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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The purpose of this research project is to characterize the newly identified potassium channel genes from *Plasmodium falciparum* and determine if they may be exploited as chemotherapeutic targets. The research plan. included: complete cloning of two *P. falciparum* K^+ channels; subcloning of channel genes for expression and epitope tagging; expression of the channel genes for biophysical, pharmacological and biochemical analyses; generation of specific antibodies to channel proteins for analysis of channels in vivo; and pharmacological analysis of specific K^+ channel blockers as anti-malaria agents. In the first year of this project we have made considerable progress in reaching these goals. We have completed the cloning and subcloning of both K^+ channel genes from *P. falciparum,* PFK1 and PFK2. We have expressed booth genes and identified protein products in bacterial and mammalian heterologous expression systems. We have generated specific antibodies using recombinant proteins as antigens. These antibodies recognize the appropriate proteins by Western blot analysis. We have screened a panel of K^+ channel blockers and have consistent data on their anti-malarial activity. We are encouraged by the progress this year and are confident of further success and reaching our stated goals as we continue with this project.

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INTRODUCTION:

The purpose of this research project is to characterize the new gene products that we have identified in *Plasmodium falciparum* as potential potassium channels and to determine if they may be exploited as chemotherapeutic targets. Our planned work included: complete cloning of two *P. falciparum* K^+ channels; subcloning of channel genes for expression and epitope tagging; expression of the channel genes for biophysical, pharmacological and biochemical analyses; generation of specific antibodies to channel proteins for analysis of channels in vivo; and pharmacological analysis of specific K^+ channel blockers as anti-malaria agents. This report describes the progress we have made towards our goals in the first year of funding.

BODY:

Approved Statement of Work

Items to be begun in year one:

1) *Complete the cloning of PFK2 cDNA and construct expression plasmids as above.*

Using both RT-PCR and PCR from *P. falciparum* genomic DNA we have successfully cloned the full length of the second identified K^+ channel gene, PFK2. We have subsequently cloned this cDNA into a variety of expression vectors for use in heterologous functional expression in mammalian cell lines and *Xenopus* oocytes. Moreover, we have clone PFK2 cDNA into vectors that result in epitope-tagged fusion proteins (FLAG-tag) enabling us to assay protein expression via commercially available antibodies raised against the FLAG epitope. For this specific aim and aims #4 & #6 (below) we have hired a post-doctoral trainee, Dr. Karena Waller, who is an expert in molecular and biochemical analysis and manipulations of *P. falciparum*. Dr. Waller will devote 100% of her research time towards this project.

2) *Express each of the four K^+ channel clones in heterologous cell systems for functional assay (electrophysiology) and biochemical detection (immunoblot of epitope tagged recombinant proteins).*

3) *Biophysical (electrophysiology) characterization of the ionic currents generated by each of the four K^+ channel clones expressed in a heterologous system.*

We are presently attempting to transfect cultured cell lines with cDNAs for either PFK1 or PFK2 for functional analysis and biochemical detection. Both CHO and HEK293 cell lines have been used for transfection. We have successfully detected protein expression by use of the anti-FLAG epitope antibody on Western analysis. In both cases (PFK1 and PFK2) we have detected appropriate sized protein bands in transfected cells. Patch clamp analysis of these transfected cells however, has yielded no K^+ currents. We are proceeding though a well-established algorithm that our laboratory employs for channel proteins that are difficult to functionally express. This includes testing a variety of other cell types, varying the temperature of cell incubation, introduction of protease inhibitors to decrease cellular degradation of heterologous proteins, and examining a variety of ionic recording conditions. Another possibility that we will consider is that the

simultaneous expression of both PFK1 and PFK21 may be necessary to obtain functionality. There have been numerous examples of this type of heterologous channel subunit assembly required for function. (1) To test for this possibility we are planning experiments to examine K^+ currents in cells that have been co-transfected with both PFK1 and PFK2.

We are also pursuing heterologous expression of PFK1 and PFK2 by injection of cRNA into *Xenopus* oocytes followed by 2-electrode voltage clamp measurements of K^+ currents. For these specific aims we have brought an M.D./Ph.D. predoctoral student, Sean McBride, into the laboratory to perform biophysical characterization of the PFK1 and PFK2 channels. Mr. McBride has considerable experience in functional expression of ion channels in Xenopus oocytes systems. This work will form part of Mr. McBride's Ph.D. thesis work.

4) *Pharmacological screening of drugs that block the cloned K^+ channel currents as measured in a heterologous expression system.*

Both PFK1 and PFK2 are most homologous to the calcium-activated K+ channel family (KCa channels). To examine the biological significance of KCa channels in *P. falciparum* we performed a drug-sensitivity screen using a variety of K^+ channel blockers that have channel-blocking activity for KCa channels from other organisms. As assessed by ^{3}H-hypoxanthine uptake and Giemsa-stained parasite density, the agents were either inactive or active anti-malarial activities in a dose-dependent fashion. Those exhibiting anti-malarial activity are all small molecule channel blockers that are known to have various degrees of membrane permeability while those failing to inhibit malarial growth are mostly membrane-impermeant peptides. The active drugs included quinidine, clotrimazole, tubocurare, trifluoroperazine, 4-aminopyradine. The peptide channel blockers lacking anti-malarial activity include apamine, charybdotoxin, and iberiotoxin. The IC_{50}s for parasite killing for the effective drugs were all comparable to the known IC_{50}s for K^+ channel block. Taken together, these data support the possibility that calcium-activated K^+ channel(s) may be a pharmacological target for anti-malarial chemotherapeutics.

Methods:

Reagents- Clotrimazole, 4- aminopyradine, haloperidol, trifluoroperazine, and tetraethylammonium chloride were purchased from Sigma. Apamine and tubocurare were purchased from Research Biochemicals International (Natick, MA). Iberiotoxin and charybdotoxin were purchased from Bachem (King of Prussia, PA). [8- ^{3}H]hypoxanthine (31 Ci/mmol) was purchased from Amersham Biosciences.

Plasmodium Cultures- Cultures of *P. falciparum* were grown in human erythrocyte suspensions using RPMI 1640 (Mediatech Inc.) medium supplemented with 10% human AB serum (Gemini Bioproducts), 0.2% glucose, 25mM Hepes, pH 7.4, and 92 μM hypoxanthine and maintained at 37 °C in 5% CO_2/ 5% O_2/ 90% N_2 environment. Parasite cultures were successfully stored and retrieved at –70 °C and subsequently, allowed to undergo one growth cycle (48 hours) before use. Cultures were maintained at 5% hematocrit and between 0.1%-5% parasitemia. Strains used were the chloroquine-sensitive strain 3D7 (kindly provided by Dr. David Fidock, Albert Einstein College of

Medicine) and the chloroquine- resistant strain FCB (kindly provided by Dr. Kasturi Haldar, Northwestern University Medical School). Human erythrocytes were collected from volunteers under protocol CCI number 00-31, MPA, M-1063 of the Committee on Clinical Investigations of the Albert Einstein College of Medicine.

Meaurement of Parasite Growth- Parasite growth was assessed by measuring incorporation of [8- ^{3}H]hypoxanthine into the nucleic acids of the parasites as described previously. (2) Effects of the various K^+ channel blockers on parasite growth were determined by measuring the reduction in incorporation of radiolabel as compared to untreated parasite controls. Assays were performed in triplicate in complete RPMI media but without hypoxanthine supplementation. Assays were done in 96- well plates, each well containing a total volume of 200 µL consisting of 100 µL *P. falciparum* culture at 2.0% hematocrit and 0.1% parasitemia and 100 µL of drug dilution (twice the final concentration) or solvent at the initiation of the experiments. Cells were incubated for 24 hours under the indicated conditions and then incubated for an additional 18 hours with 1 µCi/ well [8- ^{3}H]hypoxanthine. Each drug assay included uninfected parasites, positive control, used to indicate maximal incorporation and as well as uninfected erythrocytes, negative control, used to indicate background incorporation. The wells were harvested onto glass fiber filters using a Tomtec cell harvester, dried, sealed in a sample bag with 5 mL of scintillation fluid, and counted on a Wallac MicroBeta Trilux liquid scintillation counter. Mean incorporation and standard errors were calculated for each drug.

The effect of the K^+ channel blockers on parasite growth was confirmed by counting the parasite density on Giemsa- stained smears of the parasite cultures. Infected cultures of strains, 3D7 and FCB, at approximately 0.1% parasitemia and 1% hematocrit were cultured in the complete media without hypoxanthine, identical to the drug assay conditions, on a 12- well plate challenged with different drugs. 100 µL of each culture was removed every 12 hours for microscopic examination. Approximately half of the media in the wells were exchanged every 24 hours with fresh media containing the respective drug. The following drugs were tested: (1) complete RPMI media without hypoxanthine, (2) complete RPMI media with DMSO, (3) 1.0 µM charybdotoxin, (4) 500 µM tetraethylammonium chloride, (5) 40 µM trifluoroperazine, and (6) 400 µM tubocurare. Approximately 1000 red blood cells were visually inspected per slide. For samples with low parasitemia, an additional 4000 red blood cells were scanned to insure accurate parasite levels. All red blood cells staining for nucleic acids by Giemsa stain were counted as infected red blood cell.

Data Analysis- IC_{50} values represent the molar concentration which decreases [8- ^{3}H]hypoxanthine incorporation by 50% as compared to drug-free controls. Plots of radiolabel incorporation as a function of K^+ channel inhibitor concentrations for each compound were fit to a generalized sigmoidal function using a non-weighted least-squares criterion.[1] The function used was a hyperbolic tangent function with four parameters: $Y_i = (U\text{-}L)/2\ [\ 1 + \tanh\ (\beta \log C - \beta \log X_i)] + L$, where Y_i is the disintegrations per minute of the i^{th} sample, X_i is the concentration of compound corresponding to the i^{th} sample, U is the upper asymptote of the function (approximated by the parasite control mean), L is the lower asymptote of the function (approximated by the non- infected erythrocyte control mean), β is a scaling parameter, and C is the concentration of agent

corresponding to 50% inhibition of the uptake of [8-^{3}H]hypoxanthine . Software package, Kaleidagraph (Synergy Software), was used to fit the data to this model.

Results:

Anti-malarial activity of K^+ channel blocking drugs.

To test for the functional relevance of a BK-like channel in malaria we screened a panel of reagents that are established pore blockers of the calcium-activated K^+ channel class. We determined activity of these reagents using the ^{3}H-hypoxanthine uptake assay in two strains of cultured *P. falciparum*. The reagents that exhibited anti-malarial included the muscle relaxant tubocurare, the antipsychotic drug haloperidol, the calmodulin antagonist trifluoroperazine, the antifungal agent clotrimazole, and the K^+ channel blocker 4-aminopyradine (Figure 1). The reagents that failed to inhibit P. falciparum included the scorpion toxin iberiotoxin, the bee venom toxin apamine, the scorpion toxin charybdotoxin, and tetraethylamonium. The IC_{50} values for the effective reagents are listed in Table1. To confirm that reduction of 3H-hypoxanthine uptake corresponded to anti-malarial effects we examined Giemsa-stained blood smears at various intervals after addition of tubocurare, trifluoroperazine, TEA or charybdotoxin (figure 2). Consistent with the ^{3}H-hypoxanthine uptake data, concentrations of tubocurare and trifluoroperazine that produced maximal inhibition also abolished parasitemia within 12-24 hours. The non-inhibiting reagents charybdotoxin and TEA failed to prevent progression of parasitemia over 72 hours.

Figure 1

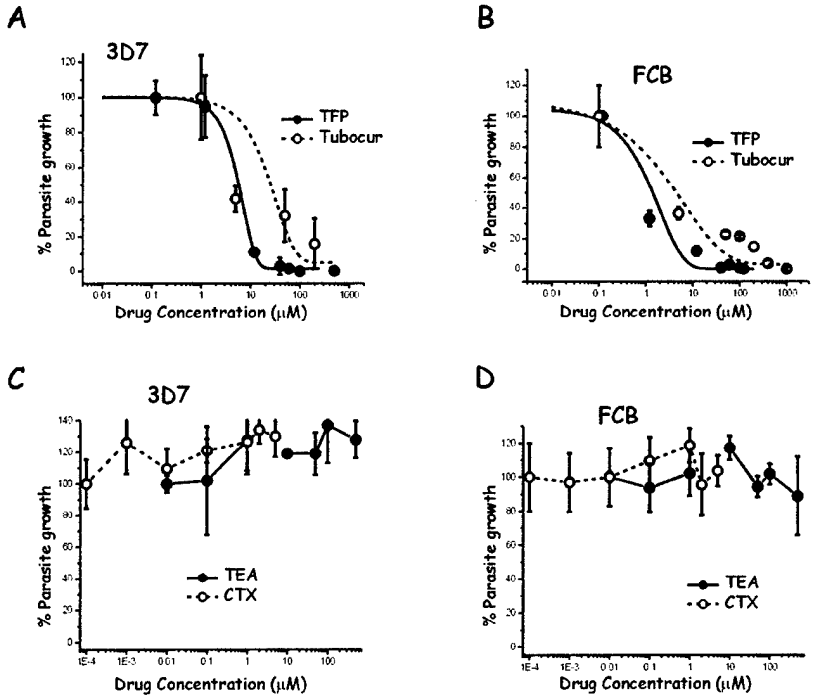
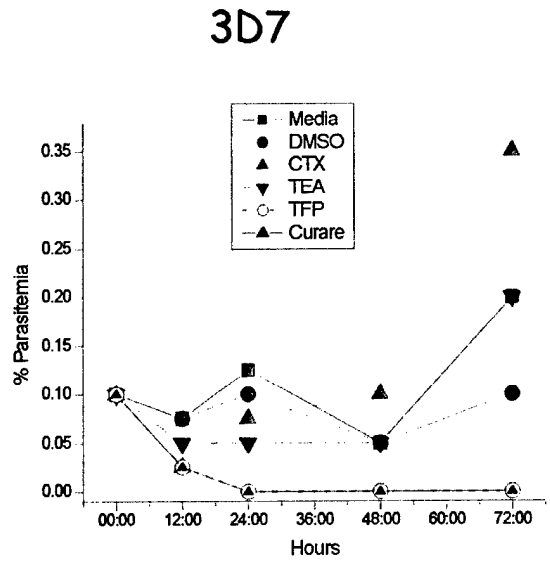


Figure 1: The effect of K^+ channel blockers on *P. falciparum* cultured in human erythrocytes as a function of drug concentration. Dose-response of chloroquine-sensitive, 3D7 (A and C), and a chloroquine-resistant, FCB (B and D) cell lines are shown. Examples of effective agents (Tubocurare and trifluoroperazine, TFP) are shown in A and B. Examples of ineffective drugs (Charybdotoxin, CTX and tetraethylammonium, TEX) are shown in C and D.

Figure 2

A



B

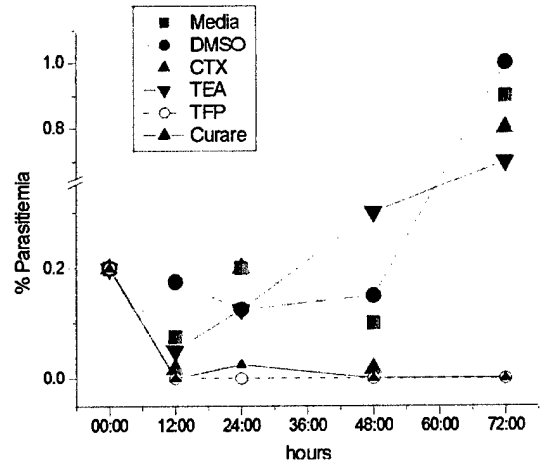


Figure 2: The effect of K^+ channel blockers on cultured *P. falciparum* parasitemia in human erythrocytes. Graphs show percent parasitemia of *P. falciparum* strain 3D7 (panel A) and FCB (panel B) in human erythrocytes measured after incubation with K^+ channel blockers shown to have no effect on parasite growth, charybdotoxin (CTX, green traingle), tetraethylammonium chloride (TEA, violet triangel), or two K^+ channel blockers shown to inhibit parasite growth, tubocurare (Curare, black triangle) and trifluoroperazine (TFP, open circle). Controls were performed with RPMI media without hypoxanthine supplementation (Media, gray square) and the same media plus DMSO (DMSO, red circle).

The data presented here show that several chemical reagents known to block calcium-activated K^+ channels also inhibit growth of cultured parasites. Taken together these results suggest that the gene products of PFK1 and/or PFK2 may be candidate targets for development of chemotherapeutic anti-malarial drugs. In support of this hypothesis is our finding that those reagents that were effect had IC50s similar to the published dose-responses for K^+ channel blocking. (3) It is interesting to note that most of the reagents that were ineffective as anti-malarial agents were peptide toxins whereas the effective drugs were small molecules. This may represent an accessibility factor where the small molecules are more likely to gain access to the parasite channel while the larger peptides may be prevented access by the red blood cell membrane. An alternative

explanation is that the peptide toxins have a high specificity for channel block due to the receptor-ligand nature of the toxin-channel interaction. Such an interaction requires specific structures and amino acid sequences and the presence of additional channel subunits that may not be conserved in the plasmodium channels.

Many mammalian tissues express a form of calcium-activated K+ channel, including the red blood cell. (4) The channel in erythrocytes belong to the intermediate conductance (IKCa) subtype that has a single channel conductance of 18-50 pS, between that of the small conductance (SKCa, i = 6-14pS) and high conductance (BKCa, i = 100-250pS) subtypes. (5) The gene (KCNN4) codes for the IKCa channel and its expression in a variety of cells results in the formation of an intermediate conductance calcium-activated K^+ channel and is alternatively termed SK4, KCa4, IK1, and IKCa1. (6) Our results do not definitively distinguish between block of endogenous erythrocyte channel block and parasite channel block, however the red cell channel is sensitive to charybdotoxin (IC50~2.5nM), an agent that failed to affect parasite growth. Future studies with additional pharmacological agents should help to confirm this distinction.

These results are presently being prepared as a manuscript to be submitted for publication in a peer-reviewed journal.

6) *Generate specific antibodies that recognize each of the four channel proteins by means of inoculating rabbits and mice with K^+ channel-GST fusion proteins.*

We have created GST-fusion vectors that encode fusion proteins of GST with various portions of PFK1 and PFK2. Four fusion proteins for each channel have been produced and inoculated into rabbits as immunizing antigens. So far sera from each rabbit immuno-reacts with appropriately sized bands on Western blot studies. These antisera are being affinity purified for use in analysis of protein expression levels, immunofluorescence and immuno-electron microscopy. These studies will allow us to determine the extent and timing of channel expression in the life-cycle of *P. falciparum.* Moreover we will be able to examine the localization of the channel protein in regards to infected human erythrocytes. These antisera will also serve as verification of heterologous channel protein expression during our functional studies.

In the second year of this project we will continue to pursue these aims as well as begin work on the subsequent approved aims listed below:

5) Biological testing of drugs identified above for anti-parasite activity in cultured Malaria (*Plasmodium falciparum.* Drugs will be assayed for effects on parasite growth, viability, ability to invade host cells, and ability to successfully exit host cells.

7) Perform immuno-biochemical and RNA analyses of the temporal and spatial expression pattern of each of the channels in the life cycle of the parasite.

8) Construct functionally dominant-negative cDNAs of all four clones and test their effects on co-expressed wild type channels in a heterologous expression system.

9) Transfect the dominant-negative channel cDNAs back into their respective parent parasites to analyze the specificity of function of each channel as assayed by parasite viability, growth and invasion.

10) Begin pilot studies for the development of high-throughput screening of large libraries channel-blocking drugs.

KEY RESEARCH ACCOMPLISHMENTS:

- Complete cloning of PFK1 and PFK2
- Epitope tagging PFK1 and PFK2
- Heterologous expression of PFK1 and PFK2 protein
- Initial drug screen identifying KCa channel blockers that inhibit P. falciparum
- Generation of specific antibodies to PFK1 and PFK2

REPORTABLE OUTCOMES:

- Complete cloning of PFK1 and PFK2
- Expression of PFK1 and PFK2
- Pharmacological evidence that KCa-type channels are essential for survival of *P. falciparum*

CONCLUSIONS:

In the first year of this project we have made considerable progress in reaching these goals. We have completed the cloning and subcloning of both K^+ channel genes from *P. falciparum,* PFK1 and PFK2. We have expressed booth genes and identified protein products in bacterial and mammalian heterologous expression systems. We have generated specific antibodies using recombinant proteins as antigens. These antibodies recognize the appropriate proteins by Western blot analysis. We have screened a panel of K^+ channel blockers and have consistent data on their anti-malarial activity. We are encouraged by the progress this year and are confident of further success and reaching our stated goals as we continue with this project.

REFERENCES:

1. J. Bradley, J. Li, N. Davidson, H. A. Lester, K. Zinn, *Proc Natl Acad Sci U S A* **91**, 8890-4. (1994); E. R. Liman, L. B. Buck, *Neuron* **13**, 611-21. (1994); P. Kofuji, N. Davidson, H. A. Lester, *Proc Natl Acad Sci U S A* **92**, 6542-6. (1995); F. Duprat *et al.*, *Biochem Biophys Res Commun* **212**, 657-63. (1995); Y. J. Liao, Y. N. Jan, L. Y. Jan, *J Neurosci* **16**, 7137-50. (1996).

2. R. E. Desjardins, C. J. Canfield, J. D. Haynes, J. D. Chulay, *Antimicrob Agents Chemother* **16**, 710-8. (1979).

3. R. Latorre, A. Oberhauser, P. Labarca, O. Alvarez, *Annu Rev Physiol* **51**, 385-99 (1989); G. J. Kaczorowski, H. G. Knaus, R. J. Leonard, O. B. McManus, M. L. Garcia, *J Bioenerg Biomembr* **28**, 255-67. (1996); V. K. Gribkoff, J. E. Starrett, Jr., S. I. Dworetzky, *Adv Pharmacol* **37**, 319-48 (1997); G. J. Kaczorowski, M. L. Garcia, *Curr Opin Chem Biol* **3**, 448-58. (1999).

4. G. Gárdos, *Biochim. Biophys. Acta.* **30**, 653-654 (1958).

5. C. Vergara, R. Latorre, N. V. Marrion, J. P. Adelman, *Curr Opin Neurobiol* **8**, 321-9. (1998).

6. W. J. Joiner, L. Y. Wang, M. D. Tang, L. K. Kaczmarek, *Proc Natl Acad Sci U S A* **94**, 11013-8. (1997); T. M. Ishii *et al.*, *Proc Natl Acad Sci U S A* **94**, 11651-6. (1997); N. J. Logsdon, J. Kang, J. A. Togo, E. P. Christian, J. Aiyar, *J Biol Chem* **272**, 32723-6. (1997); B. S. Jensen *et al.*, *Am J Physiol* **275**, C848-56. (1998).