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Study of Changes in Diacylglycerol Content on Nerve Excitation

V. V. Revin[1]*, M. A. Yudanov[1], E. S. Revina[1], G. V. Maksimov[2], and I. P. Gruniushkin[1]

[1]Department of Biotechnology, Biological Faculty, Ogarev Mordovian State University, ul. Bolshevistskaya 68, 430000 Saransk, Russia; fax: (8342) 32-4554; E-mail: biotech@moris.ru
[2]Department of Biophysics, Biological Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-1115; E-mail: maximov@biophys.msu.ru

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Abstract—Rhythmic excitation of a rabbit myelin nerve increased diacylglycerol (DAG) content from 1.53 to 2.17 μg/mg lipids. Inhibition of phosphoinositide-specific phospholipase C decreased DAG content. This suggests involvement of this enzyme in processes accompanying rhythmic excitation. The increase in membrane potential of the nerve fiber (K^+-depolarization) was accompanied by increase in DAG and phosphatidylinositol monophosphate and decrease in phosphatidylinositol triphosphate and phosphatidylinositol diphosphate content. Treatment of the nerve with DAG or a protein kinase C activator increased ^{45}Ca influx by 40%, whereas treatment with an inhibitor of this enzyme, polymyxin, inhibited this parameter by 34%. The role of phosphoinositides and protein kinase C in the regulation of Ca^{2+} transport during rhythmic excitation of the myelin nerve is discussed.

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Key words: nerve, myelin, rhythmic excitation, phospholipase C, diacylglycerol

Phospholipid metabolism plays an important role in the functioning of the nervous system [1, 2]. However, its role in myelin nerve fibers still requires detailed investigation. Myelin nerves contain phosphoinositide-specific phospholipase C (EC 3.1.4.10) [3, 4], which catalyzes hydrolysis of phosphoinositide phosphate to diacylglycerol (DAG) and inositol triphosphate and also regulates Ca^{2+} transport [5-8]. We previously demonstrated the existence of a correlation between changes in phospholipase C (PLC) activity, phosphoinositide (PI) content, and Ca^{2+} transport during rhythmic nerve excitation [9, 10]. Rhythmic excitation of myelin nerves is known to be coupled to both rapid and slow (trace hyperpolarization and depolarization) changes in membrane potential (action potential). Evidently, slow electric membrane processes are important for changes in lipid metabolism during rhythmic excitation. K^+-depolarization of neurolemma is one of factors activating brain PLC [11]. However, DAG content during rhythmic myelin nerve excitation has not been measured so far in direct experiments.

So, in this study we have investigated changes in DAG content and its role in the regulation of Ca^{2+} influx during rhythmic excitation of myelin nerve.

MATERIALS AND METHODS

Sciatic nerves from chinchilla rabbits (*Oryctolagus cuniculus*) were used in experiments. Each experiment employed from six to eight isolated nerves [12]. The isolated nerves were incubated in medium containing 100 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 3 mM NaH_2PO_4, and 30 mM glucose at 37°C for 1 h under constant oxygenation. Nerves exhibiting the same amplitude of action potential under electric stimulation with ESL-2 (impulse amplitude of 1.5 V, duration of 0.3 msec, and frequency of 200 impulses/sec) in a thermostatted chamber. Changes in action potential amplitude during nerve fiber depolarization were registered using an S1-83 oscillograph (Russia).

Lipids were isolated by the method of Bligh and Dyer [13] and Folch et al. [14] in modification of Prokhorova et al. [15]. DAGs were isolated by thin layer chromatography using triple run in the solvent mixture *n*-hexane–

Abbreviations: DAG) diacylglycerol; PI) phosphoinositides; PIP) phosphatidylinositol monophosphate; PIP2) phosphatidylinositol diphosphate; PIP3) phosphatidylinositol triphosphate; PLC) phospholipase C; PMSF) phenylmethylsulfonyl fluoride; TPA) tissue plasminogen activator.

* To whom correspondence should be addressed.

ethyl ether–acetic acid (80 : 20 : 1 v/v). Lipids were identified using standard kits from Sigma-Aldrich (USA) [16]. DAG was prepared using PLC [17]. DAG concentration was evaluated by amount of its fatty acids. Fatty acid was methylated using a solution of BF_3 in methanol [18].

Fatty acid methyl esters were analyzed using Crom-5 (Serva, Czech Republic) and Crystal-5000 (Russia) gas chromatographs using Silar-10 C (Serva) as the column phase. The rates of nitrogen, hydrogen, and airflow were 15, 17, and 150 ml/min, respectively; column temperature did not exceed 225°C.

Results were statistically treated using Microsoft Excel 2000 table and STAT 2 programs. Significance of changes was evaluated using the Student criterion.

RESULTS AND DISCUSSION

In the resting rabbit nerve, DAG content was 1.53 ± 0.07 μg/mg lipid; rhythmic excitation caused an increase in nerve DAG content to 2.17 ± 0.10 μg/mg lipid. Thus the rhythmic nerve excitation caused almost 1.4-fold increase in DAG content (Fig. 1). This increase may be attributed to activation of PI-specific PLC accompanied by increased PI hydrolysis. For evaluation of the correctness of this hypothesis, we investigated the effect of 10 mM phenylmethylsulfonyl fluoride (PMSF), an inhibitor of PI-specific PLC [19], on DAG content. Incubation of the nerve with this compound for 30 min

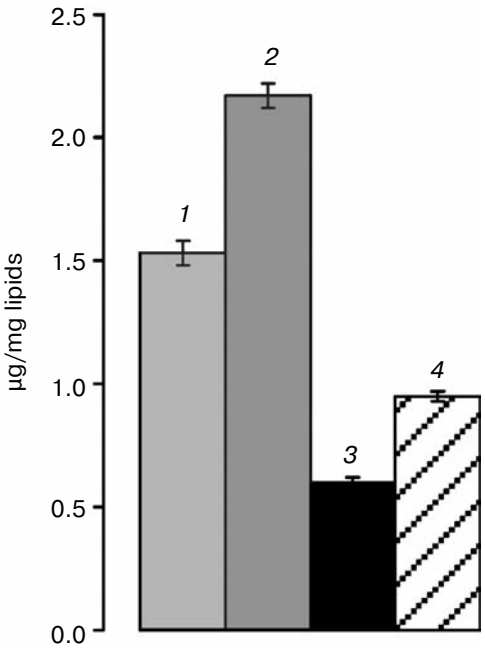

Fig. 1. DAG content in resting nerve (*1*), after rhythmic excitation at frequency of 200 Hz (*2*), in the presence of 10 mM PMSF (*3*), and after rhythmic excitation in the presence of PMSF (*4*). The ordinate shows DAG content, μg/mg total lipids.

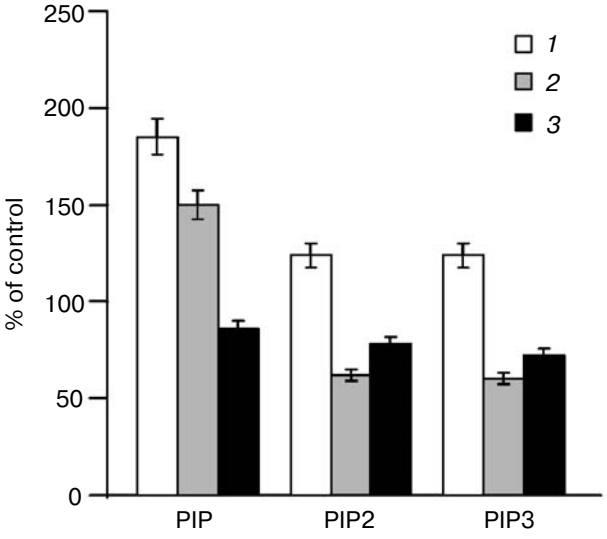

Fig. 2. Change in content of various phosphoinositides during nerve incubation: *1*) in medium with 100 mM K^+; *2*) in medium with 100 mM K^+ and 10 mM PMSF; *3*) in medium with 100 mM K^+ and 2 mM neomycin. The ordinate shows PI content, % ($p < 0.05$).

was accompanied by 2.6-fold decrease in DAG content; combined application of electric stimulation and the inhibitor caused 1.6-fold decrease in DAG content (Fig. 1). Thus, activation of PLC during rhythmic excitation of the myelin nerve may really account for the increase in DAG concentration. This is consistent with previous reports that excitation of a nerve pretreated with an inhibitor of PI-specific PLC was accompanied by the decrease in DAG content [20, 21]. For subsequent investigation of this process, we employed a specific inhibitor of PLC, neomycin, and analysis of tri-, di-, and monoinositolphosphates as substrates. These experiments revealed a decrease in PI-specific PLC during nerve incubation in the medium containing neomycin (Fig. 2). The results of these experiments suggest involvement of PI-specific PLC in the regulation of DAG content in the nerve during rhythmic excitation.

In subsequent experiments, we investigated the effect of long-term changes in nerve fiber membrane potential in the regulation of PI hydrolysis and DAG formation. The level of nerve fiber membrane potential was changed by addition of increasing concentrations of potassium ions in the medium (total osmolarity of this medium remained unchanged). Incubation of the nerve in the medium containing 25 mM K^+ caused a decrease in PIP3, PIP2, and the increase in PIP (Fig. 3). The increase in membrane depolarization amplitude by increasing K^+ concentration up to 50 mM was accompanied by subsequent decrease in PIP3 and PIP2 and accumulation of PIP. Maximal changes in the PI content were observed during nerve incubation in the medium contain-

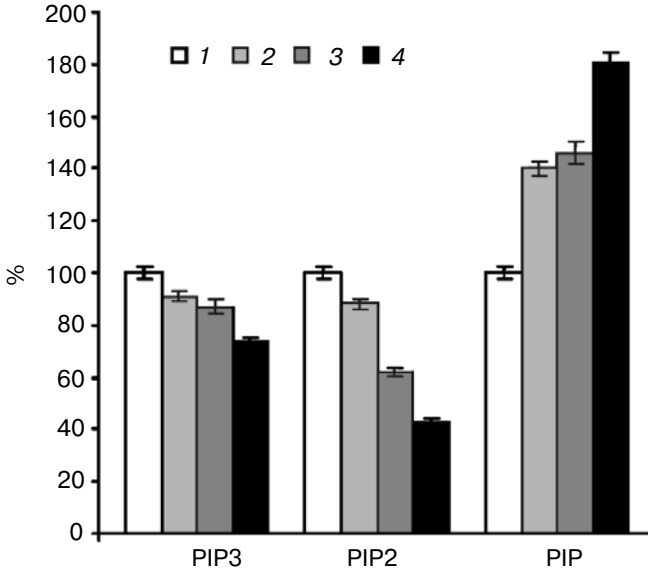

Fig. 3. Changes in PI content depending on nerve fiber membrane potential (K^+-depolarization): *1*) control; *2*) incubation with 25 mM K^+; *3*) incubation with 50 mM K^+; *4*) incubation with 100 mM K^+. The ordinate shows PI content, % ($p < 0.05$).

ing 100 mM K^+: the content of PIP3 and PIP2 decreased to 74 ± 3.6 and 43 ± 2.1% of control, respectively, whereas the content of PIP increased by 81% (Fig. 3). It is possible that the decrease in PIP3 and PIP2 during depolarization of nerve axolemma is attributed to PLC activation, whereas the increase in PIP content suggests the increase in synthesis of PIP2 and PIP3.

For evaluation of putative contribution of changes in nerve fiber membrane potential in regulation of PI-specific PLC, we investigated changes in DAG content under condition of K^+ induced nerve depolarization. Incubation of a nerve with 25 mM K^+ caused almost 3-fold increase in DAG content (Fig. 4). Subsequent increase in extracellular K^+ to 50 mM resulted in 5-fold increase in DAG accumulation, whereas 100 mM K^+ caused 5.2-fold increase in DAG content (Fig. 4). These changes in nerve DAG content were maintained for 30 min after normalization of the nerve fiber membrane potential.

For evaluation of putative activation of DAG synthesis, we blocked this process by preincubation of the nerve in medium containing 10 mM lithium chloride. The experiments revealed that that such treatment for 0.5 h caused 3-fold decrease in nerve DAG content (Fig. 5). These data support our suggestion that the increase in DAG content during long-term depolarization of a nerve fiber can be attributed to PI hydrolysis.

Thus, long-term nerve depolarization as well as rhythmic excitation causes an increase in DAG content, which suggests activation of PI metabolism. DAG accumulation in the nerve depends on depolarization of the nerve fiber membrane and is associated with PI-specific PLC functioning.

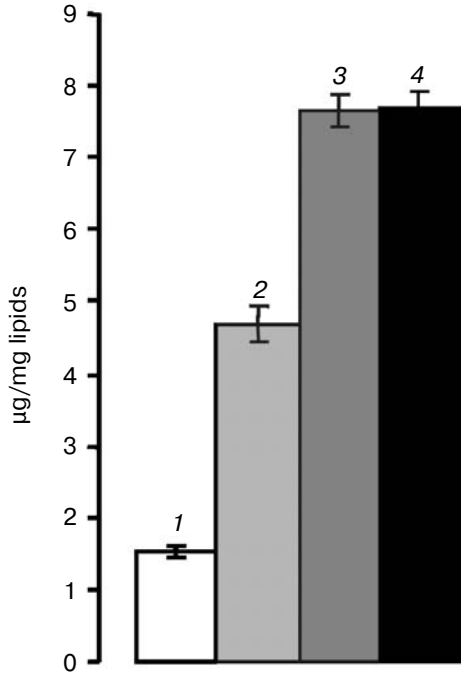

Fig. 4. Changes in DAG content depending on nerve fiber membrane potential (K^+-depolarization): *1*) medium with 4 mM K^+; *2*) medium with 25 mM K^+; *3*) medium with 50 mM K^+; *4*) medium with 100 mM K^+. The ordinate shows DAG content, µg/mg lipids ($p < 0.05$).

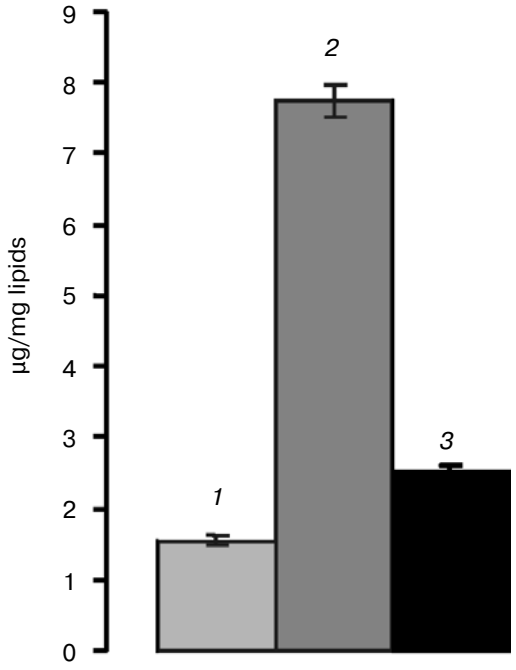

Fig. 5. Changes in DAG content depending on nerve fiber membrane potential (K^+-depolarization) and presence of LiCl: *1*) medium with 4 mM K^+; *2*) medium with 50 mM K^+; *3*) medium with 50 mM K^+ and 10 mM LiCl. The ordinate shows DAG content, µg/mg lipids ($p < 0.05$).

Evidently, changes in DAG content during axolemma depolarization can be mediated by activation of Ca^{2+} channels and Ca^{2+} influx [5, 22]. For evaluation of this suggestion, we investigated the dependence between changes in DAG content in the nerve fiber and ^{45}Ca. Being a lipophilic molecule, DAG penetrates into a cell and activates protein kinase C [23-26]. Under our experimental conditions incubation of a nerve with DAG (10^{-4} M) was accompanied by the increase in ^{45}Ca (5-7 μCi/ml) accumulation from 55 ± 2.4 to 125 ± 5.6 pmol/mg (Fig. 6). It is possible that the increase in Ca^{2+} content in the nerve during rhythmic excitation is determined by activation of Ca^{2+} channels due to depolarization as well as the decrease in DAG content and therefore protein kinase C activity.

In the next series of experiments, we investigated the effect of activator and inhibitor of protein kinase C (tissue plasminogen activator (TPA) and polymyxin, respectively) on Ca^{2+} influx [27]. TPA ($5 \cdot 10^{-6}$ M) increased Ca^{2+} accumulation in the nerve fiber from 55 ± 2.4 to 78.6 ± 3.8 pmol/mg. The maximal effect was observed during combined action of TPA and DAG (Fig. 7). Indeed, sequential incubation of the nerve with TPA and DAG increased ^{45}Ca accumulation up to 200 ± 9.4 pmol/mg. Incubation of a nerve with these compounds in the reversed order increased Ca^{2+} accumulation to 104 ± 4.7 pmol/mg [28]. Incubation of a myelin nerve in medium containing 10 μM polymyxin decreased Ca^{2+} influx by 34% versus control level, but incubation of a nerve in medium containing both polymyxin and DAG increased Ca^{2+} influx, which, however, was lower than in the presence of only DAG in the incubation medium (Fig. 7). The data suggest that regulation of Ca^{2+} influx into the nerve fiber during rhythmic excitation involves PI metabolism, particularly the product of PI hydrolysis, DAG, acting as a protein kinase C activator.

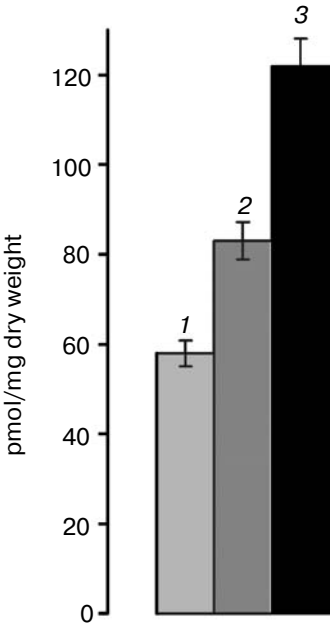

Fig. 6. Effect of DAG on Ca^{2+} influx to the nerve fiber: *1*) control; *2*) medium containing 10^{-5} M DAG; *3*) medium containing 10^{-4} M DAG. The ordinate shows ^{45}Ca content, pmol/mg dry weight ($p < 0.05$).

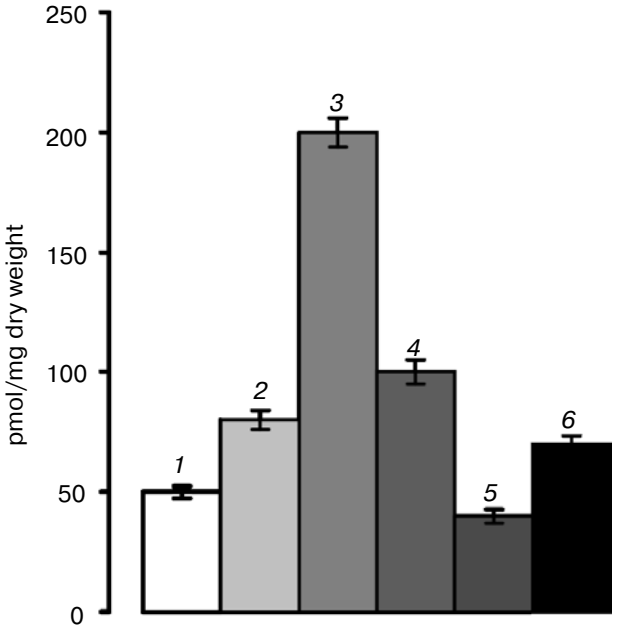

Fig. 7. Effects of DAG and activator and inhibitor of protein kinase C on the Ca^{2+} influx into a nerve fiber: *1*) control; *2*) effect of TPA ($5 \cdot 10^{-6}$ M; *3*) effect of DAG (10^{-4} M), pretreatment with TPA; *4*) effect of TPA, pretreatment with DAG; *5*) effect of polymyxin (10^{-6} M); *6*) effect of polymyxin (10^{-6} M), pretreatment with DAG (10^{-4} M). The ordinate shows ^{45}Ca content, pmol/mg nerve dry weight ($p < 0.05$).

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