Specific Inhibition of the Plasmodial Surface Anion Channel by Dantrolene

Godfrey Lisk, Myunsga Kang, Jamieson V. Cohn, and Sanjay A. Desai*

Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received 5 July 2006/Accepted 22 August 2006

The plasmodial surface anion channel (PSAC), induced on human erythrocytes by the malaria parasite Plasmodium falciparum, is an important target for antimalarial drug development because it may contribute to parasite nutrient acquisition. However, known antagonists of this channel are quite nonspecific, inhibiting many other channels and carriers. This lack of specificity not only complicates drug development but also raises doubts about the exact role of PSAC in the well-known parasite-induced permeability changes. We recently identified a family of new PSAC antagonists structurally related to dantrolene, an antagonist of muscle Ca$^{2+}$ release channels. Here, we explored the mechanism of dantrolene’s actions on parasite-induced permeability changes. We found that dantrolene inhibits the increased permeabilities of sorbitol, two amino acids, an organic cation, and hypoxanthine, suggesting a common pathway shared by these diverse solutes. It also produced parallel reductions in PSAC single-channel and whole-cell Cl$^{-}$ currents. In contrast to its effect on parasite-induced permeabilities, dantrolene had no measurable effect on five other classes of anion channels, allaying concerns of poor specificity inherent to other known antagonists. Our studies indicate that dantrolene binds PSAC at an extracellular site distinct from the pore, where it inhibits the conformational changes required for channel gating. Its affinity for this site depends on ionic strength, implicating electrostatic interactions in dantrolene binding. In addition to the potential therapeutic applications of its derivatives, dantrolene’s specificity and its defined mechanism of action on PSAC make it a useful tool for transport studies of infected erythrocytes.

* Corresponding author. Mailing address: Laboratory of Malaria and Vector Research, NIAID/NIH, Room 3W-01, 12735 Twinbrook Parkway, Rockville, Maryland 20852-8132. Phone: (301) 435-7552. Fax: (301) 402-0079. E-mail: sdesai@niaid.nih.gov.

Published ahead of print on 1 September 2006.
studies. In search of such an antagonist, we screened a library of known channel blockers for their ability to inhibit parasite-induced permeability changes. Dantrolene, a clinically used muscle relaxant that inhibits the sarcoplasmic reticulum Ca++ release channel (ryanodine receptor [RyR]), was the most potent compound identified. This finding was unexpected because RyR is not thought to share functional properties or inhibitory profiles with either PSAC or any of the proposed modified host channels. This observation has already been extended by identifying two dantrolene derivatives with substantial activity against in vitro parasite growth (26). Interestingly, both of these derivatives inhibited PSAC in single-channel and whole-cell patch-clamp recordings.

Although dantrolene may be a starting point for future antimalarial drugs, important unknowns include the range of its effects on the various parasite-induced permeability changes, its precise mechanism of inhibition, and whether it is as promiscuous as other available antagonists. Here, we found that dantrolene inhibits the full spectrum of permeability changes previously attributed to either PSAC or other putative ion channels. Electrophysiological studies revealed direct action on PSAC at an extracellular site distinct from the channel pore. Ionic strength effects on dantrolene’s affinity for this site implicate charge-charge interactions in binding and channel inhibition. Using the Xenopus oocyte expression system, we also found that dantrolene has no measurable effect on any of five divergent anion channels from other organisms, achieving a higher level of specificity for PSAC than previously demonstrated. Finally, we used this new and more specific antagonist as a tool to further substantiate a central role of PSAC in the parasite-induced uptake of sugars, amino acids, organic cations, and purines. Such mechanistic studies will be important if therapeutic agents targeting this unusual ion channel are to be successfully developed.

MATERIALS AND METHODS

Osmotic lysis assays. The kinetics of infected RBC osmotic lysis in solutions of permeant solutes was followed as previously described (59). Trophozoite-infected RBCs were enriched by Percoll-sorbitol separation, washed, and suspended at 0.5% hematocrit in test permeant solute with or without candidate antagonist. Each solution had a nominal osmolarity of ~310 mosM and contained permeant solute supplemented with only 20 mM Na-HEPES and 0.1 mg/ml bovine serum albumin, pH 7.4 (buffer A). The percent transmittance (T) of 700 nm light through this cell suspension was then continuously followed as a marker of osmotic swelling and lysis.

Radinisotope uptake. Infected RBCs were enriched, washed, and used in uptake of [1H]hypoxanthine (4 μCi/ml) at 5% hematocrit in 150 mM NaCl, 20 mM Na-phosphate, and 150 μM unlabelled hypoxanthine with indicated dantrolene concentrations. Uptake was performed at room temperature and terminated by transfer of 40 μl of cell suspension to 1 ml of tracer-free uptake buffer with 5 mM furosemide and centrifuged at 14,000 x g through dibutyl phthalate. Cell pellets were digested and counted as described previously (13). Measurements were taken in duplicate, and the counts were averaged. Parallel experiments with uninfected RBCs were identically executed.

Electrophysiology. Patch-clamp of infected RBCs was performed as described previously (1, 12) using bath and pipette solutions as indicated in each figure legend. In addition to physiological or supraphysiological concentrations of charge-carrying ions, all solutions contained 10 mM MgCl₂, 5 mM CaCl₂, and 20 mM Na-HEPES, pH 7.4 (buffer B). Where used, hypertonic salt solutions were designed to permit detection of currents through single PSAC molecules; hypertonic solutions achieve improved signal-to-noise ratios by permitting higher Cl⁻ selective currents. In some experiments, we used a specially designed chamber to permit very slow perfusion of the bath around the patch-clamped cell because the fragile seal on human erythrocytes does not tolerate the turbulence invariably associated with rapid bulk perfusion of the bath (1). The design of this chamber permitted complete solution changes in separate troughs without aggressive bulk perfusion. Under these conditions, we could sustain high resistance seals for up to 1 h on single infected erythrocytes.

Analysis. To determine mean single-channel open probabilities (Pₒ), we pooled between 24 and 65 s of recordings at a membrane potential (Vₒ) of ~100 mV from up to four single-channel patches at each dantrolene concentration in two separate patch-clamp solutions. The analysis used locally developed code that identifies transitions between channel open and closed states by the 50% threshold-crossing technique. The results were then compared to the measured Pₒ in the absence of dantrolene, 43 ± 2% (1).

Because PSAC openings at positive Vₒ are poorly resolved, we used integration of currents relative to the closed channel baseline to examine the voltage dependence of dantrolene’s effects in single-channel recordings. Here, the code determined the mean current at the closed state, subtracted this value from the trace, and then averages the residual current samples at each voltage. This open-channel current corresponds to the product of the single channel amplitude and the Pₒ. The main advantage of this integration method over the above mid-threshold algorithm is that it does not require definition of open-channel current levels.

Dwell time distributions were obtained with code that uses consecutive midthreshold crossings to determine event durations. These durations were corrected for sampling error through linear interpolation of sampled currents immediately before and after each crossing as described previously (10). We also quantified the nonzero response time of our recording equipment by measuring an overall rise time, Tᵣ, of 66.4 μs, consistent with Gaussian filtering at 5 kHz. Equipment rise times in this range are problematic for very short channel openings or closings (<30 ns), where the numbers of events are underestimated. To correct for this error, we transformed measured event durations with the equation:

\[ t_{\text{corr}} = -\frac{1}{a} \ln\left(1 - \frac{t_{\text{meas}}}{c}\right) \]

where \( t_{\text{meas}} \) and \( t_{\text{corr}} \) are the true and measured event durations, respectively; \( a \), \( b \), and \( c \) are empirical constants that depend on \( T_r \) as 0.5382 × \( T_r \), 0.837 × \( T_r^2 \), and 1.120 × \( T_r^{-3} \), respectively (9). Measurements of short events generated with small resistor capacitor circuits confirmed the validity of this approach with our equipment and filtering conditions (10). Histogram ordinate values were normalized to the percentage of the total number of events detected under each condition and are displayed on a square root-logarithmic plot (53). On these plots, an exponentially decaying probability density function, expected for any kinetic process with a single reversible transition, would be given by the equation:

\[ f(t) = c \times \exp(\ln(t) - b) \]

where \( c \) and \( r \) are constants and \( t \) is the duration of channel events. Importantly, the peak of \( f(t) \) occurs at \( \ln(t) = b \), corresponding to the time constant of the kinetic process, \( \tau \).

Heterologous expression of chloride channels. Functional studies of five divergent anion channels expressed in Xenopus oocytes were performed with the two-electrode voltage clamp method (55). CICL-1 and CIC-L5 were cloned into the pTLN vector (36) and transcribed from the Sp6 RNA promoter. CFTR and MOD-1 (modulation of locomotion chloride channel in Caenorhabditis elegans) were cloned into the PGEM 3Z vector (33) and transcribed from the T7 RNA promoter. For each heterologous channel, oocytes were microinjected with 4 ng of in vitro transcribed cRNA flanked by the 3' and 5' untranslated regions of a Xenopus β-globin gene to express cloned protein (33). After incubation at 18°C for 3 days, electrophysiological recordings were obtained in 96 mM NaCl, 2 mM KCl, 1 mM MgCl², 1.8 mM CaCl₂, 5 mM Na-HEPES, 0.1 mg/ml gentamicin, and 0.55 mg/ml pyruvate, pH 7.4, at 22°C. For endogenous Ca++-activated Cl⁻
RESULTS

Dantrolene inhibits both sorbitol-induced lysis and PSAC-mediated Cl− currents. Trophozoite-stage infected RBCs undergo osmotic lysis in isotonic sorbitol with a halftime of 7 to 10 min (Fig. 1 A, upper trace), depending on parasite maturity. This halftime is a quantitative marker of this solute’s markedly increased permeability after infection. We used a simple light-scattering assay designed to track lysis of RBCs (59) and found that dantrolene produces a concentration-dependent slowing of sorbitol-induced lysis, with a concentration of 10 μM almost completely abolishing these transmittance changes (bottom trace). Over a range of concentrations, inhibition was adequately fitted by the equation

\[ y = y_{\text{max}} \frac{K_{0.5}}{K_{0.5} + x} \]  

(4)
as expected for a 1:1 interaction between dantrolene and a single passive mechanism of sorbitol uptake. The fitted \( K_{0.5} \) was 1.2 μM (Fig. 1 B).

We then wondered if there are other pharmacological similarities between the parasite-induced permeabilities and RyR. Although azumolene, a bromo-phenyl analog of dantrolene, also inhibits both of these functionally divergent transport mechanisms, we found no other pharmacological similarities (Fig. 1C and D). Ryanodine, an agonist for skeletal muscle Ca++ release at low concentrations and an antagonist at high concentrations, had no effect on sorbitol-induced osmotic lysis. The RyR agonist caffeine, the RyR antagonist ruthenium red, and other modulators of Ca++ release channels also had no significant effects.

While polymorphisms in PSAC gating suggest it is parasite encoded (1), other channels proposed to mediate organic solute uptake after infection are believed to result from modifications of host channel proteins (18, 17, 58). Our searches of the completed P. falciparum genome database did not find any clear homologues of a short 20-residue region in RyR where dantrolene binds (45), leaving open the question of how the parasite induces a dantrolene-sensitive sorbitol permeability.

To explore possible mechanisms of dantrolene’s effects, we used cell-attached patch-clamp on trophozoite-stage-infected RBCs. Because PSAC has a small single channel conductance and fast-flickering transitions with mean open durations of only 200 μs, detection of single-channel molecule events re-

1884 LISK ET AL. EUKARYOT. CELL
quires hypertonic recording solutions containing chloride salts, seal resistances of \( \geq 100 \text{G}\Omega \), and optimized low-noise circuitry (1). Similar experiments using isotonic salt solutions reveal characteristic inward-rectifying channel noise in multichannel patches but do not permit single-channel detection (12, 1). Here, we began with low noise recordings using a total nominal chloride concentration of 1,145 mM and detected a 20-pS inward rectifying channel with fast-flickering gating (Fig. 2A, top two traces). This channel behavior was detected as one or more functional copies in about half of all successful patches. Other channel types, such as activity consistent with proposed outward rectifying channels (17), were never detected under these experimental conditions (\( n = 604 \) patches).

Identical experiments performed with up to 20 \( \mu \text{M} \) dantrolene in both pipette and bath solutions produced PSAC activity with visibly different gating behavior (Fig. 2A, bottom three traces). Dantrolene added a population of relatively long closings without affecting open-channel amplitude. This change was most apparent at negative \( V_m \) values, where nearly all of PSAC’s intrinsic closings are less than 5 ms long (10). Because PSAC openings are less frequent at positive \( V_m \), we integrated open-channel currents to determine that increasing concentrations produce progressively greater inhibition at all examined voltages (Fig. 2B). As this approach is limited to analyses of relatively short recordings from single-channel molecules, we did not attempt to quantify inhibition here. Nevertheless, these recordings provide pharmacological evidence for a mechanistic link between sorbitol-induced osmotic lysis and PSAC channel activity.

To more quantitatively determine dantrolene’s affinity for inhibition in single-channel recordings, we measured PSAC open probability (\( P_o \)) in up to 61 s of recording at each dantrolene concentration as described in Materials and Methods. Without the addition of antagonist, PSAC exhibits a mean \( P_o \) of 43 \( \pm \) 2% at a \( V_m \) of \(-100 \text{ mV} \) under these recording conditions (1). Consistent with the above averaging of open-channel currents, the mean \( P_o \) decreased as the dantrolene concentration was raised. By pooling measurements from a total of 11 single channel patches, the \( P_o \) versus dantrolene dose response was adequately fitted by equation 4 with a \( K_{0.5} \) of 4.0 \( \mu \text{M} \) (Fig. 2C). Although this value is similar to the \( K_{0.5} \) of 1.2 \( \mu \text{M} \) for inhibition of sorbitol-induced lysis (Fig. 1B), the levels of inhibition in our single-channel recordings at each dantrolene concentration were somewhat less than expected from the sorbitol-induced lysis experiments. We were further puzzled because similar measurements with furosemide (1) and phloridzin (11) have produced more precise correlations between inhibitory effects on osmotic lysis and single channel recordings. Indeed, furosemide interaction and inhibition are also precisely reproduced in independent whole-cell patch-clamp and tracer uptake experiments (1).

Why might dantrolene not have produced similarly reproducible levels of inhibition? One possibility is that uptake of sorbitol occurs via a pathway other than PSAC and that both are inhibited by dantrolene, albeit with somewhat differing \( K_{0.5} \) values. Another possibility is that dantrolene’s ability to inhibit PSAC may be influenced by the differing experimental conditions used in the lysis and single-channel transport assays. These differences include the compositions of solutions, the

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** (A) Single PSAC recordings in 1,000 mM choline-Cl, 115 mM NaCl plus buffer B, and indicated dantrolene concentrations in bath and pipette. \( V_m \) was \(+100 \text{ mV} \) for the top trace and \(-100 \text{ mV} \) for all others. Closed channel levels are marked with dashes on both sides of each trace. At a \( V_m \) of \(+100 \text{ mV} \), PSAC openings are infrequent and poorly resolved because of the channel’s intrinsic inward-rectifying voltage dependence. Notice that increasing dantrolene concentrations decrease the frequency of openings, visible as downward deflections from the closed level for \( V_m \) of \(-100 \text{ mV} \). Horizontal and vertical scale bars represent 150 ms and 2.0 pA, respectively. (B) Averaged single-channel current (\( i \)) versus imposed \( V_m \) for recordings without dantrolene (filled circles) or with 5 \( \mu \text{M} \) (filled triangles) or 20 \( \mu \text{M} \) dantrolene (open circles). Each symbol represents the mean single-channel current \( \pm \) standard error of the mean determined by integration of open-channel currents as described in Materials and Methods. At each voltage, between 2.9 and 12 s of recording was averaged. (C) Pooled single-channel \( P_o \) in the same solution with 0, 5, 10, and 20 \( \mu \text{M} \) dantrolene in both bath and pipette. Symbols represent mean \( \pm \) standard error of the mean and were calculated from up to 61 s of recording at each concentration. \( V_m \) was \(-100 \text{ mV} \) in these recordings. Solid line represents the best fit of single-channel \( P_o \) values to equation 4. The dashed line shows the profile expected based on the inhibition dose response for sorbitol-induced lysis (Fig. 1B).
temperatures used, and the membrane potentials channels encounter.

We examined the effect of solution composition on dantrolene inhibition. While the lysis experiments are performed in very low ionic strength solutions with almost no charged ions, single-channel detection of PSAC requires a supraphysiological ionic-strength solution. If dantrolene binds PSAC through interaction with charged residues on the channel protein, then increasing the solution ionic strength would be expected to decrease the affinity of block. We tested this possibility by performing single PSAC recordings in a solution with an intermediate ionic strength (Fig. 3 A, right column of traces). This solution has a nominal Cl\(^{-}\) concentration of 530 mM and produces reduced PSAC single-channel amplitudes still clearly resolved above the baseline noise. As previously reported (1), PSAC gating and voltage dependence were not affected by lowering ionic strength. We then examined the effect of 20 \(\mu M\) dantrolene under these conditions and found it produced a statistically significant greater inhibition (Fig. 3 B) (\(P\) value of 0.7%, two-tailed Student’s t test).

Because of the suboptimal signal-to-noise ratio in this lower ionic strength solution and because of the inability to detect single PSAC transitions in more physiological solutions, we performed additional experiments using the whole-cell patch-clamp configuration. The whole-cell configuration not only permits detection of PSAC in isotonic salt solutions, but it avoids concerns about conclusions based on analyses of a small number of ion channel molecules. Here, we obtained high resistance seals on infected erythrocytes in one of four different ionic strength solutions, achieved the whole-cell configuration, and used slow perfusion of the bath with the same solution supplemented with dantrolene concentrations between 2 \(\mu M\) and 40 \(\mu M\). Current-voltage profiles, recorded after stabilization at each dantrolene concentration, revealed an unambiguous ionic strength effect on dantrolene inhibition (Fig. 4 A, B, and C). We tallied the dose responses in each recording solution, fitted each with equation 4, and found that the dantrolene \(K_{0.5}\) increased monotonically as a nonlinear function of the Cl\(^{-}\) concentration (Fig. 4E). As the Cl\(^{-}\) concentration was lowered to physiological values, dantrolene inhibition approached the level seen in our osmotic lysis experiments (2.3 \(\pm\) 0.2 \(\mu M\) in physiological saline versus 1.2 \(\mu M\) in the salt-free sorbitol solution). The parallel effects observed in our single-channel and whole-cell recordings indicate that ionic strength modulates dantrolene’s affinity for inhibition of PSAC. Preliminary whole-cell recordings after addition of the impermeant anion gluconate to physiological saline yielded similar increases in \(K_{0.5}\) (data not shown), further suggesting electrostatic interactions between dantrolene and charged residues on the channel’s binding site.

**PSAC’s unique pharmacological profile.** Dantrolene block of PSAC might reflect either this channel’s unusual pharmacological profile or a previously unknown effect on a larger subset of anion channels. We distinguished these possibilities by expressing a diverse collection of anion channels or transporters on Xenopus oocytes. We tested CFTR, CIC-1, CIC-5, the oocyte’s eCaCC, and MOD-1, a serotonin-gated Cl\(^{-}\)-channel (24, 52, 61, 47). Transfected oocytes exhibited currents with mean reversal potentials between –25 and –30 mV, consistent with selective Cl\(^{-}\) conduction (not shown). Although these channels exhibited divergent voltage and ligand dependencies as well as differing levels of expression on oocytes, none were measurably inhibited by 50 \(\mu M\) dantrolene (Fig. 5), the limit of its solubility under these conditions and some 45 times its \(K_{0.5}\) for inhibition of PSAC-mediated sorbitol lysis. In contrast, previously known PSAC antagonists are less specific. Glybenclamide (50 \(\mu M\)) produced 60% inhibition of CFTR (mean of \(n = 3\) cells; data not shown), consistent with previous studies (60). NPPB is also known to have broad activity against these and other anion channels (24).

**Site and mechanism of dantrolene action on PSAC.** We next used whole-cell patch-clamp measurements on infected RBCs to examine dantrolene’s site of action on the PSAC molecule. In these experiments, rapid equilibration between the relatively small RBC cytosolic volume (~100 femtoliters) and the larger volume of the pipette solution (~10 \(\mu L\) in our experi-
ments) permits the selective application of dantrolene to one membrane face of PSAC at a time. To avoid errors due to leakage of inhibitor between bath and pipette compartments, these experiments also required high seal resistances, as previously described (1). In the absence of dantrolene, whole-cell currents were greater at negative membrane potentials (Fig. 6, left group of traces), consistent with PSAC’s voltage-dependent gating (12); we calculated a whole-cell chord conductance of 54 ± 5 nS between −100 and 0 mV under these conditions (n = 18 cells). Dantrolene (20 μM), when added to both...
intracellular and extracellular solutions, reduced this conductance by 80% (11 ± 0.9 nS, n = 8 cells). Extracellular addition alone matched this level of inhibition (11 ± 1 nS, n = 4 cells), while intracellular addition alone produced significantly less inhibition (48 ± 6 nS, n = 6 cells; P < 10⁻², two-tailed Student’s t test), suggesting that dantrolene’s binding site is on PSAC’s extracellular face.

Dantrolene inhibition of PSAC is unusual, distinguishing this channel from known human anion channels. How is this inhibition achieved? Analysis of open and closed event durations in single-channel recordings with multiple concentrations of an inhibitor can be used to categorize the agent as a simple pore blocker, a simple nonluminal inhibitor, or a compound with a more complicated mechanism (3). Pore blockers produce inhibition by directly occluding an open-channel pore. Because the resulting steric hindrance to ion flow bypasses the channel’s intrinsic gating, measured durations of open-channel events decrease as the concentration of a pore blocker is increased. In contrast, a simple nonluminal inhibitor reduces currents by binding to a site on the channel separate from the pore used by permeating solutes. Its binding there prohibits transitions to the open state by stabilizing one or more closed conformations. As a result, nonluminal inhibitors increase the durations of closed events without affecting open-channel durations. If this distant effect involves a 1:1 interaction with first-order rate constants, the nonluminal inhibitor will add a single exponentially decaying population of closings whose mean duration does not vary with inhibitor concentration. Any other combination of effects on open and closed channel durations would suggest a more complicated mechanism of inhibitor action, as commonly reported (46).

We used analysis of single PSAC recordings to probe dantrolene’s mechanism of inhibition. In the absence of inhibitors, PSAC exhibits one open and multiple closed states (10). The distribution of open-channel durations was not affected by the presence of up to 20 μM dantrolene (Fig. 7 A), excluding a simple pore-blocking mechanism. The distribution of closed durations, on the other hand, was markedly affected with the addition of a large fraction of closed events greater than 10 ms (Fig. 7 B, red distribution). This fraction was adequately fitted by the theoretical distribution for a single dantrolene-bound state with an exponentially decaying rate constant of 21 s⁻¹ for unbinding (Fig. 7 B, smooth curve). Because this rate constant did not vary with inhibitor concentration (Fig. 7 C), dantrolene’s effects on the channel can be conservatively explained by a simple nonluminal interaction with a single site on PSAC’s extracellular face that prevents the conformational changes needed for channel opening. (Although the duration of individual inhibitory event does not depend on dantrolene concentration, the frequency of these events increases with concentration. This higher frequency of blocked events produces the greater inhibition observed in macroscopic measurements.)

Although osmotic lysis, tracer flux, and electrophysiological measurements with furosemide, a known PSAC antagonist, suggest that PSAC mediates the uptake of solutes as diverse as...
anions, sugars, amino acids, and bulky organic cations (1), some workers remain skeptical because furosemide is nonspecific; a highly nonspecific antagonist might inhibit multiple different pathways induced by the parasite and falsely implicate a common route (23). Moreover, electrophysiological studies in other laboratories suggest that channels with distinct biophysical properties may also be induced by the parasite (17, 18, 58). Because dantrolene is more specific than previously characterized inhibitors, we tested whether it can also inhibit the increased permeabilities of solutes other than sorbitol and Cl⁻. Using the osmotic lysis assay, we found that it inhibited the parasite-induced permeability of two amino acids and of the organic cation phenyl-trimethyl-ammonium at concentrations that inhibit sorbitol uptake (Fig. 8 A). It also inhibited the parasite-induced component of [³H]hypoxanthine tracer uptake (Fig. 8 B). Because this purine is required for in vitro parasite growth (14), dantrolene inhibition is consistent with a role for PSAC in nutrient acquisition.

The broad inhibitory effects of dantrolene on parasite-induced permeability changes, when combined with its known inhibitory effects on parasite growth in culture (32, 26), suggest it may be a starting point for development of future antimalarial drugs that kill parasites by inhibition of PSAC. As with a number of previous antagonists of this channel, we found that dantrolene inhibition is adversely affected by the addition of serum (Fig. 8 C), presumably because of adsorption to lipids and hydrophobic proteins. Drug development programs will need to identify derivatives with sustained availability in serum to achieve in vivo PSAC inhibition and parasite killing.

FIG. 6. (A) Whole-cell currents for infected RBCs without (left group of traces) or with 20 μM dantrolene added to both bath and pipette, to bath only, or to pipette only (second, third, and fourth groups, respectively). Groups of traces represent the current in response to $V_m$ pulses from −100 to +100 mV (10-mV increments) and reveal PSAC's inward rectifying behavior (12). Bath and pipette contained 500 mM NaCl plus buffer B. Notice that addition of dantrolene to the bath alone produces marked inhibition in whole-cell currents, while addition to the pipette has a negligible effect. Scale bars, 25 ms/2,000 pA for all groups of traces. (B) Corresponding current-voltage profiles for no dantrolene (open circles) or 20 μM dantrolene in both bath and pipette (filled circles), in bath only (open triangles), or in pipette only (closed triangles). (C) Mean ± standard error of the mean of whole-cell chord conductances calculated between a membrane potential of −100 and 0 mV with indicated dantrolene concentrations (in μM). Asterisks denote statistical significance relative to measurements with no dantrolene ($n = 4$ to 18 cells under each condition).
DISCUSSION

Because PSAC does not share other properties with the skeletal RyR Ca$^{2+}$ release channels, dantrolene inhibition of PSAC is quite unexpected. Indeed, inhibitory effects of dantrolene on parasite growth, known for a number of years (32), were attributed to yet undiscovered Ca$^{2+}$ channels without consideration of possible effects on known parasite-induced channels. In light of our findings, dantrolene’s ability to inhibit in vitro parasite growth can be more conservatively explained by its effects on PSAC.

Since PSAC’s identification (12), other electrophysiological and biophysical studies (17, 18, 58, 22) have generated much controversy over whether a single ion channel could be responsible for the increased permeabilities of diverse solutes of both positive and negative charge. One of the main arguments for a single shared pathway has been nearly universal inhibition by a collection of quite nonspecific inhibitors. Furosemide (which inhibits both anion and cation channels as well as carriers), NPPB (which inhibits many different anion channels and other enzymes [5]), and phloridzin (which inhibits many diverse transporters and even affects the permeability of pure lipid bilayers [38]) were among the most used agents to argue for one pathway. Workers were rightly skeptical of these agents. Our studies indicate that dantrolene is significantly more specific (Fig. 5). Its ability to inhibit the uptake of multiple solutes in the various assays used here allays the concerns arising from poor specificity. Moreover, its parallel effects on PSAC activity detected in both single channel and whole-cell electrophysiological experiments suggest a solid pharmacological link between PSAC and the generally accepted organic solute permeability changes.

A previous screen of 165 dantrolene derivatives implicated the nitro-phenyl furan group of dantrolene in PSAC inhibition (Fig. 1 C, dashed rectangle). Here, we found that azumolene, which has a bromo-phenyl group in the place of the critical nitro-phenyl, also inhibits PSAC with comparable affinity. This observation suggests that the strong electron donating abilities of both these groups are critical for PSAC inhibition. This suggestion is also consistent with our identification of a modest, but statistically significant strength effect on dantrolene’s affinity for the channel.

Ionic strength effects on inhibitor binding have also been seen with other enzymes, including a number of ion channels (4, 19, 29, 37, 42). They represent a classical test of electrostatic interactions between fixed charges on the enzyme’s binding site and polar or charged groups on the inhibitor. In one elegant study (19), engineered mutations of a negatively charged residue on a K$^+$ channel altered the effect of ionic strength on inhibitor affinity in a predictable fashion, definitively confirming electrostatic interactions with that residue. Those workers also determined that the ionic strength effect was primarily via effects on the inhibitor association rate constant, a finding which suggested a detailed mechanism of inhibitor action on the channel. While similar studies with PSAC must wait for both the cloning of its gene(s) and the development of a suitable heterologous expression system, the ionic strength effect on dantrolene affinity is most conservatively explained by interaction with charged residues at the channel extracellular face, some of which have been identified using covalent modification with N-hydroxysulfosuccinimide esters (8). Alternative explanations are unlikely because neither ionic strength nor Cl$^-$ concentration affects the affinity of furosemide (1) or phloridzin (11). They also do not alter PSAC gating, voltage dependence, or selectivity, suggesting that there are not global changes in channel structure under high-salt conditions (12, 1).

Because dantrolene and its derivatives kill in vitro parasite cultures, they may be lead compounds for antimalarial development (26). Several observations suggest this approach should be explored actively. First, while it remains debated whether the permeability changes after infection result from a parasite-encoded protein or a modified host protein, functional and biophysical conservation on erythrocytes infected with the phylogenetically distant Plasmodium knowlesi is consistent with an important biological role after infection (35). Second, the plasma-exposed location of PSAC reduces concerns about acquired resistance through extrusion of unme-
tabolized drug from the infected erythrocyte complex, a mechanism with substantial supportive evidence for chloroquine (31, 51, 40) and possibly for other antimalarial drugs (25).

Other mechanisms of resistance, such as selection of mutations in the antagonist binding pocket, remain possible. Third, PSAC’s location on the surface of the infected RBC complex reduces drug design constraints such as those proposed by Lipinski et al. (34). These constraints propose that an ideal drug should not have a molecular weight or cLogP (a marker of aqueous and membrane solubility) outside of empirically determined ranges to facilitate access to intracellular targets. A drug binding site directly exposed to bloodstream concentrations, such as now demonstrated for the dantrolene site on PSAC, would presumably not need to meet these recommendations.

ACKNOWLEDGMENTS

We thank J. Gonzalez and K. Oades for help with screening ion channel inhibitors and T. Jentsch, D. Gadsby, and H. Horvitz for providing the Xenopus expression constructs. We thank S. Baylor and K. Swartz for helpful suggestions and their comments on the manuscript.

This research was supported by the Intramural Research Program of the NIH, NIAID.

REFERENCES

1. Alkhalil, A., J. V. Cohn, M. A. Wagner, J. S. Cabrera, T. Rajapandi, and S. A. Desai. 2004. Plasmodium falciparum likely encodes the principal anion channel on infected human erythrocytes. Blood 104:4279–4286.
2. Ancelin, M. L., M. Parant, M. J. Thuet, J. R. Philippot, and H. J. Vial. 1991. Increased permeability to choline in simian erythrocytes after Plasmodium knowlesi infection. Biochem. J. 273:701–709.
3. Arias, H. R. 1996. Luminal and non-luminal non-competitive inhibitor binding sites on the nicotinic acetylcholine receptor. Mol. Membr. Biol. 13:1–17.
4. Arias, H. R. 1996. Temperature and ionic strength dependence of quinacrine binding and quinacrine displacement elicited by high concentrations of agonists on the nicotinic acetylcholine receptor. Arch. Biochem. Biophys. 333:1–11.
5. Breuer, W., and K. L. Skorecki. 1989. Inhibition of prostaglandin E2 synthesis by a blocker of epithelial chloride channels. Biochem. Biophys. Res. Commun. 163:398–405.
6. Cabantchik, Z. I. 1990. Properties of permeation pathways induced in the human red cell membrane by malaria parasites. Blood Cells 16:421–432.
7. Caro, J. F. 1990. Effects of gliburide on carbohydrate metabolism and insulin action in the liver. Am. J. Med. 89:175–258.
8. Cohn, J. V., A. Alkhalil, M. A. Wagner, T. Rajapandi, and S. A. Desai. 2003.
Extracellular lysines on the plasmodial surface anion channel involved in Na⁻ exclusion. Mol. Biochem. Parasitol. 132:27–34.

Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single-channel records, p. 191–285. In B. Sakmann and E. Neher (ed.), Single channel recording. Plenum Press, New York, N.Y.

Desai, S. A. 2005. Open and closed states of the plasmodial surface anion channel. Nanomed. Nanotechnol. Biol. Med. 1:58–66.

Desai, S. A., A. Alkhali, M. Kang, U. Ashfaq, and M. L. Nguyen. 2005. PSAC-independent chloroquine resistance in Plasmodium falciparum. J. Biol. Chem. 280:10661–10667.

Desai, S. A., S. M. Bezrukov, and J. Zimmerberg. 12.

Ginsburg, H., and W. D. Stein. 24.

Jentsch, T. J., V. Stein, F. Weinreich, and A. A. Zdebik. 2006. Identification of a dantrolene-binding sequence on the skeletal muscle ryanodine receptor. J. Biol. Chem. 281:21515–21519.

Lorenz, C., M. Pusch, and T. J. Jentsch. 1996. Heteromultimeric CLC chloride channels with novel properties. Proc. Natl. Acad. Sci. USA 93:13362–13366.

MacKinnon, R., R. Latorre, and C. Miller. 1989. Role of surface electrostatics in the operation of a high-conductance Cs⁺-activated K⁺ channel. Biochemistry 28:8902–8909.

Melnik, E., R. Latorre, J. E. Hall, and D. C. Totosan. 1977. Phloretin-induced changes in ion transport across lipid bilayer membranes. J. Gen. Physiol. 69:243–257.

Meyer, G., S. Doppierio, P. Vallin, and L. Daffonchio. 1996. Effect of frusmide on Cl⁻ channel in rat peritoneal mast cells. Eur. Respir. J. 9:2461–2467.

Naude, B., J. A. Brzostowski, A. R. Kimmel, and T. E. Wellens. 2005. Dicoumarol discoidarum expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, Plasmodium falciparum transporter PICCRT. J. Biol. Chem. 280:25596–25603.

Nadler, C., J. Diez, P. A. Hannerstedt, M. O. Christen, N. Wierzchicki, and R. G. Garay. 1987. Inhibition of Cl⁻ exchanger by xipamide in human red blood cells. Eur. J. Pharmacol. 144:353–362.

Neuemyer, T., F. Tonelio, M. F. Dal, B. Schiffer, F. Orlik, and R. Benz. 2006. Anaxtras lethal factor (LF) mediated block of the anion protective antigen (PA) ion channel: effect of ionic strength and voltage. Biochemistry 45:3060–3068.

Overman, R. R. 1948. Reversible cellular permeability alterations in disease. In vivo studies on sodium, potassium and chloride concentrations in erythrocytes of the malaria monkey. Am. J. Physiol. 151:123–128.

Palfrey, H. C., and M. E. O’Donnell. 1992. Characteristics and regulation of the Na/K/Cl co-transporter. Cell Physiol. Biochem. 2:293–307.

Paul-Pletzer, K., T. Yamamoto, M. B. Bhat, J. Ma, N. Ikemoto, L. S. Jimenez, H. Morimoto, P. G. Williams, and J. Parness. 2002. Identification of a dantrolene-binding sequence on the skeletal muscle ryanodine receptor. J. Biol. Chem. 277:34918–34923.

Prince, R. J., R. A. Pennington, and S. M. Sine. 2002. Mechanism of tacrine block at adult human muscle nicotinic acetylcholine receptors. J. Gen. Physiol. 120:360–393.

Ranganathan, R., S. C. Cannon, and H. R. Horvitz. 2005. MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in C. elegans. Nature 438:369–393.

Riggs, H., H. Qian, and J. Zakevicius. 2004. Properties of connexin26 hemichannels expressed in Xenopus oocytes. Cell Mol. Neurobiol. 24:647–655.

Ritsikis, T. L., E. A. Kovala, and Y. N. Antonenko. 2002. Membrane dipole potential modulates proton conductance through gapicin channel: movement of negative ions across the channel. Biophys. J. 82:565–573.

Saliba, K. J., H. A. Horner, and K. Kirk. 1998. Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite Plasmodium falciparum. J. Biol. Chem. 273:10190–10195.

Sanchez, C. P., W. Stein, and M. Lanzer. 2003. Trans stimulation provides evidence for a drug efflux carrier. The mechanism of chloroquine resistance in Plasmodium falciparum. Biochemistry 42:9383–9394.

Sheppard, D. N., and M. J. Welsh. 1999. Structure and function of the CFTR chloride channel. Physiol. Rev. 79:232–345.

Siewert, F. J., and S. M. Sine. 1987. Data transformations for improved display and fitting of single-channel dwell time histograms. Biophys. J. 52:1047–1054.

Staines, H. M., B. C. Dee, M. O’Brien, H. J. Lang, H. Englert, H. A. Horner, J. C. Ellory, and K. Kirk. 2004. Furosemide analogues as potent inhibitors of the new permeability pathways of Plasmodium falciparum-infected human erythrocytes. Mol. Biochem. Parasitol. 133:315–318.

Stuhmer, W. 1992. Electrophysiological recording from Xenopus oocytes. Methods Enzymol. 206:750–757.

Tian, Y. A., G. Johnson, and S. J. Ashcroft. 1998. Sulfonylurea enhance coxycysis from pancreatic beta-cells by a mechanism that does not involve direct activation of protein kinase C. Diabetes 47:1722–1726.

Upstun, J. M., and A. M. Gero. 1995. Paroxysmic induced permeation of nucleosides in Plasmodium falciparum malaria. Biochim. Biophys. Acta 1236:249–258.

Verloo, P., C. H. Kocnic, W. A. van der, B. C. Tilly, B. M. Hogema, M.
Sinaasappel, A. W. Thomas, and H. R. De Jonge. 2004. Plasmodium falciparum-activated chloride channels are defective in erythrocytes from cystic fibrosis patients. J. Biol. Chem. 279:10316–10322.

59. Wagner, M. A., B. Andemariam, and S. A. Desai. 2003. A two-compartment model of osmotic lysis in Plasmodium falciparum-infected erythrocytes. Biophys. J. 84:116–123.

60. Yamazaki, J., and J. R. Hume. 1997. Inhibitory effects of glibenclamide on cystic fibrosis transmembrane regulator, swelling-activated, and Ca\(^2+\)-activated Cl\(^-\) channels in mammalian cardiac myocytes. Circ. Res. 81:101–109.

61. Young, G. P., J. D. Young, A. K. Deshpande, M. Goldstein, S. S. Koide, and Z. A. Cohn. 1984. A Ca\(^2+\)-activated channel from Xenopus laevis oocyte membranes reconstituted into planar bilayers. Proc. Natl. Acad. Sci. USA 81:5155–5159.