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Malaria Parasites Are Rapidly Killed by Dantrolene Derivatives Specific for the Plasmodial Surface Anion Channel

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ABSTRACT

Dantrolene was recently identified as a novel inhibitor of the plasmodial surface anion channel (PSAC), an unusual ion channel on *Plasmodium falciparum*-infected human red blood cells. Because dantrolene is used clinically, has a high therapeutic index, and has desirable chemical synthetic properties, it may be a lead compound for antimalarial development. However, dantrolene derivatives would need to preferentially interact with PSAC over the sarcoplasmic reticulum (SR) Ca^{2+} release channel to avoid unwanted side effects from antimalarial therapy. Furthermore, dantrolene's modest affinity for PSAC (K_m of 1.2 μM) requires improvement. In this study, we tested 164 derivatives of dantrolene to examine whether these hurdles can be surmounted. A simple screen for PSAC block defined the minimal scaffold needed and identified compounds with $\geq$5-fold higher affinity. Single-channel patch-clamp recordings on infected human red blood cells with two derivatives also revealed increased blocking affinity that resulted from slower unbinding from a site on the extracellular face of PSAC. We tested these derivatives in a frog skeletal muscle contractility assay and found that, in contrast to dantrolene, they had little or no effect on SR Ca^{2+} release. Finally, these blockers kill in vitro parasite cultures at lower concentrations than dantrolene, consistent with an essential role for PSAC. Because, as a class, these derivatives fulfil the requirements for drug leads and can be studied with simple screening technology, more extensive medicinal chemistry is warranted to explore antimalarial development.

The plasmodial surface anion channel (PSAC) may be an ideal target for the development of antimalarial drugs because it is located on the host erythrocyte membrane (Desai et al., 2000), has an unusual pharmacological profile (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted), and is conserved on parasite isolates from around the world (Alkhalil et al., 2004). Although the precise physiological role of PSAC is unknown, a role in nutrient acquisition for the intracellular parasite is likely (Desai et al., 2000) because this ion channel is highly permeable to purines, amino acids, vitamins, and other nutrients needed for parasite growth (Ginsburg, 1994; Kirk et al., 1994; Desai, 2004).

Although the molecular mechanism and location of the parasite-induced increased permeability were identified only recently (Desai et al., 2000), its potential value as a target for antimalarial development has been under consideration for much longer. Early studies recognized that most PSAC inhibitors kill parasites under in vitro culture conditions (Cabantchik et al., 1983; Kutner et al., 1987; Kirk et al., 1993). The major issues that should be addressed if these findings are to be successfully translated into clinically useful antimalarial drugs include 1) a demonstrated essential role of PSAC for the intraerythrocytic parasite, 2) verification that growth inhibition results from specific action on PSAC rather than on other parasite targets, 3) a clear understanding of how candidate antimalarial compounds inhibit permeation through PSAC, 4) robust inhibitory activity under physiological conditions at concentrations achieved in the human bloodstream, and 5) lack of effect on human anion channels and other essential activities. Little progress has been made on any of these major hurdles. Indeed, some may only be

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ABBREVIATIONS: PSAC, plasmodial surface anion channel; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; SR, sarcoplasmic reticulum; DMSO, dimethyl sulfoxide; RBC, red blood cell; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); *NPF*-1, 2-butyl-5-imino-6-{[5-(4-nitrophenyl)-2-furyl]methylene}-5,6-dihydro-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidin-7-one; *NPF*-2, 5-imino-6-{[5-(2-methyl-4-nitrophenyl)-2-furyl]methylene}-2-(trifluoromethyl)-5,6-dihydro-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidin-7-one.

addressed after the definitive identification of PSAC's gene(s) (Desai, 2004).

Despite these uncertainties, PSAC remains an attractive target for antimalarial development. Most programs aimed at identifying clinically useful compounds begin with known inhibitors and use medicinal chemistry approaches to search for derivatives with higher affinity and specificity for the target. Several groups have taken this approach for PSAC with some success. In one study (Kirk and Horner, 1995), 16 derivatives of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were tested for their activities against PSAC and compared with those against carrier-mediated transport in uninfected human RBCs, revealing compounds with improved but incomplete specificity for PSAC. Another study used derivatives of furosemide and achieved marked improvements in affinity for PSAC (Staines et al., 2004). Finally, the antiplasmodial activity of some chalcones was linked to inhibition of PSAC through another study of derivative compounds (Go et al., 2004). It is unfortunate that neither the mechanisms of PSAC inhibition by these agents nor the mechanism by which derivatives achieve their improved activities has been explored.

We found recently that dantrolene, a clinically used ion channel blocker with a high therapeutic index, unexpectedly inhibits PSAC (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted). This prompted us to search for derivatives that might be useful antimalarial compounds. We tested 164 dantrolene derivatives and identified compounds with both improved affinity and specificity for PSAC. Recordings from single channel molecules indicate that improved affinity results primarily from the slower unbinding of high-affinity derivatives from a common extracellular site on PSAC. These compounds were also found to have dramatically less activity against sarcoplasmic reticulum (SR) Ca^{2+} release channels, the human target of dantrolene. Because these derivatives also kill malaria parasites under in vitro conditions, further investigation of their antimalarial actions is warranted.

Materials and Methods

Screen of Dantrolene Derivatives. *Plasmodium falciparum* (Indo 1 isolate) parasites were cultured, harvested by the Percoll-sorbitol method (Aley et al., 1984), and suspended in 150 mM NaCl, 20 mM Na-HEPES, and 0.1 mg/ml BSA, pH 7.4, before use in screening 164 dantrolene derivatives (purchased from Chembridge Corp., San Diego, CA). Each compound was prepared as a 1 mg/ml DMSO stock solution and evaluated at a final concentration of 4.2 μg/ml by addition to sorbitol lysis buffer (280 mM sorbitol, 20 mM Na-HEPES, and 0.1 mg/ml BSA, pH 7.4). Lysis of trophozoite-stage infected RBCs in these solutions was then followed at a hematocrit of approximately 0.2%. Transmittance of 700 nm light through this RBC suspension was measured at 20 and 60 min to estimate fractional osmotic lysis caused by PSAC-mediated uptake of sorbitol (Wagner et al., 2003). Comparison of control suspensions with DMSO or 200 μM furosemide, which produce negligible and almost complete PSAC block, respectively, was used to calculate the percentage of PSAC block for each compound. Continuous recording of transmittance changes was also used to follow the kinetics of lysis with certain compounds.

Electrophysiology. Cell-attached single channel and whole-cell recordings on infected RBCs were performed as described previously (Desai et al., 2000) with bath and pipette solutions of 1000 mM choline chloride, 115 mM NaCl, 20 mM Na-HEPES, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.4. Seal resistances were >100 GΩ. Channel blockers, when present, were added to both compartments. Data were filtered at 5 kHz and digitized at 100 kHz.

Open and closed durations were measured as described previously (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted). Blocked channel events that resulted from the action of dantrolene or its derivatives were readily distinguished from intrinsic closings, which are significantly shorter, with mean durations of less than 1 ms (Alkhalil et al., 2004). Channel dwell durations were determined by fitting to the probability density functions for one open and four closed exponentially decaying states as described previously (Sigworth and Sine, 1987; Desai, 2005). Closed dwell durations were adequately fitted by constraining the time constants for the three states intrinsic to PSAC and determining the least-squares best estimate for a single time constant imposed by each inhibitor. Seven thousand to 90,000 events each from three separate single channel recordings were used to determine the mean dwell durations for each inhibitor.

Growth Inhibition. Synchronous parasite cultures at 1% ring-stage parasitemia were seeded at 2% hematocrit with or without channel inhibitors in RPMI 1640 supplemented with 3.6 mg/l hypoxanthine and 2% pooled human serum. Cultures were incubated at 37°C under 5% O_2/5% CO_2/90% N_2 for 48 h with medium exchange at 24 h. At 48 h, the cultures were washed in the same medium without inhibitor or hypoxanthine and grown for an additional 24 h with 2.1 μCi/ml [^{3}H]hypoxanthine. Incorporation of this label into parasite DNA and RNA, a marker of parasite growth (Chulay et al., 1983), was quantified by harvest onto filter paper and β-scintillation counting.

Frog Skeletal Muscle Contractility. Bundles of one to four intact twitch fibers were isolated by manual dissection from *Rana pipiens* leg muscles and mounted at a sarcomere length of 3 to 3.5 μm in Ringer's solution (120 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 5 mM PIPES, pH 7.1) at 16°C. The combined twitch response to a suprathreshold electrical shock applied by an external source was recorded with a tension transducer attached to one tendon end of the bundle (Baylor and Hollingworth, 2003).

Results

Screen for Higher Affinity Derivatives. The dantrolene molecule consists of a 2-(4-nitrophenyl)furan group (Fig. 1B, red scaffold) linked to hydantoin. Because high concentrations of hydantoin fail to inhibit PSAC-mediated transport (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted), we speculated that the 2-(4-nitrophenyl)furan group is responsible for PSAC inhibition. We tested this hypothesis with 164 compounds that contain this group in a modified osmotic lysis assay that tracks PSAC-mediated uptake of the sugar alcohol, sorbitol (Wagner et al., 2003). Each compound was evaluated at 20 and 60 min after addition of sorbitol (Fig. 1A) to permit identification of both modest and high-affinity PSAC inhibitors: in this endpoint assay, inhibition of cell lysis for short durations can be achieved by weak antagonists, but prolonged inhibition requires higher affinity. The sigmoidal shape of the profile in Fig. 1A graphically reflects this dual stringency of our assay.

Compounds selected from chemically unbiased small molecule libraries have a $\leq$1% probability of inhibiting PSAC (S. Desai, unpublished observations). In contrast, we found that 46% of compounds containing the 2-(4-nitrophenyl)furan group significantly inhibited lysis by $\geq$50% at the 20-min measurement, confirming that this group is responsible for PSAC inhibition. Addition of various substituents on different parts of this scaffold significantly affected the affinity of these compounds for PSAC. We selected two high-affinity

compounds, *NPF*-1 (2-butyl-5-imino-6-{[5-(4-nitrophenyl)-2-furyl]methylene}-5,6-dihydro-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidin-7-one) and *NPF*-2 (5-imino-6-{[5-(2-methyl-4-nitrophenyl)-2-furyl]methylene}-2-(trifluoromethyl)-5,6-dihydro-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidin-7-one) (Fig. 1B), for further study; in the osmotic lysis assay, these compounds exhibited dose responses with K_m values of 200 to 400 nM, significantly stronger inhibition than that seen with dantrolene (Fig. 1C).

Electrophysiological Studies with *NPF*-1 and *NPF*-2. We used both single-channel and whole-cell patch-clamp to measure the effects of these dantrolene derivatives on PSAC-mediated Cl^- transport. In these experiments, Cl^- currents were significantly inhibited by *NPF*-1 and by *NPF*-2 (Fig. 2, A and B) at concentrations similar to those used in the sorbitol uptake assay (Fig. 1C). Furthermore, both patch-clamp configurations revealed significantly greater levels of inhibition by these new inhibitors than by dantrolene, indicating that our simple screen did achieve improved affinity for PSAC. Thus, *NPF*-1 and *NPF*-2 produce parallel effects on sorbitol and Cl^- transport in infected RBCs.

The scaffold these derivatives share with dantrolene suggests that this class of antagonists inhibits PSAC through a similar mechanism (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted). To study how they achieve their higher affinity, we analyzed the durations of single channel openings and closings recorded with each inhibitor. Neither dantrolene nor the higher affinity derivatives had any effect on the channel's mean open duration (Fig. 3A), consistent with a binding site that does not involve direct blockade of the permeation pore. Instead, both dantrolene and the two derivatives added a population of long closings to our single channel recordings. The mean duration of these imposed closings, however, was significantly longer for the derivatives than for dantrolene (Fig. 3B). Because the ~4-fold increase in these block durations approximates the 3- to 5-fold higher affinity measured in our sorbitol-mediated lysis assay (Fig. 1C), these single-channel studies indicate that these derivatives achieve their increased inhibitory effects primarily through slowed unbinding from a common site on the PSAC molecule. Experiments with dantrolene have revealed that this site is on the extracellular face of PSAC (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted).

Effects on Parasite Growth. In our model, PSAC functions as the first step in a sequential diffusive pathway of nutrient acquisition by the intraerythrocytic parasite (Desai et al., 2000). Here, the high affinity inhibitors may be lead compounds for future antimalarials that starve the intracellular parasite of nutrients present in plasma. Consistent with this proposed role, we found that both dantrolene and the higher affinity derivatives quickly killed in vitro parasite cultures (Fig. 4). As also predicted by our model, we found that increased affinity of channel block correlated with killing at lower concentrations. Although qualitative concordance between affinity for channel block and growth inhibition is expected, precise correlations are not necessarily expected because of the nonlinear relationship between nutrient uptake and growth and because of potential off-target effects of these compounds.

Specificity of Derivatives for PSAC. Although previous studies identified a number of PSAC antagonists (Kutner et al., 1987; Kirk et al., 1993, 1994; Staines et al., 2004), none were acceptable candidates for antimalarial development because of lack of specificity: each of those compounds also inhibits human channels or transporters, producing unacceptable side-effects if administered for malaria (Pasvol, 2001). Do the new PSAC antagonists identified here also suffer from poor specificity? In the past, dantrolene has been thought to be specific for muscle SR Ca^{2+} release, offering some basis for optimism. Neither dantrolene, *NPF*-1, nor *NPF*-2 inhibited ClC-1 (Jentsch et al., 2002), modulation of locomotion chloride channel in *Caenorhabditis elegans* (Ranganathan et al., 2000), or the endogenous Ca^+-activated Cl^- channels on *Xenopus laevis* oocytes (data not shown), sug-

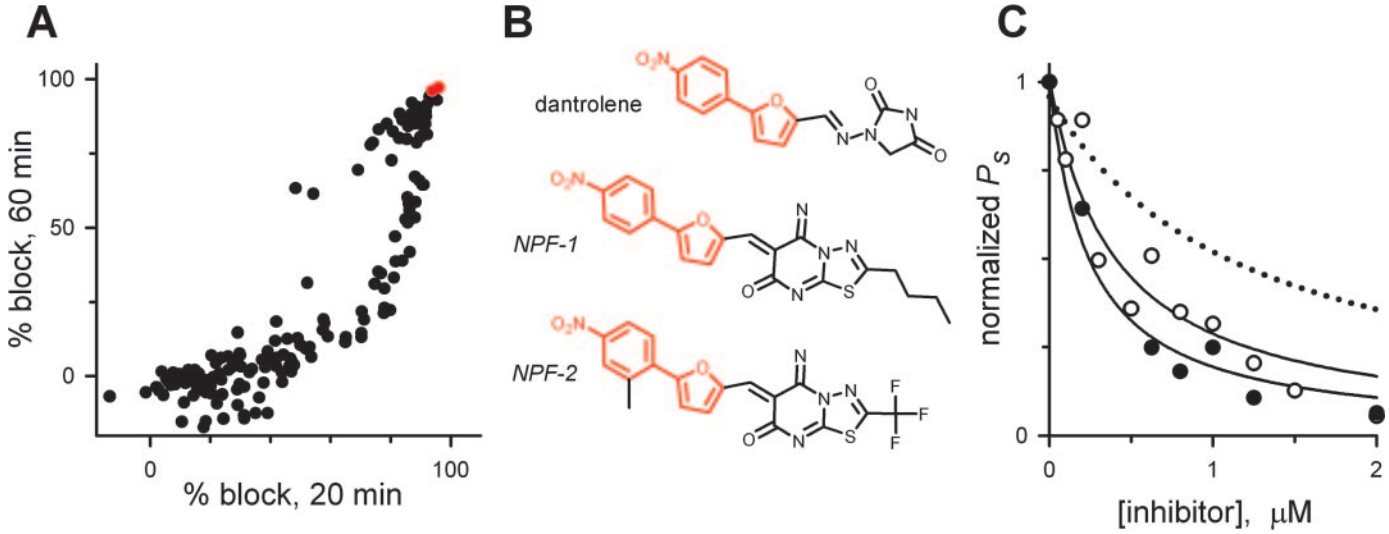

Fig. 1. Identification of high-affinity dantrolene derivatives. A, 164 derivatives were tested with a two-point osmotic lysis measurement. Each symbol represents the average of two to five measurements for a single compound. Percentage PSAC block was determined relative to control measurements without inhibitor and with 200 μM furosemide, which abolishes PSAC-mediated lysis in the time frame of this experiment (Wagner et al., 2003). Values for *NPF*-1 and *NPF*-2 are shown in red. B, structures of dantrolene, *NPF*-1, and *NPF*-2. The common scaffold of these derivatives is shown in red. C, sorbitol permeabilities, P_S, at a range of *NPF*-1 and *NPF*-2 concentrations (● and ○, respectively), normalized to the permeability without inhibitor. Solid lines represent least-squares best fits to $y = K_m/(K_m + x)$ with K_m of 0.24 and 0.40 μM for *NPF*-1 and *NPF*-2, respectively. Dotted line represents the level of inhibition seen with dantrolene (K_m of 1.2 μM; G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted).

gesting that toxic side effects from nonspecific inhibition of other anion channels are unlikely. We then examined whether these derivatives block SR Ca^{2+} release in a frog skeletal muscle contractility assay. In intact muscle fibers, dantrolene markedly decreased peak twitch tension without affecting the kinetics of the contraction (Fig. 5, A and B), consistent with its known block of SR Ca^{2+} release and no effect on the actin-myosin machinery. In contrast, *NPF*-1 had little or no measurable effect on either the peak tension reached or the kinetics of contraction, indicating that it does

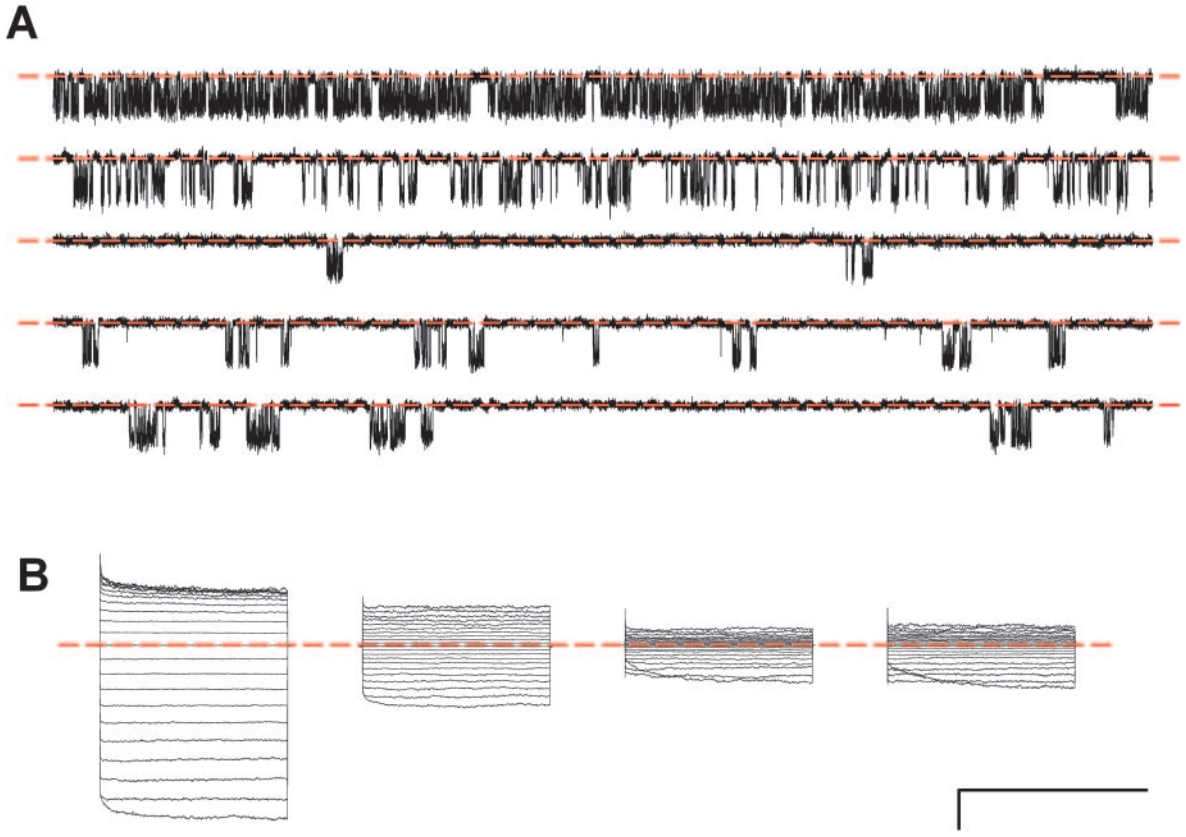

Fig. 2. Single PSAC and whole-cell patch-clamp recordings. A, recordings from infected RBC patches, each with one functional PSAC molecule without (top trace) or with 5 μM dantrolene, 5 μM *NPF*-2, 1 μM *NPF*-2, or 1 μM *NPF-1* (second, third, fourth, and fifth traces, respectively). Dashed red lines represent closed channel levels. Membrane potential, −100 mV. Notice the long closed events imposed by low concentrations of *NPF*-1 and *NPF*-2. B, whole-cell recordings without inhibitor (left group of traces) or with 10 μM dantrolene, 1 μM *NPF*-2, or 1 μM *NPF*-1 (second, third, and fourth groups of traces, respectively). In each group, traces reflect current responses to voltage pulses from −100 mV to +100 mV in 10-mV increments. Dashed red line represents the zero current level. Horizontal and vertical scale bars represent 137 ms/2.7 pA in A and 50 ms/2000 pA in B.

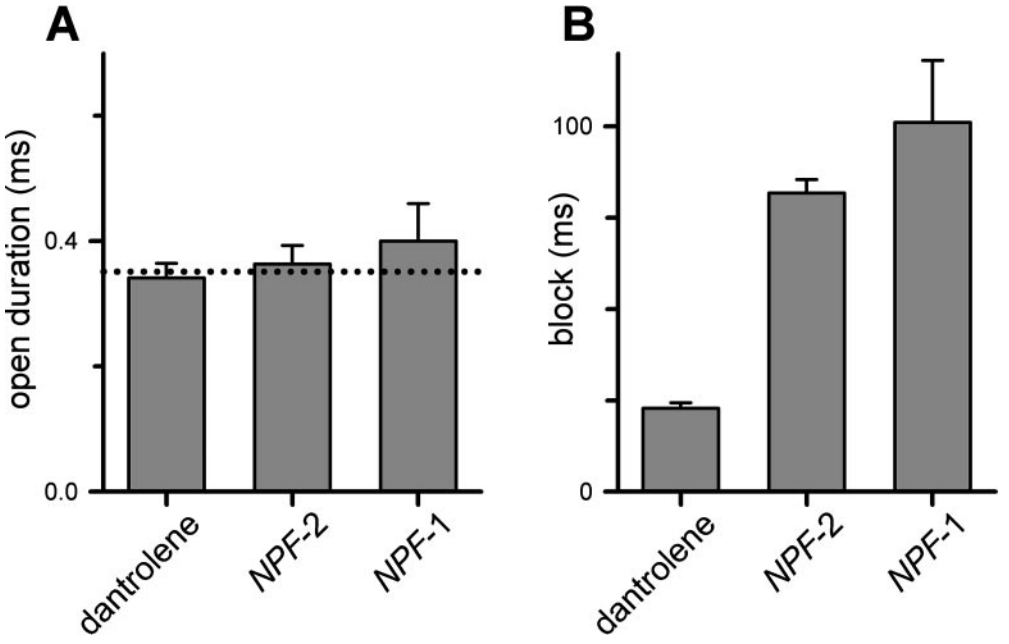

Fig. 3. Single channel open (A) and block (B) durations in the presence of inhibitors. Bars represent the mean ± S.E.M. for three single channel recordings each with 5 to 20 μM dantrolene or 0.5 to 2.5 μM *NPF*-1 or *NPF*-2. Dotted line in A represents the mean open duration of PSAC without inhibitors. *NPF*-1 and *NPF*-2 achieve their greater PSAC inhibitory effects relative to dantrolene through longer block durations without a significant change in the mean open duration. As expected for simple inhibition at a site distinct from the channel pore, mean block durations did not vary with inhibitor concentration (data not shown).

not significantly block SR Ca^{2+} release. In other experiments, fibers continued to twitch normally in *NPF*-1 for >4 h, indicating that the many processes required for excitation-contraction coupling are not compromised by this PSAC antagonist. In similar experiments, *NPF*-2 had no significant immediate effect on contractility, again suggesting a reduced ability to block SR Ca^{2+} release. However, on a longer time scale (>30 min), *NPF*-2 abruptly abolished the twitch. The mechanism underlying this adverse effect was not investigated but is probably not directly related to Ca^{2+} release. Rather, the abrupt and complete loss of the twitch suggests a sudden inability to generate an action potential. An alternative explanation (i.e., low membrane permeability of *NPF-2* producing delayed inhibition of SR Ca^{2+} release channels) cannot be formally excluded but is less likely because this should produce twitches of gradually decreasing strength rather than the observed abrupt loss.

Discussion

The aim of this study was to evaluate whether dantrolene is a suitable lead compound for the development of novel antimalarial drugs that target PSAC. PSAC offers a number of advantages over other parasite targets. Perhaps most importantly, it is a well-defined target that can be studied with multiple functional assays. Indeed, functional uptake studies performed more than 50 years ago provided evidence for the existence of PSAC (Overman, 1948). Over the years, numerous laboratories have used isotope flux (Homewood and Neame, 1974; Kirk et al., 1994; Upston and Gero, 1995), osmotic fragility (Ginsburg et al., 1985), and fluorescent assays (Cabantchik et al., 1983) to quantify and characterize its permeability properties and to identify inhibitors. The addition of single channel and whole-cell patch-clamp measurements to this armamentarium represent two recent advances

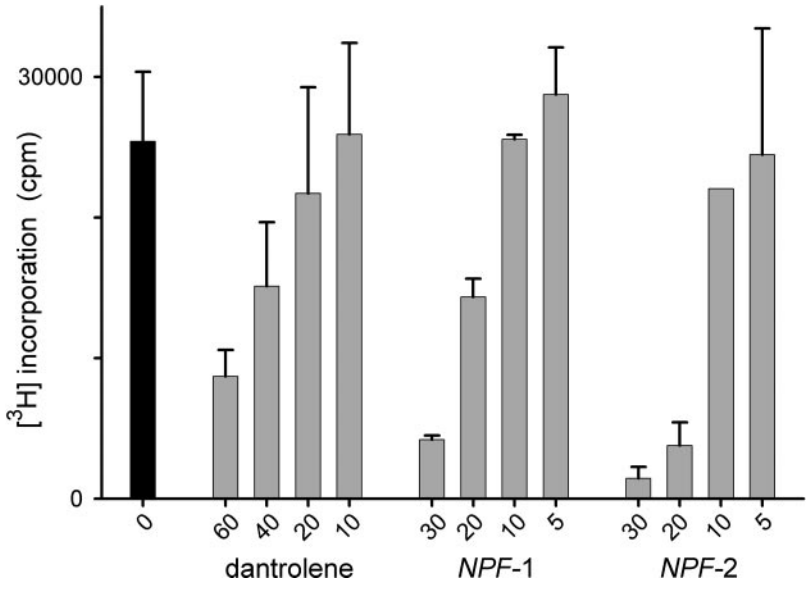

Fig. 4. Malaria parasites are killed by *NPF*-1 and *NPF*-2. Parasites were grown for 48 h in the presence of indicated concentrations (micromolar) of dantrolene or its derivatives and assayed for parasite survival. Each bar represents the mean ± S.E.M. of three measurements and was compared with DMSO controls without channel blockers (black bar). Notice that significantly less *NPF*-1 or *NPF*-2 is needed to kill parasites, consistent with their increased affinities for PSAC.

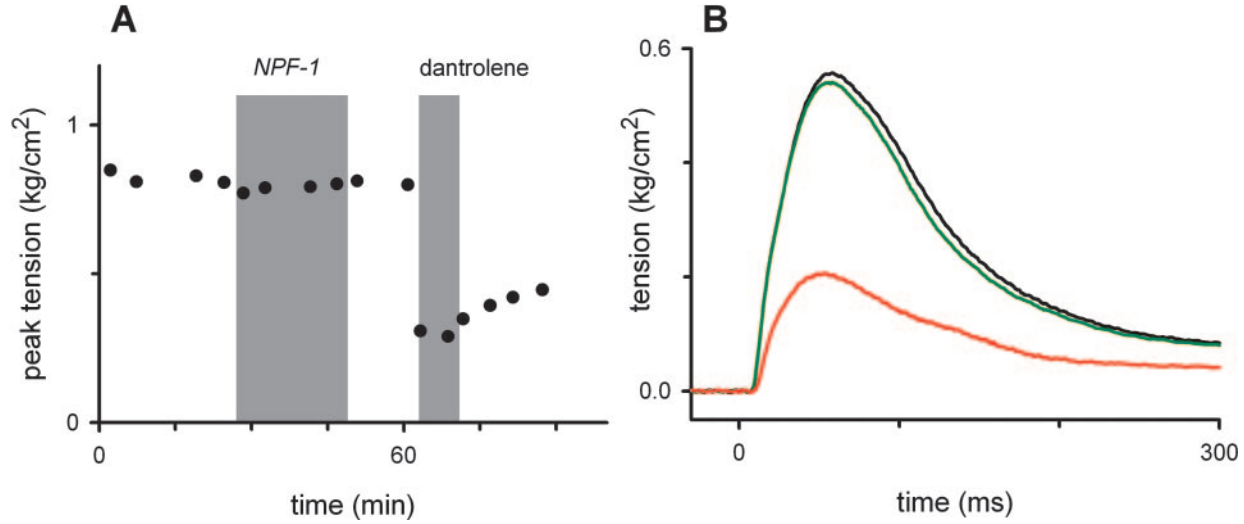

Fig. 5. Specificity for PSAC. A, comparison of *NPF*-1 with dantrolene in a frog skeletal muscle contractility assay. Measurements of peak twitch tension after electrical stimulation were repeated in the absence or presence of compounds, perfused as indicated (shaded intervals) at 5 μM concentration. Notice that *NPF*-1 had little or no effect on muscle contraction, whereas dantrolene reduced the peak tension by more than 60%. B, amplitude and kinetics of twitch responses corresponding to data points in A: black trace, no inhibitor (average of four responses, two immediately before and two after exposure to *NPF*-1); green trace, average response in the presence of *NPF*-1; red, average response in the presence of dantrolene.

(Desai et al., 2000); these have already provided fundamental new insights into the function of PSAC (Cohn et al., 2003; Alkhalil et al., 2004). We know of no other parasite target with this diverse collection of available functional assays.

Another important advantage of PSAC over other parasite targets is its location on the surface of infected RBCs. Not only does this location nullify parasite resistance mechanisms based on extrusion of antimalarial drugs from their site of action (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted), it reduces constraints on the size and nature of acceptable lead compounds. For example, a major reason for favoring low molecular weight compounds is their greater accessibility to intracellular targets (Lipinski et al., 2001). Given the extracellular location of the dantrolene binding site, it may even be possible to retain PSAC block after conjugating the active 2-(4-nitrophenyl)furan group to an impermeant high molecular weight adjunct. Such a bulky conjugate would probably need intravenous administration, but it would not produce toxic effects through interactions with intracellular targets.

How does dantrolene compare with other PSAC antagonists as a lead compound for antimalarial development? Although its affinity (K_m of 1.2 μM) is similar to those of the long known inhibitors NPPB and furosemide, it is less plagued by activity against other targets. NPPB inhibits many other anion channels (Wangemann et al., 1986; Diener and Rummel, 1989; Reddy and Quinton, 2002) as well as cyclooxygenase (Breuer and Skorecki, 1989) and would therefore not be an appropriate lead compound. Although furosemide also inhibits many diverse channels and carriers, it is still used clinically as a loop diuretic, achieving adequate therapeutic specificity as a result of active concentration at its site of action (Brater, 1998). Thus, furosemide derivatives would need to have either other modes of excretion or no action against renal transporters to be candidates for therapeutic use against PSAC. Dantrolene avoids many of these problems because it has known activity against only one type of mammalian channel. In this study, we further improved on its relative specificity for PSAC by engineering out activity against this other channel.

We found that significantly higher concentrations of dantrolene and its derivatives were required to kill parasites than to block PSAC in both our osmotic lysis and patch-clamp assays (compare Figs. 4 and 1B). A number of factors may contribute to this discrepancy. First, nutrient uptake via PSAC in the enriched RPMI culture medium presumably occurs at rates greater than needed to sustain parasite growth. Whether this uptake is more limiting under in vivo conditions, especially in malnourished children most frequently afflicted with malaria, has remained entirely unexplored. Second, these new PSAC antagonists, like some previously characterized agents, are significantly adsorbed by serum lipids and proteins, reducing their effective concentration in our in vitro growth experiments. Finally, preliminary experiments suggest that certain nutrient solutes present in our culture adversely affect these antagonists' affinity for the channel (S. Desai, unpublished observations). Drug development starting with these and other PSAC antagonists will need to critically consider these and other factors to maximize therapeutic efficacy against malaria.

The number of ion channels induced by the intracellular parasite on its host membrane remains in question. Some researchers believe that organic and inorganic solutes enter the infected RBC through two unrelated channels (Ginsburg and Stein, 2004; Huber et al., 2004; Verloo et al., 2004). Although furosemide inhibits sorbitol, lactate$^-$, and Cl^- transport with identical affinities (Alkhalil et al., 2004), it has been argued that furosemide is nonspecific and that its affinity is not sufficient to exclude models with two separate channels. Our findings with dantrolene and its derivatives provide new and quantitative evidence against these models. Not only do dantrolene, *NPF*-1, and *NPF*-2 produce parallel effects on sorbitol and Cl^- transport, but there is good agreement between their relative affinities for inhibition of osmotic lysis in sorbitol (Fig. 1C) and their relative durations of inhibition in single PSAC recordings (Fig. 3B). In light of the high specificity of *NPF*-1 and *NPF*-2 for inhibiting transport in infected RBCs, we believe the only reasonable model is a single type of channel that mediates the increased uptake of both organic and inorganic solutes. If this more conservative model is correct, the value of PSAC as an antimalarial target should be further increased.

Our study is the first to examine the submolecular site of action of a class of PSAC antagonists. Our electrophysiological studies determined that the binding site of dantrolene and its derivatives is on the extracellular face of PSAC and that it involves electrostatic interactions with charged residues (G. Lisk, M. Kang, J. V. Cohn, and S. A. Desai, submitted). Because the levels of inhibition of both single channels and whole-cell currents were not affected by membrane potential (Fig. 2 and other analyses, not shown), the binding site cannot be buried within the membrane's electric field. Finally, our single channel studies reveal that *NPF*-1 and *NPF*-2 achieve their improved affinity by having slower off rate constants from the dantrolene binding site. Similar biophysical insights into the mechanism of action of these and other PSAC antagonists should play an important role in guiding future drug development against this important target.

NPF-1 represents the first ion channel antagonist specific for PSAC. It binds with submicromolar affinity to an extracellular site on this unusual parasite ion channel, disallowing parasite resistance mechanisms based on extrusion of drug from the infected RBC. These new PSAC antagonists markedly reduce parasite burdens under in vitro conditions, consistent with the proposed role of PSAC in nutrient acquisition (Desai et al., 2000). Finally, the in vitro concentrations required are lower than the plasma dantrolene concentrations achieved in standard muscle relaxant therapy (Flewellen et al., 1983), suggesting that these compounds have adequate affinity for PSAC to be clinically useful. Additional improvements in activity should be facilitated by the ease of chemical syntheses around the 2-(4-nitrophenyl)furan scaffold and the simple screening assays described here.

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