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Regulatory Differences among Avian Ecto-ATPases

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Extracellular ATP is found to produce a variety of important biological responses. Ecto-ATPases are located on numerous cell types in many different species, regulate extracellular ATP levels and can be a key step in generating adenosine. Studies conducted on chicken ecto-ATPases from liver and cardiac and smooth muscle show a variety of differing properties including (1) different apparent K_m's, (2) lectin sensitivity, (3) responses to detergents, (4) responses to lipid mediators, and (5) responsiveness to nucleotide-mimetic affinity labels. These results suggest that although each enzyme hydrolyzes extracellular ATP, they should each be viewed as a distinct subtype of the whole ecto-ATPase family due to their differential responses, largely linked to proposed regulatory phenomenon.

Key Words: **ecto-ATPase; kinetic analysis; lectins; detergents; affinity labeling; lipid mediators.**

An extremely efficient magnesium or calcium activated externally active ATPase exists in a variety of tissues including skeletal muscle, smooth muscle, cardiac muscle, brain, lung, kidney, pancreas, liver, prostate, and oviduct (1–9). This ecto-ATPase (E-type) is characterized by insensitivity to inhibitors of the P-type, F-Type, and V-Type ATPases; ouabain, azide, and bafilomycin, respectively.

Almost as ubiquitous as the ecto-ATPase on the plasma membrane are receptors that bind either ATP or ADP (P_2-receptors) and adenosine receptors (P_1-receptors). Extracellular ATP can evoke at least two cellular responses: 1) intracellular [Ca^{2+}] increases and protein kinase C activation, 2) activation of ligand gated cation channels (10). Extracellular ATP sequentially hydrolyzed to adenosine is recognized by a P_1-receptor, leading to increases in intracellular cAMP (11). It appears that extracellular ATP produces a variety of cellular effects dependent upon the tissue type and that the role of the ecto-ATPase is quite likely to regulate extracellular ATP, ADP and adenosine. However, the connection between the ecto-ATPase and P_2- and P_1-receptors is largely unexplored.

There are few studies describing the characterization of ecto-ATPases from the same species. To better understand the physiological roles of the ecto-ATPase, the ecto-ATPases from chicken liver, smooth and cardiac muscle have been explored. Using lectin stimulation, substrate and inhibitor studies, detergents and an affinity label, we show that there appear to be three ecto-ATPase subtypes with diverse features potentially related to regulation of hydrolytic activity.

MATERIALS AND METHODS

Chicken organs were purchased from a local firm within twelve hours of slaughter. Nonidet P-40, n-octyl glucoside, digitonin and L-α-lysophosphatidylcholine, ConA, DTT, lipid mediators, and all coupled enzyme assay reagents were purchased from Sigma Chemical Company (ST. Louis, MO). TIFIAA was purchased from Molecular Probes (Eugene, OR).

Chicken tissue microsomes. Chicken smooth muscle, heart, and liver were trimmed of fat and minced and 50-100 grams were placed in 400 mL of homogenation buffer (20 mM MOPS, pH 7.4, 1 mM EDTA, 10 mM EGTA, 1 mM DTT, and 10% (w/w) sucrose). The tissue was homogenized using a Waring blender and centrifuged at 6,000 $\times$ g for 10 minutes. The pellet was discarded, and the supernatant was centrifuged at 30,000 $\times$ g for 20 minutes. The pellet was discarded, and the supernatant centrifuged at 100,000 $\times$ g for 60 minutes. The pellet was resuspended in 20 mM MOPS, pH 7.3, and containing 5% sucrose. The microsomes were further purified on a discontinuous 25%-35% sucrose density gradient and spun in a Beckman SW-27 rotor at 24,000 rpm for 6.5 hours. The white fluffy material at the interface was collected, diluted two fold with water, and centrifuged at 100,000 $\times$ g for 60 minutes. All centrifuge runs were conducted at 4° C. Unless detergent solubilization was attempted, the pellet was resuspended in storage buffer (20 mM MOPS, pH 7.3, containing 30% sucrose) and stored at −80° C until used. Specific activities for smooth muscle, liver, and heart ecto-ATPases were

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Abbreviations used: AA, arachidonic acid; ConA, concanavalin A; DMS, N,N-dimethylsphingosine; DTT, dithiothreitol; LPC, lysophosphatidylcholine; MOPS, 3-[N-morpholino]propanesulfonic acid; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PMA, 4-α-phorbol 12-myristate 13-acetate; R59022, diacylglycerol kinase inhibitor I; TIF, tetraiodofluorescein; TIFIAA, tetraiodofluorescein iodoacetamide.

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TABLE 1

Differential Tissue-Dependent Kinetic Responses to ATP

Tissue	K_m of apparent high affinity site	K_m of apparent low affinity site	ConA effect
Smooth Muscle	19 ± 9 μM	200 ± 10. μM	Conversion to a single site
Cardiac Muscle	27 ± 10. μM	385 ± 75 μM	No effect
Liver	None	340 ± 84 μM	No effect

Note. Ecto-ATPase activities were evaluated as a function of ATP concentration. Double reciprocal plots were created from these data and K_m's calculated. The coupled enzyme system described in "Methods and Materials" was used to calculate specific activity.

about 150, 110, and 60 μMol/hour/mg respectively under conditions specified below.

Detergent solubilization. Membranes were resuspended in solubilization buffer (20 mM TRIS, pH 7.4, 5 mM $MgCl_2$) containing 1% (w/w) of detergent and incubated on ice for 30 minutes. This mixture was spun at 100,000 × g for 1 hour at 4° C. The supernatants were tested for activity immediately. The pellets were resuspended in storage buffer and also tested for activity.

TIFIAA additions to partially purified membranes. Membranes were brought to pH 8.0 by the addition of 150 mM MOPS, pH 8.3. TIFIAA was added to bring about a final concentration of 10 μM. This mixture was incubated on ice for 60 minutes before being quenched DTT (200 μM final).

ATPase activities. ATPase activity was determined with a coupled-enzyme, ADP release assay. Ecto-ATPase activity was measured by incubating 5-30 μg of protein in a 1 mL assay mixture containing 20 mM MOPS, pH 7.3, 5 mM $MgCl_2$, 0.2 mM EGTA, 1.5 mM phospho(enol)pyruvate, 0.3 mg/mL NADH and non-limiting amounts of pyruvate kinase and lactate dehydrogenase at 30° C. 1 mM (final) ATP was typically added to initiate the reaction and the oxidation of NADH was monitored. To inhibit the Na,K-ATPase, the Ca-ATPase, and F_1 ATPase 100 μM vanadate and 5 mM azide were added. Previous studies have shown that the enzyme is insensitive to bafilomycin. For ConA stimulation, the indicated amount of ConA was incubated in the assay mixture for 5 minutes at room temperature prior to ATP addition.

RESULTS

Differential kinetic responses of the liver, smooth and cardiac muscle type ecto-ATPases to ATP and ConA. Biphasic double reciprocal plots consistent with negative cooperativity or interconvertable substrate binding sites that do not co-exist independently, have been shown for the skeletal muscle ecto-ATPase (1). Two of the three ecto-ATPases studied exhibit a similar response (Table 1). All three ecto-ATPases in this study exhibit similar apparent K_m's for the low affinity ATP binding site, 200-385 μM. The high affinity site for the smooth and cardiac muscle type ecto-ATPases show similar apparent K_m's ranging in value from 19-27 μM. This biphasic response has been previously abolished with lectins in the case of skeletal muscle T-tube, brain and smooth muscle types (2), suggesting that upon lectin binding, the low affinity binding site is somehow sterically blocked or abolished. Notably, ConA does not affect the heart ecto-ATPase biphasic response (data not shown).

Previous work had also established that lectins prominently stimulate the skeletal muscle T-tube, brain, and smooth muscle ecto-ATPases (1,2). Lectin stimulation of other ecto-ATPases was evaluated and the data in Figure 1 show maximum activation of the smooth muscle enzyme to be about 2.3-fold, while ConA activation of heart and liver is absent. The large error bars associated with the ConA stimulation of the smooth muscle ecto-ATPase activity are due to the variability from one membrane preparation to the next and is thought to be due to variable endogenous levels of lipid mediators. Although every smooth muscle ecto-ATPase preparation shows lectin activation, the stimulation varies moderately. Those preparations lower in initial enzyme specific activity tend to have higher ConA activations, while those higher in activity tend to have less ConA activation. Conversely, there was little difference in the liver and cardiac muscle preparations in their response to ConA.

The ecto-ATPases show varied detergent solubility. TX-100 has been shown to be an irreversible inhibitor of the skeletal, brain and smooth muscle forms at concentrations well below the CMC (1). Here, other detergents were used to investigate ecto-ATPase solubilization (Table 2). The cardiac and smooth muscle ecto-ATPases showed extreme sensitivity to detergent exposure and/or solubilization, most prominently seen with NP-40. The highest yield after detergent treatment was no higher than 21% total activity in the supernatant. In contrast, liver ecto-ATPase total activity was consistently over 90%, irrespective of the detergent used. Specific activities of the cardiac and smooth muscle typically were only 10% of the original activity,

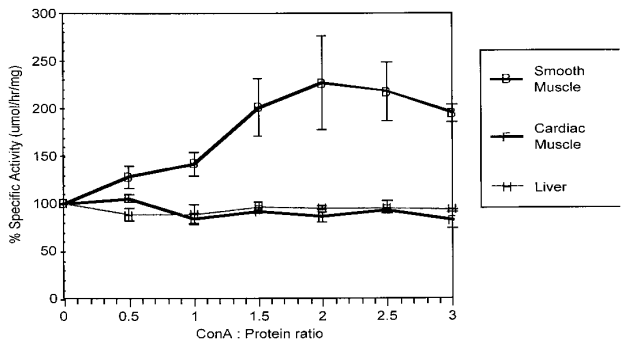

FIG. 1. ConA modulation of the ecto-ATPases from liver and smooth and cardiac muscle differ. Membranes were incubated in 1 mL reaction mixture at room temperature for 5 minutes. The mixture was then brought up to 37° C and ATP added to initiate the reaction. The coupled enzyme assay described in "Methods and Materials" was used to determine specific activity. These data represent triplicate data points from 3 different membrane preparations for each tissue.

TABLE 2

Comparison of Detergent Effects on the Ecto-ATPases from Chicken Liver, Smooth and Cardiac Muscle

Tissue	Detergent			
	NP-40	n-octyl glucoside	LPC	Digitonin
Smooth Muscle	None	4%	7%	21%
Cardiac Muscle	None	20%	17%	16%
Liver	>90%	>90%	>90%	>90%

Note. Percentages indicate the amount of activity in the supernatant divided by the total activity in the original membrane. "None" indicates that there was no activity in either the supernatant and pellet. The detergents had no effect on the coupled enzyme system.

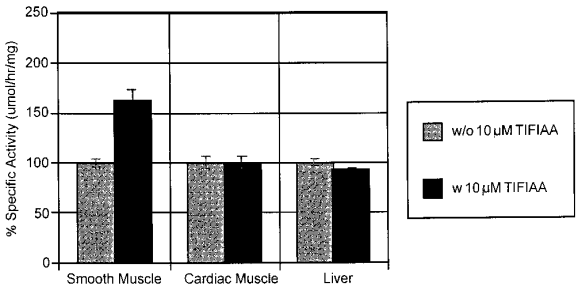

FIG. 2. TIFIAA effects on ecto-ATPase activities. Membranes from the different tissues were allowed to incubate with 10 μM TIFIAA for 60 minutes at pH 8.0 for 60 minutes. The TIFIAA was quenched to a final concentration of 200 μM DTT. Specific activities were done in triplicate with two different membrane preparations using the coupled enzyme system described in "Methods and Materials". The quenched TIFIAA had no effect on the coupled enzyme system.

while liver specific activity was generally a little higher in the supernatant than in the original membranes, typically 1.1-1.2 fold. Digitonin, LPC, and n-octyl glucoside were about equally effective in solubilizing the cardiac ecto-ATPase activity. Digitonin was the most effective detergent in solubilizing smooth muscle ecto-ATPase activity.

Lipid mediators differentially modulate ecto-ATPase activity. Lipid mediators, diacylglycerol kinase inhibitor I, and dimethyl sphingosine were tested for their ability to modulate ecto-ATPase activity (Table 3). The liver ecto-ATPase showed little modulation by these molecules with the exception of activation by DMS. The cardiac enzyme was somewhat activated by DMS, while the smooth muscle type showed the most prominent stimulation. The smooth muscle ecto-ATPase was most sensitive, in order, to PMA, AA, and OAG, with approximate K_i's of 0.5 μM, 5 μM, and 9 μM, respectively (data not shown). The cardiac muscle ecto-ATPase was somewhat less inhibited by AA and PMA, but more sensitive to OAG at 1 μM. Previous work had shown the skeletal and brain types to be sensitive to R59022 (2). The ecto-ATPase family of enzymes is unique in being the only other enzyme, other than diacylglycerol kinase, to be sensitive to R59022. However, of the three types tested here, only the smooth muscle ecto-ATPase showed significant inhibition to R59022. Interestingly, DAG kinases are not responsive to phorbol esters, in contrast to some members of the ecto-ATPase family.

TABLE 3

Comparison of Lipid Mediators and Lipid Mimetic Agents on Ecto-ATPase Activity from Different Chicken Tissues

Modulator	Smooth muscle (% Activity)	Cardiac muscle (% Activity)	Liver (% Activity)
None	100 ± 1	100 ± 3	100 ± 3
1 μM PMA	36 ± 1	82 ± 4	104 ± 6
1 μM AA	63 ± 7	82 ± 4	104 ± 3
1 μM OAG	74 ± 4	48 ± 4	107 ± 4
10 μM R59022	45 ± 2	95 ± 4	107 ± 5
10 μM DMS	144 ± 6	115 ± 5	128 ± 3

Note. The coupled enzyme assay was used to measure ATPase activity as described in "Material and Methods". Values are the average of three separate experiments ± the S.D. The % activity is the specific activity of the ecto-ATPase with the modulator divided by ecto-ATPase activity without modulator multiplied by 100. Modulators were added at the indicated final concentrations and incubated at assay temperature for 5 minutes prior to starting the assay with ATP. The modulators had no effect on the coupled enzyme system in the absence of the ecto-ATPase.

TIFIAA modulates only the smooth muscle ecto-ATPase activity. TIF, a halogenated xanthene derivative, has been shown to be a potent nucleotide-mimetic agent (12-15). TIFIAA preferentially forms a thioether with free thiol groups at pH > 7.5. In evaluating the presence of a cysteine residue in the nucleotide catalytic or regulatory site(s), affinity labeling was undertaken using TIFIAA. Only the smooth muscle ecto-ATPase showed modulation by the probe and interestingly, the enzyme showed activation instead of the expected inhibition (Figure 2). This activation was both time and concentration dependent, and not reversed after membrane washing (data not shown), suggesting covalency between the probe and the enzyme. Like ConA, TIFIAA always activates chicken smooth muscle ecto-ATPase activity. This activation varies from membrane preparation, with membrane preparations with lower ecto-ATPase activity more reactive with TIFIAA.

DISCUSSION

There have been a variety of studies which describe characterization of ecto-ATPases from numerous tissue types and species (9). However, there are few data from studies evaluating tissue differences of the ecto-

ATPases within the same organism (1,3,8). In this study we have demonstrated that chicken ecto-ATPase responses to substrate, ConA, detergents, lipid mediators, and TIFIAA vary by tissue type. Earlier findings showed that the ecto-ATPases from chicken brain, skeletal and smooth muscle showed similar responses to lectins, ATP, and regulatory lipids, indicating that they were likely the same molecular species (1).

ConA, as well as detergents and lipid mediators, can possibly influence the quaternary structure of ecto-ATPases (2). ConA, a tetrameric protein with four sugar binding sites, possibly stabilizes the ecto-ATPase in a more active oligomeric state. Earlier, it was proposed that ConA could shift the monomer → oligomeric equilibrium by stabilizing the oligomeric state of the skeletal muscle subtype (1,2,16). These data were later supported by cross-linking data (17,18). Further detailed examination of these phenomenon in smooth muscle is currently underway. In this study, the cardiac and smooth muscle types exhibited significant detergent-mediated decreases in total and specific activity and those lipid mediators of a mixed hydrophobic and polar nature were most effective against these same enzymes. These mediators were used well below their CMC values, and it is tempting to suggest that they could be interacting at the oligomer contact points, inducing a oligomer → monomer transition with a consequent reduction in enzyme activity.

TIF, a high affinity inhibitor, is a largely hydrophobic molecule with polar moieties, which has been shown to interact with adenine (di)nucleotide binding sites (12). The smooth muscle ecto-ATPase shows mixed inhibition when treated with TIF, with an approximate K_i of 1.2 μM (data not shown). Possible reasons for this mixed inhibition may be: 1) with its nucleotide-mimetic properties, TIF is undoubtedly reversibly interacting at the catalytic site, 2) TIF may also be reversibly interacting at nucleotide regulatory sites (12,13), and 3) with its mixed hydrophobic and polar nature, TIF may also be disrupting the contact points between the ecto-ATPase oligomers, by binding at oligomer stabilization/interaction sites, possibly inducing an ecto-ATPase oligomer → monomer equilibrium shift. Of significance, although TIF inhibits the enzyme, TIFIAA, contrastingly, activates the smooth muscle ecto-ATPase. It is proposed that the TIFIAA reaction may mimicking factors that induce a ecto-ATPase monomer → oligomer shift. That TIFIAA does not modulate the activities of the cardiac and liver ecto-ATPase types suggest quaternary or other differences between these enzyme subtypes.

It has been independently proposed that shifts in the oligomer ↔ monomer equilibrium of the ecto-ATPases is a potentially important regulatory feature of the ecto-ATPase family (19). Under this model, as with ConA (2), homooligomers of the ecto-ATPase exhibit a higher specific activity than the monomeric form. The following results can be interpreted in terms of oligomer state: 1) inhibition of the smooth and cardiac types to detergents, an oligomer → monomer shift, 2) activation of the smooth muscle type by ConA, a monomer → oligomer shift, 3) inhibition of the smooth and cardiac types by charged, mainly hydrophobic molecules an oligomer → monomer shift. That the liver subtype is relatively insensitive to detergents, ConA, and hydrophobic molecules with polar moieties can be explained by its existing as a very stable homooligomer. In contrast to the liver type, the cardiac muscle ecto-ATPase shows responses to lipid mediators. However, these responses are somewhat dissimilar to the smooth muscle type's responses. Further, the cardiac type is not modulated by ConA, possibly due to differential glycosylation patterns. In conclusion, regulatory data presented here and earlier (1,7,8) suggest that there are at least the following three distinct subtypes of ecto-ATPases within chicken: 1) ecto-ATPases from brain, skeletal and smooth muscle, 2) the ecto-ATPase from cardiac tissue, and 3) the ecto-ATPases from liver and oviduct.

The differences linked to putative regulatory responses exhibited by the ecto-ATPases suggest varied mechanisms in regulation of extracellular ATP. The effects of extracellular ATP upon whole cells from the studied tissue types studies is scarce and until such studies are completed, it will be difficult to fully understand the physiological functions of these ecto-ATPases. However, research into the regulatory responses of the ecto-ATPases will bring about continued insight into their function.

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