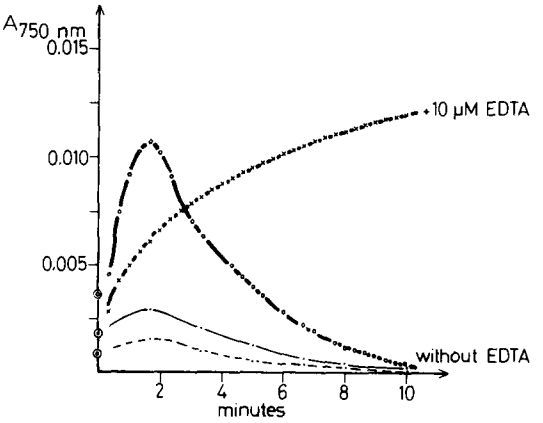

Fig. 1. Changes in absorption at 750 nm with time after addition of vanadyl. The solution contained 150 mM NaCl/20 mM Hepes, pH 7.6 at room temperature. Upper curve with 10 μM EDTA and lower curves without EDTA. At zero time 50 (·-·-·-·), 100 (·-·-·) or 200 μM vanadyl (O——O), or 200 μM vanadyl in the presence of EDTA (×——×) was added. EDTA can be replaced by EGTA or diethyldithiocarbamic acid.

If a small amount of EDTA is added to the solution, far less than the amount of vanadyl, the absorbance at 750 nm exhibits a monotonic increase (Fig. 1), probably again due to complex formation, and there is no indication of a peak at 260 nm characteristic of vanadate ion. Other chelators, such as EGTA and sodium diethyldithiocarbamic acid, are also effective in preventing the conversion of vanadyl to vanadate. These data suggest that the oxidation of vanadyl is catalyzed by traces of metal ions which can be removed by the chelating agents.

A23187 mediates the entry of vanadyl into red cells

Fig. 2a shows the electron paramagnetic resonance spectrum observed in a pellet of cells which have been exposed to 500 μM vanadyl for 15 min

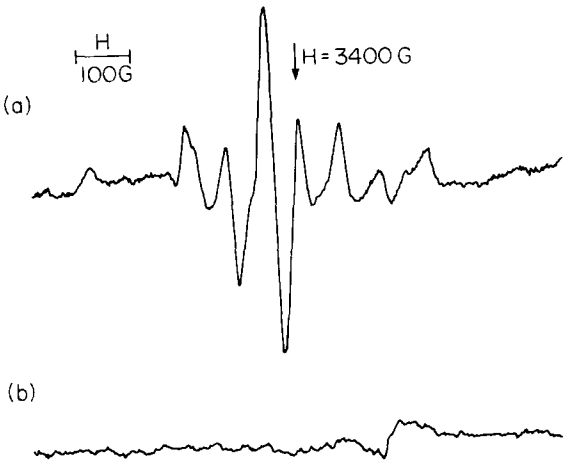

Fig. 2. (a) ESR spectrum (first derivative) of packed red cells which had been exposed to 500 μM vanadyl sulfate at 10 percent hematocrit in 150 mM NaCl, 20 mM Hepes, pH 7.6 in the presence of 10 μM EDTA and 2 μM A23187 for 15 min at 37°C. The spectrum was corrected for the background seen in cells which were not exposed to vanadyl sulfate or ionophore (b). Frequency: 9.24 GHz; power 90 mW.

at 37°C in the presence of the ionophore A23187. It is typical for the slow motion regime with rotational correlation times $> 10^{-9}$ s/rad showing the parallel and perpendicular features of the vanadyl hyperfine coupling ('powder-type' spectrum) [4]. This spectrum is seen when vanadyl is bound to intracellular components [5] and indicates that vanadyl has entered the cells. The lower spectrum (Fig. 2b) is that which is observed in the absence of vanadyl and A23187.

The same spectrum as in Fig. 2a was obtained in the presence of H_2DIDS to inhibit the anion exchange system and therefore to preclude the possibility that vanadyl might enter the cells by being oxidized to vanadate and then transported into the cell interior by the anion exchange system, where it would be reduced again to vanadyl (Table I). When A23187 was not present, the ESR signal was reduced to very low levels (Table I, compare lines 1 and 2), indicating that the uptake was dependent on the presence of the ionophore A23187. If vanadyl was added first, then EDTA, and then A23187, the uptake of vanadyl decreased as the time interval between addition of vanadyl and EDTA was increased (Table I, lines 2, 3 and 4). This result is expected on the basis of the time-course of oxidation of vanadyl to vanadate (Fig. 1), and supports the concept that the entry of vanadyl (and not vanadate) is directly mediated by the ionophore, A23187

TABLE I

ESR DETECTION OF INTRACELLULAR VANADYL IN THE PRESENCE OR ABSENCE OF A23187

Cells were washed as described in Methods and were made up to 50 percent hematocrit in 50 mM NaCl/100 mM KCl/20 mM Hepes, pH 7.4 at 37°C. Cells were incubated at 1 percent hematocrit at 37°C in the same medium, with the additions indicated in the table. When present, H_2DIDS was at a concentration of 20 μM, EDTA at 20 μM, and A23187 at 2 μM. The order of addition was cells, then 2 min later vanadyl or vanadate, then 2 min later A23187. The cells were incubated for 5 min after the last addition, then centrifuged and taken up in 50 μl capillary tubes for measurement of EPR spectra as described in Methods.

Sample	VO^{2+} (μM)	VO_3^- (μM)	H_2DIDS	Time of EDTA addition [a]	A23187	cm [b]	cm/Hb [c]	Relative cm/Hb [d]
1	100	0	+	2 min before	−	1.9	0.086	7.9
2	100	0	+	2 min before	+	23.6	1.082	100.0
3	100	0	+	15 s after	+	21.1	0.876	81.0
4	100	0	+	60 s after	+	9.0	0.377	34.8
5	0	100	+	–	−	1.8	0.083	7.7
6	0	100	+	–	+	1.2	0.048	4.4
7	0	100	−	–	−	2.8	0.109	10.1

[a] EDTA was added before or after vanadyl as indicated.
[b] Height of central peak of vanadyl powder spectrum (see Fig. 2a).
[c] Height of peak in cm divided by hemoglobin content of cells in capillary in g/100 ml.
[d] cm/Hb expressed as a percentage of the value for sample 2.

Characteristics of the potassium permeability increase in the presence of vanadyl and A23187

As shown in Fig. 3, in the presence of 10 μM EDTA, the addition of 20 μM vanadyl in the presence of 2 μM A23187 leads to a very rapid loss of potassium from the cells, with no corresponding increase in sodium uptake. The magnitude of this permeability increase is about half as great as that seen with 20 μM calcium. Like the Ca^{2+}-stimulated K^+ flux [10,11], the K^+ flux stimulated by vanadyl is strongly inhibited by quinidine and by oligomycin. The inhibition with 0.5 μg/ml oligomycin is in about the same order as with 60 μM quinidine (data not shown). The addition of 20 μM vanadate, instead of vanadyl, had no measurable effect on the rate of potassium efflux (data not shown). The lack of effect of vanadate is also evidenced by the decrease in the flux if some time elapses between the addition of vanadyl and the addition of EDTA. If vanadyl, for example, has been added 2 minutes before EDTA the effect on potassium efflux is only about 10%. This would be expected if the effect is specific for vanadyl, since under these conditions the vanadyl ion is rapidly converted to vanadate (see Fig. 1).

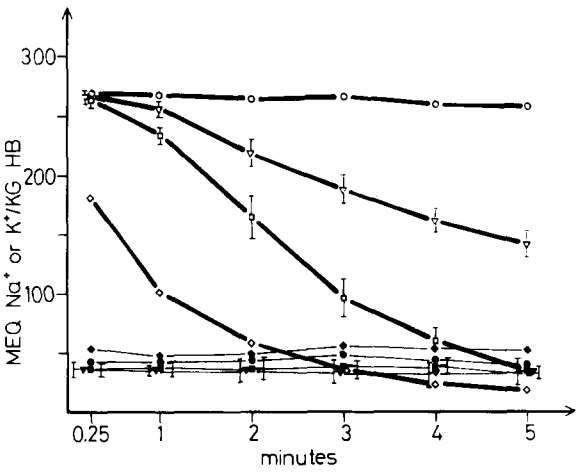

Fig. 3. Na^+ and K^+ content of erythrocytes in milliequivalents (MEQ) per kg hemoglobin (HB) as a function of time. Erythrocytes were suspended at 0.5 percent in 150 mM $NaNO_3$, 1 mM KCl or KOH, 10 μM EDTA and 20 mM Hepes solution, pH 7.4 at 37°C. At time zero 2 μM A23187 was added. Control without A23187 (○———○, K^+ and ●———●, Na^+); 60 μM quinidine and 20 μM VO^{2+} (▽———▽, K^+ and ▼———▼, Na^+); 20 μM VO^{2+} (□———□, K^+ and ■———■, Na^+); 20 μM Ca^{2+} (◇———◇, K^+ and ◆———◆, Na^+). VO^{2+} or Ca^{2+} were added together with A23187. Points with bars represent mean values of four experiments ± S.D.

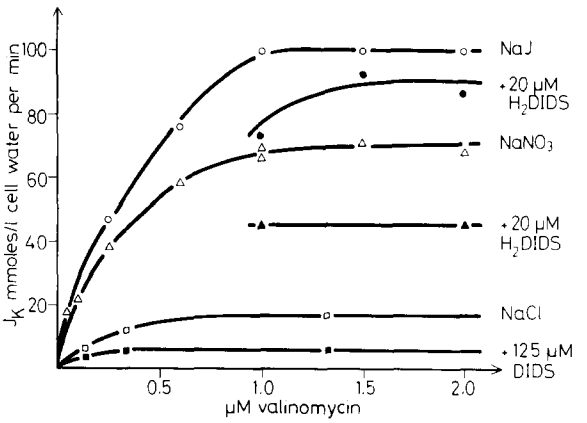

Fig. 4. Net potassium efflux (mmol K^+/l cell water per min) as a function of valinomycin concentration. Washed red cells were suspended at 0.067 percent hematocrit in 150 mM NaI/1 mM KCl/10 μM EDTA and 20 mM Hepes, pH 7.4 and 37°C without (○———○) and with 20 μM H_2DIDS (●———●); 150 mM $NaNO_3$/1 mM KCl/10 μM EDTA and 20 mM Hepes, pH 7.4 and 37°C without (△———△) and with 20 μM H_2DIDS (▲———▲); 145 mM NaCl/1 mM KCl and 20 mM Tris-HCl, pH 7.1 and 37°C without (□———□) and with 125 μM DIDS (■———■).

As previously shown in various laboratories, if the potassium permeability of the membrane is greatly increased, either by the action of intracellular calcium [20,21] or by external addition of an ionophore such as valinomycin [18,22], the rate of net KCl efflux is limited by the net permeability of the anion, chloride. For example, in Fig. 4, as the valinomycin concentration is raised in chloride medium, the efflux of potassium reaches a plateau. In nitrate media, this plateau is reached at much higher rates of net potassium efflux, indicating that the nitrate anion has a much higher net permeability than does the chloride anion, approx. 4-times as large. The net potassium efflux in the presence of vanadyl plus A23187 was about 40 mmol/l cell water per min. In nitrate media, this is within the range where the flux is nearly proportional to the potassium permeability, and is not limited by the anion permeability. In chloride media, however, at such a high potassium permeability, the net potassium efflux would be limited by the chloride permeability to a much lower value. In fact, when experiments were run in chlo-

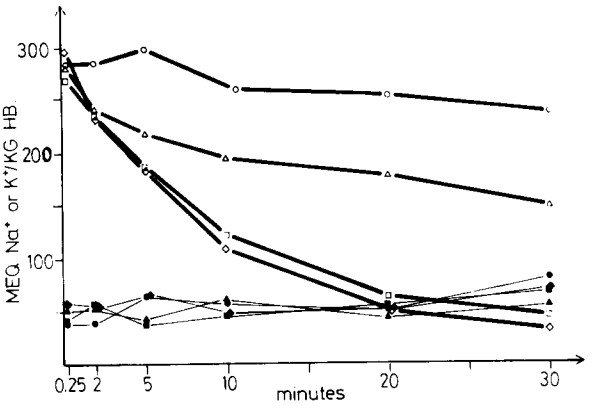

Fig. 5. Na^+ and K^+ content of erythrocytes in milliequivalents (MEQ) per kg hemoglobin as a function of time. Erythrocytes were suspended at 0.5 percent hematocrit in 150 mM $NaNO_3$/ 10 μM EDTA/and 20 mM Hepes solution, pH 6.5 at 37°C. The experiment was started with 2 μM A23187. fluxes were measured in the presence of 100 μM VO^{2+} without K^+ (○——○, K^+ and ●——●, Na^+); 100 μM VO^{2+} and 1 mM KNO_3 (△——△, K^+ and ▲——▲, Na^+); 100 μM VO^{2+} and 5 mM KNO_3 (□——□, K^+ and ■——■, Na^+); 100 μM VO^{2+} and 10 mM KNO_3 (◇——◇, K^+ and ◆——◆, Na^+).

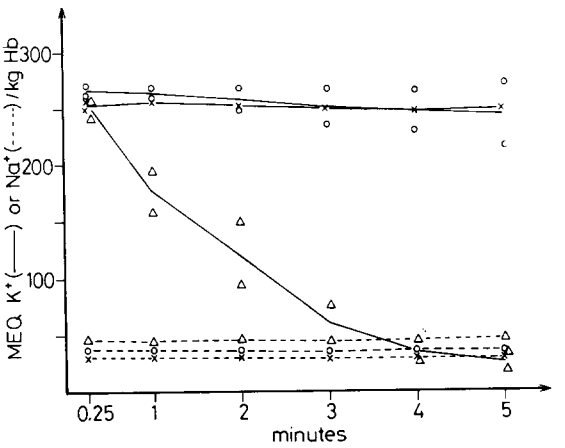

Fig. 6. Na^+ and K^+ content of erythrocytes in milliequivalents (MEQ) per kg hemoglobin as a function of time. Erythrocytes were suspended at 0.5 percent hematocrit in 160 mM $NaNO_3$ (Suprapur)/1 mM KNO_3, pH 7 at 37°C. The Ca^{2+} concentration was 0.24 μM. The experiment was started with 1 μM A23187. Fluxes were measured with A23187 alone (×——×, K^+ and ×---×, Na^+); with 100 μM VO^{2+} and 10 μM EDTA (○——○, K^+ and ○---○, Na^+); or with 5 μM $CaCl_2$ (△——△, K^+ and △---△, Na^+). The lines represent the mean of two experiments.

ride media (data not shown), a much lower flux was observed, corresponding to the plateau flux in the presence of valinomycin. Therefore, in order to measure the flux under conditions where the potassium efflux reflects changes in potassium permeability, rather than anion permeability, all of the experiments reported in this section were done in nitrate media.

The vanadyl-stimulated K^+ flux was also similar to the Gardos effect in its pH dependence (not shown), being much slower at low pH [23]. As with the Gardos effect [23], it was possible to demonstrate at low pH that the K^+ efflux was strongly affected by the presence of extracellular potassium (Fig. 5). Half-activation of the flux was seen at an extracellular potassium concentration between 2 and 5 mM. All of these close similarities to the Gardos effect could mean either that vanadyl is capable of directly stimulating the system involved in the Gardos effect or else that vanadyl in some way causes the release of calcium, which then acts on the Gardos system. Although the EDTA present in the medium in these experiments would be expected to chelate calcium, in all cases an effect with vanadyl was only seen at vanadyl concentrations in excess of the EDTA concentration. Thus, if vanadyl were to bind strongly to EDTA, it might be able to displace calcium and thereby to trigger the permeability change. To avoid this problem, experiments were carried out in media with very low calcium concentrations, but in the absence of EDTA.

Experiments in low calcium media

The results of experiments with vanadyl and A23187 in nitrate media made with ultrapure components to minimize the calcium concentration are shown in Fig. 6. The upper line shows that with A23187 alone very little potassium loss takes place, indicating that the calcium concentration in the ultrapure medium is lower than that required to trigger the Ca-stimulated increase in K^+ permeability. When 5 μM calcium was added, as in the lower trace, the potassium permeability was increased and potassium rapidly flowed out of the cells, with no corresponding increase in sodium permeability. When 100 μM vanadyl was added, in the presence of A23187 and 10 μM EDTA,

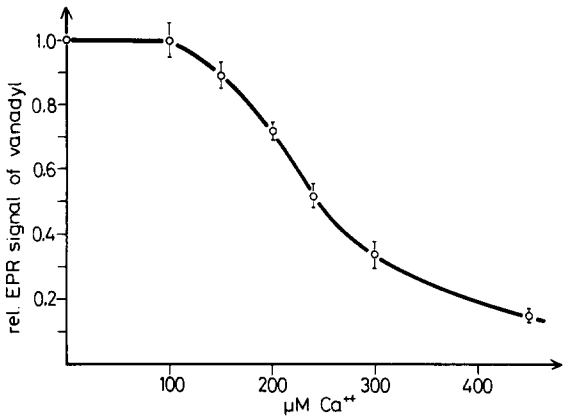

Fig. 7. Effects of added calcium on the ESR signal height (peak-to-peak) of 300 μM VO^{2+} in neutral solution (pH 7.6) at room temperature containing 150 mM NaCl/20 mM Hepes and 300 μM EDTA. The concentration of calcium chloride given on the abscisssa was added before addition of 300 μM VO^{2+}. Bars indicate standard deviation of measurements taken from the various peaks of the vanadyl spectrum.

there was no increase in Na^+ or K^+ permeability, and the results were indistinguishable from those with A23187 alone. The results strongly suggest that intracellular vanadyl by itself has no measurable effect on the potassium permeability of the membrane, but rather that the observed permeability changes are probably caused by a different mechanism, probably involving an increase in free intracellular calcium.

Other evidence for calcium involvement in the effect of vanadyl

If vanadyl by itself has no effect on potassium permeability, the very large changes in potassium permeability seen earlier when vanadyl was added to an EDTA-containing medium can be most readily explained by displacement of calcium from EDTA by vanadyl. To be effective in doing this at such low concentrations, vanadyl would have to have a very high affinity for EDTA. This was tested by making use of the electron paramagnetic resonance technique to measure vanadyl bound to EDTA. If vanadyl and EDTA are added in equal amounts, a large ESR signal characteristic of vanadyl bound to EDTA is seen. Vanadyl in excess of EDTA does not increase the magnitude of the signal, probably because such additional vanadyl forms complexes which are ESR silent [4]. Thus, the ESR signal can be used to monitor the amount of vanadyl complexed to EDTA. Fig. 7 shows such an experiment, in which an amount of vanadyl equal to the amount of EDTA was added to solutions containing various amounts of calcium. The calcium concentration required to displace half of the vanadyl from EDTA was approximately the same as the vanadyl concentration. Since under these conditions half of the EDTA is complexed with calcium and the other half with vanadyl, at nearly equal concentrations of the metal ions, this result would indicate that the affinities of EDTA for calcium and vanadyl are very similar under these conditions. This is in contrast to literature reports [3] that the affinity of vanadyl for EDTA is considerably less than that of calcium. These data therefore indicate that the proposed mechanism of action of vanadyl by displacing calcium from EDTA is reasonable in light of the relative affinities of the two ions for the chelator. In view of the fact that vanadate does not cause a significant increase in potassium flux with A23187 present, except at concentrations over 200 μM, it would seem likely that the affinity of EDTA for vanadate is much less than for vanadyl or calcium.

In addition to the ESR data, analysis of the dose-response for the effect of vanadyl on potassium efflux, in the presence of A23187 and EDTA, supports the calcium-displacement hypothesis. No significant effect of vanadyl on potassium efflux is seen until the vanadyl concentration is greater than 15 μM at 10 μM EDTA concentration. This would be expected since traces of calcium will not be displaced until there is more vanadyl than EDTA present. When the vanadyl concentration exceeds the EDTA concentration by 10 μM, the K^+ flux jumps to a very high value. Further increases in the vanadyl concentration do not increase the K^+ flux any further, but instead lead to a gradual decrease in the K^+ efflux. This could be explained if high concentrations of vanadyl compete with the traces of calcium for entry via the A23187 ionophore.

Experiments with vanadate in the presence of EDTA

If vanadate has a much lower affinity for EDTA than does vanadyl, then according to the calcium displacement hypothesis, if EDTA and vanadate

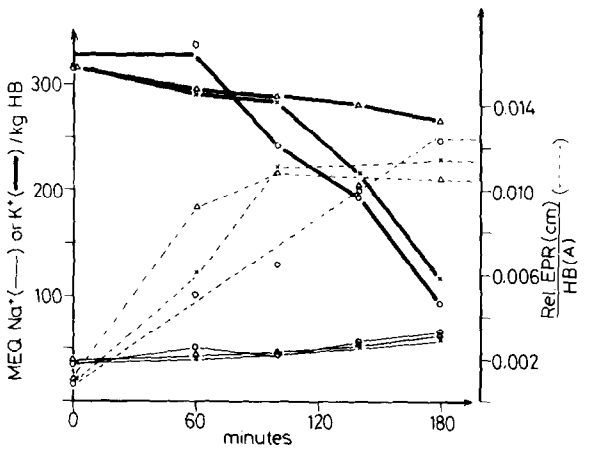

Fig. 8. Na^+ and K^+ content of erythrocytes in milliequivalents (MEQ) per kg hemoglobin (left ordinate) and relative EPR signal in cm of the central peak of the vanadyl powder spectrum per absorbance of hemoglobin (Rel. EPR (cm)/HB (*A*)) (right ordinate) as a function of time. Erythrocytes were suspended at 10 percent hematocrit in 150 mM NaCl/5 mM adenosine/2.5 mM iodoacetic acid/0.5 mM VO_3^-/20 mM Hepes, pH 7.4 at 37°C without EDTA (○▬▬○, K^+; ○——○, Na^+; ○- - -○, rel. EPR); with 10 μM EDTA (×▬▬×, K^+; ×——×, Na^+; ×- - -×, rel. EPR); or with 50 μM EDTA (△▬▬△, K^+; △——△, Na^+; △- - -△, rel. EPR). Mean of two experiments.

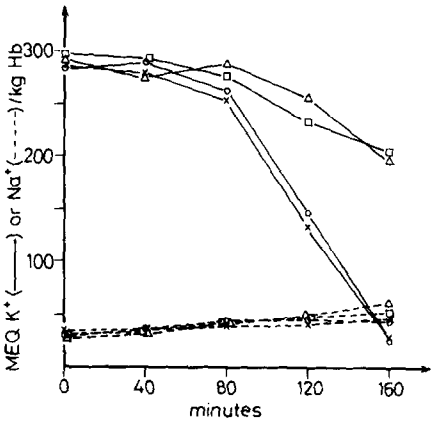

Fig. 9. Na^+ and K^+ content of erythrocytes in milliequivalents (MEQ) per kg hemoglobin as a function of time. Erythrocytes were suspended at 0.5 percent hematocrit in 150 mM NaCl/0.5 mM $CaCl_2$/2 mM adenosine/2.5 mM iodoacetic acid/0.5 mM VO_3^-/20 mM Hepes, pH 7.4 at 37°C without quinidine (×——×, ○——○, K^+ and ×- - -×, ○- - -○, Na^+); or with 100 μM quinidine (△——△, □——□, K^+ and △- - -△, □- - -□, Na^+).

are present in the medium, the EDTA should chelate traces of calcium, and vanadate will not bind to EDTA strongly enough to release the calcium. Fig. 8 shows such an experiment. With no EDTA outside, vanadate enters the cells and inhibits the calcium pump, thereby permitting the traces of calcium in the medium to cause an increase in potassium permeability. With 10 μM EDTA, this effect is reduced, and with 50 μM EDTA it is almost completely abolished. In both cases, the ESR spectra (right hand ordinate) show that vanadate has entered the cells and has been converted to vanadyl. The inhibitory effect of EDTA is therefore probably due to its ability to chelate traces of extracellular calcium, thereby preventing calcium accumulation in the cells despite the inhibition of the calcium pump by vanadate.

Influence of vanadate on the Gardos effect

If vanadate inhibits the calcium pump, then when cells are metabolically depleted and are suspended in high calcium media, vanadate should prevent calcium efflux and therefore should cause a greater accumulation of intracellular calcium than would ordinarily occur. This in turn should result in a further increase in K^+ permeability. Fig. 9 shows that 0.5 mM vanadate does stimulate K^+ efflux, with little effect on Na^+ permeability. Under these circumstances, the K^+ permeability increase in the presence of vanadate is about 8-fold larger than in the control without vanadate (data not shown). The nearly complete inhibition of this increased K^+ efflux by 100 μM quinidine, together with the specificity of the permeability change for K^+, suggests strongly that the effect of vanadate involves a stimulation of the Gardos system, and not some other nonspecific effect.

Rate of oxidation of vanadyl to vanadate

From the data presented so far, it seems that all of the effects of vanadyl and vanadate can be explained on the basis of a combination of displacement of calcium from chelators such as EDTA by vanadyl and inhibition of the calcium pump by vanadate. It would seem difficult, however, to explain on this basis why Siemon et al. [9] observed no difference between the effects of vanadate and vanadyl on K^+ efflux, even when the experiments were performed under a nitrogen atmosphere to prevent oxidation of vanadyl to

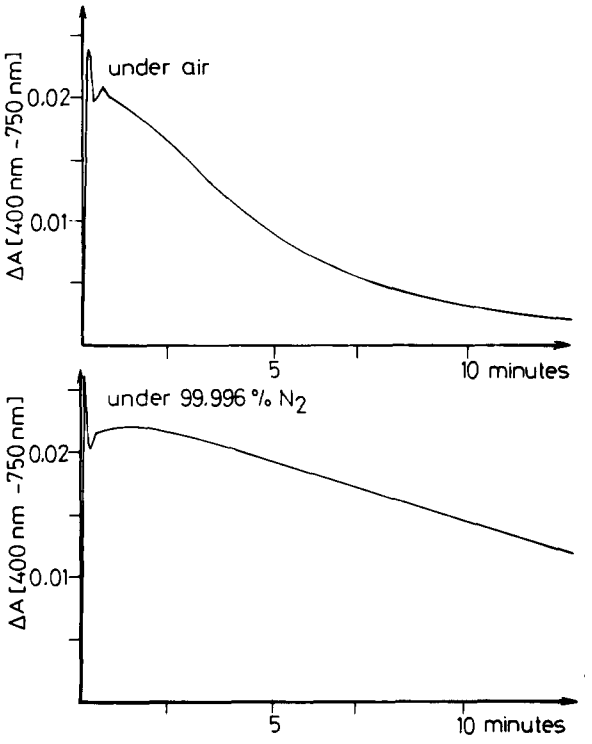

Fig. 10. Difference in absorption ΔA (400 nm − 750 nm) as a function of time. At zero time 200 μm VO^{2+} was added to 150 mM NaCl/20 mM Hepes, pH 7.4 at 38°C. The upper curve was recorded under air and the lower curve under a N_2 atmosphere.

vanadate. Under these conditions, with no chelators or ionophore present, only vanadate would be expected to have an effect on K^+ efflux.

To see whether or not some vanadate might be produced from vanadyl under the conditions of Siemon et al. [9], the conversion of vanadyl to vanadate was monitored by measuring the difference in absorption at the wavelengths of 400 nm and 750 nm (Fig. 10). The fall in this absorption difference reflects the decay of vanadyl complexes to vanadate [19]. In the presence of air, vanadyl is rapidly oxidized, so that by 10 min it is almost completely converted to vanadate. Surprinsingly, even under a nitrogen atmosphere, sufficient traces of oxygen were present to cause a less rapid but still considerable production of vanadate. Thus, in the experiments of Siemon et al. [9], even when vanadyl was added to the cells under nitrogen, sufficient vanadate was present to account for the observed effects on K^+ permeability on the basis of Ca^{2+}-ATPase inhibition, since Ca^{2+}-ATPase is half-inhibited by only 1.5–3 μM vanadate [13,14].

Discussion

Effects of vanadyl ion

The experiments presented above indicate that several apparently complex effects of vanadium ions can be explained on the basis of interactions with calcium chelators and the calcium pump.

In the case of vanadyl ion, it appears that EDTA and other chelating agents are very useful in stabilizing the vanadyl ion in the +4 oxidation state, even in the presence of atmospheric oxygen (Fig. 1). Under these conditions, the ionophore A23187 efficienctly mediates the transfer of vanadyl, as well as calcium and other divalent ions, across the red cell membrane (Fig. 2 and Table I). When EDTA is present in the solution along with A23187, vanadyl but not vanadate causes a very large increase in the potassium permeability of the membrane, which in every respect tested is similar to the Gardos calcium-stimulated K^+ flux (Figs. 3 and 5). In very low calcium media, however, vanadyl does not affect the K^+ permeability (Fig. 6).

The key which resolves this apparent contradiction is the finding that vanadyl at physiological pH has a high affinity for EDTA, in contrast to a report in the literature [3], and thus that it can displace calcium from EDTA. With A23187 present, this calcium can enter the cell and trigger the K^+ permeability increase. In ordinary (not ultrapure) solutions, A23187 alone, without added Ca^{2+}, increases K^+ permeability (data not shown). Since 10 μM EDTA prevents this increase, it must require Ca^{2+} concentrations less than 10 μM. Thus, under these conditions, the system must be in the state with high sensitivity to calcium [10,11,15].

In view of its ability to carry vanadyl ion, A23187 may provide a useful means for introducing vanadyl into the cell interior in red cells and other cells. When this technique is used, however, one must be very careful to insure that the calcium concentration is kept very low, so that the effects of vanadyl can be distinguished from those of calcium. In this regard, chelating agents are of little help, because of the ability of vanadyl to displace calcium from them.

Effects of vanadate

In the presence of calcium, vanadate has been shown to cause an increase in red cell potassium permeability in blood bank cells, presumably because of the increase in intracellular calcium following inhibition of the Ca^{2+}-ATPase [24]. We have observed (Fig. 9) a similar similar stimulation of K^+ efflux by vanadate under conditions which initiate the Gardos effect, and have found that this increased permeability is specific for K^+ with respect to Na^+ and is inhibited by low concentrations of quinidine.

Even if calcium is not added to the medium (Fig. 8), vanadate causes a significant increase in selective K^+ permeability, presumably because under these conditions the Ca^{2+}-pump is inhibited and the very small increase in internal Ca^{2+} is sufficient to stimulate the Gardos system. This effect is completely abolished when EDTA is added to the medium, indicating both that it is dependent on Ca^{2+} influx and confirming the fact that vanadyl ion, which is produced in the cells by reduction of vanadate under these conditions, itself has no measurable effect on K^+ efflux. All of these results can be readily interpreted on the basis that internal vanadate inhibits the Ca^{2+}-ATPase [13,14].

The experiment in Fig. 8 would also seem to speak against the possibility that displacement of calcium from intracellular binding sites by vanadyl plays any role in the K^+ permeability increase, since in this case vanadyl is formed within the cells and should be able to displace intracellular bound calcium. Yet no calcium-stimulated K^+ permeability increase is seen in the absence of extracellular Ca^{2+}.

Explanation of earlier results

It is of some interest to ask whether or not the results of Siemon et al. [9] can be entirely accounted for on the basis of the two vanadium effects discussed above. In the experiments with vanadate present in the external medium, an increase in K^+ efflux was seen, both in the presence and absence of 20 μM H_2DIDS, which inhibits the red cell anion exchange mechanism. In the absence of H_2DIDS, it would seem that vanadate simply enters the cells, inhibits the Ca^{2+}-ATPase, and causes an increase in intracellular Ca^{2+} concentration, which triggers the Gardos effect. Even in the presence of inhibitors of the anion exchange, the uptake of vanadate is not completely inhibited [5], so that at the high vanadate concentrations used sufficient vanadate should enter the cells to inhibit the Ca^{2+}-ATPase, if the Ca^{2+}-ATPase is half-inhibited at 1.2–3 μM vanadate [13,14].

According to this hypothesis, an effect of inhibitors of the anion exchange system should be seen if the vanadate concentration is reduced. In accordance with this prediction, we have observed that DIDS causes some inhibition of the K^+ efflux at a vanadate concentration of 0.5 mM (data not shown). Varecka and Carafoli [24] have reported that high (400 μM) DIDS concentrations dissolved in 0.5 percent dimethyl sulfoxide completely inhibit the Ca^{2+}-stimulated K^+ efflux seen in the presence of vanadate and have suggested that this is due to an effect of DIDS on net anion permeability. However, the dimethyl sulfoxide in the control experiment already exerted a strong inhibitory effect, so that the combined effect of DIDS and dimethyl sulfoxide may not be related simply to inhibition of the net anion permeability. Since we observed that DIDS only inhibits net chloride transport to a maximum of about 70 percent [18], some other effect in addition to anion permeability inhibition must be invoked to explain the almost complete inhibition of K^+ flux.

The experiments of Siemon et al. [9] with vanadyl are interpretable on the same basis if, as we have shown here (Fig. 10), most of the vanadyl is converted to vanadate within the 60-min interval before the K^+ efflux begins, even in the presence of a nitrogen atmosphere. Thus, all of the earlier observations can be accounted for on the basis of inhibition of the Ca^{2+} pump by internal vanadate, as suggested by Rega.

Sensitivity of the system to Ca^{2+} and vanadate

It should be noted that the data presented here confirm other indications that very small traces of calcium can have a very large effect on the K^+ permeability of erythrocytes. Thus, calcium effects can be seen even without added calcium, unless special precautions are taken to exclude calcium from the media. Similarly, very small amounts of vanadate, such as those which enter the cell when vanadyl is supplied to the medium and when

anion transport is inhibited by DIDS, seem to be capable of significantly inhibiting the Ca^{2+} pump. Our experiments suggest that under these conditions the Ca^{2+} pump has a very high affinity for vanadate, as has been observed in the presence of high internal K^+ concentrations [13,14,25].

If our interpretation of the results in terms of a simple effect of vanadate on the calcium pump is correct, this has the interesting implication that even in metabolically depleted cells, the calcium pump still continues to function, and that it is an important factor in determining the intracellular calcium concentration and hence the magnitude of the Ca^{2+}-stimulated K^+ permeability increase. This would seem a reasonable possibility in view of the fact that passive calcium flow across the membrane is so slow that very little ATP need be consumed by the pump to counteract this leak.

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