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Effect of Modulators of Protein Kinase C Activity on Ca^{2+} Transport in Retinal Rod Microsomes

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Abstract—The effect of modulators of protein kinase C (PKC) activity on Ca^{2+} translocation in retinal rod microsomes was studied. It is shown that PKC activators (phorbol 12-myristate-13-acetate (PMA) and diacylglycerol (DAG)) and inhibitors (chelerythrine chloride, polymyxin B, and phloretin) stimulate and inhibit ATP-dependent Ca^{2+} uptake in retinal rod microsomes, respectively. This effect is apparently due to an influence of PKC on Ca-ATPase contained in these vesicular structures. It was found that PKC inhibitors (chelerythrine chloride, polymyxin B, and phloretin) and activators (PMA and DAG) potentiate Ca^{2+} release from Ca^{2+}-loaded retinal rod microsomes. Specific and nonspecific mechanisms of Ca-release stimulation by the modulators of PKC activity are discussed.

Key words: retinal rod microsomes, Ca^{2+} transport, phorbol 12-myristate-13-acetate (PMA), 1,2-diacylglycerol (DAG), chelerythrine chloride, polymyxin B, phloretin

Ca^{2+} is known to be a versatile regulator of cell functions. It is involved in the regulation of various processes such as muscle contraction, hormone secretion, cell transformation, nerve impulse conduction, etc. [1]. However, Ca^{2+} does not mediate visual transduction. It is only involved in light adaptation of the photoreceptor cells. For example, the change in Ca^{2+} concentration in the interdisk space is currently regarded as a factor of coupling in the course of adaptation of the photoreceptor sensitivity to light [2]. Recently, a Ca-binding protein was discovered. This protein can interact with rhodopsin kinase, thereby regulating rhodopsin phosphorylation. High cytoplasmic concentrations of Ca^{2+} decrease rhodopsin kinase activity owing to the inhibitory effect of recoverin. A decrease in Ca^{2+} cytoplasmic concentration results in activation of rhodopsin kinase, because recoverin cannot inhibit the enzyme at low Ca^{2+} concentrations [3]. In view of this, study of the system of regulation of Ca^{2+} concentration in the cytoplasm of retinal cells is still a topical problem.

It is known that many cell proteins are involved in signaling after their covalent modification as a result of their phosphorylation by various protein kinases, including protein kinase C (PKC). The latter is involved in activation of many cell processes in erythrocytes, neutrophils, lymphocytes, and fibroblasts, as well as in regulation of the cardiovascular system [4, 5]. It is believed that in T-cells PKC attenuates the interaction between HIV and T4 antigen (CD4), a receptor for this virus [6]. However, insights on the nature of target proteins and the role of phosphorylation are still far from complete.

PKC activity has also been detected in photoreceptor cells. It was reported that this enzyme can phosphorylate the key proteins of the visual cascade (rhodopsin, transducin, phosphodiesterase, guanylate cyclase, and arrestin) [7-9]. It was also found that in photoreceptor cells PKC affects lipid metabolism via phosphorylation of some proteins, such as phosphatidate phosphohydrolase, phosphatidylethanolamine-N-methyltransferase, phospholipase A, and phospholipase D [10, 11]. However, the involvement of PKC in the regulation of Ca^{2+} transport in photoreceptor cells is poorly studied.

In this work we studied the effect of modulators of PKC activity on Ca^{2+} accumulation and release in retinal rod microsomes.

MATERIALS AND METHODS

In this study we used Tris from Reanal (Hungary), EDTA and EGTA from Serva (Germany), and imidazole

Abbreviations: PKC) protein kinase C; DAG) diacylglycerol; PMA) phorbol 12-myristate-13-acetate; PLC and PLD) phospholipases C and D, respectively; $InsP_3$) inositol-1,4,5-trisphosphate; $InsP_4$) inositol-1,3,4,5-tetraphosphate.

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from BDH (UK). Phloretin, polymyxin B, chelerythrine chloride, PMA, DAG, GTP, and ATP were from Sigma (USA). $^{45}CaCl_2$ and [γ-^{32}P]ATP were from Izotop (St. Petersburg, Russia). Whatman GF/F fiberglass filters were from Whatman (UK). Other reagents were of domestic production. All solutions were prepared in double distilled water.

The study was performed on rod microsomes obtained from the retina isolated from fresh bovine eyes that were preliminarily adapted to the dark for 1 h and then cooled to 0°C. Microsomal fraction was obtained by differential centrifuging. Briefly, prepared retinas were homogenized in 0.32 M sucrose containing 20 mM Tris-HCl (pH 7.6) and 1 mM EDTA. The homogenate was centrifuged at 900*g* for 10 min to separate unbroken cells and tissues. The supernatant was then centrifuged for 20 min at 11,500*g*. The resulting supernatant was centrifuged at 100,000*g* for 1 h. The pellet contained the retinal microsomal fraction. The purity of the microsomal fraction was assessed by measuring the activity of marker enzyme of the microsomal fraction, glucose-6-phosphatase [12].

All measurements were performed at 37°C in standard incubation medium containing 150 mM KCl, 20 mM imidazole-HCl (pH 7.0), 2 mM $MgCl_2$, and 0.1 mM EGTA. Accumulation and release of Ca^{2+} were recorded 10 min after initiation of the reaction on the linear portion of the kinetic curves. Accumulation and release of Ca^{2+} were studied independently under conditions optimal for each process.

Accumulation of Ca^{2+} by the membrane vesicles was monitored at 37°C in standard incubation medium containing 0.1 mM $CaCl_2$ (2 μCi/ml $^{45}Ca^{2+}$), which corresponds to 3 μM free Ca^{2+}. The accumulation of Ca^{2+} was initiated by adding 50-100 μg protein to the incubation medium. After a 10-min incubation, the reaction was stopped by applying the mixture onto GF/F Whatman fiberglass filters and subsequent washing the filter with 5 ml of ice-cold reaction medium. The filers were then dried and placed into vials with 5 ml scintillation liquid containing 0.2 mM 1,4-bis[5-phenyl-2-oxazolyl]benzene (POPOP) and 30 mM 2,5-diphenyloxazole (PPO) in toluene. The radioactivity of the specimens was measured on a Wallac 1409 DSA counter.

ATP-dependent Ca^{2+} accumulation was recorded in a similar way in the medium containing 2 mM ATP. The contribution of the ATP-dependent component was estimated by the difference in Ca^{2+} accumulation in the presence and absence of ATP (at equal concentrations of free Ca^{2+} in the incubation medium).

Calcium release was studied in $^{45}Ca^{2+}$-loaded membrane vesicles that were then washed free from external Ca^{2+}. The vesicles were loaded with $^{45}Ca^{2+}$ by incubating them in EGTA-free medium containing 0.2 mM $CaCl_2$ (4 μCi/ml $^{45}Ca^{2+}$) at 4°C for 1 h. The suspension was then centrifuged at 20,000*g* for 15 min, and the supernatant was discarded. The pellet was resuspended in Ca^{2+}-free medium and centrifuged again under the same conditions. Washed vesicles were resuspended in the incubation medium. Calcium release was induced by adding the vesicle suspension to $^{45}Ca^{2+}$-free standard incubation medium at 37°C. The reaction was stopped 10 min after its initiation by applying the reaction mixture onto GF/F Whatman fiberglass filters. The filters were rinsed twice with 5 ml ice-cold incubation medium. The radioactivity trapped on the filters was measured as described above. To quantitatively estimate Ca^{2+} release, the radioactivity was expressed in percent relative to the initial value. The figures show the residual content of $^{45}Ca^{2+}$ in the membrane vesicles after Ca^{2+} release.

Free calcium concentration was calculated using the Bio Quest 1990 software (version 1.1) developed at the Institute of Biophysics (Russian Academy of Sciences, Pushchino). The specimens of photoreceptor membranes were bleached using a 100 W lamp at the distance of 30 cm for 10 min with stirring. Protein content was determined by the method of Lowry with some modifications as described in [13]. The results are presented as the means of threeto five experiments (each made in quadruplicate) $\pm S_x$.

RESULTS AND DISCUSSION

Endoplasmic reticulum, an intracellular calcium storage site, regulates Ca^{2+} concentration in the cytoplasm of photoreceptor cells owing to its ability to effectively accumulate and release calcium ions contained in the cytoplasm of the outer and inner retinal rod segments. Transmembrane transfer of Ca^{2+} in endoplasmic reticulum occurs in response to different endogenous stimuli including the physiologically active compound 1,2-diacylglycerol (DAG), that activates PKC. The lipid bilayer of cells (including resting cells) always contains DAG as an intermediate product of membrane phospholipid exchange. It is produced as a result of hydrolysis of phospholipids mediated by phospholipases C and D (PLC and PLD).

An addition of 10 μM DAG to incubation medium containing microsomes stimulates ATP-dependent Ca^{2+} accumulation by the microsomes (Fig. 1). A similar effect is observed when 0.1 μM phorbol 12-myristate-13-acetate (PMA), an artificial analog of DAG, is added to the incubation medium (Fig. 1). Note that in control (without ATP in the incubation medium) DAG and PMA did not affect Ca^{2+} accumulation by retinal rod microsomes. This apparently indicates that DAG activates the Ca^{2+}-pump in the endoplasmic reticulum.

It is known that PLC activity is regulated by GTP-binding proteins (G proteins) that hydrolyze GTP [14, 15]. Rod outer segments contain the GTP-binding protein transducin. GTP-binding proteins, including transducin, are activated only in the presence of guanosine trisphos-

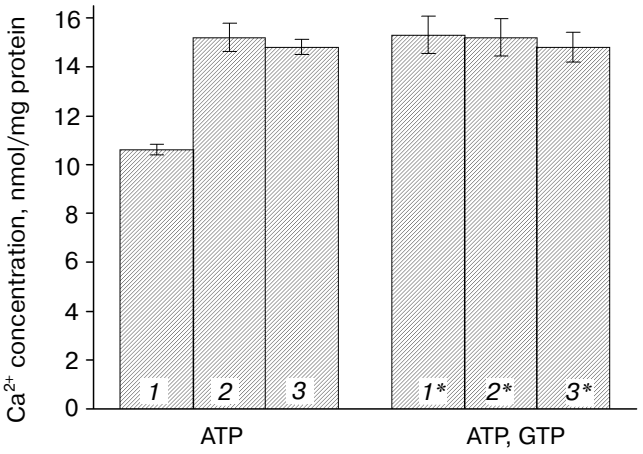

Fig. 1. Effect of PKC activators DAG (10 μM) (*2*, *2**) and PMA (0.1 μM) (*3*, *3**) on ATP-dependent Ca^{2+} accumulation by retinal rod microsomes (0.49 mg protein/ml) in the presence of 2 mM ATP (*1*, *2*, *3*) or 2 mM ATP and 100 μM GTP (*1**, *2**, *3**).

phate. It was shown that PLC activity increases most significantly at 100 μM GTP [16]. An addition of 100 μM GTP to the reaction medium stimulates ATP-dependent Ca^{2+} accumulation by the same amount as DAG and PMA (Fig. 1). An addition of DAG and PMA in the presence of GTP does not cause further activation of Ca^{2+} uptake (Fig. 1). Therefore, it can be assumed that activation of ATP-dependent accumulation of Ca^{2+} by the microsomes in the presence of GTP is mediated by DAG, whereas other products of phosphoinositide metabolism are not involved in this process. Actually, we found that neither inositol-1,4,5-trisphosphate ($InsP_3$) nor inositol-1,3,4,5-tetraphosphate ($InsP_4$) affect the ATP-dependent Ca^{2+} accumulation by retinal rod microsomes (data not shown). The absence of an effect of $InsP_3$ on ATP-dependent Ca^{2+} accumulation can be explained by the fact that the incubation medium used in these experiments contained 2 mM ATP and 0.1 mM $CaCl_2$. Under these conditions, the microsomes are able to accumulate Ca^{2+} in an ATP-dependent manner. Because $InsP_3$-regulated Ca^{2+}-channels are blocked by high concentrations of external Ca^{2+}, the rate of Ca^{2+} accumulation cannot be decreased by releasing Ca^{2+} accumulated in the vesicles. It is known that $InsP_3$-dependent Ca^{2+} release occurs at low concentrations of cytosolic Ca^{2+} (less than 0.2-0.3 μM); at higher concentrations it is blocked [17, 18]. Hence, at 0.1 mM $CaCl_2$ (which corresponds to 3 μM free Ca^{2+}) $InsP_3$-dependent Ca^{2+}-channels are blocked.

To clarify the role of PKC in ATP-dependent Ca^{2+} accumulation by the microsomes isolated from photoreceptor cells, we used PKC inhibitors chelerythrine chloride, polymyxin B, and phloretin at concentrations of 10, 20, and 200 μM, respectively. Inhibition of PKC by these inhibitors is most specific at these concentrations [19-21].

We found that addition of these inhibitors to the incubation medium suppresses ATP-dependent Ca^{2+} accumulation by retinal rod microsomes (Fig. 2). This apparently suggests that PKC is involved in phosphorylation of Ca^{2+}-ATPase contained in the vesicles, thereby activating it. It should be noted that the inhibitory effect of these compounds is retained even when ATP-dependent Ca^{2+} accumulation is stimulated by DAG and GTP added either separately or simultaneously (Figs. 2 and 3). Therefore, it can be assumed that an addition of suspension of the microsomes to the incubation medium containing 2 mM ATP and 0.1 mM $CaCl_2$ results in ATP-dependent Ca^{2+} accumulation due to Ca-pump operating in the presence of ATP and in additional activation of the Ca^{2+}-pump by PKC in the presence of Ca^{2+}. An addition of PKC inhibitors under these conditions decreases ATP-dependent Ca^{2+} accumulation due to PKC inhibition (Fig. 2). These results are consistent with the data that have been obtained recently by Militante and Lombardini in rat retina homogenate. They showed that chelerythrine inhibits Ca^{2+} accumulation, ATPase activity, and protein phosphorylation in the retina [22].

Our results indicate that PKC activators and inhibitors modulate ATP-dependent Ca^{2+} accumulation by retinal rod microsomes, apparently due to the PKC effect on Ca^{2+}-ATPase contained in these vesicular structures.

The role of PKC in Ca^{2+} mobilization from the storage sites of the photoreceptor cells remains obscure. In view of this, we studied the effect of the modulators of PKC activity on Ca^{2+} release from $^{45}Ca^{2+}$-loaded retinal rod microsomes.

Ca^{2+} is released from the vesicles along the concentration gradient. The released Ca^{2+} is not re-accumulated by the microsomes, because the incubation medium did not contain ATP required for Ca^{2+}-ATPase functioning.

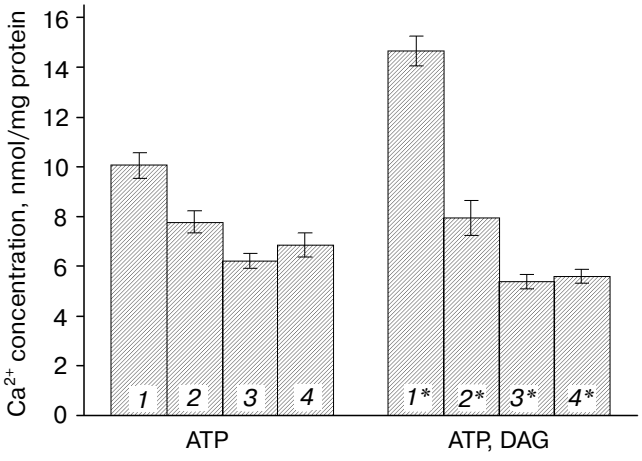

Fig. 2. Effect of PKC inhibitors chelerythrine (10 μM) (*2*, *2**), phloretin (0.2 mM) (*3*, *3**), and polymyxin B (20 μM) (*4*, *4**) on ATP-dependent Ca^{2+} accumulation by retinal rod microsomes (0.56 mg protein/ml) in the presence of 2 mM ATP (*1*, *2*, *3*, *4*) or 2 mM ATP and 10 μM DAG (*1**, *2**, *3**, *4**).

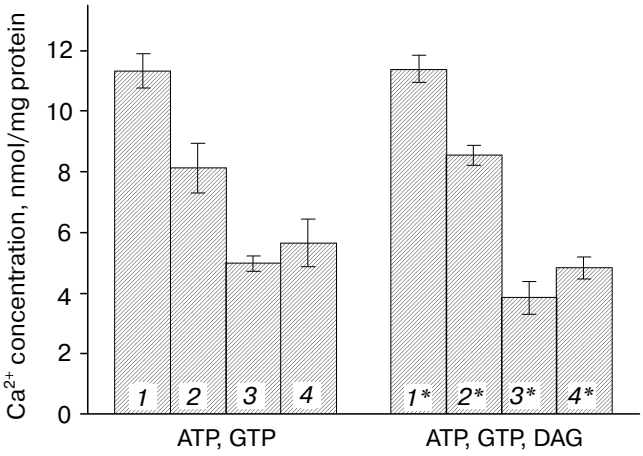

Fig. 3. Effect of PKC inhibitors chelerythrine (10 μM) (*2*, *2**), phloretin (0.2 mM) (*3*, *3**), and polymyxin B (20 μM) (*4*, *4**) on ATP-dependent Ca^{2+} accumulation by retinal rod microsomes (0.56 mg protein/ml) in the presence of 2 mM ATP and 100 μM GTP (*1*, *2*, *3*, *4*) or 2 mM ATP, 100 μM GTP, and 10 μM DAG (*1**, *2**, *3**, *4**).

An addition of chelerythrine chloride, polymyxin B, or phloretin under these conditions potentiated Ca^{2+} release from the microsomes (Fig. 4). The PKC inhibitor staurosporine exerted a similar effect on Ca^{2+} release in salivary gland cells [23]. Note that PKC inhibitors have more pronounced effect on Ca^{2+} release from the microsomes than GTP (Fig. 4).

It is known from the literature that GTP stimulates hydrolysis of membrane phosphoinositides, primarily phosphatidylinositol-4,5-bisphosphate, by PLC [24]. One of the products of PLC-catalyzed hydrolysis is water-soluble $InsP_3$. In view of this, we assume that in our study the presence of GTP in the incubation medium stimulates production of endogenous $InsP_3$, which induces Ca^{2+} release from Ca^{2+} storages [25]. The more pronounced activation of Ca^{2+} release by PKC inhibitors compared to GTP suggests that PKC may regulate Ca^{2+} release not only from the $InsP_3$-sensitive Ca^{2+} storages. This assumption is corroborated by the results obtained by Shin et al. in salivary gland cells using the fluorescent probe Fura-2. They found that in the presence of staurosporine, even after depletion of the $InsP_3$-dependent Ca^{2+} pool, the concentration of free Ca^{2+} in the cytoplasm continues to increase [23].

PKC activators DAG and PMA can also regulate Ca^{2+} release from retinal rod microsomes. Figure 5 shows that DAG and PMA potentiate Ca^{2+} release from retinal rod microsomes loaded with $^{45}Ca^{2+}$ under conditions optimal for Ca^{2+} release. This finding is in agreement with data obtained by Gomez and Nasi, who discovered that in the photoreceptor cells of invertebrates PMA increases ionic permeability of the plasma membrane and that this effect is synergistic with the $InsP_3$-dependent Ca^{2+} release [26]. It was also shown that PMA injection in dark-adapted fish visual cells mimics the effect of light on the retina [27].

At first glance, the data shown in Fig. 5 are at variance with those shown in Fig. 4, which indirectly indicate that PKC inhibits Ca^{2+} release from the microsomes. It should be taken into consideration that Ca^{2+} release may be induced by DAG not only due to direct interaction of DAG with PKC, but also by a PKC-independent mechanism. It was reported in the literature that in different cell types DAG activates the Ca^{2+}-channels of the plasma

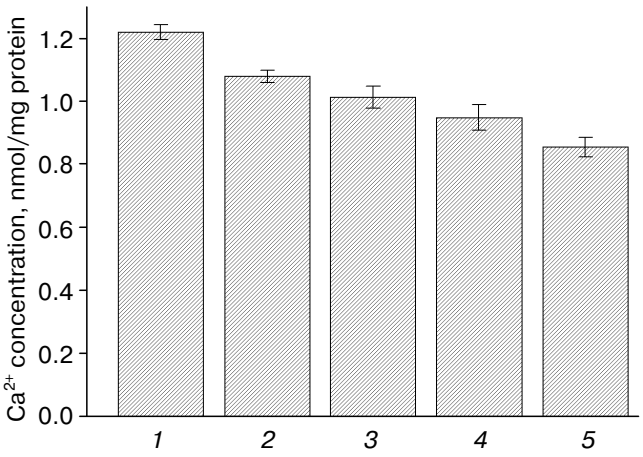

Fig. 4. Effect of PKC inhibitors and GTP on Ca^{2+} release from retinal rod microsomes (0.78 mg protein/ml): *1*) control; *2*) 100 μM GTP; *3*) 20 μM polymyxin B; *4*) 0.2 mM phloretin; *5*) 10 μM chelerythrine.

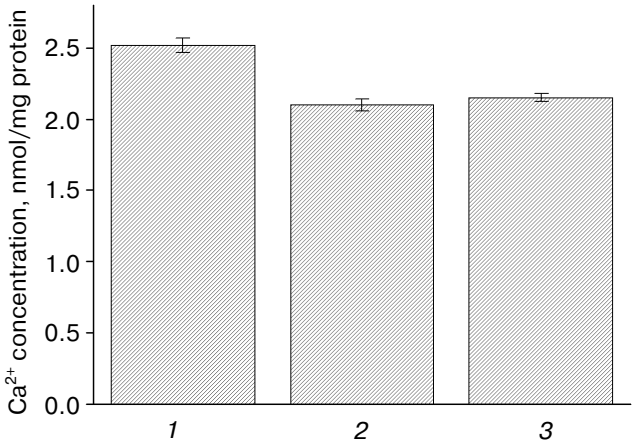

Fig. 5. Effect of PKC activators DAG and PMA on Ca^{2+} release from retinal rod microsomes (0.63 mg protein/ml): *1*) control; *2*) 10 μM DAG; *3*) 0.1 μM PMA.

membrane and Na/H-exchanger by directly interacting with them [28, 29]. It was shown that in nerve cells DAG analogs 1-oleyl-2-acetyl-glycerol (OAG) and 1,2-dioctanoyl-sn-glycerol (1,2-DiC8) modulate Ca^{2+}-currents even in the presence of PKC inhibitors [30]. Gordon et al. showed that in frog rods DAG analogs 1,2-DiC8 and 1,3-dioctanoyl-sn-glycerol inhibit cGMP-activated channel by phosphorylation-independent mechanism (via direct allosteric interaction with them) [31].

Taken together with data published in the literature, our data on Ca^{2+} release from retinal rod microsomes suggest that activation of Ca^{2+} release by DAG and PMA as a result of direct interaction of these compounds with Ca^{2+}-channels prevails over activation of PKC by DAG and PMA.

It should be noted also that the effects of PKC activators (DAG and PMA) and GTP resulting in enhancement of Ca^{2+} release from the microsomes are of similar values (Figs. 4 and 5). Perhaps all these compounds are involved in modulation of the same Ca^{2+}-channels.

Based on the results of this study, it can be concluded that PKC modulates Ca^{2+} transport in retinal rods by regulating Ca^{2+} accumulation and release in endoplasmic reticulum.

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