

Plasmodium falciparum-activated Chloride Channels Are Defective in Erythrocytes from Cystic Fibrosis Patients*

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Patrick Verloo^{‡§}, Clemens H. M. Kocken^{§¶}, Annemarie Van der Wel[¶], Ben C. Tilly[‡],
Boris M. Hogema[‡], Maarten Sinaasappel[¶], Alan W. Thomas[¶], and Hugo R. De Jonge^{‡***}

From the [‡]Department of Biochemistry, Erasmus University Medical Center, 3000 DR Rotterdam, the [¶]Department of Parasitology, Biomedical Primate Research Centre, 2280 GH Rijswijk, and [§]Sophia Children's Hospital, Erasmus University Medical Center, 3015 GJ Rotterdam, The Netherlands

An inwardly rectifying anion channel in malaria-infected red blood cells has been proposed to function as the “new permeation pathway” for parasite nutrient acquisition. As the channel shares several properties with the cystic fibrosis transmembrane conductance regulator (CFTR), we tested their interrelationship by whole-cell current measurements in *Plasmodium falciparum*-infected and uninfected red blood cells from control and cystic fibrosis (CF) patients. A CFTR-like linear chloride conductance as well as a malaria parasite-induced and a shrinkage-activated endogenous inwardly rectifying chloride conductance with properties identical to the malaria-induced channel were all found to be defective in CF erythrocytes. Surprisingly, the absence of the inwardly rectifying chloride conductance in CF erythrocytes had no gross effect on *in vitro* parasite growth or new permeation pathway activity, supporting an argument against a close association between the *Plasmodium*-activated chloride channel and the new permeation pathway. The functional expression of CFTR in red blood cells opens new perspectives to exploit the erythrocyte as a readily available cell type in electrophysiological, diagnostic, and therapeutic studies of CF.

Intraerythrocytic development of malaria parasites leads to increased nutrient uptake through parasite-induced new permeation pathways (1–4) that are considered promising targets for antimalarial chemotherapy development (5). Previous reports demonstrated that the new permeation pathways show functional characteristics of a chloride channel (1). More recent data suggested that a small conductance chloride channel, as measured with the on-cell patch clamp configuration, accounts for the increased uptake of nutrients into infected red blood cells and that thus this channel qualifies as a prime target for pharmacotherapy (6). The channel exhibits voltage-dependent gating, showing higher open probability at negative membrane potential, which is visible in the whole-cell patch clamp configuration as a strong inwardly rectifying chloride current. The

pharmacological profile of this channel shows a striking resemblance to that of the cystic fibrosis transmembrane conductance regulator (CFTR),¹ a cAMP-regulated chloride channel that is mutated in patients with cystic fibrosis (CF) (7, 8). Functional expression of CFTR in red blood cells is suggested also by the failure of CF erythrocytes to perform deformation-induced and cAMP-induced ATP release (9, 10). In the present study, direct evidence in support of this concept was obtained by patch clamp measurements of whole-cell chloride currents in CF and non-CF red blood cells. The CF defect in endogenous and parasite-induced chloride conductances was exploited to evaluate the possible contribution of red blood cell chloride channels to the new permeation pathway. Moreover, the lack of functional chloride channels in CF red blood cells offered us the unique possibility to study the putative obligatory role of the *Plasmodium*-activated chloride current in the growth and survival of *Plasmodium falciparum*.

EXPERIMENTAL PROCEDURES

RT-PCR—For RT-PCR, RNA was isolated from human erythroblasts obtained by bone marrow puncture (11) using TRIzol (Invitrogen). cDNA was prepared from 2 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen). For amplification of CFTR, PCR reactions were performed using platinum *Taq* polymerase (Invitrogen) with primers 5'-AGGGGAAGTCACCAAGCAGTACAGC-3' (sense; position 402–427 in sequence NM_000492 (GenBank™/EBI accession number); exon 4) and 5'-GCGCAGAACAAATGCAGATGAGATG-3' (antisense; position 1173–1149; exon 8). The thermocycler profile was as follows: 96 °C for 4 min followed by 11 cycles (1 min at 68 °C, 2 min at 68 °C, 1 min 45 s at 72 °C) with annealing at 68 °C, 11 cycles with annealing at 64 °C, 11 cycles with annealing at 60 °C, 17 cycles with annealing at 56 °C, and a final extension at 72 °C for 5 min. The high number of cycles was necessary due to very low amounts of CFTR mRNA. The amount of cDNA used in each reaction (1–2 µl) was normalized with respect to the amount of actin cDNA by performing PCR on serial dilutions of cDNA using primers specific for β -actin, 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' (sense) and 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' (antisense), under conditions similar to those used for the CFTR PCR but using a 25-cycle PCR program. Relative expression levels were compared with expression in T84 cells (which have high CFTR expression) by using dilutions (1000–100,000-fold) of cDNA from T84 cells. Although CFTR expression in erythroblasts was at least 1000-fold lower than in T84 cells, the product was detected in the majority of PCR reactions ($n = 5$ –8 for each cDNA sample) and was always absent in a –RT control ($n = 5$), and the identity of the 771-bp PCR product was verified by sequencing using an ABI377 automated sequencer.

***Plasmodium* Culture**—*P. falciparum* strains FCR3 (Gambian origin) and NF54 (unknown origin) were cultured essentially by the method of Trager and Jensen (12) in standard culture medium. Parasitemia was

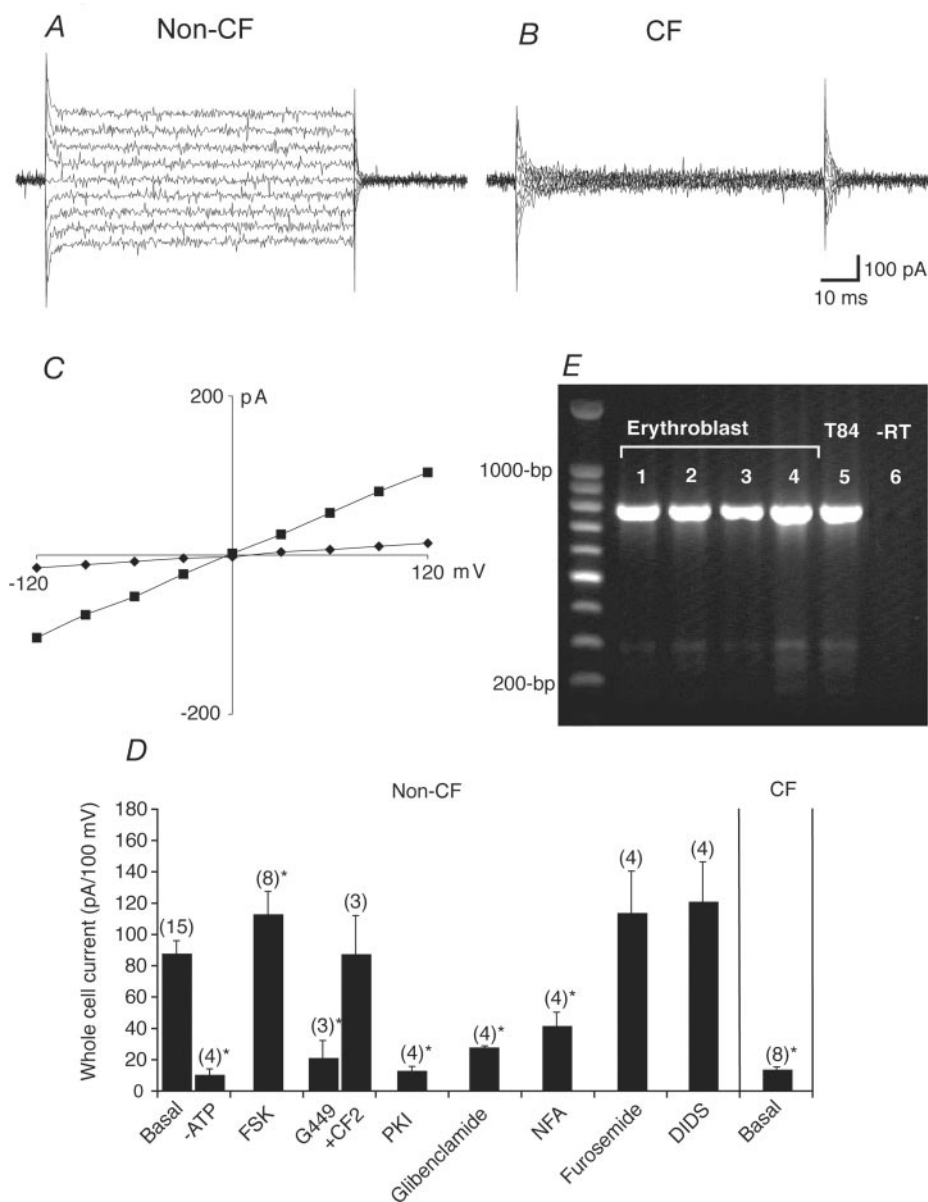
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§ Both authors contributed equally to this work.

*** To whom correspondence should be addressed: Dept. of Biochemistry, Erasmus University Medical Center, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. Tel.: 31-10-4087324; Fax: 31-10-4089472; E-mail: h.dejonge@erasmusmc.nl.

¹ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; RT, reverse transcriptase; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; I-V, current-voltage; PKA, protein kinase A; PKI, PKA inhibitor.

FIG. 1. Whole-cell recordings of chloride currents in human red blood cells. *A*, representative traces of basal chloride current in non-CF red blood cells. *B*, representative traces of chloride current in red blood cells from a CF patient (CFTR Δ Phe-508/ Δ Phe-508), demonstrating that the basal current is virtually absent in CF red blood cells. *C*, average I-V plot of the basal chloride current in non-CF (■) versus CF (◆) red blood cells (S.E. is smaller than symbol size). *D*, pharmacological profile of the basal chloride current in red blood cells. Because the basal current is linear, only the currents at positive voltages are plotted in the graph. The current is largely dependent on ATP (2 mM) in the pipette. Anti-CFTR antibody G449 (10 μ g/ml IgG), G449 preincubated with antigenic peptide (CF-2; 0.1 mg/ml) for 5 min at room temperature, and PKI (10 μ M) were added to the pipette; forskolin (FSK; 1 μ M), glibenclamide (100 μ M), niflumic acid (NFA; 100 μ M), furosemide (100 μ M), and DIDS (250 μ M) were added to the bathing solution. The number of individuals tested is given between parentheses. *, $p < 0.05$ compared with basal. *E*, CFTR RT-PCR on erythroblast RNA, showing the 771-bp PCR product. Lane 1, RT-PCR product from freshly isolated erythroblasts; lanes 2–4, RT-PCR product from erythroblasts cultured for 1, 2, and 3 days, respectively; lane 5, RT-PCR product from T84 cells for comparison; lane 6, as lane 1, but without addition of RT.



determined by evaluation of Giemsa-stained thin films. When needed, parasite cultures were synchronized using alanine treatment (13).

Invasion—Nycodenz-purified mature stages-infected red blood cells at 3% hematocrit were mixed with red blood cell suspensions of various origins at a ratio of 1:100. Triplicate 5-ml cultures of each red blood cell suspension were cultivated overnight to allow reinvasion. A sample of 150 μ l from each of the cultures was then transferred to a 96-well plate, and 25 μ l of [3 H]hypoxanthine (ICN Biomedicals Inc., Irvine, CA) in standard culture medium (1 μ Ci) was added to each of the wells. Cultures were incubated for another 22.5 h and harvested using a Titertek cell harvester (ICN Biomedicals Inc.), and incorporation of radiolabel was determined by liquid scintillation counting using a Top-Count NXT (PerkinElmer Life Sciences) as a measure of parasite viability and invasion rate. The original 5-ml cultures were maintained for 7 days with periodic addition of fresh red blood cells and culture medium (identical treatment for all cultures), and parasitemia was regularly determined.

Patch Clamp—Red blood cells were bathed in 110 mM CsCl, 5 mM MgSO₄, 3.5 mM sodium gluconate, 12 mM HEPES, 8 mM Tris-HCl (pH 7.4), and 10 mM dextrose. In the shrinkage experiments the bathing solution contained an additional 300 mM mannitol. The intracellular pipette solution contained 110 mM CsCl, 2 mM MgSO₄, 25 mM HEPES, 1 mM EGTA, 2 mM Na₂ATP, 50 mM mannitol (pH 7.4). Patch pipettes were pulled from borosilicate glass (Clark GC150T-10) and had a resistance of 8–12 megohms. All experiments were done with 2 mM ATP in the pipette unless stated otherwise. Where stated, G449 (10 μ g/ml

IgG), CF-2 (0.1 mg/ml, preincubated with G449 for 5 min at room temperature), protein kinase A (PKA) inhibitor (PKI; 10 μ M), or acid phosphatase (30 units/ml) was added to the pipette. Forskolin (1 μ M), niflumic acid (100 μ M), furosemide (100 μ M), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; 250 μ M), or glibenclamide (100 μ M) was added to the bathing solution. Cell shrinkage was triggered by increasing the osmolarity of the bath by addition of 300 mM mannitol to the standard bathing solution following seal formation. In the figures, error bars indicate S.E., and the number of individuals tested is given between parentheses.

Voltage steps from -120 to $+120$ mV were applied in 30-mV increments; holding potential was set at 0 mV. For command pulse control, data acquisition, and analysis, pCLAMP 6 software (Axon instruments Inc., Foster City, CA) was used.

Isotonic Lysis—Synchronized infected cells (enriched to a parasitemia of 5–10%) from non-CF and CFTR Δ Phe-508/ Δ Phe-508 patients ($n = 4$ different persons) were bathed in an isotonic solution containing 300 mM glucose, 150 mM choline chloride, or 300 mM alanine. The cells were incubated at 37 $^{\circ}$ C for 15 min, and cell lysis due to the passive movement of the osmolyte into the cells was measured spectrophotometrically at 580 nm. Results are plotted as percentage compared with total lysis after 2 h of incubation in 300 mM choline chloride in the absence of inhibitors. Uninfected red blood cells did not undergo lysis in response to isosmotic choline or alanine. In case of glucose, the lytic response resulting from glucose uptake through the endogenous glucose carrier in uninfected red blood cells was subtracted.

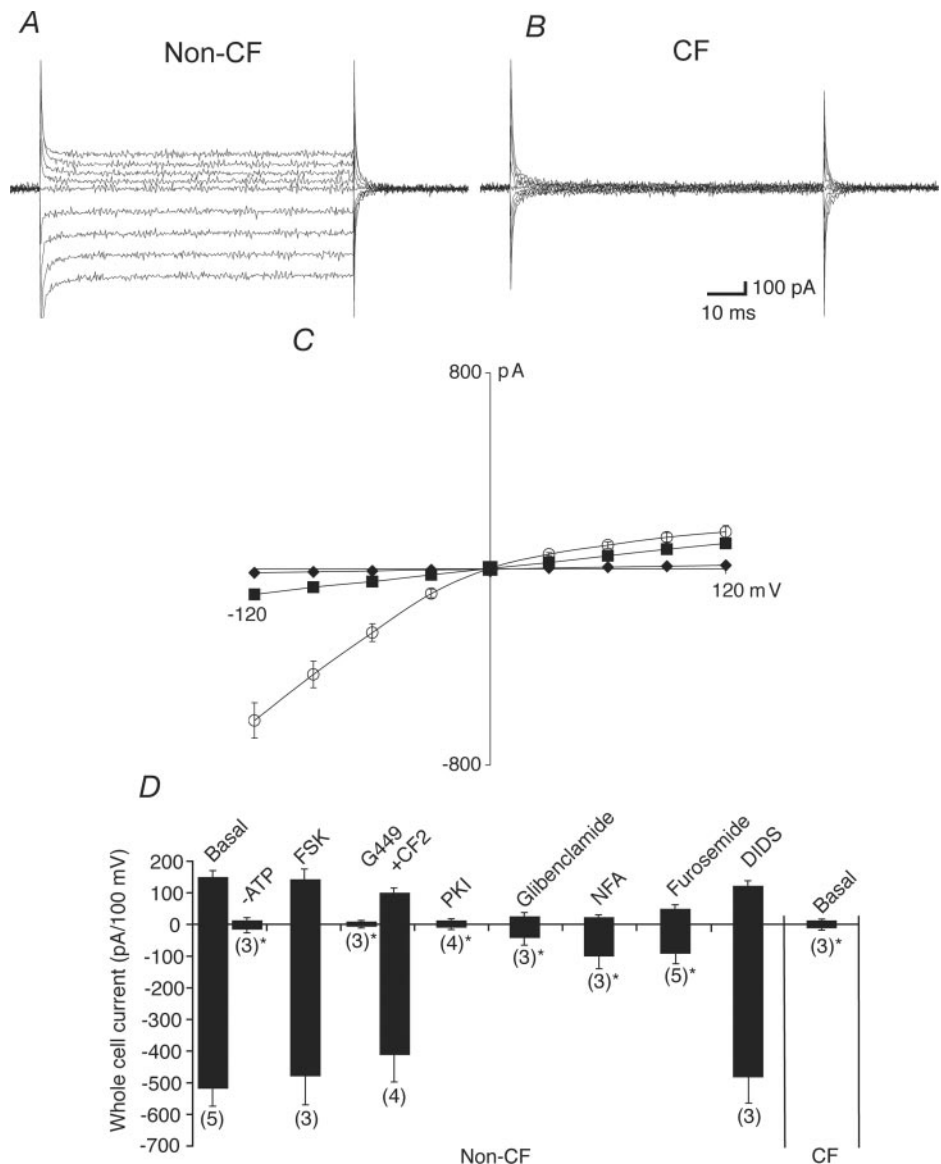


FIG. 2. Induction of an inwardly rectifying chloride conductance by cell shrinkage. *A*, representative traces of a shrinkage-induced chloride conductance in non-CF red blood cells. Following seal formation, cell shrinkage is triggered by increasing the osmolality of the bath by addition of 300 mM mannitol to the standard bathing solution. *B*, representative traces demonstrating the absence of shrinkage-induced chloride currents in CF red blood cells. *C*, average I-V plot of the inwardly rectifying chloride current in non-CF (○) versus CF (◆) red blood cells. The basal current in a non-CF cell is given for comparison (■). *D*, pharmacological profile of the shrinkage-activated current in non-CF red blood cells. Because of the inward rectifying nature of the conductance, currents at both negative and positive voltages are plotted. The main difference with the inhibition profile of the basal chloride current (Fig. 1D) is its sensitivity to furosemide, a potent inhibitor of the *P. falciparum*-activated, inwardly rectifying chloride current in malaria-infected red blood cells. *, $p < 0.05$ compared with basal; FSK, forskolin; NFA, niflumic acid.

RESULTS

To characterize the role of the malaria-induced chloride channel activity in parasite blood stage development, we performed whole-cell patch clamp experiments with control red blood cells and red blood cells from CF patients, both uninfected and infected with *P. falciparum*. As a first step, to identify CFTR in red blood cells, we studied uninfected red blood cells from healthy individuals and homozygous Δ Phe-508 patients, the most common CFTR mutation in the Caucasian population. Red blood cells were freshly isolated from heparinized blood samples and were bathed at 37 °C in a 110 mM symmetrical CsCl solution. In the presence of ATP in the pipette solution, an average whole-cell chloride conductance of 873 ± 82 pS ($n = 15$ different individuals) was observed in red blood cells from healthy volunteers (Fig. 1A), which was virtually absent from CF red blood cells (131 ± 19 pS, $n = 8$ different CF (CFTR $^{\Delta$ Phe-508/ Δ Phe-508) patients) (Fig. 1B). This basal current displayed a linear I-V relationship with a reversal potential close to 0 mV (Fig. 1C) and was virtually absent when ATP was omitted from the pipette (97 ± 41 pS, $n = 4$ healthy individuals) (Fig. 1D). The anion permeability of this pathway, determined by the observed shift in reversal potential (6), showed a selectivity sequence of $\text{Br}^- > \text{Cl}^- > \text{I}^-$. The current was slightly stimulated upon addition of the adenyl cyclase

activator forskolin ($1 \mu\text{M}$) to the bath (Fig. 1D) and was inhibited by the chloride channel inhibitors niflumic acid ($100 \mu\text{M}$) and glibenclamide ($100 \mu\text{M}$) (14). However, the nonspecific chloride channel inhibitor DIDS ($250 \mu\text{M}$), as well as the new permeation pathway-inhibiting drug furosemide ($100 \mu\text{M}$) (1, 6, 15), had no influence on this basal current. Addition of PKI to the pipette, or addition of the polyclonal antibody G449, raised against the CF-2 peptide (a cloned R-domain peptide of CFTR (residues 645–835)) (16) to the cytoplasmic side of the erythrocyte, resulted in strong inhibition, approaching the low current level seen in CF red blood cells. The latter effect was abolished by adding the CF-2 peptide as competitor (Fig. 1D), demonstrating the specificity of the inhibitory effect of the antibody. Collectively, these electrophysiological data, in particular the apparent dependence of the chloride conductance on cytoplasmic ATP and PKA, its inhibition by anti-CFTR antibody, and its absence from CF red blood cells, strongly support the notion that CFTR chloride channels are present in normal red blood cells and are tonically active under our experimental conditions. This concept is strengthened further by the finding, on the basis of RT-PCR, of low levels of CFTR mRNA in nucleated precursor cells, the erythroblasts, isolated from bone marrow punctures from healthy individuals (Fig. 1E) and by the recent demonstration of a 15-pS, PKA/ATP-activated linear

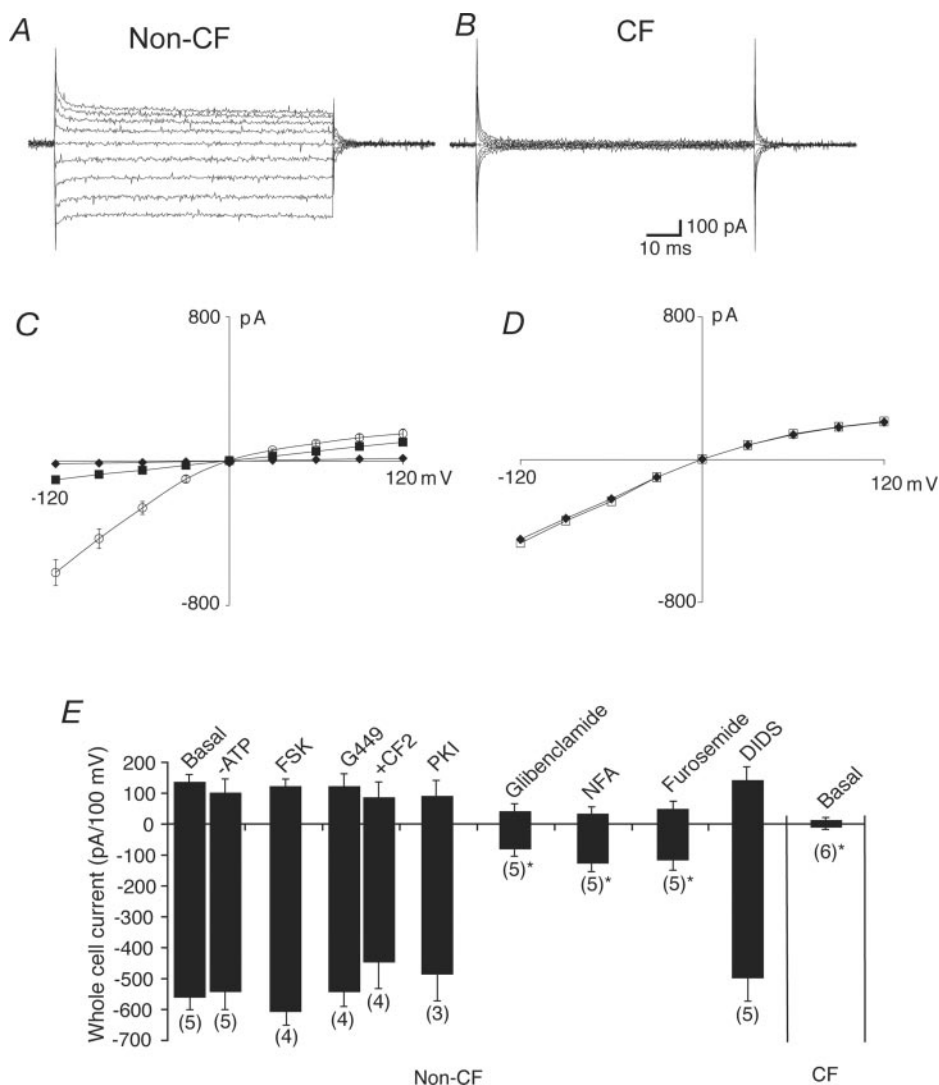


FIG. 3. Voltage clamp analysis of *P. falciparum*-induced chloride currents in human red blood cells. Conditions were similar to those in Fig. 1. *A*, representative traces of parasite-induced inwardly rectifying chloride current in infected non-CF red blood cells. *B*, representative traces demonstrating the absence of inwardly rectifying current in infected CF red blood cells. *C*, average I-V plot of the chloride current in infected non-CF (○) and CFTR Δ Phe-508/ Δ Phe-508 (◆) red blood cells. The basal current in a non-CF cell is shown for comparison (■). *D*, typical I-V plot of the inwardly rectifying current in an infected red blood cell (◆) and in the same infected red blood cell that is shrunken by the addition of 300 mM mannitol to the bath (□). Cell shrinkage did not further enhance the chloride current in infected red blood cells. *E*, pharmacological profile of the malaria-activated current in non-CF red blood cells. The inhibition profile only differs from the shrinkage-activated current (Fig. 2D) in its lack of sensitivity to PKI and G449 addition and its independence of ATP addition. *, $p < 0.05$ compared with basal; FSK, forskolin; NFA, niflumic acid.

anion conductance in uninfected non-CF red blood cells (17). Taken together, these data for the first time indicate a functional expression of CFTR in control but not in CFTR Δ Phe-508/ Δ Phe-508 red blood cells.

Deformation of the erythrocytes by osmotic shrinkage, induced by raising the osmolarity from the bath from 300 to 600 mosM by increasing the mannitol concentration, resulted in the appearance of an additional chloride conductance (Fig. 2A) of similar magnitude and with an identical I-V relationship (Fig. 2C), pharmacological profile (Fig. 2D), and anion selectivity sequence ($I^- > Br^- > Cl^-$) to that described for the *P. falciparum*-induced inwardly rectifying chloride current (6). Similar to the CFTR-like current, this conductance was dependent on ATP and showed inhibition with all CFTR inhibitors tested earlier, including the G449 anti-CFTR antibody and PKI (Fig. 2D). However, it strikingly differed from the CFTR-like current in its sensitivity to furosemide, a potent inhibitor of the new permeation pathway and of the *Plasmodium*-activated chloride conductance (6). The furosemide inhibition appeared unrelated to its known action as an inhibitor of the Na-K-Cl cotransporter because bumetanide (up to 250 μ M), a more potent Na-K-Cl cotransporter inhibitor, was unable to block the chloride current (data not shown). Interestingly this shrinkage-induced, inwardly rectifying chloride current was likewise deficient in shrunken red blood cells from CF patients (Fig. 2B), suggesting a close functional relationship with the CFTR chloride channel. Notably, basal potassium conductances monitored by whole-

cell patch-clamping in potassium gluconate-containing medium were not different between control and CF red blood cells (results not shown), arguing against a general nonspecific effect of the CF condition on ion permeabilities of the red blood cell membrane.

To directly compare the pharmacological profiles of the shrinkage-induced and the malaria-induced chloride channels, we performed patch clamp experiments on *P. falciparum* trophozoite-infected red blood cells from healthy individuals at 0.5–5% parasitemia. Consistent with the study by Desai *et al.* (6), infection resulted in the appearance of an inwardly rectifying chloride current that was fully active under isosmotic conditions (Fig. 3A). The magnitude and I-V relationship (Fig. 3C) of this malaria-induced chloride conductance were identical to the shrinkage-activated, endogenous chloride conductance. Moreover, osmotic shrinkage of infected red blood cells did not further enhance the chloride conductance of the plasma membrane (Fig. 3D), indicating that the malaria-induced current and the cell shrinkage-induced endogenous current are manifestations of the same, inwardly rectifying chloride channel. However, the pharmacological profile (Fig. 3E), although largely identical to that of the shrinkage-activated chloride conductance (Fig. 2D), indicated that the conductances do differ in at least three important aspects; whereas shrinkage activation of the chloride channel is dependent on ATP and PKA and inhibited by anti-CFTR antibody, the *P. falciparum*-activated channel functions in the absence of ATP (Fig. 3E) (6)

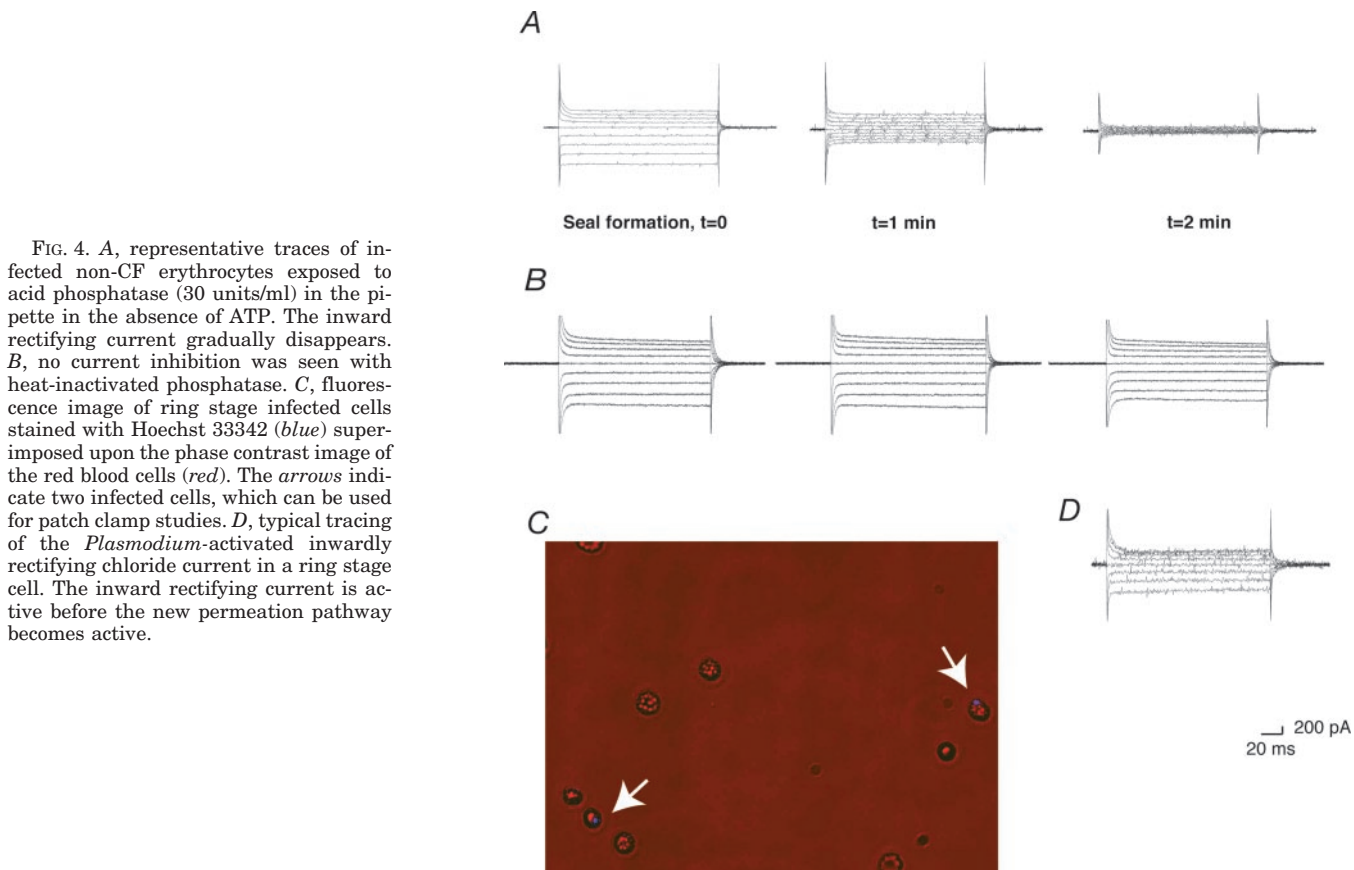


FIG. 4. A, representative traces of infected non-CF erythrocytes exposed to acid phosphatase (30 units/ml) in the pipette in the absence of ATP. The inward rectifying current gradually disappears. B, no current inhibition was seen with heat-inactivated phosphatase. C, fluorescence image of ring stage infected cells stained with Hoechst 33342 (blue) superimposed upon the phase contrast image of the red blood cells (red). The arrows indicate two infected cells, which can be used for patch clamp studies. D, typical tracing of the *Plasmodium*-activated inwardly rectifying chloride current in a ring stage cell. The inward rectifying current is active before the new permeation pathway becomes active.

and is insensitive to PKI and G449 antibody (Fig. 3E). These findings suggest that the ATP-dependent phosphorylation of the R-domain of CFTR by PKA is necessary for shrinkage activation of the inwardly rectifying chloride channel but not for *P. falciparum* activation of the channel. Alternatively, infection of red blood cells with *P. falciparum* could result in a sustained phosphorylated status of CFTR, possibly induced by excessive production of endogenous *Plasmodium* PKA (18) or cGMP-dependent protein kinase (19), which is expressed in the early intraerythrocytic stages of the parasite life cycle (20). The structurally related *Toxoplasma gondii* cGMP-dependent protein kinase was demonstrated to be important in the *in vivo* intracellular survival of the parasite (21). A critical role of protein phosphorylation in chloride channel activation is also strengthened by the observation that the *Plasmodium*-activated chloride current gradually disappears when acid phosphatase is added to the pipette ($n = 4$) (Fig. 4A). No current inhibition was observed when heat-inactivated acid phosphatase was added to the pipette ($n = 4$) (Fig. 4B). We also patch-clamped ring stage cells (2–6 h post-invasion) as identified by fluorescent microscopy following staining with the DNA dye Hoechst 33342 (Fig. 4C). There was no difference in amplitude of the inwardly rectifying chloride channel compared with trophozoite-infected cells (Fig. 4D), implying that full activation of the channel is already achieved at an early stage of infection, long before activation of the new permeation pathway ($n = 4$) (22).

CF red blood cells, lacking CFTR and CFTR-related conductances, offered us the unique possibility to study the obligatory role of this chloride channel in the survival of *P. falciparum*. If the parasite-activated channel is indeed identical to the endogenous shrinkage-activated channel, as strongly suggested by the experiments above, and the new permeation pathway is the macroscopic manifestation of this channel as suggested by De-

sai *et al.* (6), then the new permeation pathway, and thus survival of the malaria parasite, would be expected to be impaired in CF red blood cells. To test this hypothesis, blood samples from non-CF, CFTR Δ Phe-508/ Δ Phe-508 patients and obligate carriers (CFTR $^{+/\Delta}$ Phe-508) were used for *in vitro* culture of *P. falciparum* FCR3 and NF54 strains. Two strains were used to exclude strain-specific effects. On the basis of [3 H]hypoxanthine incorporation assays, no difference in invasion rates and subsequent intraerythrocytic development of the *P. falciparum* FCR3 strain parasites could be observed between non-CF, CFTR $^{+/\Delta}$ Phe-508, and CFTR Δ Phe-508/ Δ Phe-508 red blood cells (Fig. 5A). Follow-up of the individual cultures by analysis of Giemsa-stained thin films demonstrated that parasites in all cultures developed normally and had comparable growth rates, irrespective of the CF genotype. Similar results were obtained with the NF54 parasite strain, yet patch clamp analysis of the malaria-infected CF red blood cells revealed that the parasites had failed to induce the inwardly rectifying current (Fig. 3B), indicating that the CFTR-related chloride channel is not obligatory for parasite survival *in vitro*.

To further investigate whether the functional absence of the inwardly rectifying channel changes the activity of the new permeation pathway, we performed isotonic lysis experiments in *Plasmodium*-infected red blood cells to monitor the transport of choline chloride, glucose, and alanine. In these experiments the infected red blood cells are incubated in an isosmotic solution of one of the nutrients. Passive movement of the solute through the red blood cell membrane causes swelling and subsequent lysis of the cell. The lysis rate can be used to assess the new permeation pathway activity (1) and thus to monitor differences in nutrient transport across the red blood cell membrane of *Plasmodium*-infected CF and non-CF cells. The only major difference observed between infected non-CF and CF red blood cells was at the level of glucose uptake ($p < 0.01$ for all

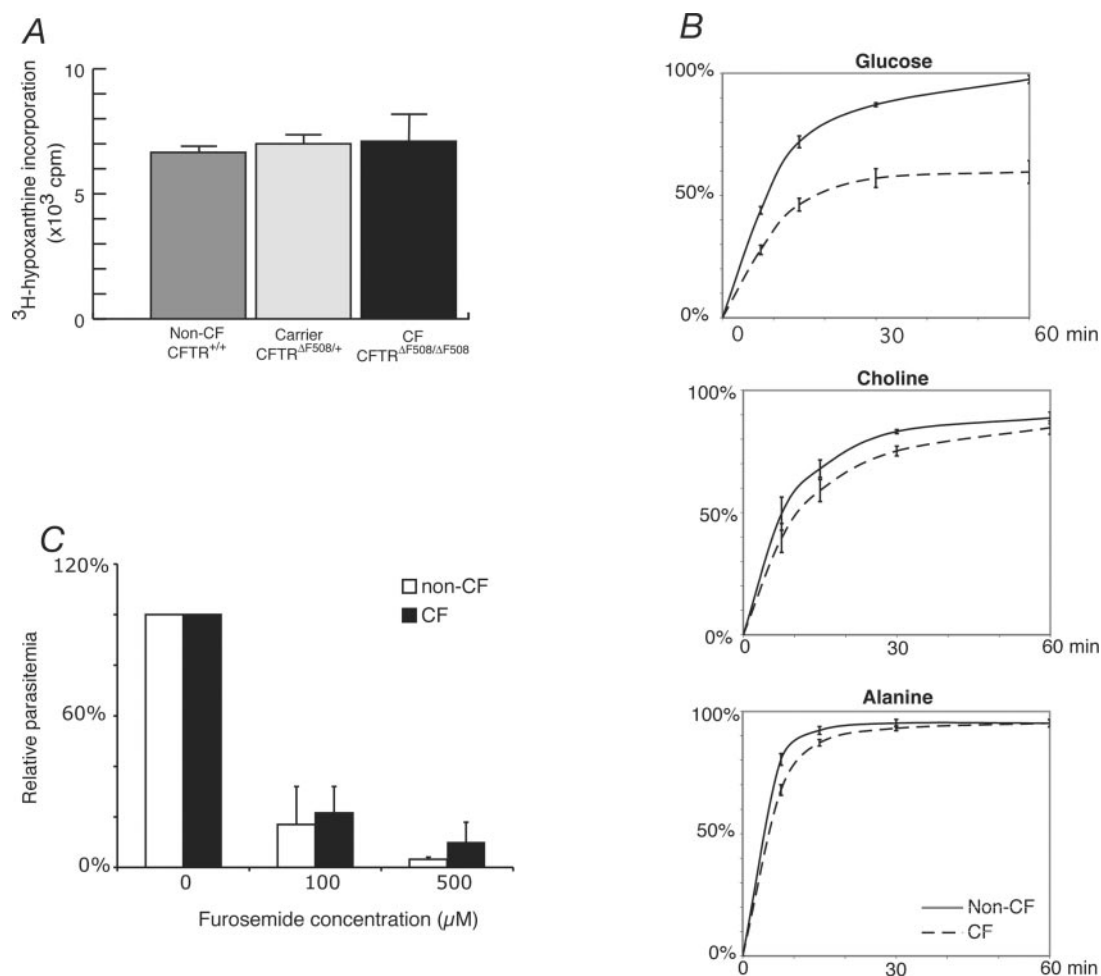


FIG. 5. A comparison of parasite survival and new permeation pathway activity in non-CF and CF red blood cells. *A*, parasite viability after invasion as determined by [^3H]hypoxanthine incorporation in red blood cells from non-CF, CFTR ^{Δ Phe-508/ Δ Phe-508} CF patients, and obligate carriers (CFTR^{+/+} Δ Phe-508). Error bars indicate S.E. values ($n = 6$). *B*, isotonic lysis (taken as a measure of new permeation pathway activity). The traces represent time-dependent lysis of infected erythrocytes bathing in isosmotic glucose, choline chloride, or alanine, demonstrating that the activity of the new permeation pathway in infected CF red blood cells (dashed lines) is only slightly inhibited compared with non-CF red blood cells (solid lines) even though the parasite-induced inwardly rectifying chloride conductance is completely absent in CF red blood cells (Fig. 3, *B* and *C*). Error bars indicate S.E. ($n = 4$). *C*, relative parasitemia of infected CF (CFTR ^{Δ Phe-508/ Δ Phe-508}, filled bars) and non-CF (open bars) red blood cells incubated for 48 h in the absence or presence of 100 or 500 μM furosemide.

time points, $n = 4$) (Fig. 5*B*). Lysis experiments additionally suggested that the influx of alanine and choline is only slightly impaired in infected CF red blood cells, whereas the maximal percentage of lysis was similar in infected CF and non-CF red blood cells. These data suggest that the *Plasmodium*-induced inwardly rectifying chloride channel only accounts for a (minor) part of the nutrient influx in infected red blood cells. Collectively these data indicate that the (slightly) reduced influx of nutrients in infected CF red blood cells had no detectable influence on parasite growth rates under standard *in vitro* culture conditions, as shown by the invasion/growth experiments (Fig. 5*A*). Importantly, the new permeation pathway-inhibiting drug furosemide blocked glucose and alanine influx completely, and choline influx partially, both in infected control and CF red blood cells (data not shown). These results indicate that the antiparasitic action of furosemide cannot be attributed to the inhibition of the inwardly rectifying chloride channel alone as this channel is only manifest in non-CF cells. This conclusion is reinforced by the observation that parasites growing in CF red blood cells remained fully sensitive to the new permeation pathway blocker furosemide, as demonstrated by the profound decline in parasitemia in both CF and non-CF red blood cells following a 48-h exposure to furosemide (Fig. 5*C*).

DISCUSSION

The results presented here demonstrate that a CFTR-like chloride conductance is functionally expressed in normal but not in CF red blood cells. Combined with our data on CFTR mRNA expression in red blood cell progenitor cells, as well as on inhibition of channel activation by a CFTR-specific, R-domain-directed antibody, our study demonstrates for the first time that CFTR is responsible for an endogenous chloride conductance in red blood cells. Moreover, our results also indicate that CFTR is additionally required for the activation of a host cell-encoded inwardly rectifying chloride current by either cell shrinkage or *P. falciparum* infection. Because the inwardly rectifying conductance differs from the CFTR-chloride channel with respect to several major electrophysiological, regulatory, and pharmacological properties (anion permeability sequence, rectifying behavior, sustained opening in the absence of ATP, and furosemide inhibition), our data collectively support a dual channel model in which CFTR and the inward rectifier constitute separate molecular identities but are tightly coupled functionally, analogous to the well established relationship between CFTR and the outwardly rectifying chloride channel (23). However, in view of recent data suggesting that ion selectivity and activation modes of CFTR in native tissues may

differ from those in cultured cells (24), the present evidence for a two- rather than one-channel model is inconclusive and awaits further identification of the inwardly rectifying conductance, preferably at the molecular level.

Taken together, our data do not support the hypothesis raised by Desai *et al.* (6) that the *Plasmodium*-activated inwardly rectifying ion channel (recently renamed PESAC (25, 26)) adequately accounts for the parasite-induced increases in permeability to a broad range of solutes. Their hypothesis was based on the highly similar pharmacological profile of the inwardly rectifying chloride conductance and the new permeation pathway (6) and on the finding that the chloride conductivity of infected red blood cells, as calculated from patch clamp analysis, matched the chloride conductance calculated from chloride flux measurements (25). Our data suggest a less vital role for the inwardly rectifying chloride channel. This is evident from the normal development of malaria parasites in CF red blood cells, in which the inwardly rectifying chloride conductance is completely lacking, and from the modest impact of the CF condition on functioning of the parasite-induced new permeation pathway, suggesting that the inward rectifier is not the target for the antiplasmodial action of furosemide. Additionally, patch-clamping ring stage cells demonstrated that the inwardly rectifying chloride conductance is activated long before the new permeation pathway becomes active (Fig. 4, C and D).

Furthermore, the closure of the parasite-induced inwardly rectifying chloride conductance following protein dephosphorylation by acid phosphatase, a broad specificity protein phosphatase, demonstrates for the first time that the "sustained" activation of the inwardly rectifying chloride conductance can be reversed. This finding suggests that the inwardly rectifying chloride conductance is activated through *Plasmodium*-induced (hyper)phosphorylation of red blood cell proteins and argues against potential irreversible modes of channel activation, *e.g.* limited proteolysis or protein oxidation, as was suggested by Huber *et al.* (27). We were also unable to demonstrate an anion channel with outward rectifying properties that is up-regulated by *Plasmodium* infection; however, it was recently demonstrated that the outward rectifying current is only manifest when the holding potential is negative or when human serum is added to the bath (28, 29). In our experiments, we use a holding potential of 0 mV, and the human serum concentration in the bath, a remnant from the human serum necessary for the *Plasmodium* culture, is less than 0.001%.

Although we demonstrate that the *Plasmodium*-induced inwardly rectifying chloride conductance is apparently identical to the endogenous shrinkage-activated inwardly rectifying chloride conductance, and therefore host- rather than parasite-encoded, our data do not reveal whether the new permeation pathway is endogenous or parasite-encoded and do not provide a clue as to the molecular identity of the malaria-induced new permeation pathway.

Finally it should be emphasized that the results presented, although arguing firmly against a crucial role of CFTR and the CFTR-related inwardly rectifying chloride conductance in new permeation pathway formation, do not exclude the possibility that chloride channel abnormalities in red blood cells could become of pathophysiological significance under *in vivo* conditions. Our finding that these channels are defective in CF offers the opportunity to test this hypothesis in *Plasmodium*-infected CF animal models. Furthermore the functional expression of CFTR in red blood cells opens new perspectives to exploit the erythrocyte as a readily available cell type in electrophysiological, diagnostic, and therapeutic studies of CF.

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