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Influence of Lead Ions on Cation Permeability in Human Red Cell Ghosts

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Summary. Intracellular Pb^{2+} ions can replace Ca^{2+} ions in stimulating the Ca-dependent K permeability of human red blood cells. In metabolically depleted resealed ghosts, the threshold for stimulation of ^{86}Rb efflux by internal Pb^{2+} is around 5×10^{-10} M, and stimulation is half-maximal at about 2×10^{-9} M, and maximal at 10^{-8} M Pb^{2+}. There is no effect on ^{22}Na efflux in this concentration range. ^{86}Rb efflux is antagonized by internal Mg^{2+} ions, and by the channel-blocking drugs quinidine and diS-$C_2(5)$, as observed for the Ca-dependent K permeability in red cells. In ghosts containing EDTA, which prevents any internal effects of Pb^{2+} ions, external Pb^{2+} increases both ^{22}Na and ^{86}Rb permeability when its concentration exceeds 6×10^{-7} M. This effect is seemingly unrelated to the Ca-dependent K permeability. This work makes extensive use of Pb^{2+} ion buffers, and gives information about their preparation and properties.

Key Words human red cells (or erythrocytes) · Ca-dependent K permeability (or K permeability) · Pb · Pb ion buffers

Introduction

Low concentrations of lead accelerate potassium efflux from the red blood cells of a number of mammalian species, including rabbit and man (Ørskov, 1935). Sodium uptake is also increased (Ørskov, 1947), and counterflow of tracer ^{42}K occurs (Joyce, Moore & Wetherall, 1954). A detailed examination of the effects of Pb on K efflux from human red cells has been carried out (Passow & Tillman, 1955; Grigarzik & Passow, 1958). At about that time, it was realized that Ca plays an important role in the control of red cell K permeability (Gárdos, 1959). The plasma membranes of many cells are now known to contain K channels that are activated by intracellular Ca^{2+} ions (Lew & Ferreira, 1978; Latorre & Miller, 1983; Marty, 1983). These channels are normally closed in human red blood cells because the Ca pump keeps intracellular free Ca^{2+} concentrations below the levels needed to open them. Many similarities between the effects of Pb and Ca on the K permeability of human red cells have been noted, but there are also some differences (Riordan & Passow, 1971, 1973; Passow, 1981).

One approach to the study of membrane permeability is to apply solutions of controlled composition to both surfaces of the cell membrane. This can readily be accomplished with human red cells by the use of resealed ghosts (Bodemann & Passow, 1972). This technique has been used to investigate the properties of the Ca-dependent K permeability mechanism (Simons, 1976*a*,*b*). The present paper examines the effects of a range of concentrations of Pb^{2+} ions on the cation permeability of nominally ATP-free human red cell ghosts. In order to do this, Pb buffers were used. These are mixtures of Pb salts with chelating agents which tend to maintain a constant concentration of Pb^{2+} ions. They operate on exactly the same principles as Ca or H ion buffers. With Pb buffers inside ghosts, the Ca-dependent K permeability is stimulated above 5×10^{-10} M Pb^{2+}, while with external Pb buffers, membrane permeability to both Na and K is increased above 6×10^{-7} M Pb^{2+}.

A preliminary report of this work has been published (Simons, 1983*a*).

Materials and Methods

Preparation of Resealed Ghosts

This was similar to previous work (Simons, 1976*a*). Four- to 14-day-old blood-bank blood was used. The cells were first depleted of ATP by 18- to 20-hr incubation at 37°C suspended in a medium containing 150 mM KCl, 2 mM (K)HEPES and 1 mM EGTA, pH 7, then washed and packed. They were lysed in 15 volumes of ice-cold hemolyzing solution which contained Pb buffers (3 mM nitrilotriacetate (NTA) or 1,2-dihydroxybenzene-3,5-disulfonate (Tiron) with 0 to 2.5 mM $Pb(NO_3)_2$), 3 mM-HEPES, tracers and sufficient KCl to give an osmotic pressure of 30 ideal mosm/liter. Five mM NaCl or sucrose were included when their tracer effluxes were being measured. From 1 to 2.1 mM $MgSO_4$ was

Table 1. Retention of Pb buffers by resealed ghosts[a]

Concentration in lysing solution (mM)			Time (min)	Concentration in ghosts (mmol/10^{13} cells)			Ratio Pb / Buffer
Pb	NTA	Tiron		Pb	Free buffer	Total buffer	
0	3	0	0	0	1.96	1.96	0
			44	0	1.78	1.78	0
1.5	3	0	0	0.94	1.08	2.01	0.47
			44	0.82	0.93	1.75	0.47
0	0	3	0	0	1.89	1.89	0
			44	0	1.40	1.40	0
1.5	0	3	0	1.13	0.93	2.06	0.55
			44	0.91	0.73	1.64	0.56

[a] Resealed ghosts were prepared and analyzed as described in the text. Each preparation was analzed after isolation of the ghosts (0 min) and after 44 min subsequent incubation at 37°C, which spans the period over which cation fluxes were measured. The results are expressed per 10^{13} cells, roughly equivalent to a liter of packed cells.

added, when needed, in order to give a final Mg^{2+} concentration of 1 mM. In much of the work ^{86}Rb was used as a tracer for K, but two experiments were done with ^{42}K (Fig. 6). The pH of the hemolyzing solutions were adjusted to 7.0 at room temperature with KOH or HCl, before they were cooled to 0°C. The cells were hemolyzed with vigorous stirring, and 3 min later an appropriate volume of 2 M KCl solution was added to bring the osmotic pressure to 200 ideal mosm/liter. The ghosts were resealed by incubation at 37°C for 30 min, then cooled in ice, centrifuged (35,000 × *g*) and washed 3 or 4 times in ice-cold "ghost medium." This contained 98 mM KCl, 3 mM (K)HEPES and 0.1 mM NTA, to bind any Pb leaking from the ghosts, and had a pH of 7.2 at 37°C. The NTA was omitted in experiments with external Pb buffers.

Chemical Analysis of Resealed Ghosts

It is important to be sure that the Pb buffers are successfully incorporated into the ghosts and retained in them for the duration of the experiments. Table 1 gives the results of an analysis of resealed ghosts prepared with Pb, NTA and Tiron. The ghost preparations were extracted with 1.5% perchloric acid (final conc.). Pb in the extracts was measured directly by atomic absorption spectrophotometry (Pye Unicam SP9). Free chelator was estimated after the addition of HEPES and neutralization to pH 7.2 with KOH, by titration with $Pb(NO_3)_2$ solutions, using the Orion Pb-sensitive electrode to indicate the end-point. The total chelator present in the ghosts is given by the sum of the Pb and the free chelator concentrations. Measurements were made with ghosts prepared with chelator and no Pb, and at a 1 : 1 chelator/Pb ratio. In both cases the total chelator in the ghosts after resealing and washing was about $\frac{2}{3}$ of that in the hemolyzing solution. Although there was some further loss of Pb and chelator upon subsequent incubation at 37°C, the ratio of Pb to chelator, which determines the free Pb^{2+} concentration, did not change.

Measurement of Membrane Permeability by Tracer Efflux

Resealed ghosts were resuspended in "ghost medium" at 0°C, and aliquots of suspension were distributed to test-tubes in an ice-bath. Drugs, or Pb buffers, were added, when required, and the suspensions incubated at 37°C for fixed times, cooled in ice ($1\frac{1}{2}$ min), centrifuged at 3500 × *g* under refrigeration ($2\frac{1}{2}$ min), and the supernatants removed for counting. ^{86}Rb and ^{22}Na were measured in a well-type scintillation counter. ^{14}C and ^{35}S samples were deproteinized with 50 g/liter trichloroacetic acid and counted by liquid scintillation. Efflux rate constants were calculated from graphs of log{1 − (counts in supernatant/counts in suspension)} against time, and a line was fitted to the points by the least-squares method. Two time patterns were used: '4 point' and '2 point'. In the '4 point' method, duplicate samples of every suspension were incubated for 8, 20, 32 and 44 min, while in the '2 point' method, samples were incubated in triplicate for 8 and 32 min. The pH of the suspensions were checked routinely while the efflux was in progress, and did not deviate outside the range from 7.10 to 7.30.

$^{35}SO_4$ efflux was measured slightly differently. The suspensions were incubated in bulk at 37°C, and 1.3-ml samples were centrifuged at 6-min intervals from 4 to 52 min, using an Eppendorf 5412 centrifuge.

Tests of Homogeneity of the Ghosts

The resealed ghosts were tested to see if they behaved as a homogeneous population. In both experiments, ghosts containing a Pb buffer were compared with ghosts containing a Ca buffer.

a) Shrinkage in Low-K Solutions

The ghosts contain 100 mM KCl, and shrink through loss of KCl when incubated in 5 mM KCl, 95 mM choline Cl media. Figure 1 shows the resulting change in volume distributions of the ghosts, measured with a ZF Coulter Counter and P64 size distribution analyzer. From these measurements, one can deduce that 66% of the Ca-containing ghosts have shrunk, 30% have remained the same volume and 3% have swollen. The corresponding figures for the Pb-containing ghosts are 43, 45 and 11%. Alternatively, if one assumes that half of the ghosts apparently remaining at the same volume have actually shrunk, and half have swollen, the maximum fraction of ghosts that could possibly have shrunk is 81% for the Ca-containing ghosts, and 66% for the Pb-containing ghosts.

After the 5 mM K incubation, samples of ghost suspension were centrifuged at 34,000 × *g* for 10 min, and divided arbitrarily into upper, middle and lower layers. The middle layers were discarded. The upper layers contained mostly swollen cells, and contained 0.8 mmol Ca and 0.7 mmol Pb, per 10^{13} cells. The lower layers, mainly of shrunken cells, contained 2.1 mmol Ca and 1.5 mmol Pb, per 10^{13} cells. These observations indicate that the swollen cells have lost much of their Ca or Pb, either because of a lack of resealing, or because of relysis. In either case, they would be highly permeable to monovalent cations, including choline (which would account for the swelling), and not contribute to tracer efflux measurements.

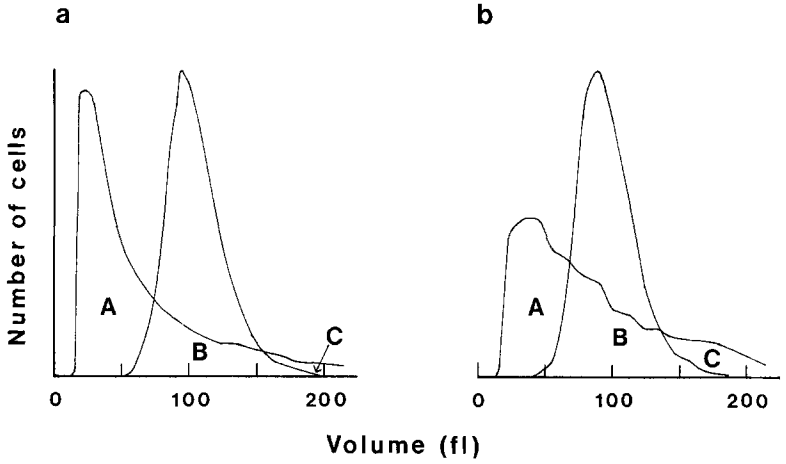

Fig. 1. Volume distribution of resealed ghosts containing (*a*) 2 mM Ca and 3 mM HEDTA and (*b*) 2 mM Pb and 3 mM NTA. The volume distributions were measured in standard 100 mM K medium, and are shown before (right-hand peak) and after (left-hand peak) a 15-min incubation at 37°C in a 5-mM K (choline) medium. They have been adjusted to represent the same total number of cells. The areas marked *A*, *B* and *C* can be considered to represent ghosts whose volumes have decreased, remained unaltered or increased, respectively, as a result of the incubation

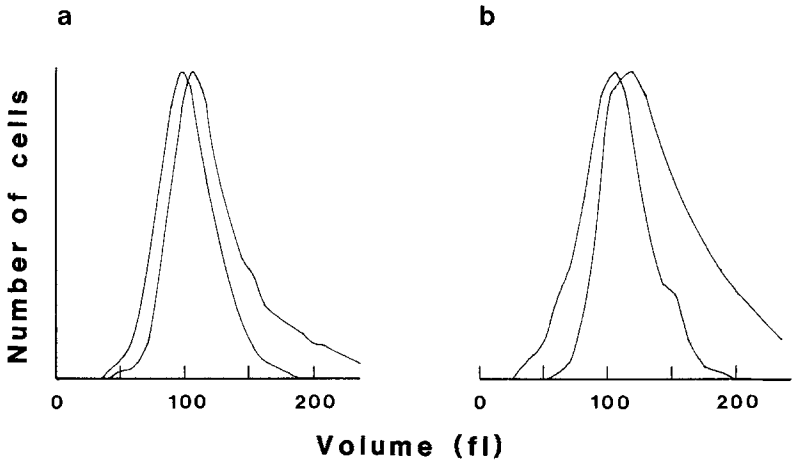

Fig. 2. Volume distributions of resealed ghosts containing (*a*) 2 mM Ca and 3 mM HEDTA and (*b*) 2.5 mM Pb and 3 mM NTA, measured before (right-hand peak) and after (left-hand peak) purification on a sucrose gradient (Bodemann & Passow, 1972). The ghosts were layered over a solution containing 98 mM KCl, 3 mM (K)HEPES and 1 M sucrose, and centrifuged for 30 min at 38,000 × *g* and 4°C. The ghosts entering the sucrose layer were discarded; those on the surface were washed two times in standard "ghost medium" before further use

b) Time-Course of ^{86}Rb Efflux, and Effects of Purification

If tracer ^{86}Rb leaves a single cellular compartment at a constant rate, the graphs of log{1 − (counts in supernatant/counts in suspension)} against time should be linear. Any upward deviation could reflect variability of efflux rates within the ghost population, or the presence of 'leaky' ghosts. Bodemann and Passow (1972) have described a technique for the removal of 'leaky' ghosts by centrifuging over a sucrose cushion. This was done for ghosts containing Ca and Pb buffers, and Fig. 2 gives their volume distributions. The purification resulted in a small reduction in mean cell volume, and the virtual elimination of cells with large volumes, above about 150 fl. These large cells were more numerous in the Pb-containing ghosts. Figure 3 compares the time-courses of ^{86}Rb efflux from the ghosts. Purification has had no effect: the curvature is unaltered. These observations, taken with those above (a), suggest that the ghosts of large volume are 'leaky' and lack both the buffer systems and ^{86}Rb. The curved ^{86}Rb efflux time-courses reflect the permeability characteristics of the resealed ghosts, and are not due to 'leaky' ghosts. Over 90% of the tracer has equilibrated, so there are unlikely to be any resealed ghosts which are not contributing to the measured tracer efflux rate.

The curvature seen in Fig. 3, in which the K channels were activated maximally, probably indicates a heterogeneity of the rates of efflux from individual cells of the population. The rate constants reported below are the slopes of straight lines fitted to the results obtained over 32- or 44-min incubation. The curvature during this period does not seriously interfere with the use of rate constants to compare cation efflux rates from different ghost preparations, as it was not very great, and was less (or even nonexistent) in ghosts containing buffers which give less than full activation of the K permeability.

Lead Buffers

Nitrilotriacetic acid (NTA), 1,2-dihydroxybenzene-3,5-disulfonate (Tiron) and citrate were used to buffer Pb^{2+} ions. All experiments and calibrations were carried out in solutions of ionic strength 0.1. The calibrations depend partly on literature values, but mainly on measurements with Orion ion-selective electrodes—model 94-82A for Pb^{2+} and 93-20 for Ca^{2+}. The Pb^{2+} electrode was calibrated in two ways: either just with unbuffered Pb solutions, or with both unbuffered and buffered solutions. The calibration of the Orion Pb^{2+} electrode by the latter method has been described (Kivalo et al., 1976). $Pb(NO_3)_2$ solutions and

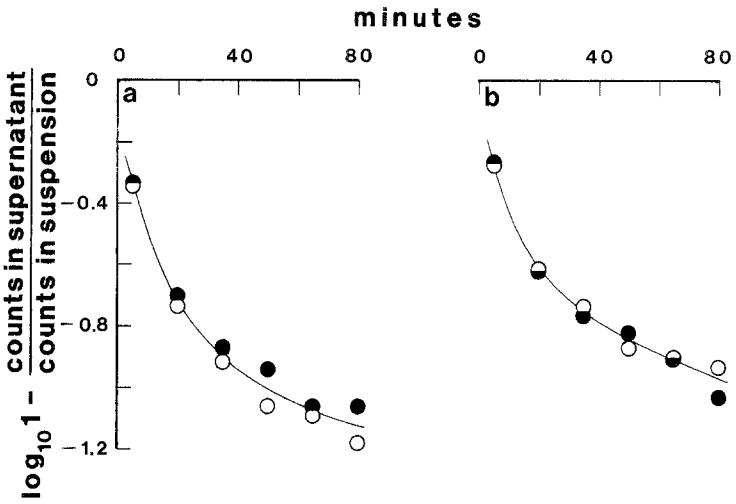

Fig. 3. Time-course of ^{86}Rb efflux from resealed ghosts containing (*a*) Ca and (*b*) Pb buffers. The ghosts are from the same preparations as in Fig. 2. Efflux was measured at 37°C, in a medium containing 98 mM KCl, 3 mM (K)HEPES and 0.1 mM NTA, either before (○) or after (●) purification of the ghosts on a sucrose gradient, as described in the legend to Fig. 2. The lines are drawn by eye

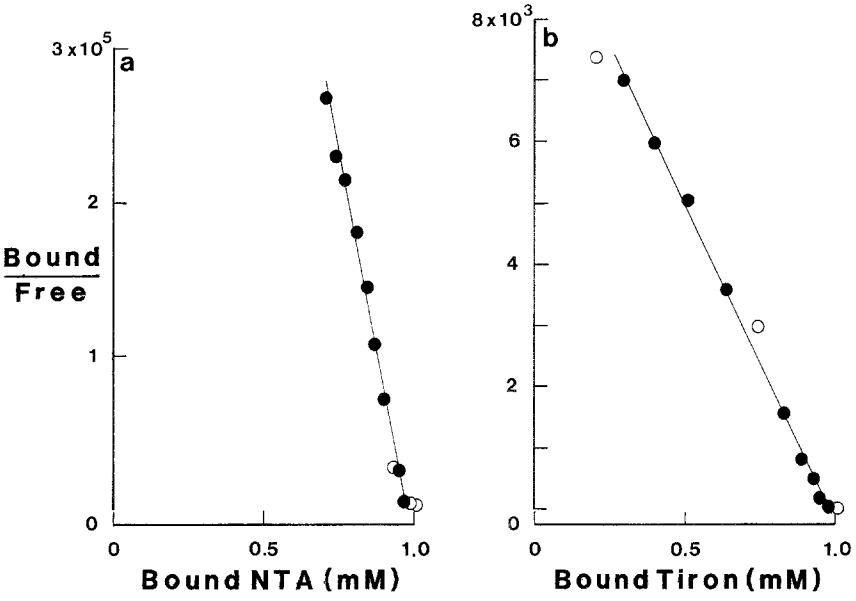

Fig. 4. Scatchard plots for the determination of the dissociation constants of (*a*) Pb-NTA and (*b*) Pb-Tiron (Bers, 1982). Free Pb^{2+} was measured in solutions containing 1 mM-NTA or Tiron, 200 to 1000 μM $Pb(NO_3)_2$, 94 mM KCl and 5 mM (K)HEPES, pH 7.2 at 25°C. The Pb electrode was calibrated as described in the text. The lines are drawn by the least-squares method, ignoring a few points (○) that are clearly "off." They have slopes (*a*) −1026, (*b*) −10.38 μM^{-1} and intercepts (*a*) 975, (*b*) 980 μM

Pb/NTA buffers are made up in 0.1 M KNO_3, and a range of free Pb^{2+} concentrations down to 10^{-12} M is obtained by varying the pH of the Pb/NTA buffers. The Pb^{2+} electrode gives a linear response in the range from 10^{-2} to 10^{-12} M Pb^{2+} (Kivalo et al., 1976), and this was confirmed in the present work (*not shown*).

Calibration of Pb/NTA Buffers

The logarithm of the equilibrium constant (K_{eq}) for Pb-NTA is given as 11.8 in 0.1 M KCl at 20°C (Schwarzenbach & Freitag, 1951) and 11.39 in 0.1 M KNO_3 at 20°C (Schwarzenbach et al., 1955). The former value has been used in the calibration of Pb^{2+} electrodes (Kivalo et al., 1976). In the present experiments the effective dissociation constant for Pb-NTA was measured at room temperature (23 to 25°C), using the method of Bers (1982). The electrode was calibrated with solutions containing 10 mM $Pb(NO_3)_2$ + 90 mM KNO_3, 1 mM $Pb(NO_3)_2$ + 99 mM KNO_3 and 0.1 mM $Pb(NO_3)_2$ + 100 mM KNO_3. Mixtures of 1 mM NTA and varying concentrations of $Pb(NO_3)_2$ were made up with 94 mM KCl and 5 mM (K)HEPES to pH 7.20, and their free Pb^{2+} concentrations measured. Sample results are given in Fig. 4(*a*); the log K_d measured in three separate experiments was 8.73, 8.80 and 9.01, mean 8.85. This value is used in the current work, and is equivalent to a log K_{eq} of 11.48, using accepted values for the H^+-NTA equilibrium constants (Schwarzenbach et al., 1955). It is not significantly different from the 11.39, given by Schwarzenbach et al. (1955) in 0.1 M KNO_3. Careful measurements with Pb-NTA mixtures in both 0.1 M KNO_3 and 0.1 M KCl failed to distinguish any significant difference in free Pb^{2+} concentration between them.

Calibration of Pb/Tiron Buffers

No appropriate values could be found in the literature, so the K_d was determined by Bers' (1982) method, exactly as described for NTA. Samples results are given in Fig. 4(*b*); the log K_d measured

Table 2. Calibration of lead/citrate buffers[a]

Total Pb (mM)	Free Pb^{2+} (μM)					Mean ± SEM
	1	2	3	4	5	
0.25			0.54	0.44	0.39	0.46 ± 0.04
0.5	0.69		1.02	0.83	0.78	0.83 ± 0.07
1.0	1.45		1.91	1.62	1.51	1.62 ± 0.10
1.5	2.19	2.69	3.02	2.51	2.69	2.62 ± 0.14
2.0	3.16		4.47	3.72	4.07	3.86 ± 0.28

[a] Solutions were made up containing 3 mM citrate, 92 mM KCl, 5 mM (K)HEPES and the stated concentrations of $Pb(NO_3)_2$, and adjusted to pH 7.2 with KOH. Their free Pb^{2+} concentrations were measured at room temperature (23 to 25°C), with the Orion electrode, calibrated by the method of Kivalo et al. (1976).

in three separate experiments was 7.00, 7.03 and 7.02, mean 7.02.

Calibration of Pb/Citrate Buffers

The free Pb^{2+} concentration at certain fixed Pb/citrate buffer ratios was measured on several different occasions, and the results are given in Table 2. This different method had to be adopted because Pb does not form a simple 1 : 1 complex with citrate, and the Bers (1982) method could not be applied.

Effect of Ca and Mg on Pb Buffers

Ca and Mg would only have been present as trace contaminants, except in experiments where Mg was added to compete with Pb. Table 3 gives values for the effective dissociation constants for the complexes of the buffers with Pb, Ca and Mg in 0.1 M KCl at pH 7.2 and 23°C. The measured Ca and Mg K_d's are in good agreement with the literature values, except for Tiron, which binds Ca and Mg very weakly, making accurate determinations difficult. In the experiments with Mg, the free Pb^{2+} and Mg^{2+} values are corrected for the competition between Pb and Mg for the buffer when NTA was used, but not when Tiron was used. The errors introduced in the latter case are negligible.

General Points

The intracellular free Pb^{2+} concentrations are calculated from the fixed buffer ratio in the hemolyzing solution, assuming a pH of 7.2. The difference between the experimental (37°C) and calibration (24°C) temperatures was ignored.

The free Pb^{2+} concentrations used in this publication are based upon the convention that the activity coefficient of Pb^{2+} is unity in 0.1 M KNO_3 (possibly ambiguous).

It is impossible to make up solutions at pH 7 with a free Pb^{2+} concentration greater than about 4 μM. Stronger solutions are either acid (e.g. Pb nitrate or acetate solutions) or metastable with respect to precipitation of lead hydroxide.

Table 3. Summary of metal ion/buffer dissociation constants[a]

	Literature values		Measured values		
	Ca	Mg	Pb	Ca	Mg
NTA	3.95[b]	2.95[b]	8.85	3.98	3.06[f]
Tiron	1.52[c]	1.41[c]	7.02	1.70	1.88[g]
Citrate	3.42[d]	3.27[d]	5.58[e]	3.45	3.27[f]

[a] This table gives the log values of the dissociation constants of the buffers for Pb, Ca and Mg in 0.1 M KCl at pH 7.2, 23°C. Literature values are calculated from (b) Irving & Miles (1966), (c) Schwarzenbach (*unpublished*, quoted in Sillen & Martell, 1964), and (d) Campi et al. (1964). Note (e) that the value for Pb-citrate is approximate as these do not form a 1 : 1 complex. Ca dissociation constants were measured with a Ca electrode and a range of Ca/buffer mixtures. Mg dissociation constants were measured either (f) with a Ca electrode, by displacement of Ca from Ca/Mg/buffer mixtures or (g) by dual-wavelength spectrophotometry with Mg/buffer mixtures, using Arsenazo III as an indicator.

Source of Materials

Isotopes were from Amersham International. All chemicals were of Analytical Grade, where possible. Tiron was supplied by B.D.H.

Results

Effect of Internal Pb on Rb Permeability

Attempts were made to incorporate several different Pb buffers into resealed ghosts. These were mostly unsuccessful: EDTA, EGTA and HEDTA bind Pb so strongly that there was no effect on cation permeability with 1 : 2 Pb/buffer mixtures inside resealed ghosts. 4′(2′-thiazolylazo)-resorcinol, 8-hydroxyquinoline and citrate could not be used as they were not retained within the ghosts. Only two substances proved useful for controlling intracellular free Pb^{2+}: NTA and Tiron. Figure 5 shows that the ^{86}Rb permeability is increased when the intracellular free Pb^{2+} concentration is about 10^{-9} M, rising to a maximum at 10^{-8} M. There is a smooth transition from results with Pb/NTA buffers to Pb/Tiron buffers. The presence of 1 mM free Mg^{2+} shifts the Pb^{2+} activation of ^{86}Rb efflux by about 0.5 log units to the right. This is similar to the effect of Mg^{2+} on Ca-induced K permeability (Porzig, 1975; García-Sancho, Sanchez & Herreros, 1982). Figure 5 also shows the effect of Ca^{2+} ions on ^{86}Rb permeability, measured with ghosts containing Ca/EGTA and Ca/HEDTA buffers. The Ca^{2+}-activation curve has a

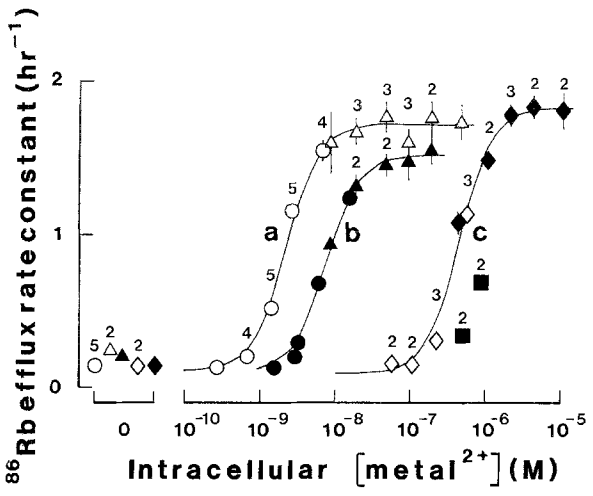

Fig. 5. ^{86}Rb permeability of resealed ghosts containing Pb and Ca buffers. Each point represents the weighted mean ± SD of 1 to 5 experiments (indicated where >1) with ghosts containing a particular buffer mixture. In each experiment the efflux rate constant was determined by the '4-point' method, and the results of different experiments were combined by weighting each according to SD^{-2}. The symbols refer to the different buffer mixtures used: (○) Pb/NTA, (△) Pb/Tiron, (●) Pb/NTA + 1 mM Mg^{2+}, (▲) Pb/Tiron + 1 mM Mg^{2+}, (◇) Ca/EGTA, (◆) Ca/HEDTA and (■) Ca/NTA. The lines are least-squares fits to the modified Hill equation, rate = $aM^2/(b + M^2) + c$, where M is the metal ion concentration in nM (Pb^{2+}) or μM (Ca^{2+}). The parameters are, for Pb^{2+} (curve *a*), $a = 1.61$ hr^{-1}, $b = 4.9$ nM, $c = 0.11$ hr^{-1}, for Pb^{2+} + 1 mM Mg^{2+} (curve *b*), $a = 1.41$ hr^{-1}, $b = 58.9$ nM, $c = 0.11$ hr^{-1}, and for Ca^{2+} (curve *c*), $a = 1.74$ hr^{-1}, $b = 0.201$ μM, $c = 0.09$ hr^{-1}

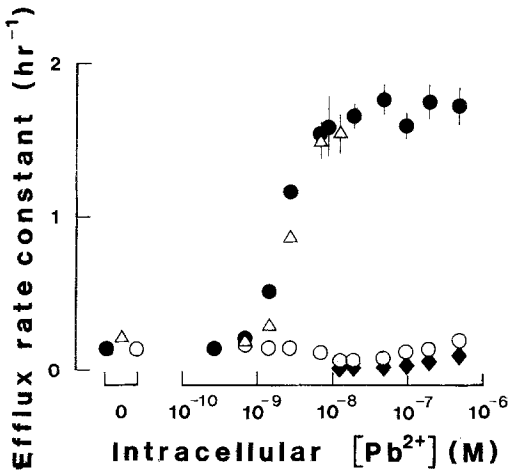

Fig. 6. ^{86}Rb (●), ^{42}K (△), ^{22}Na (○) and ^{14}C-sucrose (◆) permeabilities of resealed ghosts containing Pb buffers. The ^{86}Rb data are taken from Fig. 5. The ^{42}K results are the weighted means of two experiments for each Pb^{2+} concentration, ^{22}Na, three experiments and ^{14}C-sucrose, two experiments. Tracer efflux rates were determined by the '4-point' method

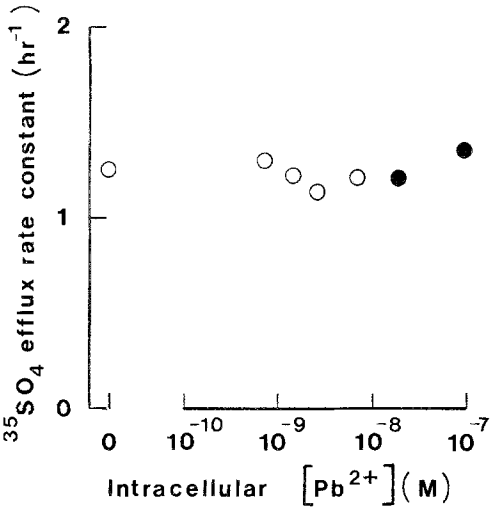

Fig. 7. ^{35}S-SO_4 permeability of resealed ghosts containing Pb/NTA (○) or Pb/Tiron (●) buffers and 3 mM sulfate. Efflux was measured into sulfate-free solutions at 37°C, as described in Materials and Methods

similar shape to the Pb^{2+}-activation curve, but lies about 2.3 log units to the right.

Selectivity of Effects of Internal Pb

The permeability of resealed ghost membranes to ^{86}Rb is raised by intracellular Pb^{2+} concentrations in the 10^{-9} to 10^{-8} M range. Figure 6 shows that ^{42}K permeability is raised similarly, but there is no effect on permeability to ^{22}Na or ^{14}C-sucrose, at least up to 10^{-7} M Pb^{2+}. The membrane permeability to these substances is much higher than the resting permeability in intact cells, because of nonspecific leaks in resealed ghosts. The Na pump does not operate, because of lack of ATP.

Internal Pb also had no effect on ^{35}S-sulfate permeability, in a single experiment (Fig. 7). It was only possible to use internal Pb^{2+} concentrations up to 10^{-7} M, because of precipitation of $PbSO_4$ at higher concentrations.

Validation of the Effects of Internal Pb on Rb Permeability

Although the stimulation of ^{86}Rb and ^{42}K permeability seems clear enough (Figs. 5 and 6), two possible sources of interference should be considered. There might be other substances in the ghosts which bind Pb, lowering the intracellular free Pb^{2+} concentration. The second possibility is that Ca^{2+} ions, either present as contaminants, or displaced from other binding sites by Pb^{2+} ions, might be partly, or even

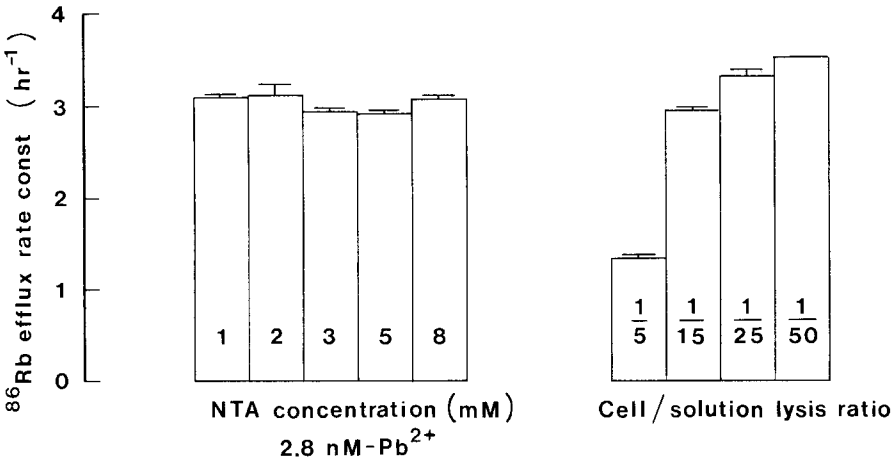

Fig. 8. ^{86}Rb efflux from resealed ghosts, varying the lysing conditions. In (*a*) the concentrations of $Pb(NO_3)_2$ and NTA were varied, keeping their ratio constant at 2 : 3 (2.8 nM Pb^{2+}). The concentration of NTA is given in the figure. In (*b*) the lysing solution contained 2 mM $Pb(NO_3)_2$ and 3 mM NTA, but the ratio of cells to lysing solution was varied as indicated. In both experiments the efflux rates were measured by the '4-point' method. The hemoglobin contents of the ghosts in (*b*) were 63, 26, 19 and 12 g/10^{13} cells, for the lysing ratios 1 : 5, 1 : 15, 1 : 25, and 1 : 50, respectively

totally responsible for the increase in ^{86}Rb permeability.

The possibility of Pb binding to other substances was investigated by choosing a Pb/NTA buffer ratio (2 : 3; 2.8 nM Pb^{2+}) which gives submaximal activation of ^{86}Rb permeability, then varying the concentration of the buffer, keeping the ratio (and hence the free Pb^{2+} concentration) constant. This had no effect on the rate of ^{86}Rb efflux (Fig. 8*a*). Varying the cell/solution lysing ratio, which alters the quantity of red cell solutes present in the ghosts, did have an effect (Fig. 8*b*). Lowering the ratio from the normal 1 : 15 to 1 : 5 reduced the rate of ^{86}Rb efflux, probably because of increased binding of Pb to cell solutes. Raising the ratio to 1 : 25 or 1 : 50 had little effect. These results suggest that cell solutes do bind Pb, but this normally does not interfere with the effect on ^{86}Rb permeability.

The possibility of contamination by Ca is harder to rule out. Most of the points on the rising phase of the curve relating ^{86}Rb permeability to intracellular free Pb^{2+} refer to experiments with Pb/NTA buffers. There was negligible Ca contamination in the $Pb(NO_3)_2$ used—0.6 μM was found in a 100-mM stock solution, but measurements should really be made on the resealed ghosts. The Pb buffers also bind Ca, so it is best to look at the one which binds Ca least well, as there will then be the greatest chance of seeing an effect of adventitious Ca. Tiron binds Ca very poorly (Table 3). Calculations indicate that with 3 mM Tiron, up to 0.5 mM Pb, and low levels of Ca (2 to 20 μM), 10% of the Ca would be bound to Tiron, and 90% would be free. Table 4 gives the results of an experiment in which both ^{86}Rb efflux and the total Ca and Pb contents of the ghosts were measured. The ghosts were prepared so that they would contain the range of free Pb^{2+} concentrations over which ^{86}Rb efflux is stimulated. The ^{86}Rb efflux rate constants are in reasonably good agreement with the values calculated from the curve in Fig. 5, and correlate well with the Pb content of the ghosts. The Ca content of the ghosts is very low—on average 2 μM—and does not correlate at all with ^{86}Rb efflux. Ca contamination could not have been responsible for the stimulation of ^{86}Rb efflux in this experiment.

Table 4. ^{86}Rb efflux from resealed ghosts containing Pb/Tiron buffers[a]

Pb in lysing solution (μM)	Theoretical free Pb^{2+} in ghosts (nM)	^{86}Rb efflux rate constant (hr^{-1})		Conc. in ghosts (μM)	
		Calculated	Observed	Pb	Ca
0	0	0.11	0.14 ± 0.02	1	4
50	1.6	0.66	0.23 ± 0.01	31	−1
100	3.3	1.22	0.96 ± 0.03	64	0
150	5.0	1.46	1.45 ± 0.07	107	3
200	6.8	1.57	1.78 ± 0.09	124	0
250	8.7	1.62	1.67 ± 0.09	182	6
300	10.6	1.65	2.03 ± 0.11	213	3

[a] ^{86}Rb efflux was measured from resealed ghosts prepared with 3 mM Tiron and 0 to 300 μM $Pb(NO_3)_2$, as indicated above. ^{86}Rb efflux rates, measured by the '4-point' method, are compared with the values calculated from the theoretical free Pb^{2+} concentrations and the curve in Fig. 5. The cation contents of the ghosts were measured by atomic absorption spectrophotometry, using extracts made in 1.5% perchloric acid. Trials showed that Pb did not interfere with Ca determinations.

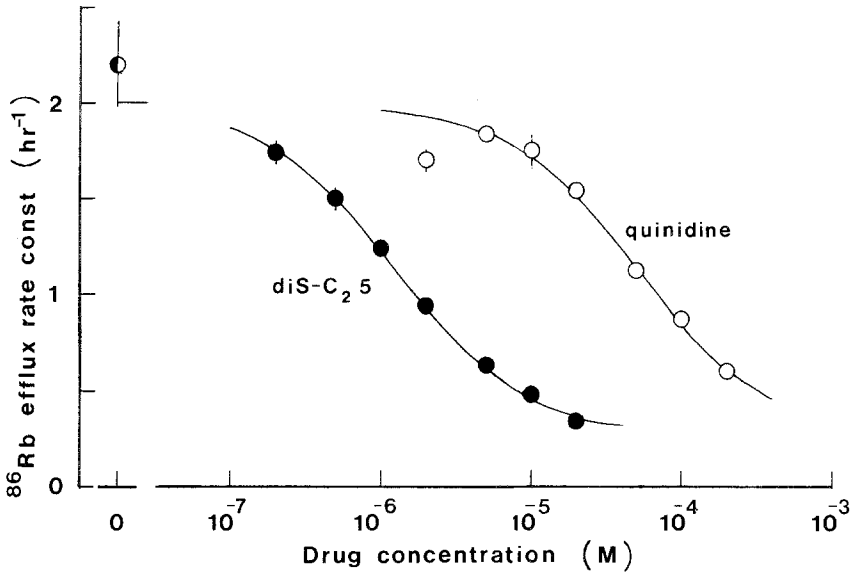

Fig. 9. Effect of inhibitors on ^{86}Rb efflux. Resealed ghosts were prepared with a 2.5 mM $Pb(NO_3)_2$/3 mM NTA buffer (7.1 nM Pb^{2+}), and ^{86}Rb efflux was measured by the '2-point' method. The drugs were dissolved in small volumes of medium (quinidine) or DMSO(diS-C_2(5), full name 3,3'-diethylthiadicarbocyanine iodide). DMSO was also added to the controls, final concentration 0.7%. The curves are least-squares fits to the equation rate = $V_o/\{1 + (I/K_i)\} + V_u$, with V_o = 1.73 hr^{-1}, V_u = 0.26 hr^{-1} and K_i = 1.3 μM for diS-C_2(5) and 51 μM for quinidine

If one now considers the experiments with ghosts containing Pb/NTA buffers (Fig. 5), one can ask how much Ca contamination would have been necessary to account for the stimulation of ^{86}Rb permeability. Calculations for the Pb/NTA buffer mixtures which give free Pb^{2+} concentrations of 1.4, 2.8 and 7.1 nM, indicate that the Ca contamination would have to be 4, 6 and 8 μM, respectively, using the measured dissociation constants (Table 3), and assuming that the free Ca^{2+} concentrations given by the line in Fig. 5 would have been needed to produce the observed ^{86}Rb efflux rates. These levels of Ca contamination are actually minimum estimates, because NTA would not be acting effectively as a Ca buffer. Metal-ion buffers, like H^+ buffers, are only effective within 1 log unit of their dissociation constants, i.e. within a range of metal/buffer ratios of 1 : 11 to 10 : 11. Two experiments were carried out with ghosts containing Ca/NTA buffers at low Ca/NTA ratios. The results, included in Fig. 5, show that these solutions are markedly less effective at stimulating ^{86}Rb efflux, compared with the well-buffered Ca/EGTA or Ca/HEDTA mixtures. These observations imply that the Ca contamination would have to be in excess of 10 μM to account for the stimulation of ^{86}Rb efflux by Pb/NTA buffers. This seems unlikely, but it is impossible to exclude the possibility that Pb^{2+} may make the K channels more sensitive to traces of Ca^{2+}.

Effect of Inhibitors of Ca-Dependent K Permeability

The Ca-dependent K permeability in human red cells is inhibited by quinidine (Armando-Hardy et al., 1975) and the carbocyanine dye, diS-C_2(5) (Simons, 1979). Figure 9 shows that these substances inhibit ^{86}Rb efflux from ghosts containing Pb/NTA buffers (7 nM Pb^{2+}), with inhibition constants of 51 and 1.3 μM (diS-C_2(5)) (quinidine). These values are comparable with observations on Ca-containing ghosts (Simons, 1979) or intact cells (Lew & Ferreira, 1978). In separate experiments, 20 μM diS-C_2(5) had no significant effect on ^{22}Na efflux from resealed ghosts containing 7.1 nM Pb^{2+} (rate constant 0.126 ± 0.027 hr^{-1}, control 0.121 ± 0.020 hr^{-1}), and 300 μM quinidine had no effect on ^{22}Na efflux at 480 nM Pb^{2+} (0.169 ± 0.017 hr^{-1}, control 0.195 ± 0.023 hr^{-1}).

Effects of External Pb

These experiments were designed to investigate whether external Pb has effects on membrane permeability independent of its action on the inner surface of the membrane. Pb permeates the red cell membrane quite rapidly, and the passive rate of Pb uptake is linearly proportional to the external free Pb^{2+} concentration (Simons, 1983*b*). In order to avoid effects of internal Pb, membrane permeability was measured with resealed ghosts prepared in 5 mM EDTA. The Pb content of the ghosts suspended in the higher Pb^{2+} concentrations were always measured at the end of the experiment. The highest value ever found (1.39 mmol/10^{13} cells) would have less-than-half-saturated the EDTA present. The K_d of Pb-EDTA at pH 7.2 is $10^{-15.1}$ M (Schwarzenbach & Freitag, 1951), so the internal free Pb^{2+} concentration would always have been below 10^{-15} M.

Figure 10 gives the results of three experiments with EDTA ghosts, in which ^{86}Rb, ^{22}Na and ^{14}C-sucrose tracer effluxes were measured while the ghosts were suspended in Pb buffers. Cation permeability is unaffected by external Pb^{2+} up to a threshold of about 6 × 10^{-7} M. At higher concentrations both ^{86}Rb and ^{22}Na effluxes are increased equally. This is not connected with the use of Pb/citrate buffers: there is no permeability increase at the lowest Pb^{2+} concentration with a citrate buffer. ^{14}C-

sucrose efflux is increased by high external Pb^{2+}, but not as much as cation efflux. This rules out the possibility that the apparent increase in cation permeability may be due to cell lysis.

In one experiment, 250 μM quinidine had no effect on ^{86}Rb and ^{22}Na effluxes stimulated by 2.6 μM external Pb^{2+}: ^{86}Rb efflux 0.92 ± 0.03 hr^{-1} (control), 0.87 ± 0.04 hr^{-1} (quinidine), ^{22}Na efflux 1.08 ± 0.03 hr^{-1} (control), 1.07 ± 0.02 hr^{-1} (quinidine).

Discussion

It has been known for many years that Pb increases human red cell K permeability (Ørskov, 1935). Similarities between the effects of Pb and Ca on K permeability have been noted (Lew & Ferreira, 1978; Passow, 1981). The possible effects of Pb on Na permeability (Ørskov, 1947) had not been investigated, and the failure of internal chelators to protect against the effects of external Pb in resealed ghosts (Riordan & Passow, 1971) had not been explained. The present work clarifies the picture. Pb has two effects on membrane cation permeability in resealed ghosts. It acts like Ca at the inner surface, stimulating a K permeability with well-defined characteristics, and it causes an increase in both Na and K permeability at the outer surface.

The Internal Pb Site

The striking feature of this work is the similarity of the curves relating ^{86}Rb efflux to intracellular free Pb^{2+} (with or without 1 mM Mg^{2+}) and Ca^{2+} (Fig. 5). The maximum velocity is the same for the three conditions. The effect of internal Mg^{2+} ions, the stimulation of K, Rb but not Na permeability, and the inhibitory actions of quinidine and diS-C_2(5) are all very similar for both Pb^{2+} and Ca^{2+} ions. Pb^{2+} probably acts directly at the same binding site(s) as Ca^{2+}. The idea that displacement of Ca^{2+} is responsible (Lew & Ferreira, 1978) seems unlikely because ^{86}Rb efflux correlates with Pb, but not Ca content in nominally Ca-free ghosts containing Pb/Tiron buffers (Table 4). The Ca^{2+} and Pb^{2+} activation curves are well-fitted by equations of the form $V = V_{max}[M^{2+}]^2/(K + [M^{2+}]^2)$, but not by $V = V_{max}[M^{2+}]/(K + [M^{2+}])$, or $V = V_{max}[M^{2+}]^2/(K + [M^{2+}])^2$. This suggests that the activation of the K channels does not depend only upon the filling of one or two noninteracting sites with Ca^{2+} or Pb^{2+}, but involves some type of cooperative effect. A highly cooperative effect is also suggested by experiments with inside-out vesicles (Lew, Muallem & Seymour, 1982).

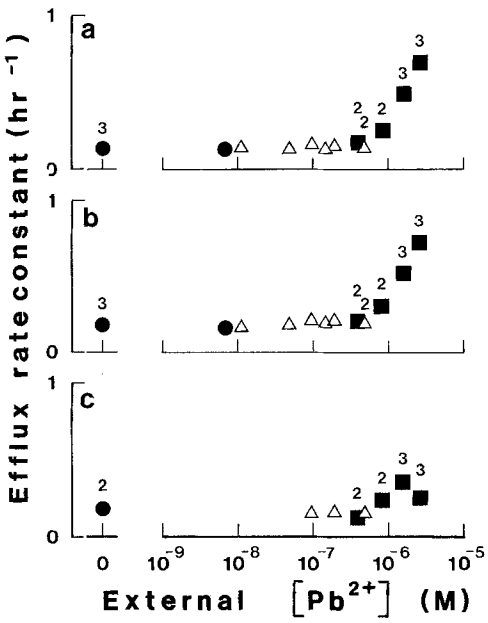

Fig. 10. Effect of external Pb buffers on membrane permeability to (*a*) ^{86}Rb, (*b*) ^{22}Na and (*c*) ^{14}C-sucrose. The ghosts contained 5 mM-EDTA and the tracers. Efflux was measured at 37°C by the 2-point method, with the ghosts suspended in standard medium supplemented with 3 mM NTA (●), Tiron (△) or citrate (■), and 0 to 2.5 mM $Pb(NO_3)_2$, sufficient to give the indicated extracellular free Pb^{2+} concentrations. The results are expressed as weighted means (weighting by SD^{-2}). The number of experiments contributing to each point is indicated when >1. ^{22}Na and ^{14}C-sucrose effluxes were measured simultaneously using the same batches of ghosts: the isotopes were separated by the channels-ratio technique of liquid scintillation counting

The affinity of the K channel for Pb is about 2.3 log units higher than for Ca. This may provide a clue to the nature of the Ca-binding site(s). All Ca-chelating agents also bind Pb, with a higher affinity, but the difference in affinities is about 7 log units for EDTA, CDTA and HEDTA (Sillen & Martell, 1964). Literature values for the Pb-EGTA dissociation constant vary widely, but preliminary observations with ion-selective electrodes suggest that the Pb-EGTA and Ca-EGTA dissociation constants differ by about 2.8 log units in 0.1 M KCl at pH 7.2. The comparison of the K channel with EGTA is not exact, because the inhibitory effects of 1 mM Mg^{2+} suggest that the K channel has a higher affinity for Mg than EGTA does, while it has a lower affinity for both Ca and Pb.

Experiments were also carried out with internal Pb/citrate buffers. These ghosts showed high permeabilities to ^{86}Rb and ^{22}Na, but the results have not been included, and are of doubtful validity, because the ghosts failed to retain reasonable quantities of Pb or citrate. It is uncertain whether this was because intracellular free Pb^{2+} concentrations

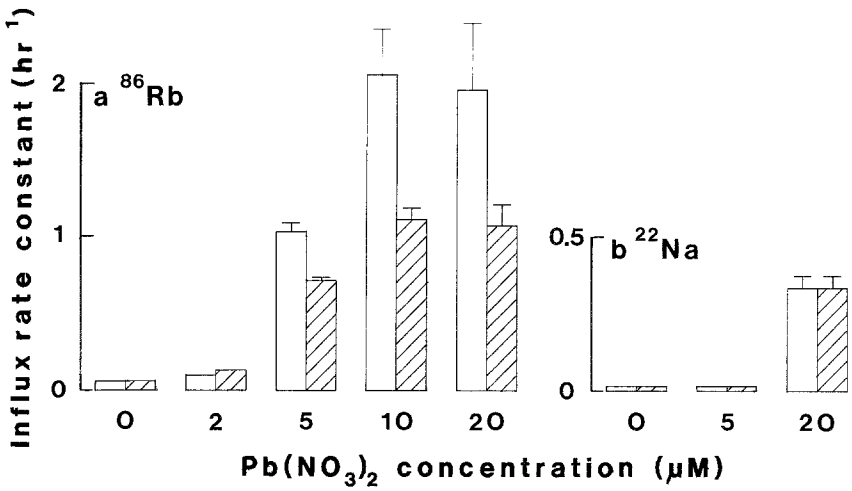

Fig. 11. (*a*) ^{86}Rb and (*b*) ^{22}Na tracer influxes into intact human red cells treated with Pb. The stated concentrations of $Pb(NO_3)_2$ were added to suspensions (9×10^{11} cells/liter) of red cells in a medium containing 100 mM KCl, 50 mM NaCl, 0.5 mM $MgCl_2$ and 3 mM (K)HEPES, pH 7.2. ^{86}Rb influx was measured by centrifuging samples of suspension through silicone oil after 0, 6, 12, 18 and 24-min incubation. The results are expressed as influx rate constants, calculated from plots of log{1 − (counts in cells/counts in cells at equilibrium)} against time. The counts in cells at equilibrium were determined after addition of 2 μM valinomycin. ^{22}Na influx was measured in a similar fashion, but samples were taken after 0, 5, 10, 20 and 40 min, and the equilibrium counts were calculated pro rata from the ^{86}Rb values. All measurements were repeated in the presence of 5 μM diS-C_2(5) (shaded columns), and are given +1 SD

above 5×10^{-7} M cause a generalized increase in membrane permeability to both anions and cations, or because high Pb^{2+} concentrations interfere with the ghost resealing process.

The External Pb Site

Stimulatory effects of external Pb on K efflux from resealed ghosts containing chelating agents have been seen before (Riordan & Passow, 1971). The present work shows that the threshold occurs at about 6×10^{-7} M Pb^{2+}. The mechanism is probably unrelated to the Ca-dependent K permeability because (i) Ca^{2+} ions do not produce similar effects, (ii) there is no selectivity between Na and Rb and (iii) drugs which block the K channel are ineffective.

Comparison with Intact Cells

Most of the previous work with Pb has involved the use of intact red blood cells, exposed to unbuffered Pb solutions. The overall effect of Pb on cation permeability will depend upon a number of factors, such as the rate of binding to the cells, permeation of the membrane and binding to cellular constituents. The threshold concentration of Pb required to see increased K efflux is about 2×10^{-8} mol Pb/g cells (Grigarzik & Passow, 1958). Most of this Pb would have been bound to the cells, mainly to hemoglobin (Barltrop & Smith, 1971). Recent measurements of Pb binding to cell proteins show that it linearly proportional to the free Pb^{2+} concentration over the 10^{-7} to 10^{-6} M range, with 15 μmol Pb bound/g hemoglobin at 1 μM Pb^{2+} (Simons, 1984). A cell Pb content of 2×10^{-8} mol/g would thus be equivalent to an intracellular free Pb^{2+} concentration of 4×10^{-9} M, assuming 0.33 g hemoglobin per g cells. The threshold in resealed ghosts is 5×10^{-10} M Pb^{2+}, which is reasonably close to the calculated value, bearing in mind that the binding of Pb to low molecular weight compounds, such as phosphates, has not been taken into account. There may also be binding to high-affinity sites.

Figure 11 gives the results of an experiment in which intact cells were exposed to low concentrations of $Pb(NO_3)_2$, and ^{86}Rb and ^{22}Na influxes were measured. The threshold for stimulation of ^{86}Rb uptake is lower than for ^{22}Na uptake. Pb-induced ^{86}Rb uptake is partly inhibited by diS-C_2(5), but this K channel blocker has no effect on ^{22}Na influx. These observations are consistent with the resealed ghost experiments, and agree with the hypothesis that Pb stimulates cation permeability by two routes: intracellular stimulation of the Ca-dependent K permeability and extracellular stimulation of a nonspecific pathway.

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