Research Article

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Diacylglycerol Signaling Pathway Modulates Membrane Potentials in Red Blood Cells

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ABSTRACT

Objective: *Membrane potential (MP) plays an active role in the excitability of neurons. Several clinical trials have shown that the membrane potentials in red blood cells (RBCs) drawn from three groups of populations including Bipolar Disorder (BD), Attention Deficit Hyperactive Disorder (ADHD) and Negatives (who are neither BD nor ADHD) are significantly different from each other. In neurons, change in potassium conductance is suggested as a plausible reason for the differences in MP among the groups. The objective of this study is to determine if the MP of RBCs respond in a similar manner as neurons to drugs that influence small conductance potassium channels.*

Methods: *Measurements of membrane potential in whole blood cells and statistical analysis were done as described in earlier publications.*

Results: *Promoters and inhibitors of the diacylglycerol (DAG) signaling pathway in the RBCs modulate the small conductance potassium (SK) channels through the critical proteins such as DAG, protein kinase C (PKC), and calmodulin (CAM).*

Conclusions: *The diacylglycerol (DAG) signaling pathway modulates the hSK channels by regulating the critical proteins along this pathway in RBCs. Since hSK family is widely distributed in neurons, the differences observed in RBCs among BDs, ADHDs and Negatives are explained by this finding. Some of the clinical observations can also be explained by this pathway.*

Keywords

Bipolar Disorder, Attention Deficit Hyperactive Disorder, Membrane Potential, Membrane Potential Ratio, RBC, DAG Signaling Pathway, Calcium Activated Potassium Channels.

Abbreviations

(*8-CPT*-cAMP): 8-(4-Chlorophenylthio) adenosine 3',5'-cyclic monophosphate; ADHD: Attention Deficit Hyperactive Disorder; AIP: Autocamtide-2-related Inhibitory Peptide; ALX: 3-[2-[4-[bis(4-Fluorophenyl)methylene]-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H) quinazolinone]; ATP: Adenosine Triphosphate; BD: Bipolar Disorder; CAK: Calcium Activated Potassium Channel; CaM: Calmodulin; cAMP: Cyclic adenosine 3',5'-monophosphate; CLTX: Clotrimazole; DAG: Diacylglycerol; DGK: Diacylglycerol Kinase; DGKH: Diacylglycerol Kinase Eta; EtOH: Ethyl Alcohol; GPCR: G-Protein Coupled Receptors; hSK: human Small Conductance Potassium Channel; IP3: Inositol Triphosphate; KCNN: Potassium Channel NN; MP: Membrane Potential; MPH: Methylphenidate; MPR: Membrane Potential Ratio; PA: Phosphatidic Acid; PIP2: Phosphatidylinositol 4,5-bisphosphate; PKA: Protein Kinase A; PKC: Protein Kinase C; PMA: Phorbol 12-Myristate 13-Acetate; RBC: Red Blood Cells; SNP: Single Nucleotide Polymorphism; uM: Micro Molar.

Introduction

It is generally well recognized that mental disorders are caused by the malfunction of the neurons in the brain [1]. Neurons communicate with each other through electro-biological signals.

These signals are generated and modulated by the membrane potential (MP) and the excitability of the neurons [1]. Recent clinical trials using human whole blood samples [2] have shown that that the MP values are significantly different among the three groups of patients namely Bipolar Disorder (BD) patients, Attention Deficit Hyperactive Disorder (ADHD) patients and the negative group who are neither BD nor ADHD (Figure 1A). Moreover, the membrane potentials of RBCs respond to effective drug treatments. What is the cause of these effects observed in RBCs and how is it related to the disorders of the brain? It is the objective of this investigation to discover the common biological pathway between the RBCs and the neurons in the brain that gives rise to the observed results.

Since the red blood cells (RBCs) form more than 99 percent of the whole blood cells, it follows that the differences observed are mainly due to the mechanisms with in the RBCs. As early as 1958 Gardos discovered that the potassium permeability in RBCs is controlled by the calcium activated potassium channel (CAK) called Gardos channel [3]. This channel has been confirmed as the KCNN4 (also called hSK4) belonging to the family of small conductance potassium channels [4]. The other members of this family of small conductance calcium activated potassium channels including hSK1, hSK2 and hSK3 are widely distributed in the human brain [5]. Grygorczyk et al. [6] showed that the net efflux of K+ is decreased significantly when RBCs are suspended in K+ free buffer, implying that the hSK4 channels are closed in the absence of extra cellular K+. We used K+ free buffers to study the behavior of K+ channels in RBCs.

The calcium activated K channel structure consists of six domains. The pore contains the voltage sensing domain and a pair of transmembrane domains involved in the Ca2+-activated regulation of the K+ conductance. A unique, large, intracellular domain acts as a sensor for the intracellular Ca2+ concentration. The pore formed by the amino acid chain is about 6-8 angstroms through which the K+ ion (4 angstroms) flows. The amino acid configuration and location regulate this flow rate, thereby regulating the excitability of this channel [7]. It is known that any mutations affecting the amino acids forming this pore would affect the K+ currents and excitability [8]. This pore is closed when RBCs are suspended in a K+ free buffer as shown by Grygorczyk et al. [6]. This reduction in the intra cellular K+ concentration hyperpolarizes the cell membrane.

DAG Signaling Pathway

In one of the biological signaling pathways, diacylglycerol functions as a second messenger signaling lipid [9]. DAG is a product of the hydrolysis of the PIP2 (phosphatidyl inositol-bisphosphate) by the enzyme phospholipase C. It produces inositol triphosphate (IP3) through the same reaction. Although IP3 diffuses into the cytosol, diacylglycerol (DAG) remains within the plasma membrane due to its hydrophobic properties. The production of DAG in the membrane facilitates translocation of PKC from the cytosol to the plasma membrane [10]. Hence, both DAG and PKC play important roles in several signal transduction cascades [11]. Calcium activated potassium channels (CAK channels of which hSK4 is a member) are activated by Calmodulin (CaM) [12]. CaM is a widespread and abundant transducer of calcium signaling in cells [13]. It can bind to and regulate a number of different protein targets, thereby affecting many different cellular functions. Calcium gating is the primary mechanism controlling the potassium flow through the pores in the small conductance CAK channels. CaM is responsible for this calcium gating [12]. CaM, in turn, is modulated by the following two important signaling pathways in the cell. The cAMP pathway and the DAG pathway are activated by the G-protein coupled receptors (GPCR) which receive the signal from external stimuli by the ligands [9]. Promoters and inhibitors of the primary proteins were used to determine the pathway controlling the MPs in RBCs.

Methods

The method employed in the current study has been reported previously [2]. A ratiometric method was developed for the purpose of measuring the membrane potential ratios. This ratio method eliminates the distortions of data caused by the photo bleaching and variations in the.

Probe loading and retention, as well as instrumental factors such as illumination stability and sensitivity of the detector. The ratio indicates the differences in the potentials among specific samples in two different buffers (a test buffer and a reference buffer) under identical conditions. The MP in the test buffer divided by the MP in the reference buffer is called the membrane potential ratio (MPR). The reference buffer contained sodium, calcium and glucose at biological concentrations with no potassium. The buffering agent HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) was also added to the buffers to maintain the pH. The test buffer contained ethyl alcohol (EtOH) in addition to sodium, calcium, glucose and Hepes. Addition of EtOH opens the pore and lets K+ ions flow out, thereby reducing the intracellular K+ concentration. Krjnevic [14] speculated that EtOH, sedatives and hypnotic drugs activated the CAK channels. Later, Mustonen et al. showed that the ethanol increased K+ efflux in CAK channels [15]. Mustonen et al. also showed that the ethanol effect was reversed by quinine, a CaK channel blocker. Our experiments with hSK4 blockers, Quinine and Clotrimazole, show that the EtOH effect is reduced by these blockers (Figure 1B). These experiments confirm that EtOH opens the hSK4 and allows the K+ to permeate out of the cell thereby hyperpolarizing the cell membrane. Briefly, whole blood cells with lipid-soluble fluorescent dye, dihexyloxacarbocyanine iodide (DiOC6 [3]) were incubated separately in the reference buffer and the test buffer for 20 minutes. After the blood cells were loaded with the dye, the blood suspension was centrifuged and the excess dye was decanted. The blood cells were resuspended in their respective buffers and distributed in 96 well plates. The membrane potentials were measured in a plate reader (BioTek FLx800) and MPR values were calculated. The statistical method, ANOVA, was used to determine significance and p-value. See Reference (2, 29) for a full discussion of these methods of measurement of the MPR and the statistical analyses used.

Results

cAMP Signaling Pathway

There are two candidate signaling pathways (cAMP signaling pathway and DAG signaling pathway) activating the potassium channel. Cyclic adenosine monophosphate (cAMP, cyclic AMP or 3'-5'-cyclic adenosine monophosphate) is an important second messenger in many biological processes. Adenosine triphosphate (ATP) is the precursor of the cAMP and the cAMP pathway is used for intracellular signal transduction in many different organisms. It works by activating the cAMP-dependent protein kinase called protein kinase A (PKA). The addition of cAMP analog 8-CPT promotes the PKA activity [16]. In order to test if the cAMP pathway is involved in the MPR test, the 8-CPT- cAMP (ENZO Life Sciences, 50 micro molar) was added to the test buffer and the MPR values were determined. There was no effect of 8-CPT on MPR as shown in Figure 1C. This result showed that the cAMP pathway is not involved in the processes affecting the MPR. Then the DAG signaling pathway was investigated by using promoters and inhibitors of the principal proteins along this pathway.

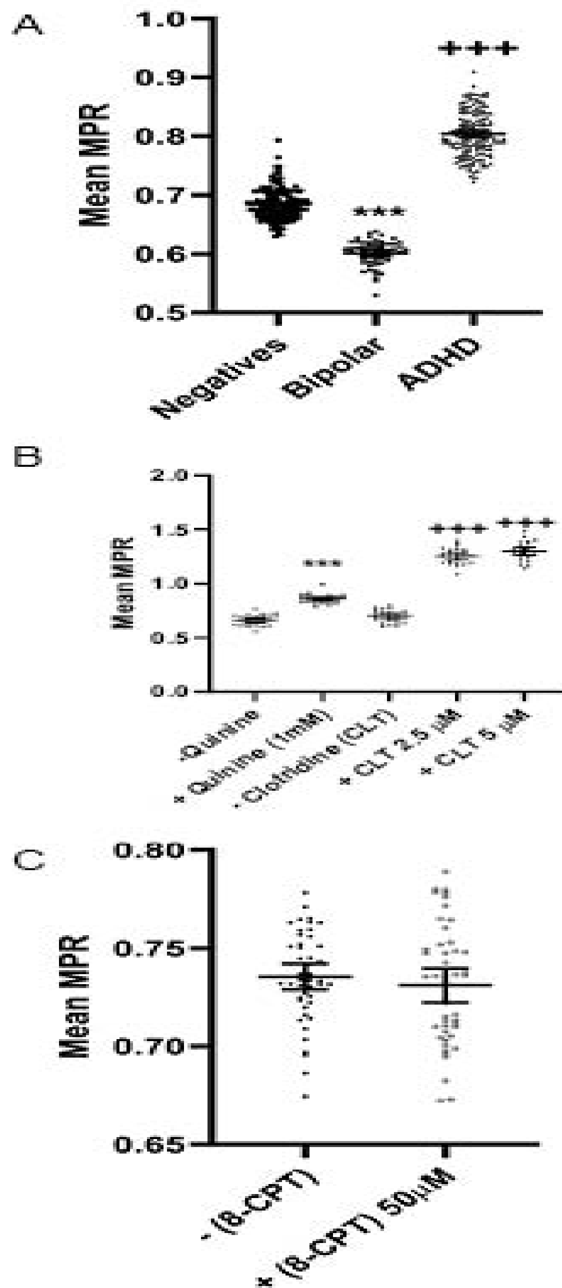

Figure 1: Changes in MPR in RBC. A. The mean values of MPR in the RBCs from the 3 groups patients, BD, ADHD and Negatives. The results showed significant differences (*** BD vs. Negatives; +++ ADHD vs. Negatives) between the groups (***, $^{+++}$ = P<0.001). B. Addition of 1 mM quinine, a CaK channel blocker to the test buffer increased the MPR compared to the buffer without addition of quinine (P<0.001). Similarly, addition of Clotrimazole (CLTX; 2.5 µM) to the test buffer increased the MPR compared to the buffer without addition of CLTX. These results suggest that hSK4 channel blockers regulate MPR in RBCs. C. Addition of 8-CPT-cAMP (50 µM) to the test buffer did not affect MPR. These results suggested that cAMP pathway did not play a role in altered MPR in RBCs.

PKC and Phorbol 12-Myristate 13-Acetate (PMA)

PMA is a diester of phorbol often employed in biomedical research to activate the signaling enzyme protein kinase C (PKC) [17]. In order to see if the activation of PKC has any effect on the MPR, PMA (ENZO Life Sciences) was added to the test buffer and the MPR was measured. As shown in Figure 2A, the MP is indeed depolarized, indicating the involvement of PKC in the hSK4 activation. This figure shows a comparison of the MPR values with 2.5 µM PMA in the test buffer with those values without PMA. PMA depolarizes the cells very effectively (Figure 2A).

Staurosporine

Staurosporine is a natural product originally isolated in 1977 from the bacterium Streptomyces Staurosporeus. The main biological activity of staurosporine is the inhibition of protein kinases, including PKC through the prevention of ATP binding to the kinase [18]. The PKC inhibitor staurosporine (Sigma-Aldrich) was added to the test buffer at three different concentrations and the MPR values were determined as shown in figure 2B. Staurosporine hyperpolarizes the membrane potentials very effectively indicating the role of PKC and the DAG pathway in membrane potentials.

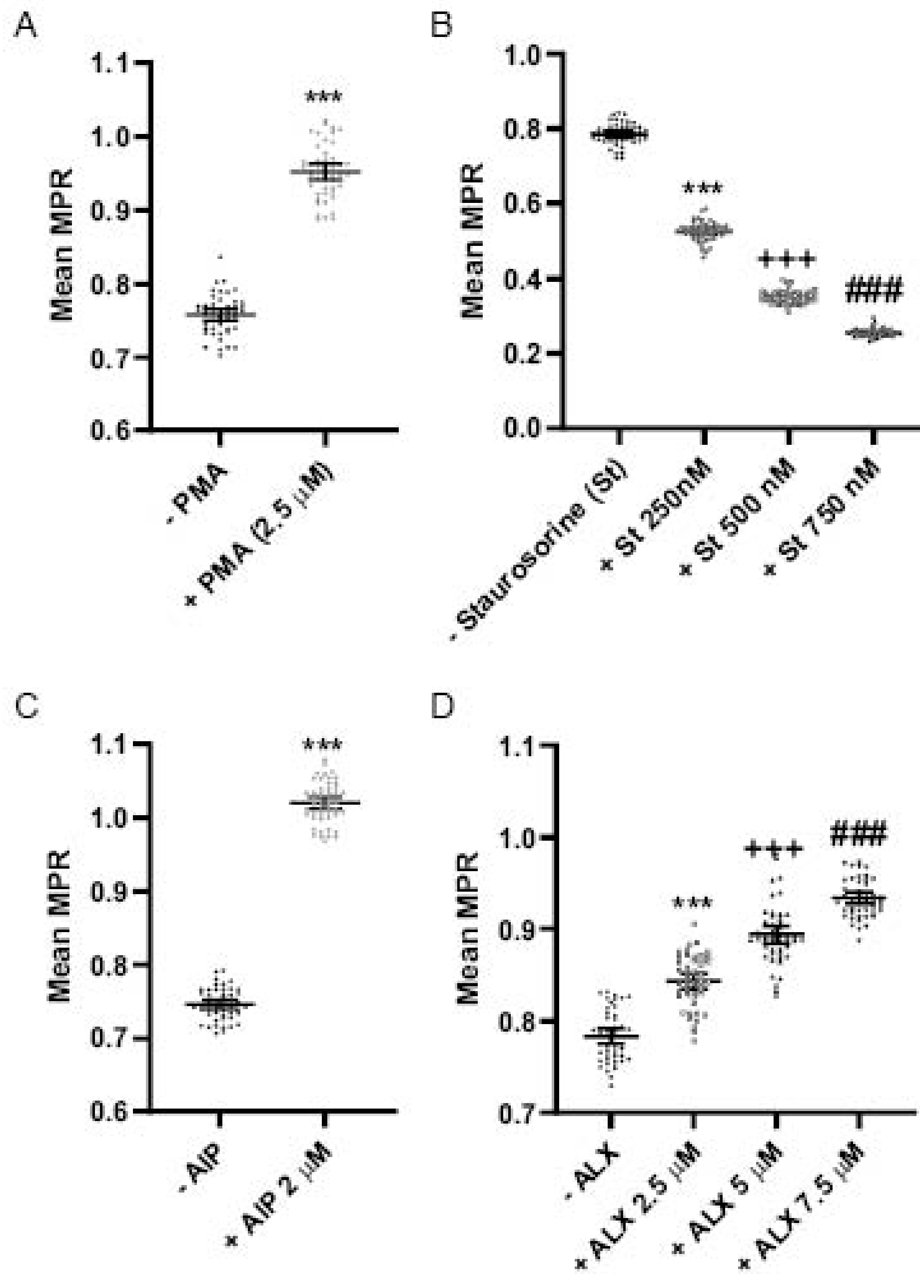

Figure 2: Regulation of MPR by DAG modulators. A. Comparison of MPR with (2.5 µM) and without Phorbol 12-Myristate 13-Acetate (PMA). PMA activates the protein kinase C (PKC) pathway and depolarizes the MPR of RBC effectively ($P<0.001$). B. Addition of PKC inhibitor, staurosporine, at 3 different concentrations [250 nM (***), 500 nM (+++) and 750 nM] hyperpolarizes the MPR of RBCs effectively. C. Addition of CaM Kinase inhibitor AIP (Autocamtide-2-inhibitor related peptide) at 2 µM, depolarizes the RBC membrane potential ($P<0.001$) suggesting a role for DAG signaling pathway in MPR. D. Addition of DAG Kinase inhibitor, ALX, depolarized the MPR ($P<0.001$).

As shown in this figure, staurosporine hyperpolarizes the MP in a concentration dependant manner.

CaM Kinase II
As discussed earlier, calcium gating in small conductance calcium activated potassium channels (CaK channels) is the primary mechanism controlling the potassium flow through the pores. CaM is responsible for this calcium gating. CaM Kinase II is regulated by the Ca2+/calmodulin complex and is involved in many signaling cascades [19]. CaM Kinase II is found in high concentrations in neuronal synapses and it may constitute up to 2% of the total protein content in some regions of the brain. The effects of Ca2+ are also important as it activates CaM Kinase II pathway, in which calcium modulated protein, calmodulin, binds to Ca2+, undergoes a change in conformation, and activates CaM Kinase II. CaM Kinase II has the unique ability to increase its binding affinity to CaM by making CaM unavailable for the activation of other enzymes. In order to see the involvement of Ca2+/CaM/ CaM Kinase II in MPR the CaM Kinase inhibitor was investigated.

CaM Kinase II Inhibitor AIP
A novel synthetic peptide AIP (Autocamtide-2-related Inhibitory Peptide), a nonphosphorylatable analog of autocamtide-2, was found to be a highly specific and potent inhibitor of calmodulin-dependent CaM kinase II [20]. AIP (myristoylated, sold by ENZO Life Sciences) is the same as AIP but is N-terminal myristoylated to increase cell permeability. AIP increases the MPR robustly at a 5 micro M concentration as shown in figure 2C again establishing the DAG pathway as the primary signaling process.

DAG Kinase Inhibitor
The conversion of DAG to phosphatidic acid (PA) utilizing ATP as a source of the phosphate is aided by the Diacylglycerol Kinase (DGK). DGK is a family of enzymes that catalyzes DAG [21]. DGK activity is low in non-stimulated cells, allowing DAG to be used for glycerophospholipid biosynthesis. DGK activity increases on receptor activation of the phosphoinositide/DAG pathway driving the conversion of DAG to PA. DGK occupies an important position as both DAG and PA are thought to function as bioactive lipid signaling molecules with distinct cellular targets. DGK effectively serves as a switch by terminating the signaling of one lipid while simultaneously activating the signaling by another [21]. DAG Kinase inhibitor, ALX-430-028, {ENZO Life Sciences, chemical name 3-[2-[4-[bis (4-Fluorophenyl) methylene]-1-piperidinyl] ethyl]-2, 3-dihydro-2-thioxo-4(1H)-quinazolinone} was found to inhibit diacylglycerol kinase in human red blood cell membranes [21]. As shown in Figure 2D, ALX depolarizes the membrane potential at three different concentrations: 2.5 uM, 5 uM and 7.5 uM.

Discussion
As shown in figure 1A, the MPR values are significantly different for the three groups of patients who participated in the clinical trials [2]. What is the cause of these differences? The DNA from the hSK4 channels from these patients were sequenced and found no mutations. This result led to the alternative mechanisms that may cause the observed differences in MPR among the 3 groups. I tested if the MPR can be altered by manipulation of Calcium activated potassium channels in RBCs. It is generally well recognized that the extracellular ligands activate the g-protein coupled receptors (GPCR) in the membrane of the target cell. The GPCRs transmit these signals into the cell through two primary signal transduction pathways. These pathways process and transmit this signal to the K+ ion channels in its membrane. These two pathways are the cAMP signaling pathway and the DAG signaling pathway [9].

The foregoing results indicate that these differences in the mean values of MPR for the negatives, BDs, and ADHDs arise from the differential modulation of the DAG signaling pathway controlling the hSK4 channels in the RBCs. The selectivity filter of the small conductance potassium channels (SK family) is directly responsible for the selective and rapid conduction of potassium whereas other parts of the protein are thought to function as a molecular gate that either permits or blocks the passage of ions. The selectivity filter of all SK channel subtypes—whether SK1, SK2, SK3, or SK4—is highly conserved and reflects the selectivity seen in any potassium channel [28]. A genome-wide association study implicated the diacylglycerol kinase eta (DGKH) and several other genes in the etiology of bipolar disorder [22]. In this study the authors found that, out of 37 single nucleotide polymorphisms (SNPs) selected for individual genotyping, the strongest association signal was detected at a marker within the first intron of DGKH. Similarly, PKC has been implicated in BD by several authors [23]. Moreover, the important role of the DAG pathway in modulating the membrane potentials and excitabilities in these disorders is a very useful tool in understanding these illnesses. Hokin [24] and his colleagues found that the hydrolysis of the membrane bound phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP2) into IP3 and DAG is promoted by lithium in brain cortex slices in species ranging from mouse to monkey. DAG signaling pathway explains how lithium works in controlling the MP and excitability in BD through the promotion of DAG in this pathway. Cholinergic agonists promote the PIP2 hydrolysis whereas the cholinergic antagonists inhibit this reaction. It is clinically observed that some antidepressants induce mania in depressed BD patients. This well-known observation can also be explained as the effect of cholinergic antagonists in this pathway [25]. Methylphenidate (MPH) is a commonly used drug for the treatment of ADHD. Ishimatsu et al. [26] found that the application of MPH to artificial cerebrospinal fluid (ACSF) produced a hyperpolarizing response. The second messengers for MPH are IP3/DAG via phospholipase C and phospholipid phosphatidylinositol bisphosphate [27]. This would explain how MPH works in ADHD.

Conclusions
The diacylglycerol (DAG) signaling pathway modulates the hSK4 channels by regulating the critical proteins along this pathway in RBCs. Since hSK family is widely distributed in neurons, the differences observed in RBCs among BDs, ADHDs and Negatives are explained by this finding. Some of the clinical observations

can also be explained by this pathway. The neurotransmitters (first messengers) activate the g-protein coupled receptors (GPCR) in the membrane. This activates the guanosine Triphosphate (GTP) which in turn activates the phospholipase C (PLC). The PLC cleaves the phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and inositol triphosphate (IP3). Thus the DAG signaling pathway connects the neurotransmitter signaling to the MP and the excitability of neurons in these disorders.

The author is the Founder of PsychNostics. There is no conflict of interest since this paper contains basic research results.

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