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SHORT COMMUNICATION

Heparin inhibits the reconstituted plasma membrane Ca^{2+}-ATPase from porcine brain synaptosome

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Heparin has been shown to be involved in the regulation of cellular Ca^{2+} by binding to many proteins with high affinity. Here we examined the effects of heparin on the plasma membrane Ca^{2+}-ATPase from porcine brain synaptosome. Our results showed that heparin dramatically inhibited the ATP hydrolysis and Ca^{2+} uptake in the presence and absence of calmodulin. Together with controlled proteolysis by trypsin, we concluded that the calmodulin-binding domain of the plasma membrane Ca^{2+}-ATPase was less important for the heparin inhibition. Excess phosphatidylserine was able to eliminate the heparin inhibition. We observed that Ca^{2+} affinity kept no obvious changes, but the ATP affinity of plasma membrane Ca^{2+}-ATPase was apparently decreased in the presence of heparin. Our results indicated that heparin had little effects on ATP or Ca^{2+} binding sites of the enzyme.
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Keywords: **calcium, calcium pump, heparin**

Abbreviations: **CaM calmodulin, PC phosphatidylcholine, PS phasphotidylserine, PMCA plasma membrane Ca^{2+}-ATPase.**

Introduction

The plasma membrane Ca^{2+}-ATPase (PMCA) is a P-type ATPase that plays a crucial role in regulation of cell calcium homeostasis [1,2]. Its function is to extrude Ca^{2+} from the cytosol to the extracellular space to maintain the resting low intracellular calcium concentration and to prevent cells from a lethal overload of calcium. Unlike other P-type ATPases, the PMCA is regulated by calmodulin (CaM) [3,4] and acidic phospholipids [5]. The CaM-binding domain of the pump has been identified in the carboxy-terminal region [6–8], and acts as an autoinhibitory domain, binding to 'receptor' sites in the second and third cytoplasmic units of the pump, in the absence of CaM. The interaction of CaM would remove the binding site from its 'receptor', permitting full expression of pump activity. An activation of the PMCA by acidic phospholipids has been ascribed to the interactions of lipids with C-terminal CaM-binding site, and also phospholipid-binding domain, locating at the C-terminal segment of the loop between transmembrane helices 2 and 3 of the pump (A_L region) [9,10].

Heparin is a highly sulfated linear polysaccharide and implicates in modulating intracellular events by binding many proteins with high affinity [11]. Heparin binds to the α1-subunit of the L-type Ca^{2+} channel [12] and blocks uptake of $^{45}Ca^{2+}$ through this channel at an extracellular site [13]. Intracellular heparin also inhibits binding of inositol 1,4,5-triphosphate (IP_3) to its receptor and the subsequent release of intracellular Ca^{2+} [14], and is therefore known as an IP3 receptor antagonist. A recent study has shown that heparin fragments bearing a C4-C5 unsaturation at the nonreducing end of the molecules promote a decrease of the intracellular calcium, and suggested that the fragments might act upon Na^+/Ca^{2+} exchanger promoting the extrusion of Ca^{2+} [15].

Since PMCA is responsible for the extrusion of calcium from cells and heparin is involved in regulation of cell calcium, we examined the effect of heparin on purified PMCA from porcine brain synaptosome. Our results show that heparin inhibits the

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activity of the enzyme. The inhibitory effect observed may relate to the interaction of heparin with the phospholipid-binding domain of PMCA.

Materials and methods

Materials

Calmodulin-Sephorose CL-4B, Dextran sulphate and Dextran were from Pharmacia. Phosphatidylcholine and phosphatidylserine were from Avanti Polar Lipids Inc. Heparin, ATP, NADH, pyruvate kinase, lactate dehydrogenase, phospho(enol)pyruvate, A23187, trypsin type II-S from porcine pancreas, trypsin inhibitor type I-S from soybean were from Sigma. Bio-beads were from Bio-Rad. Other reagents were commercially available in China and were of AR grade.

Purification of plasma membrane (Ca^{2+} + Mg^{2+})–ATPase from pig brain

The purified PMCA was obtained by the method of Salvador and Mata [16]. Fresh pig brain (approximately 80 g) was homogenized in 10 vol. of 10 mM HEPES/KOH, pH 7.4, 0.32 M sucrose, 0.5 mM $MgCl_2$, 0.2 mM PMSF and 5 mM 2-mercaptoethanol (Buffer I). After low speed centrifugation (2,000 g, 10 min), the supernatant was collected. Microsomes were isolated from the supernatant with high speed centrifugation (30,000 g, 30 min). Then the pellet was collected in Buffer I, loaded in a 20–40% (W/V) sucrose gradient, and centrifuged at 63,000 g for 45 min. The protein at the interface was collected and centrifuged at 30,000 g for 30 min. The pellet was resuspended and lysed on ice for 40 min in 10 mM HEPES/KOH, pH 7.4, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 mM PMSF. The suspension was centrifuged at 30,000 g for 30 min to get the synaptic plasma membrane vesicle (SPMV) in the pellet. The SPMV fraction was resuspended in 10 mM HEPES/KOH, pH 7.4, and 0.34 M sucrose, and stored at -70°C. SPMV at a protein concentration of 6 mg/ml in 20 mM HEPES/KOH, pH 7.4, 300 mM KOH, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mM PMSF, 10 mM 2-mercaptoethanol, and 15% (W/V) glycerol was solubilized by the addition of 0.6% (W/V) Triton X-100 and agitated for 15 min on ice. After centrifugtion at 125,000 g for 30 min, the solubilized protein was loaded onto a calmodulin affinity column and washed with a 50 μM Ca^{2+} buffer. PMCA was eluted from the column by including 2 mM EDTA instead of Ca^{2+} in the buffer. After the chromatography column, fractions containing maximum protein concentration and ATPase activity were pooled. The active fractions containing EDTA were neutralized by the addition of $MgCl_2$ to a final concentration of 1 mM. $CaCl_2$ was added to a final concentration of 100 μM. The purified ATPase was divided into appropriate aliquots, quickly frozen in liquid nitrogen and stored at -80°C. The protein concentration was measured by using the slight modification of the Lowry et al. procedure [16,17] to avoid interference by Triton X-100 and 2-mercaptoethanol, using bovine serum albumin as a standard.

Reconstitution of the isolated ATPase by the Bio-Beads Triton X-100 removal method

The reconstitution protocol was that described by Niggli et al. [5], except that 80 mg/ml Bio-Beads were added to the mixture of phospholipids solution and ATPase every hour for three times and the mixture was agitated slowly at room temperature.

Determination of Ca^{2+}-ATPase activity

The enzyme activity was measured spectrophotometrically at 340 nm with the aid of a coupled enzyme assay [18]. Briefly, microsomes plus a certain concentration of inhibitor were added to the reaction mixture that contained, in a final volume of 1 ml, 40 mM HEPES/KOH, pH 7.4, 120 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 0.15 mM NADH, 0.42 mM Phospho(enol)pyruvate, 10 IU of pyruvate kinase, and 28 IU of lactate dehydrogenase. After 2 min incubation at 37°C, the reaction was started by the addition of 1 mM $CaCl_2$ (10 μM free $CaCl_2$). It should be noted that a preliminary incubation of 3 μg of protein with 3 μg phosphatidylcholine (equivalent to 1mg of lipid/mg of protein) for 5 min at 37°C was necessary. The Ca^{2+}-ATPase activity was obtained after subtraction of the Mg^{2+}-dependent activity, measured in the presence of 1 mM EGTA. Defined concentrations of free Ca^{2+} were established with the aid of $CaCl_2$ and EGTA solutions as described by Bers et al. [19].

Measurement of calcium uptake by the proteoliposomes

Ca^{2+} uptake activity was measured at 30°C by dual wavelength spectrophotometer using arsenazo III as Ca^{2+} indicator as described by Gould et al. [20]. Ca^{2+} uptake was followed by monitoring the differential (675 versus 685 nm) absorption changes which were linearly proportional to changes of the Ca^{2+}concentration in the external medium. The reaction mixture contained 100 mM KCl, 40 mM HEPES-K^+, pH 7.4, 5 mM $MgCl_2$, 20 μM arsenazo III, and 25–30 μg of proteoliposomal protein per ml. Additional $CaCl_2$ (40 μM) was added after obtaining the absorption base line in order to standardize the absorption changes. Then the reaction was started by the addition of 0.5 mM ATP. When the absorption had no changes, 10 μM A23187 was added to the mixture.

Limited trypsin proteolysis of the purified PMCA

The protocol for tryptic digestion was that described by Benaim et al. [21]. Proteosomes were incubated on ice for 30 min in the presence of 50 μg/ml trypsin. The proteolytic medium also contained 40 mM HEPES/KOH, pH 7.4, 100 mM KCl, 10 μM $CaCl_2$, and 100–200 μg/ml protein. Sample digestion was stopped with a 10 fold excess of trypsin inhibitor.

Analysis of results

The different values of K_m were determined using the computer programe Microsoft Origin 7.0. The values of K_m for ATP were determined using OneSiteBind plot and the values of K_m for Ca^{2+} were determined using DoseResp plot. The values shown in figures are means ± S.D. for n different experiments, using different enzyme preparations.

Results and discussion

In order to study the effect of heparin on the plasma membrane Ca^{2+}-ATPase, the purified enzyme from porcine brain synaptosome was reconstituted into phosphatidylcholine liposomes to retain the basal enzyme activity. As shown in Figure 1A, the Ca^{2+}-ATPase activity was decreased on increasing heparin concentrations. Similar inhibitory effect of Ca^{2+} uptake was also observed (Figure 1B). The half-maximal inhibitory concentration (IC_{50}) was approximately 2.2 ± 0.25 μg/ml of heparin. The effect of heparin on the affinity of PMCA for ATP or Ca^{2+} was studied at maximal inhibitory concentration of 30 μg/ml heparin, respectively (Figure 2). It may be observed that heparin decreased the affinity of the enzyme for ATP ($K_m = 23.2 \pm 0.42$ μM) and V_{max} ($V_{max} = 0.95$ μmolPi/min · mg) with respect to the control (without heparin) ($K_m = 9.4 \pm 0.4$ μM, $V_{max} = 1.5$ μmolPi/min · mg) (Figure 2A). The heparin inhibition can not be reversed by the increasing ATP concentration. It is clearly indicated that heparin and ATP do not compete for the same binding sites. In contrast, the enzyme's affinity for Ca^{2+} showed no significant changes in the presence of 30 μg/ml heparin ($K_m = 200 \pm 10$ nM) with respect to that without heparin ($K_m = 245 \pm 10$ nM) (Figure 2B), but the V_{max} ($V_{max} = 1.05$ μmolPi/min · mg) in the presence of heparin was smaller than that in the absence of heparin ($V_{max} = 1.5$ μmolPi/min · mg). This result suggests that the heparin inhibition does not involve the Ca^{2+} binding sites.

The PMCA is stimulated by the binding of CaM to the CaM-binding domain in the C-terminal region. To understand whether the heparin inhibition is related to CaM-binding domain of the enzyme, we thus examined the effect of heparin on the PMCA in the presence of CaM (Figure 3). It was found that the heparin inhibition was maintained after CaM stimulation. The IC_{50} for heparin in the presence of CaM was 1.8 ± 0.2 μg/ml, which was similar to that observed in the absence of CaM (2.2 ± 0.25 μg/ml). This result implied that heparin might not interact with the CaM-binding domain. It has been reported that the CaM-binding domain of the PMCA could be removed by limited proteolysis, such as trypsin [22,23], calpain [24,25], and the digested enzyme sufficed for a CaM-like activation [26]. The effect of heparin on the PMCA stimulated by trypsin treatment was thus examined, with the understanding that limited trypsin treatment liberates the CaM-binding domain from the PMCA, causing its activation independently of CaM. As can be seen in Figure 3, the heparin inhibited the activity of the trypsinized enzyme in a similar manner as observed in the presence of CaM. The IC_{50} for heparin was 1.7 ± 0.2 μg/ml with the trypsin-treated enzyme. This result demonstrated that heparin is an inhibitor of PMCA, and this inhibition is independent of the CaM-binding domain of PMCA. This result also ruled out the interaction of heparin with CaM causing the decreases of enzyme activity in the presence of CaM.

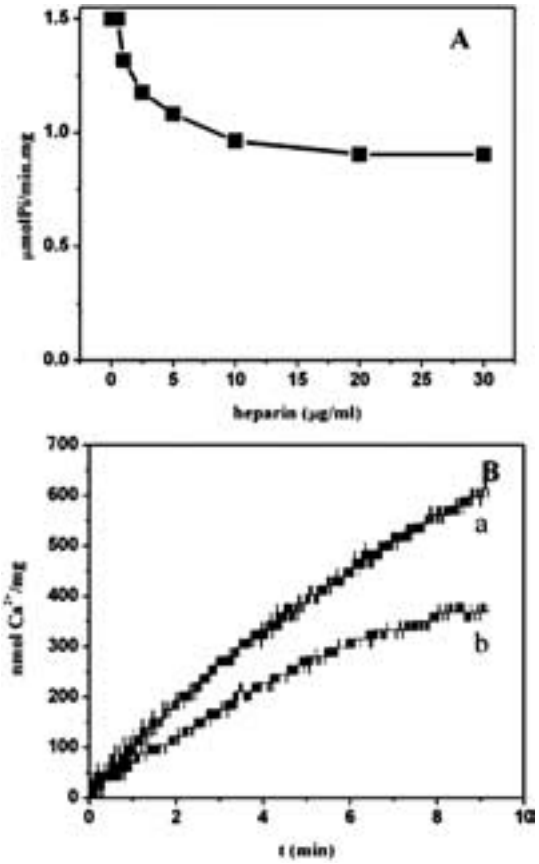

Figure 1. *Inhibition of ATP hydrolysis (A) and Ca^{2+} uptake (B) of the purified PMCA reconstituted in phosphatidylcholine by heparin.* (A): ATP hydrolysis. The reaction mixture contained, in a final volume of 1 ml, 40 mM HEPES/KOH, pH 7.4, 120 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 0.15 mM NADH, 0.42 mM Phospho(end)pyruvate, 10 IU of pyruvate kinase, and 28 IU of lactate dehydrogenase. After 2 min incubation at 37°C, the reaction was started by the addition of 1 mM $CaCl_2$ (10 μM free $CaCl_2$).The reaction mixture contained different concentration of heparin (0–30 μg/ml). Each point represents the means of three independent determinations. (B): Ca^{2+} uptake. The purified ATPase was reconstituted in phosphatidylcholine. The reaction mixture contained 100 mM KCl, 40 mM HEPES-K^+, pH 7.4, 5 mM $MgCl_2$, 20 μM arsenazo III, and 25–30 μg of proteoliposomal protein per ml. Additional $CaCl_2$ (40 μM) was added after obtaining the absorption base line in order to standardize the absorption changes. Then the reaction was started by the addition of 0.5 mM ATP. Experiments were carried out at 30°C in the absence (a) or in the presence (b) of 30 μg/ml heparin.

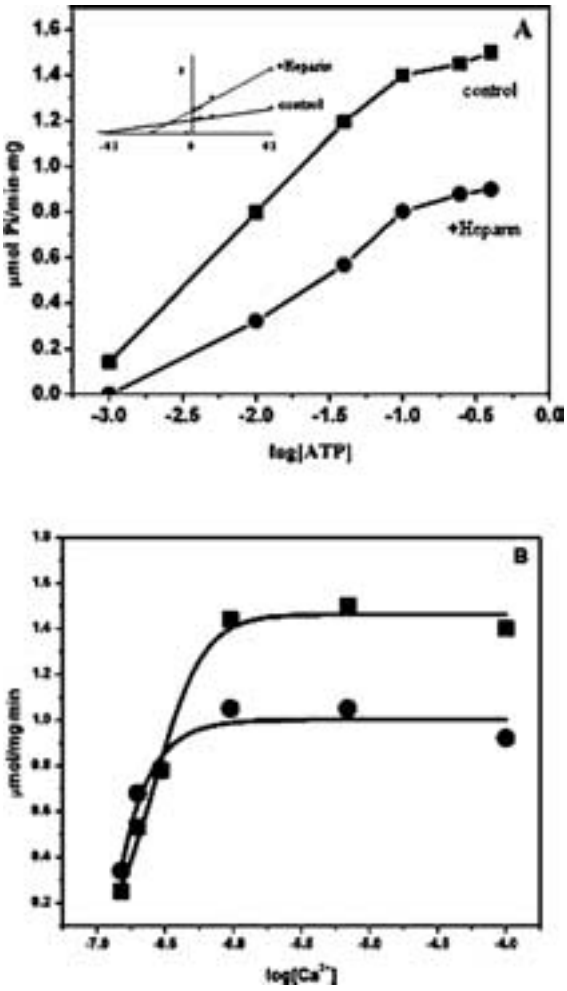

Figure 2. *Effect of heparin on PMCA activity as a function of ATP (A) and Ca^{2+} concentrations (B).* The purified ATPase was reconstituted in phosphatidylcholine. (A): The PMCA's affinity for ATP. The reaction medium contained different ATP concentrations (0–400 μM). Enzymatic activity was assayed at 37°C in the absence (■) or presence of 30 μg/ml heparin (●). The inset shows Lineweaver-Burk plots of the data in Figure 2a in the absence of heparin or in the presence of 30 μg/ml heparin. (B): The PMCA's affinity for Ca^{2+}. The reaction medium contained different free Ca^{2+} concentrations. The rate of ATP hydrolysis was measured at 37°C in the absence (■) or in the presence of 30 μg/ml heparin (●). Each point represents the means of three independent determinations. Other experimental details are given in Materials and methods.

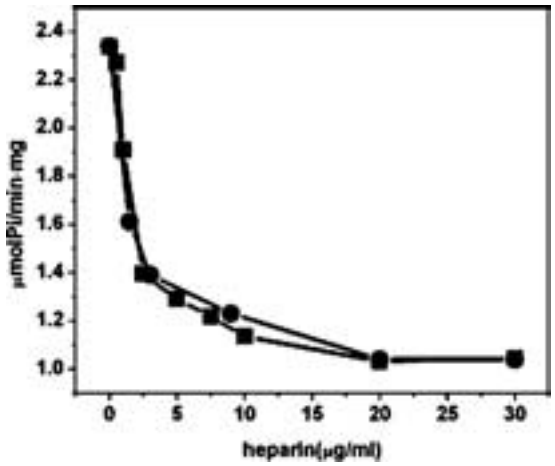

Figure 3. *Inhibition by heparin of PMCA activated by CaM or by trypsin treatment.* The purified ATPase was reconstituted in phosphatidylcholine. The reaction mixture contained different concentration of heparin (0–30 μg/ml). Experiments were carried out at 37°C in the presence of 5 μg/ml calmodulin (■). Enzyme activity data (●) correspond to samples previously subjected to controlled trypsin digestion and measured in the absence of exogenous calmodulin. Each point represents the means of three independent determinations. Other experimental details are given in Materials and methods.

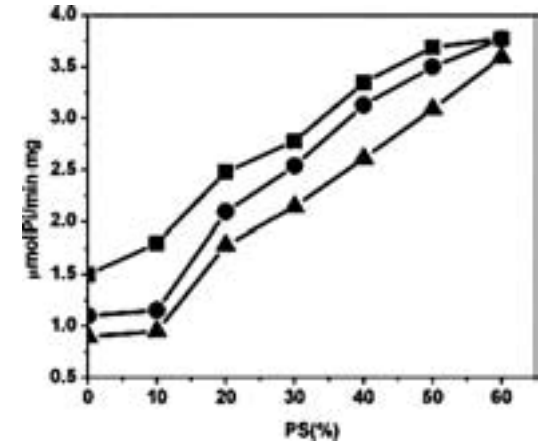

Figure 4. *Recovery of the ATPase activity in mixtures of phosphatidylcholine and phoaphatidylserine.* The purified ATPase was reconstituted into mixtures of phosphatidylcholine (PC) and phosphotidylserine (PS) at the given weight ratio of PS. ■–■, activity of PMCA in the absence of heparin. ●–●, activity of PMCA in the presence of 2.5 μg/ml heparin. ▲–▲, activity of PMCA in the presence of 30 μg/ml heparin. IC_{50} for PS was the PS concentration at which activation percent were 50% ((V_{max}(0.6 PS)–V_{min}(0 PS))/2). Each point represents the means of three independent determinations. Other experimental details are given in Materials and methods.

Besides the C-terminal CaM-binding domain, the C-terminal segment of the loop between transmembrane helices 2 and 3 (A_L region) of the enzyme has been identified to activate the PMCA by binding to acidic phospholipids [9,10]. Since the CaM-binding domain is not critical for the heparin inhibition, we then examined the A_L region, and studied whether excess phosphatidylserine (PS) would eliminate the heparin inhibition. Figure 4 shows that the PMCA activity was completely recovered by addition of PS. The half-maximal activation concentrations (IC_{50}) for PS at heparin concentrations of 0 μg/ml, 2.5 μg/ml, 30 μg/ml were 0.24 ± 0.01, 0.27 ± 0.01, 0.36 ± 0.01 (weight ratio) respectively. The IC_{50} for PS increased in proportion to heparin concentration. Heparin would thus appear

to inhibit the PMCA activity by affecting the enzyme itself, and this inhibition may be eliminated by excess PS. Our results above indicate that (1) heparin affects sites other than the CaM-binding domain of the PMCA and inhibits activity; (2) the heparin inhibition was eliminated by excess of PS.

A wide range of proteins interact with heparin, and it is expected that there should be a common structural theme that defines the specific molecular contacts between these binding partners [27]. Hence, the motifs (XBBXBX) and (XBBBBXXBX) have been identified [28], where B designates a basic amino acid and X any other amino acid. By sequencing the A_L region [7,10], it was found that the A_L region contained PKKEKS, consistent with the heparin binding motif (XBBXBX). We then speculated that heparin would bind to this A_L region of the PMCA to inhibit the enzyme. Our preliminary result supported this suggestion. It was found that a synthetic peptide of 10 amino acids (KVPKKEKSVL) in the A_L region containing PKKEKS motif could recover heparin inhibited activity of the PMCA to about 87% with IC_{50} of 12 ± 2 μM in the presence of 30 μg/ml of heparin.

The PMCA along with the Na^+/Ca^{2+} exchanger are the mediators of the Ca^{2+} extrusion after cell activation, but the Na^+/Ca^{2+} exchanger mediates the Ca^{2+} efflux predominately in cardiac cells [29] whereas the PMCA does so in human erythrocytes [30]. It was reported that heparin inhibited the Ca^{2+} transport ATPase in the membrane of red blood cells [31], although the mechanism remained to be elucidated. Recent studies have shown that heparin is able to produce up to 80% reduction of the cytosolic calcium by binding to the Na^+/Ca^{2+} exchanger from smooth muscle cells [15]. Obviously, how heparin affects the Ca^{2+} extrusion in the PMCA predominant cells should be undertaken for future work.

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