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PROSTAGLANDIN H SYNTHASE ACTIVITY IN THE PRESENCE OF CALCIUM IONS AND MOUSE SKIN *IN VITRO*

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Using preparation of microsomes obtained from bovine vesicular gland, the prostaglandin H synthase (PGHS, EC 1.14.99.1) activity was studied at a wide range of Ca^{2+} concentrations. It was demonstrated that at calcium concentrations lower than 0.005 mM, the activity was not changed significantly. Increase in calcium concentration resulted in enzyme inhibition and at the same time decreased the constant of enzyme inactivation during the reaction. The storage stability of lyophilized enzyme preparations obtained with and without calcium ions was evaluated. The effect of calcium ions to stabilize the PGHS, reported early for the enzyme solutions, was not observed. Moreover, the PGHS activity and PGE_1 as well as PGE_2 concentration were monitored in the presence of mice skin (1 cm^2) applied as piece or in the homogenized form. It was observed that preincubation with skin at 37°C for more than 54 minutes slowly decreased the enzyme activity and prostaglandin concentration.

Prostaglandin H synthase (PGHS, EC 1.14.99.1) is the first and rate-limiting enzyme in the transformation of polyunsaturated fatty acids into prostanoids [1–3], which are the physiologically active substances important for the regulation of functioning of human and mammalian cells in normal and pathological conditions [4, 5]. The enzyme exhibits two enzymatic activities (cyclo-oxygenase and peroxidase) and requires participation of heme as prosthetic group and three different substrates: polyunsaturated fatty acid, oxygen and an electron donor. The specific characteristic of PGHS is its fast and irreversible inactivation in the course of catalysis [2, 3] that is accompanied with formation of hemoprotein radicals followed by dramatic changes in the protein‘s structure.

Recently, it was demonstrated that the enzyme application can markedly accelerate healing of skin incisional wounds in a mouse model as well as the orthodontic tooth movement in cats [5, 6]. Thus, this enzyme is undoubtedly of pharmacological interest, since it can be applied to regulate the level of a wide array of prostanoids.

The PGHS activity may be inhibited by non-steroidal anti-inflammatory agents (such as aspirin, indomethacin, ibuprofen, etc.). This regulation of enzymatic activity is described by competitive mechanisms [7]. Moreover, endogenous inhibitors of enzyme activity were found in blood serum, in placenta and human amniotic fluid [8, 9]. Haptoglobin was identified as the endogenous inhibitor present in cytosol of primary cells from sheep vesicular glands [9]. Recently, it was found that calcium ions increased the PGHS activity and stability during the enzyme storage in solution and preparations immobilized on silica gel [10, 11]. However, the effect of calcium ion on enzymatic activity was not described at a wide range of ion concentrations. Considering the calcium as the second messenger regulated by many different ligands as well as by arachidonic acid (PGHS substrate) [12], it is important to define the effects of this ion to enzyme activity.

Description of the regulation of enzyme activity by calcium is also interesting due to the possibility of ion application for precipitation of microsomal fraction during PGHS extraction [13]. Lyophilization of this preparation lead to ion concentration. The activity and stability of lyophilized preparations obtained by using of calcium ions is not defined, and this is a crucial point to enzyme application in therapeutical practice.

The stability of physiologically active compound during its application *in vivo* is of great importance to predict possibility of its use as pharmacological preparation. Normally, it is considered that the pharmacological preparation must be stable in the tissues for at least 0.5 h to cause the physiological response [14]. So, the stability of PGHS during its application as well as of the prostaglandins that can be synthesized by enzyme action must be characterized previously to study the mechanism of their action.

In this paper we report on: i) spectrophotometrical determination of the PGHS activity under a wide range of Ca^{2+} concentrations (from 0 to 6 mM) using arachidonic acid as substrate; ii) characterization of storage stability of lyophilized enzyme preparations obtained by differential centrifugation and by application of calcium ions for microsome precipitation; iii) spectrophotometrical monitoring of the PGHS activity and PGE_1 as well as PGE_2 concentration in the presence of mice skin.

MATERIALS AND METHODS

Preparations of microsomal fractions containing PGHS were obtained from bovine vesicular glands by two different methods: precipitation by using calcium ions and ultracentrifugation [13, 15]. Beckman J2-HS and XL-90 centrifuges with JA-10 and 55.2 Ti rotors, respectively, were used for centrifugation. The following reagents were purchased from Sigma Chemical Company (USA): Trizma, diethyldithiocarbamate sodium salt (DEDTC), Tween-20, $CaCl_2$, $NaClO_4$, HCl, arachidonic acid, L-adrenaline, hemine. EDTA disodium salt and ethanol were from Baker Analyzed (USA).

The PGHS activity and protein concentration in obtained preparations were analyzed. The protein concentration was determined according to the method of Bradford [16]. Spectrophotometric assay [13, 15] of PGHS cyclooxygenase plus peroxidase activities was performed in 2 ml of 50 mM Tris-HCl buffer, pH 8, containing 0.05% Tween-20. The reaction mixture contained an aliquot of solubilized microsomes, 0.03 ml of 100 mM L-adrenaline as an electron donor and 0.02 ml of 0.02 mM hemin as a prosthetic group. To begin the reaction 0.03 ml of 15 mM arachidonic acid was added. The kinetics of L-adrenaline oxidation in the PGHS catalyzed reaction was monitored at 480 nm (Cintra-20) at 22°C.

The effect of calcium ions on PGHS activity was studied by the same technique using the 50 mM Tris-HCl buffer, pH 8, containing the same concentration of nonionic surfactant and the different concentrations of calcium chloride from 0 to 8 mM. The assay was carried out without preincubation.

Microsomal fractions obtained by ultracentrifugation and by precipitation with calcium ions were suspended in double distilled water and lyophilized. Storage stability of PGHS of these lyophilized microsomal preparations was monitored by the same technique after incubating of the preparations for different time at room temperature (23–25°C), at 4°C and at –14°C. The detection of kinetics of PGHS catalyzed reaction was performed after resuspension of 0.005 g of lyophilized preparation in 1 ml of Tris-HCl buffer, pH 8.

To study the PGHS activity and PGEs concentration in the presence of mouse skin, 1 cm^2 of skin was applied immediately after its extraction as piece or in the homogenized form. Preincubation of the mixture of skin and prostaglandin as well as PGHS preparation in physiological solution (1:1) was performed for 0 to 54 minutes. The aliquot of 0.06 ml of the mixture was applied to assay for activity determination at the same manner as described above. Monitoring of PGE_1 and PGE_2 concentration was performed by spectrophotometric assay based on reaction of their transformation to PGBs under pH 10 in medium containing ethanol : water (5:1). The PGBs are characterized by light absorbance at 278–280 nm and molar extinction coefficient equal to 23,000 M^{-1} cm^{-1}. The prostaglandin concentration was calculated as increase of absorbance at 278–280 nm divided by coefficient of molar extinction.

RESULTS AND DISCUSSION

The microsomal fractions revealing prostaglandin H synthase activity (PGHS, EC 1.14.99.1) were obtained from bovine vesicular glands by differential centrifugation and by precipitation with calcium ions [13, 15]. Using the preparation obtained by differential centrifugation, the PGHS activity was studied spectrophotometrically at a wide range of Ca^{2+} concentrations (from 0 to 8 mM) using

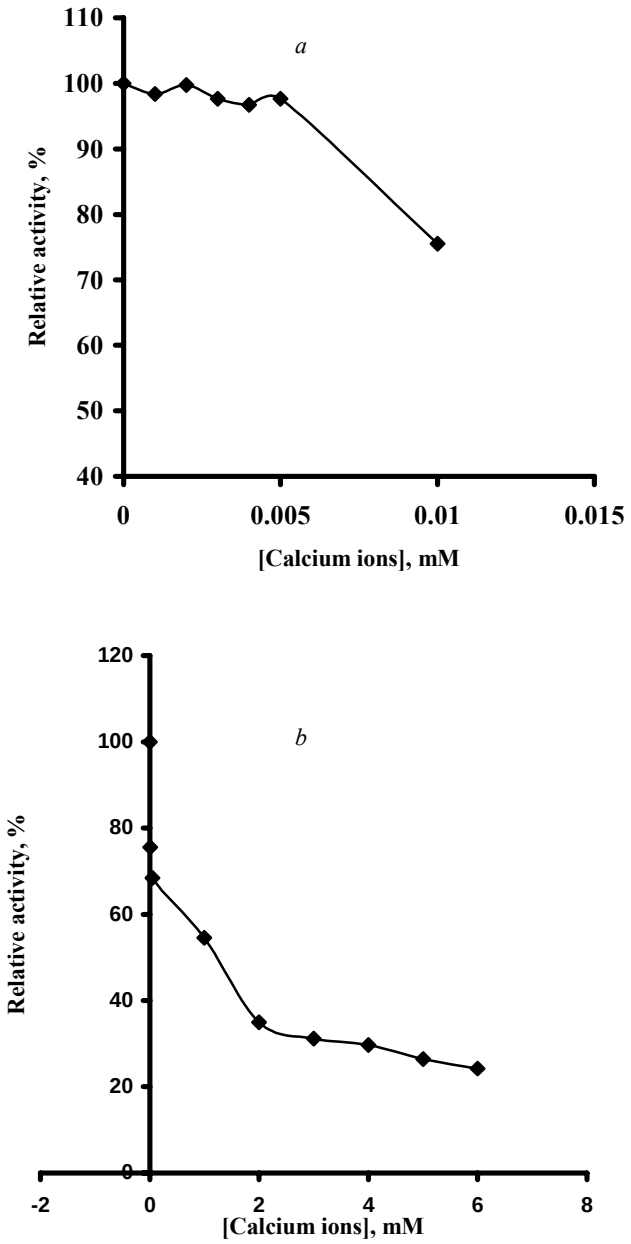

Fig. 1. Relative prostaglandin H synthase activity of microsome preparation in the presence of different concentrations of calcium ions: *a* – concentration from 0 to 0.01 mM; *b* – concentration higher than 0.005 mM. Enzyme activity at 0 mM of calcium ions was used as reference of 100% activity

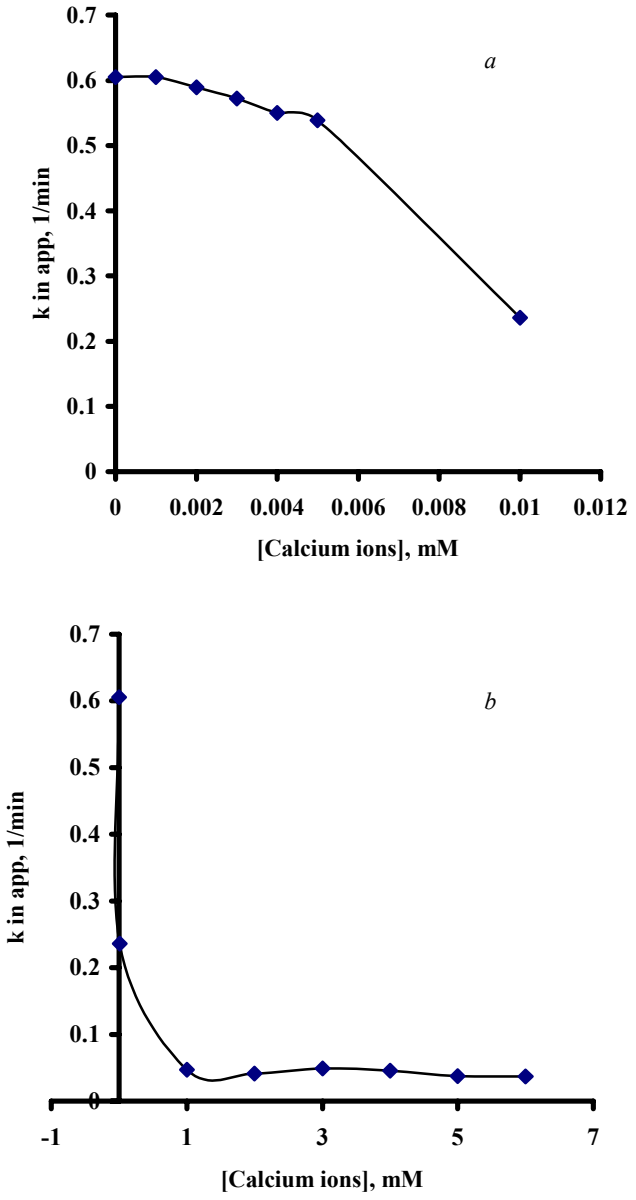

Fig. 2. Constant of inactivation of prostaglandin H synthase in the course of the reaction in the presence of different concentrations of calcium ions: *a* – concentration from 0 to 0.01 mM; *b* – concentration higher than 0.005 mM

arachidonic acid as substrate. The initial rate (Fig. 1) and constant of the substrate-induced enzyme inactivation (Fig. 2) were evaluated. It was observed that at calcium concentration lower than 0.005 mM, the activity was not changed significantly (Fig. 1, *a*). Increasing the calcium concentration inhibited enzyme activity and at the same time decreased the constant of enzyme inactivation in the course of the reaction (Fig. 1, *b*). At 8 mM calcium concentration the enzyme was inhibited completely.

It is probable that regulation of PGHS activity by calcium ions may take place during cell response to different concentrations of arachidonic acid, as well as of other ligands that induced the change of concentration of this second messenger in the cells [12, 17]. Moreover, it is important to consider these results in PGHS activity studies since calcium ion presence influence detected values.

It was reported previously that calcium ions stabilized the enzyme during its storage in solution and in the immobilized form in silica gel [10, 11]. In the present study the storage stability of lyophilized enzyme preparations obtained by ultracentrifugation (without calcium ions) and by microsome precipitation in the presence of calcium ions was evaluated (Fig. 3). Comparison of enzyme stability of both preparations stored at different temperatures showed that use of calcium ions did not favor the stability of lyophilized PGHS preparations. The higher storage stability was observed at lower temperature (–14°C). This data is in accordance with the results obtained previously with PGHS from sheep vesicular glands [18] and confirmed that the lower temperature is appropriate for enzyme storage for more than 1 month (Fig. 3). Moreover, the PGHS activity and PGE_1 as well as PGE_2 concentration were monitored spectrophotometrically in the presence of mouse skin (1 cm^2) applied immediately after its extraction as piece or in the homogenized form. It was observed that the enzyme activity and prostaglandin concentration were decreased slowly, when the enzyme and prostaglandins preparations were preincubatied with skin at 37°C for more than 54 minutes (Fig. 4). The presence of skin increased the enzyme activity (Fig. 4, *a*). It may be related to the presence of skin having low concentrations of peroxide forms that induced the ciclooxigenase activity of PGHS [2]. The PGEs concentration decreased after skin addition. In the presence of

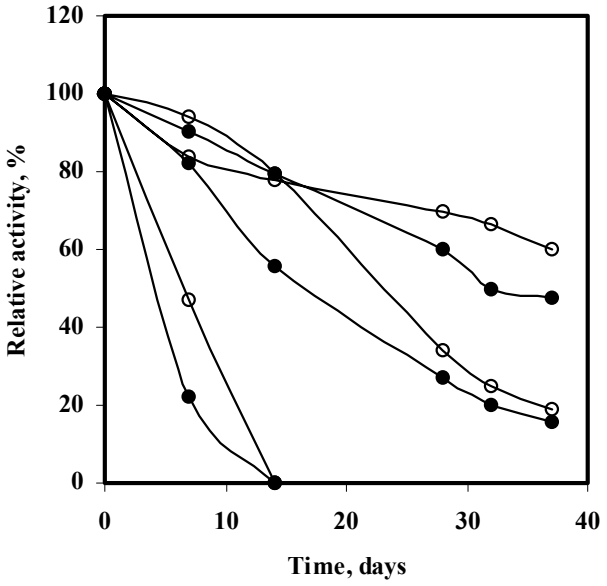

Fig. 3. Storage stability of prostaglandin H synthase from microsome preparations, obtained by ultracentrifugation (o) and by precipitation with calcium ions (•), at room temperature (25°C), at 4°C and at –14°C: bottom curves, middle cures and top curves, respectively

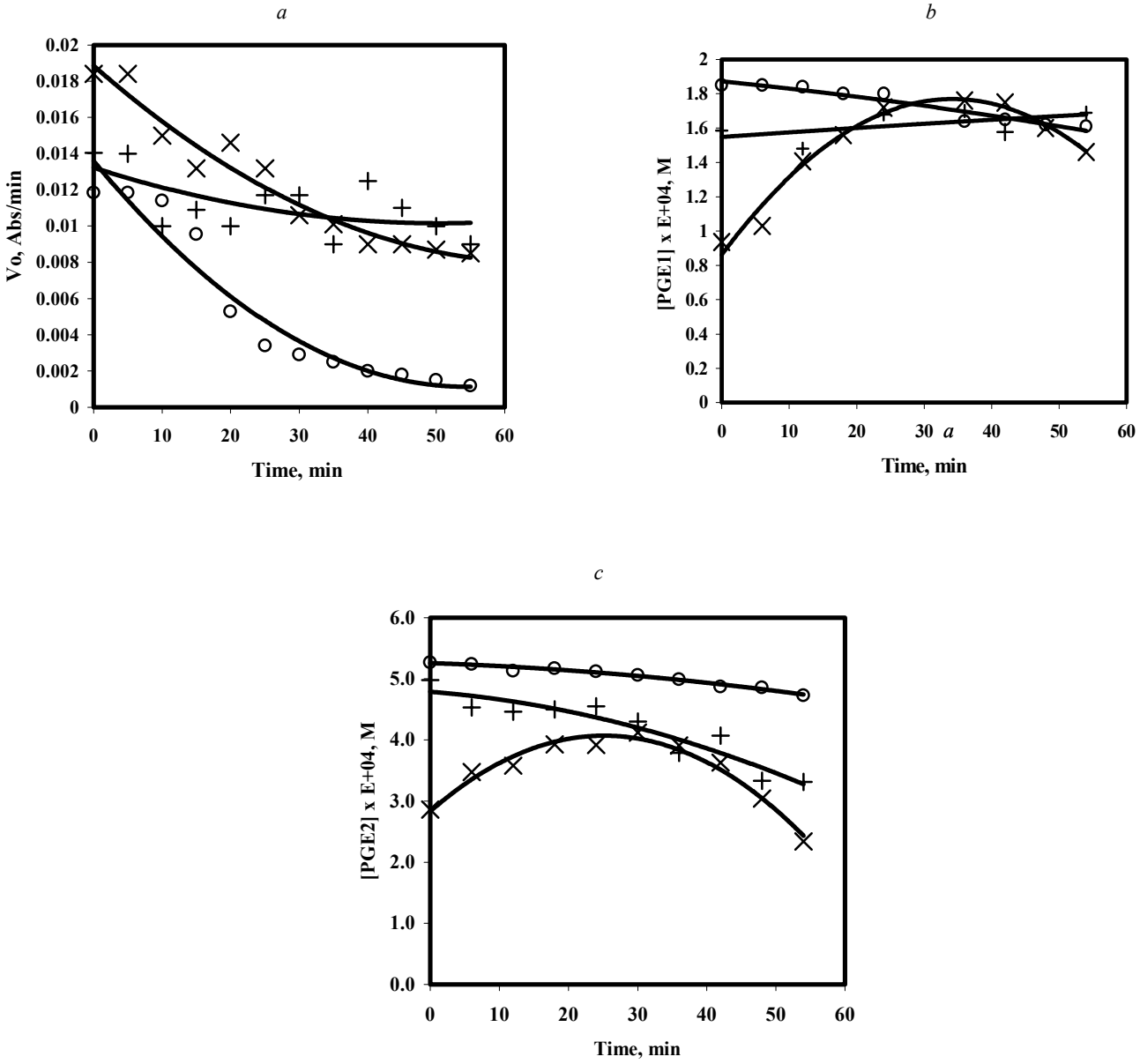

Fig. 4. Monitoring of: *a* – prostaglandin H synthase activity, *b* – PGE_1 concentration; *c* – PGE_2 concentration after their preincubation at 37°C in the presence of skin using skin piece (x) and skin homogenized in physiological solution (+). The control monitoring (o) was performed without skin at the same conditions

skin piece, partial absorption of prostaglandins was observed initially followed by their liberation in the solution during incubation (Fig. 4, b, c). After 54 min the concentration of PGE_1 was similar to that of control without skin, but PGE_2 concentration was lower than control at 27% and 46 % in mixture with homogenized skin and piece of skin, respectively.

The obtained data clearly demonstrates that the prostaglandin and PGHS preparations applying for wound treatment in skin *in vivo* will have a sufficient life-time and stability to develop the physiological response [14]. Further, the present study describes the properties of PGHS and prostaglandins that must be considered during their application for the treatment of skin wounds.

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АКТИВНОСТЬ ПРОСТАГЛАНДИН СИНТАЗЫ В ПРИСУТСТВИИ ИОНОВ КАЛЬЦИЯ И МЫШИНОЙ КОЖИ *IN VITRO*

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Исследовано влияние ионов кальция на активность простагландин синтазы (PGHS, КФ.1.14.99.1) в микросомах, выделенных из везикулярных желез быка. При концентрации ионов кальция < 0,005 мМ активность практически не изменялась. Увеличение концентрации кальция приводило к ингибированию и к уменьшению константы скорости инактивации фермента в процессе реакции. Определена активность лиофилизованных препаратов фермента, выделенных в присутствии и в отсутствие ионов кальция. Описанный ранее стабилизирующий эффект ионов кальция в процессе хранения фермента в растворе не наблюдался для лиофилизованных препаратов. Были измерены активность фермента и концентрация простагландинов E_1 и E_2 в присутствии 1 $см^2$ кожи мыши (в виде среза или гомогената). Было обнаружено, что предынкубация фермента и простагландинов в присутствии кожи в течение 54 мин при 37°С приводит к медленному уменьшению как активности фермента, так и концентрации простагландинов.