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# Passive $Ca^{2+}$ Transport and $Ca^{2+}$-Dependent $K^+$ Transport in *Plasmodium falciparum*-Infected Red Cells

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**Abstract.** Previous reports have indicated that *Plasmodium falciparum*-infected red cells (pRBC) have an increased $Ca^{2+}$ permeability. The magnitude of the increase is greater than that normally required to activate the $Ca^{2+}$-dependent $K^+$ channel ($K_{Ca}$ channel) of the red cell membrane. However, there is evidence that this channel remains inactive in pRBC. To clarify this discrepancy, we have reassessed both the functional status of the $K_{Ca}$ channel and the $Ca^{2+}$ permeability properties of pRBC. For pRBC suspended in media containing $Ca^{2+}$, $K_{Ca}$ channel activation was elicited by treatment with the $Ca^{2+}$ ionophore A23187. In the absence of ionophore the channel remained inactive. In contrast to previous claims, the unidirectional influx of $Ca^{2+}$ into pRBC in which the $Ca^{2+}$ pump was inhibited by vanadate was found to be within the normal range (30–55 μmol $(10^{13}$ cells · hr$)^{-1}$), provided the cells were suspended in glucose-containing media. However, for pRBC in glucose-free media the $Ca^{2+}$ influx increased to over 1 mmol $(10^{13}$ cells · hr$)^{-1}$, almost an order of magnitude higher than that seen in uninfected erythrocytes under equivalent conditions. The pathway responsible for the enhanced influx of $Ca^{2+}$ into glucose-deprived pRBC was expressed at approximately 30 hr post-invasion, and was inhibited by $Ni^{2+}$. Possible roles for this pathway in pRBC are considered.

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*Abbreviations:* pRBC, parasitized red cells; $[Ca^{2+}]_i$, free internal calcium concentration; $[Ca^{2+}/Ni^{2+}]_o$, external calcium/nickel concentration; NPP, new permeation pathways; DBP, dibutylphthalate; HBS, HEPES-buffered saline; DICAP, depletion-induced calcium permeability.

**Key words:** Calcium transport — Potassium transport — Red cells — *Plasmodium falciparum*

## Introduction

The total $Ca^{2+}$ content of normal human red cells is less than 5 μmol (l cells)$^{-1}$ (Harrison & Long, 1968; Bookchin & Lew, 1980; Engelmann & Duhm, 1987). Of this amount less than 1 μmol (l cells)$^{-1}$ can be extracted using ionophores in the presence of extracellular chelators (Bookchin & Lew, 1980). This condition is maintained throughout the life-span of the cells by five factors: a low intrinsic $Ca^{2+}$ permeability (Lew et al., 1982), a powerful $Ca^{2+}$ extrusion pump (Schatzmann, 1983), minimal cytoplasmic $Ca^{2+}$ buffering capacity (Ferreira & Lew, 1976; Tiffert & Lew, 1997*b*), a total absence of $Ca^{2+}$-accumulating compartments of endoplasmic reticulum or mitochondrial origin (Williamson et al., 1992), and a minimal presence of endocytic inside-out vesicles (Lew et al., 1985). The pump-leak turnover of $Ca^{2+}$ in red cells suspended in autologous plasma is about 30–50 μmol (l cells · hr)$^{-1}$ (Lew et al., 1982; Desai, Schlesinger & Krogstad, 1991; Tiffert et al., 1993) and the physiological free $Ca^{2+}$ level, $[Ca^{2+}]_i$, is less than 100 nM (Lew et al., 1982; Scharff & Foder, 1986; Murphy et al., 1986; Engelmann & Duhm, 1987; David-Dufilho, Montenay-Garestier & Devynck, 1988; Tiffert & Lew, 1997*a*).

Invasion of red cells by *Plasmodium falciparum* induces profound changes in the $Ca^{2+}$ homeostasis of the cells. External $Ca^{2+}$ is required for both the invasion and subsequent development of the parasite (Wasserman, Alarcón & Mendoza, 1982; Raventos Suarez et al., 1982), and there is a net uptake of $Ca^{2+}$ by parasitized cells (pRBC) as the parasite matures (Bookchin et al.,

1980; Tanabe, Mikkelsen & Wallach, 1982; Adovelande et al., 1993), with the bulk of the intracellular $Ca^{2+}$ being localized within the parasite (Shalev et al., 1981; Leida, Mahoney & Eaton, 1981; Tanabe et al., 1982). The increased uptake of $Ca^{2+}$ by pRBC has been attributed to a marked, stage-dependent elevation in the $Ca^{2+}$ permeability of the red cell (Tanabe et al., 1982; Krishna & Ng, 1989; Kramer & Ginsburg, 1991; Desai et al., 1996), though the mechanism underlying this increase is unclear.

Increased $Ca^{2+}$ influx is characteristic of other red cell disorders. In deoxygenated sickle cell anemia red cells the $Ca^{2+}$ permeability is increased about 3–5-fold by the reversible formation of a poorly cation-selective pathway, which is generated from the interaction of deoxy-haemoglobin S polymers and the red cell membrane (Bookchin & Lew, 1981; Etzion et al., 1992). The consequent increase in $[Ca^{2+}]_i$ is sufficient to trigger activation of $Ca^{2+}$-sensitive $K^+$ channels ($K_{Ca}$ or Gardos channels, (Gardos, 1958)) in sickle cell subpopulations (Lew, Ortiz-Carranza & Bookchin, 1997). In normal red cells a less than tenfold elevation in $Ca^{2+}$ permeability, induced experimentally with the use of ionophores is sufficient to elicit $K_{Ca}$ channel activation (Tiffert, Spivak & Lew, 1988). Yet in pRBC, reportedly showing a much higher increase in their passive $Ca^{2+}$ permeability (Kramer & Ginsburg, 1991; Desai et al., 1996) direct $^{86}Rb^+$ flux measurements have ruled out activation of $K_{Ca}$ channels (Kirk et al., 1992).

The aim of the present work was to investigate the origin of this apparent discrepancy. In so doing we have investigated the functional status of the $K_{Ca}$ channel in pRBC, as well as the relationship between $K_{Ca}$ channel activity, $Ca^{2+}$ permeability and metabolic status of these cells.

## Materials and Methods

### *Plasmodium falciparum* Cultures

Human red blood cells (type O) infected with the ITO4 line of *P. falciparum* (Berendt et al., 1989) were cultured under 1% $O_2$, 3% $CO_2$, 96% $N_2$ in RPMI 1640 culture medium, supplemented with D-glucose (10 mM), glutamine (2 mM), HEPES (40 mM), gentamicin sulfate (25 mg $(l)^{-1}$), and human serum (8.5% v/v, pooled from different blood donors). Most experiments were carried out using infected cells with mature, trophozoite-stage parasites (36–40 hr post-invasion), synchronized by a combination of sorbitol hemolysis (Lambros & Vanderberg, 1979) and gelatin flotation (Pasvol et al., 1978). Parasitized cells were harvested from culture immediately prior to experimentation by centrifugation on Percoll for 10 min at 400 × *g*, as described elsewhere (Kirk et al., 1996). For 100 ml, 66 ml of Percoll were diluted with 9.1 ml of 10 × phosphate buffered saline (containing (g $(l)^{-1}$) 1.44 $KH_2PO_4$, 90 NaCl and 7.95 $Na_2HPO_4 \cdot 7H_2O$, pH 7.4 (Gibco)) and 24.9 ml of water to a density of 1.090 (approx. 66% v/v) and an osmolality of 320 mOsm (kg $H_2O)^{-1}$. This method yielded suspensions of 80–96% parasitemia.

In experiments comparing infected with uninfected cell suspensions, uninfected red cells from the same donor were incubated in parallel with pRBC cultures under identical conditions for at least 24 hr prior to the experiment. To ensure that the infected and uninfected cell suspensions were strictly comparable, the uninfected cells were harvested (as above) prior to experimentation. Uninfected cells from the malaria-infected culture were obtained by subjecting trophozoite-stage infected cultures to a sorbitol lysis (as described above) leaving uninfected cells with less than 5% immature ring-stage infected cells.

Experiments in which $Ca^{2+}$ influx into pRBC was measured over the parasite's 48 hr life cycle required highly synchronized cultures. This was achieved by a combination of consecutive sorbitol lysis and gelatin flotation steps over the week prior to the experiment. At the beginning of the experiment the culture was harvested by gelatin flotation at approximately the time of schizogony and placed onto fresh red cells immediately (this step removed any early ring-stage parasites). Approximately 5 hr later the culture was subjected to a sorbitol lysis (to remove remaining schizonts). This process allowed the parasitized cells to be synchronized to within 4–6 hr of each other. Harvesting for experimentation was performed using 65–80% v/v Percoll (produced as described above), depending on the age of the culture, as the density of infected erythrocytes decreases with age.

Cell counts were made using either a Coulter Multisizer or an improved Neubauer counting chamber. Parasitemia was estimated from methanol-fixed Giemsa-stained smears.

### Composition of Media

Detailed compositions of the media used in this study are shown in the figure legends. All solutions were adjusted to pH 7.4 using 2 M NaOH and the osmolality adjusted to 300 ± 3 mOsm (kg $H_2O)^{-1}$ (with distilled water or the dominant salt, usually NaCl), measured with a freezing-point osmometer (Roebling, Germany).

### Efflux Experiments

Estimates of the unidirectional efflux of $K^+$ were made from the efflux of $^{86}Rb^+$ used as a congener for $K^+$. All such measurements were carried out at 37°C. Cell suspensions (1 ml, 10% hematocrit) were preincubated in microcentrifuge tubes for 1 hr at 37°C in growth medium containing $^{86}Rb^+$ at an activity of 10 μCi $(ml)^{-1}$ in the presence of 0.1 mM ouabain, which was added 10 min before loading, and 0.01 mM bumetanide (to inhibit the $Na^+/K^+$ pump and the Na-K-2Cl cotransporter, respectively). Under these conditions infected cells were able to load with $^{86}Rb^+$ via the parasite-induced new permeation pathways (NPP), which are permeable to $^{86}Rb^+$ (Kirk et al., 1994), with minimal tracer incorporation into uninfected cells from the malaria-infected culture. Following the loading period, to remove extracellular radioactivity, the cells were washed three times in ice-cold efflux saline. Following the third wash, the efflux was commenced with the addition to the cell pellet of 1 ml of prewarmed saline containing the $Ca^{2+}$ ionophore A23187 and various inhibitors as indicated (*see* Fig. 1 legend). At predetermined intervals 0.1 ml aliquots were removed from the suspension and transferred to microcentrifuge tubes containing 0.5 ml of ice-cold 'stopping solution' (to inhibit further efflux) layered over 0.3 ml of dibutylphthalate (DBP). The tubes were centrifuged immediately (10,000 × *g*, 30 sec) to remove the red cells below the oil layer and then 0.5 ml of each supernatant solution was transferred to scintillation vials for counting using a β-scintillation counter. At the end of the efflux time course single 0.1 ml aliquots of the remaining suspensions were transferred into microcentrifuge tubes containing 0.1% v/v Triton X-100 (0.3 ml), to lyse the cells and 5% w/v

trichloroacetic acid (0.3 ml), to deproteinize the sample. The tubes were then centrifuged (10,000 × *g*, 10 min) and 4 × 0.1 ml aliquots of the supernatant solution were transferred to scintillation vials for counting, thus enabling an estimate of the initial $^{86}Rb^+$ content of the cells.

### Influx Experiments

Estimates of the unidirectional influx of $K^+$ and $Ca^{2+}$ were made from the influx of $^{86}Rb^+$ and $^{45}Ca^{2+}$. All such measurements were carried out at 37°C. In all cases, cells were washed (×4) then resuspended in HEPES-buffered saline (HBS) containing additional solutes (as specified in the text and figure legends) before the commencement of a flux.

In carrying out influx experiments, washed cell suspensions were dispensed into microcentrifuge tubes and allowed to equilibrate to temperature for at least 10 min. Influx commenced with the addition to the suspension of the radioisotope, to give final activities between 1 and 10 μCi $(ml)^{-1}$ (as specified in the figure legends), a final cell concentration of 1–4 × $10^8$ cells $(ml)^{-1}$, and a final sample volume of 0.5–1.5 ml. At predetermined time intervals aliquots of suspension (100–200 μl) were removed and placed into microcentrifuge tubes, which contained 0.3 ml of DBP. In $^{45}Ca^{2+}$ influx experiments a 'stopping solution' (ice-cold HBS containing 2 mM EGTA (1 ml) and layered over DBP) was used to stop further influx, remove externally bound radiolabel and dilute the total radiolabel to reduce the extracellular radioactivity in the cell pellet. These were centrifuged immediately (10,000 × *g*, 30 sec) to sediment the cells below the oil. The aqueous supernatant solution was removed by aspiration and the radioactivity remaining on the walls of the tube removed by rinsing the tubes (×4) with water, removing the DBP on the final wash. Alternatively, for $Ca^{2+}$ uptake experiments under physiological conditions the aliquots of suspension were washed (×4) by addition and then centrifugation (10,000 × *g*, 10 sec) of ice-cold HBS containing 0.1 mM EGTA (1 ml per wash) to stop further influx and remove externally bound radiolabel. The cell pellets were then processed by the addition of 0.1% v/v Triton X-100 (0.5 ml) and 5% w/v trichloroacetic acid (0.5 ml). After centrifugation (10,000 × *g*, 10 min), the supernatants were transferred into scintillation vials for counting.

For samples in which cells were spun through a DBP layer, the extracellular space in the cell pellet was estimated from the amount of radiolabel in pellets derived from samples taken within a few seconds of combining the cells with radiolabel.

Throughout this study influx rates were estimated from the amount of radiolabel taken up within a fixed incubation period that fell within the initial, linear phase of the uptake time course (characterized either in the course of this work or previously). For $^{86}Rb^+$ influx experiments a single time point of 20 min was used (Kirk et al., 1991). For $^{45}Ca^{2+}$ influx experiments a single time point of 25 min was used. For these influx measurements the flux was terminated as described above, taking triplicate samples in each case.

In some experiments vanadate was used to inhibit the plasma membrane $Ca^{2+}$ pump of red cells, allowing the study of their passive $Ca^{2+}$ permeability properties (Desai et al., 1991; Tiffert & Lew, 1997*b*). Vanadate was used at concentrations between 1 and 5 mM to ensure over 99.5% pump inhibition (Tiffert & Lew, 1997*b*). Stock vanadate solution was added to the appropriate flux medium and the pH was adjusted to 7.4. The medium containing vanadate was then boiled until clear to remove polyanionic forms of vanadate and the osmolality adjusted to 300 ± 3 mOsm $(kg\ H_2O)^{-1}$.

All fluxes were expressed in mol (or subunits) per $10^{13}$ cells per hr $(mol\ (10^{13}\ cells \cdot hr)^{-1})$. $10^{13}$ cells represents the approximate number of cells in one liter of packed cells. This form of expressing fluxes allows straightforward comparisons (albeit approximate) between fluxes in infected and uninfected cells, where fluxes are traditionally reported in mol per liter normal cells per hour or mol per 340 g hemoglobin (the mean hemoglobin content of one liter of normal red cells) per hour.

### ATP Depletion

Infected red cells deplete their stores of ATP within a matter of minutes in the absence of external glucose, as they consume glucose up to 100 times faster than uninfected cells (Roth, Jr., 1990; Kirk, Horner & Kirk, 1996). In this study parasitized cells were depleted of their intracellular ATP by incubation with glucose-free media (compositions are stated in the text and figure legends) or by replacing glucose in the media with 5 mM 2-deoxy-D-glucose. 2-Deoxy-D-glucose was found to induce a rapid depletion of ATP, even in the presence of low levels of glucose.

In contrast to infected cells, glucose-deprivation of uninfected cells has little effect on the cells' ATP levels in the short-term (Lew, 1971). ATP depletion of uninfected cells was achieved by preincubation at 5% hematocrit for 3 hr at 37°C in media containing iodoacetamide (6 mM) and inosine (10 mM) (Lew, 1971; Ferreira & Lew, 1977; Desai, Schlesinger & Krogstad, 1991; Desai et al., 1996). Control red cells (i.e., not ATP-depleted) were incubated (in the presence of 10 mM glucose) in parallel with red cells undergoing ATP depletion.

### Materials

$^{86}Rb^+$, $^{45}Ca^{2+}$ and [$^3$H]-hypoxanthine were obtained from Du Pont New England Nuclear. RPMI 1640 growth medium, gentamicin sulfate, and 1× and 10× phosphate-buffered saline were from Gibco BRL, Life Technologies (Paisley, Scotland). Human serum was supplied by National Blood Services (South West, Bristol). Ouabain, bumetanide, furosemide, quinine, $d_6$-dimethyl sulfoxide (DMSO), ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), ATP bioluminescent assay kit, buffers and sodium vanadate were from Sigma Chemical (Poole, Dorset). $NiCl_2$ was from Aldridge Chemical (Gillingham, Dorset). Clotrimazole and charybdotoxin were from Calbiochem-Novabiochem (Beeston, Nottingham). Nitrendipine was a gift from the late Dr. M. Wolowyk (Faculty of Pharmacy and Pharmaceutical Science, University of Alberta). All inhibitors were added to cell suspensions as a stock solution in DMSO. All reagents were of analytical grade.

## Results

### Is the $K_{Ca}$ Channel in a Functional State in pRBC?

The function of the $K_{Ca}$ channel in pRBC was investigated by measuring the $Ca^{2+}$-induced $^{86}Rb^+$ efflux from $^{86}Rb^+$-preloaded, infected cells. The $^{86}Rb^+$-loading procedure consisted of a brief exposure of the cells to high specific activity $^{86}Rb^+(K^+)$ in the presence of ouabain and bumetanide and was designed to label the parasitized cells via the new broad-specificity permeation pathways (NPP) induced by the parasite in the host cell (Kirk et al., 1994; Ginsburg & Kirk, 1998), with minimal tracer incorporation into uninfected cells. Typical results of $^{86}Rb^+$ efflux experiments are shown in Fig. 1. In the presence of ouabain, bumetanide and furosemide (inhibi-

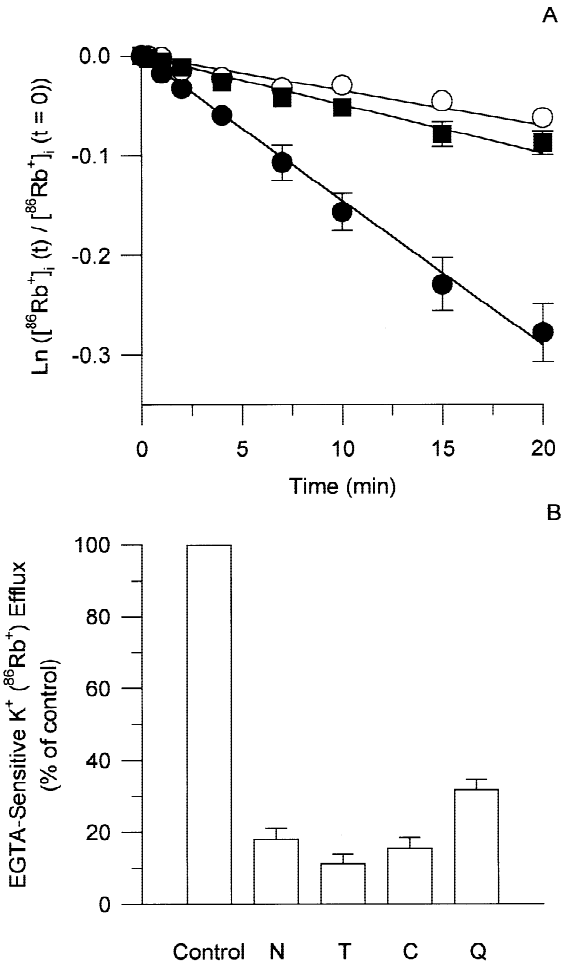


**Fig. 1.** Effect of (*A*) $[Ca^{2+}]_i$ and (*B*) $K_{Ca}$ channel inhibitors on the efflux of $K^+$ ($^{86}Rb^+$) from pRBC at the mature trophozoite-stage. Cells were preloaded with $^{86}Rb^+$, washed with ice-cold efflux medium to remove external $^{86}Rb^+$ and packed (*see* Materials and Methods). The efflux medium contained (mM) KCl 5, NaCl 150, HEPES 10, glucose 10, $MgCl_2$ 0.24 and $CaCl_2$ 0.40 (pH 7.4, 300 ± 3 mOsm (kg $H_2O)^{-1}$). Efflux measurements were initiated by mixing packed cells with prewarmed (37°C) efflux medium. All parasitemias were above 80%. Points and bars represent means from single experiments on red cells from three different donors. Error bars indicate SEM and, where not shown, fall within the symbols. (*A*) unfilled circles, no additions; filled circles, 10 μM A23187; filled squares, 10 μM A23187 and 1 mM EGTA. (*B*) 100% corresponds to the EGTA-sensitive $K^+$ ($^{86}Rb^+$) efflux at 20 min for cells treated with 10 μM A23187; "NTCQ", abbreviations for nitrendipine (10 μM), charybdotoxin (10 nM), clotrimazole (2 μM) and quinine (0.1 mM), respectively.

tors of the $Na^+/K^+$ pump, the Na-K-2Cl cotransporter and the NPP, respectively), $^{86}Rb^+$ loss was approximately exponential with time for ≥20 min under all conditions tested. Addition of the ionophore A23187, in the presence of 0.4 mM $[Ca^{2+}]_o$, induced a significant increase in $^{86}Rb^+$ efflux relative to controls (Fig. 1*A*). This increase was largely but not completely reversed in the presence of excess EGTA. The incomplete reversal is attributable to a minor component of the $^{86}Rb^+$ release being due to ionophore/$Ca^{2+}$-induced hemolysis.

The $Ca^{2+}$-dependent component of the A23187-induced $^{86}Rb^+$ efflux was inhibited by charybdotoxin, nitrendipine, clotrimazole and quinine (Fig. 1*B*), all inhibitors of the red cell $K_{Ca}$ channel. The mean rate constant of the $Ca^{2+}$-dependent, $K_{Ca}$ channel inhibitor-sensitive, $^{86}Rb^+$ efflux was 0.62 ± 0.09 $hr^{-1}$ (mean ± SEM; $n = 3$).

### Do $K_{Ca}$ Channels Participate in the $K^+$ ($^{86}Rb^+$) Turnover of pRBC at Physiological $[Ca^{2+}]_o$ Levels?

In the absence of ionophore, the $K_{Ca}$ channels of pRBC remain inactive at the $[Ca^{2+}]_o$ of 0.4 mM normally used for in vitro cultures (Kirk, Elford & Ellory, 1992). This indicates that $K_{Ca}$ channel activity is not necessary for normal parasite growth in vitro, but does not rule out $K_{Ca}$ channel participation in the $K^+$ turnover of parasitized red cells at the higher $Ca^{2+}$ concentrations found in the plasma (typically 1.3 mM). It has been reported that the $Ca^{2+}$ permeability of pRBC is much higher than that of normal red cells, and, furthermore, that $Ca^{2+}$ influx into pRBC increases steeply with $[Ca^{2+}]_o$ (Kramer & Ginsburg, 1991). It was therefore investigated whether the $K_{Ca}$ channel was activated in pRBC suspended in medium having a $[Ca^{2+}]_o$ similar to that found in vivo.

$^{86}Rb^+$ influx into infected cells was measured in media with 1.3 mM $[Ca^{2+}]_o$, with and without transport inhibitors, both in the presence and absence of glucose. In the presence of glucose (Fig. 2*A*, clear columns), $^{86}Rb^+$ influx was about 7 mmol ($10^{13}$ cells · hr)$^{-1}$. This was reduced to about 1.4 mmol ($10^{13}$ cells · hr)$^{-1}$ by a combination of ouabain, bumetanide, and furosemide. About thirty percent of the influx of $^{86}Rb^+$ into glucose-fed cells was inhibited by ouabain and is therefore attributable to the $Na^+/K^+$ pump. Approximately 60% was via the NPP, inhibited by furosemide, and the rest was via Na-K-2Cl cotransport, inhibited by bumetanide (*not shown*). The further addition of $K_{Ca}$ channel blockers nitrendipine or clotrimazole had no significant effect on $^{86}Rb^+$ influx.

The activity of the $K_{Ca}$ channels was also investigated in glucose-deprived infected cells (Fig. 2*A*, filled columns), under conditions in which the $Ca^{2+}$ content of the cells was increased (*see* Figs. 2*B* and 3*B*). Glucose-deprivation of cells with mature trophozoite-stage parasites causes rapid depletion of ATP (Kanaani & Ginsburg, 1989; Roth, Jr., 1990; Kirk et al., 1996). This condition resembles that of the "Gardos effect" in uninfected cells (Gardos, 1958) in which rapid ATP depletion is induced by the combined effects of metabolic substrates and inhibitors (Lew, 1971, 1974). It can be seen (Fig. 2*B*) that glucose-deprivation caused increasing $Ca^{2+}$ uptake with a lag period similar to that of metabolic depletion treatments used previously to study the Gardos channel in uninfected cells (Lew, 1971). However, the

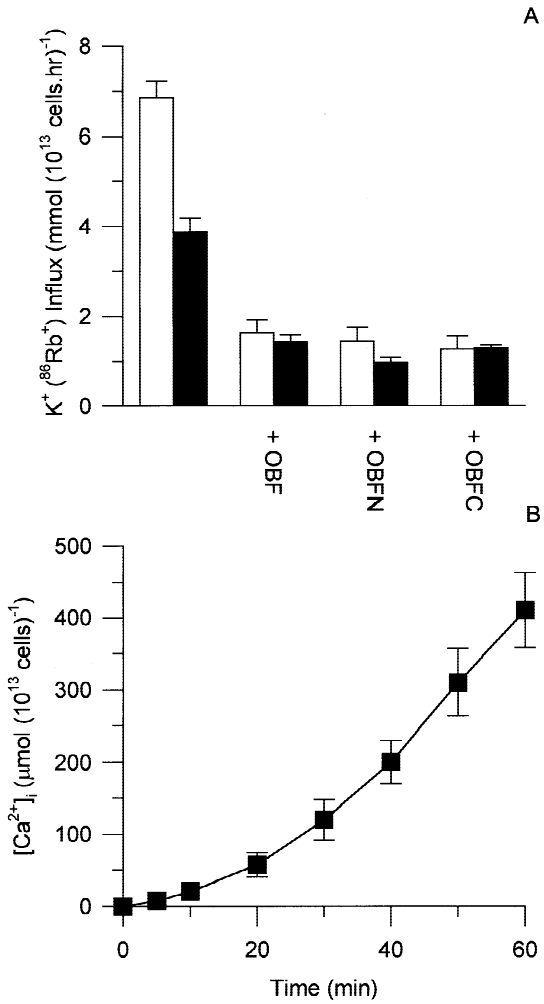


**Fig. 2.** The effect of glucose deprivation on (*A*) the influx of $K^+$ ($^{86}Rb^+$) and (*B*) the time course of $^{45}Ca^{2+}$ uptake in pRBC at the mature trophozoite-stage. (*A*) $K^+$ ($^{86}Rb^+$) influx measurements were initiated by addition of tracer (1 μCi (ml)$^{-1}$) after a 60-min incubation of the cells at 37°C in efflux medium (*see* legend of Fig. 1) with 1.3 mM $CaCl_2$, and either with or without 10 mM glucose. Unfilled columns, glucose-fed cells; filled columns, glucose-deprived cells; "OBFNC", abbreviations for ouabain (0.1 mM), bumetanide (10 μM), furosemide (0.1 mM), nitrendipine (10 μM) and clotrimazole (2 μM), respectively. (*B*) $^{45}Ca^{2+}$ uptake measurements were initiated by addition of packed, washed cells to prewarmed (37°C), glucose-free efflux medium containing 1.3 mM $CaCl_2$ with tracer (2 μCi (ml)$^{-1}$). Values were recalculated equivalent to 100% parasitemia. Points and bars represent means from single experiments on red cells from three different donors. Error bars indicate SEM and, where not shown, fall within the symbols.

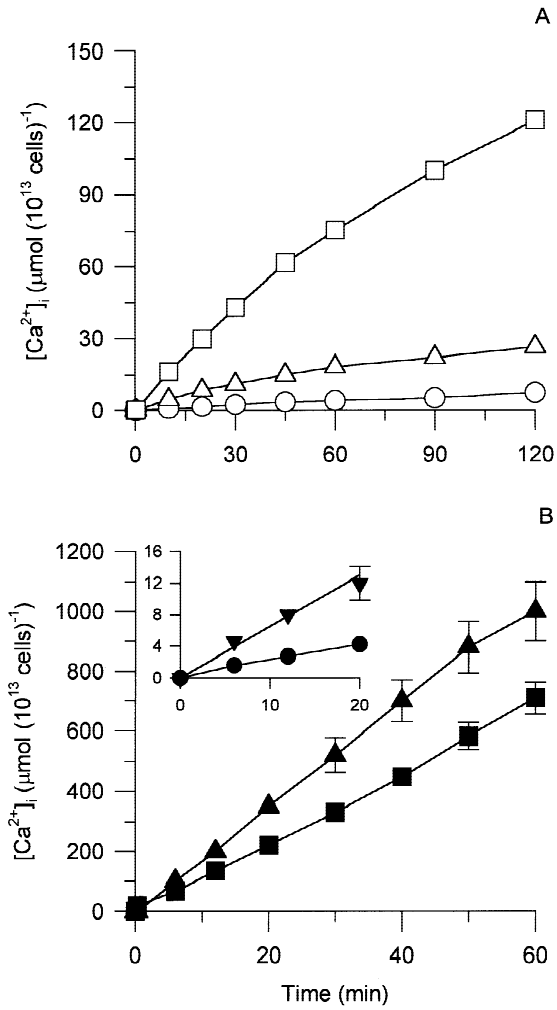


**Fig. 3.** The effect of metabolic perturbation on the time courses of $^{45}Ca^{2+}$ uptake into (*A*) uninfected and (*B*) pRBC at the mature trophozoite-stage. $^{45}Ca^{2+}$ influx measurements were initiated by addition of tracer (10 μCi (ml)$^{-1}$ to uninfected and glucose-fed infected cells and 2 μCi (ml)$^{-1}$ to glucose-deprived infected cells) together with unlabeled $CaCl_2$ (final concentration of 1.3 mM) to prewarmed (37°C) cells in influx media. The media contained (mM) KCl 80, NaCl 50, sucrose 30, HEPES 20 for uninfected red cells and NaCl 130, sucrose 30, HEPES 20 for infected red cells (both media were pH 7.4, 300 ± 3 mOsm (kg $H_2O$)$^{-1}$). Values were recalculated equivalent to 100% parasitemia. Points represent means from single experiments on red cells from three different donors. Error bars indicate SEM and, where not shown, fall within the symbols. (*A*) Unfilled circles, uninfected cells in the presence of 10 mM glucose (i.e., metabolically normal); unfilled triangles, uninfected cells, which were ATP-depleted by preincubation for 3 hr at 37°C in media with 6 mM iodoacetamide, 10 mM inosine; unfilled squares, ATP-depleted uninfected cells in the presence of 5 mM vanadate. (*B*) Filled triangles, glucose-deprived infected cells (i.e., ATP-depleted); filled squares, glucose-deprived infected cells in the presence of 5 mM vanadate. Inset: filled circles, infected cells in the presence of 10 mM glucose (i.e., metabolically normal); filled inverted triangles, glucose-fed infected cells in the presence of 1 mM vanadate.

final $Ca^{2+}$ uptake rate of glucose-starved cells was orders of magnitude larger than that observed in either glucose-depleted uninfected cells, or glucose-fed pRBC (*see* Fig. 3). The nature and origin of this high $Ca^{2+}$ permeability state is addressed below. Here we consider the effects of glucose deprivation on $^{86}Rb^+$ fluxes.

As shown in Fig. 2*A* (filled columns), in the absence of inhibitors, $^{86}Rb^+$ influx into glucose-depleted pRBC was about 4 mmol ($10^{13}$ cells · hr)$^{-1}$ (lower than the rate seen in glucose-fed cells, consistent with ATP-depletion causing inactivation of the $Na^+/K^+$ pump). Treatment of the cells with ouabain, bumetanide, and furosemide

reduced the flux to approximately 1.4 mmol ($10^{13}$ cells · hr)$^{-1}$. As was the case in glucose-fed pRBC, $K_{Ca}$ channel blockers were without significant effect. Thus, in the glucose-deprived parasitized cells, the large $Ca^{2+}$ gains observed did not trigger $K_{Ca}$ channel activation.

The results show that $K_{Ca}$ channels do not participate in the $K^+$ ($^{86}Rb^+$) turnover of glucose-fed infected cells at physiological $[Ca^{2+}]_o$ levels, and that they remain inactive in glucose-deprived, ATP-depleted cells under conditions of large net cell $Ca^{2+}$ gains.

## Is the $Ca^{2+}$ Permeability of pRBC Under Metabolic Control?

Figure 3 shows typical $^{45}Ca^{2+}$ uptake results in uninfected and infected red cells in different metabolic conditions, with and without $Ca^{2+}$ pump inhibition by vanadate. The external $Ca^{2+}$ concentration was 1.3 mM. $Ca^{2+}$ uptake was linear for at least 20 min under all conditions. Similar results were obtained in uninfected cells isolated from *P. falciparum*-infected cell cultures, as well as uninfected cocultured cells (*not shown*).

Glucose-fed cocultured uninfected cells took up $Ca^{2+}$ at a rate of 4 ± 1 μmol ($10^{13}$ cells · hr)$^{-1}$ (mean ± SEM; $n = 3$; Fig. 3*A*). In fresh red cells, $Ca^{2+}$ uptake under similar conditions is immeasurably small (Ferreira & Lew, 1977; Schatzmann, 1983). $Ca^{2+}$ uptake by uninfected cells predepleted of ATP by the combined use of iodoacetamide and inosine was increased to 20 ± 2 μmol ($10^{13}$ cells · hr)$^{-1}$ (mean ± SEM; $n = 3$; Fig. 3*A*). Residual $Ca^{2+}$ pump activity fueled by low remaining ATP levels limits $Ca^{2+}$ gain under these conditions (Harbak & Simonsen, 1987; Alvarez, García-Sancho & Herreros, 1988). Vanadate, at concentrations above 1 mM, inhibits the $Ca^{2+}$ pump of intact red cells by over 99.5% (Tiffert & Lew, 1997*b*) and under these conditions (i.e., ATP-depleted and vanadate-treated) $Ca^{2+}$ uptake was 113 ± 12 μmol ($10^{13}$ cells · hr)$^{-1}$ (mean ± SEM; $n = 6$; Fig. 3*A*), 2–3 times higher than previous estimates of the rate in fresh red cells under the same conditions (Lew et al., 1982; Desai et al., 1991). Thus, for uninfected cocultured cells or uninfected cells isolated from the malaria-infected culture, the general $Ca^{2+}$ uptake pattern followed that observed before in fresh red cells under different experimental conditions, with a tendency to moderately elevated values.

The situation in pRBC was significantly different (Fig. 3*B*). $Ca^{2+}$ influx in glucose-fed pRBC, without and with vanadate was 15 ± 1.2 and 37 ± 6.3 μmol ($10^{13}$ cells · hr)$^{-1}$, respectively (Fig. 3*B*, inset), whereas in glucose-deprived cells, without and with vanadate, $Ca^{2+}$ influx was increased to 1043 ± 86 and 758 ± 54 μmol ($10^{13}$ cells · hr)$^{-1}$, respectively. This increase was almost an order of magnitude greater than that seen under equivalent conditions in uninfected cells. The small, apparent inhibition of $Ca^{2+}$ uptake by vanadate in glucose-deprived infected cells observed in Fig. 3*B* was not found to be significant ($P > 0.2$, paired *t*-test) when the data were analyzed with data from further experiments (*see* Table 2).

In glucose-fed, vanadate-treated cells, $Ca^{2+}$ uptake approximates the true passive $Ca^{2+}$ permeability of the infected, metabolically competent cells, with minimal effects of residual $Ca^{2+}$ pump activity. If the sole effect of glucose deprivation (with or without additional vanadate) had been to inhibit the $Ca^{2+}$ pump, the $Ca^{2+}$ uptake of glucose-deprived cells would have been similar to that observed in glucose-fed, vanadate-treated cells. Therefore, the progressive increase in $Ca^{2+}$ uptake, which develops during glucose deprivation (Figs. 2*B* and 3*B*), represents the formation or activation of a new $Ca^{2+}$ permeability that is absent or inactive in glucose-fed infected cells (Fig. 3*B*).

These data are consistent with the hypothesis that in pRBC, unlike in uninfected cells, the $Ca^{2+}$ permeability is under metabolic control, increasing in response to a depletion of cellular ATP. To characterize further the depletion-induced $Ca^{2+}$ permeability (referred to henceforth as DICAP) we compared its kinetic properties and inhibitor-sensitivity with those of the passive $Ca^{2+}$ permeation pathway of uninfected, cocultured cells.

## External $Ca^{2+}$ Concentration-Dependence of Passive $Ca^{2+}$ Permeation Pathways in Uninfected and pRBC

$Ca^{2+}$ influx was measured in uninfected and infected cells at different $[Ca^{2+}]_o$. All cells were ATP-depleted. Uninfected cells were depleted by inosine-iodoacetamide treatment and $Ca^{2+}$ uptake was measured in the presence of vanadate to block residual $Ca^{2+}$ pumping. Infected cells were depleted by a 1 hr preincubation in glucose-free media. Figure 4 shows $Ca^{2+}$ uptake as a function of $[Ca^{2+}]_o$. It can be seen that $Ca^{2+}$ uptake in infected cells was much higher than that in uninfected cells (Fig. 4, inset) at all $[Ca^{2+}]_o$, and that this difference was contributed by both, saturable and linear components.

The continuous lines in Fig. 4 describe fits of the experimental points by equations with Michaelis-Menten and linear terms. The fitted parameters are given in Table 1. In the uninfected cells, the saturable component retained a $K_m$ value of 0.7 mM, similar to that of fresh red cells (0.8 mM, (Tiffert et al., 1984; McNamara & Wiley, 1986)), but the slope of the linear component was significantly higher. In infected cells the $K_m$ of the saturable component was approximately 3.6 mM, and its maximal capacity was over twentyfold that of the uninfected cells. At physiological $[Ca^{2+}]_o$ levels, the major difference in $Ca^{2+}$ uptake between infected and uninfected cells is therefore attributable to the saturable com-

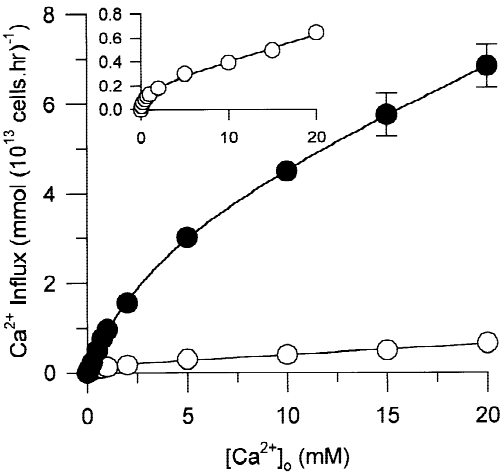


**Fig. 4.** The effect of $[Ca^{2+}]_o$ on the influx of $^{45}Ca^{2+}$ in ATP-depleted uninfected cell and ATP-depleted pRBC at the mature trophozoite-stage. $^{45}Ca^{2+}$ influx measurements were initiated by addition of washed cells to prewarmed (37°C) influx media (*see* legend of Fig. 3) containing tracer (2 μCi (ml)$^{-1}$) and varying amounts of $CaCl_2$. Values were recalculated equivalent to 100% parasitemia. Points represent means from single experiments on red cells from three different donors. Error bars indicate SEM and, where not shown, fall within the symbols. Unfilled circles, ATP-depleted uninfected cells in the presence of 5 mM vanadate; filled circles, glucose-deprived infected cells. Inset: shows a more detailed view of the concentration-dependence of $Ca^{2+}$ influx in ATP-depleted uninfected red cells.

ponent of the DICAP-mediated transport; at higher $[Ca^{2+}]_o$ the dominant effect is through the linear component which is also increased substantially. The DICAP is therefore via one or more pathways with kinetic properties, differing somewhat from those of the passive $Ca^{2+}$-permeation pathway of cocultured uninfected cells.

Unlike the broad-specificity NPP, induced by the intracellular parasite in the host cell membrane (for recent review *see* Ginsburg & Kirk, 1998), the DICAP is not functional in normally metabolizing pRBC (*see* Fig. 3*B*). Involvement of the NPP in DICAP was ruled out by measuring $Ca^{2+}$ uptake through DICAP as a function of $[Ca^{2+}]_o$ in the presence and absence of furosemide, a potent inhibitor of the NPP (Kirk et al., 1994). As anticipated, furosemide (0.1 mM) was without effect on DICAP-mediated $Ca^{2+}$ uptake over the $[Ca^{2+}]_o$ range 0.1 to 20 mM (*not shown*).

### Effect of $Ni^{2+}$ on the Passive $Ca^{2+}$ Uptake of Uninfected and pRBC

Divalent cations are known to act as partial inhibitors of passive $Ca^{2+}$ transport pathways in different cells, including red cells (Varecka & Carafoli, 1982). Preliminary experiments indicated that $Ni^{2+}$ was an effective inhibitor of DICAP. Its relative effects on the passive $Ca^{2+}$ permeability of infected red cells are shown in Table 2. $Ni^{2+}$, at a concentration of 0.1 mM, had relatively little effect on $Ca^{2+}$ uptake into ATP-depleted, uninfected cells, with or without vanadate (*not shown*). In infected cells it had no effect in the presence of glucose, whereas it inhibited up to 90% of the $Ca^{2+}$ influx in the glucose-deprived cells. The inhibition of DICAP by $Ni^{2+}$ appeared to be partially antagonized by vanadate. The reason for this is unknown.

The concentration-dependence of the effects of $Ni^{2+}$ on DICAP is shown in Fig. 5. The half-maximal inhibitory potencies of $Ni^{2+}$ in the absence and presence of vanadate were 6 and 15 μM, respectively. $Ni^{2+}$ exerted its maximal inhibitory effect at concentrations of between 0.1 and 1 mM. At a concentration of 1 mM, $Ni^{2+}$ inhibited >90% of the depletion-induced $Ca^{2+}$ uptake. $Ni^{2+}$ inhibition was partially prevented or antagonized by vanadate, reaching only 70% inhibition at 1 mM.

### Activity of the DICAP During the 48 Hour Intraerythrocytic Phase of the *P. falciparum* Life Cycle

The developmental stage-dependence of DICAP was investigated, by measuring the $Ni^{2+}$-sensitive $Ca^{2+}$ uptake into pRBC depleted of ATP by preincubation for 1 hr in glucose-free media. The $Ca^{2+}$ influx rates are shown in Fig. 6, and the efficiency of the ATP-depletion procedure throughout the relevant stages of parasite development is reported in Table 3. All measurements were performed using highly synchronized parasite cultures, prepared as described in Materials and Methods. The results in Fig. 6 show that the DICAP was expressed only after 28 hr post-invasion, was fully inducible by ATP depletion at 35 hr, and remained so at least up to 43 hr. Table 3 shows that inducibility was not related to the ATP depletion efficiency of glucose-deprivation, since this procedure was equally effective at 28 hr, when the DICAP was not yet inducible, as at all subsequent times thereafter. Thus, the DICAP is a late-comer relative to the NPP of normal cultures, which is reported to be induced within the range of 6 to 15 hr post-invasion (Elford et al., 1985; Kutner et al., 1985).

The results in Table 3 deserve separate consideration. The ATP content of parasitized cells was obtained by subtracting the contribution of the uninfected cells present (assumed to be the same as cocultured cells, the ATP content of which was unaffected by 1 hr of glucose deprivation) and thereby correcting to 100% parasitemia. Error propagation affected the estimates of residual ATP in the glucose-deprived pRBC much more than in the glucose fed pRBC, reflected in a 6-fold increase in the relative errors of the ATP content estimates in ATP-depleted pRBC (20%) relative to fed pRBC (3%). With such large errors, the residual ATP content of glucose-

**Table 1.** Parameters from curve fits of $Ca^{2+}$ uptake as a function of $[Ca^{2+}]_o$ in uninfected (RBC) and parasitized (pRBC) red cells

| | $K_m$ (mM) | $V_{max}$ (mmol ($10^{13}$ cells · hr)$^{-1}$) | $k_d$ (mmol ($10^{13}$ cells · hr · mM)$^{-1}$) |
|---|---|---|---|
| RBC | 0.7 ± 0.04 | 0.185 ± 0.019 | 22.4 ± 1.6 |
| pRBC | 3.63 ± 0.42 | 3.98 ± 0.65 | 205 ± 40 |
| *P* (paired *t*-test) | <0.01 | <0.01 | <0.02 |

The data (*see* legend of Fig. 4 for the conditions used) were fitted to the equation: *Influx rate* = $((V_{max} \times [Ca^{2+}]_o)/(K_m + [Ca^{2+}]_o)) + (k_d \times [Ca^{2+}]_o)$ using Sigmaplot software (Jandel Scientific). Data fits without the linear component were found to contain much larger errors (*data not shown*). Least mean squares parameter values from single experiments on cells from three different donors are shown as the mean ± SEM. The *P* values report the statistical significance of the difference between the parameters of $Ca^{2+}$ transport between uninfected and malaria-infected red cells.

**Table 2.** The effect of $Ni^{2+}$ on the influx of $^{45}Ca^{2+}$ in pRBC at the mature trophozoite-stage

| ATP-depleted | Vanadate | $Ni^{2+}$ | Influx (μmol ($10^{13}$ cells · hr)$^{-1}$) | $Ni^{2+}$-induced inhibition (%) |
|---|---|---|---|---|
| − | − | − | 15.4 ± 0.5 | −4 |
| − | − | + | 16.0 ± 0.8 | |
| − | + | − | 40.4 ± 5.7 | 32 |
| − | + | + | 27.5 ± 3.4 | |
| + | − | − | 1070 ± 49 | 88 |
| + | − | + | 125 ± 27 | |
| + | + | − | 1019 ± 56 | 61 |
| + | + | + | 400 ± 30 | |

$^{45}Ca^{2+}$ influx measurements were performed (*see* legend of Fig. 3 for the conditions used) in the absence and presence of 0.1 mM $Ni^{2+}$. Values were recalculated equivalent to 100% parasitemia and are shown as the mean ± SEM, from single experiments on cells from three different donors.

deprived pRBC may not be significantly different from zero. In the experiments giving rise to Fig. 2*A* it was found that in pRBC subjected to glucose-starvation there was negligible effect of ouabain on $K^+$ ($^{86}Rb^+$) influx (*not shown*). This is consistent with ATP depletion (at least in the red cell compartment) being sufficiently complete to cause inactivation of the $Na^+/K^+$ pump.

### Is the DICAP Required for Normal *P. falciparum* Growth In Vitro?

The conditions of glucose deprivation required for DICAP activation suggest that it is not required for normal parasite growth. This was tested directly using $Ni^{2+}$ to inhibit the DICAP. A growth assay (in 10% v/v human serum) was carried out in parallel with dose-response experiments to study the effect of $Ni^{2+}$ on $Ca^{2+}$ uptake in glucose-deprived infected cells, in the presence and absence of 10% v/v human serum. Figure 7 shows the dose-response curves for the effect of $Ni^{2+}$ on [$^3$H]-hypoxanthine incorporation (a measure of parasite growth) and DICAP-mediated $Ca^{2+}$ uptake. The serum reduced the ability of $Ni^{2+}$ to block DICAP, shifting the dose-response curve to the right. Nevertheless, 1 mM $Ni^{2+}$ inhibited DICAP-mediated $Ca^{2+}$ uptake by approximately 90%, both in the presence and absence of serum. At the same concentration it had little effect on parasite growth, consistent with DICAP not playing an important role in the growth of *P. falciparum* in vitro.

## Discussion

The aim of this study was to clarify the apparent discrepancy between reports that pRBC show a highly increased $Ca^{2+}$ permeability (Kramer & Ginsburg, 1991; Desai et al., 1996) and the report that the $K_{Ca}$ channel remains inactive in these cells (Kirk et al., 1992). The present results confirmed $K_{Ca}$ channel inactivity at physiological $[Ca^{2+}]_o$ levels, even in ATP-depleted cells in which the $Ca^{2+}$ influx rate was elevated to a level well above that sufficient to activate $K_{Ca}$ channels in normal, uninfected red blood cells and in sickle cells.

The only condition in which $K_{Ca}$ channel activation could be elicited in parasitized cells with mature-stage parasites was by $Ca^{2+}$ permeabilization with the ionophore A23187. The mean rate constant for the iono-

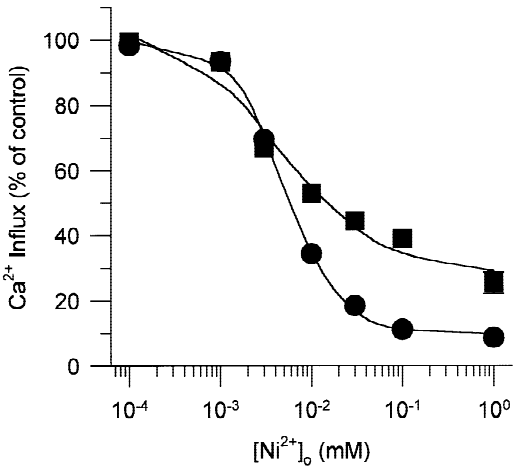


**Fig. 5.** Dose-response curves for the effect of $Ni^{2+}$ on the influx of $^{45}Ca^{2+}$ in glucose-deprived pRBC at the mature trophozoite-stage. $^{45}Ca^{2+}$ influx measurements were initiated by addition of tracer (2 μCi $(ml)^{-1}$) and unlabeled $CaCl_2$ (final concentration of 1.3 mM) to prewarmed (37°C) cells in influx media (*see* legend of Fig. 3) containing varying amounts of $NiCl_2$. All parasitemias were above 80%. $Ca^{2+}$ influx measurements are expressed as percent of the values obtained in $Ni^{2+}$-free controls with the same parasitemia. Bars represent means from single experiments on red cells from three different donors. Error bars indicate SEM and, where not shown, fall within the symbols. Filled circles, glucose-deprived infected cells; filled squares, glucose-deprived infected cells in the presence of 5 mM vanadate.

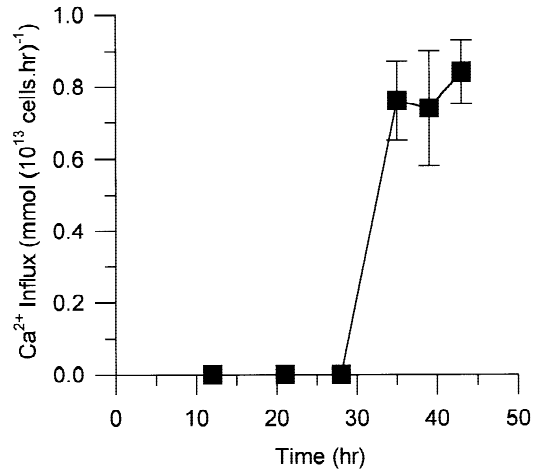


**Fig. 6.** Influx of $^{45}Ca^{2+}$ into glucose-deprived pRBC over the intraerythrocytic phase of the life cycle of the parasite. Cultures were synchronized as detailed in Materials and Methods. At the indicated times aliquots of the infected culture were enriched using Percoll density separation and then the cells were incubated at 37°C in glucose-free media for 1 hr before experimentation. $^{45}Ca^{2+}$ influx measurements were initiated by addition of tracer (2 μCi $(ml)^{-1}$) and unlabeled $CaCl_2$ (final concentration of 1.3 mM) to prewarmed (37°C) cells in influx media (*see* legend of Fig. 3). Values were recalculated equivalent to 100% parasitemia. Points represent means from single experiments on red cells from two different donors. Error bars indicate SEM and, where not shown, fall within the symbols.

phore-induced $^{86}Rb^+$ efflux was 0.62 ± 0.09 $hr^{-1}$. Although direct comparisons between infected and uninfected cells were not feasible because the $K^+$ pools and driving forces for $^{86}Rb^+$ efflux are substantially different, it is relevant to note that under equilibrium exchange conditions, at high external $K^+$ concentrations, maximal tracer equilibration rates in resealed ghosts (Simons, 1976) and intact (uninfected) red cells (Lew & Ferreira, 1976) are in the range 3 to 10 $hr^{-1}$. $^{86}Rb^+$ efflux from infected cells in a 5 mM $[K^+]_o$ medium is predominantly by tracer exchange, because the host cell $K^+$ pool is substantially reduced (Lee et al., 1988). The rate of efflux of $^{86}Rb^+$ from ionophore-treated parasitized cells is therefore substantially less than that expected for normal, uninfected cells, though whether this is due to an impairment of $K_{Ca}$ channel function in parasitized cells, or to the compartmentalization of much of the preloaded $^{86}Rb^+$ within the intracellular parasite is unclear.

Although the $^{86}Rb^+$ efflux data are consistent with the hypotheses that the $K_{Ca}$ channels of parasitized red cells operate with reduced activity and $Ca^{2+}$-sensitivity, uncertainties concerning the effect of $Na^+$-$K^+$ gradient dissipation on $K_{Ca}$ channel function, and the extent to which different treatments induce elevated $[Ca^{2+}]_i$ in the host cell cytosol, do not allow firm conclusions to be drawn on the true state of the $K_{Ca}$ channels in pRBC. More detailed information on cytoplasmic $Ca^{2+}$ buffering and compartmentalization, on host cell $Ca^{2+}$ pump function and $[Ca^{2+}]_i$ levels during controlled $Ca^{2+}$ permeabilization, and on the dynamics of parasite-host $Ca^{2+}$ and $K^+$ exchange will be required to establish the extent of $K_{Ca}$ channel function conservation or alteration. The only firm conclusion that can be drawn from the $^{86}Rb^+$ flux data is that $K_{Ca}$ channel activity is neither elicited nor required during normal parasite growth at the $[Ca^{2+}]_o$ present in vitro or in vivo.

The most significant findings in the present study are: (i) that the $Ca^{2+}$ permeability of glucose-fed pRBC remains comparable with the normal levels found for cocultured uninfected cells throughout the whole asexual developmental cycle of the parasite in red cells; and (ii) that the high $Ca^{2+}$ permeability of infected cells reported previously (Kramer & Ginsburg, 1991; Desai et al., 1996) probably represents the late expression, between 28 and 35 hours after invasion, of a $Ni^{2+}$-sensitive $Ca^{2+}$ permeability that is only activated under conditions of ATP-depletion. Whether DICAP mediates the influx of $Ca^{2+}$ into the red cell cytosol, or perhaps provides a direct ("parallel") route from the external medium into the intracellular parasite (*see* Gero & Kirk, 1994; Ginsburg & Kirk, 1998) remains to be resolved.

To stress the fact that the novel $Ca^{2+}$ permeability becomes measurable only after ATP depletion, it was named the depletion-induced $Ca^{2+}$ permeability, DICAP. The failure of $Ni^{2+}$ to inhibit the in vitro growth of the parasites, at concentrations sufficient to cause >90% in-

**Table 3.** The ATP contents of cocultured, uninfected cells and pRBC over the intraerythrocytic phase of the life cycle of the parasite

| Time (post-invasion; hr) | | 28 | 35 | 39 | 43 | Mean ± SEM |
|---|---|---|---|---|---|---|
| parasitemia (%) | | 36 | 49 | 69 | 78 | |
| | Uninfected | | | | | 1.07 ± 0.10 |
| [ATP] | Glucose-fed | 1.72 | 2.14 | 2.22 | 2.54 | |
| (mmol ($10^{13}$ cells)$^{-1}$) | (100% parasitemia) | 2.87 | 3.25 | 2.73 | 2.95 | 2.95 ± 0.09 |
| | Glucose-deprived | 0.86 | 0.79 | 0.46 | 0.44 | |
| | (100% parasitemia) | 0.49 | 0.50 | 0.18 | 0.26 | 0.36 ± 0.07 |

Glucose-deprived cells were incubated at 37°C in glucose-free media for 1 hr before the ATP assay. Cultures were synchronized as detailed in Materials and Methods. At the indicated times aliquots of the infected culture were enriched to the indicated parasitemias using Percoll density separation (*see* Materials and Methods). Note the improved enrichment efficiency with the stage of the culture. The firefly luciferase method was used to measure ATP levels (Brown, 1982) and adapted as described elsewhere (Kirk et al., 1996). Similar results were obtained in an experiment on cells from a different donor.

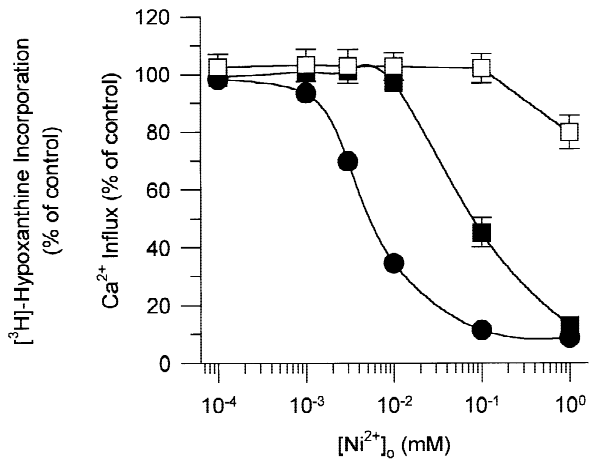


**Fig. 7.** Dose-response curves for the effect of $Ni^{2+}$ on the influx of $^{45}Ca^{2+}$ (closed symbols) and [$^3$H]-hypoxanthine incorporation (open symbols) in pRBC at the mature trophozoite-stage. $^{45}Ca^{2+}$ influx measurements were initiated by addition of tracer (2 μCi (ml)$^{-1}$) and unlabeled $CaCl_2$ (final concentration of 1.3 mM) to prewarmed (37°C) cells in influx media (*see* legend of Fig. 3) containing varying amounts of $NiCl_2$. Filled circles, glucose-deprived infected cells; filled squares, glucose-deprived infected cells in the presence of 10% human serum (for this condition 5 mM 2-deoxy-D-glucose was also present in the medium to ensure ATP-depletion despite the presence of glucose in the serum). All parasitemias were above 80%. $Ca^{2+}$ influx measurements are expressed as percent of the values obtained in $Ni^{2+}$-free controls with the same parasitemia. Points represent means from single experiments on red cells from three different donors. Error bars indicate SEM and, where not shown, fall within the symbols. The effect of $Ni^{2+}$ on parasite growth (unfilled squares) was tested using [$^3$H]-hypoxanthine incorporation as a marker of parasite growth (Desjardins et al., 1979) adapted as described elsewhere (Kirk et al., 1993). Points represent means from 8 experiments on cells from a single donor. Error bars indicate SEM. Note that 10% human serum was present during the growth assay.

hibition of DICAP is consistent with the DICAP not playing a role in parasite development under culture conditions. The question remains, however, of whether DICAP has a functional role in vivo.

One possibility is that the DICAP may represent the activation of a capacitative $Ca^{2+}$ channel. In the in vivo situation, at the developmental stage in which DICAP becomes expressed, most pRBC are sequestered in the blood capillaries. In some vessels, parasitized cell density may become high enough to transiently reduce plasma glucose levels. Glucose deprivation, and the consequent ATP-depletion, may prevent the operation of the $Ca^{2+}$ pumps responsible for maintaining intracellular $Ca^{2+}$ stores within the parasite (Garcia et al., 1996; Passos & Garcia, 1997, 1998), resulting in $Ca^{2+}$ store depletion. It may be this, rather than ATP depletion *per se* that triggers the activation of the DICAP. In this context DICAP might provide a route for the rapid refilling of the parasite's $Ca^{2+}$ stores.

Alternatively, the DICAP might conceivably play a role in killing the parasite under conditions where its continued survival would threaten the host. If the density of pRBC in the microcirculation becomes high enough to reduce or block circulation and compromise the glucose supply to the highly metabolically active parasitized cells, the DICAP would be activated. This would result in the increased uptake of $Ca^{2+}$, together with other local alterations, leading to the rapid demise of the parasite and host cell. This may ultimately act to limit damage to the host organism.

It is important to stress that our finding that the passive $Ca^{2+}$ permeability, in pRBC under physiological conditions, is within the range found in normal, uninfected cells and is not at odds with the previous finding that pRBC show a marked increase in their total $Ca^{2+}$ content (Bookchin et al., 1980; Leida et al., 1981; Tanabe et al., 1982). The intracellular parasite may be able to take up significant quantities of $Ca^{2+}$ across its plasma membrane and to accumulate $Ca^{2+}$ within its organelles, without necessarily inducing a significant increase in host cell $[Ca^{2+}]_i$. The mechanisms by which it does so, as well as those involved in the DICAP phenomenon described here, are yet to be clearly defined.

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