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In Vitro Action of Matrix Metalloproteinases 2 and 9 Inhibitors on Na^+/K^+-ATPase, H^+/K^+-ATPase and PMCA Activities in the Osmoregulatory Epithelia of Climbing Perch (*Anabas testudineus* Bloch)

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Abstract

Matrix metalloproteinases 2 and 9 (MMP2 and MMP9) are involved in the extracellular matrix (ECM) remodeling. We tested the short-term *in vitro* action of inhibitors of MMP2 and MMP9 on P-type ion transporter function in organ explants of climbing perch (*Anabas testudineus)* to understand how these ECM remodeling components influence the ion transporter function in the osmoregulatory epithelia of fish. Graded doses (10^{-8}, 10^{-7} and 10^{-6} M) of inhibitors of MMP2 and MMP9 were administered *in vitro* to explants of gills, kidney and intestine, kept for either 15 or 30 min and the activities of P-type ATPase such as Na^+/K^+-ATPase (NKA), H^+/K^+-ATPase (HKA) and plasma membrane Ca^{2+}-ATPase (PMCA) were quantified. We found that the inhibitors of MMP2 and MMP9 produced dose- and time-dependent modulation in the activities of NKA, HKA and PMCA in the tested tissue explants. Incubation of MMP2 and 9 inhibitors at the highest dose (10^{-6} M) for 15 and 30 min produced substantial rise in NKA activity. Likewise, HKA activity that showed significant rise after incubation of 10^{-7} and 10^{-8} M inhibitors in gills and kidney explants, decreased at the lowest dose (10^{-8} M) of inhibitors. The lower doses of both inhibitors, while increasing PMCA activity in kidney and intestinal explants inhibited its activity in gill explant. These differential tissue-responsive actions of MMP2 and MMP9 inhibitors indicate that these ECM remodeling components can modify the function of the membrane-bound P-type ion transporters in the osmoregulatory tissues of fish.

Keywords: MMP, fish, ECM, Na^+/K^+-ATPase, PMCA, H^+/K^+-ATPase, Metalloproteinase, *Anabas testudineus*

1. Introduction

Extracellular matrix (ECM) components that are secreted by the cells form a complex network and mediate biological processes during tissue formation and function particularly in remodeling processes to establish physiological homeostasis[1,2]. Numerous biochemical and mechanical interactions operate during ECM-cell communication where many components, including integrin- and discoidin receptors, are involved in the regulation of migration, differentiation and proliferation of cells during normal development and also in pathological conditions[3.1]. The ECM components maintain tissue integrity via heterogeneous protein components and undergo changes in response to cellular stimuli, and these changes range from dynamic homeostasis to remodeling[4]. For example, the major macromolecules in the ECM such as proteoglycans maintain cell-matrix dynamics and the collagen, elastins, laminins and fibronectins as the fibrous proteins maintain tissue integrity[5,6]. These networks of macromolecules present in the ECM help to resist several stresses through continuous remodeling, and it is important for normal functioning of all tissues[7]. ECM carries out several regulatory and instructive

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roles on cellular behavior by providing a dynamic cellular micro-environment for cells[8].

Dynamic remodeling of ECM that maintains normal physiological homeostasis implies its synthesis and deposition due to proteolytic degradation of its own components[5]. Several proteolytic enzymes are involved in the degradation of ECM components, most notably matrix metalloproteinases (MMPs) that cleave the peptide bonds of ECM component proteins and provide tissue homeostasis[9]. MMPs are zinc-binding endopeptidases coming under metazins family and have the capacity for degrading the ECM proteins and basement membranes[10,11]. Among MMPs, a subgroup called gelatinases that include MMP2 (Gelatinase A) and MMP9 (Gelatinase B) have gained considerable focus due to their involvement in degrading collagen IV present in the basement membrane and facilitate normal and pathological remodeling[12-14]. MMP2 and MMP9 are mainly produced by the inflammatory cells including neutrophils, macrophages and endothelial cells[15,16,12].

In fresh water teleost fish, the major osmoregulatory organs, that include gills, kidney and intestine, play important roles in ionoregulation accomplished by the dynamic modulation of ion-transport proteins[17,18]. These osmoregulatory organs of fish are rich in various ion pumps that include Na^+/K^+-ATPase (NKA), H^+/K^+-ATPase (HKA) and plasma membrane Ca^{2+}-ATPase (PMCA) for maintaining proper hydromineral and ion homeostasis[18-21]. The P-type ATPase family members have a common kinetic mechanism where the active transport of specific ions operates across the basolateral plasma membrane through ion-activated ATP hydrolysis[22,23]. NKA and HKA ion pumps are homologous which maintain ionic-balances via transporting sodium ions and protons and are driven by ATP hydrolysis to carry out numerous cellular functions[24,25]. Likewise, PMCA that transports plasma membrane Ca^{2+} ions from the cells and become a fine-tuner of cytosolic Ca^{2+} level, is important for carrying out several Ca^{2+}-dependent cellular functions especially cell signaling and apoptosis[26,27]. Studies revealed that PMCA regulates the Ca^{2+} ion concentration in microdomains of cells and the pump modulates its activity, along with low affinity and high capacity Ca^{2+} exchanger (NCX), and transduces the Ca^{2+} message to cells to carry out cellular functions[28].

Ion pumps in osmoregulatory epithelia maintain ion gradients across the basolateral plasma membrane and provide driving force for cellular and systemic ionoregulation. On the other hand, remodeling of ECM matrix is necessary for normal physiological processes such as development, maintenance of homeostasis and tissue repair in fish[29]. Several studies have shown special functions of gelatinases (MMP2 and MMP9) in fishes including degradation of ECM in muscle tissue and inflammation of immune response[31-37]. However, little information on the role of MMPs in ionocyte function in the osmoregulatory epithelia of fish is available. We, therefore, hypothesized that ECM remodeling in fish osmoregulatory tissues might alter the ionoregulatory performance of ATPase-driven ion pumps. An *in vitro* approach using the inhibitors of MMP2 and MMP9 was explored in this study to understand how MMP2 and MMP9 influence the plasma membrane-bound ion transporters in the osmoregulatory tissue explants of the test species *Anabas testudineus* Bloch.

2. Materials and Methods

2.1 Fish Handling Conditions

Climbing perch *Anabas testudineus* Bloch, commonly known as 'koi', belonging to order Perciformes and family Anabantidae, was used as the experimental model. As an omnivorous freshwater fish that exhibits air-gulping behavior, this fish can thrive well in low-lying water bodies utilizing its well-developed physiological and biochemical mechanisms[38-40]. Healthy adult fish, in their post-spawning phase, were collected from the wild and provided with fish feed at 1.0% body weight (30 ± 5g). Fish were maintained at laboratory conditions for three weeks under natural photoperiod (12 h L: 12 h D) at water temperature ranging from 28° to 29°C and with a mean water pH of 6.4. For static experimentation, the fish were transferred to 50 L glass tanks for two weeks before sampling. There was no mortality of fish under all conditions during experimentation and the fish fed upon the meals provided. The regulations of Animal Ethical Committee of the University were followed.

2.2 Experimental Design

2.2.1 Effects of Varied Doses of MMP2 and MMP9 Inhibitors in Fish

The short-term *in vitro* action of the inhibitors of MMP2 or MMP9 was tested on tissue explants of gills, kidney and intestine of the fish. The activities of P-type ion transporters viz. Na^+/ K^+-ATPase (NKA), H^+/ K^+-ATPase

(HKA) and plasma membrane Ca^{2+}-ATPase (PMCA) in these tissues were quantified. For *in vitro* experiment, twenty-four laboratory-acclimated fish were randomly assigned among four groups of six each. These fish were briefly anesthetized in 2-phenoxyethanol (SRL, Mumbai) solution and the blood was collected from the caudal artery using a 22 gauge heparinized syringe. The blood-free tissue explants were prepared from the gills of second gill arch, posterior kidney and anterior intestine. The tissue explants were cut into small pieces (5 mm) that provided enough surface area for the absorption of the added inhibitors. These tissue explants were washed thrice in perfusion medium to remove the tissue debris before the treatment. These tissue explants were then held for 10 min for equilibration in perfusion medium on an orbital shaker platform at room temperature (28°C). MMP2 or MMP9 inhibitor at graded concentrations (10^{-8}, 10^{-7}, or 10^{-6} M) was added *in vitro* to the incubation medium that contained the tissue explants for either 15 or 30 min. Similarly, control fish which lacked the inhibitors were maintained concurrently. After incubating the explants for specified durations, the incubation was terminated by keeping the explants at 4°C and were thoroughly washed with ice-cold perfusion medium for several times. The explants were then kept in SEI buffer (0.05 M pH 7.1) that contained 0.25 M sucrose, 10 mM Na_2EDTA, and 0.1 M imidazole and stored at -80°C until analysis.

Frozen tissue explants (gills, kidney, and intestine) were thawed on ice quickly, weighed and homogenized (1:10, w:v) in SEI buffer (0.05 M; pH 7.1) using glass homogenizer fitted with Teflon pestle. The homogenates were centrifuged at 700 x g for 10 min at 4°C (Eppendorf 5430R) and the supernatant fraction (Ho) was collected after removing cell debris. The oubain-specific Na^+/K^+-ATPase (NKA), and SCH 28080-sensitive gastric H^+/K^+-ATPase (HKA) activities were quantified in the Ho fraction. Vanadate-sensitive plasma membrane Ca^{2+}-ATPase (PMCA) activity was quantified in mitochondria-free supernatant which was collected after centrifuging the Ho fraction at 10,000 x g for 10 min at 4°C. Protein concentrations of these fractions were measured using modified Biuret assay using bovine serum albumin as the standard[41].

2.3 Ouabain-sensitive Na^+/K^+-ATPase (NKA) Specific Activity

The hydrolytic activity of NKA in membrane fractions of gill, kidney and intestine explants was quantified adopting the method described for microplate assay[40]. Briefly, each tissue samples (1.0 µg protein) was assayed in duplicates. Samples were added to a 96 microplate with saponin (0.2 mg $protein^{-1}$) that increased the substrate accessibility. Samples were then incubated in buffer containing 100 mM NaCl, 30 mM imidazole (pH 7.4), 0.1 mM EDTA and 5 mM $MgCl_2$, where 0.13 mM KCl was used as promoter and 0.14 mM ouabain was used as inhibitor. The reaction was initiated by the addition of 0.3 mM ATP, and incubated for 15 min at 37°C and terminated by adding 8.6% ice-cold TCA. The inorganic phosphate released was measured in microplate reader (Synergy HT Biotek, USA) at 700 nm against sodium phosphate as the standard. The difference of reaction rate between total ATPase activity and ouabain-sensitive activity was calculated and expressed as µmoles of Pi liberated per hour per mg protein.

2.4 SCH 28080–sensitive H^+/K^+-ATPase (HKA) Specific Activity

The hydrolytic activity of HKA in membrane fractions of gills, kidney and intestine were quantified based on NKA microplate assay[40]. Each plasma membrane fraction from tissue samples (1.0 µg protein) were added to a 96 microplate after mixing the samples with saponin (0.2 mg $protein^{-1}$) and assayed in duplicates. As inhibitor, 0.1 mM SCH28080 [2-methyl-8-(phenylmethoxy) imidazole [1,2-a] pyridine-3-acetonitrile], was used whereas 0.13 mM KCl was used as promoter. The reaction was initiated by the addition of 0.3 mM ATP followed by incubation for 15 min at 37°C. After adding 8.6% ice-cold TCA the inorganic phosphate production was assayed in microplate reader. The rate of enzyme activity was expressed as micromoles Pi liberated per hour per mg protein.

2.5 Vanadate-sensitive Plasma Membrane Ca^{2+}-ATPase (PMCA) Specific Activity

The rate of PMCA in gill, kidney and intestine explants was quantified based on NKA microplate assay[40]. As inhibitor, vanadate (mM) was used, whereas $CaCl_2$ (mM) was used as promoter. The reaction was started by the addition of 0.3 mM ATP following an incubation period for 15 min at 37°C. The inorganic phosphate released after adding 8.6% ice-cold TCA was measured in Synergy HT Biotek microplate reader and the enzyme activity was expressed in µmole of Pi liberated per hr per mg protein.

2.6 Data Analysis

Statistical analysis was performed by means of one-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test, and data were expressed as mean ± SE. Data were collected from eight fishes in each group and significance between the groups were analyzed with the help of Graphpad software (Graphpad Instat-3, San Diego, USA) and the level of significance was accepted if $P< 0.05$.

3. Results

3.1 *In vitro* Action of MMP2 and MMP9 Inhibitors on NKA Activity

Dose- and time-dependent increase in NKA activity after 15 and 30 min of *in vitro* exposure of medium (10^{-7}) and higher (10^{-6}) doses of MMP2 inhibitor were found in gill explants (Figure 1A). Similarly, exposure of gill explants to medium and higher doses of MMP9 inhibitor after 15 min incubation produced a significant increase in NKA activity (Figure 1B). Likewise, 30 min incubation of MMP9 inhibitor produced a significant increase in NKA with all doses tested (Figure 1B). In kidney explants, exposure to the higher dose of MMP2 inhibitor for 15 and 30 min produced significant increase in NKA activity, whereas MMP2 inhibitor treatment at medium dose at these time intervals produced significant decrease in NKA activity (Figure 1C). Incubation of higher dose of MMP9 inhibitor for 15 min produced a marked increase in NKA activity but the activity decreased after incubation with the lower and medium doses, though 30 min incubation produced a significant increase of NKA activity at all tested doses (Figure 1D). In intestinal explants, MMP2 inhibitor incubation at the medium and higher doses for 15 min increased the NKA activity, whereas the activity decreased after the lower dose of MMP2 inhibitor (Figure 1E). MMP9 inhibitor produced a significant decrease in NKA activity at the lower dose, though its activity shoot up after exposure to the higher dose of MMP9 inhibitor for 15 min (Figure 1F). The MMP9 inhibitor exposure for 30 min, however, did not evoke any response in intestinal NKA activity (Figure 1F).

3.2 *In vitro* Action of MMP2 and MMP9 Inhibitors on HKA activity

Gill explants, upon exposure to a higher dose of MMP2 inhibitor for 15 min, produced a significant increase in HKA activity but its activity decreased after incubation with the lower and medium doses of inhibitor for 15 min but incubation with medium and higher doses for 30 min produced significant increase in its activity (Figure 2A). Incubation of MMP9 inhibitor for 15 min and 30 min at the medium and higher doses produced significant increase in HKA activity in gill explants (Figure 2B). In kidney explants, HKA activity showed decrease after 15 min incubation of MMP2 at the lower and medium doses, but it showed an increase after a higher dose of MMP2 inhibitor (Figure 2C). The HKA activity in kidney explants showed an increase after 30 min incubation of MMP2 inhibitor at the medium and higher doses, and the MMP9 inhibitor exposure increased the HKA activity to significant levels at all the tested doses for 30 min (Figure 2C). On the contrary, 15 min incubation of the higher dose of this inhibitor significantly increased HKA activity but it was significantly decreased at the lower and medium doses (Figure 2D). In intestinal explants, MMP2 inhibitor at the medium and higher doses for 15 min increased the HKA activity in a dose-dependent manner, though a decrease in its activity was found after exposure to all the three doses of MMP2 inhibitor for 30 min. MMP9 inhibitor exposure significantly activated the intestinal HKA activity after 15 min of incubation at all three doses, whereas 30 min incubation produced an increase in its activity at the higher dose (Figure 2F).

3.3 *In vitro* Action of MMP2 and MMP9 Inhibitors on PMCA Activity

A significant decrease in PMCA activity was found after 15 min and 30 min incubation with the higher dose of MMP2 inhibitor in gill explants (Figure 3A). Likewise, PMCA activity decreased in gill explants after 30 min incubation with all the three doses of MMP9 inhibitor. On the contrary, the higher dose of MMP9 inhibitor for 30 min incubation increased the PMCA activity in gill explants (Figure 3B). The PMCA activity in kidney and intestine explants, however, did not show any response to 30 min of MMP2 inhibitor incubation (Figure 3B). After 15 min exposure with MMP2 inhibitor, a gradual dose-dependent increase in PMCA activity was found in kidney explants (Figure 3C). Incubation of MMP9 inhibitor for 30 min produced significant increase of the PMCA activity in kidney explants at all three doses (Figure 3D). In intestine explants, exposure to the higher dose of MMP2 inhibitor for 15 min produced increase of the

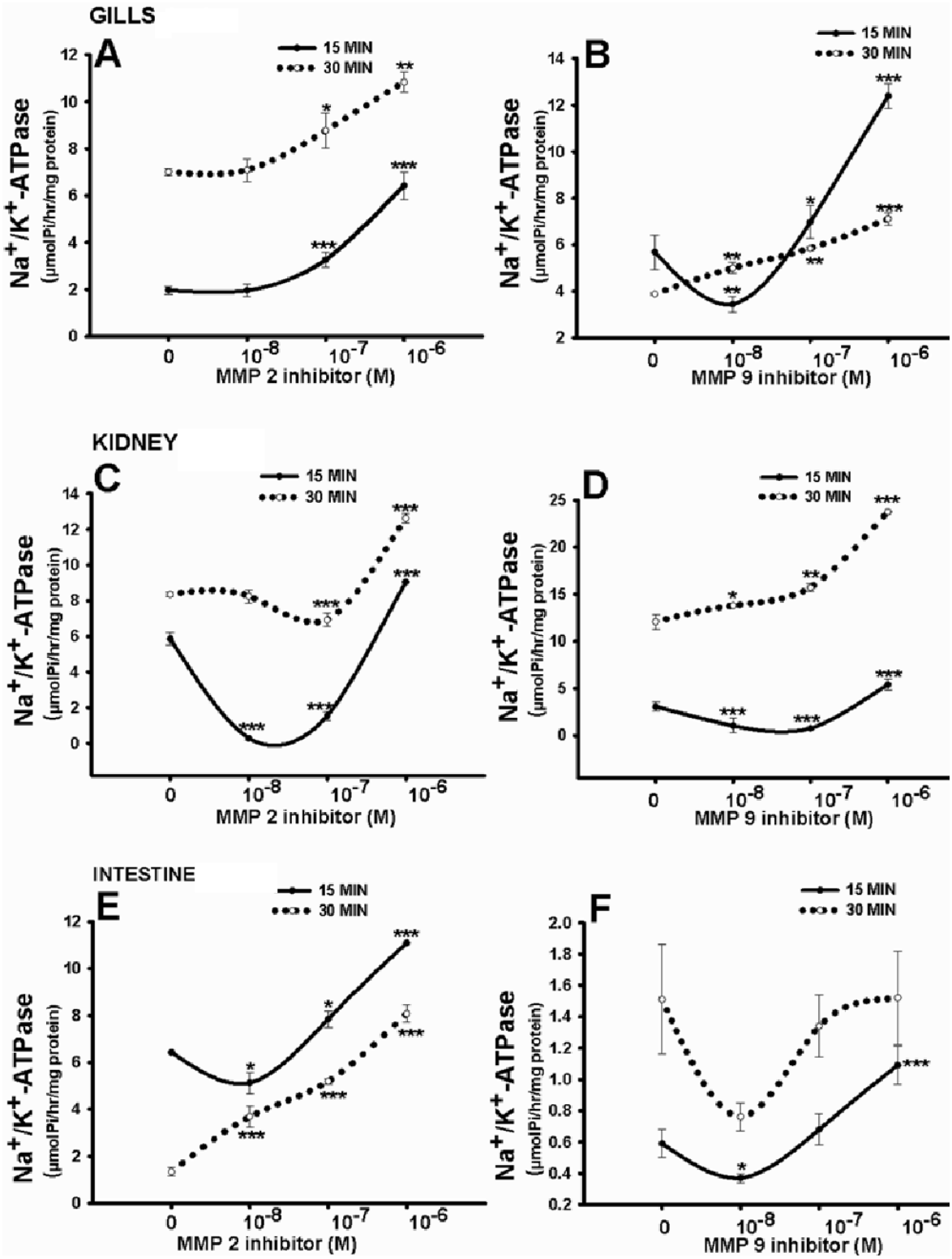

Figure 1. (A and B), (C and D) and (E and F) Showing the *in vitro* effects of varied doses of MMP2 and MMP9 inhibitor (MMP2/MMP9; 10^{-8}, 10^{-7}, 10^{-6} M) for 15 min and 30 min on Na^+/K^+-ATPase activity in the gills, kidney and intestine of fish. Each point is mean ± SE for six fish. The significance levels are represented as "*" ($P < 0.05$), "**" ($P < 0.01$) and "***" ($P < 0.001$) compared to control fish.

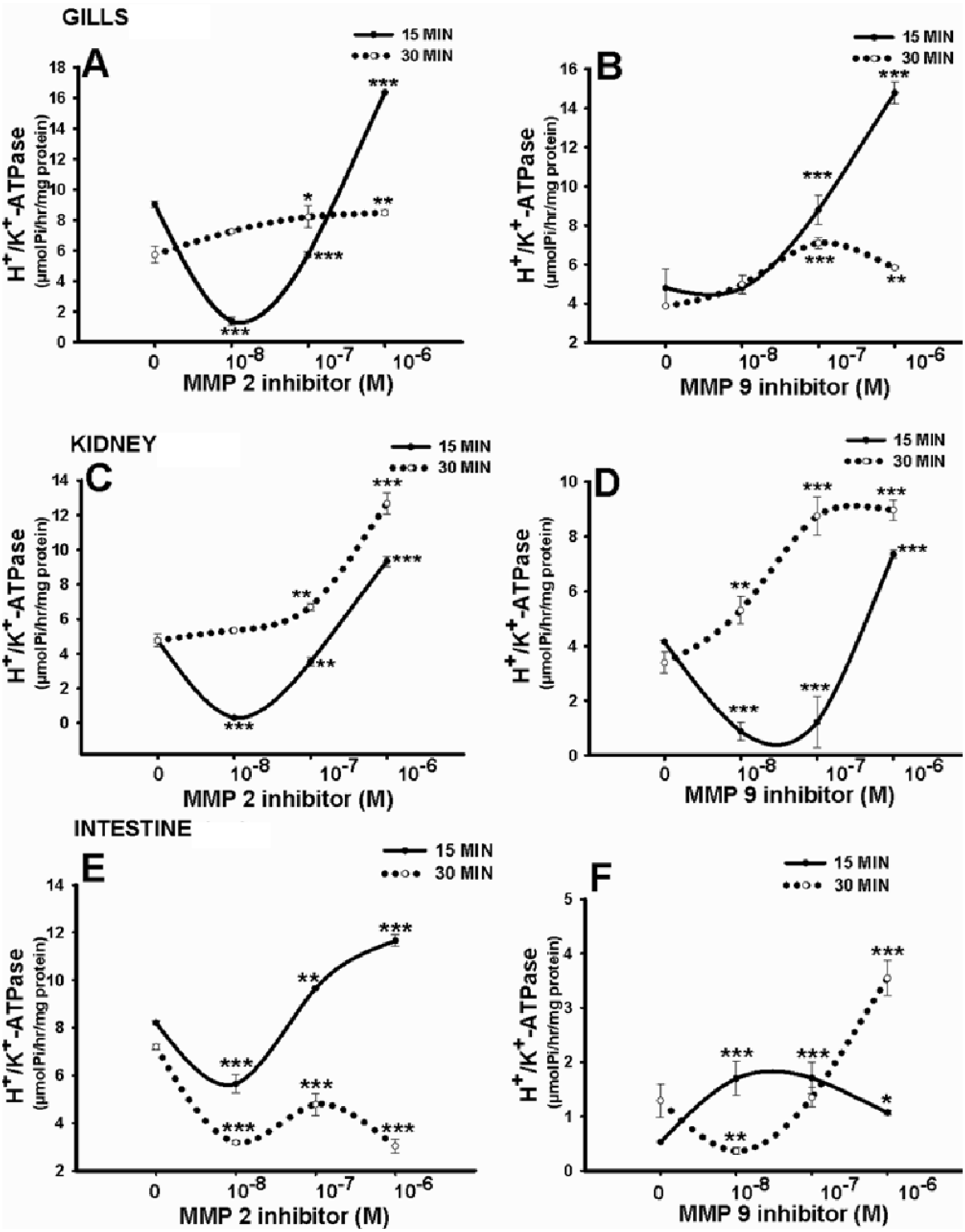

Figure 2. (A and B), (C and D) and (E and F) Showing the *in vitro* effects of varied doses of MMP2 and MMP9 inhibitor (MMP2/MMP9; 10^{-8}, 10^{-7}, 10^{-6} M) for 15 min and 30 min on H^+/K^+-ATPase activity in the gills, kidney and intestine of fish. Each point is mean ± SE for six fish. The significance levels are represented as "*" ($P < 0.05$), "**" ($P < 0.01$) and "***" ($P < 0.001$) compared to control fish.

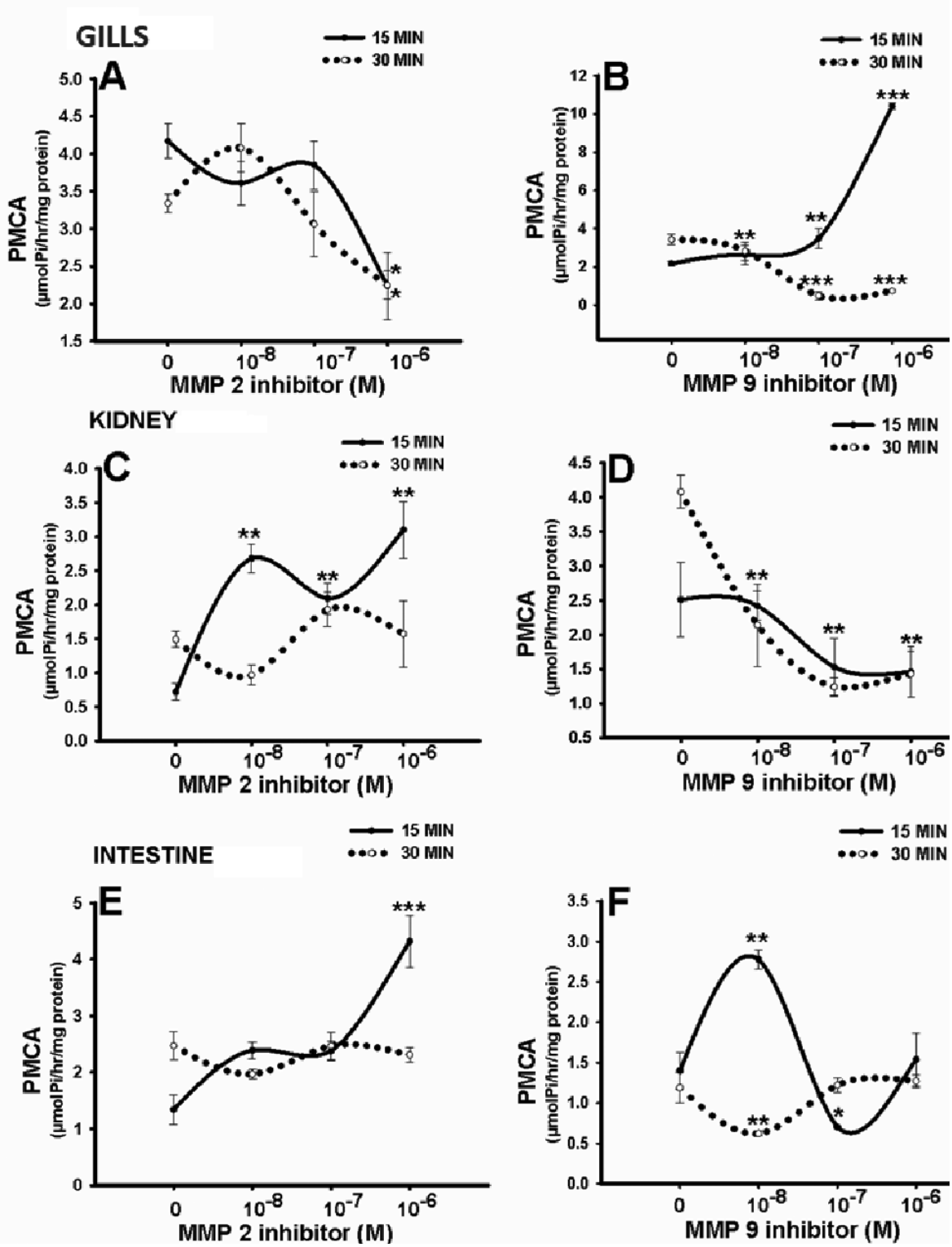

Figure 3. (A and B), (C and D) and (E and F) Showing the *in vitro* effects of varied doses of MMP2 and MMP9 inhibitor (MMP2/MMP9; 10^{-8}, 10^{-7}, 10^{-6} M) for 15 min and 30 min on fish plasma membrane Ca^{2+}-ATPase activity (PMCA) in the gills, kidney and intestine of fish. Each point is mean ± SE for six fish. The significance levels are represented as "*" ($P < 0.05$), "**" ($P < 0.01$) and "***" ($P < 0.001$) compared to control in the intestine of fish.

PMCA activity, whereas the low dose of MMP9 inhibitor decreased its activity (Figure 3E). In intestine explants, MMP9 inhibitor incubation at varied doses for 30 min produced significant decrease in PMCA activity but it showed activation after 30 min incubation of higher dose of inhibitor (Figure 3F).

4. Discussion

The ECM is continuously remodeled in response to both intracellular and extracellular stimuli that modulate the physiological and pathological processes. The degree of remodeling, however, depends on the time and course of exposure to stimuli[42,43]. The roles of MMP2 and MMP9 are well studied in mammals where they are involved in the breakdown of ECM components and thus facilitate tissue remodeling during normal and pathological conditions[44-46]. However, the physiological role of MMPs on ion transport function has not yet been examined in fish.

Fishes are unique among vertebrates and have well developed mechanisms to regulate osmotic and ionic homeostasis through several ion transporters that are important for maintaining normal cellular and physiological activities[47,40]. The physiological action of MMPs on ion transport function has not yet been studied in fishes. In the present study, we selected lower doses of inhibitors of MMPs in nanomolar concentration to evoke physiological response that does not reveal any toxicity as suggested earlier[48]. We used tissue explants for studying the action of inhibitors of MMPs since earlier investigators have extensively explored *in vitro* cell line studies[48,49].

In fresh water teleosts, the ionic and osmotic balances are maintained through various ion pumps, especially P-type ion transporters such as NKA, HKA and PMCA present in the osmoregulatory organs[18,19,21]. As the major osmoregulatory organs of teleost fish gills, kidney and intestine play major roles in integrating ionic and osmotic homeostasis that are sensitive to stressful environmental conditions[50,17,18,40,51]. The role of ECM components, particularly MMP2 and MMP9, in ion transporter functions that regulate ion homeostasis is not yet understood in fishes. The specific inhibitors of tissue MMP2 and MMP 9 used in this study clearly indicate that the inhibitors of MMP2 and 9 might have lowered its tissue contents in the tested tissues that would lead the modulation of the ion transporter activity. The elevated NKA activity in all tested explants after inhibitor treatments indicate a role for MMP2 and 9 in Na^+ homeostasis. In freshwater teleost fish, the active uptake of Na^+ ion from freshwater is mainly carried out by the several channels and sodium hydrogen (NHE) antiporters present in the apical membrane. The concurrent excretion of Na^+ ion into plasma occurs due to the coordinating functions of ion channels and transporters including NKA, Na^+/Cl^- transporters (NCC), Na/K/Cl co-transporters (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels[52-55].

The dose and time-dependent action of MMP2 and MMP9 inhibitors on NKA activity in the organs playing role in osmoregulation point to a direct action of these inhibitors on remodeling proteases such as gelatinase A and B. Similar response has been found in HKA activity which also showed a dose-dependent increase after treatment of inhibitor. It is likely that structural similarity between NKA and HKA would also contribute to its similar response to the tested inhibitors[26]. The present *in vitro* model thus confirms the temporal and spatial action of these inhibitors on the tested ion transporter functions in the osmoregulatory epithelia. This further indicates that remodeling of ECM component would demand involvement of gelatinases A and B as major extracellular regulators that can control membrane-bound ion transport function. In fishes, MMPs are known for involvement in follicular development, oocyte maturation, embryogenesis and immune response[4,56-60]. Furthermore, MMP2 and MMP9 inhibitors have been shown to modulate the migration of lens epithelial cells during their development under normal conditions[60,61]. Several studies used MMP2 and MMP9 inhibitors to examine vascular smooth muscle migration after vascular injury, progression of various cancers, and remodeling of lungs during hypoxia[62-65]. It is clear that a pool of NKA that resides in the plasma membrane is activated by the modified MMP2 and 9 distributions due to the inhibitor treatment in the tested tissue explants. This, further, points to the cross-talks that exist between ECM components and ionocytes present in the osmoregulatory epithelia of fish.

The diffusion loss of ions and the osmotic influx of water are balanced in freshwater teleosts via absorption of ions across the gills and excretion of large amounts of urine through the kidneys[66]. The ion transporters present in the osmoregulatory organs carry out these functions and maintain ionic equilibrium across the plasma membrane, utilizing energy derived from ATP hydrolysis. Plasma membranes of osmotic epithelia that possess NKA, HKA and PMCA actively pump Na^+/H^+ and Ca^{2+}

ions that play major roles in cellular and systemic ion homeostasis[33,67,28,68,40]. It appears that during the incubation of tissue explants with the inhibitors of MMP2 and MMP9 there could be disturbed synthesis of MMP2 and MMP9 that would ultimately loosen the ECM architecture that holds the ionocytes. The elevated ion pump activities, therefore, account for the disturbed MMP2 and MMP9 synthesis. Our data further indicate that the higher dose of inhibitor could loosen the ionocytes that would augment the rate of activity of NKA and HKA tested in tissue explants. Furthermore, it appears that the concentration and duration of exposure of MMP inhibitor would bring about substantial alteration in ECM architecture leading to a modulated NKA and HKA activities. These findings are in agreement with the previous studies that reported major role of ion transporters in normal and pathological conditions as it can regulate ionic mechanisms of cells via pH-dependent and Ca^{2+}– dependent regulation of cell volume[69]. Further, our results imply that MMP2 and MMP9 are critical in regulating these membrane-bound ion pumps and thus support the notion that ionocyte function is in tune with ECM components, pointing further to the integration of ion transporter functions during ECM remodeling.

The data thus support the hypothesis that experimental remodeling using the inhibitors of MMP2 and MMP9 would modify the performance of P-type ion pumps in osmoregulatory epithelia of fish. This study, first of its kind, thus, explores the integrative action of MMP2 and MMP9 as ECM remodeling components on the ion transporter functions in osmoregulatory epithelia of fish.

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