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Solute-Inhibitor Interactions in the Plasmodial Surface Anion Channel Reveal Complexities in the Transport Process

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ABSTRACT

Human red blood cells infected with the malaria parasite *Plasmodium falciparum* have markedly increased permeabilities to diverse organic and inorganic solutes. The plasmodial surface anion channel (PSAC), recently identified with electrophysiological methods, contributes to the uptake of many small solutes. In this study, we explored the effects of known PSAC antagonists on transport of different solutes. We were surprised to find that the transport of two solutes, phenyltrimethylammonium and isoleucine, was only partially inhibited by concentrations of three inhibitors that abolish sorbitol or alanine uptake. Residual uptake via endogenous transporters could not account for this finding because uninfected red blood cells (RBCs) do not have adequate permeability for these solutes. In infected RBCs, the residual uptake of these solutes could be abolished by higher concentrations of specific and nonspecific PSAC antagonists. Adding sorbitol or alanine, permeant solutes that do not exhibit residual uptake, could also abolish it. The residual uptake did not exhibit anomalous mole fraction behavior and had a steep activation energy. These observations exclude uptake via unrelated pathways and instead point to differences in how PSAC recognizes and transports various solutes. We propose a possible model that also may help explain the unique selectivity properties of PSAC.

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Plasmodium falciparum, the most virulent human malaria parasite, infects 300 to 500 million people each year and is the primary cause of death for 1 to 3 million people. Most clinical sequelae are the direct result of the intraerythrocytic stages of the parasite. During these stages, the parasite remodels its host RBC by producing marked changes in erythrocyte deformability, ultrastructure, and permeability. Many of the permeability changes can be accounted for by a recently identified broad permeability ion channel more permeable to anions than to cations (Desai et al., 2000). This channel, the plasmodial surface anion channel (PSAC), has now been studied with a number of different methods, including tracer flux (Kutner et al., 1985; Kirk et al., 1994), osmotic lysis assays in solutions of permeant solutes (Ginsburg et al., 1985; Wagner et al., 2003), and both single-channel and whole-cell patch-clamp of infected RBCs.

Although some studies suggest that there may also be other parasite-induced channels (Huber et al., 2002; Staines et al., 2003; Verloo et al., 2004; Bouyer et al., 2006), the strongest evidence that PSAC mediates the uptake of diverse solutes such as sugars, amino acids, purines, some vitamins, and some organic cations as well as halide anions comes from studies with reversible inhibitors. With each known inhibitor, parallel effects on tracer uptake and osmotic lysis have been reported (Ginsburg et al., 1985; Kirk et al., 1994). Where examined, single PSAC patch-clamp has also produced concordant data on inhibitor effects (Alkhalil et al., 2004; Desai et al., 2005). Furthermore, some recently identified antagonists do not inhibit other channels or carriers (Kirk and Horner, 1995; Kang et al., 2005; Lisk et al., 2006) and are therefore specific for PSAC. Thus, both specific and nonspecific antagonists support a central role for PSAC in the uptake of these diverse solutes. A PSAC mutant generated by in vitro selection confirms this central role because erythrocytes infected with this mutant have globally altered permeability properties (Hill et al., 2007).

How a single ion channel type can mediate this broad collection of permeabilities and yet maintain strong selectivity against certain solutes is not well understood. For example, although PSAC is permeable to water-soluble organic

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ABBREVIATIONS: RBC, red blood cell; PSAC, plasmodial surface anion channel; $PhTMA^+$, phenyltrimethylammonium ion; PhTMA-Cl, phenyltrimethylammonium chloride; BSA, bovine serum albumin; 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; R^+, residual producing; R^-, nonresidual producing.

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reagents with molecular masses over 600 Da (Cohn et al., 2003) and to various organic cations (Staines et al., 2000), it effectively excludes the small Na^+ ion. Although electrostatic repulsion of Na^+ by pore mouth positive charges on PSAC can account for part of this discrimination (Cohn et al., 2003), the channel almost certainly has other features that contribute to its unusual selectivity profile.

In this study, we identify solute-inhibitor interactions within PSAC that reveal its ability to discriminate between permeant solutes. One explanation for our findings is that PSAC may have two parallel routes for solute transport. Because similar behavior has been reported in other channels that transport divergent solutes (Wadiche and Kavanaugh, 1998; Ryan and Vandenberg, 2005), we propose that PSAC may also use two distinct routes for permeating solutes to achieve its unusual selectivity properties. The presence of more than one route for uptake of nutrient precursors also has important implications for the discovery and development of antimalarial drugs that target PSAC.

Materials and Methods

Osmotic Lysis Experiments. *P. falciparum* parasites (Indo 1 isolate) were cultured by standard methods, harvested at the trophozoite stage, and enriched to 96 to 99% parasitemia by the Percoll-sorbitol method for osmotic lysis experiments as described previously (Wagner et al., 2003). Osmotic lysis experiments were performed at ~0.3% hematocrit in sorbitol, phenyltrimethylammonium chloride (PhTMA-Cl), isoleucine, or alanine solutions with nominal osmolarities of 280 to 290 mOsm. Each of these four solutions was additionally buffered with 20 mM Na-HEPES and 0.1 mg/ml BSA to pH 7.45. Where present, PSAC antagonists were added from concentrated DMSO stocks except for phloridzin, which was directly added to the buffered lysis solutions. Osmotic swelling and lysis resulting from PSAC-mediated solute uptake was tracked by measuring transmittance of 700 nm light through the cell suspension in a DU640 spectrophotometer with a six-cuvette holder (Beckman Coulter, Fullerton, CA). Transmittance in each cuvette was typically sampled every 15 s; manual resuspension of erythrocytes at 7-min intervals was used to prevent cell settling with minimal mechanical shearing of cells. Unless indicated, solutions were prewarmed to 37°C and maintained at this temperature with a Peltier controller throughout lysis measurements. Anomalous mole fraction experiments used defined mixtures of the above buffered sorbitol and PhTMA-Cl solutions supplemented with 50 μM furosemide to expose residual lysis rates. Although lysis in $PhTMA^+$-containing solutions requires the concomitant uptake of Cl^- to maintain electroneutrality, PSAC's markedly greater Cl^- permeability (Kirk et al., 1994) ensures that $PhTMA^+$ uptake is rate-limiting and the primary determinant of lysis kinetics. Transport rates were quantified from lysis time courses based on the inverse relationship between the solute's permeability and the time to hemolysis (Wagner et al., 2003).

In some experiments, infected cells were preloaded with one permeant solute before measuring lysis kinetics in another solute. Preloading was achieved by incubation in one of the above osmotic lysis solutions supplemented with 145 mM NaCl for 30 min at room temperature. In this hypertonic solution, infected RBCs initially shrink and then regain their initial volumes through the uptake of the permeant solute. They are then stable in these solutions, without measurable lysis, for at least 4 h because of the low Na^+ permeability of the infected RBC (Cohn et al., 2003). Control light scattering experiments in these hypertonic solutions revealed that shrinkage and recovery is completed within 30 min (data not shown), as expected from reversibility considerations for PSAC-mediated diffusion. Osmotic lysis was then initiated by replacing the extracellular NaCl with a second permeant solute (290 mOsm). This final lysis solution contains 290 mOsm each of the preloaded solute and of the test solute buffered with 20 mM Na-HEPES and 0.1 mg/ml BSA to pH 7.45. Because the preloaded solute does not have a concentration gradient across the erythrocyte membrane under these conditions, it does not undergo net transport during the lysis time course. This approach permits examination of lysis mediated by one solute with another permeant solute on both sides of the RBC membrane.

Tracer Flux. Infected RBCs were enriched, washed, and used in uptake of [^{14}C]sorbitol, [^{3}H]alanine, [^{14}C]PhTMA, and [^{3}H]isoleucine. Except where indicated, each solute was added at 5 mM concentration (2.5 μCi/ml) to infected or uninfected RBCs at 2% hematocrit in uptake buffer (150 mM NaCl, 20 mM Na-phosphate, and 0.1 mg/ml BSA, pH 7.4). Uptake was performed at 37°C and terminated by transfer of 50 μl of cell suspension to 1 ml of tracer-free uptake buffer with 2 mM furosemide, and centrifuged at 14,000*g* through dibutyl phthalate. This approach produced minimal extracellular trapping, which was not subtracted. Cell pellets were digested and counted as described previously (Desai et al., 1991).

Electrophysiology. Whole-cell voltage-clamp of trophozoite stage-infected RBCs was performed as described previously (Alkhalil et al., 2004). Pipettes were pulled from quartz glass to tip diameters less than 0.5 μm and resistances of 1 to 4 MΩ. High-resistance seals (generally >100 GΩ) were obtained in NaCl containing bath solutions that ensure osmotic stability of the cell. Brief electrical pulses were then used to achieve the whole-cell configuration. Bath solution changes were subsequently performed using a new chamber that achieves complete solution changes without damaging the fragile seal on human RBCs (Lisk and Desai, 2006). All experiments shown used symmetric bath and pipette solutions containing buffer A (5 mM $CaCl_2$, 10 mM $MgCl_2$, and 20 mM Na-HEPES, pH 7.4) supplemented with indicated concentrations of charge carriers. Recordings were filtered at 5 kHz with an eight-pole Bessel filter and digitized at 100 kHz.

Results

Residual Uptake of $PhTMA^+$ and Isoleucine. Human RBCs infected with trophozoite-stage *P. falciparum* parasites undergo osmotic lysis in permeant solutes because of uncompensated solute uptake via PSAC and concomitant water entry through aquaporins. Light scattering measurements through suspensions of infected RBCs can track the kinetics of the lysis process (Wagner et al., 2003). It is noteworthy that they yield estimates of solute permeability coefficients that match those obtained with tracer flux and whole-cell patch-clamp, indicating that these simple light scattering measurements are quantitative.

The light scattering assay has also been quantitatively validated with furosemide and phloridzin, two classic PSAC antagonists that produce concordant dose-responses for inhibition of lysis in sorbitol and for decreases in open probability of single PSAC patch-clamp recordings (Alkhalil et al., 2004; Desai et al., 2005).

Figure 1 shows osmotic lysis experiments with four solutes presumed to enter infected RBCs via PSAC (Ginsburg et al., 1985; Staines et al., 2000) and presents an unexpected finding. As described previously, osmotic lysis in sorbitol proceeded with a half-time ($t_{1/2}$) of ~7 min. In this experiment, it was almost completely abolished by addition of 200 μM furosemide (Fig. 1A, left), consistent with simple Michaelian inhibition and a K_m of 2.7 μM (Alkhalil et al., 2004). Although lysis in isotonic alanine was also abolished by 200 μM furosemide, lysis in two other solutes with similar permeation rates, phenyltrimethylammonium ($PhTMA^+$) and isoleucine, were incompletely inhibited (Fig. 1A). The residual

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lysis in each of these solutes was reproducible and statistically significant (Fig. 1B, $P < 10^{-6}$ for each pairwise comparison to sorbitol and alanine, two-tailed Student's t tests).

The observed residual lysis in either $PhTMA^+$ or isoleucine could be abolished with higher concentrations of furosemide (2 mM, Fig. 1A). It could also be significantly reduced by addition of other PSAC antagonists—5-nitro-2-(3-phenylpropylamino)benzoic acid (Kirk and Horner, 1995), glybenclamide (Kirk et al., 1993), and 2-butyl-5-imino-6-{[5-(4-nitrophenyl)-2-furyl]methylene}-5,6-dihydro-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-7-one (*NPF*-1) (data not shown). Because *NPF*-1 is a new PSAC antagonist with submicromolar affinity and high specificity (Kang et al., 2005), we used it in independent experiments and found that it also permitted lysis in $PhTMA^+$ and isoleucine at a concentration that abolishes sorbitol or alanine uptake. As seen with furosemide, a 10-fold higher *NPF*-1 concentration could largely abolish this residual lysis (Fig. 1C). Hereafter, we refer to $PhTMA^+$ and isoleucine as R^+ solutes (for residual positive) and to sorbitol and alanine as R^- solutes.

We found that there was negligible osmotic lysis of uninfected human RBCs in either R^+ solute, which rules out their uptake via pathways constitutively active in the host RBC membrane (Fig. 1D).

R^+ Phenotype Is Also Seen with Tracer Flux and Electrophysiology. Although osmotic lysis measurements have been quantitatively correlated with other methods (Wagner et al., 2003; Alkhalil et al., 2004), they are limited to isotonic concentrations of permeating solutes and can only indirectly measure transmembrane transport. We therefore examined possible pharmacological differences between transport of R^+ and R^- solutes with tracer flux and electrophysiological methods.

Tracer influx measurements using 5 mM concentrations of each solute reproduced the pattern observed in osmotic lysis experiments: 200 μM furosemide essentially abolished [^{14}C]sorbitol and [^{3}H]alanine uptake, but a higher 2 mM concentration was required to produce similar inhibition of [^{14}C]$PhTMA^+$ uptake (Fig. 2A). Furosemide at 2 mM was also required to abolish the parasite-induced increases in

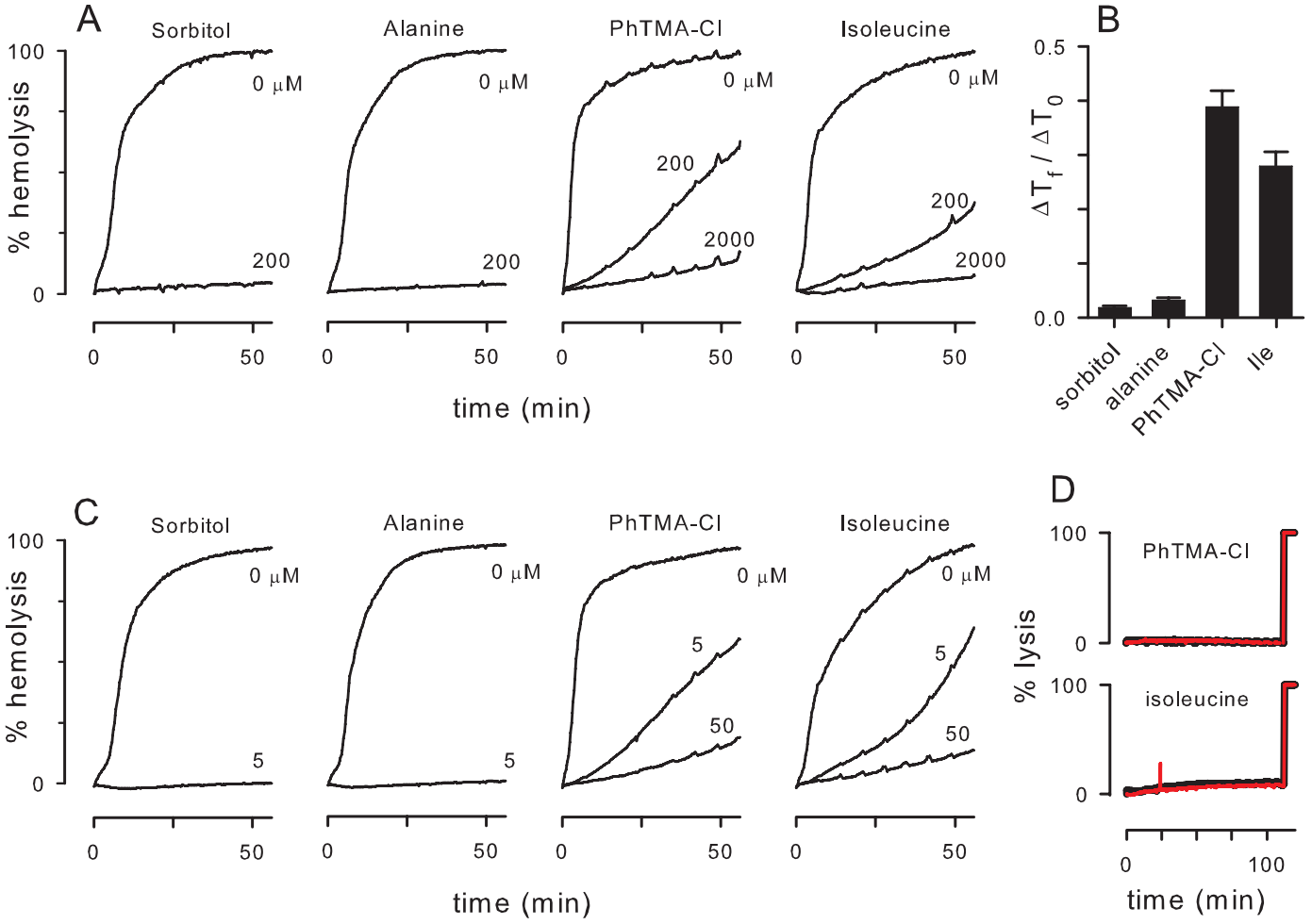

Fig. 1. Osmotic lysis reveals differing patterns of uptake by infected RBCs. A, kinetics of infected erythrocyte lysis in isotonic sorbitol, alanine, PhTMA-Cl, or isoleucine with 0, 200, or 2000 μM furosemide, as indicated. Although lysis in sorbitol or alanine is almost completely abolished by 200 μM furosemide, that in PhTMA-Cl or isoleucine requires higher concentrations. B, comparison of the residual lysis in these solutions, calculated as the mean transmittance change with 200 μM furosemide at 50 min divided by that without furosemide ($\Delta T_f/\Delta T_0$, mean ± S.E.M. for n = 12–28 each solute). C, infected RBC lysis kinetics in each solute with indicated concentrations of *NPF*-1 (in micromolar). Higher concentrations of this specific PSAC antagonist are also required to abolish $PhTMA^+$- and isoleucine-mediated lysis. D, osmotic stability of uninfected human RBCs in PhTMA-Cl or isoleucine, determined with the transmittance assay. In each graphic, black and red traces represent measurements with 0 and 200 μM furosemide, respectively. The abrupt increases at ~110 min correspond to hemolysis upon addition of detergent (1% saponin); this large increase confirms that RBCs were intact and permits normalization to 100% lysis. Neither solution produces significant uninfected RBC lysis, despite the greater duration than in A.

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[^{3}H]isoleucine uptake, yielding a low rate that matched carrier-mediated uptake in uninfected RBCs (Fig. 2B). These tracer uptake experiments indicate that the R^+ phenotype was not an artifact of the high solute concentrations required for osmotic lysis experiments. Instead, they suggest that there are clear differences in how R^+ and R^- solutes are transported across the infected erythrocyte membrane.

We then performed whole-cell voltage-clamp of infected RBCs to further examine the R^+ phenotype. With symmetric Cl^--containing solutions, whole-cell currents in the absence of inhibitors were larger at negative membrane potentials than at positive values, consistent with voltage-dependent gating of PSAC (Desai et al., 2000). Furosemide (200 μM), applied with perfusion conditions that preserve seal quality (Lisk and Desai, 2006), abolished these PSAC-mediated currents when NaCl or choline chloride were the predominant species in the bath and pipette solutions (Fig. 2C). With PhTMA-Cl solutions in both compartments, however, 200 μM furosemide produced only partial inhibition. Because increasing the furosemide concentration to 2 mM could reversibly inhibit the residual currents, these voltage-clamp experiments reproduce the observations made with osmotic lysis and tracer uptake experiments.

Because our osmotic lysis and voltage-clamp measurements required high concentrations of R^+ solutes for adequate signal-to-noise ratios, we worried that these high concentrations might adversely affect PSAC structure and thereby reduce furosemide affinity. We explored this possibility with tracer uptake using 45 μM [^{14}C]PhTMA$^+$, a low concentration estimated from the finite isotope specific activity. Despite a ~3000-fold reduction in $PhTMA^+$ concentration from the 145 mM used in osmotic lysis, these conditions reproduced the central observation of incomplete inhibition of R^+ solute uptake by 200 μM furosemide (Fig. 3). Raising

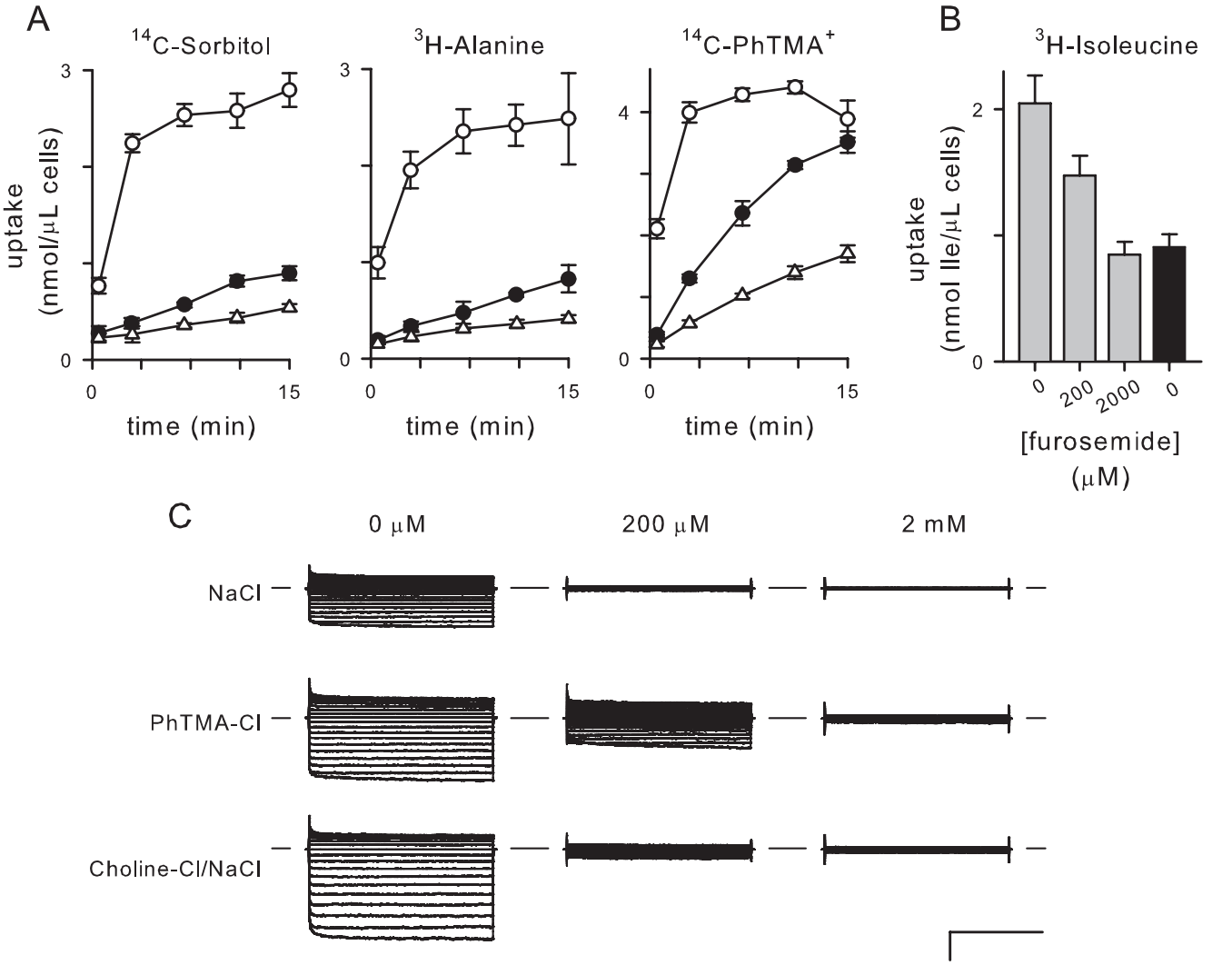

Fig. 2. The R^+ phenotype is also apparent in tracer flux and whole-cell patch-clamp. A, tracer uptake kinetics at 37°C for indicated solutes at 5 mM concentration without (○) or with furosemide (200 μM, ●; 2 mM, △). B, uptake of [^{3}H]isoleucine performed as in A, except that only a single timepoint (3 min) is shown. Gray and black bars represent uptake by infected and uninfected RBCs, respectively. Furosemide concentrations are indicated below each bar. Values in A and B represent mean ± S.E.M. of three to five measurements at each timepoint. C, whole-cell voltage-clamp current responses to voltage steps from a holding potential of 0 mV to values between −100 mV and +100 mV in 10-mV increments. Each row of traces represents measurements on a single cell before or immediately after application of furosemide at concentrations indicated above each column. Bath and pipette solutions contained buffer A plus either 500 mM NaCl, 500 mM PhTMA-Cl, or 1000 mM choline-Cl + 115 mM NaCl (top, middle, and bottom rows, respectively). Dashes to each side of traces represent the zero current levels. Scale bars represent 25 ms (horizontal) and 3 nA (vertical) for all traces. Notice that 200 μM furosemide does not abolish currents in PhTMA-Cl. Furosemide washout completely restored the currents (data not shown), as described previously (Lisk and Desai, 2006).

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the furosemide concentration by 10-fold produced near-complete inhibition, consistent with the pattern observed in both osmotic lysis and whole-cell voltage-clamp measurements. Thus, the reduced efficacy for inhibition of $PhTMA^+$ transport is not a byproduct of immersing channels in a high concentration of PhTMA-Cl. Instead, we propose that the observed differences in inhibitor sensitivity reflect differences in how R^+ and R^- solutes are transported by PSAC.

R^- Solutes Increase the Furosemide Sensitivity of R^+ Solute Transport. We tested our hypothesis with osmotic lysis experiments using infected erythrocytes preloaded with permeant solutes. Figure 4 shows the 12 possible pairwise experiments that involve preloading cells with one solute followed by measurement of osmotic lysis as a result of uptake of another solute. In this figure, each panel represents one of these 12 permutations. Each experiment was carried out to allow direct comparison of lysis rates with and without preloading. The experiments were also performed in the presence and absence of 200 μM furosemide to examine the relative effect on the residual transport component. Because the preloaded solute is maintained at its steady-state distribution throughout the lysis experiment, it does not undergo net transport or directly contribute to cell swelling and lysis (see *Materials and Methods*).

In the absence of furosemide, preloading had at most a modest slowing effect on osmotic lysis in all 12 permutations (Fig. 4, green trace versus black trace), consistent with PSAC's weak saturability in previous single channel and whole-cell patch-clamp experiments [$K_{0.5} > 700$ mM calculated from data in Alkhalil et al. (2004)].

When 200 μM furosemide was included in these experiments, preloading produced a more complex pattern of effects. R^- solute uptake was abolished by furosemide independent of preloading with any other solute (Fig. 4, A–F, red and blue traces). R^+ solutes exhibited more interesting patterns: their uptake could be abolished by 200 μM furosemide if the cells were first preloaded with any R^- solute (Fig. 4, G, H, J, and K, arrows). If the other R^+ solute was used for preloading, lysis rates were unchanged, indicating preserved residual uptake (Fig. 4, I and L).

The differing effects of preloading with R^- and R^+ solutes cannot be explained by competition within the channel pore for two reasons. First, we expected competition to be similar for each of the 12 permutations because of the similar permeabilities of these four solutes (Fig. 1A), rather than strongly dependent on which solute is preloaded. Second, competition as a result of solute preloading should have similar effects on transport kinetics whether furosemide is present or absent. Because competition between solutes cannot produce the complex patterns we observed, these findings instead implicate an allosteric modulatory role of R^- solutes on the transport of R^+ solutes. R^+ solutes do not have comparable effects on R^- solute transport.

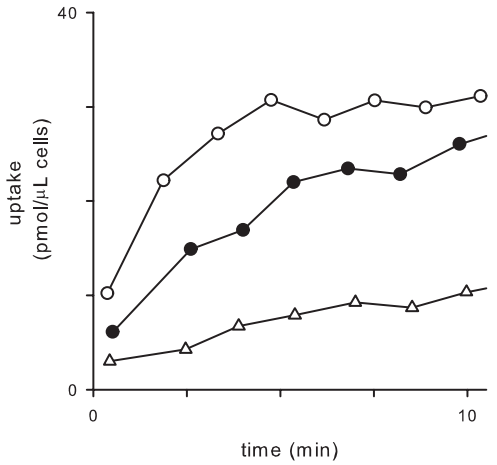

Fig. 3. Reduced furosemide sensitivity is also present at low solute concentrations. Uptake of 45 μM [^{14}C]PhTMA was followed at 37°C without (○) or with furosemide (200 μM, ●; 2 mM, △).

We next examined this effect of R^- solutes with whole-cell voltage-clamp. We determined the mean whole-cell currents in 500 mM PhTMA-Cl after addition of 200 μM furosemide to the bath solution (Fig. 5, ●). When measured with 200 mM alanine added to both bath and pipette solutions, 200 μM furosemide abolished this residual current (△). The reduction in current was statistically significant ($P = 0.005$; Student's t test using currents at −100 mV), consistent with an allosteric modulatory effect of alanine on PhTMA-Cl transport.

Similar Patterns with Other Antagonists. The residual $PhTMA^+$- and isoleucine-mediated lysis phenotype observed with 5 μM *NPF*-1 (Fig. 1C) was also abolished by preloading with either sorbitol or alanine (all permutations tested, data not shown).

Because both furosemide and NPF-1 inhibit by binding to the extracellular face of PSAC at sites functionally distinct from the channel pore (Desai et al., 2005; Kang et al., 2005), we wondered whether the observed patterns are unique to inhibitors acting within a defined extracellular domain of the channel. We tested this possibility with phloridzin, a lower affinity antagonist whose site of action is on the intracellular channel face (Desai et al., 2005). As seen with furosemide and *NPF*-1, phloridzin was less effective at inhibiting R^+ solute uptake; the residual lysis components here were also eliminated by sorbitol preloading (Fig. 6).

Possible Models for Transport of Various Solutes. Our findings implicate distinct mechanisms of transport for R^+ and R^- solutes through PSAC. Two of the simplest models that can account for both the differing sensitivity to antagonists and the modulatory effects of permeating solutes are illustrated in Fig. 7. In one model, the channel has a single multioccupancy pore (Fig. 7A). When one or more critical sites in the pore are occupied by an R^- solute, distant allosteric effects are proposed to improve furosemide, *NPF*-1, and phloridzin affinity at their separate binding sites. Because these binding sites are on opposite channel faces (Desai et al., 2005), these allosteric effects would presumably involve global conformational changes in PSAC upon sorbitol or alanine binding.

In the second model, there are two parallel routes that solutes can take through a single transport complex (Fig. 7B). R^+ solutes can traverse the membrane through either route. In contrast, R^- solutes are permitted to use only one of the routes, drawn in Fig. 7B as a continuous aqueous pore spanning the membrane (i.e., a channel-type route). In addition, when an R^- solute is bound in the channel pore, it exerts an inhibitory effect on the alternate route available only to R^+ solutes. (Although the alternate route is drawn in Fig. 7B as a carrier, it could exhibit either channel or carrier-type behavior.) Both routes are inhibited by furosemide,

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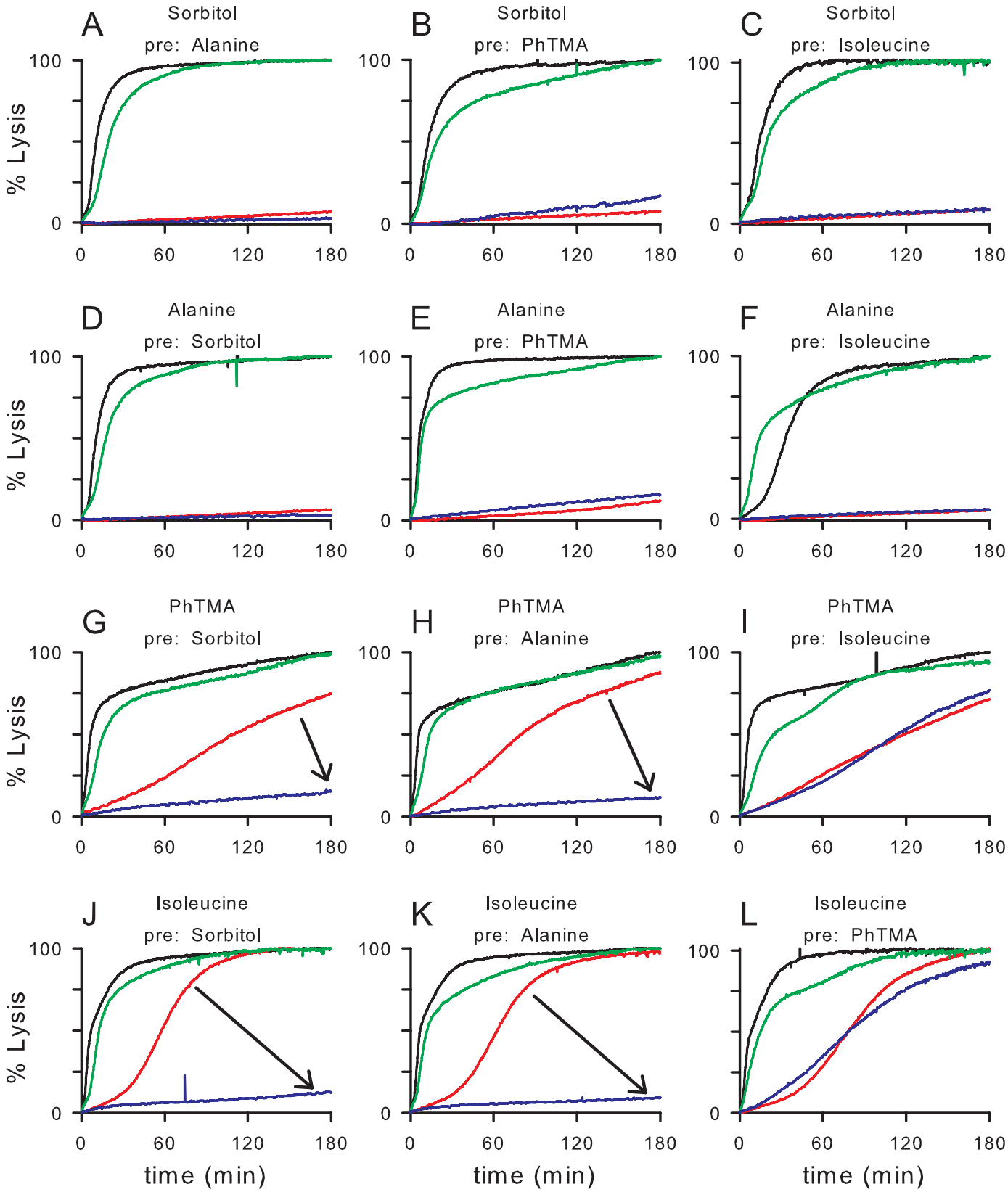

Fig. 4. Residual lysis phenotype is abolished by preloading R^- solutes. Each panel shows kinetics of osmotic lysis mediated by one solute without (black and red) and with (blue and green) preloading another solute. Headings for each panel indicate the solute that produced lysis followed by the preload solute (pre:). To ensure that preloading does not have other adverse effects, infected RBCs that were not preloaded underwent sham incubations in PBS. Lysis was followed without (black and green traces) or with 200 μM furosemide (red and blue traces) for 180 min to increase detection of slow lysis. The modulatory effect of R^- solute preloading on R^+ solute transport is indicated with arrows in G, H, J, and K; there was markedly less effect of preloading in all other permutations.

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NPF-1, and phloridzin, but with ~10-fold higher concentrations required to inhibit the alternate route.

These two models share modulation of R^+ solute transport by R^- solutes bound to a site such as a selectivity filter within the channel pore. Although a separate site for R^- solute binding on one or the other exposed face of the channel could produce similar effects in either of the two possible models, it would need a number of features already present in the pore; parsimony favors modulation through binding of R^- solutes within the pore.

Can the two models be distinguished experimentally? One observation commonly used to support multioccupancy pores is anomalous mole fraction behavior, a test based on measuring transport rates with defined mixtures of two permeant solutes. When the behavior is present, it typically produces lower-than-expected flux in solute mixtures because transport of individual solute molecules depends on which solutes are in adjacent sites within the pore (Hille, 2001). In contrast, the model with two parallel routes (Fig. 7B) should not exhibit anomalous mole fraction behavior because each pathway need only accommodate one solute at a time to explain our findings. To perform this test, we used defined mixtures of the PhTMA-Cl and sorbitol solutions and measured lysis rates in the presence of 50 μM furosemide, which produces slow lysis in sorbitol but robust residual lysis in PhTMA-Cl. Figure 8 shows that PSAC does not exhibit anomalous mole fraction behavior under these conditions. Because multioccupancy channels are not required to exhibit anomalous mole fraction behavior, this result does not definitively exclude either of the two proposed models.

We devised another test of the two models based on possible differences in temperature dependence. Solutes in a single shared route encounter similar energy barriers at each step in their transport. Thus, the multioccupancy pore model, shown in Fig. 7A, predicts similar temperature dependences for transport of R^- and R^+ solutes. In contrast, solutes moving through separate but parallel pathways may well encoun-

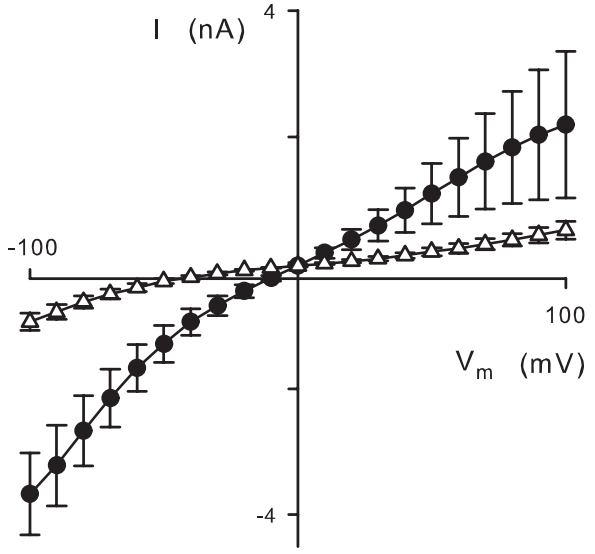

Fig. 5. Alanine reduces the residual whole-cell currents in PhTMA-Cl. Current-voltage profiles showing whole-cell currents with symmetric bath and pipette solutions of buffer A plus 500 mM PhTMA-Cl and 200 μM furosemide (●). △, experiments in which 200 mM alanine was additionally present in both compartments. Symbols represent the mean ± S.E.M. of currents at each voltage (n = 3 or 4 cells each). To avoid osmotic lysis in these permeant solutes, the whole-cell patch-clamp configuration was achieved in a bath solution containing buffer A plus 500 mM NaCl before extracellular perfusion of permeant solutes. All currents could be reversibly abolished by perfusion of 2 mM furosemide (not shown).

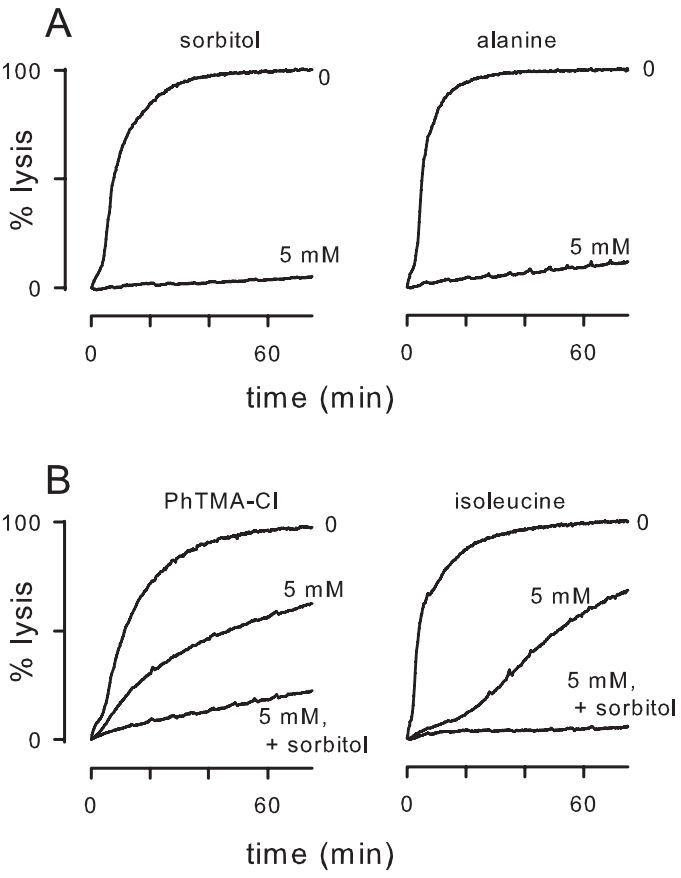

Fig. 6. Phloridzin also produces residual osmotic lysis that can be abolished by sorbitol preloading. A, lysis time courses in indicated R^- solutes with 0 or 5 mM phloridzin (upper and lower traces). B, R^+ solute-mediated lysis kinetics without inhibitor (top traces) or with 5 mM phloridzin (middle and bottom traces). Bottom traces reflect the time course after preloading with 290 mM sorbitol.

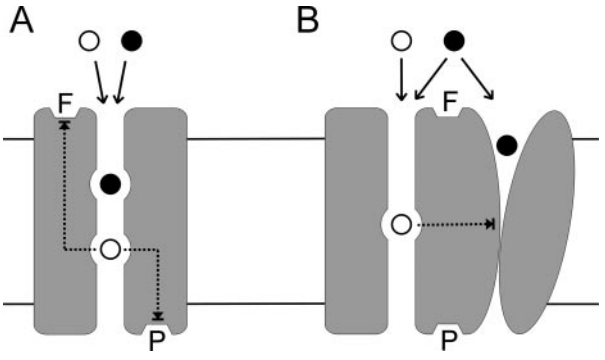

Fig. 7. Two possible models for the observed solute-inhibitor interactions. A, a multioccupancy channel. Here, binding of R^- solute (○) within the pore produces distant effects on furosemide (F) and phloridzin (P) affinity, whose binding sites are on opposite sides of the membrane. If R^- solutes are absent, higher concentrations of these inhibitors are required to inhibit transport of R^+ solutes (●). B, two parallel routes model. In this model, R^+ solutes can permeate both routes, shown here as a channel (left) and a carrier (right). In contrast, R^- solutes can only pass through the channel. When an R^- solute binds in the channel, the other route is inhibited. Although the other route is shown here as a carrier with alternating access to the two membrane faces, similar behavior can be achieved with two parallel channel-type routes. In both models, the modulatory effects of R^- solutes are shown with dashed lines.

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ter different energy barriers because of differences in protein conformational changes required for each transport cycle. The model in Fig. 7B therefore permits marked differences in the temperature dependence of R^- and R^+ solute transport. We therefore evaluated the temperature dependences of sorbitol and $PhTMA^+$ transport using osmotic lysis rates. In the absence of furosemide, both solutes exhibited weakly temperature dependent uptake over the range of 13.5 to 44.5°C with Q_{10} values of 1.7 ± 0.2 (data not shown), near predictions for aqueous phase diffusion (Kholodenko and Douglas, 1995). In contrast, the residual component of $PhTMA^+$ uptake, measured in the presence of 100 μM furosemide, was steeply temperature dependent (Q_{10} = 3.5 ± 0.2) with measurements at or below room temperature producing negligible residual lysis (Fig. 9, A and B). Furosemide (3 μM) added to sorbitol uptake experiments had a significantly smaller effect (Q_{10} = 2.1 ± 0.2; Fig. 9B), excluding a nonspecific effect of this inhibitor. Uptake of both [^{14}C]$PhTMA^+$ and [^{3}H]isoleucine in the presence of furosemide were also steeply temperature dependent (data not shown), confirming a direct effect of temperature on the residual transport of R^+ solutes. Because a single shared route for $PhTMA^+$ and sorbitol transport is difficult to reconcile with such marked differences in temperature dependence, these results suggest a model with two parallel routes through PSAC (Fig. 7B).

We explored the temperature dependence further with dose responses for furosemide inhibition. At both 37°C and 13.5°C, inhibition of sorbitol transport was adequately fitted by the Langmuir isotherm (Fig. 9C, solid lines), suggesting a single transport component inhibited by furosemide with a 1:1 stoichiometry. At 37°C, inhibition of $PhTMA^+$ uptake was not consistent with a single component because of a poor fit by the Langmuir isotherm. It was, however, satisfactorily fitted by an equation that describes two components with differing $K_{0.5}$ values (dotted line; see legend for equation), further supporting the proposed model of two parallel routes through PSAC. At 13.5°C, the furosemide dose response for inhibition of $PhTMA^+$ uptake was better approximated by the Langmuir isotherm, consistent with a steep temperature dependence for the residual component. It also more closely resembled the dose response determined with osmotic lysis in sorbitol.

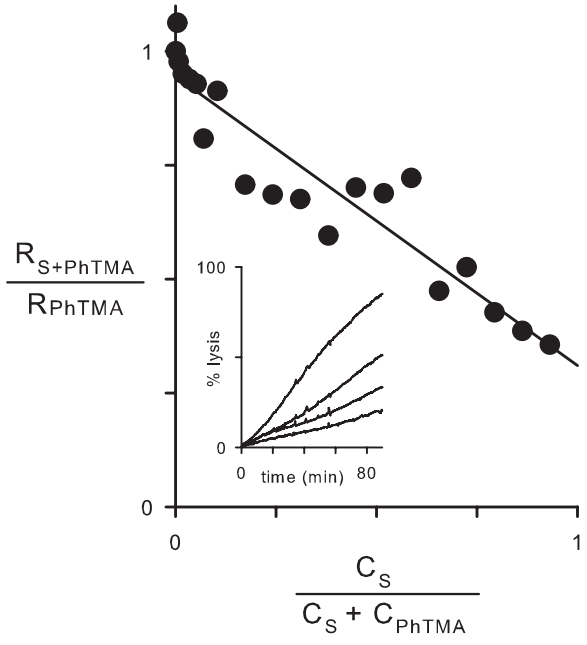

Fig. 8. Absence of anomalous mole fraction behavior. Symbols represent the rate of residual lysis in fractional mixtures of the sorbitol and PhTMA-Cl solutions with 50 μM furosemide. Each rate was normalized to the rate in the same solution without furosemide and then adjusted to 1.0 for the residual lysis in $PhTMA^+$ alone ($R_{S+PhTMA}/R_{PhTMA}$). The abscissa represents the mole fraction of sorbitol in each solution using standard notation (Hille, 2001). Solid line represents the least-squares best fit to a straight line, indicating the absence of anomalous mole fraction behavior. Inset shows lysis time courses in $C_S/(C_S + C_{PhTMA})$ = 0.0, 0.31, 0.52, and 1.0 (top to bottom traces, respectively) in the presence of 50 μM furosemide.

Discussion

We report quantitative differences in inhibitor affinity and temperature dependence for uptake of four organic solutes by *P. falciparum*-infected RBCs. These differences were unexpected because multiple previous studies suggest these solutes share a common transport mechanism (Kirk et al., 1994; Desai et al., 2000; Staines et al., 2000). Possible explanations for these differences include 1) uptake via separate ion channels, 2) a multioccupancy ion channel that permits simultaneous binding by two or more permeating solutes, and 3) two parallel routes through a single ion channel complex.

Although not observed in our electrophysiological studies (Alkhalil et al., 2004), some reports suggest that there may be more than one ion channel induced by the malaria parasite (Huber et al., 2002; Staines et al., 2003; Bouyer et al., 2006). Could the differences in uptake of R^+ and R^- solutes be explained by two separate ion channels, only one of which is PSAC? To account for our findings, these two channels would both require inhibition by the various inhibitors used here, but with differing affinities. Because *NPF*-1 does not inhibit other ion channels (Kang et al., 2005), activity against two unrelated ion channels induced by the parasite would be a remarkable coincidence. Both putative channels would also require permeability to $PhTMA^+$ and isoleucine, whereas only one could pass sorbitol and alanine. The more selective channel would then also need to be inhibited by both sorbitol and alanine to account for the modulatory effects of R^- solutes on R^+ solute transport (Figs. 4 and 5); it would also require a steep temperature dependence. These various constraints are quite complicated; two separate parasite-induced channels cannot easily account for the differences in uptake of R^- and R^+ solutes.

A single multioccupancy pore also has difficulty accounting for our observations. Most importantly, differences in temperature dependence are hard to reconcile with movement of these solutes through a single shared route. Because this channel's voltage dependence results from gating of the aqueous pore, the weaker rectification of PhTMA-Cl currents in the presence of furosemide also suggests transport via a route not subject to the same gating (Figs. 2C and 5).

Although more complicated models for PSAC may also explain our findings, we consider two separate routes through a single ion channel complex to be the most conservative model. This model has precedence in some other ion channels, where supportive evidence has come from both functional and crystallographic studies (Sonders and Amara, 1996; Yool and Weinstein, 2002; Vandenberg and Ryan, 2005). If PSAC does have two parallel routes for solutes, one

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must exhibit channel-type kinetics, given previous single channel measurements (Alkhalil et al., 2004; Desai, 2005; Desai et al., 2005; Kang et al., 2005). The other route has a steep temperature dependence and a relatively slower transport rate; it has also not been detected at the single channel level in our cell-attached recordings. Each of these observations inconclusively favors a carrier-type mechanism. Thus, the general architecture of PSAC may be similar to that of other transport proteins exhibiting both channel and carrier-type behaviors (Wadiche et al., 1995; Cammack and Schwartz, 1996; Quick et al., 2001; Carvelli et al., 2004).

Why might PSAC have two parallel but functionally distinct routes for transport? Although methods to address this question are not currently available, having two parallel routes may help PSAC achieve its broad and unusual selectivity. PSAC must permit rapid flux of anions, sugars,

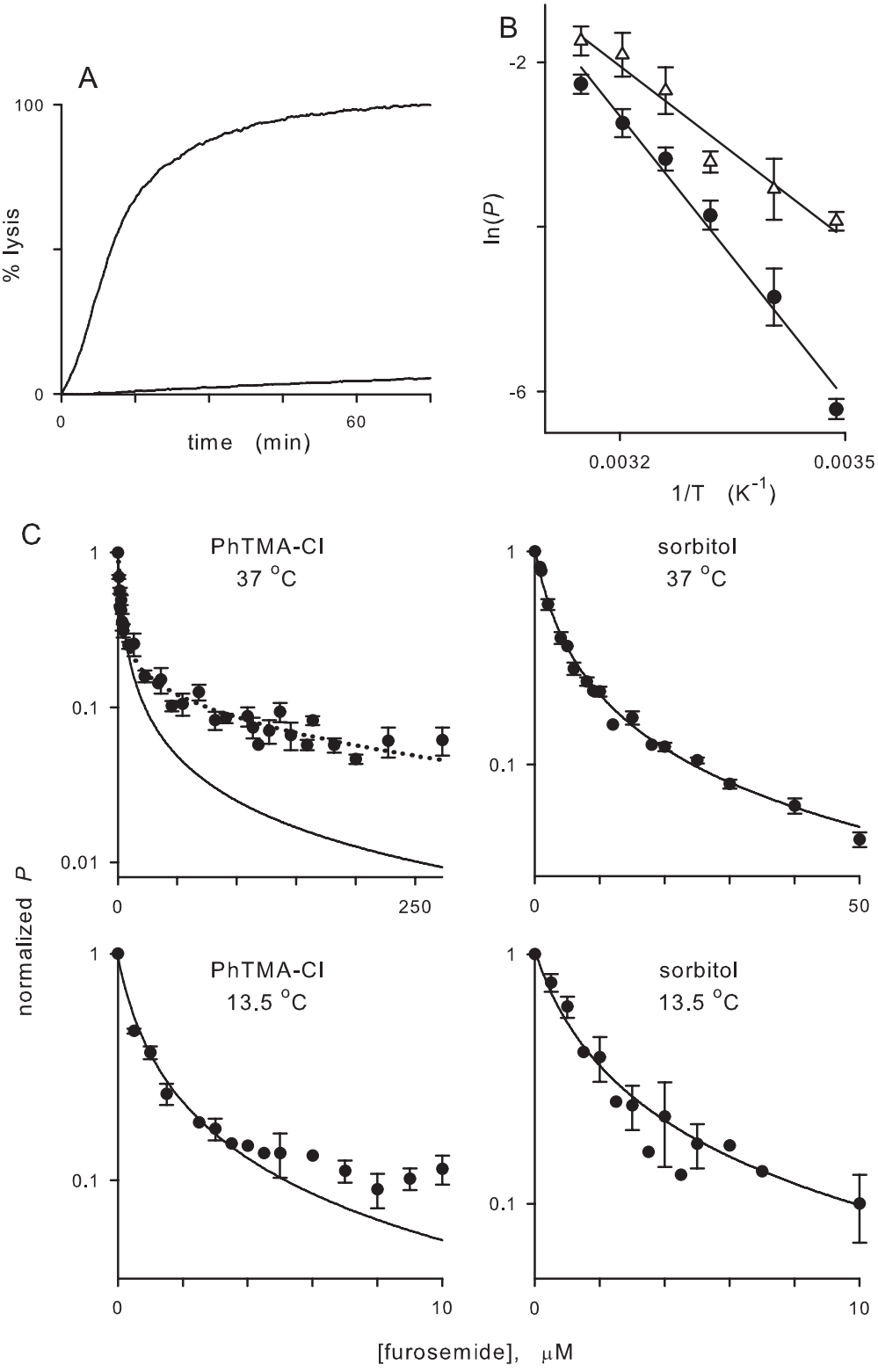

Fig. 9. Temperature dependence. A, lysis time course at 20.5°C in PhTMA-Cl with 0 or 100 μM furosemide (upper and lower traces, respectively). Residual lysis in isoleucine was also abolished at this temperature (not shown). B, Arrhenius plot of apparent permeability coefficients (P) calculated as the reciprocal of the time to 20% of complete lysis in sorbitol with 3 μM furosemide (△) or PhTMA-Cl with 100 μM furosemide (●). Symbols represent the mean ± S.E.M. of up to seven experiments each. C, furosemide dose responses for inhibition of lysis in PhTMA-Cl or sorbitol at indicated temperatures. At each furosemide concentration, mean ± S.E.M. permeability coefficients were determined as described under *Materials and Methods* from up to eight independent experiments and normalized to 1.0 without furosemide. Ordinates are shown on a logarithmic scale to emphasize transport remaining at high furosemide concentrations. The solid lines represents the best fit to $y = K_m/(K_m + x)$. Notice that this equation does not produce an acceptable fit of the dose response in PhTMA-Cl at 37°C; in that panel, the dotted line represents the best fit to two components: $y = (a \times K_1/(K_1 + x)) + (1 - a) \times K_2/(K_2 + x)$.

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amino acids, purines, some organic cations, and some vitamins, many of which are required for intraerythrocytic parasite growth. Despite this list of diverse permeant solutes, the channel effectively excludes Na^+ ions by some 100,000-fold relative to Cl^- (Cohn et al., 2003). This combination of selectivity properties may be less difficult to achieve with two separate routes in a single PSAC complex. Studies are under way to determine whether both routes function under physiological conditions and to explore whether they are accessible to other solutes with increased permeability after infection.

PSAC antagonists should interfere with nutrient acquisition by the intracellular parasite (Desai et al., 2000) and are therefore being actively pursued in antimalarial drug discovery programs. The solute-inhibitor interactions identified here will need to be considered when selecting antagonists for advancement in these programs. We predict that antagonists that effectively inhibit both routes through this channel should be more potent antimalarial agents than currently available PSAC antagonists.

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