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# Cation-dependent uptake of zinc in human fibroblasts

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**The influence of $K^+$ and $Ca^{2+}$ on $Zn^{2+}$ transport into cultured human fibroblasts was investigated. $Zn^{2+}$ uptake was markedly reduced in the presence of both valinomycin and nigericin (electrogenic and electroneutral $K^+$ ionophores, respectively), and by reduction in the transmembrane $K^+$ gradient produced by replacement of extracellular $K^+$ with $Na^+$, suggesting that $Zn^{2+}$ may be driven by a $Zn^{2+}/K^+$ counter-transport system. To test the counter-transport hypothesis, we used $^{86}Rb$ as an analog of $K^+$ for efflux studies. The rate of $Rb^+$ efflux was 3760 times that of $Zn^{2+}$ uptake, thus the component of $K^+$ involved in the $Zn^{2+}$ counter-transport system was only a small proportion of the total $K^+$ efflux. In investigating the effect of $Ca^{2+}$ on $Zn^{2+}$ uptake, we identified two components: (1) a basal $Zn^{2+}$ uptake pathway, independent of hormonal or growth factors which does not require extracellular $Ca^{2+}$ and (2) a $Ca^{2+}$-dependent mechanism. The absence of $Ca^{2+}$ decreased $Zn^{2+}$ uptake, while increasing extracellular $Ca^{2+}$ stimulated $Zn^{2+}$ uptake. The effect was mediated by $Ca^{2+}$ influx as the ionophores A23187 and ionomycin also stimulated $Zn^{2+}$ uptake. We could not ascribe the $Ca^{2+}$ effect to known $Ca^{2+}$ influx pathways. We conclude that $Zn^{2+}$ uptake occurs by a $K^+$-dependent process, possibly by $Zn^{2+}/K^+$ counter-transport and that a component of this is also $Ca^{2+}$-dependent.**

**Keywords:** calcium, fibroblasts, potassium, transport, zinc

## Introduction

The mechanism of $Zn^{2+}$ accumulation by cells has not been clearly elucidated. Physiologically, $Zn^{2+}$ occurs largely bound to various ligands, including serum proteins and amino acids. Although there have been many $Zn^{2+}$ uptake studies on a variety of cells, the identification of the form in which $Zn^{2+}$ is taken up has only been made in red blood cells and human skin fibroblasts.

In red blood cells, the uptake was via an anion transport system (Kalfakakou & Simons 1986). $Zn^{2+}$ uptake was stimulated by bicarbonate ions and was by reduced by DIDS and furosemide, agents known to inhibit anion transport. Later it was shown that a major transport route for $Zn^{2+}$ across the red blood cell membrane was via the bicarbonate/chloride anion exchanger, as a $[Zn(HCO_3)_2Cl]^-$ complex (Torrubia & Garay 1989). Another ionic $Zn^{2+}$ transporting mechanism which requires thiocyanate or salicylate ions has been described in red blood cells (Kalfakakou & Simons 1990). In the latter study, it was observed that replacement of extracellular $Na^+$ by $K^+$ inhibited $Zn^{2+}$ uptake. This phenomenom, however, could not be explained in terms of the known properties of the anion exchanger.

In the fibroblasts, none of the serum $Zn^{2+}$-binding ligands were found to provide the substrate for $Zn^{2+}$ uptake. Rather, the uptake has been correlated with the 'free' $Zn^{2+}$ concentration, suggesting that $Zn^{2+}$ is taken up by cells as an ion rather than as a $Zn^{2+}$–ligand complex (Ackland *et al.* 1988).

$Zn^{2+}$ uptake by the body is homeostatically regulated. The amount of $Zn^{2+}$ absorbed by the gut depends on the body $Zn^{2+}$ requirement (Cotzias & Papavasiliou 1964). In the gut, which has been the major focus of studies investigating the factors which regulate $Zn^{2+}$ uptake, dietary proteins, amino acids and minerals, and various forms of stress such as infection and fasting, have been found to influence $Zn^{2+}$ uptake (Evans & Johnson 1980, Solomons *et al.* 1983). The intracellular protein metallothionein is thought to be significant in the regulation of $Zn^{2+}$ uptake

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in the gut. Low metallothionein levels are found in the intestines of $Zn^{2+}$-depleted rats (Richards & Cousins 1976) and metallothionein gene expression is increased in response to $Zn^{2+}$ (Menard *et al.* 1981). There is evidence that the liver also is important for $Zn^{2+}$ homeostasis. Studies of cultured rat hepatocytes show that $Zn^{2+}$ accumulation is stimulated by hormones such as glucocorticoids and that this effect is accompanied by an increase in metallothionein mRNA levels (Hager & Palmiter 1981).

Apart from the role of metallothionein, little is known about the cellular mechanisms which may be involved in regulating cellular $Zn^{2+}$ uptake. Many functions of a cell in response to stimulation are immediate and rapid and therefore do not involve protein synthesis. Given the importance of $Zn^{2+}$ in cellular metabolism, it is likely that there are such regulatory mechanisms for $Zn^{2+}$ uptake.

In this study with cultured human fibroblasts, we investigate the possibility of $Zn^{2+}$ transport via the bicarbonate/chloride anion exchange mechanism. We also investigate the possibility that other ions, including $K^+$, $Na^+$ and $Ca^{2+}$, are involved in the transport of $Zn^{2+}$ into cells. We also consider whether second messenger events have any direct effect on the regulation of $Zn^{2+}$ uptake. The short time intervals used for our uptake experiments (30 min or less) precludes transcriptional events. In this study we use human fibroblasts, as they have been shown to be actively growing with requirements for $Zn^{2+}$ which have been previously determined (Ackland *et al.* 1988).

## Materials and methods

### *Materials*

$BaCl_2$, $CsCl_2$, TEAC (tetraethylammoniumchloride), tolbutamide, ionomycin, verapamil, nifidipine, diltiazem, dynorphin A, phe-met-arg-phe amide, phorbol 12-myristate 13-acetate (TPA), 4a-phorbol (4aP), W13, protein kinase inhibitor, staurosporine and calmodulin were obtained from Sigma (Castle Hill, NSW, Australia). BRL 38227 was generously donated by Beecham Research Laboratories (Dandenong, Victoria, Australia) and glibenclamide was kindly donated by Hoechst (St Kilda, Victoria, Australia). A23187 was obtained from Boehringer Mannheim (Castle Hill, NSW, Australia). These compounds were dissolved to form a stock solution in either water, ethanol or dimethylsulfoxide (DMSO) depending on their aqueous solubility. Ethanol and DMSO controls were included in the experiments.

### *Cells*

Normal human fibroblasts were used. They were initially established from human forearm skin by the scratch technique (Fowler 1984) and were cultured in Eagle's Basal Medium (BME; Flow, Sydney, Australia), with 10% fetal bovine serum (FBS, CSL, Parkville, Victoria, Australia), (growth medium). Cells were grown in 3.5 cm diameter culture dishes for 4 days to near confluence.

### *Incubation media*

BME/10% FBS was used for uptake experiments where the ionic composition of the media was not being studied. To test the effect of extracellular $Ca^{2+}$ on $Zn^{2+}$ uptake, Hank's balanced salt solution (HBSS) with 100 $\mu$M albumin and 5 $\mu$M $ZnCl_2$ was used, either containing 1.3 mM $CaCl_2$ or $Ca^{2+}$-free. For the $Mg^{2+}$ concentration curve, HBSS with 100 $\mu$M albumin and 5 $\mu$M Zn containing $MgCl_2$ up to 8 mM was prepared. BME/10% FBS was also used as incubation medium to test $Ca^{2+}$ channel blockers and agents which affected protein kinases. This medium could not be rendered $Ca^{2+}$-free without substantially altering other components. Therefore in this medium, agents could not demonstrated unequivocably to be acting through $Ca^{2+}$ pathways.

For experiments where the extracellular $K^+$ concentration was increased, the incubation medium used was HBSS with 10 $\mu$M albumin, containing 1 $\mu$M $ZnCl_2$ and 2 $\mu$Ci ml$^{-1}$ $^{65}$Zn. The ionic strength of the medium was maintained by substituting KCl for NaCl.

### *Zinc uptake*

$^{65}$Zn was obtained from New England Nuclear (Broadbeach, QLD, Australia). BME/10% FBS or HBSS, prepared as described above, was equilibrated with $^{65}$Zn (2.5 $\mu$Ci ml$^{-1}$) for 3 h. For uptake experiments, the cells were washed three times in 1 ml PBS. For the EGTA wash, cells were first rinsed in 1 ml of 1 mM EGTA followed by two PBS washes. Cells were then incubated in culture medium with $^{65}$Zn, containing inhibitors or ionophores. Following incubation, they were washed three times in phosphate buffered saline (PBS) then incubated with 1 mg ml$^{-1}$. Pronase at 4°C for 20 min. The cells were transferred to centrifuge tubes and spun in a Beckman microfuge for 15 s. The supernatant was retained as the fraction containing membrane-bound $Zn^{2+}$. The pellet, containing the Pronase resistant component of $Zn^{2+}$ uptake, was resuspended in 500 $\mu$l of 2 M NaCl and DNA determined fluorimetrically as outlined below. The $^{65}$Zn in the Pronase-sensitive and the Pronase-resistant fractions was determined with a LKB $\gamma$-counter. The $Zn^{2+}$ uptake was calculated from the specific activity of the $^{65}$Zn and the $Zn^{2+}$ concentration measured by flame atomic absorption spectrophotometry.

### *Rubidium efflux*

$^{86}$Rb was used as an analog of $K^+$ for measuring $K^+$ efflux from fibroblasts. $^{86}$Rb was obtained from Du Pont (Australia). The labeled medium containing 5 $\mu$Ci ml$^{-1}$ $^{86}$Rb was prepared as for the $^{65}$Zn. For efflux experiments the cells were labeled with $^{86}$Rb in BME/10% FBS overnight. After the experiment the culture medium was removed and the cells were washed as described above for the $^{65}$Zn protocol. The culture medium was then counted in a $\gamma$-counter and the cells were assayed for DNA.

The $K^+$ efflux rate from the cells was calculated from the specific activity of the $Rb^+$. To determine specific activity of the $Rb^+$, the intracellular $K^+$ concentration of

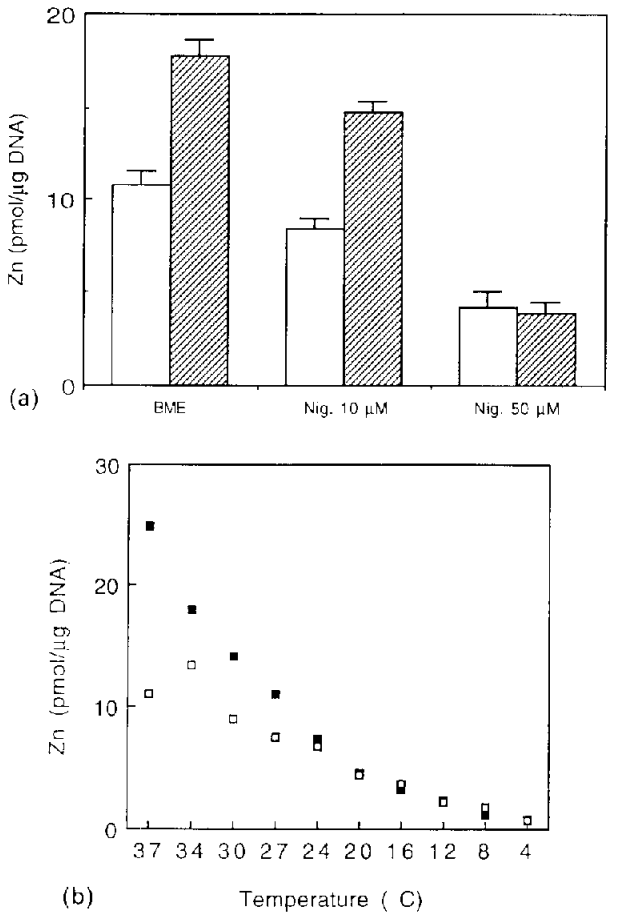


**Figure 1.** **(a)** Uptake of $Zn^{2+}$ by fibroblasts incubated in $^{65}Zn$-labeled BME/10% FBS with 10 and 50 $\mu$M nigericin for 30 min (□) and 60 min (▨). Results are mean (±SEM) from one of three similar experiments. **(b)** Uptake of $Zn^{2+}$ at different temperatures, by fibroblasts incubated in $^{65}Zn$-labeled BME/10% FBS (control, ■) and with 50 $\mu$M nigericin (□). Each point is the mean of three determinations.

the fibroblasts and the cell volume were determined. To measure the cellular $K^+$ concentration, the cells from 3.5 cm diameter culture dishes were rinsed, Pronase-treated as above, then spun down in a microfuge. The supernatant was removed and the cells rinsed in suprapur NaCl 0.9%, spun down and resuspended in 200 $\mu$l of deionized water. They were sonicated 20 times and spun for 20 min in a Beckman airfuge at 100 000 *g*. The supernatant was removed, made up to 2 ml with deionized water and the $K^+$ concentration was measured by flame photometry.

The cell volume was estimated from the amount of tritiated water incorporated into the fibroblasts. Cells were incubated at 37 C with BME/10% FBS containing 0.05 $\mu$Ci ml$^{-1}$ tritiated water. After 2, 5, 10 and 30 min (similar uptakes were recorded for all these equilibration times), the supernatants were removed, the cells rinsed in PBS at 4°C and 1 mg ml$^{-1}$ Pronase added for 20 min. The cells plus supernatants were counted in a Beckman $\beta$-counter LS 3801. The volume of the cells was calculated from the specific activity of the tritiated water in the incubation medium and d.p.m. of the cell pellets.

*DNA estimation*

DNA was measured by the method of Labarca & Paigen (1980). An aliquot of 500 $\mu$l was sonicated and then made up to 1 ml in 2 M NaCl to a final concentration of 1 $\mu$g ml$^{-1}$ Hoechst 33258. Fluorescence was measured at an excitation wavelength of 356 nm and an emission wavelength of 458 nm.

## Results

*Effect of $K^+$*

The effect of a range of inhibitors and ionophores on transport of $Zn^{2+}$ into fibroblasts was investigated. $Zn^{2+}$ uptake was not affected by ouabain or amiloride (at concentrations up to $10^{-3}$ M), nor by the anion transport inhibitors, furosemide or DIDS (data not shown). Monensin was previously shown to have no effect on $Zn^{2+}$ uptake (Ackland *et al.* 1988).

$Zn^{2+}$ uptake was, however, inhibited by $K^+$ ionophores. Nigericin reduced $Zn^{2+}$ uptake in a dose-dependent manner (Figure 1a), with the effect increasing with increasing incubation time. At temperatures less than 24°C, this effect was abolished (Figure 1b). Valinomycin had a similar, although less marked effect (Figure 2).

When cells were incubated with nigericin in the presence of 150 mM extracellular $K^+$, the $Zn^{2+}$ uptake was reduced relative to that in $K^+$-free medium (Figure 3). A similar effect was observed in cells incubated with valinomycin in the presence and absence of 150 mM $K^+$ (Figure 4).

The data indicates a role for $K^+$ ions in transport of $Zn^{2+}$ across the fibroblast membrane and suggests that the $K^+$ gradient may be important. We tested this directly by

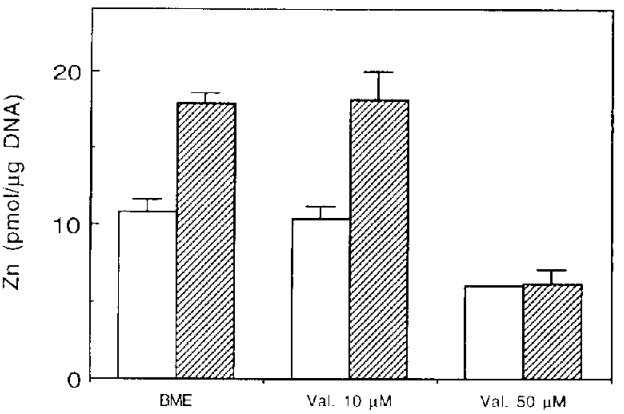


**Figure 2.** Uptake of $Zn^{2+}$ by fibroblasts incubated in $^{65}Zn$-labeled BME/10% FBS with 10 and 50 $\mu$M valinomycin for 30 min (clear bars) and 60 min (▨). Results are mean (±SEM) of three similar determinations.

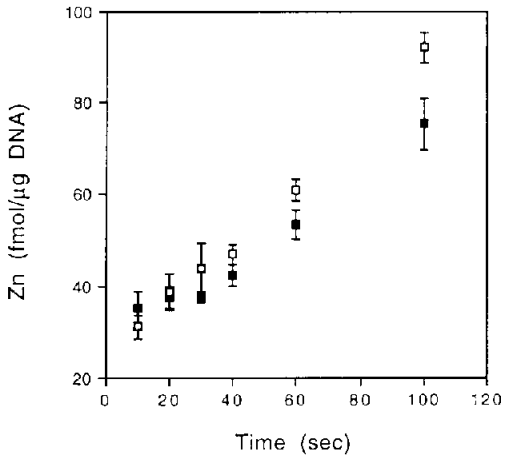


**Figure 3.** Knitial $Zn^{2+}$ uptake by fibroblasts incubated with $^{65}Zn$-labeled HBSS, 100 µM albumin and 50 µM nigericin with either 150 mM $K^+$ (■) or no $K^+$ (□). Each point represents the mean (±SEM) of three determinations.

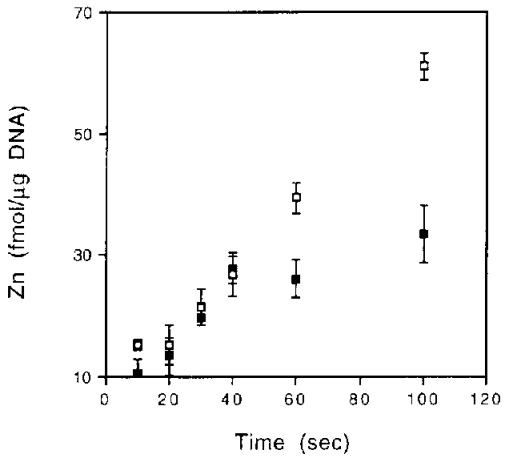


**Figure 4.** Initial $Zn^{2+}$ uptake by fibroblasts incubated with $^{65}Zn$-labeled HBSS, 100 µM albumin and 50 µM valinomycin with either 150 mM $K^+$ (■) or no $K^+$ (□). Each point represents the mean (±SEM) of three determinations.

incubating the cells in the presence of increasing extracellular $K^+$ concentrations for 15 min. As shown in Figure 5, $Zn^{2+}$ uptake decreased as the extracellular $K^+$ concentration was increased.

The dependence of $Zn^{2+}$ uptake on the $K^+$ gradient was also seen when cells were incubated with and without extracellular $K^+$. After 15 min, cells incubated in the absence of extracellular $K^+$ had taken up 9.1 pmol $Zn^{2+}$ µg $DNA^{-1}$, whereas cells in 150 mM $K^+$ had accumulated only 5.5 pmol $Zn^{2+}$ µg $DNA^{-1}$ (Figure 6), significantly less (Student's $t$-test $P=0.02$).

A counter-transport system in which $Zn^{2+}$ ions move into the cell in exchange for $K^+$ ions moving out could account for our observations. We next tested for such a $K^+/Zn^{2+}$ counter-transport system. Cells were loaded with rubidium, as a substitute for $K^+$ ions, and the efflux rate of $Rb^+$ was measured. The initial efflux rate from the cells was 1128 pmol µg $DNA^{-1}$ $min^{-1}$ (Figure 7a). This is approximately 3760 times greater than the measured $Zn^{2+}$ uptake rate (0.3 pmol mg $DNA^{-1}$ $min^{-1}$) (Figure 7b). Assuming that the transport is electroneutral, the amount of $K^+$ ions being transported through this carrier is only 0.05% of the total efflux. This is too small to be measured against such a large background. Indeed we could measure no $Zn^{2+}$-dependent change in $Rb^+$ efflux from the cells either with increasing or decreasing extracellular $Zn^{2+}$ (data not shown).

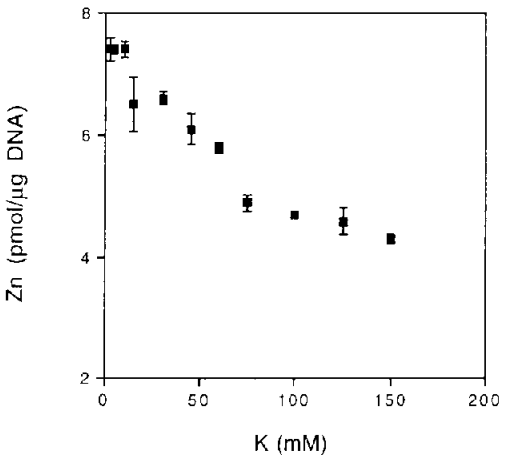


**Figure 5.** $Zn^{2+}$ uptake over 15 min by fibroblasts incubated with $^{65}Zn$-labeled HBSS containing 100 µM albumin in which KCl was substituted for NaCl. Each point represents the mean (±SEM) of three replicate observations.

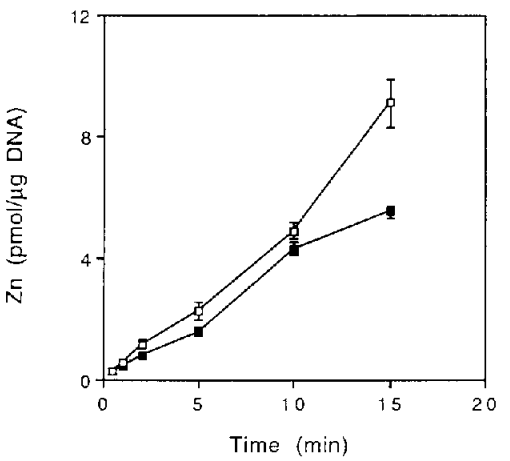


**Figure 6.** $Zn^{2+}$ uptake over 15 min by fibroblasts incubated with $^{65}Zn$-labeled HBSS containing 100 µM albumin in the presence of 150 mM $K^+$ (■) and without $K^+$ (□). Each point represents the mean (±SEM) of three replicate observations.

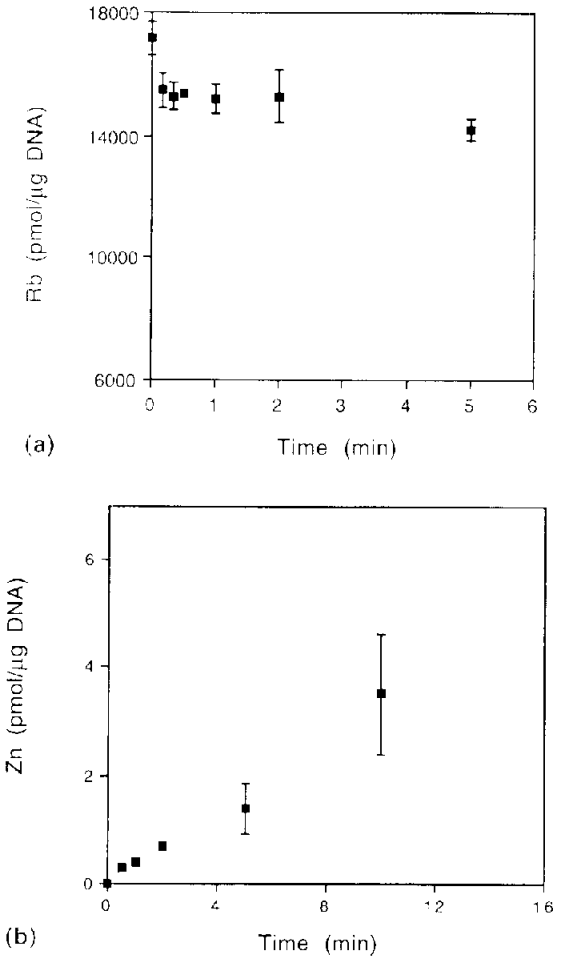


**Figure 7.** **(a)** Initial $^{86}Rb$ efflux from fibroblasts after overnight labeling with BME/10% FBS containing $^{86}Rb$. Each point represents the mean (±SEM) of triplicate observations. **(b)** Initial uptake of $Zn^{2+}$ from $^{65}Zn$-labeled BME/10% FBS. Each point represents the mean (±SEM) of triplicate observations.

To decrease the background release of $Rb^+$, a number of $K^+$ channel blocking agents were used. Although $Ba^{2+}$ (Quayle *et al.* 1988), TEAC (Standen *et al.* 1989), $Cs^{2+}$ (Hu *et al.* 1989), tolbutamide (Amoroso *et al.* 1990), 4-aminopyridine (Gandolfo *et al.* 1989) and glibenclamide (Daut *et al.* 1990) are known to block $K^+$ channels in several systems, they had no effect on $Rb^+$ efflux from fibroblasts (data not show). Both nigericin and valinomycin, however, markedly decreased intracellular $Rb^+$ (Figure 8).

Most counter-transport systems are reversible. We therefore preloaded the cells with $^{65}Zn$ and measured the initial rate of efflux of $^{65}Zn$ in the presence of increasing extracellular $K^+$. No change in the rate of $^{65}Zn$ efflux was detectable.

### *Effect of $Ca^{2+}$*

Pronase-resistant $Zn^{2+}$ uptake (intracellular zinc) was reduced by 50% in the absence of $Ca^{2+}$ after 15 min incubation (Figure 9). This effect was more marked when an EGTA wash preceded the addition of the $Ca^{2+}$-free medium. The control, consisting of an EGTA wash followed by incubation with medium containing $Ca^{2+}$ showed a reduction in $Zn^{2+}$ uptake of 15%. Pronase-sensitive $Zn^{2+}$ binding was not affected by the presence or absence of $Ca^{2+}$ ions.

A concentration curve for extracellular $Ca^{2+}$ after 15 min incubation showed a pronounced increase in Pronase-sensitive $Zn^{2+}$ binding at $Ca^{2+}$ concentrations above 6 mM. At 9 mM there was a 2-fold increase in $Zn^{2+}$ uptake and at 13 mM a 4-fold increase, relative to the uptake at 1.3 mM, the normal physiological $Ca^{2+}$ concentration (Figure 10a). The Pronase-resistant zinc uptake increased gradually

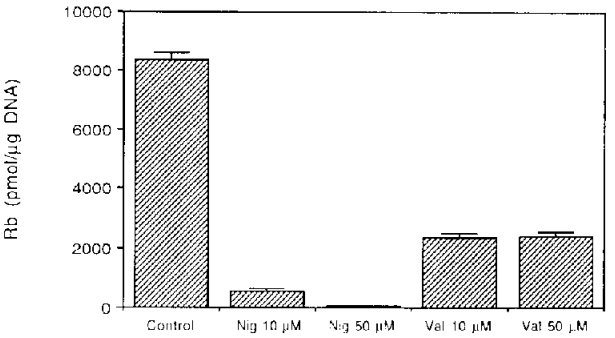


**Figure 8.** $^{86}Rb$ efflux from fibroblasts after overnight labeling with BME/10% FBS containing $^{86}Rb$. Efflux of $^{86}Rb$ was measured after 30 min in the presence of 10 and 50 µM nigericin and with 10 and 50 µM valinomycin. Each point represents the mean (±SEM) of three observations.

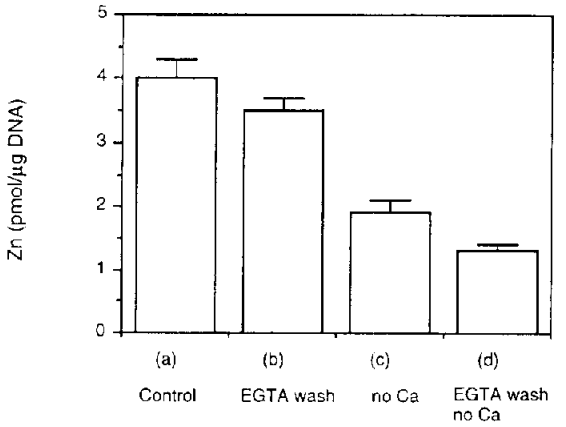


**Figure 9.** Uptake of Pronase-resistant $Zn^{2+}$ after 15 min by fibroblasts incubated in $^{65}Zn$-labeled HBSS with 100 µM albumin and 5 µM $ZnCl_2$. **(a)** Control. Cells had PBS washes and 1.3 mM $Ca^{2+}$ in incubation medium. **(b)** EGTA wash. Cells were washed in 1 mM EGTA then PBS and incubated with 1.3 mM $Ca^{2+}$. **(c)** No $Ca^{2+}$. Cells had PBS washes and were incubated in $Ca^{2+}$-free medium. **(d)** EGTA wash, no $Ca^{2+}$. Cells were washed in 1 mM EGTA, then PBS and incubated in $Ca^{2+}$-free medium. Results are mean (±SEM) of three determinations.

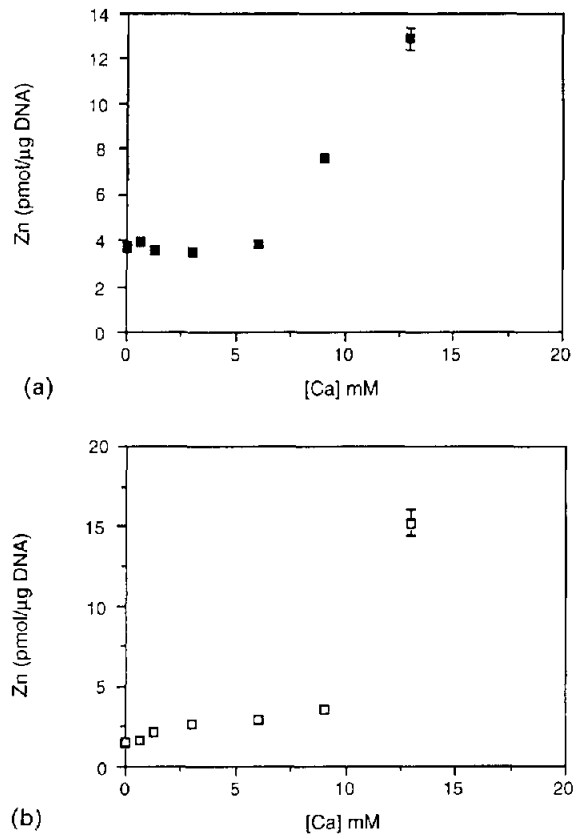


**Figure 10.** (**a**) Pronase-sensitive $Zn^{2+}$ uptake after 15 min by fibroblasts incubated in $^{65}Zn$-labeled HBSS with 100 $\mu$M albumin and 5 $\mu$M $ZnCl_2$ with increasing concentrations of $Ca^{2+}$. Results are mean ($\pm$SEM) of three determinations. (**b**) Pronase-resistant $Zn^{2+}$ uptake after 15 min by fibroblasts incubated in $^{65}Zn$-labeled HBSS with 100 $\mu$M albumin and 5 $\mu$M $ZnCl_2$ with increasing concentrations of $Ca^{2+}$. Results are mean ($\pm$SEM) of three determinations.

over the $Ca^{2+}$ concentration range from 0 $Ca^{2+}$ (1.8 pmol $\mu$g $DNA^{-1}$) to 9 mM $Ca^{2+}$ (3.6 pmol $\mu$g $DNA^{-1}$) and then increased rapidly to 15.2 pmol $\mu$g $DNA^{-1}$ at 13 mM $Ca^{2-}$ (Figure 10b).

To test if the effect was specific for calcium, rather than a divalent cation effect, we examined uptake of $Zn^{2+}$ in the presence of varying $Mg^{2+}$ concentrations. Over a range of $Mg^{2+}$ concentrations which extended to 10 times the normal physiological concentration of 0.8 mM, there was no change in either Pronase-sensitive binding or Pronase-resistant $Zn^{2+}$ uptake (data not shown).

We determined whether $Ca^{2+}$ influx could stimulate $Zn^{2+}$ uptake by adding different $Ca^{2+}$ ionophores. A23187 at 10 $\mu$M significantly increased Pronase-resistant $Zn^{2+}$ uptake from 6.5 pmol $\mu$g $DNA^{-1}$ in the control to 16.5 pmol $\mu$g $DNA^{-1}$ in the presence of $Ca^{2+}$ and to 12 pmol $\mu$g $DNA^{-1}$ in the absence of $Ca^{2-}$ (Figure 11a). The $Ca^{2-}$ ionophore ionomycin, at 20 $\mu$M, produced a small but significant increase in Pronase-resistant $Zn^{2+}$ uptake from 4.9 pmol $\mu$g $DNA^{-1}$ in the absence of $Ca^{2+}$ to 6.4 pmol $\mu$g $DNA^{-1}$ in the presence of $Ca^{2+}$ (Figure 11b).

We next investigated whether this $Ca^{2+}$-dependent $Zn^{2+}$ uptake was mediated directly through known $Ca^{2+}$ influx pathways or alternatively through pathways which are known to be regulated by $Ca^{2+}$. Specific inhibitors for the three classes of L-channels (verapamil, nifidipine and diltiazem) had no effect on Pronase-resistant $Zn^{2+}$ uptake when used at 5 or 20 $\mu$M in HBSS (data not shown).

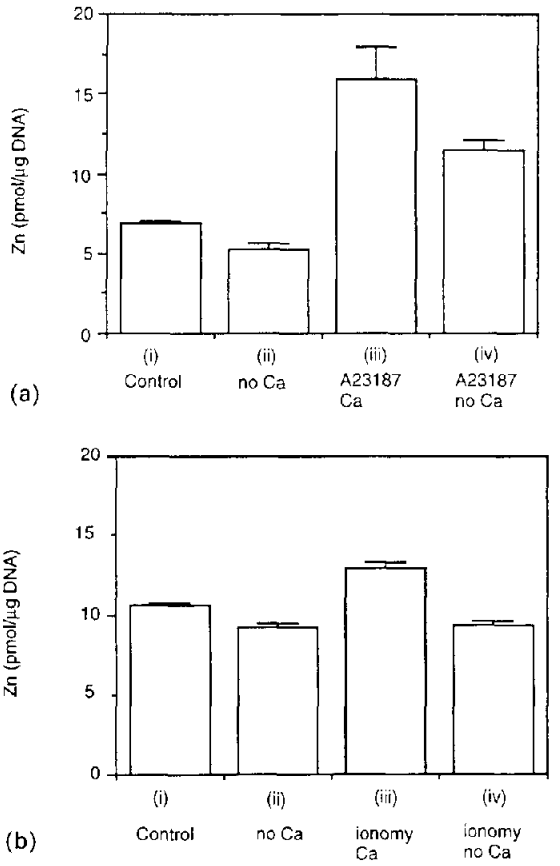


**Figure 11.** (**a**) Pronase-resistant $Zn^{2+}$ uptake after 15 min by fibroblasts incubated in $^{65}Zn$-labeled HBSS with 100 $\mu$M albumin and 5 $\mu$M $ZnCl_2$. (i) Control. Cells had 1.3 mM $Ca^{2+}$ in incubation medium. (ii) No $Ca^{2+}$. Cells had no $Ca^{2+}$ in the incubation medium. (iii) A23187 $Ca^{2+}$. Cells had 10 $\mu$M A23187 and 1.3 mM $Ca^{2+}$ in the incubation medium. (iv) A23187 no $Ca^{2+}$. Cells had 10 $\mu$M A23187 and no $Ca^{2+}$ in the incubation medium. Results are mean ($\pm$SEM) of three determinations. $Zn^{2+}$ uptake was significantly higher in cells treated with A23187 in the presence of $Ca^{2+}$ relative to the control (A23187 and no $Ca^{2+}$). $P=0.05$. (**b**) Pronase-resistant $Zn^{2+}$ uptake after 15 min by fibroblasts incubated in $^{65}Zn$-labeled HBSS with 100 $\mu$M albumin and 5 $\mu$M $ZnCl_2$. (i) Control. Cells had 1.3 mM $Ca^{2+}$ in incubation medium. (ii) No $Ca^{2+}$. Cells had no $Ca^{2+}$ in the incubation medium. (iii) Ionomy $Ca^{2+}$. Cells had 20 $\mu$M ionomycin and 1.3 mM $Ca^{2+}$ in the incubation medium. (iv) Ionomy no $Ca^{2+}$. Cells had 20 $\mu$M ionomycin and no $Ca^{2+}$ in the incubation medium. Results are mean ($\pm$SEM) of three determinations. $Zn^{2+}$ uptake was significantly higher in cells treated with ionomycin in the presence of $Ca^{2+}$ relative to the control (ionomycin and no $Ca^{2+}$). $P=0.002$.

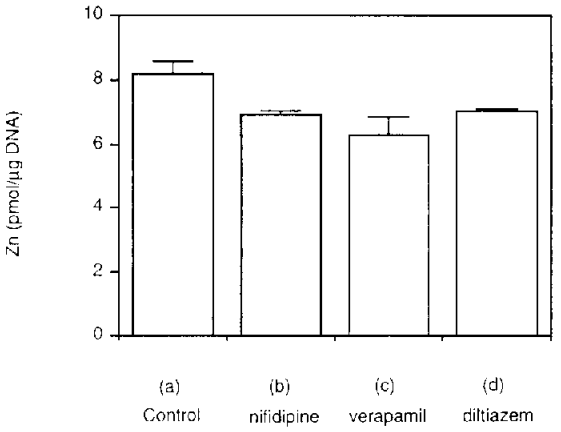


**Figure 12.** Pronase-resistant $Zn^{2+}$ uptake after 15 min by fibroblasts incubated with $^{65}Zn$-labeled BME/10% FBS alone (control) and containing 20 $\mu$M nifidipine, 20 $\mu$M verapamil and 20 $\mu$M diltiazem. Each point represents the mean (±SEM) of three replicate observations. $Zn^{2+}$ uptake was significantly lower in cells treated with the L-channel blockers relative to the control. [a] $P = 0.03$. [b] $P = 0.04$. [c] $P = 0.03$.

However, when the L-channel inhibitors were used in the presence of BME/10% FBS, which contains hormones and growth factors, there was a significant reduction in Pronase-resistant $Zn^{2+}$ uptake (Figure 12). N-channel blockers dynorphin A (5 and 20 $\mu$M) and phe-met-arg-phe amide (40 and 80 $\mu$M) had no obvious effect on Pronase-resistant $Zn^{2+}$ uptake when presented to the cells either in HBSS or in BME/10% FBS.

$Zn^{2+}$ uptake could also be regulated by a number of $Ca^{2+}$-dependent processes. To see if protein kinase C was involved, we used TPA 0.1 and 1 $\mu$M, with 4aP as a control. There was no effect on Pronase resistant $Zn^{2+}$ uptake over 30 min. Protein kinase C could still be important in $Zn^{2+}$ uptake, but if it was maximally stimulated in this system we would not see any change in $Zn^{2+}$ uptake when TPA was added. We tested whether protein kinase C was involved by incubating the cells in TPA overnight, removing the TPA and incubating with $^{65}Zn$. The removal of TPA would cause a down-regulation of protein kinase C. We did not, however, see any changes in the $Zn^{2+}$ uptake from serum-free or serum-containing medium. Several other inhibitors of protein kinase C, staurosporine (50 nM) and lithium (5 and 20 $\mu$M) also had no effect on $Zn^{2+}$ uptake. The calmodulin inhibitor W13 at 50 $\mu$M had no effect.

Protein kinase A appeared not to be involved in regulating $Zn^{2+}$ uptake in this system as protein kinase A inhibitor (10 $\mu$M) and dibutyryl cyclic AMP (up to 100 $\mu$M), a stimulator of protein kinase A, did not affect $Zn^{2+}$ uptake over a period of 30 min. Dynorphin A, an inhibitor of adenylate cyclase activity, was without effect.

Similarly, neither adrenaline nor dexamethasone, a glucocorticoid, changed $Zn^{2+}$ uptake over 30 min.

## Discussion

The data presented in this study shows clearly that, in fibroblasts, $Zn^{2+}$ uptake is not dependent upon $Na^+$ ions, nor is there, as has been shown for red blood cells, a $Cl^-/HCO_3^-$-dependent mechanism. In contrast, transport into fibroblasts appears to operate through a $K^+$-dependent process.

Zinc uptake was reduced in the presence of the $K^+$ ionophores nigericin and valinomycin. This effect is likely to be through their capacity to change the $K^+$ gradient across the cell since it was observed only at temperatures greater than 24° C, approximately that of the membrane phase transition temperature. The inhibitory effects of nigericin and valinomycin on $Zn^{2+}$ uptake were also considerably reduced when the $K^+$ gradient was abolished by incubating the cells in 150 mM $K^+$, compared with no extracellular $K^+$. This is further evidence that $Zn^{2+}$ uptake is dependent on the $K^+$ gradient.

The data could be explained by postulating the presence of a $Zn^{2+}/K$ counter-transport system in which $Zn^{2+}$ moves into the cell in exchange for $K^+$ efflux. If this was the case, we would expect that cellular $Zn^{2+}$ uptake was dependent on the extracellular $K^+$ concentration. We indeed found that over a period of 15 min, $Zn^{2+}$ uptake was progressively reduced in the presence of high extracellular $K^+$ relative to the rate in $K^+$-free medium. Furthermore, when cells were incubated with increasing concentrations of $K^+$, there was an inverse relationship between $Zn^{2+}$ uptake and the extracellular $K^+$ concentration.

We considered the possibility that changes in $K^+$ fluxes produced by the ionophores altered the transmembrane potential and that $Zn^{2+}$ uptake was actually voltage dependent. However, several pieces of evidence suggest that this is not the case. Firstly, nigericin, a charged carboxylic ionophore, forms a neutral complex with cations and effects a cation for proton exchange — an electroneutral process. Valinomycin, on the other hand, is electrogenic, as it forms a charged cation complex which is transported across the membrane (Pressman 1976). Despite these differences, both these ionophores had an inhibitory effect on $Zn^{2+}$ transport. Secondly, changing the membrane potential by different methods, using $Na^+$ ionophores, for example, or changing the *trans*-membrane $Na^+$ gradient by substituting choline for $Na^+$ (data not shown), did not have any effect on $Zn^{2+}$ transport.

To further investigate the possibility of a $Zn^{2+}/K^+$ counter-transport system operating in fibroblasts, we used $Rb^+$ as a tracer for measuring $K^+$ fluxes. The rate of $K^+$ efflux from the cell was found to be 3760 times greater than the $Zn^{2+}$ uptake rate, which suggested that the amount of $K^+$ involved with $Zn^{2+}$ influx was only a small proportion (less than 0.05%) of the total $K^+$ efflux. It is not surprising, therefore, that changes in the extracellular $Zn^{2+}$ concentration did not significantly alter $Rb^+$ efflux. Given that most of the intracellular $Zn^{2+}$ is protein bound, and that the free $Zn^{2+}$ is only a very small proportion of the total intracellular metal, it is also not surprising that alterations in extracellular $K^+$ did not change $Zn^{2+}$ efflux.

To reduce the $Rb^+$ efflux, we added a variety of $K^+$ channel blockers, which have been shown to be effective in smooth (Daut *et al.* 1990) and skeletal (Quayle *et al.* 1988) muscle cells, renal tubules (Kone *et al.* 1989), lymphocytes (Price *et al.* 1989) and in the brain (Gandolfo *et al.* 1989). None of these made any significant difference to $Rb^+$ efflux or indeed to $Zn^{2+}$ uptake. It is possible, however, that $K^+$ channels sensitive to these inhibitors may not be present in skin fibroblasts. A metal-dependent $K^+$ efflux pathway which is insensitive to several $K^+$ inhibitors has been identified in kidney cells (Kone *et al.* 1990).

We investigated whether there were similarities in the mechanism of $Zn^{2+}$ uptake between fibroblasts and red blood cells. In red blood cells, two $Zn^{2+}$ uptake mechanisms have been identified, one in which $Zn^{2+}$ is taken up via the $[Cl^-/HCO3^-]$ anion exchanger as a complex $[Zn(HCO_3)_2Cl]^-$ (Torrubia & Garay 1989), the other where $Zn^{2+}$ is taken up possibly as a neutral complex in the presence of thiocyanate or salicylate ions (Kalfakakou & Simons 1990). Neither of these pathways would appear to be operating in human fibroblasts, since we found that inhibitors of the $[Cl^-/HCO3^-]$ anion exchange did not affect $Zn^{2+}$ uptake.

Evidence for the existence of a $Zn^{2+}/K^+$ counter-transport system has been found in a study on microvilli membrane vesicles prepared from human placenta, where $Zn^{2+}$ uptake was inhibited with increasing extravesicular $K^+$ concentrations (Aslam & McArdle, 1992). In the presence of both valinomycin and nigericin, an outwardly directed $K^+$ gradient stimulated $Zn^{2+}$ uptake. The transporter was also shown to work in the opposite direction. Preloading the vesicles with $Zn^{2+}$ and imposing an inwardly directed $K^+$ gradient resulted in $Zn^{2+}$ efflux, the rate of which was proportional to the $K^+$ gradient. The data suggest that there is a $K^+$-dependent $Zn^{2+}$ transporter in vesicle membranes rather than a voltage-dependent uptake system for $Zn^{2+}$.

Our results show that a component of $Zn^{2+}$ uptake, up to 50%, is dependent on extracellular $Ca^{2+}$, in serum-free incubation medium. Increasing extracellular $Ca^{2+}$ up to 6 mM gave a linear increase in the amount of $Zn^{2+}$ taken up, above 6 mM $Ca^{2+}$, the rate of increase accelerated markedly. The effect at lower more physiological, concentrations does suggest, however, that $Ca^{2+}$ plays a modulating role in $Zn^{2+}$ uptake. The stimulation appears to occur as a result of increased $Ca^{2+}$ influx. This is supported by the positive effect of the $Ca^{2+}$ ionophores A23187 and ionomycin on $Zn^{2+}$ uptake. Although A23187 binds a range of metal ions, and has an affinity for $Zn^{2+}$ which is 100 times greater than that for $Ca^{2+}$ (Chapman *et al.* 1987), the importance of the $Ca^{2+}$ effect is demonstrated by the reduction in $Zn^{2+}$ uptake when A23187 is added without $Ca^{2+}$ and by the fact that ionomycin also has an effect. The results can be explained either by a direct effect of $Ca^{2+}$ influx on $Zn^{2+}$ uptake, or through $Ca^{2+}$-regulated intracellular mechanisms.

We used blockers of different $Ca^{2+}$ channels to determine which of these, if any, were involved in $Ca^{2+}$-dependent $Zn^{2+}$ uptake. None of them had any effect on $Zn^{2+}$ uptake.

$Ca^{2+}$ may be exerting its effect on $Zn^{2+}$ uptake by acting on intracellular pathways. However, the calmodulin inhibitor W13 and protein kinase C inhibitors and stimulators all had no effect on $Zn^{2+}$ uptake. Similarly, dibutyryl cyclic AMP and protein kinase inhibitor, adrenaline and dexamethasone all had no effect. It has been shown that dexamethasone, adrenaline and glucagon can stimulate $Zn^{2+}$ uptake in cultured liver cells (Weiner & Cousins 1983, Cousins 1985), but the effect is almost certainly a consequence of increased metallothionein synthesis rather than a direct effect (Failla & Cousins 1978).

In conclusion, $Zn^{2+}$ uptake occurs by a $K^+$-dependent process, the most likely system we would suggest being a $Zn^{2+}/K^+$ counter-transport system. There are at least two components of $Zn^{2+}$ uptake in fibroblasts, a $Ca^{2+}$-independent component which would seem to be a basal rate, independent of extracellular $Ca^{2+}$ and not regulated by second messenger pathways, and a $Ca^{2+}$-dependent component which is stimulated by extracellular $Ca^{2+}$ and inhibited in the absence of $Ca^{2+}$. This $Ca^{2+}$-dependent component is not mediated by known voltage-dependent $Ca^{2+}$ channels or by second messenger pathways. Its mechanism is unknown. There may also be a third component to $Zn^{2+}$ uptake which is hormone or growth factor-regulated and mediated through $Ca^{2+}$ L-channels.

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