Ca^{2+}-activated K^+ Channel and the Activation of Ca^{2+} Influx in Vanadate-treated Red Blood Cells

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Abstract. The mechanism by which K^+ inhibits vanadate-induced $^{45}Ca^{2+}$ influx by human red blood cells (RBC) was studied using several independent approaches The following results were found

1 The inhibitory effect of K^+ was absent when RBC were loaded with a Ca^{2+}-chelator This treatment at the same time inhibited the vanadate-induced K^+ efflux, and the membrane hyperpolarization induced by Ca^{2+} in vanadate-treated cells

2 The potency of K^+, Rb^+, and Cs^+ to inhibit vanadate-induced Ca^{2+} influx corresponded to their ability to depolarize the RBC membrane via the Ca^{2+}-activated K^+ channel (K(Ca))

3 Inhibition of the vanadate-induced $^{45}Ca^{2+}$ influx by a protonophore proceeded in parallel with the inhibition of the vanadate-plus-Ca^{2+}-induced membrane hyperpolarization

4 Valinomycin in part released the inhibition of the vanadate-induced Ca^{2+} influx by known K(Ca) inhibitors (quinine, oligomycin, 4-aminopyridine) but not by inhibitors of the Ca^{2+} channel (Cu^{2+}, HS-reagents organic Ca^{2+} channel blockers)

5 K^+ did not inhibit the vanadate-induced Ca^{2+} influx in dog RBC which have K(Ca) but no transmembrane K^+ gradient

The inhibition of the vanadate-induced Ca^{2+} influx by external K^+ appears to be due to the elimination of the electrical component of the Ca^{2+}-motive force imposed by opening of the K(Ca) This implies that the Ca^{2+} carrier mediating the influx of Ca^{2+} in the presence of vanadate is of uniport type, and that the activity of K(Ca) may serve as a supporting element for Ca^{2+} influx

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Abbreviations: DTNB 5,5′-dithiobis-(dinitrobenzoic acid), FCCP – fluorocarbonyl cyanophenylhydrazone, K(Ca) Ca^{2+}-activated K^+ channel OM oligomycin, RBC red blood cells, TPP tetraphenylphosphonium, TCS 3 3′4′ 5 tetrachlorosalicylanilide, VM valinomycin

Introduction

Although the role of the Ca^{2+} activated K^+ channels (K(Ca)) in excitable cells is widely recognized, their presence and function in non-excitable cells is much less understood In red blood cells (RBC) the activity of the K(Ca) was discovered by Gardos as Ca^{2+}-sensitive K^+ efflux (further referred to as the Gardos effect) from metabolically poisoned red cells several decades ago (Gardos 1958) Since then it has been thoroughly studied (see Lew and Ferreira 1978 Schwartz and Passow 1983 for review) and shown that the Gardos effect is closely linked to the Ca^{2+} influx as suggested by the concommitant appearance of Ca^{2+} influx and K^+ efflux using different experimental procedures such as ATP-depletion (Gardos 1958 Ferreira and Lew 1977) treatment with propranolol (Mannmen 1970 Szasz and Gardos 1974 Szasz et al 1977) vanadate (Varečka and Carafoli 1982) or menadione (Fuhrmann et al 1985) On the other hand, the Ca^{2+} influx into RBC loaded with Ca^{2+} chelators like BAPTA (Lew et al 1982), or quin-2 (McNamara and Wiley 1986, Pokudin and Orlov 1986), has not been reported so far to be accompanied by K(Ca) activation, and by the Gárdos effect

The mechanism(s) of activation of the Ca^{2+} influx observed during the activation of the Gardos effect and the properties of the Ca^{2+} influx pathway(s) observed by the above methods are mostly obscure and difficult to compare because no comprehensive data to be compared are available It seems probable that Ca^{2+} influx induced by ATP-depletion, vanadate, or Ca^{2+} chelator entrapment is mediated by a carrier although its detailed description could only be obtained in vanadate-treated cells(Varečka and Carafoli 1982 Varečka et al 1986 Varečka and Peteriajova 1990)

The link of Ca^{2+} influx to K^+ efflux which is the crucial aspect of the activation of the Gardos effect, does not seem to exist if the Ca^{2+} influx is induced by the entrapment of a Ca^{2+} chelator This is indicated by the fact that the Ca^{2+} influx induced by Ca^{2+} chelator entrapment is insensitive to the ionic composition of the suspension medium (McNamara and Wiley 1986 Pokudin and Orlov 1986) unlike that induced by ATP depletion (Ferreira and Lew 1977), or vanadate (Varečka and Carafoli 1982), where it was found to be inhibited by increasing concentrations or by dissipation of the K^+ gradient by ionophores The difference in the sensitivity to

extracellular K^+, if compared with the different capabilities of the above methods to activate K(Ca), indicates that K(Ca) activation is a prerequisite for the inhibition of Ca^{2+} influx by extracellular K^+ This implies that it is the specific action of K(Ca) which contributes to the driving force of the Ca^{2+} influx in vanadate-treated, or ATP-depleted RBC, and thereby promotes the influx of Ca^{2+} into the cytoplasm There is evidence for this suggestion in propranolol-treated cells (Szász et al 1977) and in ATP-depleted cells (Gárdos et al 1980, Szász et al 1981) We used vanadate-treated RBC as a model to test the above suggestion and found evidence to support it

Materials and Methods

Red blood cell suspension

Blood from healthy volunteers of both sexes was withdrawn by venipuncture into EDTA-containing anticoagulant (5 mmol/l), and was used within 3 days, stored at 0–4 °C Red blood cells (RBC) were isolated after centrifugation of the blood (10 min at 600 × *g*) and aspiration of the supernatant with the buffy coat, and three-fold washing with and, finally, suspending into a medium containing (in mmol/l) 20 Tris-HCl, pH 7 3, 130 NaCl, 5 KCl, 10 glucose (further referred to as the suspension medium), to the final haematocrit of 30%, and immediately used for experiment Dog blood was withdrawn by venipuncture from non-medicated dogs raised in the vivarium of the Drug Research Institute in Modra, Slovakia, by the same procedure Dog red cells were, however, used for experiments on the same day

Red cell loading with permeant Ca chelators

The 30% suspension was loaded with 75 μmol/l (if not indicated otherwise in the Figures) BAPTA/AM (or quin-2/AM) (and controls with 0 5% DMSO) for 30 min at 37 °C in the presence of 0 2 mmol/l EGTA, cooled to 25 °C, centrifuged, and adjusted to the same volume of medium of desired composition Test and control suspensions were handled as pairs in individual experiments

Vanadate-induced Ca^{2+} influx

The influx of Ca^{2+} was measured with the radionuclide ^{45}Ca, after repetitive washing to remove extracellular radioactivity, as described previously (Varečka and Carafoli 1982) Aliquots of 30% suspension were preincubated with 1 mmol/l $NaVO_3$ for 15 min at 25 °C, and $^{45}CaCl_2$ (2 5 mmol/l) was added and incubated for 60 min at the same temperature unless indicated differently The incubation was stopped by addition of the same volume of the stopping medium containing (in mmol/l) 20 Tris-HCl, pH 7 3, 75 KCl, 60 NaCl, 10 glucose, and 1 EDTA (further referred to as the stopping medium), and by rapid centrifugation of the sample in

a microcentrifuge. The supernatant was sucked off, and the pellet was washed with the stopping medium three more times. Finally, the pellet was precipitated with 10% trichloroacetic acid (TCA) containing 20 mmol/l $LaCl_3$, the precipitate was centrifuged and the pellet was taken for liquid scintillation counting. Control cells without vanadate were treated in parallel. When inhibitors were tested, the same volume of solvent (DMSO, methanol, max. 0.5% V/V) was added to the control samples. All samples were run in duplicates, and the average value of parallel samples (+/− standard error) is given in the Figures. The standard error is indicated by bars when exceeding the size of the symbols. Experiments illustrated in the Figures were typical of at least two (as a rule, three) separate experiments.

Measurement of $^{40}Ca^{2+}$-induced $^{45}Ca^{2+}$ efflux

The total volume of the RBC suspension to be used in the experiment was preloaded with $^{45}Ca^{2+}$ in the presence of vanadate as described above, for 60 min, chilled on ice, and kept in an ice-water mixture until complete removal of external radioactivity by repetitive washing with the stopping medium (two washings) and two washings in the suspension medium containing 0.2 mmol/l EGTA. Finally, the suspensions were centrifuged at 0–4 °C for 5 min at 2000 × *g*, the supernatants were quantitatively aspirated, and the pellets were adjusted to the original volume with the suspension medium containing 0.2 mmol/l EGTA. Aliquots of this suspension kept on ice were pipetted into test tubes pre-warmed to 25 °C for exactly 5 min and $^{40}CaCl_2$ was added to the test suspensions. No addition was made to the control suspensions. At the time shown (usually 0, 2, 5, 10, 20 and 50 min), 0.5 ml aliquots were withdrawn and immediately centrifuged through a silicone oil layer. The radioactivity of both the supernatants and the pellets was measured after TCA precipitation of proteins. All measurements were done, and the results processed as described in the preceding paragraph.

Measurement of the Gárdos effect

The Gárdos effect was monitored either by measuring the net K^+ efflux by flame photometry, or by the release of $^{86}Rb^+$ from cells pre-equilibrated with it. Net K^+ efflux was measured in the vanadate-treated cell suspension as described above. At time zero, $^{40}CaCl_2$ (2.5 mmol/l) was added, and aliquots of the suspension were withdrawn after a 50 min incubation at 25 °C, and after spinning down RBC through a silicone oil layer the supernatant was used for flame photometry. Control test tubes were treated in parallel. When ^{86}Rb was used as tracer, 2 MBq of the carrier-free radionuclide was incubated with 7.5 ml of whole blood overnight at 0 °C, and RBC were isolated as described previously. Other steps were identical with those used for the measurement of net K^+ efflux except that radioactivity was measured in the supernatant. Values shown in Figures correspond to what is described in the preceding paragraphs.

Measurement of changes in ^{3}H-TPP$^+$ distribution

EGTA (0 2 mmol/l) was added to the suspension of RBC (pre-treated with BAPTA/AM, or controls with DMSO, as indicated in the Figure) Aliquots of the 20% suspension were supplemented with 1 mmol/l $NaVO_3$ and 25 μmol/l ^{3}H-TPP$^+$ (approx 20,000 cpm/assay), and were equilibrated for 25 min At time zero, aliquots were withdrawn and centrifuged immediately through a layer of dibutylphthalate (DBP), and the phases were separated quantitatively from each other immediately after the centrifugation At 5 min, $CaCl_2$ (2 7 mmol/l) was added After 13 min and 55 min aliquots were withdrawn and treated as above Control cells were treated similarly The radioactivity from pellets was extracted into ethanol, and separated from the cell debris by centrifugation Correction of medium and pellet radioactivity for the radioactivity trapped in dibutylphthalate did not significantly influence the obtained values The radioactivity of the supernatants was measured in parallel, and this is presented in the Figures

Chemicals

^{3}H-Tetraphenyl phosphonium chloride from Radiochemical Centre, Amersham (Bucks, England), $^{45}CaCl_2$ from Radiochemical Works (Swierk, Poland), ^{86}RbCl from Isocommerz (Dresden, Germany) valinomycin, ionomycin, quin-2/AM, and BAPTA/
AM from Calbiochem (Luzern, Switzerland), quinine and oligomycin from Sigma (St Louis USA), 4-aminopyridine from Fluka (Buchs, Switzerland), Tris base, FCCP, DTNB and dibutylphthalate from Serva (Heidelberg, Germany), 3,3′,4′,5-tetrachloro-salicylanilide (TCS) from Eastman-Kodak Comp (Rochester, USA), and the methyl-phenyl silicone oil from Lučební závody Kolín (Czech Republic) $NaVO_3$ was from Reachim, (Moscow, Russia) Other chemicals (all of analytical grade) were purchased from Lachema, (Brno, Czech Republic)

Results

Ca^{2+} chelator entrapment prevents the inhibition of Ca^{2+} influx by K^+, and simultaneously blocks the Gardos effect and accompanying membrane hyperpolarization

Fig 1 shows the effect of the substitution of Na^+ ions for K^+ on the vanadate-induced $^{45}Ca^{2+}$ influx which was observed earlier (Vaĭečka and Carafoli 1982), and its modification by cytoplasmic Ca buffering This was brought about by preincubation of cells with tetraacetoxymethyl esters of Ca chelators BAPTA/AM or quin −2/AM, as described by other authors (Lew et al 1982, McNamara and Wiley 1986, Pokudin and Orlov 1986) Treatment of RBC with 75 μmol/l quin-2/AM abolished the inhibition of the vanadate-induced $^{45}Ca^{2+}$ influx by high extracellular K^+ (Fig 1*A*) When the cells were treated with lower concentrations of the permeant chelator some inhibition of Ca^{2+} influx by K^+ was observed (not shown)

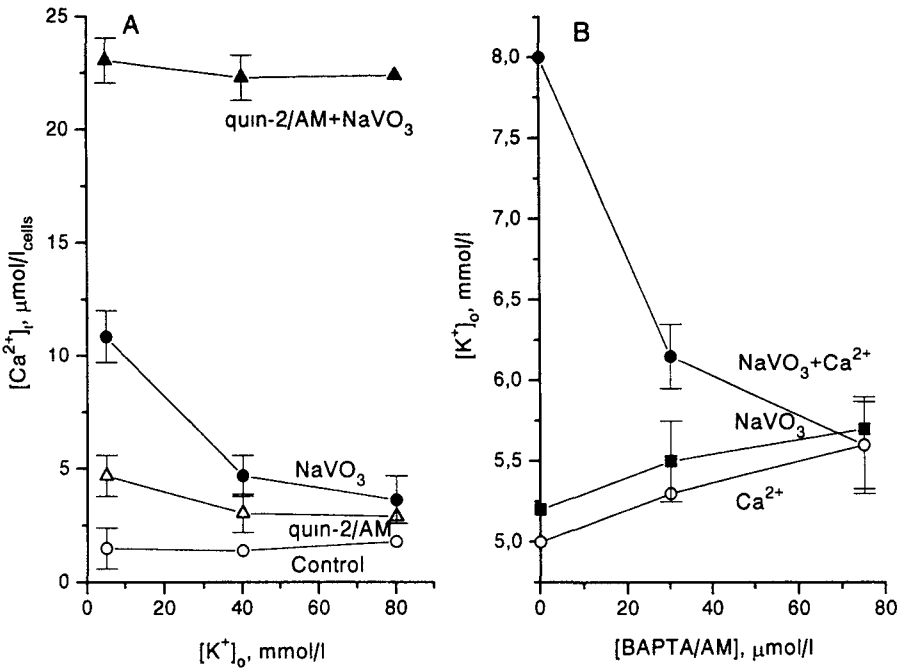

Figure 1. Effects of Ca^{2+} chelator treatment on the (*A*) K^+ gradient dependence of the vanadate-induced $^{45}Ca^{2+}$ influx, (*B*) vanadate and Ca^{2+}-induced K^+ efflux (the Gardos effect) *A* Cells loaded with 75 µmol/l quin-2/AM (triangles), control cells without chelator (0 5% v/v DMSO) (circles) 1 mmol/l $NaVO_3$ (closed symbols), controls without vanadate (open symbols) *B* RBC pre-treated with BAPTA/AM and with 1 mmol/l $NaVO_3$ (closed symbols) $^{40}Ca^{2+}$ (2 5 mmol/l) added (circles) Controls without Ca^{2+} (squares) and without $NaVO_3$ (open circles)

Treatment with the permeant Ca chelator stimulated the influx both in the absence, and in the presence of vanadate The increment of the influx caused by the presence of the Ca chelator in the presence of vanadate exceeded that in the control without vanadate (Fig 1*A*) Under the same conditions, Ca^{2+}-buffering significantly inhibited the vanadate *plus* Ca^{2+}-induced Gárdos effect (Fig 1*B*) The highest concentration of BAPTA/AM used (75 µmol/l) completely inhibited the K^+ efflux In order to localize the site of the action of the Ca^{2+}-chelator, the effect of the BAPTA/AM treatment on membrane potential changes induced by the opening of K(Ca) was investigated Membrane potential changes were monitored by changes of ^{3}H-labelled tetraphenyl-phosphonium (^{3}H-TPP$^+$) distribution These indicated hyperpolarization of the RBC membrane triggered by Ca^{2+} in vanadate-treated cells as compared with control without vanadate, and its inhibition by the BAPTA/AM treatment (Fig 2*A*) This was not due to a decrease of Ca^{2+}-ATPase

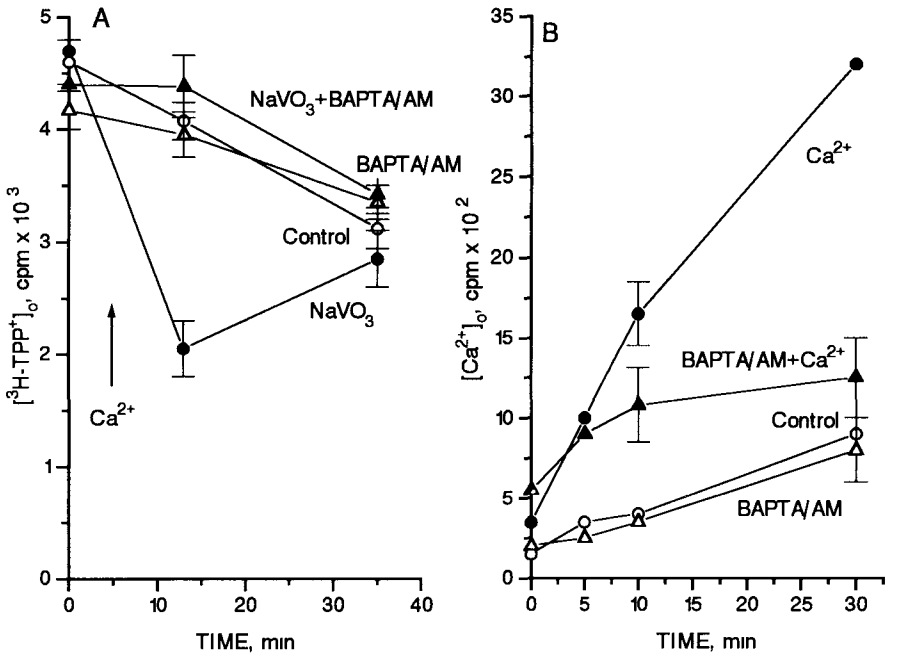

Figure 2. Effects of BAPTA/AM treatment on the (*A*) ^{3}H-TPP$^+$ influx during the Gárdos effect, and (*B*) $^{40}Ca^{2+}$-induced $^{45}Ca^{2+}$-efflux from vanadate-loaded human RBC *A* RBC loaded with BAPTA/AM (triangles), controls with DMSO (circles) and supplemented with $NaVO_3$ and ^{3}H-TPP$^+$ 2 7 mmol/l $^{40}Ca^{2+}$ added at 5 min (closed symbols), no addition in controls (open symbols) Only radioactivity of supernatants is presented *B* RBC preincubated with BAPTA/AM (triangles), or DMSO (circles) and subsequently loaded with $^{45}Ca^{2+}$ by means of $NaVO_3$, and washed out of extracellular radioactivity as described in Materials and Methods $^{40}Ca^{2+}$ added (closed symbols) or no addition made (open symbols) Only the supernatant radioactivity is shown

inhibition which was not always complete in our experimental conditions (Varečka and Carafoli 1982, Varečka et al 1986) by the Ca chelator The corresponding evidence was provided by the measurement of $^{40}Ca^{2+}$-induced $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded RBC This showed that the radioactivity pumped out of the cells in the initial phase of the exchange process was not significantly different in either vanadate or vanadate plus BAPTA-loaded RBC (Fig 2*B*) The loss of sensitivity of the vanadate-induced Ca^{2+} influx to the increase of extracellular K^+ concentration upon the buffering of cytoplasmic Ca^{2+} suggests that the rise of cytoplasmic Ca^{2+} concentration and the subsequent opening of K(Ca) are prerequisite for the inhibition of the Ca^{2+} influx by K^+ In order to corroborate this suggestion by independent evidence, we studied the effect of the experimental varying of the membrane potential during the vanadate-induced $^{45}Ca^{2+}$ influx and the use of

"mutant" RBC species lacking Na^+, K^+ATPase

Alkali metal cations influence the vanadate-induced $^{45}Ca^{2+}$ influx and the membrane potential in correlation with their selectivity for K(Ca)

The relative permeabilities of human RBC K(Ca) to monovalent cations are Rb^+ (1 0), K^+ (0 67), Cs^+ (0 05) (Simmons 1976) We assumed that replacement of Na^+ ions with other alkali metal cations display different degrees of inhibition as compared with K^+, and that the efficiency of the inhibition can be expected to correlate with the permeability of the individual ions through K(Ca) if their inhibitory effect is due to the change of the membrane potential Such an assumption seems to be justified because in isotonic solutions, the net transport of KCl after the opening of K(Ca) was shown to be limited by chloride efflux (Schubert and Sarkádi 1977) In our experiments the concentration of chloride was kept constant This assumption is valid for human RBC which have a negligible activity of the Ca^{2+}-activated Na^+/H^+ exchanger (Escobales and Canessa 1985) and no known active Na^+ channel as compared with other cells As shown in Fig 3*A*, the order of potency of K^+, Rb^+ and Cs^+ in inhibiting the vanadate-induced Ca^{2+} transport follows the order of their known permeabilities in K(Ca) (Simmons 1976), unlike with impermeant choline which stimulated Ca^{2+} influx (Fig 3*A*) The measurement of the ^{3}H-TPP$^+$ distribution changes induced by Ca^{2+} in vanadate-treated RBC in media with different degrees of substitution of Na^+ with K^+, Rb^+, and Cs^+, respectively, showed that depolarization was maximal in Rb^+ media followed by K^+ media and, finally, by Cs^+ media (Fig 3*B*) These results are in accordance with the notion that the influence of alkali metal ions is mediated by a K(Ca)-imposed membrane potential

Uncoupler inhibits the vanadate-induced Ca^{2+} influx and dissipates the membrane potential in a Ca^{2+}-dependent manner

It was found previously (Varečka and Carafoli 1982) that the uncoupler, FCCP, strongly inhibited the vanadate-induced Ca^{2+} influx and stimulated the Gárdos effect Because FCCP and derivatives are known to interact with HS-groups (Drobnica and Šturdík 1979), and the vanadate-induced Ca^{2+} influx is sensitive to HS-inhibitors (Varečka et al 1986), another inhibitor, 3,3′,4′,5-tetrachlorosalicylanilide (TCS) was used in these experiments As shown in Fig 4*A*, TCS also suppressed the Ca^{2+} influx when present in a concentration of 10 μmol/l At the same time, it also strongly (but not completely) inhibited hyperpolarization of the membrane potential induced by Ca^{2+} and vanadate (Fig 4*B*) Thus, effects of TCS on both vanadate-induced $^{45}Ca^{2+}$ influx and membrane potential changes suggest that to a significant extent the inhibition of $^{45}Ca^{2+}$ influx is due to the collapse of the membrane potential change induced by the opening of K(Ca)

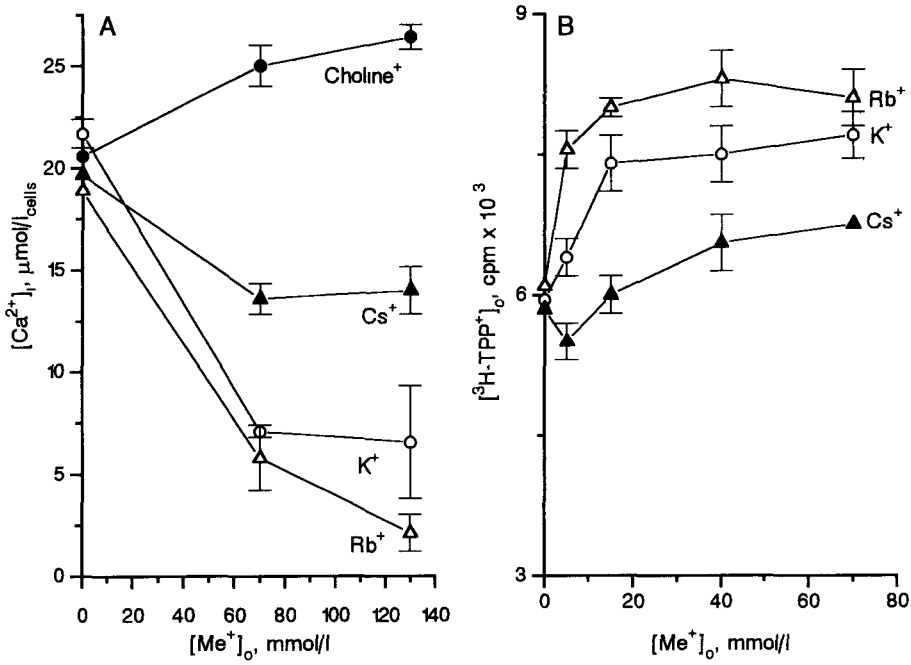

Figure 3. The substitution of Na^+ by other monovalent cations affects similarly the vanadate-induced $^{45}Ca^{2+}$ influx (*A*), and change of the ^{3}H-TPP$^+$ distribution (*B*) *A* RBC (90 % haematocrit) pretreated with 2 5 mmol/l $NaVO_3$ transferred by a positive displacement pipette into test tubes containing media with increasing concentrations of KCl (open circles), RbCl (open triangles), CsCl (closed triangles) and choline chloride (closed circles) instead of NaCl (final concentrations indicated in the Figure, 5 mmol/l KCl was present in all test tubes), and the isotonicity was kept constant Controls without vanadate were treated as above, their values did not differ from each other, and were less than 1 μmol/l_{cells} *B* RBC treated with $NaVO_3$ and transferred into KCl (open circles), RbCl (open triangles), and CsCl (closed triangles) media (final concentrations indicated) 2 7 mmol/l $^{40}Ca^{2+}$ added ^{3}H-TPP$^+$ activity in the supernatant after 8 min incubation after addition of Ca^{2+} is shown No differences caused by substituents were found in controls without vanadate (not shown)

The inhibition of vanadate-induced Ca influx by K(Ca) inhibitors is released by valinomycin

It is conceivable that the inhibition of K(Ca) by its specific inhibitor should eliminate the shift of the membrane potential induced by K(Ca) activation It was found previously that quinidine, an inhibitor of the K(Ca) (Armando-Hardy et al 1975), inhibited the vanadate-induced $^{45}Ca^{2+}$ influx (Varečka and Carafoli 1982) This may be a consequence of the inhibition of either K(Ca) or of the Ca^{2+} car-

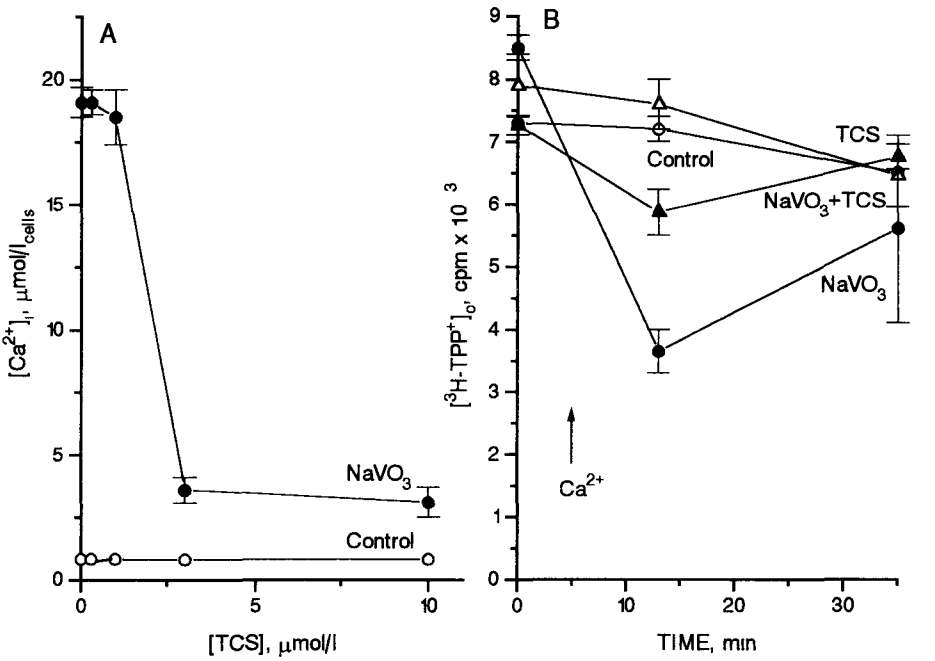

Figure 4. The effects of uncoupler on the vanadate-induced $^{45}Ca^{2+}$ influx (*A*), and on the vanadate plus Ca^{2+}-induced membrane potential changes (*B*) *A* The $^{45}Ca^{2+}$ influx in the suspension medium in the presence of TCS (3,3,4,5 – tetrachloro salicyl anilide) with 1 mmol/l $NaVO_3$ (closed circles) Controls without $NaVO_3$ (open circles) *B* The ^{3}H-TPP^{+} activity in the medium induced by Ca^{2+} in vanadate-treated cells in the presence of 3 μmol/l TCS (triangles), or methanol (0 5% v/v) (circles) $^{40}Ca^{2+}$ (2 7 mmol/l) added at 5 min (closed symbols), controls without Ca^{2+} (open symbols)

ner, because the specificity of quinidine has not been yet tested Later, also other compounds known as inhibitors of K(Ca), such as oligomycin (Blum and Hoffman 1972), or 4-aminopyridine (Thomsen and Wilson 1983, Christé et al 1995) were tested Both suppressed the vanadate-induced $^{45}Ca^{2+}$ influx (and also the vanadate-induced Gárdos effect) It was assumed that the addition of the K^{+}-specific ionophore valinomycin (VM) to cells containing K(Ca) inhibitors would restore the membrane potential and reverses the inhibition provided that it has been caused by the inhibition of the K(Ca) but not of the Ca^{2+} carrier, and that the Ca^{2+} carrier is uniporter As control substances, Ca^{2+} channel blockers, such as verapamil, or nifedipin, or divalent ions, as well as HS-reagents which were found to inhibit the vanadate-induced $^{45}Ca^{2+}$ influx (Varečka et al 1986), were tested It was found that valinomycin never reversed the inhibition of the vanadate-induced $^{45}Ca^{2+}$ influx in the group of $^{45}Ca^{2+}$ influx inhibitors but increased it in the group

Table 1. Inhibition of vanadate-induced $^{45}Ca^{2+}$ influx by inhibitors of Ca^{2+} channels and of K(Ca), and reversal of the inhibition by valinomycin

	$^{45}Ca^{2+}$ influx, (μmol/l_{cells})	
	Control	Inhibitor
Quinine 1 5 mmol/l ($n = 3$)		
Control	36 1 ± 0 6	8 0 ± 0 5
+VM (1 μg/ml)	34 7 ± 1 6	14 9 ± 0 6
4-aminopyridine, 10 mmol/l ($n = 2$)		
Control	24 1 ± 1 1	11 1 ± 1 0
+ VM (1 μg/ml)	20 0 ± 1 0	14 8 ± 0 7
Oligomycin 10 μg/ml ($n = 4$)		
Control	29 8 ± 0 9	10 9 ± 0 7
+ VM (1 μg/ml)	27 6 ± 0 5	15 0 ± 0 4
Nifedipin, 60 μmol/l ($n = 4$)		
Control	19 9 ± 1 9	5 0 ± 0 2
+ VM (1 μg/ml)	17 0 ± 1 5	4 7 ± 0 5
Cu^{2+} 10 μmol/l ($n = 3$)		
Control	27 0 ± 1 4	13 3 ± 0 0
+ VM (1 μg/ml)	23 0 ± 0 7	12 3 ± 0 6
DTNB 25 μmol/l ($n = 1$)		
Control	34 9 ± 1 6	12 9
+ VM (1 μg/ml)	26 8 ± 0 8	7 6
Verapamil 140 μmol/l ($n = 1$)		
Control	5 5	1 8
+ VM (1 μg/ml)	4 9	1 5

Presented are results from typical experiments (the total number of experiments performed is shown in parentheses) where several concentrations of inhibitors were used The effects of verapamil and DTNB were tested in pilot experiments only Each value is an average from duplicate assays ± S E except in the experiment with DTNB where only single assays were done in the presence of the inhibitor

of inhibitors of the K(Ca) (Table 1) Valinomycin slightly inhibited the influx in control test tubes (Table 1), although on several occasions also a small (about 10%) stimulation was observed (not shown) In some experiments no stimulatory effect of VM was observed even in the presence of K(Ca) inhibitors but no stimulation of the influx by VM inhibited by the Ca^{2+} channel inhibitors was ever

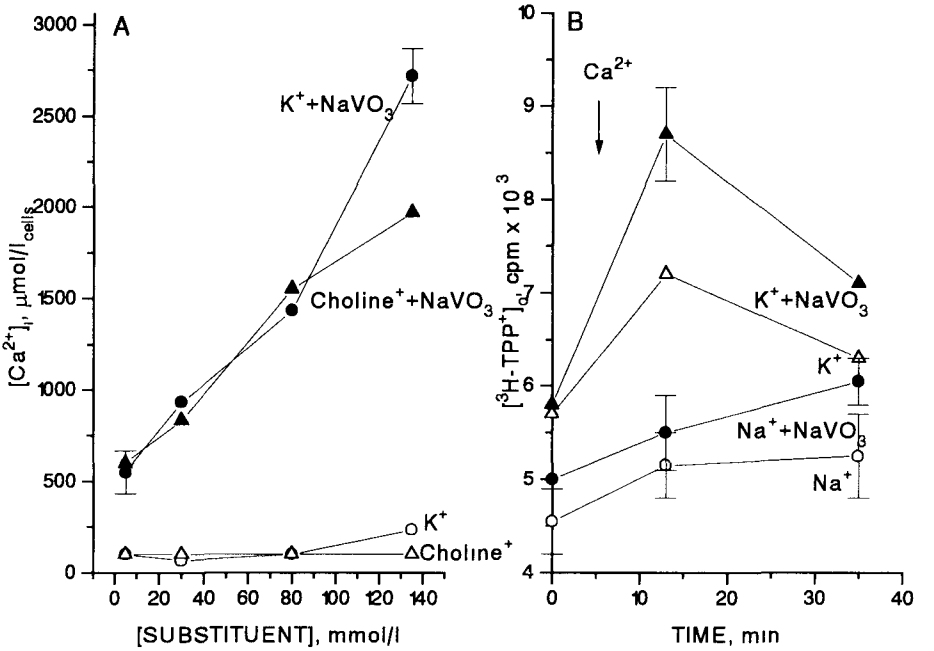

Figure 5. The $^{45}Ca^{2+}$ influx induced by vanadate in dog RBC in media with various ionic compositions (*A*) and the changes of the ^{3}H-TPP^{+} distribution induced by Ca^{2+} (*B*) *A* RBC pre-loaded with 1 mmol/l $NaVO_3$ in the Na^{+}-medium which was subsequently replaced by K^{+} (closed circles), or choline^{+} medium (closed triangles) containing 1 mmol/l $NaVO_3$ so that their final concentration was as indicated Control suspensions (open symbols) were treated in parallel *B* RBC suspended (20% haematocrit) in the Na^{+}-rich (circles) or K^{+}-rich medium (triangles) and the suspensions were supplemented with 0 2 mmol/l EGTA Vanadate (0 1 mmol/l) added to all samples followed by ^{3}H-TPP^{+} $^{40}Ca^{2+}$ (2 7 mmol/l) was added at 8 min (closed symbols)

observed Thus, the dual effect of VM on the $^{45}Ca^{2+}$ influx inhibited by both Ca^{2+} channel inhibitors and inhibitors of K(Ca) supports the possibility that the K(Ca) inhibitors eliminate a part of the Ca^{2+}-motive force during their actions, which could be released by valinomycin

Open Ca^{2+}-activated K^{+} channel without K^{+} gradient cannot mediate the inhibition of the vanadate-induced $^{45}Ca^{2+}$ influx with K^{+}

Dog RBC do not possess Na^{+},K^{+}-ATPase in their membrane and have no transmembrane gradients of monovalent ions (Parker 1977) but vanadate was able to induce $^{45}Ca^{2+}$ influx also in these cells Its potency in dog RBC exceeded that in human RBC by about one order of magnitude At 1 mmol/l vanadate, i e , under conditions used in experiments with human RBC, the influx was so massive

that it almost led to an equilibration of Ca^{2+} across the membrane (Fig 5A) The difference could not be ascribed to any experimentally introduced factor, and it represents a true interspecies difference Also the $^{45}Ca^{2+}$ influx was found to be saturatable with respect to Ca^{2+} and perhaps, like in human RBC, is mediated by a carrier (not shown) The vanadate-induced $^{45}Ca^{2+}$ influx in dog RBC was not reduced when Na^+ were stoichiometrically substituted with either K^+, or choline$^+$ In contrast, it was stimulated regardless of the substituent (Fig 5A) When most of the Na^+ were substituted the influx also increased in controls without vanadate added (Fig 5A) Probably, both responses are due to the reversal of the Na^+/Ca^{2+} antiporter activity known to be present in the dog RBC membrane The same response of dog RBC was obtained when 0 1 mmol/l $NaVO_3$ was used as inducer with the extent of the influx only being about 20% (not shown) No changes in the transmembrane distribution of ^3H TPP$^+$ induced by Ca^{2+} in vanadate treated cells and in controls in Na^+ rich medium (i e suspension medium) were found (Fig 5B) However in a K^+ rich medium the release of ^3H TPP$^+$ in the presence and to some extent also in the absence of vanadate was observed This could be explained by the proposal that K(Ca) is also activated in dog RBC by the increase of the cytoplasmic Ca^{2+} concentration Direct evidence supporting this proposal was

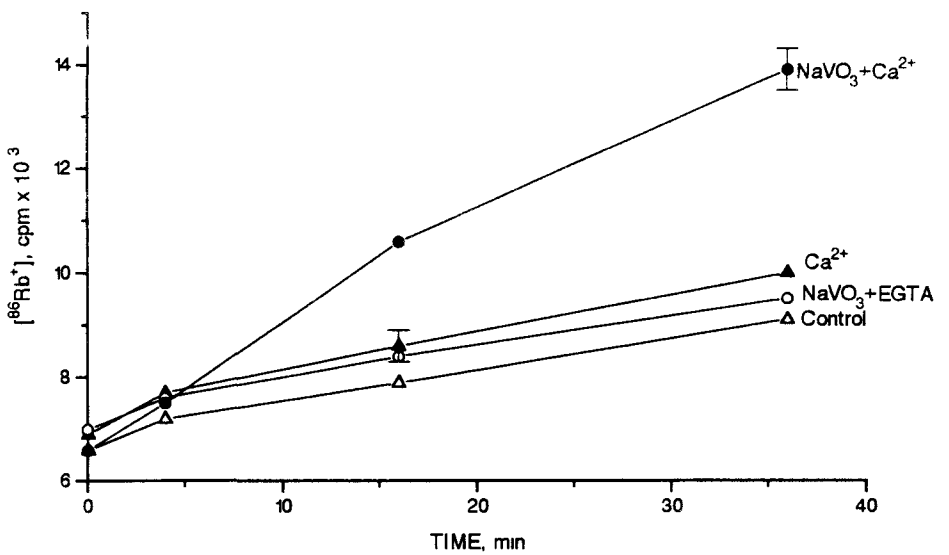

Figure 6. The effect of vanadate and Ca^{2+} on the $^{86}Rb^+$ efflux from dog RBC Whole blood was loaded with ^{86}RbCl and RBC were isolated as described in Materials and Methods, and were supplemented with 0 2 mmol/l EGTA 0 1 mmol/l vanadate (closed symbols), no vanadate (open symbols) After 15 min at 25 °C, $^{40}Ca^{2+}$ (2 7 mmol/l) (circles), and EGTA (2 5 mmol/l) (triangles) were added Radioactivity in the external medium is shown

obtained using $^{86}Rb^+$ as a tracer of the K^+ movement In the Na^+-rich medium where the K^+ concentration was approximately equal on both sides of the membrane, $^{86}Rb^+$ release was observed if both Ca^{2+} and vanadate were present in the suspension, but not in controls with vanadate and EGTA and with Ca^{2+} in the absence of vanadate (Fig 6) Thus, the experiments with dog RBC show that the opening of K(Ca) without subsequent membrane hyperpolarization is not sufficient to mediate the inhibition of the vanadate-induced $^{45}Ca^{2+}$ influx by high K^+ and that a steep transmembrane K^+ gradient is indispensable for this effect to occur

Discussion

The entrapment of the Ca chelator in RBC cytoplasm changed profoundly the properties of vanadate-induced $^{45}Ca^{2+}$ influx (Fig 1*A*) First, the $^{45}Ca^{2+}$ influx increased both in the presence and in the absence of vanadate The increase of the influx could be explained either by an increase in the concentration (osmotic) component of the Ca^{2+}-driving force due to the buffering of the cytoplasmic Ca^{2+} concentration below the resting level, or by an increase of the passive permeability during the treatment with permeant chelator Assuming that the minimum concentration of trapped chelator of 100 μmol/l (present in our conditions according to Pokudin and Orlov (1986) lowers the cytoplasmic Ca^{2+} concentration down to about 100 200 nmol/l (as computed by the Bound and Determined programm of Brooks and Storey (1992)), a value which is higher than that in the resting state if data by Lew (1990) are adopted, but it is approximately equal with the data published by Simmons (1976) Thus, the chelator loading seems to decrease the „resting" cytoplasmic Ca^{2+} concentration only marginally and therefore, the increase of the $^{45}Ca^{2+}$ influx in the chelator-treated cells might be better explained by an increase of membrane permeability Remarkably, the increment of the $^{45}Ca^{2+}$ influx induced by the quin-2 loading in the presence of vanadate was greater than in controls without vanadate (Fig 1*A*) Because both tests were performed under otherwise identical conditions, this may indicate that the vanadate treatment also induced an increase of passive Ca^{2+} permeability This possibility is supported by experimental evidence (Varečka et al 1997) Second, the $^{45}Ca^{2+}$ influx became insensitive to external K^+ concentration changes This fact and the parallel inhibition of both the Gárdos effect and the membrane hyperpolarization suggest that it is opening of the K(Ca) which is a prerequisite for the obtaining the $^{45}Ca^{2+}$ influx inhibition by K^+ The involvement of K(Ca) in this process could also be supported by the evidence obtained in experimental conditions where K(Ca) was open, i e , in experiments with monovalent ions and TCS (Fig 3, 4), by a parallelism between the inhibition of $^{45}Ca^{2+}$ influx and the depolarization potency The inhibitory effects of both monovalent ions and TCS on the vanadate-induced $^{45}Ca^{2+}$ influx could be explained by their influence on membrane potential

The opening of K(Ca) without generating a membrane potential does not seem to cause any inhibitory effect of K^+ on the vanadate-induced $^{45}Ca^{2+}$ influx This could be demonstrated in experiments with dog RBC which lack the Na^+,K^+ ATPase (Parker 1977) but possess the K(Ca) (Richhardt et al 1979) (Figs 5,6) However, the ability of these cells to take up $^{45}Ca^{2+}$ in the presence of vanadate has not been impaired by this fact, and was apparently driven by the osmotic component of the Ca^{2+}-motive force mediated by a high-capacity transporting mechanism

The results show that either the prevention of K(Ca) opening (Figs 1,2) or the elimination of membrane hyperpolarization induced previously by the K(Ca) opening (Figs 3,4) inhibit $^{45}Ca^{2+}$ influx, and this implies that K(Ca) blockade could mimic inhibition of the Ca^{2+} carrier The action of valinomycin on $^{45}Ca^{2+}$ influx (Table 1) confirmed this notion, and differentiated between inhibitors of K(Ca) and inhibitors of the Ca^{2+} carrier which could also inhibit the Gárdos effect induced by vanadate (Varečka et al 1986) Valinomycin could not be used as a tool in this respect in experimental models where the voltage activated Ca^{2+} channel represent the Ca^{2+} influx pathways

The experimental approaches used to explain the mechanism of the vanadate-induced $^{45}Ca^{2+}$ influx inhibition in human RBC by external K^+ brought results which deny the role of the osmotic component of the electrochemical potential of K^+ and identify the elimination by K^+ of membrane hyperpolarization imposed by opening of K(Ca) as the causative factor of the inhibition Such a model implies that a Ca^{2+} uniporter is a transporting species operating in our experimental conditions, (i e , in the presence of vanadate) and that the membrane potential change elicited by the opening of K(Ca) increases the total Ca^{2+}-motive force across the RBC membrane Thus, K(Ca) activity (and the subsequent Gárdos effect) participate in facilitating the Ca^{2+} influx in our experimental model Our results support the earlier suggestion of Szász et al (1981) and Gárdos et al (1980) based on data obtained with ATP-depleted cells, or La^{3+}-treated RBC (Gárdos et al 1980), or RBC treated with propranolol (Szász et al 1977, Gárdos et al 1980) These authors used inhibition of the anion channel by the stilbene derivative SITS or dipyridamol (Gárdos et al 1980) which stimulated $^{45}Ca^{2+}$ influx as a tool This approach yielded similar results also in vanadate-treated RBC (Varečka and Carafoli 1982) but could not identify the component of the K^+ electrochemical potential effective in the inhibition of the $^{45}Ca^{2+}$ influx by extracellular K^+ The inhibition of the anion channel also blocks the Gárdos effect and preserves the K^+ gradient Consequently, the stimulation of the $^{45}Ca^{2+}$ influx could be due to the increase of the membrane potential (if the Ca^{2+} carrier is a uniporter) or to the maintaining of the K^+ gradient (if the Ca^{2+} carrier is a $Ca^{2+}/2K^+$ antiporter) Our results, however, seem to resolve this ambiguity

In our previous paper (Varečka and Carafoli 1982) we suggested that the dia-

matic shift in $[K^+]$ at both sides of the RBC membrane caused by the Gárdos effect which led to the dissipation of the K^+ gradient acts as negative feedback mechanism preventing the overload of cytoplasm by Ca^{2+} Presented results are not in contradiction with this suggestion The stimulation of the Ca^{2+} influx occurs in the early phase after K(Ca) opening (few minutes after Ca^{2+} addition) when the membrane hyperpolarization is maximal (Fig 2*A*) The membrane potential change fades rapidly (Fig 2*A*) whereas $[K^+]_o$ increases gradually and reaches the steady-state after 30–45 min (Varečka and Carafoli 1982) Thus, both effects are temporally separated Such a dual effect of K^+ has been recently described in synaptic processes (Matyushkin et al 1995)

The suggestion that the activity of K(Ca) promotes the $^{45}Ca^{2+}$ influx and thereby contributes to the total Ca^{2+}-motive force contains a contradiction The K(Ca) could contribute to the total Ca^{2+}-motive force only after Ca^{2+} accumulate in the cytoplasm but the accumulation is only promoted after opening of the K(Ca) This contradiction could be explained by proposing that a transient period occurs which starts after the inhibition of the Ca^{2+} ATPase and ends after the opening of the K(Ca) During this period Ca^{2+} accumulate by a mechanism responsible for the resting Ca^{2+} cycling Another possibility could be that vanadate exerts a dual (or multiple) effect on the RBC membrane, affecting both the Ca^{2+} influx mechanism and K(Ca) in a coordinate fashion Our results published in the accompanying paper (Varečka et al , 1997) support the second possibility Finally our results convincingly explain the loss in the sensitivity of the Ca^{2+} influx to the medium composition observed when Ca-chelator trapping procedures were used (McNamara and Wiley 1986, Pokudin and Orlov 1986) These procedures prevented the K(Ca) opening and thereby the membrane potential change and the Gardos effect The RBC membrane retained its extremely low and almost identical K^+ and Na^+ permeability (Lew and Beauge 1979) which precluded the "sensing" of the changes in the medium composition unlike other procedures such as vanadate treatment (Varečka and Carafoli 1982) or ATP depletion (Ferreira and Lew 1977, Szasz et al 1977, 1981, Lew and Ferreira 1978), which are accompanied by the Gardos effect

The stimulating role of K(Ca) opening on Ca^{2+} influx is not restricted to RBC Recently, a similar phenomenon was observed during an analysis of the immunoglobulin-induced $^{45}Ca^{2+}$ influx by basophilic leukemia cells (Labrecque et al 1991) Other observations have been made in a variety of cells where the Ca^{2+} influx induced by receptor agonists was inhibited by a decrease of the membrane potential (Oettgen et al 1985 Sage and Rink 1986 DiVirgilio et al 1987, Mohr and Fewtrell 1987, Penner et al 1988 Savage et al 1989 Luckhoff and Busse 1990, Pittet et al 1990) However, the role of the K(Ca) has not yet been experimentally investigated It may be interesting to mention that Macara and Gray (1987) made this observation also in vanadate-treated A431 epidermal carcinoma cells These

data suggest that vanadate may mimic some Ca^{2+}-mediated receptor agonist action(s) in the cell membranes which frequently occur in cell membranes (Račay and Lehotský 1996) This is not necessarily true for other inducers of the Gárdos effect For example, vanadate and fluoride trigger the Gárdos effect probably by different mechanisms as indicated by the extent of $^{45}Ca^{2+}$ influx and changes in Na^+ permeability elicited by these agents (Varečka et al 1994, 1995)

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