

Evidence for the involvement of *Plasmodium falciparum* proteins in the formation of new permeability pathways in the erythrocyte membrane

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Summary

The intraerythrocytic developmental stages of the malaria parasite *Plasmodium falciparum* are responsible for the clinical symptoms associated with malaria tropica. The non-infected human erythrocyte is a terminally differentiated cell that is unable to synthesize proteins and lipids *de novo*, and it is incapable of importing a number of solutes that are essential for parasite proliferation. Approximately 12–15 h after invasion the parasitized cell undergoes a marked increase in its permeability to a variety of different solutes present in the extracellular milieu. The increase is due to the induction in the erythrocyte membrane of ‘new permeability pathways’ which have been characterized in some detail in terms of their transport and electrophysiological properties, but which are yet to be defined at a molecular level. Here we show that these pathways are resistant to trypsin but are abolished by treatment of intact infected erythrocytes with chymotrypsin. On resuspension of chymotrypsinized cells in chymotrypsin-free medium the pathways progressively reappear, a process that can be inhibited by cytotoxic agents, and by brefeldin A which inhibits protein secretion. Our results provide evidence for the involvement of parasite encoded proteins in the generation of the pathways, either as components of the pathways themselves or as auxiliary factors.

Introduction

As part of its complex life cycle *Plasmodium falciparum*, the causative agent of the most severe form of human malaria, invades differentiated erythrocytes. The parasite’s intraerythrocytic development is completed within 48 h. It involves cell growth, stage differentiation, and replication, and thus a high nutritional demand. The parasite draws a range of essential nutrients from the extracellular milieu. For some of these (e.g. glucose) the capacity of the endogenous erythrocyte membrane transporters is sufficient to meet the requirements of the parasite (Kirk and Saliba, 2006). For others, such as the essential vitamin pantothenic acid, however, the primary route of entry into the parasitized cell are the ‘new permeability pathways’ (NPP) induced by the parasite in the host erythrocyte membrane some 12–15 h after invasion (Saliba *et al.*, 1998; Saliba and Kirk, 2001; Kirk and Saliba, 2006). The parasite-induced NPP are permeable to low molecular weight solutes, including amino acids, sugars and other polyols, nucleosides, vitamins and a wide range of other organic and inorganic cations and anions (Cabantchik, 1990; Kirk, 2001; Ginsburg and Stein, 2004). They show a general preference for anions over cations, but have a broad specificity, accommodating physiological and non-physiological molecules alike. The pathways are inhibited by a variety of well-known anion transport pathway inhibitors, including furosemide and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) (Kirk *et al.*, 1994). They are also both permeable to, and inhibited by, certain biotin derivatives which are presumed to bind irreversibly either to the proteins comprising the pathways, or to auxiliary proteins essential for maintaining the pathways (Nyalwidhe *et al.*, 2002; Baumeister *et al.*, 2003; Cohn *et al.*, 2003).

Electrophysiological measurements on *Plasmodium*-infected erythrocytes have revealed novel conductances not seen in non-infected cells under physiological conditions (Desai *et al.*, 2000; Egée *et al.*, 2002; Huber *et al.*, 2002; Duranton *et al.*, 2003; 2004; Staines *et al.*, 2003; 2004). Detailed analyses of these conductances have led to the proposal that these are the electrophysiological correlates of the NPP. Whether there is a single class, or several classes, of ion channels involved is a

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matter of ongoing debate (Alkhalil *et al.*, 2004; Ginsburg and Stein, 2004; 2005; Huber *et al.*, 2005). Similarly, the question of whether the channels comprising the NPP are derived from the host erythrocyte, or the intracellular parasite, or whether parasite proteins function synergistically with host proteins, is yet to be resolved. Several studies have reported that exposure of uninfected cells to particular stresses [oxidation (Duranton *et al.*, 2002; Huber *et al.*, 2002) or stimuli (Egée *et al.*, 2002; Verloo *et al.*, 2004)] result in the activation of conductances/transport pathways with functional characteristics similar to those seen in infected cells, consistent with the pathways being derived from host cell proteins. However, Desai and colleagues have recently reported strain-specific variations in the electrophysiological characteristics of the parasite-induced conductance, and have interpreted this as indicating that the channel proteins are derived from the parasite rather than the host (Alkhalil *et al.*, 2004).

The substrate-selectivity and pharmacological properties of the NPP are consistent with the involvement of anion-selective channels and the electrophysiological data are in support of this view. The major endogenous anion transport pathway in the erythrocyte membrane is the 'band 3' anion exchanger (AE1), of which there are some 10^6 copies per cell (Frazar *et al.*, 2003). Although band 3 is not a classical anion channel (its major role is, rather, as an anion transporter), band 3 proteins from species other than humans have been shown to function as anion channels which, moreover, are permeable to a range of both anionic and electroneutral organic solutes (Fievet *et al.*, 1995; Garcia-Romeu *et al.*, 1996). The possibility has long been considered that the NPP might involve either the native, or perhaps more likely, a modified form of band 3. Estimates of the number of copies of the channels comprising the NPP range from 16 to 8000 (Ginsburg and Stein, 2005). Thus, if the NPP were formed, at least in part, by modified band 3 protein, only a small fraction of 1% of the total population of band 3 proteins present in an erythrocyte would need to undergo the required modifications. In infected erythrocytes a proportion of band 3 proteins is known to undergo a distinct conformational change in at least one extracellular domain (Winograd and Sherman, 2004; Winograd *et al.*, 2005), though the consequence of this for the transport function of the protein are unclear.

The aim of this study was to investigate a possible role of the band 3 protein in the appearance of the NPP. Here we show that chymotrypsin treatment of infected cells cleaves the band 3 protein and results in the disappearance of the NPP. In the absence of the protease, the NPP reappear within 4 h. This reappearance requires live parasites and therefore leads us to conclude that band 3 cannot be responsible for this pathway.

Results

Band 3 and the NPP are both susceptible to chymotrypsin

The previous finding that the NPP induced by the parasite in the host erythrocyte membrane are blocked by biotin derivatives (Baumeister *et al.*, 2003; Cohn *et al.*, 2003) is consistent with the NPP being comprised of one or more channel proteins. To explore further the properties of the protein(s) involved we tested a range of proteases for their effect on the NPP. The activity of the NPP was assessed by monitoring the haemolysis of IRBC suspended in approximately iso-osmotic solutions of the NPP substrate L-alanine.

Untreated IRBC suspended in an iso-osmotic L-alanine solution for 15 min (at 37°C) underwent approximately 60% haemolysis, due to the influx of L-alanine via the NPP (Fig. 1A). Addition of the NPP blocker furosemide (100 µM) reduced haemolysis to below 10%. Pretreatment of the IRBC with trypsin had no significant effect on the haemolysis of the IRBC in the L-alanine solution. By contrast, pretreatment of the IRBC with chymotrypsin completely abolished the furosemide-sensitive haemolysis of IRBC, indicating a complete disruption of the NPP. The same results were obtained when haemolysis was induced using sorbitol (data not shown). Chymotrypsinization also inhibited the parasite-induced influx of L-[¹⁴C]-glutamate into infected cells, reducing it to the same extent as the NPP-inhibitor furosemide (Fig. 1B). Infected cells remained intact during protease treatment as determined by the lack of haemoglobin in the incubation solution.

The effects of chymotrypsin on the NPP were both concentration and time dependent (Fig. 2A) and were accompanied by proteolytic cleavage of band 3 (Fig. 2B). The major proteolytic cleavage product is a polypeptide of ~65 kDa (Cabantchik and Rothstein, 1974). The ~45 kDa band seen in the control and in the protease treated sample is a degradation product of band 3 that appears quickly after haemolysis (Czerwinski *et al.*, 1988). Incubation of infected cells with chymotrypsin at different temperatures revealed an interesting differential effect of chymotrypsin on the NPP and on the integrity of the band 3 protein (Fig. 2C and D). Chymotrypsin treatment at 22°C resulted in a complete cleavage of the full-length band 3 molecule to the < 66 kDa product (Fig. 2D). However, at this temperature chymotrypsin had no significant effect on NPP activity (Fig. 2C). At 37°C both, the full-length band 3 protein and NPP activity disappeared, whereas for IRBC treated at 22°C for 30 min, followed by 30 min at 37°C there was an intermediate effect on the NPP activity (but full cleavage of band 3), consistent with the time-dependent inactivation of the NPP by chymotrypsin seen in Fig. 2A.

These results are, to our knowledge, the first to dem-

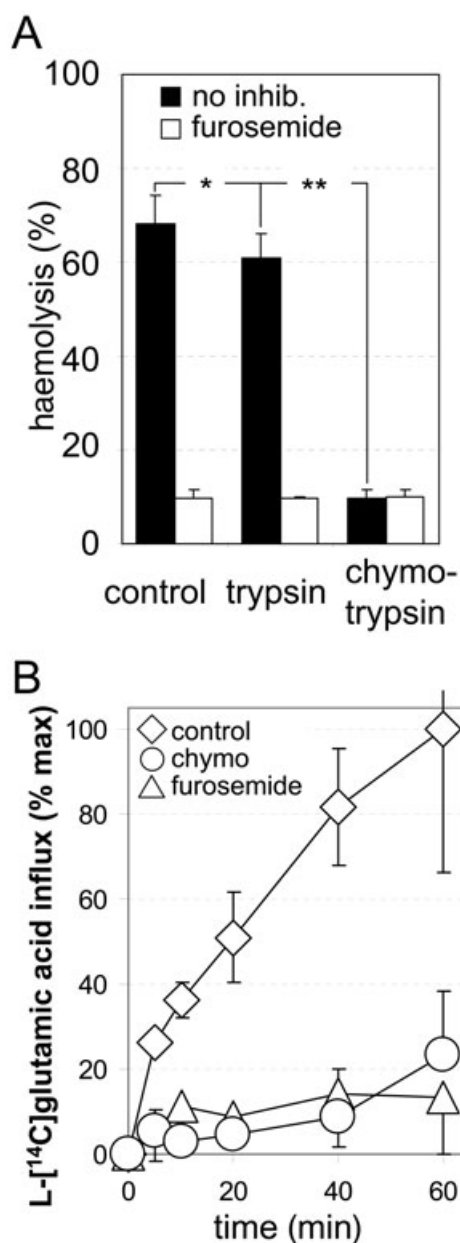


Fig. 1. NPP activity is sensitive to chymotrypsin.

A. Trophozoite stage IRBC (parasitaemia 95%) were incubated with trypsin or with chymotrypsin. Control cells were left untreated. Subsequently, the cells were washed three times and subjected to the L-alanine haemolysis assay either in the absence or in the presence of the NPP inhibitor furosemide (100 μ M). The amount of haemoglobin released from the same number of IRBC after lysis in water was defined as 100%. The results are mean values of five independent experiments, and the error bars indicate standard deviations. *P*-values from unpaired *t*-tests: **P* = 0.12; ***P* = 0.001; ****P* = 0.001. The respective confidence intervals are: -0.006 to 0.099, 0.275–0.380 and 0.228–0.333. *P*-values after ANOVA: **P* = 0.07; ***P* = 0.001; ****P* = 0.001.

B. Chymotrypsinized IRBC were incubated in the presence of L-[¹⁴C]-glutamic acid for up to 60 min. At the indicated time points cells were harvested and the radiolabelled amino acid in the cellular fraction was quantified. Non-chymotrypsinized cells, suspended either in the absence or presence of 100 μ M furosemide served as controls. The results are mean values of three independent experiments.

onstrate that the NPP involve a protease-susceptible protein. Moreover, the reduction in the amount of full-length band 3 protein does not correlate with a decrease in NPP activity. The differential effects on the band 3 protein and NPP activity indicate that proteins other than band 3, either alternatively or synergistically, contribute to the NPP.

NPP are restored during parasite development

Treatment of IRBC with the biotin derivative sulfo-NHS-LC-biotin results in the blockade of the NPP, presumably by covalent binding of the derivative to accessible lysine residues of the channel protein(s) (Nyalwidhe *et al.*, 2002; Baumeister *et al.*, 2003; Cohn *et al.*, 2003). If cells pre-treated with the biotin derivative are incubated in the absence of the compound for a prolonged period the activity of the NPP is gradually restored (Baumeister *et al.*, 2003).

A similar recovery of NPP activity was seen following treatment of IRBC with chymotrypsin. When late ring-stage parasitized erythrocytes (i.e. 12 h post invasion) were either treated with chymotrypsin or biotinylated the relatively low level of haemolysis seen in these cells was further reduced. On maturation of the parasitized cells there was a substantial increase in the level of haemolysis, in both treated and untreated cells, consistent with the appearance of functional NPP in IRBC that had been subjected to biotinylation or chymotrypsinization (Fig. 3A). Because erythrocyte proteins cannot be regenerated after protease treatment, these results suggest that if host proteins are involved in the formation of the NPP they become susceptible to the protease only upon their activation.

To investigate the recovery of the NPP following chymotrypsinization of IRBC at a more advanced developmental stage IRBC, approximately 24 h post invasion, were treated with chymotrypsin and then cultured in RPMI 1640 medium, either with or without human plasma. As shown in Fig. 3B, for the first hour after chymotrypsinization the degree of haemolysis of cells in an iso-osmotic L-alanine solution remained low, but increased significantly over the next 3 h, consistent with the reappearance of the NPP. The rate of reappearance was unaffected by the presence of human serum in the medium. The high degree of synchronization of the cultures used in this experiment (as evident from the fact that 80% of the cells underwent haemolysis under control conditions, with a recovery of haemolysis levels to almost 40% within the 4 h timescale of the experiment) argues against an alternative explanation, namely that NPP development is not synchronized and that the apparent reappearance of NPP activity is the result of the activation of previously inactive channels in a young subpopulation of the cells. Further-

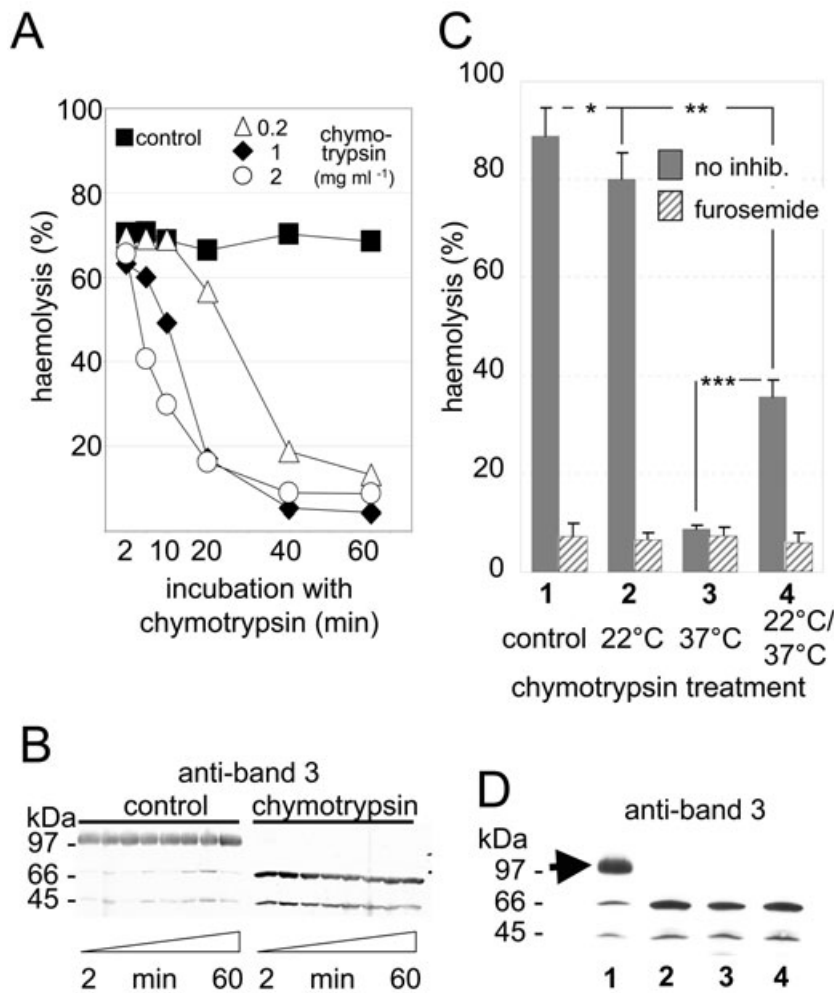


Fig. 2. Band 3 and NPP are susceptible to chymotrypsin in a time- and concentration-dependent manner. Infected erythrocytes were incubated with various concentrations of chymotrypsin. Control cells were left untreated.

A and B. At each one of several time points two aliquots of the cells were harvested and either (A) subjected to the iso-osmotic haemolysis assay or (B) processed for the analysis of the band 3 by immunoblotting. The samples shown in B (right panel) were obtained from IRBC after treatment with 0.2 mg ml⁻¹ chymotrypsin, including two additional time points of 30 and 50 min respectively.

C and D. Intact IRBC (parasitaemia 95%) were incubated in PBS containing chymotrypsin (2 mg ml⁻¹) for 60 min at 22°C (2) or at 37°C (3) or first at 22°C for 30 min and subsequently at 37°C for another 30 min (4). Control cells (1) were incubated in PBS at 37°C without chymotrypsin. The cells were washed free of protease and then subjected to the L-alanine haemolysis assay either in the absence or the presence of the NPP inhibitor furosemide (C) or processed for the analysis of band 3 by immunoblotting (D).

For A and C the amount of haemoglobin released from the same number of IRBC after lysis in water was defined as 100%. *P*-values from unpaired *t*-tests: **P* = 0.13; ***P* = 0.0003; ****P* = 0.0003. The respective confidence intervals are: -0.038 to 0.218, 0.357–0.569, -0.3388 to -0.2111. *P*-values after ANOVA: **P* = 0.27; ***P* = 0.011; ****P* = 0.009.

more, it is important to note that the time course of reappearance does not reflect events at a single cell level; the data provide no insight into whether the reappearance of NPP in an individual IRBC occurs suddenly or whether it is a gradual process.

When chymotrypsinized IRBC were cultivated in the continued presence of chymotrypsin, the NPP did not reappear (Fig. 4A). Similarly, the reappearance of the NPP was prevented when the chymotrypsinized cells were cultured (in the absence of chymotrypsin) in medium containing either the cytotoxic protein kinase inhibitor Tpen or the protein synthesis inhibitor cycloheximide (Fig. 4B). These results demonstrate that parasite viability/protein synthesis is essential for the reappearance of NPP.

The fungal metabolite brefeldin A (BFA) inhibits protein secretion in most eukaryotic cells (Lippincott-Schwartz *et al.*, 1989) including *P. falciparum* (Benting *et al.*, 1994a,b; Knuepfer *et al.*, 2005). Although this drug is cytotoxic when applied over a prolonged period (Benting *et al.*, 1994a) it does not affect parasite viability within 4 h (Knuepfer *et al.*, 2005). Recently we generated and char-

acterized a BFA resistant *P. falciparum* line (Baumgartner *et al.*, 2001; Wiek *et al.*, 2004). When erythrocytes infected with BFA-sensitive or BFA-resistant parasites were treated with chymotrypsin NPP activity disappeared to the same extent. When after removal of the protease IRBC were cultivated in the presence of BFA at a concentration (5 µg ml⁻¹) sufficient to inhibit protein secretion in the BFA-sensitive, the NPP reappeared in the BFA-resistant but not in the BFA-sensitive parasites (Fig. 4C). On removal of the BFA from the BFA-sensitive parasites the parasites were able to complete their intraerythrocytic development and to reinvade at normal rates (data not shown), thus excluding the possibility that the effects of the BFA treatment were due to a killing of the parasites. Lower concentrations of BFA that do not affect protein secretion (≤ 1 µg ml⁻¹) (Benting *et al.*, 1994a) had no effect on the reappearance of the NPP in the susceptible line.

In a recent study it was shown that following treatment of IRBC with BFA the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), a parasite encoded variant protein exposed on the erythrocyte surface, requires approxi-

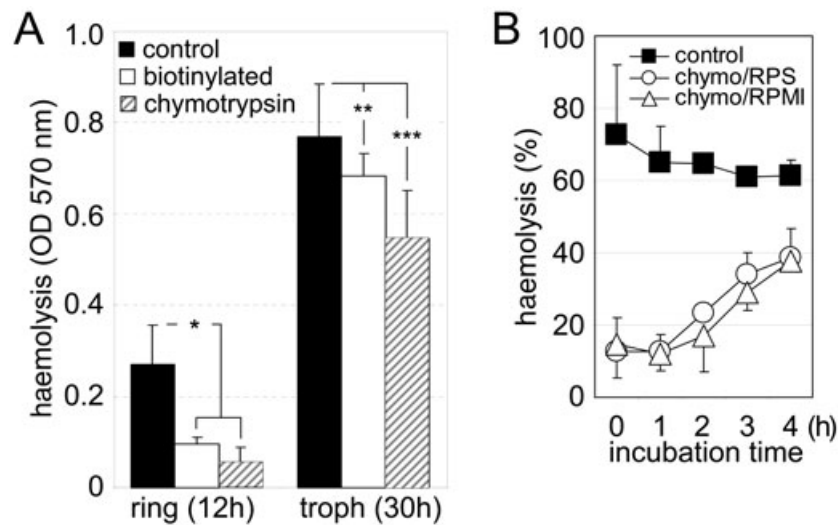


Fig. 3. NPP are restored during parasite development.

A. Ring-stage parasites (~12 h post invasion; parasitaemia 12%) were biotinylated or treated with chymotrypsin. Control cells were left untreated. Subsequently, parasites were cultivated at a haematocrit of 2% under standard conditions. Immediately after the treatment and later, at the trophozoite stage (30 h post invasion), aliquots of the cells were collected and subjected to the L-alanine haemolysis assay. Ring-stage IRBC cannot be enriched to the same parasitaemias as trophozoite IRBC. Because these cultures contain a high proportion of non-infected cells which are not susceptible to L-alanine treatment, haemolysis of the infected cell is indicated by direct OD₅₇₀ values and not in relative values as in the other figures.

B. Trophozoite IRBC were treated with chymotrypsin and subsequently incubated at 37°C in RPMI-1640 medium either in the presence (RPS) or in the absence (RPMI) of 10% human plasma. Control cells were left untreated. After several time points aliquots of the cells were subjected to the L-alanine haemolysis assay.

The results are mean values from four (A) and three (B) independent experiments. *P*-values by unpaired *t*-tests: **P* = 0.05; ***P* = 0.029; ****P* = 0.06.

mately 9 h to reach its final destination (Kriek *et al.*, 2003). The reappearance of the NPP occurs after a shorter period suggesting that transport of the molecules involved is more rapid. PfEMP-1 forms protein–protein complexes on its route to the erythrocyte surface (Papakrivovs *et al.*, 2005) which may explain the differences in the transport kinetics.

Chymotrypsin treatment blocks parasite-induced anion conductance

According to whole-cell electrophysiological recordings of human erythrocytes infected with *P. falciparum* infection induces a large outwardly rectifying anion current which has been attributed to the channels underlying the NPP

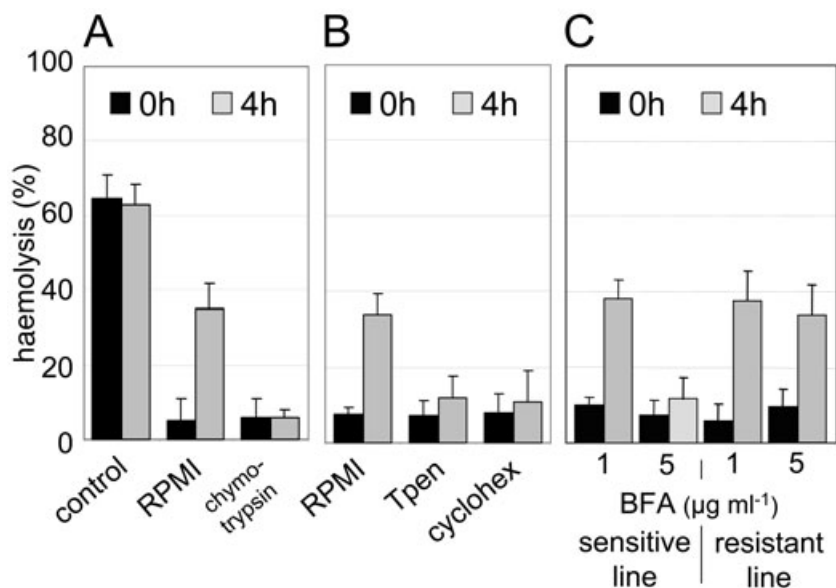


Fig. 4. Inhibition of the reappearance of the NPP following chymotrypsinization. IRBC were left untreated (control) or were treated with chymotrypsin and subsequently incubated in RPMI-1640 medium alone (RPMI) or in the presence of chymotrypsin (A), in the presence of Tpen or cycloheximide (B), or in the presence of various concentrations of BFA (C). In the experiment shown in (C) a BFA resistant line (Baumgartner *et al.*, 2001) was included. Cells were collected immediately after the chymotrypsin treatment, and 4 h later, and subjected to the L-alanine haemolysis assay. The amount of haemoglobin released from the same number of IRBC after lysis in water was defined as 100%. The results are mean values of three to five independent experiments and the error bars indicate standard deviations.

(Huber *et al.*, 2002; 2004; Staines *et al.*, 2003; Duranton *et al.*, 2004).

Under control conditions, using a K-gluconate pipette solution with a NaCl bath solution, the currents in mature *P. falciparum*-infected cells exhibited outward rectification and a reversal potential of approximately -50 mV. This negative reversal potential, close to the Cl^- equilibrium potential, confirmed the Cl^- selectivity of the induced currents (Fig. 5A and C).

The whole-cell current recorded under control conditions showed a progressive run-down with a fast current decay of about 35% of the initial value followed by a slower current decline thereafter (Fig. 5B, closed circles). Application of chymotrypsin (2.5 mg ml^{-1} at 30°C) after 20 min of recording, induced a fast and almost complete decline of the current amplitude (Fig. 5A and B, open circles). Previous studies have reported the activation of at least two anion channel types, an inwardly and an outwardly anion channel (Desai *et al.*, 2000; Egée *et al.*, 2002; Huber *et al.*, 2002; 2004; Staines *et al.*, 2003), as well as a cation channel (Duranton *et al.*, 2003). There is evidence that the outwardly rectifying anion channel is permeable to organic osmolytes (Duranton *et al.*, 2004). The chymotrypsin-inhibited current fraction (i.e. the difference between circles and diamonds in Fig. 5C) showed out-

wardly rectifying characteristics pointing to preferential inhibition of the outward rectifier. The chymotrypsin-resistant current fraction (Fig. 5C, diamonds) exhibited a linear current-voltage relationship and a more positive reversal potential than the chymotrypsin-sensitive current fraction suggesting a lower Cl^- selectivity.

Wash-out of chymotrypsin did not restore the whole-cell currents within 20 min of continuous recording. Moreover, applying serum albumin which reportedly activates the outwardly rectifying anion current in infected cells (Staines *et al.*, 2003) had no effect on the current of chymotrypsin-treated and washed cells.

In contrast to the inhibitory effect of chymotrypsin at 30°C , treatment of infected cells with chymotrypsin at 21°C had no significant effect on the observed current ($n = 4$; not shown) ($n = 4$; not shown) indicating a temperature dependence of the chymotrypsin effect similar to that observed in the isosmotic haemolysis experiments.

Chymotrypsin treatment blocks parasite development

Ring-stage parasites and the developmentally more advanced trophozoites are affected to different degrees by chemical treatments and by changes in temperature. In the presence of BFA or at low temperature, the devel-

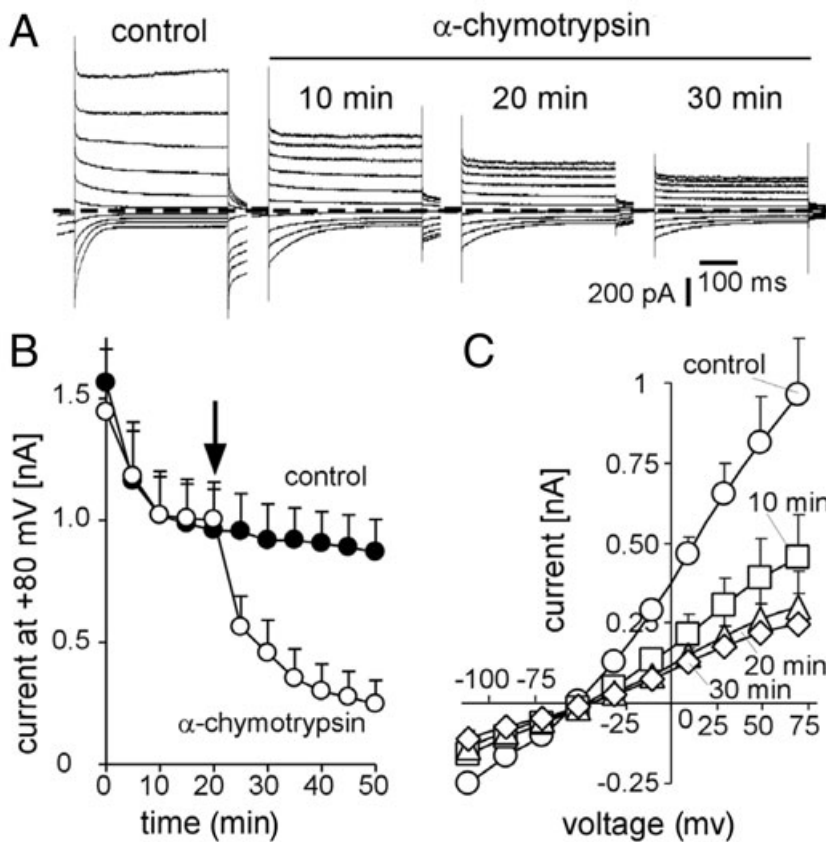


Fig. 5. Effect of chymotrypsin on the infection-induced whole-cell current in human erythrocytes.

A. Whole-cell currents traces recorded with K-gluconate in the pipette and NaCl in the bath from *Plasmodium falciparum* (late trophozoite)-infected erythrocytes before (control) and 10, 20 and 30 min after addition of α -chymotrypsin (2.5 mg ml^{-1}) to the bath solution. The control record was measured 20 min after reaching the whole-cell configuration (dash line indicates zero current).

B. Time dependence of the mean outward current at $+80$ mV voltage (\pm SE; $n = 3-4$) recorded over 50 min. Currents were obtained in the absence of α -chymotrypsin (closed circles, $n = 3$) or after addition of the enzyme (2.5 mg ml^{-1} ; open circles, $n = 4$ arrow) at 20 min of recording (arrow).

C. Current-voltage relationships (mean \pm SE) of paired experiments recorded as in A before (circles, $n = 5$) or after 10 (squares), 20 (triangles), or 30 min (diamonds) of incubation with chymotrypsin (2.5 mg ml^{-1}).

opment of ring-stage parasites is delayed until the drug is removed or the temperature is restored, after which the parasites continue their normal development without a loss of viability or infectivity (Crary and Haldar, 1992; Benting *et al.*, 1994b). By contrast, under the same conditions, trophozoites begin to lose their viability after 4 h (Benting *et al.*, 1994a). Likewise, surface biotinylation of ring-stage IRBC results in a delay of parasite development whereas biotinylation of schizont-stage erythrocytes terminates parasite development (Baumeister *et al.*, 2003).

When chymotrypsin was added to ring-stage IRBC for 18 h, parasites developed more slowly when compared with untreated IRBC and IRBC cultured in the presence of trypsin (Fig. 6A–C). Upon removal of the chymotrypsin, parasites continued their development, and reinvasion occurred at a normal rate, albeit with a developmental delay of 4–6 h (Fig. 6C). By contrast, when chymotrypsin treatment of the cultures was continued for a longer period, the morphology of the young trophozoites deteriorated. On removal of the protease at a later time point, these deteriorated parasites were unable to reinvade (Fig. 6E). Continuous treatment of infected cells with trypsin had no effect on parasite development but inhibited

re-invasion, as described previously (Fig. 6D) (Breuer *et al.*, 1983). The observation that chymotrypsin-treatment of late ring/early trophozoites caused developmental arrest rather than parasite death is consistent with other studies using inhibitors that interfere with metabolic functions (Crary and Haldar, 1992; Benting *et al.*, 1994a). The ring/early trophozoite stage parasites are metabolically less active than the more advanced trophozoite stages. It remains to be shown whether the primary effects of the blockade of the NPP are restrictions in nutrient acquisition and/or the disposal of waste products or whether the blockade affects parasite metabolism at a more general level.

Discussion

The induction of the NPP in *P. falciparum*-infected erythrocytes is thought to play a key role in the delivery of nutrients to the growing intraerythrocytic parasite, as well as in mediating the efflux of metabolic waste products and the altered ion composition of the host cell (reviewed by Kirk 2001). While the physiological properties of NPP have been studied in considerable detail, the proteins

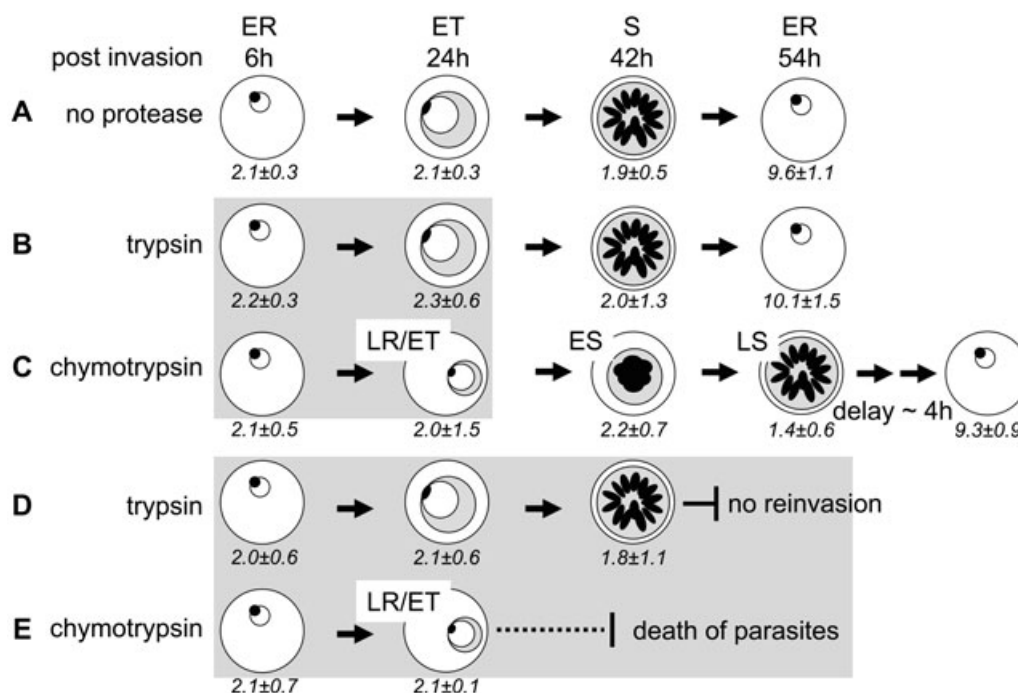


Fig. 6. Chymotrypsin treatment of IRBC affects parasite development. Cultures of highly synchronized early ring-stage infected erythrocytes (6 h post invasion) were treated either with trypsin or with chymotrypsin for 18 h. Parasitized cells were analysed on Giemsa stained blood films, harvested, and recultivated with untreated non-infected erythrocytes. Recultivation was either in the presence or in the absence of the respective proteases. Blood films were taken after various time points and analysed by light microscopy. At least 1000 cells were analysed in each case. In cultures in which reinvasion occurred, the multiplication of the parasitaemia was four- to fivefold. In cultures that were kept in presence of the proteases no ring-stage infected cells were detectable. In IRBC that were treated with chymotrypsin for 18 h and then cultivated in the absence of the protease, mainly schizonts were still present 54 h post invasion. Reinvasion occurred with a delay of approximately 4 h. The shaded boxes indicate the periods of cultivation in the presence of proteases. ER, early ring-stage IRBC; LR, late ring-stage IRBC; ET, early trophozoite stage IRBC; ES, early schizont stage IRBC; S, schizont stage IRBC; LS, late schizont stage IRBC. The numbers indicate the percentage parasitaemias.

involved in their formation are still unknown, and there is ongoing debate about whether they originate from the parasite or are endogenous to the host cell. It has been demonstrated previously that following blockade of the NPP using biotin derivatives, there is a gradual re-appearance of the pathways, implying that the NPP are a result of parasite activity (Baumeister *et al.*, 2003). The recent finding of strain-specific variation in the electrophysiological characteristics of a channel underlying the increased conductance of the mature *P. falciparum*-infected erythrocyte is consistent with this view (Alkhalil *et al.*, 2004), and at least one possible candidate protein has been identified from the *P. falciparum* genome data base (Martin *et al.*, 2005). However, other authors, including some of us, have reported the induction of NPP-like pathways and conductances in uninfected erythrocytes exposed to particular stresses or stimuli, consistent with the idea that endogenous erythrocyte proteins are sufficient to mediate these pathways (Duranton *et al.*, 2002; Egée *et al.*, 2002; Huber *et al.*, 2002; Decherf *et al.*, 2004; Verloo *et al.*, 2004).

The most abundant erythrocyte membrane protein is the band 3 anion exchanger. A fraction of the band 3 molecules are known to undergo covalent modification following *P. falciparum*-infection (Winograd and Sherman, 2004; Winograd *et al.*, 2005). Band 3, perhaps in its modified form, has long been considered a candidate for the NPP. In this study we have compared the effect of protease-treatment on the band 3 protein and on the activity of the NPP. The integrity of the band 3 protein and the activity of the NPP were both unaffected by treatment of intact IRBC with trypsin (Fig. 2). By contrast, as has been shown previously (Cabantchik and Rothstein, 1974), chymotrypsin treatment of intact cells resulted in the proteolysis of band 3, in both infected and non-infected erythrocytes. Chymotrypsin treatment of intact infected cells also resulted in the abolition of NPP activity, and of the increased anion conductance of parasitized erythrocytes. However, there were significant differences in the response of band 3 and of NPP activity to chymotrypsinization. First, whereas treatment of intact infected erythrocytes with chymotrypsin resulted in complete cleavage of the band 3 protein within 2 min there was no effect on NPP activity until >10 min. Second, when the temperature of the chymotrypsin treatment was reduced to 22°C, the band 3 protein showed the same cleavage pattern as was seen at 37°C; however, the NPP and the parasite-induced conductance were not affected. The resistance of the NPP to chymotrypsin under conditions in which the band 3 molecule was degraded argues for the inhibition of the NPP by chymotrypsinization being unrelated to the observed cleavage of band 3. Furthermore, when chymotrypsinization was carried out at 37°C leading to complete cleavage of the band 3 protein and

the disappearance of the NPP, the pathway was restored upon removal of the protease (Fig. 3), consistent with the generation and maintenance of the NPP not being dependent on the presence of intact band 3 protein in the host erythrocyte membrane.

The compounds cycloheximide, Tpen and BFA all inhibited the reappearance of NPP activity in the hours following chymotrypsinization (Fig. 4). Cycloheximide and Tpen are immediately cytotoxic to the parasite, whereas BFA inhibits protein secretion from the parasite, with cytotoxic effects occurring only after a prolonged period of treatment (Benting *et al.*, 1994a; Knuepfer *et al.*, 2005) that exceeds the time required for the reappearance of NPP. The finding that two cytotoxic compounds completely abolished the reappearance of the NPP underscores the requirement for parasite viability for their reappearance, while the inhibitory effects of BFA, seen in BFA-sensitive but not in BFA-resistant parasites, strongly indicate a contribution of secreted parasite proteins to the formation of the pathways.

This study represents, to our knowledge, the first demonstration of the protease sensitivity of the parasite-induced NPP. Our results underscore the need for parasite viability, and the likely requirement for secreted parasite proteins, to generate the NPP. The close similarity between the effects of chymotrypsin on the NPP underlying the parasite-induced influx of alanine and those on the outwardly rectifying anion current observed in infected cells is consistent with the hypothesis proposed previously (Duranton *et al.*, 2004) that the same channels are responsible for these two phenomena. However, this does not exclude the possibility that there are one or more distinct chymotrypsin-sensitive pathways. Whether the channels are themselves directly sensitive to chymotrypsin, or whether the chymotrypsin sensitivity resides in one or more auxiliary proteins that are exposed at the infected cell surface and which are required to maintain channel function is not clear. Similarly, it remains to be established whether the channel proteins originate from the parasite or the host erythrocyte. While the simplest explanation for our results is that the channel proteins are secreted by the parasite (via BFA-sensitive pathways) and transported to the infected cell surface, where they are susceptible to proteolysis by extracellular chymotrypsin, the possibility remains that the channels are endogenous to the erythrocyte and either: (i) are activated and maintained by parasite-derived, chymotrypsin-sensitive auxiliary proteins exposed at the erythrocyte surface or (ii) become chymotrypsin-sensitive only once they have been activated by parasite-encoded proteins. On exposure to oxidative stress, a (low) proportion of non-infected erythrocytes also become susceptible to iso-osmotic lysis as a result of the activation of pathways showing some similarities to the NPP (Huber *et al.*, 2002). This is consistent

with there being endogenous channel proteins capable of contributing to NPP formation. Whether these proteins do play a role in the NPP, and show the same sensitivity to chymotrypsinization, awaits further work. The resolution of the issue of the genetic origin and molecular identity of the proteins comprising the NPP, and the question of whether distinct pathways – some of which are mimicked by the oxidation of non-infected cells – operate in parallel, remain the most important challenges for future studies.

Experimental procedures

Materials

The proteases chymotrypsin (No. C4129) and trypsin (No. T8003) were from Sigma. The biotin derivative sulfo-succinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin) was from Pierce Chemicals, USA. Furosemide, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), Phenylmethanesulphonyl fluoride (PMSF), Cycloheximide and N,N,N',N'-Tetrakis(2-pyridyl)methylethylenediamine (Tpen) were from Sigma. A monoclonal antibody against the human band 3 protein was purchased from Sigma. Secondary antibodies against mouse IgG conjugated to alkaline phosphatase or to horseradish peroxidase were obtained from DAKO A/S, Denmark.

Cell culture

Plasmodium falciparum (isolate FCBR) and a BFA resistant line of the FCBR isolate (Baumgartner *et al.*, 2001) were cultivated in human erythrocytes of blood group A+, at a haematocrit of 2%. The culture medium was RPMI-1640 (Life Technologies), containing 25 mM HEPES and 8.1% NaHCO₃, supplemented with 10% heat inactivated (56°C, 30 min) plasma from blood group A+ donors. The culture conditions and the enrichment of trophozoite-infected erythrocytes by the plasmagel procedure are described in detail elsewhere (Pasvol *et al.*, 1978; Ansorge *et al.*, 1996).

Protease treatment of intact infected erythrocytes

Plasmodium falciparum-infected erythrocytes (IRBC; 1×10^8) were washed three times in phosphate-buffered saline supplemented with 0.6 mM CaCl₂ and 1 mM MgCl₂ (PBS-2) and then incubated with the different proteases in PBS-2 for 30 min at 37°C, routinely using the following concentrations; 2 mg ml⁻¹ for chymotrypsin, and 1 mg ml⁻¹ for trypsin. For experiments investigating the recovery of the NPP, protease treatment and subsequent cultivation were carried out in RPMI 1640 medium instead of PBS-2.

Iso-osmotic haemolysis

The parasite-induced transport of L-alanine and sorbitol were monitored using a semi-quantitative haemolysis method as

described by Kirk *et al.* (1994). Protease-treated as well as -untreated IRBC (5×10^7 ; parasitaemia 80–90%) were collected by centrifugation and resuspended in 150 µl of approximately iso-osmotic sorbitol (290 mM sorbitol/5 mM HEPES/NaOH, pH 7.4) or L-alanine (350 mM L-alanine/5 mM HEPES/NaOH, pH 7.4) solutions for 15 min at 37°C in the presence or in the absence of the NPP inhibitors furosemide or NPPB (each at 100 µM). Haemolysis was quantified by determining the release of haemoglobin from IRBC which was measured spectrophotometrically at 570 nm.

For experiments testing the effects of biotinylation or protease treatment on NPP activity during the development of the parasite from ring- to trophozoite-stage, the haemolysis assay was modified as follows. Approximately 12 h post invasion, ring-stage infected erythrocytes were treated with sulfo-NHS-LC-biotin or with chymotrypsin. Subsequently, cultures were adjusted to a parasitaemia of 12% at a haematocrit of 2%. An aliquot of the culture, containing 2×10^7 IRBC, was sedimented and washed twice with PBS-2, then the cells were resuspended in either sorbitol or L-alanine solutions and the amount of lysis quantified, as above. The remaining cells were cultured for 18 h under standard conditions, and the haemolysis assay was repeated with the trophozoite-stage infected erythrocytes.

Uptake of radiolabelled glutamic acid

To determine the influx of L-[¹⁴C]-glutamic acid (Amersham Bioscience, UK), 2×10^8 trophozoite-infected erythrocytes were incubated at 37°C in 1 ml of RPMI-1640 medium containing 0.5 µCi ml⁻¹ of the radiolabelled amino acid. Incubations were carried out with chymotrypsinized or untreated infected erythrocytes, either in the presence or absence of 100 µM furosemide. At various time points, aliquots of 100 µl were harvested, centrifuged through a cushion of 250 µl dibutyl phthalate and processed as described elsewhere (Baumeister *et al.*, 2003).

Detection of disappearance/reappearance of NPP activity

The reappearance of NPP activity following biotinylation of IRBC was analysed as described previously (Baumeister *et al.*, 2003). Ring-stage parasite-infected erythrocytes (1×10^9 cells with a parasitaemia of 12%) were washed three times in PBS-2 and then incubated with sulfo-NHS-LC-biotin in PBS-2, routinely at a concentration of 1 mg ml⁻¹ for 30 min at 4°C. The incubation medium contained an NPP inhibitor (either furosemide or NPPB) which was added to the cells (at a concentration of 100 µM) 2 min prior to the addition of the biotin derivative. Cells were sedimented by centrifugation (10 000 g, 15 s, 4°C) and the haemoglobin content in the biotinylation solution was determined spectrophotometrically at 570 nm in order to detect and quantify any haemolysis that might have occurred during the 30 min incubation. Cells were washed three times in culture medium to block and remove unbound sulfo-NHS-LC-biotin. They were then returned to culture for 18 h under standard conditions before being harvested, suspended in the iso-osmotic sorbitol or L-alanine solutions, and the extent of haemolysis (over 15 min at 37°C) was quantified (as above).

A similar protocol was followed in experiments designed to monitor the reappearance of the NPP after treatment of IRBC with chymotrypsin. Ring-stage parasite-infected erythrocytes (1×10^9 ; 12 h post invasion) were treated with 2 mg ml^{-1} chymotrypsin, washed with complete medium to remove the protease and then returned to culture for 18 h under standard conditions. They were then harvested and subjected to the same isosmotic haemolysis analysis.

In other experiments to assess the reappearance (over 4 h) of NPP activity following chymotrypsin-treatment of IRBC, cells were treated with chymotrypsin and subsequently incubated in RPMI-1640 medium without or with 10% human plasma (culture medium). In parallel, cells were incubated in culture medium containing chymotrypsin (2 mg ml^{-1}), Tpen ($100 \mu\text{M}$) or cycloheximide ($100 \mu\text{g ml}^{-1}$). The parasitaemia was 90% and the haematocrit 0.6%. At predetermined time points aliquots of IRBC (5×10^7) were sampled and the activity of the NPP assessed using the iso-osmotic haemolysis assay.

Analysis of the band 3 protein

Protease treated IRBC (2×10^7 cells) were resuspended in SDS-PAGE sample buffer and heated for 10 min at 95°C . Following separation by SDS-PAGE, proteins were transferred onto nitrocellulose filters which were blocked for 1 h at room temperature with 2% BSA in washing buffer (10 mM Tris/HCl, 150 mM NaCl, pH 7.4). Band 3 was detected with a monoclonal antibody using standard procedures.

Patch-clamp experiments

Patch-clamp whole-cell recording from *P. falciparum*-infected human erythrocytes was performed as described elsewhere (Huber *et al.*, 2002). Briefly, pipettes with 8–12 M Ω resistance were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech, Port Washington, NY) and by using Pulse software (HEKA). For current measurements, cells were held at a holding potential of -30 mV and 400 ms square pulses from -100 to $+80 \text{ mV}$ were applied in increments of $+20 \text{ mV}$. At each voltage the whole-cell currents (acquisition frequency of 5 kHz) were determined by averaging the current values between 350 and 375 ms of the individual voltage square pulse. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces. Data were corrected for the liquid junction potentials between bath and pipette solution, calculated according to (Barry and Lynch, 1991). The NaCl bath solution used for whole-cell recording contained (in mM) 115 NaCl, 10 MgCl₂, 5 CaCl₂, 20 HEPES/NaOH (pH 7.4). The pipette solution contained (in mM) 140 K-gluconate, 10 NaCl, 1 MgCl₂, 1 EGTA, 1 Mg-ATP, 5 HEPES/NaOH (pH 7.4).

Data analysis and statistics

Data are means \pm SE; n = number of experiments. Differences between means were estimated by unpaired *t*-test (two-tailed) or ANOVA (two-way) using StatsDirect software (StatsDirect).

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