Membrane Potential of *Plasmodium*-infected Erythrocytes

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ABSTRACT The membrane potential (E_m) of normal and *Plasmodium chabaudi*-infected rat erythrocytes was determined from the transmembrane distributions of the lipophilic anion, thiocyanate (SCN), and cation, triphenylmethylphosphonium (TPMP). The SCN- and TPMP-measured E_m of normal erythrocytes are -6.5 ± 3 mV and -10 ± 4 mV, respectively. The TPMP-measured E_m of infected cells depended on parasite developmental stage; "late" stages (schizonts and gametocytes) were characterized by a $E_m = -35$ mV and "early" stages (ring and copurifying noninfected) by a low E_m (−16 mV). The SCN-determined E_m of infected cells was −7 mV regardless of parasite stage.

Studies with different metabolic inhibitors including antimycin A, a proton ionophore (carbonylcyanide *m*-chlorophenylhydrazone [CCCP]), and a H^+-ATPase inhibitor (*N,N'*-dicyclohexylcarbodiimide, [DCCD]) indicate that SCN monitors the E_m across the erythrocyte membrane of infected and normal cells whereas TPMP accumulation reflects the E_m across the plasma membranes of both erythrocyte and parasite. These inhibitor studies also implicated proton fluxes in E_m-generation of parasitized cells. Experiments with weak acids and bases to measure intracellular pH further support this proposal. Methylamine distribution and direct pH measurement after saponin lysis of erythrocyte membranes demonstrated an acidic pH for the erythrocyte matrix of infected cells. The transmembrane distributions of weak acids (acetate and 5,5-dimethyloxazolidine-2,4-dione) indicated a DCCD-sensitive alkaline compartment. The combined results suggest that the intraerythrocyte parasite E_m and ΔpH are in part the consequence of an electrogenic proton pump localized to the parasite plasma membrane.

Cells expend energy to maintain specific transmembrane ion gradients and appropriate intracellular ion concentrations necessary for nutrient transport (e.g. Na^+-dependent amino acid transport, see reference 1) and cellular enzymatic processes (e.g. protein synthesis, see reference 2). Parasites such as the *Plasmodia* present an intriguing problem in this regard since they can apparently adjust to the very different ionic environments of the erythrocyte cytoplasm and host plasma. The intraerythrocytic forms develop from ring to schizont stage in the relatively high K^+ environment of the erythrocyte whereas the end products of this development, the merozoites and gametocytes, are viable for at least a short time in the high Na^+ of extracellular plasma.

In this publication, the membrane potential (E_m) of *Plasmodium*-infected erythrocytes has been estimated from the transmembrane distributions of radiolabeled lipophilic anion, [^{14}C]thiocyamate (SCN), and lipophilic cation, [^{3}H]triphenylmethy. phosphonium (TPMP). This method has been used to measure E_m of erythrocytes (3, 4), lymphocytes (3, 5), and bacteria (6). E_m is calculated from the ion distributions according to the equation,

$$E_m = -RT/F \log \frac{[TPMP]_i}{[TPMP]_o} = -RT/F \log \frac{[SCN]_o}{[SCN]_i}$$

At high negative E_m (relative to cytoplasm), TPMP is accumulated in cells and SCN is excluded from the intracellular space. Hoek et al. (7) have taken advantage of this reciprocal relationship between TPMP and SCN transmembrane distributions to estimate simultaneously the E_m across the plasma membrane and mitochondrial membranes of hepatocytes. Here we use SCN to monitor the erythrocyte E_m and TPMP to evaluate parasite E_m.

MATERIALS AND METHODS

Preparation of Cells

Maintenance of Sprague-Dawley rats, infection with *P. chabaudi*, and isolation of erythrocytes are described in the accompanying article (8).

Measurement of E_m

E_m is measured by modification of methods used for lymphocytes (5). Erythrocytes (2–10 × 10^7 cells/ml) are incubated at 37°C in 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES (Na^+ salt) at pH 7.4 and with 1 μCi/ml [^{3}H]H_2O for total pellet volume or 0.5 μCi/ml [^{3}H]polyethylene glycol (4,000 mol wt) as marker for extracellular space. Samples are also incubated with either 0.1 μCi/ml [^{3}H]TPMP or [^{14}C]SCN. At designated times, 200-μl samples are removed, layered over 500 μl of silicone oil, and centrifuged at 10,000 *g* for 30 s in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA). A 10-μl aliquot is removed from the cell-free supernatant to determine extracellular radioactivity, the remaining supernatant and silicone oil are removed, and the cell pellet is placed directly into scintillation vials.

Measurement of ΔpH

The method used by Friedman et al. (9) for *P. falciparum* was adapted to measure erythrocyte matrix pH. Cells (5 × 10^8 cells/ml) are incubated for 60 min at 37°C in the same buffer as utilized for E_m determination, and washed twice with 0.15 M NaCl by centrifugation for 15 s at 10,000 *g* in a Beckman microfuge. After dilution of cell pellet to 500 μl of 0.15 M NaCl, an equal volume of 0.04% saponin in 0.15 M NaCl is added and the cells are incubated for 2 min at 37°C. The lysate is cleaned of parasites by centrifugation at 10,000 *g* for 30 s in the microfuge. The pH of the lysate is measured with a Corning 11 pH meter (Corning Glass Works, Corning, NY). Parasites collected from the pellet remain intact after this treatment, as observed by light microscopy.

The transmembrane distributions of lipophilic weak acids (5,5-dimethyloxazolidine-2,4-dione [DMO] and acetate) which accumulate in alkaline compartments and of weak base (methylamine [MA]) which concentrates in acid compartments are monitored to determine intracellular pH (10, 11). The experimental technique is identical to that used for E_m measurement. Cells are incubated at 37°C for 60 min with 0.1 μCi/ml DMO, acetate, or MA and collected by centrifugation through silicone oil. Pilot experiments indicate that weak acids and bases equilibrate within 5 min and that these distributions are stable for at least 60 min.

Materials

Reagents and their suppliers are [^{3}H]H_2O (1.0 Ci/gm), [^{3}H]polyethylene glycol (4,000 mol wt; 2 mCi/gm) [^{3}H]TPMP (40 Ci/mmol), [^{3}H]DMO (60 mCi/mmol) [^{14}C]acetate (3 mCi/mmol) and [^{14}C]MA (60 mCi/mmol) from New England Nuclear (Boston, MA), [^{14}C]SCN (15 mCi/mmol from ICN, Irvine, CA), SF1250 silicone oil (General Electric Co., West Point, NY) antimycin A, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), HEPES, saponin, and 1-ethyl-3-(3-dimethylamiopropyl) carbodiimide (EDAC) (Sigma Chemical Co., St. Louis, MO), metrizamide (Nyegaard, Oslo, Norway) and *N,N'*-dicyclohexylcarbodiimide (DCCD) (Aldrich Chemical Co., Metuchen, NJ).

RESULTS

The intracellular H_2O space for parasitized rat erythrocytes is 0.37 ± 0.05 μl per 10^7 cells (10 cell preparations with parasitemia between 15 and 60%) and does not vary with parasite developmental stage or differ significantly from that of noninfected erythrocytes (0.35 ± 0.04 μl per 10^7 cells, five cell preparations).

The E_m of Normal and Infected Erythrocytes (Fig. 1)

With uninfected cells, an equilibrium level of TPMP accumulation is achieved at ~40 min and this level of uptake is stable for at least 90 min. For infected cells an apparent plateau for TPMP uptake at ~30 min is followed by maximal uptake at 60–75 min. Further incubation of infected cells results in decreased TPMP accumulation, possibly because of reduced parasite viability.

SCN uptake by normal and infected cells is biphasic, with initial rapid uptake during the first 5 min followed by slow uptake over the next hour. The latter phase has been attributed to SCN oxidation to SO_4 or non-E_m binding. This component

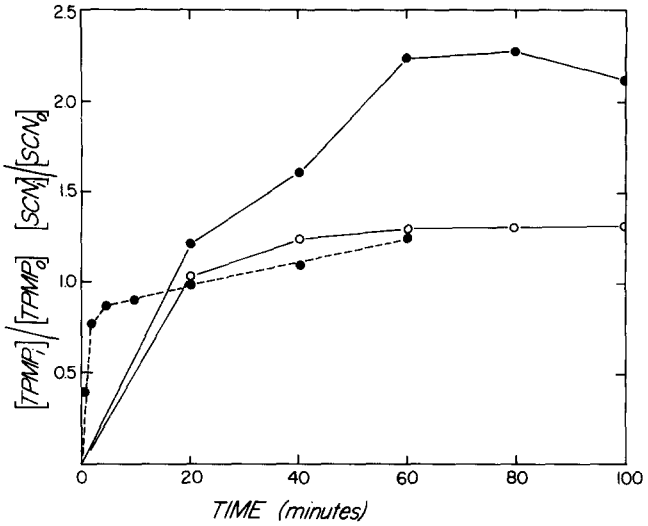

FIGURE 1 TPMP and SCN accumulation by normal and infected erythrocytes. Methods are described in the text. The data for SCN accumulation by normal erythrocytes are not shown since this uptake effectively overlaps that of infected cells. Parasitemia of infected cell population was 43.5%. The data points represent the mean of triplicate samples, with maximal standard error between samples of 10%. (●——●) TPMP-infected cells; (○——○) TPMP normal cells; (●- - - -●) SCN-infected cells.

of SCN uptake is extrapolated to zero time to obtain the SCN transmembrane distribution (3, 5).

For normal erythrocytes, E_m estimates of −10 ± 3 mV (10 cell preparations) and −6.5 ± 3 mV (three cell preparations) have been determined with TPMP and SCN, respectively. The apparent discrepancy between these E_m estimates is probably due to E_m-independent TPMP/SCN binding (3, 5). The value obtained with SCN approximates that observed for human erythrocytes (3).

An E_m of −20 ± 5 mV has been calculated from TPMP distribution of *P. chabaudi*-infected cells (five preparations, parasitemia 43 ± 5%). The 10 mV range in E_m estimates of different cell preparations probably reflects differing degrees of parasite maturity of the cell populations (see Table I). With SCN as probe, the E_m of infected cells is −6.6 ± 0.5 mV (two cell preparations), a value that does not differ significantly from that obtained for normal erythrocytes. As discussed below, the apparent discrepancy observed with SCN and TPMP estimates of the infected cell E_m stems from TPMP monitoring the E_m across both erythrocyte and parasite membranes whereas SCN distribution exclusively reflects the Donnan potential of the erythrocyte membrane.

To determine E_m-independent TPMP binding, zero-voltage correction at high extracellular K^+ levels has been attempted (3–5). At 70 mM K^+, 70 mM Na^+, a 30% reduction in TPMP accumulation by infected cells is observed with 1 μM valinomycin present. At higher K^+ concentrations, lysis of infected cells occurs with valinomycin present.

E_m and Stage of Parasite Development (Table I)

Parasite-infected erythrocytes are fractionated according to stage of parasite development on metrizamide gradients (8). Metrizamide treatment does not alter parasite viability, transport of ionic constituents such as Ca^{2+} (8), or the E_m of normal erythrocytes.

Although absolute separation of parasite stages is not

TABLE I

E_m of P. chabaudi-infected Erythrocytes after Metrizamide Separation and Measurement with TPMP

Fraction		Parasitemia	Uninfected	Differential Counts				E_m
				Ring	Troph	Sch	Gam	
	%	%						
1	0–14.6, metrizamide interface	94.6	5.4	6.8	29.2*	44.2	15.4	−39.2 ± 0.2
2	14.6–16.5, metrizamide interface	82.7	17.3	16.0	40.3*	26.4	0	−34.4 ± 1.3
3	16.5–18.3, metrizamide interface	54.7	45.3	16.2	34.0§	4.5	0	−15.9 ± 2.5
4	18.3, metrizamide interface pellet	48.2	51.8	28.8	18.3§	1.1	0	−12.6 ± 4.5
	Original	54.8	45.2	15.3	30.4	9.0	0.1	−17.3 ± 0.7
Normal (uninfected)								
Original								−6.4 ± 1.2
18.3, metrizamide pellet								−6.9 ± 1.4

* Predominantly late trophozoites by microscopic examination.
§ Predominantly early trophozoites by microscopic examination.

achieved, the results of Table I suggest that acquisition of a substantial E_m occurs in late stages of intraerythrocytic growth. Fractions 1 and 2 are characterized by relatively greater proportions of gametocytes, schizonts, and late trophozoites compared to fractions 3 and 4, and their respective E_m estimates are approximately identical (−39 versus −34 mV). In contrast, fraction 3 is essentially void of schizonts and its trophozoite population (34% of total) is predominantly early forms. The E_m of fraction 3 (−16 mV) is one-half that of fractions 1 and 2, suggesting that during the late trophozoite/early schizont stages a substantial negative inside E_m is established. Whether the earlier stages of parasite development also express a more negative E_m relative to normal cells is difficult to judge; however, fraction 4 consistently demonstrates greater TPMP uptake relative to erythrocytes from uninfected animals.

After multistep metrizamide gradient fractionation, recovery of schizonts is low. For further experiments a single-step metrizamide gradient (16.5%, $P = 1.076$) is used to separate "late" from "early" stages and uninfected cells (8). The E_m estimates are: "late" fraction (−27.8 to −45.6 mV) and "early" fraction (−10.1 to −17.7 mV). No difference in E_m of "late" and "early" fractions is observed with SCN (−7.2 ± 1.1 mV).

Antimycin A and CCCP Effects on E_m (Table II)

The E_m of "late" stage erythrocytes is markedly depolarized (~60%, five cell preparations) by 10 μM CCCP. The E_m of "late" erythrocytes after incubation with CCCP approximates that observed for normal erythrocytes.

Maximal depolarization is observed at 5 μM antimycin A only with "late" fractions containing a high percentage of gametocytes. For example, at 0.1% gametocyte, no depolarization of late fractions is observed, and at 4.0% gametocytes a 14% depolarization (−29.5 to −25.1 mV) is found.

With SCN as E_m probe, antimycin A or CCCP has no significant effects on the measured E_m of normal or infected erythrocytes. Intracellular H_2O spaces of normal and infected cells are not altered by incubation with antimycin A or CCCP.

Glucose Deprivation and DCCD Treatment (Table III)

Several investigations (reviewed in reference 12) demonstrate that glycolysis is the major energy-producing metabolic pathway for intraerythrocytic *Plasmodia*, the possible exceptions being gametocyte forms and avian *Plasmodia*, both of which contain cristate mitochondria. Glucose deprivation depolarizes E_m and treatment with 2-deoxyglucose, an inhibitor of glycolysis, further inhibits the generation of a TPMP-measured E_m. Equivalent results have been obtained with two other cell preparations of approximately identical gametocyte content (<1%). Glucose deprivation with or without 2-deoxyglucose elicits no change in SCN-determined E_m or intracellular H_2O space.

DCCD is a lipophilic reagent that blocks protonated carboxyl groups and inhibits proton-translocation ATPases (13, 14). With TPMP as the E_m probe, maximal depolarization

TABLE II

Effects of CCCP and Antimycin A on E_m Measured with TPMP *

Treatment	E_m (mV) ± SEM		
	Normal	Late	Early
None	−6.9 ± 1.4	−29.5 ± 0.6	−9.5 ± 0.6
0.1% ethanol	−8.4 ± 2.0	−27.8 ± 0.3	−12.1 ± 1.3
1 μM CCCP	−10.4 ± 2.2	−10.3 ± 0.2	−8.0 ± 2.0
10 μM CCCP§	−10.5 ± 0.6	−13.2 ± 0.4	−11.4 ± 1.0
5 μM antimycin A§	−7.9 ± 2.4	−25.1 ± 0.9	−10.2 ± 2.0

* Results are the mean of triplicate samples from single preparations of normal and infected cells after metrizamide fractionation. Gametocyte count was 4% in this preparation.
§ CCCP and antimycin A were dissolved in ethanol and, after addition to cells, the final ethanol concentration was 0.1% (vol/vol).

TABLE III

Glucose Deprivation and DCCD Treatment of "Late" Stage Infected Erythrocytes *

Treatment	E_m(mV) ± SEM	
	TPMP	*SCN*
+ glucose	−32.1 ± 0.5	−7.5 ± 0.2
− glucose	−22.2 ± 0.8	−7.6 ± 1.4
− glucose + 10 mM 2 deoxy glucose	−17.0 ± 0.6	−7.2 ± 1.5
+ glucose + 1 μM DCCD	−21.4 ± 1.0	−7.0 ± 0.8
+ glucose + 5 μM DCCD	−12.3 ± 0.9	−6.9 ± 0.9
+ glucose + 10 μM DCCD	−12.8 ± 0.3	−8.1 ± 1.1
+ glucose + 20 μM DCCD	−11.7 ± 0.3	−7.4 ± 0.6

* Experiment was performed with a single cell preparation. Comparable results were obtained with two other cell preparations (−glucose) and six cell preparations (+DCCD). Cells were incubated identically for 60 min except for the presence or absence of glucose and 10 mM 2-deoxyglucose and DCCD.

(~60%) is achieved at 5 μM DCCD with half-maximal inhibition at ~1 μM DCCD. Depolarization at low DCCD concentrations is observed irrespective of gametocyte count (six cell preparations, gametocyte count range 0.1–9%) and DCCD has no effect on SCN-determined E_m or intracellular H_2O space. TPMP-measured E_m is insensitive to 1–50 μM EDAC, a hydrophilic carbodiimide that does not react with H^+-ATPases (reference 14, data not shown).

Transmembrane ΔpH of Infected Erythrocytes (Table IV)

The depolarization observed with CCCP or DCCD implicates proton fluxes in E_m generation. We have thus measured intracellular pH of "late" stage erythrocytes with lipophilic weak acids (acetate and DMO) and base (MA), and by direct determination after saponin lysis.

The pH of normal rat erythrocyte saponin lysates is ~0.1 pH unit lower than the extracellular pH 7.3. Small transmembrane pH gradients have also been found for human erythrocytes by both weak acid (3) and saponin lysis methods (9). The pH of saponin lysates prepared from late-stage *P. chabaudi*-infected rat erythrocytes is relatively acidic (pH 6.6) with a transmembrane pH gradient of ~0.6 unit formed after incubation for 60 min at 37°C. Formation of a pH gradient (acidic inside, 0.4 pH unit) has been observed with *P. falciparum*-infected human erythrocytes and attributed to the high glycolytic rate of parasitized erythrocytes (9).

With MA as pH probe, an acidic pH for the intracellular space of infected erythrocytes is found (pH_e = 7.2 ± 0.04; pH_i = 6.41 to 6.96; three cell preparations). Treatment with 10 μM DCCD further acidifies the intracellular space monitored by MA, suggesting that DCCD treatment causes the release of protons or that an alkaline compartment of the infected cell becomes more acid. That the latter is an appropriate explanation is supported by experiments with weak acid probes. With DMO or acetate, an alkaline intracellular pH (7.51 to 7.91, six cell preparations) is measured which shifts to acid (pH shift-0.3 to 0.6 pH unit, three cell preparations) upon addition of 10 μM DCCD. The combined results suggest that pH measurement of saponin lysate or by MA distribution monitors the host-cell cytoplasmic pH whereas weak acids concentrate in the parasite alkaline compartment of the infected cell. Treatment with DCCD inhibits a parasite H^+-ATPase necessary for maintenance of an alkaline intraparasite compartment.

TABLE IV

Transmembrane pH *

Method	pH_e	pH_i ± SEM	pH
Normal erythrocytes			
Saponin lysis	7.31 ± 0.02	7.22 ± 0.02	−0.09
Infected erythrocytes			
Saponin lysis	7.21 ± 0.02	6.60 ± 0.05	−0.61
DMO	7.21 ± 0.02	7.51 ± 0.06	+0.30
Acetate	7.21 ± 0.02	7.62 ± 0.07	+0.41
MA	7.21 ± 0.02	6.96 ± 0.05	−0.25
Saponin lysis + 10 μM DCCD	7.21 ± 0.02	6.72 ± 0.05	−0.49
DMO + 10 μM DCCD	7.21 ± 0.02	7.22 ± 0.04	+0.01
MA + 10 μM DCCD	7.21 ± 0.02	6.67 ± 0.03	−0.54

* Results are from single cell preparations with pH_i determinations performed in triplicate. Qualitatively identical results were obtained with two other infected-cell preparations. Cells were incubated for 60 min (±10 μM DCCD) before pH_i measurements.

DISCUSSION

The experimental data presented here indicate that in the late trophozoite-early schizont stages of intraerythrocyte *Plasmodium* development a large, negative E_m is established across the parasite + parasitophorous vacuole membranes. This interpretation is based on evidence showing that TPMP equilibrates across plasma membranes of both erythrocyte and *Plasmodium* in an E_m-dependent fashion. Although application of a zero-voltage method to correct for E_m-independent TPMP binding (K^+ titration in the presence of valinomycin) is not completely successful due to cell lysis, the substantial depolarization observed with TPMP at low concentrations of CCCP or DCCD and by glucose deprivation suggests that TPMP accumulation is mostly E_m-dependent.

Other investigations (8, 9) using TPMP and either lengthy incubation (~2 h) or coincubation with tetraphenylboron to facilitate TPMP diffusion have determined an E_m of −20 mV for normal human erythrocytes. This value is significantly more negative than that observed for rat erythrocytes and is equivalent to that found for unfractionated *P. chabaudi*-infected cells at 44% parasitemia. No discrepancy between E_m determinations of human and rat erythrocytes is observed when SCN is the E_m probe. Although species differences may account for this apparent discrepancy in TPMP-determined E_m of normal human and rat erythrocytes, a more likely explanation stems from the relatively short (60 min) incubation used in our experiments and necessitated by reduced parasite viability upon prolonged incubation at 37°C. We have not included tetraphenylboron in the incubation buffer to shorten the TPMP transmembrane equilibration time since this agent alters K^+ permeability (e.g., see reference 9) and can lead to misleading E_m estimates (e.g., see reference 10). Thus, our short incubation period may lead to an underestimate of E_m. However, the basic finding of a more negative E_m of infected cells relative to normal cells remains valid because the TPMP-measured E_m of "late" fractions is of greater magnitude than that of unfractionated infected and normal cells and is sensitive to metabolic inhibitors and proton ionophore found not to affect the TPMP-measured E_m of normal cells.

With normal rat erythrocytes, TPMP and SCN distribution measurements provide approximately identical E_m estimates. The transmembrane distributions of both probes across normal membranes are also insensitive to antimycin A, CCCP, DCCD, and glucose deprivation. The E_m estimate obtained with SCN for infected cells does not differ from that of normal cells in magnitude or sensitivity to these treatments, in contrast to the observations with TPMP. The combined results indicate that SCN monitors the E_m across the erythrocyte membrane of infected cells whereas TPMP accumulation reflects the total E_m of the encapsulated parasite and erythrocyte membrane.

As mentioned in Results, an additional consideration in interpreting the experimental data is the presence of cristate mitochondria in gametocyte forms. We have shown that the E_m measured with TPMP of late-stage infected cells does not vary with gametocyte content (Table II) but that the extent of antimycin A inhibition of enhanced TPMP accumulation appears to parallel the relative percent of gametocytes in each cell population. Importantly, the depolarizations observed with DCCD or CCCP did not follow this trend, indicating that the E_m determined at low gametocyte counts is not entirely mito-

chondrial in origin. A relevant point in this regard is that mitochondria from schizont and trophozoite stages are few in number and thus their relative volume contribution to the parasite and to total TPMP accumulation is small. Mitochondria of these stages are also acristate and lack TCA cycle enzymes (5). Furthermore, depolarization observed with antimycin A may not entirely be the result of mitochondrial membrane depolarization but can reflect mitochondrial ATP-dependent electrogenic activities at the *Plasmodium* plasma membrane.

Several lines of evidence suggest that proton fluxes are an important aspect of the intraerythrocytic parasite E_m. The proton ionophore CCCP collapses the TPMP-measured E_m of infected cells, and DCCD at micromolar concentrations that react with the H^+-ATPase of bacteria (14) and yeast (13) is equally effective. EDAC, a hydrophilic carbodiimide that does not inhibit the H^+-ATPase of bacteria, does not depolarize the TPMP- or SCN-measured E_m of parasitized erythrocytes. Moreover, weak acid accumulation indicates an alkaline intracellular compartment in infected erythrocytes that is sensitive to DCCD. These results are consistent with the proposal that the *Plasmodium* plasma membrane of "late" stages contains an electrogenic H^+-ATPase pump that in part generates an E_m and transmembrane pH.

Several investigations have demonstrated the presence of a DCCD-inhibitable H^+-ATPase in the plasma membranes of procaryotic and eucaryotic unicellular organisms (13, 14). For example, the H^+-ATPase of *Neurospora crassa* plasma membranes is electrogenic and uses the chemical energy derived from ATP hydrolysis to generate a transmembrane E_m and alkaline inside pH gradient (15). The active transport of nutrients (e.g. glucose, reference 16) and ions (e.g. Ca^{2+}, reference 15) is coupled to the proton motive force established by the H^+-ATPase. In the accompanying publication (8) we demonstrate that Ca^{2+} transport of *P. chabaudi*-infected erythrocytes is both CCCP- and DCCD-sensitive, suggesting that Ca^{2+} transport in *Plasmodia* is coupled to a H^+-ATPase.

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