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# USE OF PHARMACOLOGICAL AGENTS TO IMPLICATE A ROLE FOR PHOSPHOINOSITIDE HYDROLYSIS PRODUCTS IN MALARIA GAMETE FORMATION

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**Abstract**—The kinetics of phosphoinositol 4,5 bisphosphate hydrolysis products in activated *Plasmodium falciparum* gametocytes suggests a role for inositol trisphosphate [Ins(1,4,5)$P_3$] and diacylglycerol (DAG) in the signal transduction pathway of malaria gametocytes. To investigate further this role, compounds that have an effect on the metabolism and biologic functions of these second messengers were tested in an *in vitro* system. Gentamycin, 2,3 diphosphoglycerate (2,3 DPG) and magnesium ion ($Mg^{2+}$), inhibitors of Ins(1,4,5)$P_3$ 5′phosphatase, all stimulated gametocytes to exflagellate in suspended animation buffer, pH 7.4, at room temperature. In addition, methylxanthines, caffeine and theobromine, calcium ionophore (A-23187), and external calcium also stimulated exflagellation. In contrast, neomycin, an aminoglycoside that inhibits phospholipase C activity, and heparin, an antagonist of Ins(1,4,5)$P_3$ binding to its receptor, inhibited microgamete formation. Quinine and chloroquine which can inhibit both phospholipase A and C activity also inhibited gametocyte exflagellation. The consistent manner in which these various compounds affect gametocyte activation further implicates phosphoinositol turnover in the signal transduction pathway of falciparum gametocytes.

The sexual cycle of the malaria parasite commences when activated gametocytes transform into motile male microgametes and non-motile female macrogametes. Even though this process, termed exflagellation, can take place *in vitro*, it is the formation of gametes in the gut of a vector mosquito that is a key to the transmission of this disease [1]. We recently showed that elevated temperature (37°) is primarily responsible for holding mature gametocytes inactive in culture and the drop in temperature that follows exposure to ambient conditions triggers exflagellation [2]. However, the mechanisms by which this temperature change is transduced across the membranes of gametocytes still remain undefined. Experiments in our laboratory have also identified the production and rapid degradation of second messenger molecules associated with the phosphoinositol 4,5 bisphosphate (PI¶) cycle in activated gametocytes [3]. In order to define further a role for PI hydrolysis products in the malaria gametocyte signal transduction pathway, the effect on exflagellation of compounds that modulate key intermediates of the PI cycle has been evaluated.

Figure 1 is a schematic representation of a receptor mediated PI-linked signal transduction system. The receptor (R), upon binding its specific ligand, activates membrane associated phospholipase C (PL-C) via G-proteins. PL-C then cleaves PI releasing inositol trisphosphate [Ins(1,4,5)$P_3$] and diacylglycerol (DAG) into the cell cytosol [4, 5]. Ins(1,4,5)$P_3$ binds to its receptor and releases membrane bound calcium [6–8] and the subsequent rise in intracellular calcium [$CA^{2+}$]$_i$ initiates cell shape changes, secretion, proliferation and/or endocytosis [9]. Similar cell functions can alternatively be accomplished through DAG which activates the cell via protein kinase C [10, 11]. The biological effects of DAG can be reproduced by its lipid soluble analog, 1-oleoyl-2-acetyl-glycerol, (OAG) [12]. Once Ins(1,4,5)$P_3$ is formed and transduces the signal, it is immediately degraded into non-physiologically active inositol phosphates by two separate pathways [13]. In the first pathway, Ins(1,4,5)$P_3$ 5′-phosphatase dephosphorylates the second messenger molecule into Ins(1,4)$P_2$ and finally to Ins(4)P. Magnesium ($Mg^{2+}$), 2,3-diphosphoglycerate (2,3 DPG) and gentamycin have been used in other systems to inhibit breakdown by Ins(1,4,5)$P_3$ 5′-phosphatase [14]. The second pathway, unlike the first, is dependent on the action of a 5′-kinase which further phosphorylates Ins(1,4,5)$P_3$ into inositol tetrakis-phosphate (Ins(1,3,4,5)$P_4$). In some systems the tetrakis-phosphate augments the calcium signal by recruiting $Ca^{2+}$ from Ins(1,4,5)$P_3$ insensitive to sensitive pools [15]. Calcium ionophore A-23187

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¶ Abbreviations: PI, phosphoinositol 4,5 bisphosphate; PL-C, phospholipase C; Ins(1,4,5)$P_3$, inositol trisphosphate; DAG, diacylglycerol; [$Ca^{2+}$]$_i$, intracellular calcium; [$Ca^{2+}$]$_o$, extracellular calcium; OAG, 1-oleoyl-2-acetyl-glycerol; PL-A, phospholipase A; COM, centers of movement; PDE, phosphodiesterase; Ins(1,3,4,5)$P_4$, inositol tetrakis-phosphate; GPIP$_2$, glycerophosphatidylinositol 4,5 bisphosphate.

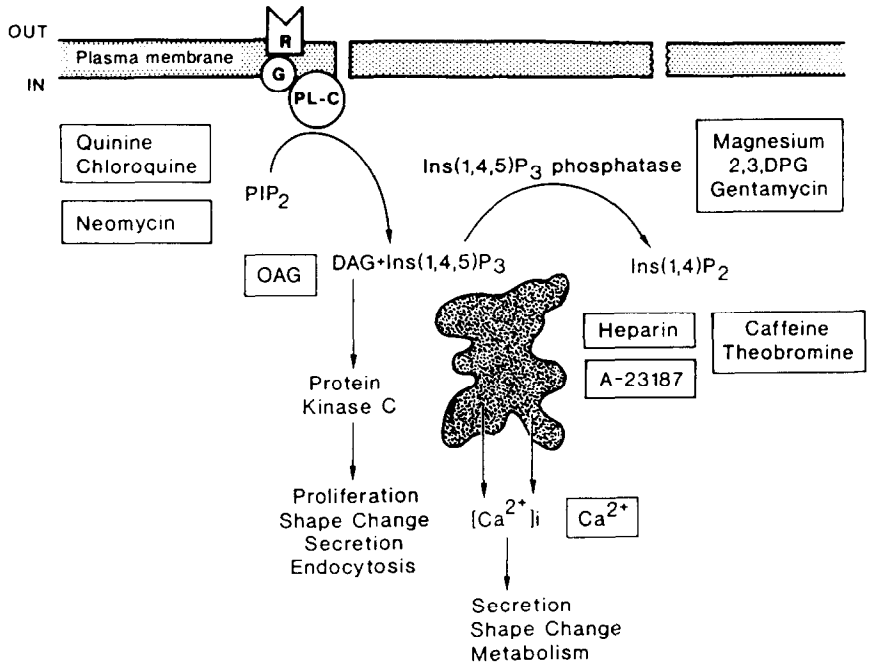


Fig. 1. Scheme of a receptor activated phosphoinositide-linked signal transduction system.

[16] can release membrane bound $Ca^{2+}$ while heparin [17] abrogates its release by competitively binding to the receptor of $Ins(1,4,5)P_3$. Neomycin inhibits the action of PL-C [18–20] and the formation of either second messenger. Glycerophosphatidylinositol 4,5 bisphosphate ($GPIP_2$) has also been detected in activated malaria gametocytes [3]. However, unlike the other metabolites which are released by the action of PL-C on phosphoinositides, this product results from the removal of both fatty acids by PL-$A_2$ and -$A_1$ leaving the glycerol backbone with a *sn*-3 phosphodiester linkage to inositol or its polyphosphates. Antimalarial drugs chloroquine and quinine, which have been described as having PL-A [19] and PL-C [21] inhibitory activity were used to inhibit the formation of $GPIP_2$, and $Ins(1,4,5)P_3$, respectively.

In the event that these unrelated compounds affect exflagellation in a consistent manner, it would serve as further evidence for a role for PI hydrolysis products in the signal transduction pathway of falciparum gametocytes.

## MATERIALS AND METHODS

*P. falciparum*, NF54, K-67 or JP 119, strains were cultured *in vitro* by the method of Trager and Jensen [20] as modified by Ifediba and Vanderberg [22]. Blood group O positive erythrocytes were used to set cultures at 6% hematocrit, 0.2% parasitemia in 25-$cm^2$ canted neck flasks flushed with a gas mixture of 3% $CO_2$, 5% $O_2$ and 92% $N_2$ (East Africa Oxygen, Nairobi, Kenya) and incubated at 37°. Parasites were maintained in culture for approximately 2 weeks in RPMI 1640 with L-glutamine (Gibco Laboratories, Grand Island, NY, U.S.A.), 25 mM Hepes, 25 mM sodium bicarbonate, 50 μg/mL hypoxanthine (the Sigma Chemical Co., St Louis, MO, U.S.A.) and 10% heat inactivated human serum with daily medium changes.

*Induction of suspended animation.* Suspended animation buffer containing RPMI 1640, 25 mM Hepes, 1 μM $CaCl_2$ and 1% decarbonated serum, pH 7.4, was prepared and warmed to 37°. All test solutions and dilutions were made using this buffer. Gametocytes in culture medium were tested daily for exflagellation by exposure to ambient conditions in a petri dish.

When good exflagellation was noted, spent medium was removed as per routine medium change and warm suspended animation buffer immediately added so as to achieve at least a 1:100 times dilution. Gametocytes were spun at 37° and the pellet resuspended in warm suspended animation buffer. Gametocytes prepared in this manner can be held for over 24 hr at 37° before testing without loss of exflagellating capacity. The pH of all test solutions was tested and adjusted with 1 N sodium hydroxide or hydrochloric acid just prior to use. Aliquots of gametocytes to be tested were placed in 3.0-mL borosilicate tubes, rapidly spun at 500 *g* for 30 sec in a Dade serofuge and supernatant decanted. The gametocyte pellet was suspended in 2 mL of the test solution and respun for 30 sec. The test solution was decanted, a hanging drop prepared on a Vaseline rimmed cover slip and placed on a microscope slide for examination.

*Scoring of exflagellation.* Slides were then viewed under phase contrast microscopy (40×) with closed circuit television. For TV viewing, a 1 kappa S-VHS

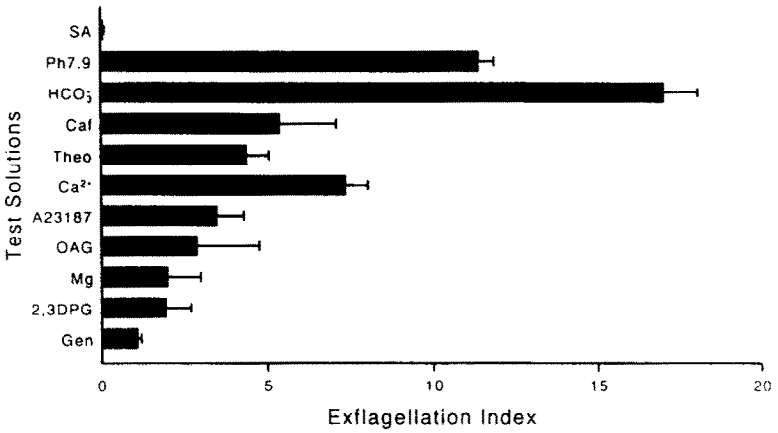


Fig. 2. Rank order of triggers of exflagellation using *P. falciparum* (K67) gametocytes, pH 7.4, means ± SD.

TV camera was connected to a Carl Zeiss Axioskop research teaching microscope and a CFM/15/2 high resolution 14″ color monitor with S-VHS input. More than one investigator counted the number of centers of movement (COM) per unit screen for 10 random screens per slide. The following scoring system was used: 0, no COM per 10 screens; 1, an occasional COM per 10 screens; 5, 0–1 COM per screen; 10, 1–2 COM per screen; 20, 2–3 COMs per screen; 40, 3–4 COMs per screen; and 80, >4 COMs per screen. A COM can indicate the presence of either a single microgamete or an exflagellating body with several microgametes. Each slide was scored sequentially every 5 min for 30 min and the average of the highest three scores taken as the exflagellation index. After each experiment, slides with a score of zero were thoroughly scanned for the presence of an occasional COM, the presence of which changed the score from 0 to 1. Each experiment was repeated at least three times before being accepted.

## RESULTS

### *Ins*(1,4,5)$P_3$ *5′-phosphatase inhibitors*

2,3 DPG (2.5 mM), Fig. 2, gentamycin (0.05–2 mM), and $Mg^{2+}$ (0.5–2 mM), Fig. 3, activated gametocytes held in suspended animation solution, pH 7.4. Magnesium ion induced exflagellation in a biphasic manner and the reason for this observed biphasic response is presently unknown.

### *Compounds that modulate* $[Ca^{2+}]_i$

The exflagellation index increased with increasing extracellular calcium concentrations $[Ca^{2+}]_o$ up to an optimum of 2.5 mM, Fig. 3. Thereafter, further increases in $[Ca^{2+}]_o$ resulted in a decrease in the exflagellation index. Since $[Ca^{2+}]_o$ concentrations above 10 mM were lytic to red cells, the decrease in exflagellation index above this concentration could have been due to gametocyte toxicity. Heparin (10 μg/mL), attenuated the ability of triggers to induce exflagellation, Fig. 4. A combination of heparin with bicarbonate, caffeine and gentamycin caused partial inhibition while with 2,3 DPG and magnesium inhibition was complete. This was probably by blocking the release of intracellular membrane bound $Ca^{2+}$. Calcium ionophore A-23187 (2 μM), theobromine (5.0 mM) and caffeine (5.0 mM) activated gametocytes in suspended animation buffer, pH 7.4, Fig. 2. Even though the ionophore activated gametocytes at micromolar concentrations, the exflagellation index was lower than that observed with the methylxanthines.

### *Compounds that inhibit PI hydrolysis*

Addition of neomycin (2 mM), which inhibits PL-C activity [21, 23, 18], did not completely suppress exflagellation caused by the triggers, but, rather downgraded the effectiveness of the triggers to induce gamete formation, except for gentamycin where there was 100% inhibition, Fig. 4. In contrast to gentamycin, addition of neomycin to suspended animation buffer did not activate gametocytes, suggesting that neomycin and gentamycin may act at different points in the signal transduction pathway of gametocytes. Alternatively, chloroquine (0.5 mM), and quinine (0.25 mM), data not shown, completely obliterated the ability of triggers to induce exflagellation. In order to eliminate the possibility that this inhibition was due to antimalarial drug toxicity, gametocytes were incubated for 1 hr in suspended animation buffer containing similar concentrations of each drug. Thereafter, gametocytes were washed with warm suspended animation buffer and activated using alkaline pH and bicarbonate ion as triggers. Not only did the washed gametocytes respond to these triggers but they also exflagellated in suspended animation buffer.

### *DAG analog*

Lipid soluble DAG analog, OAG, induced gamete formation at 1 μM concentration in suspended animation solution, pH 7.4, Fig. 2. The exflagellation indices were low and comparable to those observed with A-23187.

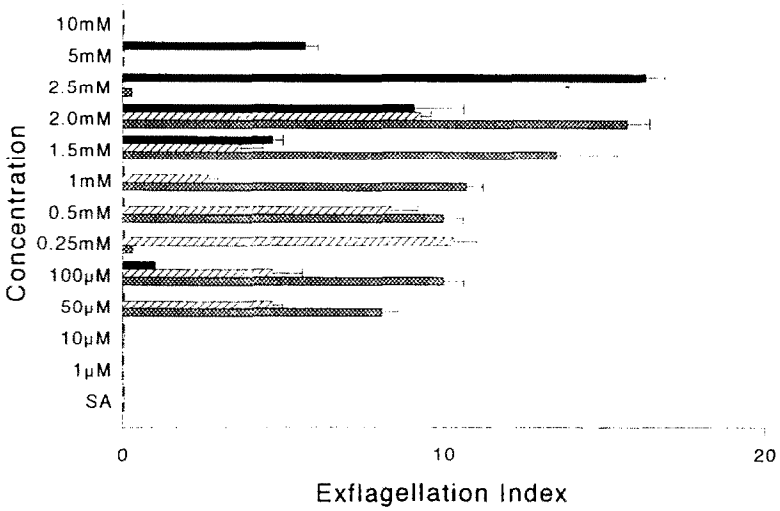


Fig. 3. Effect of calcium ion (■), magnesium ion (□) and gentamycin (□) on exflagellation of *P. falciparum* gametocytes, pH 7.4, means ± SD.

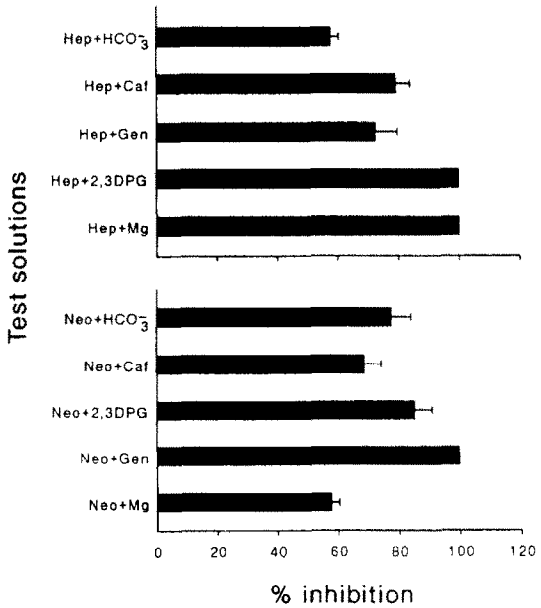


Fig. 4. Effect of heparin and neomycin on exflagellation of *P. falciparum* gametocytes, pH 7.4, means ± SD.

*Ranking of triggers of exflagellation*

Any compound or condition that activated gametocytes in suspended animation buffer, pH 7.4 was considered a trigger for exflagellation. The triggers were ranked by comparing their exflagellation indices in one isolate K67 where three replicate tests were done, Fig. 2. Our results indicate that alkaline pH and bicarbonate ion, physiologic triggers, were the most effective, and Ins(1,4,5)$P_3$ 5′-phosphatase inhibitors the least effective triggers of malaria gametogenesis. The other triggers, $[Ca^{2+}]_o$, the methylxanthines, A-23187, and OAG ranked intermediate between the physiologic triggers and the 5′-phosphatase inhibitors.

## DISCUSSION

Martin *et al.* [3] had earlier demonstrated that both Ins(1,4,5)$P_3$ and DAG are present in activated falciparum gametocytes and the kinetics of their metabolic products were consistent with a second messenger function. Our present results provide further evidence that PI hydrolysis products may indeed be playing a role in gametocyte signal transduction. There are major drawbacks to using chemical compounds and drugs to probe biochemical pathways, since these compounds may affect the given system in different ways than intended. Nevertheless, these tools can be used to provide preliminary, suggestive or corroborative evidence to implicate a specific biochemical pathway. For example, gentamycin and neomycin that belong to the same class of drugs, aminoglycosides, act at different points in the metabolism of PI [18, 21, 23]. Hence, the observation that these two aminoglycosides affect exflagellation in a manner consistent with their action on the PI cycle is suggestive of a role for the PI cycle in *P. falciparum* gametogenesis. Even though most of our data maintain this internal consistency, there are some results that are more difficult to explain. For instance, methylxanthines, caffeine and theobromine are cAMP phosphodiesterase (PDE) inhibitors and are not known to have direct actions on PI metabolism. They were included in this study because of their known action on *P. gallinaceum* gametocytes [24]. Despite the fact that these methylxanthines are triggers for exflagellation in both *P. gallinaceum* and *P. falciparum* gametocytes, the preponderance of evidence is against a second messenger role for

cAMP [25]. Other cAMP-PDE inhibitors like 8-bromo-cAMP and Squibb 20009 stimulate gametocytes, but dibutyryl cAMP has no action on gallinaceum [24] and berghei [25] gametocytes. Therefore, the above cAMP-PDE inhibitors may have activities different from their PDE inhibitory action by which they affect gametocytes. We do not know the nature of these mechanisms but have grouped the methylxanthines with compounds that modulate $[Ca^{2+}]_i$ because caffeine induces tetany in muscle by releasing bound calcium from the sarcoplasmic reticulum [26].

In other signal transduction systems, Ins(1,4,5)$P_3$ modulates cellular functions by releasing bound calcium and increasing $[Ca^{2+}]_i$. Our data is consistent with this action since, heparin competes at the Ins(1,4,5)$P_3$ receptor to inhibit $Ca^{2+}$ release and also downgrades exflagellation in falciparum gametocytes. Additionally, gametocytes exflagellate in suspended animation buffer when $[Ca^{2+}]_o$ is increased indicating that a simple rise in $[Ca^{2+}]_i$ is sufficient to activate gametocytes. In some systems (e.g., human erythrocytes) a high level of $Ca^{2+}$ could itself lead to PI breakdown and the formation of Ins(1,4,5)$P_3$ [23].

Kawamoto *et al.* report that A-23187 blocks the activation of *P. falciparum* gametocytes at pH 8.0 [25] whereas in our experiments, gametocytes were activated by this ionophore at pH 7.4. The reason for this discrepancy may lie in the use of multiple triggers. Since alkaline pH is a potent trigger of falciparum gametogenesis, the addition of another trigger, A-23187, can engender unpredictable results. We observed a similar occurrence in our mixed trigger experiments. The combination of magnesium and bicarbonate ion is one of the most potent inducers of falciparum gametocyte exflagellation at pH 7.4. However, at pH 8.0, magnesium and bicarbonate ion completely inhibit exflagellation. In view of these findings, we tested each potential trigger one at a time at pH 7.4 in the complete absence of bicarbonate ion.

Chloroquine and quinine completely and neomycin partially block triggers from activating gametocytes. Even though these antimalarials have been described as having PL-C activity [18], they are also regarded as PL-A inhibitors [19]. Previous experiments in our laboratory had provided preliminary evidence of PL-A activity in activated gametocytes [3] by showing high levels of $GPIP_2$ in samples taken at the peak of exflagellation. $GPIP_2$ is the product of PL-$A_1$ and PL-$A_2$ enzyme action on PI. Arachidonic acid, the other product of PL-$A_2$ activity in PI is the substrate for the biosynthesis of eicosanoids and these biologically active lipids are known to modulate key local cellular functions [27]. Even though our data may be suggestive, proof for the involvement of these biochemical pathways in plasmodial gametocyte signal transduction pathways must await direct measurement of the relevant mediators in activated gametocytes.

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