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Improved Perfusion Conditions for Patch-clamp Recordings on Human Erythrocytes

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Abstract

Various configurations of the patch-clamp method are powerful tools for examining the transport of charged solutes across biological membranes. Originally developed for the study of relatively large cells which adhere to solid surfaces under *in vitro* culture, these methods have been increasingly applied to small cells or organelles in suspension. Under these conditions, a number of significant technical problems may arise as a result of the smaller geometry. Here, we examined these problems using human erythrocytes infected with the malaria parasite, *P. falciparum*, a system where experimental differences and the technical difficulty of erythrocyte patch-clamp have hindered universal agreement on the properties of the induced ion channels. We found that patch-clamp recordings on infected erythrocytes are especially susceptible to artifacts from mechanical perturbations due to solution flow around the cell. To minimize these artifacts, we designed a new perfusion chamber whose geometry allows controlled solution flow around the fragile erythrocyte. Not only were recordings acquired in this chamber significantly less susceptible to perfusion artifacts, but the chamber permitted rapid and reversible application of known inhibitors with negligible mechanical agitation. Electrophysiological recordings then faithfully reproduced several findings made with more traditional methods. The new perfusion chamber should also be useful for patch-clamp recordings on blood cells, protoplasts, and organelles.

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Abbreviations used in this paper:

PSAC	plasmodial surface anion channel
NPPB	5-nitro-2-(3-phenylpropylamino)-benzoate
PVM	parasitophorous vacuolar membrane
V_m	membrane potential
γ	chord conductance
R_s	seal resistance
R_a	access resistance

INTRODUCTION

The extracellular patch-clamp method, originally developed to detect flux through single ion channel molecules [1], revolutionized the study of ion movement across biological membranes. With refinement to permit measurements in various single channel and single cell configurations [2], these methods have permitted the mechanistic dissection of the conformational changes required for channel opening and closing (known as “gating”), how channels distinguish permeant from non-permeant ions (known as “selectivity”), the step-by-step path taken by solutes as they navigate the channel pore, and the detailed mechanism of action of physiological modulators and pharmacological agents.

Nevertheless, patch-clamp methods have two important limitations. First, successful recordings require both the direct physical accessibility of the relevant membrane surface and the ability to form a poorly understood high resistance seal. Based on calculations using the bathing solution resistivity, formation of these seals almost certainly involves genuine chemical reactions between the pipette glass and the cell's lipid bilayer. Second, although they can produce quantitative and temporal resolution unmatched in the biological sciences, these methods are also prone to errors such as spurious currents unrelated to membrane transport [3,4], electrical and mechanical noise [5], and altered behavior of ion channels due to clamping [6-8].

While more commonly used to study relatively large cells (> 20 μm diameter) which adhere to solid surfaces under *in vitro* culture, they have also been used to study small cells in suspension [9-11]. The first published application of patch-clamp to the study of *P. falciparum*, the causative agent of the most severe form of human malaria, identified large conductance ion channels on the parasitophorous vacuolar membrane (PVM) around intraerythrocytic trophozoites (cell diameter 3-5 μm) [12]. This PVM channel, which may be involved in nutrient and/or macromolecule trafficking between the parasite and RBC cytosol [13], has continued to escape quantitative study with more traditional transport assays because of its intracellular location and close proximity to the underlying parasite plasma membrane.

Subsequent patch-clamp of the host erythrocyte membrane identified the plasmodial surface anion channel (PSAC), a small conductance channel that prefers anions over cations [14]. The timing of PSAC appearance on the erythrocyte membrane, its selectivity sequence for anions, and block by specific and non-specific inhibitors suggest it may account for the broad parasite-induced increases in permeability studied for decades with isotope uptake and osmotic fragility [15-17]. Over the past 4 years, patch-clamp has been undertaken in a number of laboratories [18-22]. Unfortunately, the findings in the studies from these 5 groups have produced inconsistent results, with each group proposing one or more new ion channels on the host membrane.

One explanation for these discrepant findings relates to the technical difficulty of patch-clamping the small, deformable human erythrocyte. This difficulty has led to subtle, but important experimental differences in how each group obtains and analyzes recordings. One important difference is the use of bulk solution perfusion around the clamped cell. While changing the bathing solution can provide important biological information, we have heretofore resisted continuous perfusion because our preliminary observations suggested it could damage the fragile seal on the infected erythrocyte [22].

Here, we examined the effects of solution perfusion in detail and found that standard perfusion produces marked adverse effects on seal quality. Visual examination of patch-clamped cells suggests that seal deterioration results from mechanical agitation of the small and deformable erythrocyte at the pipette tip. To explore if these adverse effects could be circumvented, we designed and fabricated a perfusion chamber that minimizes solution turbulence. Experiments

with this chamber indicate that rapid extracellular solution changes can be reproducibly achieved without either significant agitation of the erythrocyte or reductions in seal integrity. This perfusion chamber represents an important technical advance in patch-clamp of not only human RBCs, but also for other small, fragile cells or organelles in suspension because it permits rapid extracellular solution changes without seal damage.

MATERIALS AND METHODS

Osmotic lysis assays

Sorbitol-mediated osmotic lysis of *P.falciparum*-infected human RBCs was performed as previously described [23]. In brief, trophozoite-infected RBCs, grown by standard culture methods, were harvested and enriched to 95-99% by percoll/sorbitol separation [24], washed, and resuspended in PBS (150 mM NaCl, 10 mM Na-phosphate, 0.1 mg/ml BSA, pH 7.4). Osmotic lysis was initiated by suspending these cells at 0.25% hematocrit in 280 mM sorbitol, 20 mM Na-HEPES, 0.1 mg/ml BSA, pH 7.5 at 37 °C. PSAC antagonists were added as described in the figure legend. The kinetics of osmotic lysis was then followed by continuously tracking the transmittance of 700 nm light, %T, through the suspension (DU640 spectrophotometer, Beckman Coulter, Fullerton, CA).

These measurements are based on detecting the decreasing turbidity of the suspension as infected cells undergo osmotic lysis.

Electrophysiology

Cell-attached recordings on trophozoite-stage infected RBCs were obtained as previously described [14] in symmetric bath and pipette solutions of (in mM): 1000 choline-Cl, 115 NaCl, 10 MgCl₂, 5 CaCl₂, 20 Na-HEPES, pH 7.4 (solution A). This hypertonic solution increases the signal-to-noise ratio for single PSAC detection by permitting higher rates of Cl⁻ flux through open channels and by reducing pipette RC noise. Our previous studies indicate that this solution does not alter PSAC's voltage-dependent gating, selectivity, or inhibition by several classes of inhibitors [22,25-27]. Where used, the whole-cell patch-clamp configuration was obtained with brief electrical pulses (500-800 mV) to disrupt the membrane patch. These experiments used symmetrical bath and pipette solutions with osmolarities that ranged from isotonic to that of solution A, as indicated in the figure legends. In many whole-cell patch-clamp experiments, we observed a gradual decrease (or "run-down") in whole-cell currents without a change in voltage-dependence or inhibitor sensitivity. Where the effects of known inhibitors or putative modulators were evaluated quantitatively, we used recordings only after currents reached a stable plateau to minimize errors.

Recordings in both configurations utilized quartz pipettes pulled to tip diameters < 0.5 µm and resistances of 1-3 MΩ in solution A. Our experience indicates that raising the cell from the bottom of the dish facilitates formation of high resistance seals. It has the added advantage of reducing capacitive noise in both cell-attached and whole-cell recordings. All recordings were lowpass filtered at 5 KHz (8-pole Bessel) and digitized at 100 KHz; some whole-cell traces were also digitally filtered in Clampfit 9.0 (1 to 2.5 KHz, 8-pole Bessel). Measurements of single-channel amplitudes and whole-cell chord conductances (γ, calculated between -100 and 0 mV) were carried out with home-written code.

The new perfusion chamber described here was machined from a single block of Delrin, a chemically resistant acetal polymer. A 48 x 65 mm coverglass was glued to the underside of the chamber, preventing contamination of solutions between troughs while permitting uncompromised optical imaging in inverted microscopy. Perfusion of each trough in the

chamber was under independent control and was carried out with small bore tubing and a multi-channel variable speed peristaltic pump.

RESULTS

We began by evaluating the effect of bulk solution perfusion on recording quality in the cell-attached patch-clamp configuration. This configuration permits the precise tracking of seal quality by providing a channel-independent baseline current; it also permits the most sensitive detection of seal deterioration events. Figure 1A shows measurements from a typical seal on an infected human RBC membrane. As the rate of bulk solution perfusion was increased, transient current deflections from the baseline increased in frequency, duration, and absolute magnitude. Because these deflections were not of reproducible magnitude, they were easily distinguished from currents due to ion channel opening events. Transitions between open and closed currents for single channels have reproducible amplitudes because ion flux is highly regulated and is analogous to the defined rates of other proteinaceous enzymes.

Irreproducible current deflections are generally referred to as “seal breaks”, reflecting the dynamic nature of the interaction between the patch-clamp pipette and the cell surface. Of note, the seal breaks induced by perfusion were often much larger than single channel currents through PSAC, the predominant ion channel activity seen on infected RBCs (Fig. 1B). We often found that seal breaks are voltage-dependent, contrary to the assumption of ohmic flux through defects in the pipette-cell surface interface. This voltage-dependent leak, combined with its transitory nature, suggests that caution should be used in interpreting patch-clamp data acquired with bulk solution perfusion under standard conditions. Other experimental variables, such as pipette geometry and bath solution depth, may accentuate the adverse effects of perfusion in ways that are difficult to predict without systematic evaluation.

Perfusion of the bath around the clamped cell presumably degrades seal quality through mechanical stress to the seal on the fragile cell. We evaluated this by imaging infected cells under our patch-clamp conditions (supplementary movie file, Fig. S1). With perfusion, unambiguous and severe agitation of the cell was apparent, consistent with mechanical stress on the seal. This degree of agitation likely results from the small, deformable nature of the RBC and the small area of contact with the pipette tip. With standard positioning of perfusion inlet and outlet ports, we found that visible mechanical agitation of the clamped cell increased dramatically with perfusion rates greater than 100 $\mu\text{l}/\text{min}$, correlating with rates that produced significant numbers of seal break events in our cell-attached recordings (Fig. 1A). Thus, the patch-clamp seal on a human erythrocyte does not tolerate perfusion conditions generally considered safe for larger, adherent cells.

Perfusion under conditions that preserve seal quality would permit quantitative evaluation of solution composition effects, more sophisticated studies of inhibitors, and increased productivity. We therefore designed, machined and tested the perfusion chamber in Figure 2A. Its main design feature is a single large open area connected to six separate long troughs. We envisioned that patch-clamp seal formation could be achieved in the open area and that the pipette with attached erythrocyte could then be moved sequentially into each of the six troughs. The composition of the bathing solution in each trough would be under independent control because of perfusion inlets at the end of the trough.

Control experiments with the chamber verified the significant merit of the long narrow trough geometry. This geometry required almost undetectable rates of perfusion at the inlet to prevent contamination by solutions of differing composition in other troughs. We achieved slow and precisely quantified perfusion rates with a multi-channel peristaltic pump and small bore compressible tubing. We found that these slow perfusion rates, when combined with our

chamber design that minimizes solution turbulence, produced negligible mechanical agitation of single patch-clamped erythrocytes (supplementary movie file, Fig. S2).

We then evaluated the effect of this slow perfusion on high-resistance cell-attached recordings. Figure 2B shows recordings from a patch containing two functional PSAC molecules. The first trace, acquired while perfusing with an inhibitor-free solution (top trace), revealed a stable baseline without seal break events. This cell was then sequentially moved into troughs containing 1 and 3 μM NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate), a known PSAC antagonist [28] (middle and bottom traces, respectively). We found that NPPB produced unambiguous decreases in channel currents by progressively reducing opening events as its concentration was raised. Inhibition in the cell-attached configuration requires NPPB entry into the infected cell and diffusion to its site of action on PSAC molecules in the patch; its high calculated logP of 5.1 suggests this entry could occur either via partitioning in the lipid bilayer or via channels or carriers. Detailed analysis with single channel recordings will be needed to explore NPPB's mechanism of action as previously examined with furosemide [29] and phloridzin [25].

Sorbitol uptake via PSAC is abruptly inhibited upon addition of NPPB or furosemide (Fig. 3A). Indeed, rapid inhibition kinetics of small molecule inhibitors are generally expected because of low activation energy barriers for interaction with most inhibitor binding pockets on ion channels. In whole-cell patch-clamp experiments, we found inhibition occurred almost instantaneously when the cell was moved into a trough with a saturating concentration of furosemide (Fig. 3B), in good agreement with the osmotic lysis measurements. Moreover, this cell could be repeatedly moved between the inhibitor-free and high [furosemide] troughs with excellent reproducibility of single cell currents (Fig. 3C), confirming rapid reversibility of PSAC-furosemide interactions.

We worried that our slow perfusion rates may produce delayed penetration of unstirred layers around the infected RBC-patch pipette complex. We explored this theoretical concern with washout of a very high furosemide concentration in whole-cell experiments. By recording responses to short 50 ms voltage pulses applied in rapid succession immediately after moving the cell into an inhibitor-free trough, we found that washout is not instantaneous, but that it occurs rapidly with single exponential kinetics and a time constant of less than 30 sec (Fig. 3D).

The quantitative reproducibility of whole-cell currents is a good marker of whether perfusion and other stressors have adversely affected seal quality. We therefore evaluated reproducibility in multiple whole-cell experiments that used bath and pipette solutions ranging from physiological osmolarities to hypertonic solutions required for detection of single ion channel currents. In each solution, we applied one or more known PSAC antagonists in concentrations that produce incomplete inhibition. We moved each cell between multiple troughs, recorded whole-cell currents in each condition, and evaluated the reproducibility of each measurement by comparing chord conductances in sequential applications of the same solution (Fig. 4). The clustering of these measurements along the diagonal reflects the high level of reproducibility in these measurements.

We recognized an important additional advantage of our perfusion system is that it increases productivity by allowing multiple solution changes to be applied to a single cell. To date, up to 65 solution changes have been successfully applied to a single cell without deterioration of seal quality. Such an approach also provided a stable inhibitor-free baseline that can be used as an internal control for a given manipulation. Although we found that marked changes in bathing solution composition are well tolerated, we continue to match the bath solution's total osmolarity to that of the pipette solution because significant water gradients across the RBC

membrane may damage the patch-clamp seal. Future experiments with this perfusion chamber may determine if this constraint is necessary.

DISCUSSION

The increased permeability of RBCs infected with plasmodia has been known for decades and has classically been studied with tracer flux and osmotic lysis in solutions of permeant solutes. Because these methods require measurements on populations of cells, they can provide only limited insights into the molecular mechanism(s) of transport. To overcome this limitation, various groups have undertaken single cell, and in some cases, single molecule patch-clamp studies. The universal finding of increased conductive permeabilities in all of these studies has succeeded in excluding a number of mechanisms considered possible in previous macroscopic studies.

While these various patch-clamp reports agree that one or more ion channels can account for the long known increased permeability, fundamental debates remain because there is little agreement on either the electrophysiological properties of the putative channels or on their genetic origin. The present study contributes to this debate by identifying and characterizing important variables that influence the reliability and reproducibility of patch-clamp measurements on human RBCs. Our observations indicate that patch-clamp of human RBCs is complicated by their small size and their fragility. We found that with attention to the variables itemized below, it is possible to obtain single channel and whole-cell recordings that are highly concordant with osmotic lysis and/or tracer flux measurements. Achieving such a concordance should be considered a critical test of patch-clamp studies because patch-clamp, osmotic lysis, and tracer flux methods aim to measure the same parasite-induced transport mechanisms on infected erythrocytes.

There are several important technical insights we gained from the present studies. First, perfusion of the bath as routinely accepted for larger cells is not tolerated by the seal on the fragile erythrocyte. The turbulence associated with perfusion invariably produces a reduced seal quality, presumably through mechanical stress at the annulus of contact between the pipette tip and the cell surface. For some non-adherent cells, it may be possible to reduce perfusion-associated stress by chemical treatments that promote adherence to the patch-clamp dish (e.g. via pretreatment of the dish with polylysine). Such treatments may alter the properties of ion channels under study [30], complicating the interpretation of recordings. For human erythrocytes, our experience indicates that polylysine also lead to sub-optimal patch-clamp seals, possibly via transmission of mechanical vibrations from the dish during the critical seal formation process. Such vibrations are only partially reduced through the use of a vibration isolation table. Finally, this approach also has the disadvantage that the patch pipette and clamped erythrocyte can no longer be raised to reduce capacitive noise resulting from excessive submersion of the pipette in the bath solution.

Another important observation we made is that the seal resistance on small geometry cells is often reduced in the process of obtaining the whole-cell configuration with either suction or high voltage electrical pulses. We could minimize this effect by carefully zeroing the hydrostatic pressure at the pipette tip; this pressure results from the balance of the bath solution height, the height of the solution in the pipette, and capillary action within the pipette. It is additionally important to verify that the accessory port on the pipette holder is vented to ambient pressure to avoid transmitting exogenous pressure to the cell at the time of patch rupture.

Finally, our studies also revealed that rapid solution changes around the infected cell can be implemented while using only slow perfusion of the bath. This presumably reflects a small unstirred layer around the infected cell, a desirable byproduct of the small RBC size and its relative lack of cell surface irregularities like a meshwork of extracellular matrix proteins.

Indeed, we found that PSAC inhibition occurs almost instantaneously after application of antagonist in both a continuous light scattering assay for PSAC-mediated osmotic lysis and in our whole-cell recordings. This observation not only implicates a small unstirred layer around the infected cell, but it is consistent with the fast on and off rate constants measured in single PSAC recordings. (Studies with furosemide and phloridzin indicate that these rate constants are all below 100 ms [25,29].)

To quantitatively explore the experimental variables that affect patch-clamp recordings on infected RBCs, we considered the equivalent circuit diagrams for cell-attached and whole-cell patch-clamp configurations (Fig. 5, panels A and B respectively). A critical variable that has already received some attention is the seal resistance (R_s), a quantitative measure of the current that leaks through the gap between the pipette and the membrane. While formation of high R_s values was the fundamental discovery that led to the development of patch-clamp technology some 30 years ago [1], still little is known about what must be a chemical reaction between the pipette and the surface of a cell. Our empirical observations with human RBCs indicate that attention to pipette and solution cleanliness, use of pipettes immediately after fabrication, and minimal mechanical agitation during seal formation are critical. With our small geometry pipettes pulled from quartz capillaries, we routinely achieve R_s values greater than 100 gigaohms and occasionally obtain values approaching 1 teraohm [22].

For both of the equivalent circuit diagrams in Fig. 5, the fraction of the measured total current that actually flows through transport proteins on the RBC membrane (I_m/I_{tot}) is given by:

$$I_m / I_{tot} = R_s / (R_s + R_m + R_e).$$

Thus, $R_s \gg (R_m + R_e)$ is required for the measured current to correctly reflect permeation through ion channels on the membrane. If this requirement is not satisfied, much of the measured current will result from the biologically uninteresting leak between the pipette and the cell surface. Our experiments reveal that mechanical agitation of the cell after seal formation (e.g. though bulk solution perfusion) can lead to marked reductions in R_s , producing currents that may be erroneously attributed to transmembrane ion movement. This is especially problematic in the whole-cell configuration, where there is no closed-channel baseline that can be used to track changes in R_s .

A second important variable that has not received as much attention is the pipette access resistance (R_a). R_a is determined by pipette geometry, the composition of the pipette solution, and by any debris that may enter and obstruct the pipette. While the pipette resistance measured before contact with a cell is often recorded and published, empirical observations suggest that it underestimates R_a by several fold. The effect of R_a on measured transmembrane currents in the whole-cell configuration is given by:

$$I_m = I_{true} * R_s * R_m / [R_a * (R_s + R_m) + R_s * R_m],$$

where I_{true} is the current that would be measured with a perfect, non-resistive pipette. From this equation, it is apparent that conditions yielding $R_a \ll R_m \ll R_s$ produce the most faithful reproduction of transmembrane currents. A non-zero R_a also has the effect of distorting the voltage-dependence of identified ion channels in a complicated, but mathematically predictable way. We strive to maintain low R_a values by fabricating pipettes with rapidly tapering geometries, filtering all solutions to avoid clogging the pipette with debris, and using pipettes immediately after fabrication.

There are additional resistive elements not shown in our simplified equivalent circuit that may further degrade the reliability of measured currents. For example, the ground electrode in the

bath increases the series resistance in the circuit; the magnitude of this resistance should be kept to a minimum. For this reason, we found it is best to avoid using a salt agar bridge, but instead prefer to use a ground electrode immersed directly in the bath solution near the clamped cell. It is also important to apply thick and uniform AgCl deposits on both the silver grounding wire and the pipette electrode wire to produce low resistance connections with minimum junction potentials.

There are also capacitive elements not shown in our equivalent circuit that demand special attention to achieve low noise recordings. We have used quartz instead of borosilicate glass for fabrication of our pipettes because it produces less capacitive noise [5]; short geometry pipettes and pipette holders also produce noticeable improvements in single PSAC recordings. A thick coating of sylgard polymer applied to the outside of the pipette as near the tip as possible without occluding or dirtying further reduces capacitive noise in our hands. Finally, maintaining a minimum bath solution depth reduces noise resulting from a larger than necessary surface of pipette submersion. With these manipulations combined with high resistance seals, we routinely achieve baseline rms noise values of less than 200 fA at a 5 kHz bandwidth in the cell-attached configuration on infected RBCs. Values as low as 120 fA have been measured in our laboratory. Because our AxoPatch 200B amplifier produces 60 fA baseline noise without a pipette holder or pipette attached (similar to manufacturer specifications), additional manipulations to reduce resistive and capacitive noise will have only modest effect without improvements in amplifier design. This low baseline noise is important because it permits higher bandwidth recording and permits detection of brief single channel events, especially relevant in light of PSAC's gating behavior with a mean open duration of only about 200 μ s [29].

With attention to these various sources of error and appreciation that the small size and deformability of human erythrocytes require technical modifications not needed with larger cells, patch-clamp methods continue to hold promise for providing fundamental mechanistic insights into how the parasite remodels its host erythrocyte.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Perfusion can damage the patch-clamp seal on infected RBCs. (A) After a 70 G Ω cell-attached seal on an infected RBC was obtained, perfusion at gradually increasing rates was applied without changing composition. Traces shown are with 0.16, 0.22, and 0.35 mL/min perfusion with the clamped cell $\sim$ 1 cm from the perfusion inlet. As perfusion rate was increased, the frequency and magnitude of transient seal breaks increased with eventual complete loss of seal integrity. Note that the baseline current gradually increased from the zero current level (dashed lines on both sides of each trace), reflecting a decreasing seal resistance. For these traces, V_m was +100 mV. (B) A cell-attached patch with a single active PSAC molecule is shown for comparison of PSAC currents to those due to perfusion artifacts. This patch's high seal resistance (370 G Ω) produces a stable, low-noise baseline almost indistinguishable from the zero current markers. V_m was +100 mV and -100 mV (upper and lower traces respectively). For (A) and (B), recordings were with solution A in both bath and pipette. Scale bars represent 5 pA (vertical) and 100 ms (horizontal).

Figure 2.

A new chamber for controlled perfusion of clamped erythrocytes, as seen from above. The chamber has six troughs of 0.35 cm width x 2.9 cm length separated by black Delrin barriers (top to bottom, left half of chamber). This design produces no detectable contamination of a trough's solution by those in adjacent troughs despite very slow perfusion. Arrows show the 6 sites of solution entry and a single common site for solution removal. A silver-chloride coated ground wire is immersed directly in the bath, as shown in the bottom right corner. This geometry allows the facile movement of a patch-clamped RBC from one trough into any other trough to produce bath solution changes in any desired order. (B) A two PSAC patch recorded in the cell-attached configuration with continuous perfusion of solution A at 0.1 mL/min (top trace). Notice the stable baseline and the absence of seal break events. This cell was then perfused with the same solution containing 1 μ M and then 3 μ M NPPB (middle and bottom traces, respectively). Notice the decreased frequency of openings for these two independent channels with increasing [NPPB]. $V_m = -100$ mV and scale bars = 100 ms/2 pA (horizontal/vertical) for all traces. Dashes to the right and left of each trace indicate zero current levels. (C) Corresponding all-points histograms in 0 and 1 μ M NPPB. The area under the 0 μ M NPPB histogram is shaded for clarity. For each group, 20.9 seconds of data acquired at 100 KHz was binned in 20 fA increments to identify open and closed states. The level corresponding to both channels closed (labeled c) increased significantly with NPPB, whereas the levels for one open channel (o_1) and two open channels (o_2) decreased reflecting this antagonist's inhibitory effects.

Figure 3.

Rapid, reproducible PSAC inhibition by applied antagonists and complete recovery upon washout. (A) Osmotic lysis experiment showing kinetics of PSAC-mediated lysis in sorbitol without inhibitor (top trace). Lower two traces show the effect of addition of either 50 μ M NPPB or 200 μ M furosemide (traces superimposed and not distinguishable) from DMSO stock solutions at the time indicated by the arrow. Inhibition of PSAC by these agents occurs more rapidly than the temporal resolution of these osmotic lysis measurements. (B) Whole-cell currents on a trophozoite-infected RBC before and after PSAC inhibition (left and right groups of traces), achieved by moving the clamped cell into a trough perfused with 2 mM furosemide. The second group of traces was recorded only 47 seconds after the first, indicating inhibition of currents is also rapid. Much of the intervening time was spent moving the cell from one trough into another. Bath and pipette solutions were 155 mM NaCl, 10 mM glucose, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Na-HEPES, pH 7.4. Groups of traces reflect the current responses to V_m from -100 mV to + 100 mV in 10 mV increments. Scale bars represent 200 pA/20 ms. (C) Whole-cell chord conductances (γ) vs. time of recording for the cell in (B) with repeated movements between the same two troughs. Symbols represent the mean $\pm$ S.D. of 5 or 6 files in the furosemide-free or -containing troughs (filled and open circles, respectively). We applied and removed furosemide 6 times in approximately 12.5 minutes with unchanging mean conductances in each trough, reflecting the reproducibility of these currents. (D) Rapid kinetics of inhibitor washout. Whole-cell chord conductances (γ) for sequential files recorded on an infected cell in a furosemide-free trough (filled circles) or in a trough with 2 mM furosemide (open circles). Bath and pipette contained Solution A. The horizontal line represents the mean conductance before application of furosemide; the smooth curve represents the best fit to single exponential recovery of currents upon washout of the very high [furosemide]; the fitted time

constant was less than 30 sec. Recovery of PSAC-mediated currents was complete because the plateau conductance matches the measurements before furosemide application.

Figure. 4.

Reproducibility of whole-cell chord conductances (γ) in various solutions. Conductances were calculated from experiments on 23 separate cells recorded in symmetric salt solutions with total nominal osmolarities ranging from 295 to 2300 mosm. For each cell, various non-saturating concentrations of furosemide or dantrolene (known PSAC antagonists) were applied by moving the cell between perfusion troughs. Each symbol reflects the correlation between a given measurement (abscissa) and the subsequent application of the same solution (ordinate) with intervening application of at least one other solution.

Figure 5.

Equivalent circuit diagrams for the cell-attached and whole-cell patch-clamp configurations, (A) and (B) respectively. The circuit shows the main resistive components through which currents flow when an electrochemical gradient is applied: R_a , access resistance; R_m , membrane resistance (which for infected RBCs corresponds primarily to flux through PSAC); R_e , exit resistance; R_s , seal resistance. R_e reduces to zero in the whole-cell configuration. The cell and pipette are superimposed in grey outline for clarity; the small gap between the cell and pipette tip reflects an imperfect seal, through which ions may leak.