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All or None Cell Responses of Ca^{2+}-Dependent K Channels Elicited by Calcium or Lead in Human Red Cells Can Be Explained by Heterogeneity of Agonist Distribution

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Summary. We have studied the all or none cell response of Ca^{2+}-dependent K^+ channels to added Ca in human red cells depleted of ATP by incubation with iodoacetate and inosine. A procedure was used which allows separation and differential analysis of responding and nonresponding cells. Responding (H for heavy) cells incubated in medium containing 5 mM K lose KCl and water and increase their density to the point of sinking on diethylphthalate (specific gravity = 1.12) on centrifugation. Nonresponding (L for light) cells do not lose KCl at all. There is no intermediate behavior. Increasing the Ca concentration in the medium increases the fraction of cells which become H. No differences in the sensitivity to Ca^{2+} of the individual K^+ channels were detected in inside-out vesicles prepared either from H or from L cells. The Ca content of H cells was higher than that of L cells. Cells depleted of ATP by incubation with iodoacetate and inosine sustain pump-leak Ca fluxes of about 15 μmol/liter cells per hour. ATP seems to be resynthesized in these cells at the expense of cell 2,3-diphosphoglycerate stores at a rate of about 150 μmol/liter cells per hour. Inhibition of 2,3-diphosphoglycerate phosphatase by tetrathionate increased 6–8 times the measured rate of uptake of external ^{45}Ca. This was accompanied by an increase in the fraction of H cells. All or none cell responses of Ca^{2+}-dependent K channels have also been evidenced in intact human red cells on addition of Pb. They have the same characteristics as those in responding and nonresponding cells. The detailed study of the kinetics of Pb-induced shrinkage of red cells suspended in medium containing 5 mM K showed that changes of Pb concentration changed not only the fraction of H cells but also the rate of shrinkage of responding cells. H cells generated by Pb treatment contained significantly more lead than L cells. The above results suggest that the two all or none cell responses studied here can be explained by heterogeneity of agonist distribution among cells. Since pump-leak fluxes exist in both cases, differences of agonist distribution could be generated by heterogeneity of pumping among cells. This interpretation turns interest from K channels to Ca pumps to explain the heterogeneous behavior of red cells in response to a uniform stimulus.

Key Words K channels · Ca^{2+} dependence · lead effects · heterogeneity · all or none · red cells

Introduction

Many animal cells possess in their membranes K (Rb)-selective channels which are activated by intracellular Ca^{2+} (Sarkadi & Gárdos, 1985). In red cells Pb^{2+} can substitute for Ca^{2+} in the activation of K channels (Passow, 1981; Grygorczyk & Schwartz, 1983; Shields et al., 1985; Simons, 1985). Ca-dependent K channels have been found to respond, under certain conditions, in an "all or none" fashion in intact red cells (Riordan & Passow, 1973; Szász, Sarkadi & Gárdos, 1974), resealed ghosts (Knauf et al., 1975; Heinz & Passow, 1980) or inside-out vesicles derived from red cells (Garcia-Sancho, Sanchez & Herreros, 1982; Lew, Muallem & Seymour, 1982*a*): At a given Ca^{2+} (Pb^{2+}) concentration some cells (ghosts, vesicles) become activated and equilibrate at maximal rate with external K (Rb) whereas the others do not activate at all maintaining its characteristic low permeability to K (Rb).

In inside-out vesicles, where the mean number of channels per vesicle is close to one (Lew et al., 1982*a*; Alvarez & Garcia-Sancho, 1987), the all or nothing behavior has been proposed to reflect the properties of individual channels. Several papers dealing with different aspects of this matter have been published (Lew et al., 1982*a*; Garcia-Sancho et al., 1982; Alvarez, Garcia-Sancho & Herreros, 1986). In intact cells the all or none response cannot be entirely explained in terms of the properties of individual channels. This behavior would additionally require that the activation thresholds of all the channels belonging to the same cell were identical, and different from those belonging to another cell (Lew, Muallem & Seymour, 1983). Coordination of

thresholds within each cell would strongly suggest the existence of modulating mechanisms whose degree of operation would differ from cell to cell, even though the entire population is exposed to identical conditions. This heterogeneity of behavior could have important implications if it should apply to agonist-induced physiological responses.

The simplest explanation for all or none cell responses is heterogeneity of agonist distribution among cells. It has been shown recently that, when a uniform Ca influx is imposed to inosine-fed red cells using the divalent cation ionophore A23187, two extremely different cell subpopulations evolve. Some cells take up Ca to near equilibrium levels whereas others balance the imposed leak by an increase of the Ca extrusion through the Ca pump with little modification of their intracellular Ca content. This behavior can be explained by heterogeneity of the Ca-pumping capacity among cells (Garcia-Sancho & Lew, 1988*a*).

We examine here the all or none cell response to added Ca in ATP-depleted erythrocytes (Szász et al., 1974) and the all or none cell responses of intact red cells to added Pb (Riordan & Passow, 1973; Shields et al., 1985). The subpopulations of responding (permeable to K) and nonresponding cells have been separated and their contents analyzed and compared. In both cases the all or none behavior of Ca-dependent K transport has been confirmed, but, contrary to previous suggestions (Riordan & Passow, 1973), it can be explained by heterogeneity of agonist (Ca or Pb) distribution among cells. The results could be rationalized in terms of differences in the rates of calcium or lead pumping among cells which take place, although at very different rates, in both cases. This conclusion does not solve the problem of the heterogeneity of the response of individual cells but displaces the origin from the K channels to the Ca pumps.

Materials and Methods

Blood was obtained by venous puncture using heparin as the anticoagulant. Cells were sedimented by centrifugation and, after removing plasma and buffy coat, they were washed three times with at least 10 volumes of standard incubation medium, which had the following composition (mM): NaCl 145, KCl 5, Na-HEPES (pH 7.4) 10. For experiments with ATP-depleted cells the erythrocytes were resuspended at 20% hematrocrit, and 10 mM inosine, 2.5 mM sodium iodoacetate and several amounts of $CaCl_2$ or EGTA, as indicated in each experiment, were added. Samples of the cell suspension were taken after different periods, and the cells were sedimented by centrifugation at 12,000 × *g* during 1 min over phthalate oils (*see below*). The supernatant solutions were used to measure K or pyruvate and the cell pellets were processed for determination of Na and K, ^{45}Ca, hemoglobin, Pb or ATP contents as described below.

Separation of Heavy (H) and Light (L) Cells

On activation of Ca^{2+}-dependent K^+-channels KCl is lost from the cells with the subsequent decrease in cell volume and increase in cell density. This property was used to separate the two subpopulations of cells which evolve in the all or nothing responses described here: heavy (H) cells, which have shrunk by KCl loss, and light (L) cells which remain at their normal volume and density, below 1.1 g/ml. Samples containing 20–100 mg of cells suspended in 0.5–1 ml of incubation medium were placed over 0.4 ml of phthalate oil in a 1.5 ml Eppendorf tube and centrifuged at 12,000 × *g* for 30 sec. In each case two identical aliquots of the cell suspension were placed either over dibutyl-phthalate (DBP, sp. gr. 1.043), to obtain a cell pellet containing all the cells (H + L), or over diethyl-phthalate (DEP, sp. gr. 1.2), to obtain a cell pellet containing H cells only. In some cases the cells which floated over DEP were recovered and recentrifuged over DBP to obtain a sample of L cells only, but in most instances the contents of L cells were estimated by difference between data from all the cells (DBP pellet) and H cells (DEP pellet). Other details on this separation procedure have been provided before (Garcia-Sancho & Lew, 1988*b*).

After centrifugation the aqueous supernatant and most of the oil were aspirated, the walls of the tube were cleaned with cotton swabs and the pellet was lysed in distilled water over a vortex mixer. The lysate was centrifuged during 5 min at 12,000 × *g* and samples of the supernatant were used for measurement of hemoglobin or, after suitable deproteinization, for determination of Na, K, ^{45}Ca, ATP or lead (*see below*).

The fraction of dense cells (FD) was estimated from the ratio hemoglobin in H cells (DEP pellet)/hemoglobin in all cells (DBP pellet). The other data in H cells were referred to hemoglobin content. Data in L cells were usually obtained by difference: Value in L = (Value in all − Value in H × FD)/(1 − FD).

Measurements of Cell Volume Changes by Light Scattering

In some experiments the changes in cell volume were followed by measuring the changes of light transmittance in the cell suspension at 650 nm. The changes in cell volume were due to net K loss through Ca-dependent K channels. When Ca^{2+}-dependent K^+ channels are maximally activated the permeability to K^+ (P_K) becomes much larger than P_{Cl} so that net K^+ loss is limited by Cl^- movement. Under these conditions the half-time for KCl loss is 15–30 min. In the present experiments we replaced a part of the medium Cl^- for the more permeant anion SCN^-. External SCN^- exchanges within a few seconds for internal Cl^- and, when P_K is activated, SCN^- exits quickly with K^+ without limiting its rate of loss. In this way the half-time for net K loss is reduced to less than 1 min. A SCN^- concentration of 15–20 mM is enough to allow K movements without any limitation imposed by anion permeability (Garcia-Sancho & Lew, 1988*b*).

For light-scattering measurements cells were suspended in medium containing 150 mM NaCl and 10 mM K-HEPES (pH 7.5). Just before the measurements 2 ml of cell suspension (usually at

10% hematocrit) were mixed with 1 ml of a solution containing 150 mM NaSCN and 10 mM K-HEPES (pH 7.5) in a spectrophotometer cuvette and transmittance at 650 nm was adjusted to 100%. Activation of Ca^{2+}-dependent K^+ channels was started by adding $Pb(NO_3)_2$ from a concentrated stock solution, and the changes of T_{650} with time were recorded. In some experiments cells treated with Pb in SCN^--containing medium were separated through phthalate oils as described above.

Loading with Benz2

Calcium chelator benz2 was loaded in red cells following previous guidelines (Lew et al., 1982*b*). Briefly, cells were incubated with 0.2 mM benz2 acethoxymethyl-tetraester during 90 min at 37°C in a medium of the following composition (mM): NaCl 75, KCl 75, K-HEPES (pH 7.5) 10, $MgCl_2$ 0.2, EGTA 0.1. Sodium pyruvate, 10 mM, was added to prevent ATP depletion of the cells (Garcia-Sancho, 1985). By the end of this incubation period the cells were washed twice and resuspended in the adequate medium.

Experiments with One-Step Inside-Out Vesicles

One-step inside-out vesicles were prepared separately from H or L cells. In these cases separation of H and L cell fractions were performed in aqueous medium. For these purposes one volume of 20% cell suspension was centrifuged for 10 min at 4000 rpm over one volume of a solution containing; 5% hypaque, 2% ficoll, 17.7% metrizamide, 5 mM KCl and 5 mM Na-HEPES, pH 7.4 (sp. gr. 1.11). A compact pellet of cells formed at the bottom of the tube (H cells), and a net band floated over the cushion (L cells). A few cells remaining along the cushion were discarded. The two cell bands were recovered and incubated during 2 hr at 37°C in a high K-medium containing 150 mM KCl, 10 mM K-HEPES, pH 7.5, and 1 m $CaCl_2$. During this incubation shrunken cells swell to about their initial volume (Szász et al., 1974). Finally, the cells were washed and incubated during 2 hr at 37°C in Ca^{2+}-free (2 mM EDTA) medium in order to remove Ca from the cells. The cell pellets obtained after this treatment were used to prepare one-step inside-out vesicles.

One-step inside-out vesicles were prepared as described previously (Garcia-Sancho et al., 1982; Lew et al., 1982*a*, Alvarez, Garcia-Sancho & Herreros, 1984). Briefly, packed cells were lysed at 4°C with 40 volumes of a solution containing 0.1 mM EGTA and 2.5 mM K-HEPES, pH 7.5, at 4°C. The lysate was centrifuged in the cold during 15 min at 27,000 × *g*. The pellet was resuspended in about 2 volumes of medium and incubated during 45 min at 37°C to allow vesiculation. After vigorous vortexing the vesicles were washed and resuspended in an incubation medium of the following composition (mM): EGTA 0.1, KCl 16, K-HEPES 16.5; pH 7.5.

For measurements of Ca^{2+}-dependent ^{86}Rb uptake, aliquots of the inside-out vesicle suspension were mixed with incubation medium containing ^{86}Rb and different amounts of Ca and EGTA to give the required Ca^{2+} concentrations. Samples were taken after 10, 20, 30 and 40 min and passed through Dowex-50 columns to remove extravesicular radioactivity. The activity in the eluent was measured by Cerenkov counting. The fraction of activated vesicles at each Ca^{2+} concentration was calculated as described previously (Garcia-Sancho et al., 1982; Alvarez et al., 1984).

Analytical Procedures

Hemoglobin concentration in cell lysates was estimated from absorbance measurements at 540 nm (Tiffert, Garcia-Sancho & Lew, 1984). Na and K were measured by flame photometry. Pyruvate was measured by a NADH-coupled fluorimetric assay (Lowry & Passoneau, 1972). ATP was determined in neutralized perchloric acid extracts by a radioenzymatic procedure (Gonzalez & Garcia-Sancho, 1981). Pb was measured in trichloroacetic acid extracts of the cells using a Mod. 2380 Perkin-Elmer atomic absorption spectrophotometer equipped with an HG-400 graphite furnace. ^{45}Ca was determined in perchloric acid extracts by liquid scintillation counting. Results were always standardized with reference to the hemoglobin content and referred to liter of original cells.

Chemicals and Radiochemicals

Benz2-AM was obtained from Dr. V.L. Lew, The Physiological Laboratory, Cambridge, U.K. Metrizamide (Amipaque®) was a gift from Laboratories Alter, S.A. Phthalate oils were obtained from BDH Chemicals, Ltd. Tetrathionate was obtained from Fluorochem Ltd. All other chemicals were obtained either from E. Merck, Darmstadt or from Sigma Chemical Co. ^{45}Ca and ^{86}Rb were purchased from Amersham International, Buckinghamshire, England.

Results

All or None Cell Response in ATP-Depleted Erythrocytes Incubated with Different External Ca Concentrations

For these experiments cells were incubated at about 20% hematocrit in standard medium containing iodoacetate and inosine and different Ca concentrations. The activation of Ca-dependent K channels was followed by measuring the increase of K concentration into the medium (Fig. 1, upper panel). When the incubation medium contained no Ca almost no K had been released from the cells after 5 hr of incubation at 37°C. With 10 mM Ca the K concentration of the medium increased during the first 2 hr, indicating a release of K from the cells. It did not change during the next 3 hr. Higher Ca concentrations did not increase further the release of K (*not shown*). With 1 mM Ca the release K was completed after about 3 hr of incubation, to give a value which was 55–60% of that obtained with Ca concentrations giving maximal release.

Determination of the dense cell fractions in the same experiment (Fig. 1, lower panel) showed that H cells were not formed in the absence of Ca; at 10

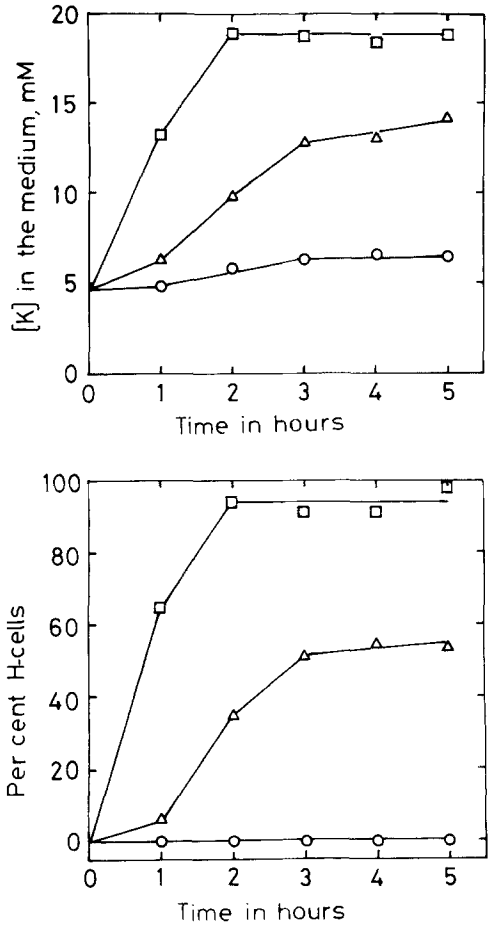

Fig. 1. Release of cell K and dense cell formation induced by external Ca in human red cells depleted of ATP by incubation with iodoacetate and inosine. Cells were suspended at 20% hematocrit in standard medium containing 2.5 mM sodium iodoacetate and 10 mM inosine and either 1 (triangles) or 10 mM Ca (squares) or 1 mM EGTA (circles). The cell suspension was then incubated at 37°C and samples were taken at the indicated times for determination of the K concentration in the incubation medium (upper panel) and the H cell fraction (lower panel)

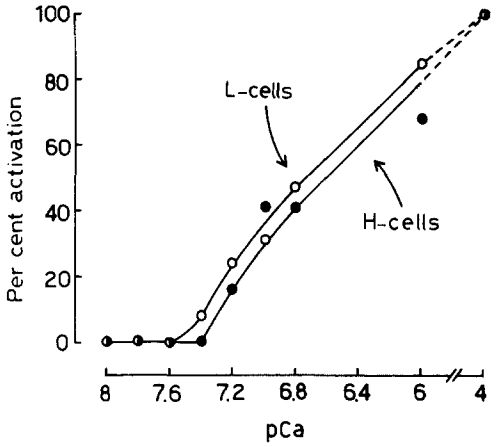

Fig. 2. Activation of ^{86}Rb uptake by Ca^{2+} in one-step inside-out vesicles prepared either from H or from L cells. Cells were incubated at 20% hematocrit in standard medium containing iodoacetate and inosine and 1 mM $CaCl_2$ for 4 hr at 37°C. Then H and L cell fractions were separated by centrifugation in aqueous medium and one-step inside-out vesicles were prepared as described in Materials and Methods. The yield of everted membrane surface, measured by acetylcholine-esterase accessibility (Steck & Kant, 1974), was 41% for vesicles prepared from L cells and 34% for vesicles prepared from H cells. Both values are within the usual range obtained in vesicles prepared from fresh cells. The measurements of uptake of ^{86}Rb by the vesicles were performed and quantified as described previously (Garcia-Sancho, Sanchez & Herreros, 1982; Alvarez, Garcia-Sancho & Herreros, 1984)

mM Ca more than 90% of the cells became dense after 2 hr, and with 1 mM Ca 50–60% of the cells became dense after 3 hr. The tight relation between K release and H cell formation suggests that the K loss takes place only from a subpopulation of cells, which become H, the others remaining unmodified with regard to their volume and K content. In order to check this point, the K content was determined within each cell fraction and referred to the hemoglobin content. The K content of H cells, obtained by incubation either with 10 mM Ca or with 1 mM Ca, decreased from 250 to 50–60 mmol/kg hemoglobin whereas the K content of L cells remained at levels not significantly different from the control values (*data not shown*). It seems, then, that a true all or none response applies: some cells lose all their K whereas the remaining do not show any loss. Results of six additional experiments with different bloods were qualitatively similar: Maximum K release, accompanied by 90–100% H cell formation was obtained with 10–20 mM Ca, and it was completed within 2–3 hr. Half-maximal K release was obtained with 0.5–2.5 mM Ca and was completed within 3–4 hr. The measured H cell fractions were always consistent with K losses.

In order to check whether the all or none cell response to added Ca depended on differences in the threshold to Ca^{2+} between the K channels belonging to H or L cells we prepared one-step inside-out vesicles from each cell subpopulation. Uptake of ^{86}Rb at different Ca^{2+} concentrations by the vesicles obtained from both cell fractions is compared in Fig. 2. The sensitivity to Ca^{2+} of ^{86}Rb uptake was similar in the vesicles prepared from both cell fractions, 50% activation being obtained at a *p*Ca of about 6.8. Similar results were obtained in another two experiments performed with different bloods. It is therefore likely that the all or nothing behavior observed in ATP-depleted cells is not due to the

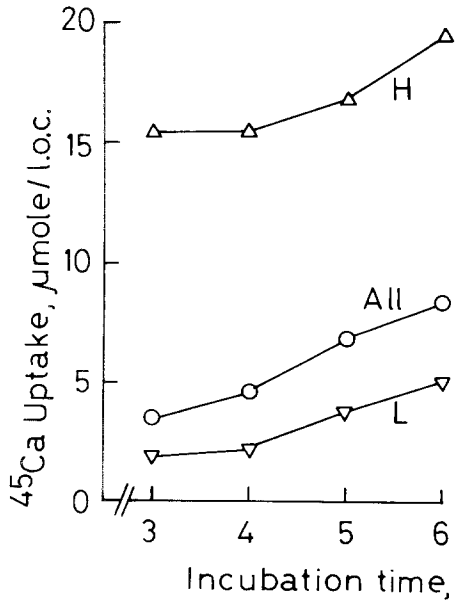

Fig. 3. Uptake of ^{45}Ca by human red cells depleted of ATP by incubation of iodoacetate and inosine. Cells were suspended at 20% hematocrit in medium containing 1 mM $CaCl_2$ at 37°C and samples were taken at 3 to 6 hr for separation of H and L fractions. The fraction of H cells was 20–25% in this experiment. Uptake of ^{45}Ca is expressed as μmol/liter original cells, calculated with reference to the hemoglobin content

heterogeneous distribution of channels with intrinsically different Ca sensitivity among the responding and nonresponding cells. The vesiculation procedure may have created, however, a uniformity which did not exist in the original cells. This is not ruled out by the present experiments.

In order to check possible differences in Ca contents between H and L cells, erythrocytes were incubated at 37°C in standard medium containing iodoacetate and inosine and 1 mM $^{45}CaCl_2$. Duplicate samples were taken 3 to 7 hr after the start of the incubation and centrifuged over DEP and over DBP and the ^{45}Ca content of each cell fraction was measured. The results are shown in Fig. 3. In this particular experiment the H cell fraction amounted to 20–25% of the cells. At all times most of the ^{45}Ca taken up by the cells was associated with the H cell fraction. For example, after 4-hr incubation the ^{45}Ca content of H cells was (in μmol/liter original cells; mean ± SD; $n = 4$) 15.6 ± 0.4 compared to 2.2 ± 0.3 in the L fraction. These results prove that there is a heterogeneity of Ca distribution among H and L cell fractions, which could explain the apparent all or none behavior of the K channels.

How is the heterogeneity of Ca distribution generated? In fed cells in which a uniform Ca influx is imposed using the ionophore A23187 a heterogeneous Ca distribution arises as a result of the heterogeneity of Ca pumping among cells (Garcia-Sancho & Lew, 1988*a*). But Ca-pumping ability should be severely restricted by ATP depletion in the experiment of Fig. 3, where measured mean cell ATP levels were about 1 μmol/liter original cells. However, when these cells were washed and resuspended in medium of the same composition (including 1 mM $CaCl_2$) except that ^{45}Ca was omitted, cellular ^{45}Ca was lost at a rate of 4.4 μmol/liter original cells per hour (*not shown*), much larger than the measured rate of uptake (1.6 μmol/liter original cells per hour, Fig. 3). These results suggest that there is some active Ca pumping from the cells. In these cells glycolysis is blocked at the glyceraldehyde-3-phosphate dehydrogenase level by iodoacetate, but still some ATP could be derived from 2,3-diphosphoglycerate. Fig. 4(*A*) shows the effects of inhibiting 2,3-diphosphoglycerate phosphatase with tetrathionate (Duhm, Deuticke & Gerlach, 1968) on the uptake of ^{45}Ca by cells incubated with iodoacetate and inosine. The uptake of ^{45}Ca was accelerated 7–8 times, from 2.1 μmol/liter original cells per hour in the controls to 15.9 μmol/liter original cells per hour in the tetrathionate-treated cells. Simultaneously the formation of H cells increased to give near 100% during the first hour of incubation with tetrathionate (Fig. 4*B*).

The increased ^{45}Ca uptake produced by tetrathionate could be attributed either to an increase of the passive permeability to Ca or to an inhibition of Ca extrusion. To test this point we measured the extrusion of ^{45}Ca from cells first incubated during 4 hr in low-K medium containing iodoacetate and inosine and 1 mM $^{45}CaCl_2$ and then washed and resuspended in the same medium containing 1 mM $CaCl_2$ without tracer. Under these conditions the rate of extrusion of ^{45}Ca measured during the first 15 min of incubation was decreased from 5.5 to 1.4 μmol/liter original cells per hour by the presence of tetrathionate (Fig. 5).

In order to check directly the effects of tetrathionate on the rate of uptake of ^{45}Ca without interference of possible effects on pumping, the uptake was measured in cells first loaded with the Ca chelator benz2. Under these conditions Ca taken up does not increase the cell Ca^{2+} levels to the point of activating the Ca pump. Consequently the true rate of influx can be approached. The results are shown in Fig. 6. Comparison of curves *a* and *d* shows again the stimulating effect of tetrathionate in cells not loaded with benz2. The rate of uptake of ^{45}Ca by benz2-loaded cells (curve *c*) was increased 3–4 times over the control value, obtained in cells not loaded with the chelator, and the presence of te-

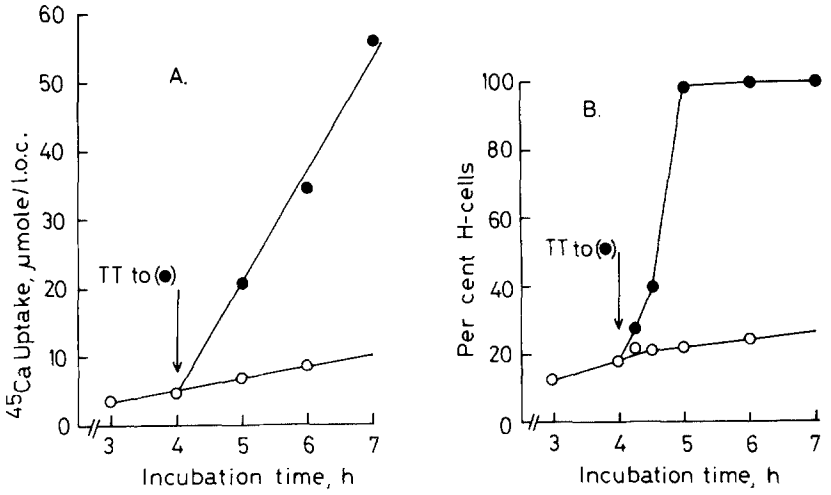

Fig. 4. Effects of tetrathionate on the uptake of ^{45}Ca (*A*) and the H cell fraction (*B*) of human red cells depleted of ATP by incubation with iodoacetate and inosine. Experimental details as in Fig. 3. Sodium tetrathionate (final concentration, 5 mM) was added to one aliquot of the cell suspension (filled circles)

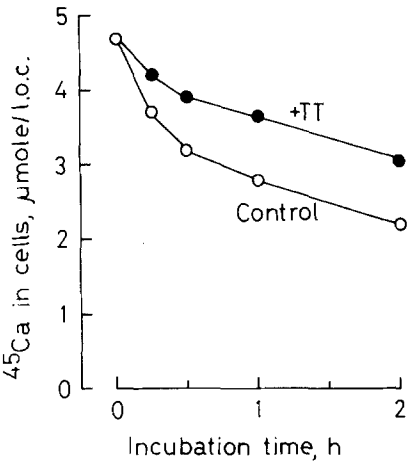

Fig. 5. Effects of tetrathionate on the exit of ^{45}Ca from ATP-depleted human red cells. Cells were first incubated at 20% hematocrit in standard medium containing 2.5 mM sodium iodoacetate, 10 mM inosine and 1 mM $^{45}CaCl$ for 4 hr at 37°C. Then the cells were washed twice and resuspended at 20% hematocrit in medium containing iodoacetate and inosine and 1 mM $CaCl_2$ and divided into two aliquots to one of which (filled circles) 5 mM sodium tetrathionate was added. Both cell suspensions were then incubated at 37°C and samples were taken after different times for determination of ^{45}Ca content of the cells. Other details as in Fig. 3

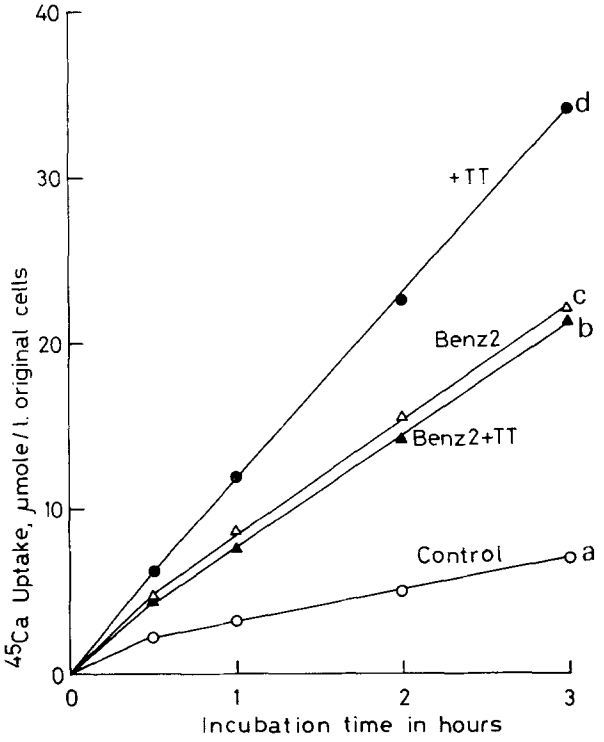

Fig. 6. Effects of tetrathionate and preloading with benz2 on the uptake of ^{45}Ca by human red cells depleted by ATP by incubation with iodoacetate and inosine. Cells suspended at 20% hematocrit were loaded with benz2 by incubation with benz2-acethoxymethyl tetraester, 0.2 mM, for 1 hr at 37°C. Control cells were incubated similarly without the benz2 derivative. Then both cell suspensions were washed twice and resuspended at 20% hematocrit in standard medium containing 2.5 mM sodium iodoacetate, 10 mM inosine and 0.1 mM EGTA. After 4-hr incubation at 37°C cells were washed and resuspended at 20% hematocrit in standard medium containing iodoacetate and inosine. Each suspension was divided into two aliquots and sodium tetrathionate (5 mM) was added to one of them (closed symbols). Immediately 1 mM $^{45}CaCl_2$ was added and samples for determination of the ^{45}Ca content of the cells were taken after the indicated times of incubation at 37°C. Other details as in Fig. 3

trathionate did not additionally increase the rate of uptake of ^{45}Ca by benz2-loaded cells (curve *b*). These results demonstrate that the increased rate ^{45}Ca uptake induced by tetrathionate in ATP-depleted cells is not due to an increased passive permeability to Ca, but to the inhibition of Ca extrusion through the Ca pump. On the other hand, it seems clear that the rate constant for ^{45}Ca uptake estimated from the time course of the uptake by ATP-depleted cells is an underestimate of the true rate constant for influx. This is due to the extrusion of

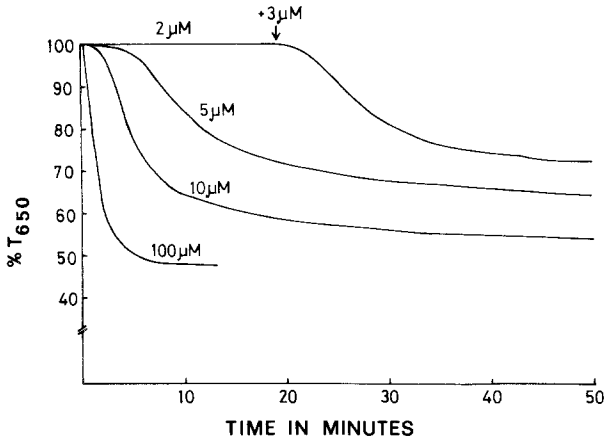

Fig. 7. Volume changes of intact human red cells induced by different Pb concentrations. Crude records of the transmittance of the cell suspensions at 650 nm are shown. Pb was added at t = 0 at the concentrations shown in the Figure. Hematocrit was 5%. For details *see* Materials and Methods

Ca by the Ca pump, even in ATP-depleted cells. With regard to the difference observed between the uptake of ^{45}Ca by tetrathionate-treated ATP-depleted cells (curve *d*) and by benz2-loaded cells with or without tetrathionate (curves *c* and *b*) we do not have a clear explanation. Perhaps membrane hyperpolarization subsequent to the activation of Ca^{2+}-dependent K^+ channels, which should be largest in the tetrathionate-treated cells not loaded with benz2, accelerates ^{45}Ca uptake.

In order to estimate the rate of ATP production derived from 2,3-diphosphoglycerate in cells treated with iodoacetate and inosine, the rate of pyruvate output with and without tetrathionate was measured. Pyruvate was produced at a rate of 327 μmol/liter cells per hour in the control and the rate was reduced to 150 μmol/liter cells per hour by the addition of tetrathionate (*data not shown*). This result suggests that 2,3-diphosphoglycerate is metabolized at a rate of at least 177 μmol/liter cells per hour in ATP-depleted cells, which implies the production of the same amount of ATP from ADP.

All or None Cell Response of Human Red Cells to Added Pb

As stated in the introduction, the addition of lead to a suspension of human red cells produces an increase of K permeability which takes place through the same pathway activated by Ca (Riordan & Passow, 1973; Passow, 1981). The uptake of lead by red cells is extremely quick (Simons, 1983, 1986*a*,*b*). This represents a technical advantage for the study of the all or nothing response. Lead-induced activation of Ca^{2+}-dependent K channels was estimated from the changes of cell volume of red cells suspended in low-K SCN^--containing medium, followed by scattering measurements. Figure 7 shows the changes of T_{650} induced by the addition of several amounts of Pb. At 100 μM Pb a maximal response was obtained. At the smaller concentrations of Pb there was a measurable lag between the addition of Pb and the start of shrinkage and, after an initial shrinkage, volume stabilized at a level which depended on the concentration of Pb. These results are consistent with previous reports (Riordan & Passow, 1973; Shields et al., 1985) and suggest that the loss of KCl induced by Pb has an all or none nature. The presence of two subpopulations of cells was first suggested by measurements of the osmotic resistance of lead-treated human erythrocytes. Passow and Tillmann (1955) showed that at lead concentrations that caused about 50% K loss the frequency distribution of lysed cells at different tonicities showed two peaks of about equal size (*see also* Riordan & Passow, 1973). In order to check this hypothesis we separated cell subpopulations by centrifugation over phthalate oils after treatment with Pb and found that: a) The percent of H cells in each case was the same as the decrease of T_{650} if that was expressed as percent of the decrease observed with the concentration of lead giving maximal effect (*data not shown*). b) The cells which did not sediment through DEP (L cells) had in all cases the same K content as the controls, not treated with Pb, whereas H cells sedimenting through DEP had lost K (Table 1).

The use of Pb is tricky and a few technical remarks are pertinent. We confirmed previous reports (Simons, 1984) showing that Pb is much more efficient (3–5 times) to activate K permeability if the cells are depleted of inorganic phosphate by preincubation with inosine. We also found that preincubation of the cells before Pb addition rendered them less susceptible to the effect of Pb on K permeability. This effect was due to release of inorganic phosphate from the cells to the medium and was faster the higher the temperature of preincubation. For example, the effect of 30 μM Pb was decreased by 50% by a 60- to 70-min preincubation at 25°C and by 100% by a 120-min preincubation (*data not shown*). The inhibitory effect could be avoided by resuspending the cells in fresh medium. These observations may be relevant for the interpretation of results obtained using long incubation periods.

Kinetics of Pb-induced K release were studied in some detail. Figure 8 shows a logarithmic plot of the change of transmittance at 650 nm referred to the change of transmittance at $t = \infty$. Table 2 shows a summary of the results of 18 similar experiments.

Table 1. Potassium contents of H and L cell fractions after treatment of human red cell suspensions with different Pb concentrations[a]

Added Pb (μM)	Percent dense cells	K content (mmol/kg hemoglobin)	
		L cells	H cells
4	13.8	308	177
5	19.1	306	178
6	36.0	302	163
7	34.3	298	154
8	51.9	300	132
10	71.3	305	106

[a] Cells were suspended at 5% hematocrit in a solution containing 75 mM of each NaCl and NaSCN, and 10 mM of K-HEPES, pH 7.5. To aliquots of this cell suspension the amounts of Pb indicated below were added. After 20-min incubation at 20°C samples were taken and centrifuged over either diethyl-phthalate or dibutyl-phthalate oil and the hemoglobin and K contents of the cell pellets were determined. K content in L cell fraction was estimated by difference. Each value shown below is the mean of two individual determinations differing by less than 10%. The K content of control cells, not treated with Pb, was 305 mmol/kg hemoglobin.

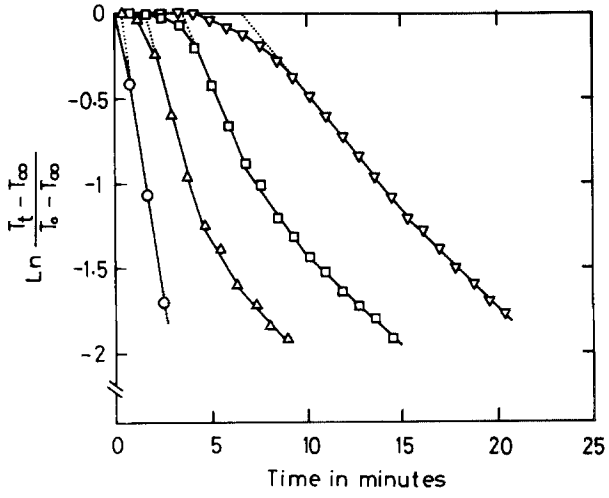

Fig. 8. Kinetics of the changes of volume induced by different Pb concentrations in intact human red cells. Transmittance records as those shown in Fig. 7 have been linearized by plotting $\mathrm{Ln}\{(T_t - T_\infty)/(T_o - T_\infty)\}$, where the subfixes indicate the time of measurement. Dotted lines are extrapolations to calculate the time lag before maximal rate of shrinkage is reached. Hematocrit was 5%. Pb concentrations were: 100 (circles), 30 (triangles), 10 (squares) or 5 μM (inverted triangles)

At Pb concentrations higher than 50 μM 100% of the cells became H and shrinking was extremely fast, with a half-time of about 0.9 min. Even in these conditions there was a lag after Pb addition of 0.4–0.6 min before the rate of shrinkage reached its maximal value. At smaller Pb concentrations the fraction of cells which shrunk decreased and the rate of shrinkage of these cells also decreased (Table 2). In addition, the time lag before maximal rate of shrinkage was reached increased. These results are consistent with previous observations (Shields et al., 1985) and suggest that the response to Pb is not strictly all or none since Pb concentration modifies not only the fraction of cells which respond but also the rate at which K is lost.

Table 2. Kinetic parameters of lead-induced swelling of human red cells incubated in low-K medium containing SCN^-[a]

Added Pb (μmol/liter cell suspension)	(n)	Lag (min)	K_{max} (min^{-1})	Percent activation
100	(3)	0.4 – 0.6	0.80 ± 0.02	100
50	(2)	0.4 – 0.8	0.79 – 0.85	100
30	(2)	1.5	0.36 – 0.45	91 – 94
10	(7)	4.0 ± 1.4	0.21 ± 0.06	81 ± 6
5	(4)	6.5 ± 0.6	0.13 ± 0.02	58 ± 7

[a] All the experiments were performed at about 5% hematocrit with similar design as those shown in Figs. 7 and 8. n stands for number of determinations. Values are given as mean ± SD or range of individual determinations. Lag was estimated by extrapolation of the maximum slope portion of the lines to the initial volume, as shown by discontinuous lines in Fig. 8.

Analysis of the Pb content of H and L cell subpopulations was also attempted. Table 3 shows the results of two experiments. On the average H cells contained 65% more lead than L cells. By comparing the mean cell content of lead (all the cells) with that associated with the H cell fraction using the paired t-test the difference was significant ($P < 0.01$) in spite of the large variability of individual data.

Discussion

Human red cells incubated with iodoacetate and inosine take up Ca from the medium at a very slow rate of about 1–2 μmol/liter cells per hour. We have presented evidence, however, that unidirectional fluxes of Ca are much faster suggesting that most of the Ca entering from the medium is extruded through the Ca pump. To stress this discrepancy between net and exchange fluxes we shall refer below to a "pump-leak steady state" even though a true steady state is not established in these cells since net flux never becomes zero. This pump-leak steady state is established at transmembrane exchange rates of Ca of about 15 μmol/liter cells per

Table 3. Lead distribution in H and L cell fractions of human red cells incubated with different Pb concentrations[a]

Exp. no.	Added Pb (μM)	%H	Pb contents (μmol/liter original cells)		
			All cells	H cells	L cells
1	2.0	21	15	32	11
	2.5	54	24	23	25
	2.5	40	25	35	19
	2.5	47	29	41	18
	3.0	63	28	33	20
	3.0	44	28	49	13
2	2.2	21	17	14	18
	2.2	28	17	15	18
	2.5	46	17	23	12
	2.5	43	20	29	13
	2.7	58	22	29	12
	2.7	56	21	18	24

[a] Cells were first incubated during 2 hr at 37°C in inosine-containing medium in order to reduce their inorganic phosphate content. This has been shown to decrease the amount of Pb bound to the cell by nonspecific mechanisms (Simons, 1984). Then the cells were resuspended at 6.2 (Exp. 1) or 7.9% (Exp. 2) hematocrit in inosine-containing medium and the amounts of Pb indicated below were added. After 10 min at 37°C NaSCN was added from a 0.15 M solution to give a final concentration of 30 mM in the suspension. After 20 more min of incubation duplicate aliquots of the cell suspension were centrifuged over dibutyl-phthalate and over diethyl-phthalate oils and the Pb content of cell extracts was determined by atomic absorption spectrophotometry and normalized with reference to the hemoglobin content. The Pb content of L cells was estimated by differences. The treatment with Pb did not decrease the ATP content of the cells by more than 15%.

hour. This exchange rate is not very different from that measured in fed cell (Lew et al., 1982*b*) but it should require higher intracellular Ca^{2+} levels in order to activate the Ca pump, depressed because of the low ATP levels. The increased cytoplasmic Ca^{2+} concentration is enough, in some cells, to activate the K channels. Although cell ATP goes as low as 1 μm, turnover rates as high as 150–300 μmol/liter cells per hour should take place according to our measurements of pyruvate output. Most of ATP formation is supported by hydrolysis of 2,3-diphosphoglycerate. The values obtained here are in accordance with turnover rates for ATP of about 200 μmol/liter cells per hour measured previously in red cells poisoned with iodoacetamide plus inosine (Glynn & Lew, 1970) and for maximum hydrolysis rates estimated for 2,3-diphosphoglycerate (Momsen, 1981).

It has been shown previously that, in inosine-fed red cells, the imposition of a uniform Ca influx with the ionophore A23187 leads to the development of an extremely heterogeneous Ca distribution in steady state. This behavior is due to heterogeneity of the Ca-pumping capacity among cells (Garcia-Sancho & Lew, 1988*a*). Since, as discussed above, ATP-depleted cells also maintain a pump-leak steady state for Ca, the heterogeneity of Ca distribution found here could be attributed to the same reason: differences of Ca-pumping ability among cells.

The all or none cell response of Ca-dependent K channels can then be attributed to heterogeneity of Ca distribution. There is no need then of postulating a coordination of channels threshold within each individual cell nor differences of the threshold sensitivity to Ca^{2+} from cell to cell. Direct comparison of the Ca^{2+} sensitivity to the K channels in inside-out vesicles derived from H and L cell fractions (Fig. 2) showed no differences in their sensitivity to Ca^{2+}. It could be argued, however, that differences among cells are lost by the release of cytoplasmic contents or other modifications produced by the vesiculation procedure. The membrane hyperpolarization resulting from activation of the K channels could exaggerate the differences of Ca content between H and L cell fractions since hyperpolarization has been reported to increase Ca uptake by red cells (Szász, Sarkadi & Gárdos, 1978). Should this situation apply, a positive feedback mechanism would be established which would sharpen the all or none cell response and the heterogeneity of Ca distribution between H and L cell fractions.

We confirm in this paper the basic characteristics of the all or none cell response to Pb described previously (Riordan & Passow, 1973; Shields et al., 1985). The rate of shrinkage of responding cells changed with Pb concentration, suggesting that the response is not strictly "all or nothing." Heterogeneity of lead distribution between the responding (H) and nonresponding (L) cells was also detected, although it was smaller than that described above for Ca. It has been reported recently that Pb can be extruded from red cells through the Ca pump (Simons, 1984). Individual differences in pumping capacity among cells, could therefore also explain the heterogeneity of Pb distribution.

The experimental evidence presented suggests that the all or none cell response of the red cell K channels can be explained by heterogeneity of agonist distribution among cells in the two cases studied here. The Ca pumps rather than the K channels would seem to generate the heterogeneous response of the individual cells. The all or none cell response of Ca^{2+}-dependent K channels reported for red cell ghosts loaded with Ca buffers (Heinz & Passow,

1980) remains now the only case which cannot be attributed to heterogeneity of agonist distribution.

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