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ATPase and ADPase activities in synovial membrane of equine metacarpophalangeal joint

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Abstract

ATPase and ADPase activities capable of hydrolyzing nucleoside di- and triphosphates in the presence of Ca^{2+} are present in synovial membrane of metacarpophalangeal joint mainly associated to membrane fractions. These hydrolytic activities have been considered involved in the inflammatory process where ATP and ADP are inflammatory mediators while adenosine counteracts this effect. Both, subcellular localization and kinetic properties of these nucleotidase activities, suggest that could correspond to single enzyme called ATP-diphosphohydrolase or apyrase. The comparison of the activity on ATP-Ca and ADP-Ca from normal and pathological equine synovial membrane did not show significant differences either in the subcellular fraction distribution or in the enrichment of each subcellular fraction. Neither differences on 5′-nucleotidase activity present in the microsomal fraction were observed.

Keywords: ATPase; ADPase; ATP-diphosphohydrolase; Apyrase; 5′-nucleotidase; Synovial membrane; Metacarpophalangeal joint

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Introduction

Equine metacarpophalangeal joints are often subjected to overstress and thus susceptible to experience traumas and lesions, inducing synovitis and capsulitis, and also causing pain mainly in the joint capsule [1]. In cases of arthritis or synovitis/capsulitis, the pain stimuli seem to be originated from the synovial membrane and joint capsule innervation [1]. Synovial joints comprise functional units that facilitate the fluid exchange, the adequate blood flow being important for the supply of nutrients to the tissue under compressive load [2]. Cartilage nutrients must cross the blood-joint barrier, which consists of a fenestrated capillary endothelium both in series with the overlying synovial intima and in parallel with the synovium interstitium [2]. The exchange properties of this barrier can be modified by alterations in synovial fluid composition, in capillary and synovial membrane permeability, and in microthrombus formation, which occur through changes in blood flow and innervation [2]. Periarticular edema can be found in articular inflammation indicating an imbalance between vascular permeability and tissue clearance, producing tissue swelling [3,4].

Synoviocytes release various chemical mediators during an inflammatory process, such as lysosomal enzymes, metalloproteinases, cytokines, prostaglandins, and free radicals [5]. In the inflammatory microenvironment, relatively high concentrations of ATP may be released both by various circulating cells, such as erythrocytes, platelets and by locally damaged cells [6–8]. There are reports of adenine nucleotide-dependent mechanisms acting through purinergic receptors, where the role of extracellular nucleotides as ATP and ADP in inflammatory reactions involve a cytotoxic reaction by the production of oxygen free radicals [6].

Extracellular purine concentrations, in great part, are determined by ecto-nucleotidases (membrane-bound enzymes with its active site directed to the external side of the plasma membrane) that degrade nucleoside mono, di, and triphosphates [9–11]. A degrading ADP and ATP enzyme, called ATP-diphosphohydrolase or apyrase has been found in various tissues and cells [9–19]. This enzyme together with 5′-nucleotidase (also an ecto-nucleotidase), which dephosphorylate AMP to adenosine, could sequentially dephosphorylate ATP to adenosine [20]. Adenosine displays anti-inflammatory properties, inhibiting the O_2^- release [6,21]. ATP-diphosphohydrolase corresponds to CD39, a membrane glycoprotein present in activated lymphocytes B, lymphocytes T and natural killers, where probably protects these cells from the potential cytotoxic effect of the extracellular ATP released from their target cells [22]. Ca^{2+}-mobilizing ATP receptors have been identified in neutrophils, monocytes and macrophages, upregulating the surface expression of certain integrin-type receptors of these cells. This is associated to the endothelial surfaces with enhanced adhesion of circulating leukocytes and enhanced migration of the inflammatory cells into peripherical tissue spaces [23]. In addition, certain macrophages also have ATP receptors which induce the formation of non-selective pores that are permeated by ions and small organic molecules, the sustained activation of these receptors being cytotoxic [23]. Therefore, the existence of an enzyme that removes ATP and ADP together with 5′-nucleotidase thus facilitating the production of adenosine, may contribute to an anti-inflammatory action on lesioned equine metacarpophalangeal joints.

The present study aims to determine the presence and subcellular distribution of ATPase–ADPase activities in synovial membrane specimens collected from normal or inflamed equine metacarpophalangeal joints.

Methods

Biological Samples

Twenty normal and twenty congestive samples of synovial membrane were aseptically dissected from metacarpophalangeal joints of standardbred horses obtained from the slaughterhouse. The synovial fluid obtained previously by arthrocentesis was evaluated subjectively for color and viscosity for the classification of normal and inflammatory samples.

Subcellular Fractionation

Synovial membranes washed three times with 0.15 M NaCl were minced and immediately homogenized at 4 °C with an Ultraturrax homogenizer in 4 volumes of 0.25 M sucrose, 50 mM Tris–HCl pH 7.4 and 0.1 mM *p*–phenylmethyl sulphonyl fluoride (protease inhibitor dissolved in dimethylsulfoxide). The homogenate was centrifuged at 3,000×g for 10 min and the supernatant was called crude fraction. The crude fraction was centrifuged at 12,000×g for 20 min, the pellet corresponded to the mitochondrial fraction and this second supernatant was centrifuged at 100,000×g for 1 h (Beckman centrifuge, Model L90). The last pellet and the supernatant constituted microsomal and cytosolic fractions, respectively [24].

Protein Determination

Protein was determined by the Lowry method using bovine serum albumin as standard [25].

Enzyme Assays

ATP-diphosphohydrolase activity was measured with ATP (ATPase activity) or ADP (ADPase activity) as substrates at a final concentration of 2 mM (saturating concentration) in the presence of 5 mM $CaCl_2$ (enough amount to obtain the maximum enzyme activity for complexing all nucleotide present) in 100 mM Tris–HCl, pH 8.0 (optimum pH for the activity); inorganic phosphate (Pi) released was determined according to ref. [26]. The characteristic enzymatic markers to identify the different subcellular fractions used were: succinate dehydrogenase (SDH), 5′-nucleotidase (5′-AMPase) and lactate dehydrogenase (LDH) measured as described in refs. [27–29], respectively. A unit of activity (U) is defined as 1 μmol of product (Pi or reduced methylene blue or NAD) liberated per min at 30 °C.

Effect of Inhibitors

The ATPase and ADPase activities were measured both in presence and absence of several inhibitors at concentrations described as effective inhibitors both of ATP-diphosphohydrolase (50 mM lidocaine) [28], and of ATPases or phosphatases (5 mM ouabain, 100 mM phenylalanine, 0.1 mg/ml oligomycin) [24,28].

Statistical Analysis

The analytical determination of both protein and enzymatic activities of each sample was done at least in duplicate. ATPase and ADPase results are the mean of samples from 20 normal specimens and 20 pathological specimens, and 5′-AMPase data are the mean of 12 normal and 12 pathological samples. SDH, LDH and 5′AMPase distribution in the different subcellular fractions were determined in 10 normal and 10 pathological samples. Differences between the means of control and pathological samples were evaluated by use of unpaired Student's *t*–test.

Results

ATP-diphosphohydrolase Subcellular Distribution

The purity of the subcellular fractions was checked by the presence of the enzyme markers (expressed as Units/mg of protein, specific activity). Data summarized in Table 1, from an average of 10 normal and 10 pathological samples randomly chosen, indicate that the mitochondrial fraction contains higher enrichment of its enzymatic marker, the succinate dehy-

Table 1
Distribution of Enzymatic Markers of Subcellular Fractions. Assay conditions are described in "Methods". Specific activity values (Units/mg protein) correspond to the average values ± SEM of 10 normal and 10 pathological samples

Fraction	SDH (U/mg prot)	5′AMPase (U/mg prot)	LDH (U/mg prot)
Normal samples			
Crude fraction	0.33 ± 0.05	2.95 ± 0.44	2.49 ± 0.19
Mitochondrial fraction	2.50 ± 0.38	0.93 ± 0.16	0.89 ± 0.02
Microsomal fraction	0.45 ± 0.05	2.67 ± 0.38	1.86 ± 0.03
Cytosolic fraction	0.86 ± 0.07	0.60 ± 0.06	4.10 ± 0.67
Pathological samples			
Crude fraction	0.29 ± 0.07	3.51 ± 0.64	2.69 ± 0.28
Mitochondrial fraction	3.00 ± 0.35	1.50 ± 0.40	0.95 ± 0.02
Microsomal fraction	0.42 ±.0.04	2.60 ± 0.37	1.96 ± 0.03
Cytosolic fraction	0.81 ± 0.05	0.90 ± 0.16	4.73 ± 0.49

drogenase. The microsomal pellet shows a higher specific activity of 5′-nucleotidase (marker of plasma membrane) and finally, lactate dehydrogenase, a cytosolic enzyme, is more concentrated in the cytosolic fraction.

The distribution of ATPase–ADPase activities expressed as total units/g tissue together with the protein concentration (mg/g tissue) of the different subcellular fractions obtained from an average of 10 normal and 10 pathological samples are summarized in Table 2. No statistically significant differences in protein concentration and ATPase–ADPase activities per g of tissue (on wet basis) were found between the two groups studied (normal synovial membrane and pathological samples) when crude, mitochondrial, microsomal and cytosolic fractions were compared.

Nevertheless, the highest specific activity of ATPase–ADPase activities distributed in the three subcellular fractions clearly corresponded only to the microsomal fractions isolated from normal and pathological synovial membranes (Fig. 1). In Fig. 1 is also shown the specific activity of 5′-nucleotidase (5′-AMPase) determined in normal and pathological samples. In the normal samples, the average of increase in specific activity of the microsomal fraction versus the crude fraction was 4 times for both ATPase and ADPase activities, and 2.8 times for 5′-AMPase. On the other hand, a lower enrichment was observed in the pathological synovial samples, 2.3 and 2 times for ATPase and ADPase activity, respectively, and 1.7 times for 5′-AMPase.

Effect of Inhibitors

In order to confirm that the ATPase–ADPase activities can be attributed to an ATP–diphosphohydrolase, we determined in 5 normal samples the effect both of some common

Table 2
Distribution of ATPase and ADPase Activities among the Different Subcellular Fractions. Assay conditions are described in "Methods". The analytical determinations of each normal (n = 10) and pathological samples (n = 10) were performed at least in duplicate. Protein and activity values correspond to average values ± SEM (standard error of the mean)

Fraction	Protein mg/g tissue	ATPase Units/g of tissue	ATPase distribution (%)	ADPase Units/ g of tissue	ADPase distribution (%)
Normal samples					
Crude fraction	3.25 ± 0.40	2.19 ± 0.27	100	1.68 ± 0.24	100
Mitochondrial fraction	0.45 ± 0.06	0.46 ± 0.04	21.0	0.30 ± 0.03	17.8
Microsomal fraction	0.29 ± 0.03	0.67 ± 0.05	31.0	0.51 ± 0.07	30.3
Cytosolic fraction	1.68 ± 0.18	0.39 ± 0.16	17.8	0.26 ± 0.04	15.5
Pathological samples					
Crude fraction	2.60 ± 0.36	2.79 ± 0.29	100	2.08 ± 0.25	100
Mitochondrial fraction	0.32 ± 0.02	0.39 ± 0.04	14.0	0.31 ± 0.04	14.9
Microsomal fraction	0.23 ± 0.02	0.54 ± 0.04	19.4	0.38 ± 0.06	18.3
Cytosolic fraction	1.47 ± 0.17	0.49 ± 0.20	17.6	0.39 ± 0.15	18.8

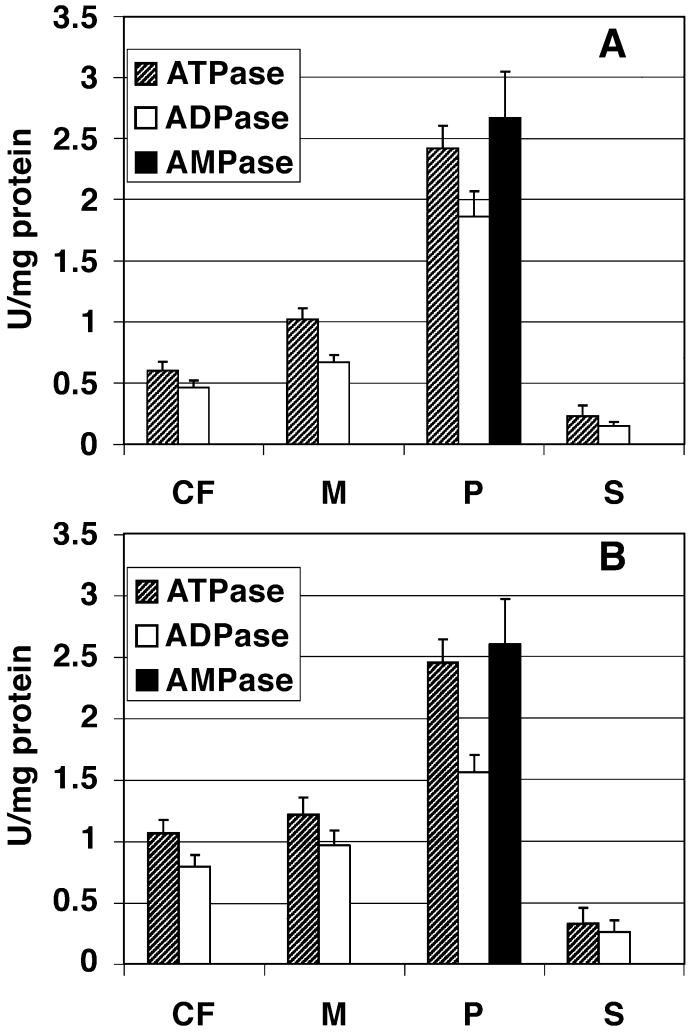

Fig. 1. ATPase–ADPase specific activity in the different subcellular fractions, and 5′nucleotidase in microsomal fraction. Data correspond to average values ± SEM. Plot **A**: Normal samples, n = 20 for ATPase–ADPase activities, and n = 12 for AMPase activity; Plot **B**: Pathological samples, n = 20 for ATPase–ADPase activities, and n = 12 for AMPase activity. **CF**: crude fraction, **M**: mitochondrial fraction, **P**: microsomal fraction and **S**: cytosolic fraction.

inhibitors of ATPases and phosphatases on normal synovial samples (crude and microsomal fractions), and of an specific inhibitor of ATP-diphosphohydrolase [28]. ATPase and ADPase activities showed insensitivity to ouabain, a (Na^+, K^+)-ATPase specific inhibitor, and to oligomycin, a potent mitochondrial ATPase inhibitor. The lack of effect of phenylalanine, an alkaline phosphatase inhibitor excludes that the hydrolytic activity observed on ATP or ADP could be due to the presence of this enzyme. Only lidocaine reduced

significantly in a parallel way both hydrolytic activities, when tested on samples chosen at random. This drug reduced the ATPase and ADPase activity in the crude fraction up to 29.5% and 31% of the initial activity, respectively, while a larger inhibitory effect was detected in the microsomal fraction with a residual activity of 20.5% and 21.5% of ATPase and ADPase activity, respectively.

Discussion

The existence of ATPase–ADPase activities in equine has only been reported in epididymal spermatozoa membrane, which has been attributed to ATP-diphosphohydrolase [30]. Assay conditions used, like pH 8.0, and 2 mM substrate with 5 mM $CaCl_2$ gave the maximum activity similar to the optimum conditions previously described for mammalian ATP-diphosphohydrolase [12,14,17]. Under these conditions, the insensitivity of both hydrolytic activities towards ATPase (mitochondrial and Na^+, K^+-ATPase) and alkaline phosphatase inhibitors discards that the same proportion of the ATPase activity measured under the conditions used could be due to some extent to any of these hydrolytic enzymes. The similar inhibitory effect of lidocaine, a specific inhibitor of ATP-diphosphohydrolase recently reported by our group [28], and the subcellular distribution in the microsomal fraction of both ATPase–ADPase activities suggest that they correspond to a single enzyme, the ATP-diphosphohydrolase. The higher concentration of ATPase–ADPase activities in the 100,000×g pellet also agree with the previously described plasma-membrane localization of this enzyme [9,12–19]. The ATPase–ADPase activities found in the mitochondrial fraction could also be due to ATP-diphosphohydrolase localized in this organelle. It has been recently shown that in human placenta this activity is associated also to the inner mitochondrial membrane [31]. On the other hand, the activity remaining in the cytosolic fraction could be accounted for a release of the enzyme from membranes during the fractionation procedure.

No statistical significant differences were observed both in the distribution and in the ATPase–ADPase specific activities between the two groups of samples under study, normal and pathological joints with visual inflammatory processes. Both the ATPase–ADPase and 5′-nucleotidase activities localized in the microsomal fraction could be important in regulating the nucleotide levels in the extracellular space of equine joints. Depending on the tissue, this enzymatic activity has been related to different physiological functions, such as neurotransmitter catabolism considering the parallel release of neurotransmitter and ATP, control of the platelet aggregation due to its hydrolytic activity on ADP, nucleotide that induced platelet aggregation [9,13,16,18,32]. All these functions correspond to regulation of the extracellular catabolism of ATP and ADP. Several signal transduction mechanisms in cellular responses are based on the interaction of nucleotides (ATP, ADP) through P_2 receptors or adenosine through P_1 purinoceptors [23,33]. It has been proposed that ectonucleotidases presumably serve at least two major roles [23]. The first one is to terminate with ATP/ADP-induced signal transduction, and the second role is to catalyze the generation of adenosine that, in addition to bind to it purinergic receptor, can be incorporated into the cells through specific transporter thus recovering the purine ring.

Enzymatic degradation of extracellular ATP to ADP to AMP, and to adenosine (antiaggregatory signal and down-regulation of inflammation) would transform and reverse the proinflammatory environment brought about by interaction with purinergic receptors on blood cells and vascular endothelium [34,35]. A role for this enzyme been proposed in inflammatory processes [36], however, the ATP-diphosphohydrolase activity can be reduced under certain non-physiological conditions. It has been reported loss of ATP-diphosphohydrolase activity during activation of endothelial cells in vitro, during ischemia–reperfusion injury and with discordant xenograft rejection in vivo [34]. This reduction in activity was associated with the generation of oxidative stress (free oxygen radicals), leukocyte infiltration and platelet deposition. The same group using *cd39* knockout mice has shown that augmentation of this activity could be an important adaptive response for graft survival [35]. These antecedents move us to study the presence of the ATPase–ADPase activities in the equine synovial membrane, considering the presence of inflammatory processes due to overstress of equines. A reduced apyrase-like activity during an inflammatory process would also affect the blood flow due to its ADPase activity that inhibited ADP-induced platelet aggregation [6]. Platelet activation as consequence of vascular injury is counteracted by antithrombic endothelial cells responses, which includes the release of eicosanoids, nitric oxide (endothelium-derived relaxing factor) and the presence of ectonucleotidase on the endothelial cells, which metabolized the released ADP [37,38].

Adenosine release is important for its anti-inflammatory and anti-aggregating properties [20,39]. In addition, this nucleoside stimulates a purinoceptor A_{2b} in cultures synoviocytes decreasing collagenase expression, which is usually overexpressed in rheumatoid processes [40]. It has been reported that during episodes of inflammation polymorphonuclear leukocyte transendothelial migration has the potential to disturb the vascular barrier function and give rise to intravascular fluid extravasation and edema [4]. AMP and adenosine, modulate this endothelial paracellular permeability, thus limiting potentially deletereous increase of this permeability [40].

Nevertheless, some oxidative metabolites of oxygen are released in an inflammatory environment [41]. which could produce a partial loss of nucleotidases activities. However, we did not find a significant reduction of ATPase, ADPase and 5′-nucleotidase activities in pathological samples with 2.46 U/mg of ATPase, 1.56 U/mg of ADPase, and 2.6 for 5′-AMPase activities, values very similar to the normal tissue (2.42 of ATPase, 1.86 of ADPase, and 2.6 U/mg of 5′AMPase activity, respectively). These results indicate that these enzymatic activities are still able to regulate the level of extracellular nucleotides. This is useful for the maintenance of the adequate platelet aggregation rate to avoid or reduce the microthrombus formation observed in some inflammatory processes altering the extraction efficiency of nutrients [2]. Due to the protective effect of both an apyrase-like activity and 5-nucleotidase activity on the inflammatory damage produced by ATP and ADP, findings of a mechanism which increase these activities or their expression would provide an adequate therapeutic treatment to alleviate the inflammatory process in the metacarpophalangeal joints. In addition, the local use of lidocaine, inhibitor of ATPase–ADPase activities (but not 5′-nucleotidase, ref. [28]) could produce the opposite of the desired effect.

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