Protein Kinase-Regulated Inwardly Rectifying Anion and Organic Osmolyte Channels in Malaria-Infected Erythrocytes

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Abstract: The intraerythrocytic amplification of the malaria parasite *Plasmodium falciparum* induces new pathways of solute permeability in the host cell's membrane. These pathways play a pivotal role in parasite development by supplying the parasite with nutrients, disposing of the parasite's metabolic waste and organic osmolytes, and adapting the host's electrolyte composition to the parasite's needs. During the last ten years, electrophysiological investigations strongly supported earlier evidence obtained by transport and pharmacological studies that this new permeability pathway, which is induced by the parasite in the host cell membrane, is constituted by anion-selective channels. This review surveys the evidences acquired using the patch-clamp technique and discuss the hypothesis that protein kinase A is an effector of the signalling pathway leading to the activation of endogenous channels upon infection.

Keywords: Erythrocyte, *Plasmodium*, new permeation pathways, protein kinase A, ion channel.

INTRODUCTION

It is agreed that the red cell membrane is relatively impermeable to cations compared to anions, and consequently that the membrane potential is identical to the Nernst potential for chloride, about -10 to -12 mV [1]. This assumption was confirmed using fluorescence technique on both Amphiuma and human red cells [2] and using selective cation ionophores, such as gramicidin and valinomycin, it was shown that the net salt efflux from the red cells could be anion rate limited, which led to the concept of the two-components anion transport system: a large exchange component and a much smaller electrogenic component [3-5]. Furthermore, several tracer flux studies reported biophysical and biochemical evidence that anion channels, corresponding to a permeability of ~10^{-7} cm.s^{-1}, are present in the red cell membrane and account for 10^{-4} to 10^{-6} of the total Cl^- exchange [6]. The functional significance of such chloride conductance would be to set the resting potential at E_{Cl} so there is no electrochemical gradient for anion movement, which eventually will optimize the role of the Cl^-/HCO_3^- exchange mechanism, for the conversion of tissues CO_2 into blood-trapped HCO_3^- [7]. The patch-clamp techniques have been successfully used on erythrocytes membrane since 1981 [8, 9], and during the past three decades, electrophysiological studies on human red blood cells (RBCs) have resulted in documented description of two different cation channels: an intermediate conductance Ca^{2+}-activated K^+ channel, known as the Gárdos channel and voltage-dependent non-selective cation channels [10-14]. In contrast, knowledge on chloride conductances are more recent and have arisen with the use of patch-clamp techniques to decipher the molecular identity of the New Permeation Pathways (NPPs) elicited after malaria infection.

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PLASMODIUM FALCIPARUM-INFECTED ERYTHROCYTE MEMBRANE PERMEABILITY

Intracellular parasitism provides shelter for the pathogen against the host's immune response, but in this situation, the parasite has to face a hostile environment, at least regarding chemical concentrations (predominantly Na^+, K^+ and Ca^{2+}) [15] with an unusual extracellular medium and redox state [16]. Moreover, in the case of *P. falciparum*, the completion of the intraerythrocytic cycle in 48 h implies the supply of essential nutrients such as panthotenate [17], isoleucine [18] and glutamate [19] that have to be taken from the extracellular medium of the host cell, and metabolic waste end products have to be removed in order to avoid self-poisoning [20]. Furthermore, it was shown that *Plasmodium falciparum* parasites consume most of the host cell haemoglobin, far more than they require for protein biosynthesis. If not removed, the amino acids produced in excess could be deleterious due to changes in the intracellular osmotic pressure that might be hazardous for the parasite survival [21, 22]. Consistently, RBCs have marked alterations in membrane transport properties during intraerythrocytic parasite development. Not only the activity of endogenous transporters, such as the NaK-ATPase pump

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or the band 3 anion exchanger, undergo modifications upon infection [15, 23, 24], but 12–15 h after invasion by merozoites, the so-called New Permeation Pathways (NPPs) can be detected, reaching a plateau after 36 h post-invasion [25, 26]. The NPPs show permeability for monosaccharide sugars and other small polyols, amino acids, peptides, nucleosides, various monocarboxylates, small quaternary ammonium compounds, and monovalent inorganic anions and cations [20, 27] when studied by means of tracer fluxes and isosmotic haemolysis experiments [20]. More importantly it has been shown that they are non-saturable, they have an activation energy lower than carrier-mediated transports and they are blocked by numerous anion transport inhibitors (e.g. furosemide and 5-nitro-2(3-phenylpropylamino) benzoic acid (NPPB)), leading to the conclusion that NPPs have some of the characteristics of anion channels [28-30]. This led at the turn of the century to a large body of experiments using the patch-clamp technique, which is the best tool to study ion channels.

COMPREHENSION OF NPPS AND CHLORIDE CONDUCTANCES IN INFECTED CELLS USING THE PATCH-CLAMP TECHNIQUE

In a milestone article in 2000, Desai and co-workers demonstrated for the first time that the very low spontaneous conductance (below 50 pS) of non-infected cells is increased by a factor of 100 to 150 in infected cells [31]. The infection-induced currents are anion selective and show a permeability sequence for monovalent anions of $SCN^- > I^- > Br^- > Cl^-$ > acetate$^-$ > lactate$^-$ >glutamate$^-$, indicating that SCN^- is the most permeable anion through the channels. The whole-cell membrane currents display a strong inward rectification. These membrane currents were attributed to the activity of a unique anion channel with very low unitary conductance (20 pS conductance in symmetrical 1.1 M Cl^-) [31]. Since the channels responsible for the increased membrane currents in infected cells were never observed in non infected cells, it was assumed that this channel was parasite-encoded and it was later called the Plasmodial erythrocyte Surface Anion Channel (PSAC) [32]. However Huber *et al.* [33] described, using whole-cell recordings, anion conductances in non-infected cells, and pointed out that only a fraction of the anion currents are inhibitable by DIDS (4,4′-diisothio-cyanato-stilbene-2,2′-disulfonic acid) suggesting that at least two different types of anion channels are present in the red cell membrane, in good agreement with experiments performed with valinomycin-induced changes in membrane potential in intact cells and in membrane vesicles prepared from RBC ghosts [34, 35]. A study on chicken red cells infected by *Plasmodium gallinaceum*, where clear upregulation of endogenous channels in infected cells was shown, also points toward an endogenous origin of the active anion channels in infected cells [36].

Moreover, rapidly three independent studies on infected cells revealed a much more complex system than expected regarding the number and the origin of the channels involved in the generation of the membrane currents activated upon infection [37-39] (Fig. **1A**, **B** and **C**). Two of these studies confirmed the inwardly rectified nature of the whole-cell conductance induced after infection [38, 39], and the third one found a phenotypically and pharmacologically different current in addition to these inwardly rectified anion currents [37]. Indeed, the third study observed an outwardly rectified current at the trophozoite stage. Subsequently it was shown that this phenomenon is elicited when traces of serum (remaining from the culture medium) are present in the bathing solution surrounding the cells. This serum effect might be linked to a direct interaction of the channels with albumin [40]. Moreover, a particular time-dependent fast inactivation of the inward currents appears when negative holding potentials are applied between the ramps of voltage [41]. Nevertheless, today, there appears to be a consensus that membrane conductance of infected cells, in absence of traces of serum and without holding resting potential applied to the membrane, is predominantly inwardly rectified and due to the activity of anion channels [42]. Moreover, the majority of the authors consider today that membrane currents are due to up-regulation of endogenous channels that are quiescent in the mature erythrocytes, although some evidences tend to prove a parasite origin of the ion channels responsible for the increase of membrane currents observed in infected RBCs [42]. Indeed, subtle change in PSAC gating was reported using two distinct parasite isolates (7G8 and Indo 1), grown in RBCs from a single donor [43], and a spontaneous blasticidin resistant parasite line showed altered PSAC selectivity profile and pharmacology [44]. Moreover, application of chymotrypsin to the patch clamp bathing solution inhibits membrane currents and this affects preferentially the outward currents confirming that plasmodial exported proteins are necessary for NPPs generation [45]. Nevertheless, in a recent attempt to unify the different data it was shown that solute transport via NPPs is not consistent with a single channel model [46, 47]. For example, sorbitol cannot enter infected RBC via a channel with a low open-state probability at positive membrane potential that commonly explains the inward rectification in whole-cell configuration in the absence of traces of serum [48]. Moreover, recent data showed that the inwardly rectified membrane conductance can be accounted for by the activity of at least two different types of channels [49, 50].

MOLECULAR IDENTITY OF INDUCED CHANNELS IN INFECTED ERYTHROCYTES

Among the hundred putative transport proteins that was predicted in the *P. falciparum* genome [51], only a few proteins were assigned as putative channels, and none as homologues of known eukaryotic anion channels. This seems to rule out the parasite origin hypothesis of the NPPs, except if the channels are constituted by an unknown type of anion channel or by a multimeric protein assemblage encoded by different genes and that would be addressed to the host membrane where they could form the pathways. Nevertheless, if so, these proteins have to be encoded by multigenic gene families in order to escape the host immune system, since membrane transporters are transmembrane proteins with extracellular loops.

If one assumes that the channels responsible for the malaria induced currents are endogenous and upregulated upon infection, studies on uninfected erythrocytes provide a way to discriminate between channels and to characterize their properties using both whole-cell recordings and single channel recordings.

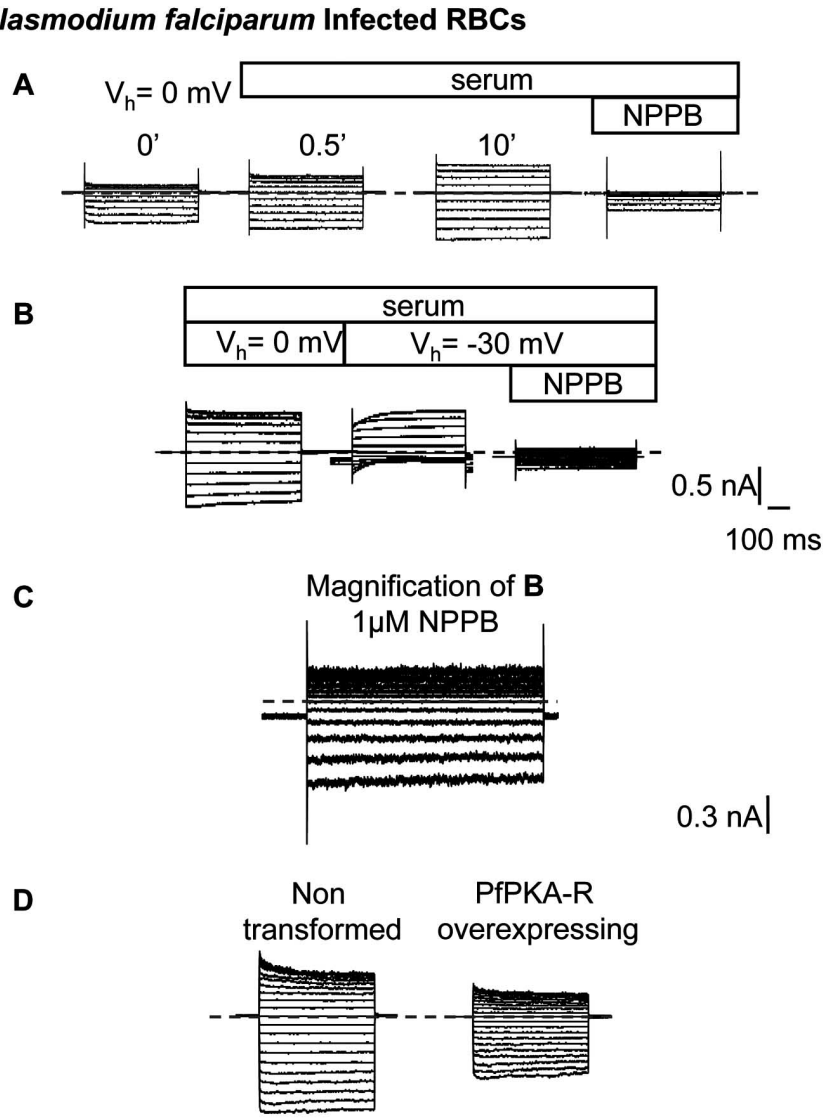

Fig. (1). Traces of whole-cell recording of *P. falciparum* infected erythrocytes.

A: left traces show a representative experiment performed on *P. falciparum*-infected RBC (held at 0 mV) exhibiting a typical inwardly rectifying anion current (bath and pipette containing no permeant cations (N-Methyl D-glucamine)). Middle traces represent the behaviour of currents when 0.4% (*v/v*) human serum is added to the bath solution (30 seconds and 10 minutes after addition). This evokes rapid changes in the observed current recordings, which reaches maximum after a further 10 min (third trace). Serum induced an increase in the whole-cell conductance at both positive and negative potentials. Finally, addition of the anion channel blocker NPPB (10 µM) inhibited the plasma-stimulated current and part of the Inwardly Rectified current (right traces). (*all records were realized according to references [35-38]*). **B:** Typical traces of non-washed cells (where traces of serum remain). The plasma-stimulated current does not exhibit any voltage dependence when cells are held at 0 mV holding potential (V_h)(left traces), but inactivates in a time-dependent fashion at hyperpolarizing voltages when held at –30 mV (middle traces). At –30 mV holding potential, in contrast, the plasma-stimulated current is strongly outwardly rectified. All records were realized according to the reference [35]. Finally, (right traces), addition of the anion channel blocker NPPB (1 µM) inhibits the plasma-stimulated current and only a small fraction of the inwardly rectified current as seen on washed cells in A left panel. **C:** Magnification of the residual currents after 1µM NPPB, showing that the inward current fraction is less sensitive to NPPB. (*all records were realized according to the references [35-38]*). **D:** Current traces recorded on non-transfected and PfPKA-R overexpressing parasite-infected erythrocytes showing that overexpression of PfPKA-R down-regulates the parasite-induced currents. All records were realized according reference [84]. Recording conditions. Seal resistances were 4-20 GOhm. Patch pipettes (tip resistance 10-20 MOhm) were prepared from borosilicate glass capillaries (GC150 TF-10, Clark Medical Instruments, PHYMEP, France) pulled and polished on a Werner Zeitz DMZ programmable puller (Augsburg, Germany). The ruptured patch whole-cell configuration was used to record whole-cell currents. Whole-cell currents were recorded using a RK400 (Biologic, France) amplifier, with voltage command protocols generated and the currents analyzed using the WCP Software (WCP V3.3.3. Software, Strathclyde, UK) by evoking a series of potentials from -100 to +100 mV in 10 mV steps for 500 ms from a holding potential (V_h). All recordings were filtered at 3 kHz with an 8-pole Bessel filter and digitized at 48 kHz.

Indeed, the main obstacle that was encountered for the characterization of the anion channels is the lack of very specific inhibitors of anion transporters [52]. Actually, even dantrolene which was firstly reported as a very specific inhibitor of PSAC [53, 54] inhibits the serum-dependent outwardly rectified component of the malaria-induced currents. Moreover, it is difficult to compare the different IC_{50} (50% inhibitory concentration) obtained using the different configurations of the patch-clamp techniques, with the values acquired using fluxes experiments or isosmotic haemolysis techniques. One has to keep in mind that in fluxes experiments, the membrane potential is the normal resting membrane potential, while in isosmotic haemolysis the cells are depolarised due to the replacement of extracellular charges by sorbitol. Similarly, the pharmacology profiles obtained using patch-clamp technique are measured at potentials far above or below the normal resting membrane potential, rendering their comparisons with other techniques difficult. Furthermore, the efficiency of a compound to inhibit channel can be different if used on the extracellular or the intracellular face of the channels ; and we cannot rule out that the differences observed regarding the IC_{50} (e.g. for NPPB and furosemide) [37, 41] for the inward and outward currents correlate to voltage dependent effects of such compounds, meaning that the potency of the compound is dependent on the driving force.

However to sum up knowledge on the different anion conductances in infected cells, strong evidences suggest that the channels responsible for the outwardly rectifying current phenotype are responsible for the passage of organic osmolytes [40, 55-58] and that these channels have common characteristics with the outwardly rectifying chloride channels (ORCC), previously described in non-infected RBC [38, 49, 58]. Furthermore, it has been shown that at least two different types of channels generate the inwardly rectifying currents [50, 59]. One type resembles the chloride channels 2 (ClC2) and the other the cystic fibrosis transmembrane regulator (CFTR), a well known cAMP dependent channel, or a channel apparently dependent on the cAMP signalling pathway called originally IRC for Inwardly Rectified Channel.

In cell-attached configuration, among the three types of channels identified in infected RBC's membrane [49], the small conductance channel SCC displays in supra-physiological ionic concentrations the same conductance and type of gating as PSAC (as reported by Desai and co-workers [60]) indicating that PSAC is therefore the supra-physiological correlate of the SCC. Moreover, the channels carrying the whole-cell current in supra-physiological conditions (*i.e.* SCC-PSAC), are inhibited by Zn^{2+} suggesting that SCC-PSAC is a ClC-2 channel [59, 61]. In physiological solutions 80% of the membrane conductance in infected cells can be accounted for by IRC and 20% by SCC whereas in supra-physiological conditions (1.1M Cl^-) the membrane conductance is almost exclusively carried by SCC (PSAC) as the IRC is functionally turned off. The channels carrying the whole-cell current in physiological conditions (predominantly IRC) display a higher sensitivity to NPPB and furosemide than the channels carrying the whole-cell current in supra-physiological conditions (*i.e.* SCC-PSAC-ClC2).

POSSIBLE REGULATION MECHANISMS OF ENDOGENOUS ANION CHANNELS

The parasite seems to have found an efficient way to activate dormant anion channels of the red cell membrane, and if the complete signalling pathway is still elusive, important leads have been pursued. Two different facets of the same hypotheses have been suggested. The first proposes that oxidative stress caused by the parasite activates and/or modulates the channels. Indeed, strikingly similar, if not identical, channels can be activated by simply imposing oxidative stress on non-infected RBC [37, 57, 62, 63]. In this review we will focus on the second facet, which proposes that a sudden change in the phosphorylation state of quiescent channels induces their activation. It is well known that post-infection changes in the phosphorylation state of certain host plasma membrane and cytoskeleton proteins occurs [64-66]. Drastic unspecific dephosphorylation is able to reduce the membrane conductance to the level of non infected cells [39, 67]. For now the most relevant kinase seems to be the c-AMP dependent Protein Kinase (PKA). Indeed, inwardly rectified anion current can be activated in whole-cell configuration in non infected cells when PKA and ATP are present in the patch pipette [38].

THE CAMP-DEPENDENT SIGNALLING PATHWAY HYPOTHESIS

The cyclic-AMP (cAMP)-dependent protein kinase, or Protein Kinase A (PKA), belongs to the serine-threonine kinase family and was first characterized as mediating the effects of elevated cAMP levels, which arose after stimulation of cell membrane by beta-adrenergic agonists [68, 69]. The cAMP-dependent signalling pathway is generally activated by the binding of a ligand to a membrane receptor, which then causes dissociation of heterotrimeric G-proteins. One of the monomeric G-protein (presumably Gαs [70]) activates a membrane-bound adenylate cyclase, which causes an increase in intracellular cAMP concentration. cAMP binds to PKA, dissociating the inactive holoenzyme and inducing phosphorylation of downstream targets. In unstimulated cells, the catalytic subunit PKA-C is bound to an inhibitory regulatory subunit (PKA-R) in a tetrameric complex composed of two PKA-R and two PKA-C molecules. Binding of cAMP to the regulatory subunits, each of which containing two cAMP-binding sites, releases the PKA-C subunits, which results in their activation. PKA-C substrates include transcription factors and other proteins involved in developmental processes. Several hundreds of PKA substrates have now been identified in nucleus and in the cytoplasm [71-74]. PKA phosphorylation can trigger any aspect of target protein function such as activity, localization or stability [75], and consequently PKA effects are usually complex.

In addition to serving as inhibitors of the catalytic subunit and receptors for cAMP, the R subunits function as adapters that link the catalytic moiety via a dimerization/docking (D/D) domain to scaffold proteins termed A-kinase anchoring proteins (AKAPs). These AKAPs target PKA-C to specific subsites within the cell. The heat-stable protein kinase inhibitor (PKI) represents another class of physiological

inhibitors of the catalytic subunit. Like the regulatory subunits, PKI binds with high affinity to the free catalytic subunit. PKA-AKAP complexes are multiprotein structures that contain the components to provide exquisite signal feedback control. In addition to binding PKA, most AKAPs also bind phosphodiesterases, which tightly regulate cAMP levels as part of a complex feedback loop. PKA-AKAP complexes are typically localized nearby PKA-regulated targets, including membrane channels and receptors.

It has been known for some time that non-infected erythrocytes have a PKA activity and regulatory RI and RII subunits, with the RI subunit being associated with the plasma membrane [76]. Moreover, recent evidences have been given that the complete cAMP dependent signalling pathway is present in mature erythrocyte [77]. Indeed, the controlled release of ATP from erythrocytes in response to both physiological and pharmacological stimuli has been proven to be dependent on the presence of CFTR in the membrane [78] and involves the signalling cascade including G proteins, adenylate cyclase and PKA [77, 79-83].

Moreover, single channel activity of the IRC on non infected cells can be elicited by activation of the adenylate cyclase using forskolin in the cell-attached configuration, and by exposure to the catalytic subunit of cAMP-dependent protein-kinase in the excised inside-out patches [38, 84]. Since the same manoeuvres also induced, in the membrane of Cystic Fibrosis red cells, activation of Cl^- channels with similar conductance, pharmacology and ability to transport ATP, it was concluded that these data support a dual channel model in which CFTR and the recorded activities constitute separate molecular identities but are coupled functionally and that cAMP signalling pathway should play a pivotal role in their activation. Nevertheless, the channels found in control and CF RBCs differed by their respective kinetics and gating properties [84]. This fits with the observation by Verloo and co-workers of low level of CFTR mRNA in nucleated precursor cells [39] and the reduced number of CFTR molecules in CF cells [85, 86].

In infected cells, dephosphorylation abolishes malaria-induced membrane currents and the natural heat-stable inhibitor of PKA is able to ablate part of the membrane currents [87], thus confirming the implication of this pathway in the induction of membrane currents upon infection.

In this context, it must be noted that *P. falciparum* also possesses a cAMP-dependent signalling pathway. In contrast to mammalian cells that have several PKA-C and PKA-R

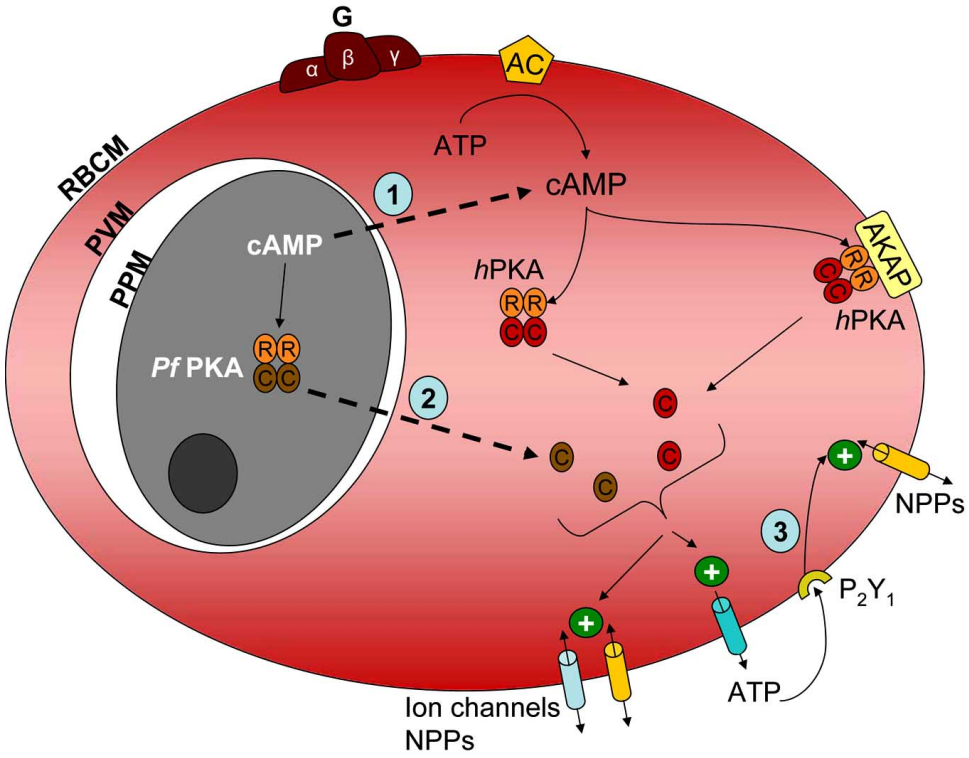

Fig. (2). Model for activation of NPPs via PKA-dependent signalling.

Erythrocyte and parasite both possess elements of the cAMP signalling pathway. During infection, different scenario could involve this pathway in NPPs activation. (1) cAMP, abundant in the parasite cytoplasm, could be exported to the erythrocyte cytoplasm and lead to the activation of hPKA-C. (2) Even if lacking a PEXEL motif, *Pf*PKA-C could be exported to the host cell cytoplasm via specific transporters. These two alternatives lead to a pool of active PKA-C (human or plasmodial). They could directly phosphorylate endogenous channel becoming the active NPPs. In a sequential model (3), these active kinases could then phosphorylate a channel that facilitate ATP release, that would act as an autocrine signal and lead *via* purinergic receptors (likely P2Y1) and intracellular cascade to the activation of the NPPs. *Abbreviations*: G, Heterotrimeric G-Protein; AC, adenylate cyclase; AKAP, A-kinase anchoring protein; ATP, adenosine triphosphate; cAMP, 3'5'-adenosine monophosphate; RBCM, red blood cell membrane; PVM, parasitophorous vacuolar membrane; PPM, parasite plasma membrane; hPKA, human protein kinase A; PfPKA, *P. falciparum* protein kinase A; R, regulatory subunit; C, catalytic subunit; NPPs, new permeation pathways.

subunits, the cAMP signalling in *P. falciparum* is extremely reduced, as the parasite possesses only a single regulatory subunit in addition of its single PKA-C subunit [87-89].

Indeed some experiments have shown the implication of the plasmodial cAMP-dependent pathways in regulation of the host cell membrane conductance. Overexpression of the *P. falciparum* regulatory subunit in transgenic parasites, or addition of recombinant PfPKA-R to the pipette in patch-clamp experiments, significantly down-regulates the conductance of one of the erythrocyte plasma membrane anion channels that has been previously described at the single channel level (Fig. **1D**).

To explain the mechanism of the indirect activation of anion conductance several hypotheses are possible. First, PfPKA-C is exported to the erythrocyte cytosol, where it phosphorylates proteins of human or parasite origin. These proteins could be transporter activators, or inhibitors, or other members of an intracellular signalling cascade that results in anion transport activation. Another possibility is that part of the cAMP produced by the parasite is exported into the host cytosol via a specific transporter and acts directly on the host PKA. Thus, after overexpression PfPKA-R could reduce available cAMP levels, not only within the parasite, but also eventually in the erythrocyte cytosol, as cAMP in infected RBC is mostly synthesized by the parasite. In this scenario, overexpression of PfPKA-R acts as a cAMP sink, as observed in the case of cGMP and the cGMP-specific phosphodiesterase PDE5 in mammals [90], thereby reducing overall erythrocyte PKA activity. Overexpression of PfPKA-R consistently leads to anion conductance inhibition and reduced parasite growth as well as delayed the half time of isoosmotic sorbitol haemolysis. In all the above hypotheses, PfPKA appears to be a key regulator of *P. falciparum* development and anion transport across the erythrocyte membrane. Whatever the exact mechanism leading to channels activation, oxidation and phosphorylation are related phenomena, as in numerous eukaryotic cells generation of reactive oxygen intermediates triggers the activation of signalling pathways via kinases [91, 92]. This is particularly relevant as some of these channels are usually associated with the release of ATP as part of an autocrine or paracrine signalling pathway involving purinergic receptors. In this context, induction of osmolyte permeability in *Plasmodium*-infected erythrocytes involves autocrine purinoreceptor signalling (P2Y1 receptor) [93, 94], which is present in the RBC membrane [95-97] (Fig. **2**). Thus, the cAMP-dependent signalling pathway and PKA are of high interest since they could be a potential therapeutic target by blocking the NPPs function and thus preventing parasites blood stage amplification.

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