Regulation of transverse tubule ecto-ATPase activity in chicken skeletal muscle

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Transverse tubule (T-tubule) ecto-ATPase from chicken skeletal muscle is an integral membrane glycoprotein that seems to exist as a homodimer and exhibits unusual properties. Treatment of T-tubule membranes with concanavalin A (Con A) did not significantly affect the thermal variation of the fluorescence anisotropy of vesicles labelled with 1,6-diphenyl-1,3,5-hexatriene or trimethylammonium-1,6-diphenyl-1,3,5-hexatriene. Cross-linking of membrane components with glutaraldehyde elicited effects on ecto-ATPase activity very similar to those of Con A treatment: a severalfold increase in activity, a decrease in Triton X-100 sensitivity and a requirement to be present before ATP to exert its action. In addition, glutaraldehyde and Con A normalized the temperature dependence and the kinetic behaviour of the enzyme. Membrane-perturbing agents (detergents, alcohols and cholesterol oxidase), with the sole exception of digitonin, caused a marked decrease in ecto-ATPase activity; the

prior presence of Con A prevented this inhibition, whereas when the lectin was added after the membrane perturbing agent, recovery of the activity was not always possible. The addition of nucleotides before Con A led to a suppression of ecto-ATPase stimulation; it occurred when the nucleotide was hydrolysed (ATP or UTP) and when it was not (adenosine 5'-[β , γ -imido]triphosphate) and even in the presence of 3 mM P_i . A model is proposed for the complex regulatory mechanisms of chicken T-tubule ecto-ATPase that involves the occurrence of two different catalytic states in an equilibrium modulated by lectins and cross-linking agents, by the structure of the membrane and by the presence of ligands for a regulatory site.

Key words: concanavalin A, ecto-nucleoside triphosphate diphosphohydrolase, fluorescence anisotropy, glutaraldehyde.

INTRODUCTION

Many cell types and tissues have the ability to metabolize extracellular nucleotides by surface-located enzymes named ecto-nucleotidases. Among them, E-type ATPases (also referred to as E-NTPDases or ecto-nucleoside triphosphate diphosphohydrolases) are proteins characterized by the following: (1) a very active nucleotide hydrolysis site situated on the exterior of the cell, (2) millimolar bivalent cation dependence, (3) low substrate specificity (hydrolysis of nucleoside 5'-triphosphates and nucleoside 5'-diphosphates), and (4) insensitivity to classic inhibitors of the P-type, F-type and V-type ATPases [1,2]. So far, three different types of phylogenetically related cell-surface-located E-type ATPases have been identified and characterized to some extent: E-NTPDase1 (CD39 or ecto-apyrase) hydrolyses ATP and ADP about equally well, E-NTPDase2 (CD39L1 or ecto-ATPase) has a high preference for hydrolysis of ATP over ADP, and E-NTPDase3 (CD39L3 or HB6) hydrolyses ATP and ADP at a ratio of approx. 3:1 [2]. These integral membrane glycoproteins have broad and overlapping tissue distributions. Multiple functions have been proposed for E-type ATPases but the physiological roles have not been clearly established, nor is it known at present why the existence of different enzymes is necessary to catalyse the hydrolysis of extracellular ATP.

The muscle tissue is a rich and easily available source of E-type ATPase activities [1]. In skeletal muscle, a very efficient E-type ATPase activity, first designated as Mg^{2+} -ATPase or 'basic'-ATPase, is associated with transverse tubule (T-tubule) membranes [3]. This activity was extensively characterized in rabbit and chicken skeletal muscle T-tubule vesicles [4–6]. The avian E-type ATPase activity exhibited some very interesting

properties, including non-Michaelis–Menten kinetics, unusual temperature dependence and stimulation by specific lectins. A comparative study showed that the E-type ATPase activities present in membranes isolated from chicken brain, gizzard and skeletal muscle exhibited very similar responses to lectins, ATP and regulatory lipids, suggesting that they might correspond to the same molecular species [7]. In contrast, the E-type ATPase activities of chicken liver, heart and gizzard membranes showed a variety of differing properties, pointing to the existence of three enzyme subtypes with diverse features [8].

Avian skeletal and smooth-muscle ecto-ATPase activity can be modulated by a variety of compounds. One of the most interesting features is the response to concanavalin A (Con A) and other lectins. Con A stimulates the activity of the membrane-bound enzyme 6–10-fold, abolishes the negative co-operativity of ATP, yielding classical Michaelis–Menten kinetics, changes the unusual temperature dependence curve of activity to a linear relationship up to 45 °C, and eliminates inhibition by Triton X-100 and saponin [5–7,9]. Moulton et al. [5] proposed that these effects occur through the modulation by Con A of a low-affinity nucleotide regulatory site where ATP and Triton X-100, as well as a variety of lipophilic and amphipathic agents [10], could interact. However, the existence of such a regulatory site with combined adenine nucleotide and lipid/Triton X-100 binding properties is not easily conceivable. Furthermore, the possibility of membrane perturbations by lipophilic compounds and non-ionic detergents cannot be dismissed.

In contrast, the fact that the chicken gizzard ecto-ATPase is stimulated by chemical cross-linking with lysine-specific reagents in a similar way to that of Con A [11] led to the proposal that Con A activates the enzyme through modulation of its oligo-

Abbreviations used: p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; Con A, concanavalin A; DPH, 1,6-diphenyl-1,3,5-hexatriene; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; TMA-DPH, trimethylammonium-1,6-diphenyl-1,3,5-hexatriene; T-tubule, transverse tubule.

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merization state [12]. In support of this hypothesis, the formation of active high-molecular-mass aggregates has been found when the chicken T-tubule ecto-ATPase was solubilized with digitonin after preincubation of the membranes with Con A [13]. Nevertheless, this model does not explain why Con A is unable to exert the stimulatory effect when added after ATP, because this small molecule should not be able to hinder the binding of Con A to the sugar moiety of the ecto-ATPase and, consequently, the formation of the activated oligomer.

All the above results point to the existence of a complex regulatory mechanism that seems to be exclusive of the ecto-ATPase because the ecto-apyrases are not affected by Con A. To gain insights into the regulation of the chicken T-tubule ecto-ATPase, we have investigated the stimulatory effects of Con A and glutaraldehyde along with the inhibition elicited by perturbation of the membrane on the enzyme's activity. In addition, the requirements for suppression of Con A stimulation were explored. A unifying model was formulated to assemble previous and present results.

MATERIALS AND METHODS

Materials

Benzyl alcohol, ethanol, glutaraldehyde and Triton X-100 were from Merck. CHAPS was purchased from Calbiochem. Malachite Green, the other detergents and enzymes, and all the lectins and nucleotides were obtained from Sigma. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich and trimethylammonium-1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) from Molecular Probes. All other chemicals were of analytical grade.

Isolation of T-tubule membranes

The procedure for preparing T-tubule membranes from breast muscle of 8–12-week-old chickens by a Ca^{2+} -loading technique was identical with that described elsewhere [6]. Protein concentration was estimated by the method of Lowry et al. [14].

Nucleotidase assays

ATPase activity was determined at 37 °C by either a coupled-enzyme spectrophotometric assay that followed ADP release under conditions described previously [6] or a colorimetric method that measured the amount of P_i liberated. The colorimetric assay was performed in 0.1 ml of a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA, 5 mM MgCl_2 and 0.5–1.5 μg of protein. The reaction was started with 3 mM ATP (final concentration); after 5 min of incubation at 37 °C, it was stopped with 50 μl of ice-cold 10% (w/v) trichloroacetic acid. The P_i produced was determined with the Malachite Green procedure [15]. Controls containing trichloroacetic acid and the appropriate quantity of T-tubule membranes were always included to correct for non-enzymic hydrolysis of the substrates and background levels of P_i . All assays were performed in duplicate. Although the activity determined at 37 °C is approx. 50% of that obtained at 25 °C, it was decided to assay the ATPase at 37 °C because that temperature is more physiological (chicken body temperature is 43 °C), it is the usual assay temperature for other ecto-nucleotidases and it is the temperature at which lectin stimulation is observed.

Fluorescence depolarization measurements

DPH and TMA-DPH were incorporated into T-tubule vesicles by incubating T-tubule membranes (30 μg protein) for 1 h at

37 °C with a stable sonicated dispersion of the fluorescent probe (1 nmol of DPH or TMA-DPH) in 0.9 ml of 25 mM Mops, pH 7.3, containing 5 mM MgCl_2 and 0.2 mM EGTA. Thereafter, 0.1 ml of the same buffer, either with or without 100 μg of Con A, was added to the sample, which was then incubated for 15 min at 37 °C. Fluorescence anisotropy measurements were performed on an SLM Aminco 8000 spectrofluorimeter (Urbana, IL, U.S.A.) equipped with 10 mm Glan-Thompson polarizers. The fluorescence emission was measured at 425 nm for excitation at 365 nm, after equilibration of the samples at each required temperature. The slit widths for both the excitation and emission beams were 4 nm. The optical path of the cells was 0.4 cm.

Treatments of T-tubule membranes

The different compounds used in this study were added directly to the enzyme reaction mixtures, except for glutaraldehyde and cholesterol oxidase. Thus, for Con A stimulation, unless stated otherwise, the indicated amount of Con A was incubated in the assay mixture for 10 min at 37 °C before the addition of nucleotide. For cross-linking assays, T-tubule membranes (0.1–0.4 mg/ml protein) were incubated at 0 °C for 30 min with 25 mM glutaraldehyde in a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA and 5 mM MgCl_2 . Aliquots of the incubated mixture were removed and diluted with assay medium to stop the cross-linking reaction before ATPase activity was assayed. No significant differences were observed when the cross-linking reaction was terminated by the addition of hydrazine to a final concentration of 125 mM.

For treatment with cholesterol oxidase (EC 1.1.3.17), intact or Con A-treated (1 mg/ml) T-tubule membranes (0.1–0.2 mg/ml protein) were incubated at 37 °C for 5–60 min with 2 units/ml of cholesterol oxidase from *Brevibacterium* sp. in 25 mM Mops, pH 7.0, containing 0.32 M sucrose and 1 mM MgCl_2 . At the indicated times, aliquots of the reaction mixture were removed and diluted for assay of ATPase activity by the colorimetric procedure.

Statistical analysis

Data are presented as means $\pm$ S.D. for three or four experiments performed with at least three independent preparations. Differences were evaluated for statistical significance by using Student's *t* test for paired or unpaired data, as required. Derived probability (*P*) values less than or equal to 0.05 were considered significant.

RESULTS

Modification of T-tubule membrane fluidity by Con A

Because ecto-ATPase is an integral membrane protein, the physical state of membrane lipids might modulate its activity. We studied whether the Con A-mediated enhancement of ATP hydrolysis could be related to a change in membrane fluidity. Figure 1 shows the effect of Con A on the thermal variation of the fluorescence anisotropy of DPH-labelled and TMA-DPH-labelled T-tubule vesicles. For both probes, the fluorescence anisotropy values decreased with increasing temperature and were relatively large, probably reflecting the high rigidity of T-tubule membranes. For TMA-DPH, a cationic derivative of DPH and considered to be anchored in proximity to the lipid bilayer surface, no differences due to treatment with Con A were found over the temperature range studied (15–50 °C). In contrast, preincubation of T-tubule membranes with Con A elicited a small increase in anisotropy values of DPH (approx. 4% at

37 °C) that was greater at higher temperatures. These results indicate that, although the fluidity of the polar/non-polar interface of the membrane was unmodified, the lipid hydrophobic core of T-tubules was slightly less fluid when the membranes had been treated with Con A.

Cross-linking with glutaraldehyde

To examine the relationship between lectin-mediated stimulation of ecto-ATPase and the ability of lectins to cross-link glycoproteins, glutaraldehyde, a non-specific cross-linking reagent that is able to react with amine groups of proteins and lipids, was used. The preincubation of chicken T-tubule membranes with 25 mM glutaraldehyde at 0 °C for different durations caused a fast and marked enhancement in ATP hydrolysis rate: a maximum was observed after 5–10 min of incubation, was maintained for at least 90 min and decreased with longer incubation periods (results not shown). The effect of T-tubule preincubation with various concentrations of glutaraldehyde on ecto-ATPase activity is shown in Figure 2. The activity increased with increasing concentration of cross-linker, reaching 4–5-fold stimulation with 25 mM glutaraldehyde; the use of higher concentrations of glutaraldehyde resulted in slightly lower enhancements of the ecto-ATPase activity. The effects of glutaraldehyde and Con A were not additive: when Con A (25 µg/ml) was added to the assay medium of glutaraldehyde-treated T-tubule membranes, the ecto-ATPase activity detected (404 ± 98 µmol/h per mg) was no higher than that corresponding to the same membranes treated with 25 mM glutaraldehyde but assayed in the absence of the lectin (490 ± 77 µmol/h per mg).

It has been demonstrated that low concentrations of the non-ionic detergent Triton X-100 substantially inactivate the T-tubule ecto-ATPase and that preincubation with Con A is able to protect the enzyme from inactivation by Triton X-100 [5]. Similarly, when chicken T-tubule membranes were incubated with glutaraldehyde before the addition of Triton X-100, the cross-linking agent was also capable of protecting the ecto-ATPase from inactivation by detergent, as shown in Figure 2. In addition, it was verified that, if T-tubules were incubated with ATP (3 mM, 5 min at 37 °C) before the addition of glutaraldehyde, ecto-ATPase stimulation was not observed (see Table 2). These results indicated that cross-linking of chicken T-tubule membrane components with glutaraldehyde had the following effects: (1) it increased 4–5-fold the ecto-ATPase activity; (2) it avoided the Con A-mediated ecto-ATPase stimulation; (3) it protected the ecto-ATPase from detergent inactivation; and (4) the presence of ATP before that of the cross-linker abolished the glutaraldehyde-mediated ecto-ATPase stimulation.

Perturbations of the lipid environment

Membrane-bound enzymes require a hydrophobic environment supplied by the lipid bilayer, the characteristics of which usually modulate the properties of membrane proteins. We have examined whether alterations in membrane integrity or membrane components would affect the stimulating action of Con A on ecto-ATPase.

Amphiphilic compounds such as alcohols and detergents are commonly employed to perturb membrane properties. Alkyl and aryl alcohols are neutral molecules that can readily partition into membranes from aqueous solution; they have been reported to increase membrane fluidity and to modify the activities of membrane-bound enzymes [16,17]. Ethanol and benzyl alcohol inhibited the chicken T-tubule membrane ecto-ATPase activity in a dose-dependent manner. The inhibitory effect increased with the hydrophobicity of the alcohol; the IC_{50} values were 10 mM

Figure 1 Effect of Con A on the temperature dependence of fluorescence anisotropy values for DPH and TMA-DPH incorporated into chicken T-tubule membranes

After DPH (○, ●) or TMA-DPH (▽, ▲) was incorporated into T-tubule vesicles, the membranes were incubated for 15 min at 37 °C either with (○, ▽) or without (●, ▲) Con A before measurement of fluorescence anisotropy values (*r*) at the indicated temperatures, as described in the Materials and methods section. Results are means for four different preparations.

for benzyl alcohol and 600 mM for ethanol (results not shown). As illustrated in Table 1, the preincubation of T-tubule membranes with Con A before the addition of alcohol protected, to a great extent, the ecto-ATPase activity from the inhibitory effects of the alcohol. However, when the lectin was added after the alcohol, only for ethanol was the stimulation of the enzyme activity partly preserved; with benzyl alcohol, the subsequent addition of Con A failed to recover the ecto-ATPase activity.

Muscle ecto-ATPases exhibit extreme sensitivity to detergent exposure and/or solubilization [5,8,18]. The effect of increasing concentrations of several detergents on chicken T-tubule ecto-

Figure 2 Effect of glutaraldehyde on ecto-ATPase activity

T-tubule membranes (0.1–0.2 mg protein/ml) were incubated for 30 min in a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA, 5 mM $MgCl_2$ and the indicated concentrations of glutaraldehyde at 0 °C. Thereafter, 10 µl aliquots of the pretreated membranes were employed to measure ATP hydrolysis activity by the colorimetric assay, either in the absence (●) or in the presence (○) of 0.2 mg/ml Triton X-100. Activity is expressed as a percentage of control activity (111 ± 18 µmol/h per mg of protein) determined without additions for T-tubule membranes incubated under the described experimental conditions.

Table 1 Effect of ethanol and benzyl alcohol on stimulation of ecto-ATPase activity by Con A

T-tubule membranes (0.2–0.7 μg of protein) were incubated at 37 °C in 0.1 ml of 25 mM Mops, pH 7.3, containing 0.2 mM EGTA and 5 mM MgCl_2 with reaction components as indicated in the section Time and order of addition; after a further 10 min the reaction was started with 3 mM ATP. The concentrations employed were: Con A, 100 $\mu\text{g}/\text{ml}$; ethanol, 1 M; benzyl alcohol, 30 mM. ATPase activity was determined by the colorimetric method and is expressed as a percentage of control activity determined without additions (104 ± 34 $\mu\text{mol}/\text{h}$ per mg of protein).

Time and order of addition		
0 min	10 min	ATPase activity (% of control)
T tubule	—	100
T tubule + Con A	—	635 ± 116
T tubule + Con A	+ ethanol	562 ± 46
T tubule + Con A	+ benzyl alcohol	455 ± 18
T tubule + ethanol	—	31 ± 9
T tubule + ethanol	+ Con A	462 ± 48
T tubule + benzyl alcohol	—	24 ± 12
T tubule + benzyl alcohol	+ Con A	69 ± 20

Figure 3 Effect of detergents on ecto-ATPase activity

T-tubule membranes (4–6 $\mu\text{g}/\text{ml}$ protein) were incubated in a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA and 5 mM MgCl_2 for 10 min at 37 °C with various concentrations of the indicated detergents, before measurement of ATPase activity by the spectrophotometric procedure. Symbols: ●, digitonin; ○, saponin; △, CHAPS; ◆, Triton X-100; □, C12E9. Results are expressed as percentages of control activity (98 ± 14 $\mu\text{mol}/\text{h}$ per mg of protein) determined in the absence of detergents.

ATPase activity is shown in Figure 3. As expected, low concentrations (below the critical micellar concentration) of the non-ionic detergents Triton X-100 and C12E9 [(polyoxyethylene)₉-lauryl ether] brought about a substantial inactivation of T-tubule ecto-ATPase, whereas the zwitterionic detergent CHAPS caused a gradual concentration-dependent loss of activity. In contrast, only a partial decrease in enzyme activity was observed when T-tubule membranes were incubated with low concentrations of the mild permeabilizing detergents saponin and digitonin; the use of higher concentrations of these detergents elicited the recovery and a 2-fold stimulation of ecto-ATPase activity respectively.

When chicken T-tubule membranes were preincubated with Con A (10 $\mu\text{g}/\text{ml}$) before the addition of detergent (0.2 mg/ml), a partial (4–5-fold) stimulation with Triton X-100 and C12E9, and a maximal (7–8-fold) enhancement of ecto-ATPase activity with CHAPS, saponin and digitonin were achieved, probably

Figure 4 Effect of treatment with cholesterol oxidase on ecto-ATPase activity

Intact (○, ●) or Con A (1 mg/ml)-treated (▽, ▼) T-tubule membranes (0.1–0.2 mg protein/ml) were incubated at 37 °C without (○, ▽) or with (●, ▼) 2 units/ml cholesterol oxidase in 25 mM Mops, pH 7.0, containing 0.32 M sucrose and 1 mM MgCl_2 . At the indicated times, 5 μl aliquots were removed from the incubation mixture for assay of the ATPase activity by the colorimetric procedure.

because lectin binding renders the enzyme less sensitive to detergent action (results not shown). However, in similar experiments (results not shown), when Con A was added after the preincubation of T-tubules with Triton X-100 or C12E9, no recovery of activity was observed, whereas with CHAPS and saponin there was a 4–5-fold stimulation of ecto-ATPase activity. For digitonin-treated T-tubules, the subsequent addition of Con A (10 $\mu\text{g}/\text{ml}$) had no significant effect on enzymic activity but an increase in lectin concentration to 200 $\mu\text{g}/\text{ml}$ achieved a 4–5-fold stimulation of ecto-ATPase activity, suggesting that the glycan moiety of digitonin might have been sequestering Con A and therefore hindering the action of the lectin on the ecto-ATPase. In consequence, when the detergent did not completely abolish the enzyme activity, subsequent treatment with Con A resulted in a significant stimulation of the activity.

Another method of altering the physical properties of the membrane is the use of enzymes to modify the chemical structure of its lipids. Because T-tubule membranes are particularly enriched in cholesterol [3] and it has been reported that the oxidation of membrane cholesterol increases membrane fluidity [19], we studied the effects of cholesterol oxidase treatment on ecto-ATPase activity. Figure 4 shows a complete loss of enzymic activity after a 30 min treatment of chicken T-tubules with cholesterol oxidase. When T-tubule membranes were incubated with Con A before treatment with the lipid-modifying enzyme, the lectin was able to protect the stimulated ecto-ATPase activity to a great extent. It is interesting that the treatment of brain synaptic membranes with cholesterol oxidase was shown to increase membrane fluidity and to decrease ecto-ATPase activity [19], whereas the thermal stability of the enzyme was increased in bovine cardiac microsomes enriched with cholesterol [20].

Modification of temperature and substrate dependence by stimulating agents

Among the major distinguishing properties of chicken T-tubule ecto-ATPase are an unusual temperature dependence and a

Figure 5 Effect of stimulatory agents on the temperature dependence of ecto-ATPase activity

T-tubule membranes (3–6 $\mu\text{g}/\text{ml}$ protein) were incubated for 10 min in 25 mM Mops, pH 7.3, containing 0.2 mM EGTA and 5 mM MgCl_2 at the indicated temperatures without (●) or with 0.5 mg/ml digitonin (▽) or 25 $\mu\text{g}/\text{ml}$ Con A (○), before measurement of ATPase activity by the spectrophotometric procedure. With regard to glutaraldehyde (▼), T-tubule membranes (0.3 mg/ml protein) were pretreated in the described medium for 30 min at 0 °C with 25 mM glutaraldehyde before 10 μl aliquots were taken for assay of ATPase activity at the indicated temperatures.

complex kinetic behaviour; both of these characteristics are abolished by Con A [5]. The stimulating capacity of glutaraldehyde and digitonin on ecto-ATPase activity led us to evaluate these agents with regard to both properties.

The effect of glutaraldehyde and digitonin on the temperature dependence of ecto-ATPase activity is illustrated in Figure 5. The anomalous bell-shaped temperature response curve observed in the absence of Con A and the typical temperature dependence detected in the presence of the lectin are included for comparative purposes. The treatment of T-tubules with glutaraldehyde elicited a temperature response curve very similar to that observed for membranes incubated with Con A; although enzyme activity was lower for glutaraldehyde-treated membranes than for lectin-treated membranes, the ratios of activity at 37 °C to that at 25 °C were similar (Table 2). In the presence of digitonin, the anomalous temperature-dependence characteristic of ecto-ATPase was still observed but the response curve was slightly shifted to the right, in such a way that the ratio of activity at 37 °C to that at 25 °C was higher for the enzyme incubated with digitonin than for that in control T-tubules (Table 2). Although it is known that the

ecto-ATPase activity of chicken muscle membranes is labile to heat [13,21], its negative temperature dependence is not due to irreversible enzyme denaturation or to a temperature-dependent K_m [5]. Con A and glutaraldehyde counteracted this anomalous behaviour, pointing to the importance of cross-linking either of the enzyme molecules themselves or of other membrane components.

Figure 6 shows the dependence on ATP concentration of the chicken T-tubule ecto-ATPase activity in the presence of glutaraldehyde or digitonin. In either the absence or the presence of activators, the ecto-ATPase activity continued to increase over all concentrations of ATP tested (5 μM to 10 mM) (Figure 6A). In agreement with previous studies [5,7,9], the ecto-ATPase by itself exhibited biphasic double-reciprocal plots, consistent with negative co-operativity or interconvertible substrate-binding sites that do not coexist independently; in the presence of Con A this behaviour was eliminated, producing simple Michaelis–Menten kinetics (Figure 6B) with both increased K_m and increased V_{max} . Although digitonin (0.5 mg/ml) enhanced ecto-ATPase activity at all ATP concentrations used, the biphasic response was not abolished and apparent K_m values for the high-affinity and low-affinity ATP-binding sites did not change significantly. In contrast, the treatment of T-tubules with glutaraldehyde produced a kinetic behaviour similar to that brought about by Con A, suggesting that on membrane cross-linking the low-affinity binding site was somehow sterically blocked or abolished.

A summary of the main effects elicited by ecto-ATPase activators is presented in Table 2. Digitonin, glutaraldehyde and Con A needed to be present in the assay medium before ATP to be able to increase ecto-ATPase activity; the prior addition of ATP seemed to ‘freeze’ the enzyme in a state non-responsive to activators. A comparison of the effects on temperature dependence and kinetic parameters indicates that digitonin increased the activity but did not abolish the anomalous responses of the chicken enzyme, whereas glutaraldehyde-treated ecto-ATPase exhibited a behaviour very similar to that observed in the presence of Con A.

Requirements for the suppression of Con A stimulation

As stated above, the order of addition of the reaction components to the T-tubule membranes strongly influenced the full expression of ecto-ATPase activity. It has previously been reported that the ATP-dependent suppression of stimulation by Con A required the presence of Mg^{2+} and that the hydrolysis of the nucleotide was not required for this phenomenon, because the presence of Mg^{2+} /adenosine 5'-[β , γ -imido]triphosphate (p[NH]ppA) prevented activation by Con A [5]. To explore further the requirements for the nucleotide-dependent suppression of stimulation

Table 2 Comparison of ecto-ATPase activators: influence of addition order, effects on temperature dependence and on kinetic parameters for ATP

Reagents were added to the reaction mixture in the order indicated, with a 10 min preincubation between additions. The temperature dependence is defined as the ratio of activity at 37 °C to that at 25 °C. For the kinetic parameters, ecto-ATPase activity was evaluated as a function of ATP concentration; double-reciprocal plots were created from these results and apparent K_m values and V_{max} were calculated. Assay concentrations: digitonin, 0.5 mg/ml; Con A, 25 $\mu\text{g}/\text{ml}$. T-tubule membranes (0.3 mg/ml protein) were pretreated with 25 mM glutaraldehyde before measurement of ATPase activity in 10 μl aliquots. Values given are means $\pm$ S.D. for at least three separate experiments.

Activator	ATPase activity for addition order (%)		Temperature dependence	Kinetic parameters		
	Activator then ATP	ATP then activator		K_m , high affinity (μM)	K_m , low affinity (mM)	V_{max} ($\mu\text{mol}/\text{h}$ per mg)
None	100	100	0.46 ± 0.11	8 ± 3	2.2 ± 0.7	215 ± 4
Digitonin	236 ± 43	93 ± 7	0.70 ± 0.08	8 ± 4	1.5 ± 0.9	268 ± 65
Glutaraldehyde	421 ± 76	105 ± 8	1.45 ± 0.36	72 ± 23	—	407 ± 115
Con A	726 ± 104	103 ± 11	1.56 ± 0.22	53 ± 7	—	754 ± 28

Figure 6 Effect of stimulatory agents on the substrate dependence of ATP hydrolysis by chicken T-tubule membranes

Ecto-ATPase activity shown as a function of ATP concentration (**A**) and a double-reciprocal plot of the same data (**B**). T-tubule membranes (3–6 μg/ml protein) were incubated in 25 mM Mops, pH 7.3, containing 0.2 mM EGTA and 5 mM MgCl₂ for 10 min at 37 °C without (●) or with 0.5 mg/ml digitonin (▽) or 25 μg/ml Con A (○), before initiation of the reaction with the indicated concentration of ATP. With regard to glutaraldehyde (▼), T-tubule membranes (0.3 mg/ml protein) were pretreated in the described medium for 30 min at 0 °C with 25 mM glutaraldehyde before 10 μl aliquots were taken for assay of ATPase activity. ATP concentrations were varied from 5 μM to 10 mM with MgCl₂ in a 5 mM excess over ATP. Enzymic activity was determined by the spectrophotometric procedure.

Table 3 Effect of P_i, PP_i and ATP analogues on stimulation of ecto-ATPase activity by Con A

T-tubule membranes (4–5 μg/ml protein) were incubated for 5 min at 37 °C with the compound and concentration indicated in 1 ml of the reaction medium for the enzyme-coupled assay. Thereafter the reaction mixture was incubated at 37 °C for 10 min, either in the absence (—) or in the presence of Con A (25 μg/ml) before starting the assay with ATP 1 mM. Results are expressed as percentages of the control activity (94 ± 23 μmol/h per mg of protein) determined without additions and in the absence of Con A. Abbreviation: p[CH₂]ppA, adenosine 5'-[β,γ-methylene]triphosphate.

Compound	ATPase activity (% of control)	
	— Con A	+ Con A
None	100	727 ± 101
UTP (1 mM)	126 ± 23	112 ± 27
P _i (3 mM)	98 ± 6	167 ± 19
PP _i (3 mM)	94 ± 8	487 ± 92
pp[CH ₂]pA (1 mM)	99 ± 19	736 ± 84
p[NH]ppA (1 mM)	33 ± 8	33 ± 6

by Con A of chicken T-tubule ecto-ATPase, we used different compounds (Table 3). Preincubation with UTP, a nucleotide known to be hydrolysed by chicken T-tubules at an even higher rate than ATP [13] prevented stimulation by Con A. Surprisingly, 3 mM P_i avoided activation by Con A without affecting ecto-ATPase activity, whereas the presence of 3 mM PP_i in the preincubation medium produced only a partial decrease (approx. 30 %) in stimulation by Con A. Two non-hydrolysable analogues of ATP exhibited very different behaviours: p[NH]ppA inhibited ecto-ATPase activity and eliminated activation by Con A but, unexpectedly, adenosine 5'-[β,γ-methylene]triphosphate had no significant effect on either enzymic activity or stimulation by lectin. It was verified that the two ATP analogues were not hydrolysed under the assay conditions used. p[NH]ppA, an inhibitor of the chicken ecto-ATPase activity, was also shown to be a competitive inhibitor of ATP hydrolysis by rat skeletal-muscle Mg²⁺-ATPase [18], suggesting that this analogue binds to ATP-binding sites and, probably, it needs to be bound to prevent activation by Con A. In contrast, it seems unlikely that adenosine 5'-[β,γ-methylene]triphosphate, which did not inhibit ecto-ATPase activity and was not capable of suppressing stimulation by Con A, binds to the ATP-binding sites of chicken ecto-ATPase.

The fact that the suppression of stimulation by Con A required a nucleotide and Mg²⁺ and also that it occurred in the presence of 3 mM P_i suggested the possible involvement of membrane protein phosphorylation processes. In addition, in the deduced amino acid sequence of chicken muscle ecto-ATPase there are two consensus sites for phosphorylation located in its ecto-domain [12]; ecto-protein kinases have also been detected in muscle cells [22]. We therefore tried to investigate whether protein phosphorylation could be underlying the ATP-dependent suppression of the stimulation of chicken ecto-ATPase activity by Con A; however, neither the presence in the ecto-ATPase reaction mixture of two known inhibitors of intracellular protein kinases (genistein and H-7) nor the treatment of ATP-incubated T-tubule vesicles with alkaline phosphatase resulted in an improvement in activation by Con A (results not shown). It had previously been observed that neither the activity nor the stimulation by Con A of chicken T-tubule ecto-ATPase was altered under phosphorylating conditions in experiments with exogenous rat brain protein kinase C [10].

DISCUSSION

The results presented here and in other studies [5–7,11,21] indicate that the chicken muscle ecto-ATPase is an enzyme that can be finely regulated and that exhibits several unusual enzymic properties. With the sole exception of chicken brain enzyme [7], these uncommon properties of the avian muscle ecto-ATPase are not shared by ecto-NTPDases from other chicken tissues [8,21] or by the ecto-ATPases present in membrane preparations isolated from the skeletal muscle of other species [4,18,23]. Of the modulators that affect chicken ecto-ATPase, Con A is of particular interest because it was found to abolish its anomalous behaviour, converting the enzyme to Michaelis–Menten-type properties [5]. The stimulatory effect of Con A is dose-dependent, saturable, rises with increasing temperature and with increasing substrate concentration and disappears after the prior addition of ATP ([5,24], and this study).

Lipid environment

One of the mechanisms proposed for the activation of chicken ecto-ATPase by Con A is the modulation of membrane lipid

mobility and the resulting lipid–protein interactions [3]. However, fluorescence anisotropy measurements indicated that the treatment of T-tubule membranes with Con A caused only a slight decrease in the lipid fluidity of the hydrophobic core of the bilayer. It is not known whether this small change in lipid motion in the central region of T-tubule membranes might have a direct influence on chicken ecto-ATPase activity and hence it might be related to the stimulatory effect of Con A. In this regard it must be pointed out that studies on the relationship of alcohol-induced changes in Mg^{2+} -ATPase activity of rabbit intestinal brush border membrane to changes in the fluidity of its lipid bilayer have demonstrated that decreases in the enzyme's activity were clearly correlated with decreases in the fluorescence anisotropy of DPH and not with those of TMA-DPH [17]. In addition, T-tubule membranes are peculiar and they might be more sensitive to changes in membrane fluidity than other cell membranes. A distinguishing feature of T-tubule membranes, even in comparison with other surface membranes, is their high contents of total lipid and cholesterol, associated with a very low lipid fluidity [3]. The consequent restriction on mobility of the membrane components seems to be very important for T-tubule ecto-ATPase activity. In fact, all the compounds (alcohols, detergents and cholesterol oxidase) and conditions (temperature) used in this study to perturb the lipid moiety of chicken T-tubule membranes, all of which are known to increase membrane fluidity, caused a marked decrease in ecto-ATPase activity. In any case, the previous presence of Con A prevents the described inhibition, whereas when the lectin is added after the lipid-perturbing agent the recovery of ecto-ATPase activity is not always possible, probably depending on the maintenance of the basic structure of the membrane. Thus the avian enzyme's catalytic activity depends to a large extent on the lipid environment. A hydrophilicity analysis of the chicken muscle ecto-ATPase sequence indicates that it contains a single transmembrane domain at each end of the protein and that most of the protein (including the active site) is located on the exterior of the cell membrane [25]. It is therefore conceivable that the membrane lipid disturbances might affect the enzyme's catalytic domain through effects on the transmembrane domains, i.e. we propose that there might be a transmission of information between the transmembrane and catalytic domains of the chicken ecto-ATPase.

As an exception to the behaviour of detergents, digitonin was capable of activating chicken skeletal muscle ecto-ATPase. Even at high concentrations of glycoside, the activation was markedly lower than that caused by Con A; digitonin did not abolish the complex response of the enzyme to $MgATP^{2-}$ and to temperature. In contrast, it has been reported that in chicken gizzard and brain membranes digitonin eliminated the negative co-operativity of ecto-ATPase [7]. The reason for this apparent discrepancy is not clear but it could be related to the high content of cholesterol in T-tubule membranes [3]. The addition of high concentrations of Con A to digitonin-treated T-tubule-membranes boosted ecto-ATPase stimulation, suggesting that digitonin activates ecto-ATPase in a different way than that caused by Con A. Analysis of the effects of digitonin is complicated by its ability to interact with cholesterol molecules of the membrane and, probably, to be bound by Con A because of its glycan moiety.

Oligomeric state

In contrast, Con A has been proposed to promote or stabilize the oligomerization state of the ecto-ATPase [18]. With regard to the oligomeric state, E-NTPDases are known to exist as homo-

oligomers stabilized by non-covalent bonds [11,26–29]. In mammalian ecto-apyrase CD39 it has been demonstrated that the enzymic activity of tetramers is greater than that of monomers and that detergents decrease the activity of the enzyme by promoting its dissociation to monomers [28]. The quaternary structure and enzymic activity of E-NTPDases seem to be dependent on proper contacts between the transmembrane domains of the monomers [28]; moreover, different quaternary structure contexts seem to influence the mechanisms of ATP and ADP hydrolysis by CD39 [30]. Chicken muscle ecto-ATPase is proposed to be a homodimer [11], although the possible existence of higher oligomerization states or the association of the ecto-ATPase with other ecto-enzymes cannot be totally discounted [13]. The finding that most of the Con A-stimulation of chicken ecto-ATPase was not evident with succinyl-Con A (the bivalent form of the quadrivalent Con A) [5,24], and the fact that the treatment of chicken muscle membranes with lysine-specific cross-linkers increased ecto-ATPase activity in parallel with the increase in an immunoreactive band corresponding to the homodimer [11], indicate the importance of oligomerization state for avian enzymic activity. Interestingly, the effects elicited on chicken ecto-ATPase activity as a result of Con A treatment were also produced by glutaraldehyde, including the elimination of the abnormal bell-shaped temperature response curve, the abolition of unusual kinetic behaviour and a lack of effect when the cross-linker was added after the nucleotide. Con A should therefore not be considered to have a unique role as an activator or regulator of the T-tubule ecto-ATPase activity. Because the simultaneous presence of Con A and glutaraldehyde did not yield an additive response, both compounds seem to be working through a common mechanism, not specific for the lectin, and probably related to the ability to cross-link membrane components. However, Con A might further exert a specific action on the enzyme through its carbohydrates.

The role of the sugar chain(s) in the chicken ecto-ATPase is still obscure, although it has been suggested that glycosylation might be important for the maintenance of enzymic activity and the homo-oligomerization of all or most ecto-NTPDases [29]. In this regard, elimination of the sialic acid residues by neuraminidase elicited a 40% decrease in chicken T-tubule ecto-ATPase activity but caused no effect on stimulation by Con A (G. Moro and A. Megías, unpublished work). Stimulation of the avian enzyme by lectin was shown to occur through direct binding of the lectin to oligosaccharide residues, which might form part of the ecto-ATPase structure, because after desialylation no activation with wheatgerm agglutinin was observed and α -methylmannoside prevented stimulation by Con A [5,6]. Furthermore, glycosylation is crucial for chicken muscle ecto-ATPase, because expression of the enzyme in mammalian cells in the presence of tunicamycin resulted in an inactive, unfolded protein [31].

Two states

Con A and glutaraldehyde induced a prominent kinetic alteration in chicken ecto-ATPase, reflecting the existence of two conformational states with different catalytic properties. Thus in the isolated T-tubule membranes the enzyme exhibited a low-activity state characterized by apparent negative co-operativity, abnormal temperature dependence (maximum activity at 25–28 °C) and a particularly high sensitivity to detergents and membrane-perturbing agents. Nevertheless, after treatment of T-tubule membranes with lectins or covalent cross-linking agents, the chicken ecto-ATPase presented a high-activity state, showing a simple Michaelis–Menten kinetics (with increased K_m and V_{max}),

Scheme 1 Proposed model for the regulation of chicken T-tubule ecto-ATPase activity

This model is consistent with the experimental results and attempts to explain some of the peculiarities of the avian enzyme. (A) The chicken muscle ecto-ATPase seems to exist in isolated membrane preparations as a homodimer, each monomer possessing a catalytic (C) and a regulatory (R) site (low-activity state). (B) The addition of cross-linking agents stabilizes the dimer and causes a conformational change in the enzyme (high-activity state). (C) The addition of membrane-perturbing agents might elicit either (1) a severe alteration of the lipid bilayer accompanied by total inactivation of the ecto-ATPase or (2) a mild modification of the membrane, with only partial loss of activity and preservation of the possibility to be shifted towards the high-activity state by cross-linking. (D) If the regulatory site is occupied before the cross-linking agent is added, no conformational change is produced.

a normal temperature response and a considerably higher resistance to lipophilic and amphiphilic substances. It has been proposed that, in addition to the substrate catalytic site, another binding site exists for the substrate and that conformational changes are related to the occupancy of this putative regulatory (inhibitory) site [5]. In this regard, purified rat heart Mg^{2+} -ATPase was found to bind adenosine 5'-[γ - ^{35}S]thiotriphosphate with two affinities [32]; several ecto-ATPases, including that of chicken muscle, use $MgATP^{2-}$ as substrate with two apparent K_m values for the high-affinity (10–100 μM) and low-affinity (0.2–8 mM) ATP-binding sites ([8,33], and this study). In the chicken T-tubule ecto-ATPase, Con A and glutaraldehyde exerted no effect if added after ATP (Table 2), suggesting that when the regulatory site is occupied the enzyme loses its ability to respond to these compounds and that it remains in the low-activity state, although it can be assumed that cross-linking of the membrane components would not be avoided by such a small molecule as ATP. Therefore cross-linking is a necessary but not sufficient condition to lead to the high-activity state of the enzyme, because it depends on the previous non-occupancy of the regulatory site. In contrast, the presence of Con A or glutaraldehyde before ATP seems to shift the conformational equilibrium towards the high-activity state and either to hinder the access of ATP to the regulatory binding site or to block the communication between the regulatory and catalytic sites. Alternatively, the catalytic and regulatory sites might be on opposite chains of the homodimeric enzyme, in such a way that

binding of the ligand to one site affects ATP hydrolysis at the second site.

The identity of the physiological ligand(s) for the putative regulatory site has not been established. It was postulated that the regulatory site exhibited combined adenine-nucleotide-binding and lipid-binding properties and that it might interact directly with ATP, p[NH]ppA, Triton X-100, phorbol esters and diacylglycerols [5,10]. However, when analysing the ability of different compounds to suppress stimulation by Con A (Table 3), it can be concluded that the regulatory site is not specific for purine (ATP) or pyrimidine (UTP) nucleotides and not even for nucleotides, because P_i and (in part) PP_i also prevented Con A activation, suggesting that the interactions with the regulatory site are largely dependent on the phosphate moiety of the ligand. In addition, our results indicated that protein phosphorylation processes did not seem to be involved and that hydrolysis of the nucleotide was not necessary (p[NH]ppA). If we take into account that the extracellular concentration of P_i is in the low millimolar range, it could be expected that in the muscle the ecto-ATPase regulatory site was at least partly occupied.

Model

By taking together all the above results, the model illustrated in Scheme 1 is proposed for the regulation of ecto-ATPase activity in chicken T-tubule membranes. It assumes that the enzyme exists in isolated membrane preparations as a homodimer,

possesses a catalytic site and a regulatory site (symbolized by C and R respectively) and presents two conformational states detected by a marked change in its catalytic properties (depicted in the model by round and square shapes). The relative proportion of high-activity and low-activity forms seems to depend on the enzyme's oligomerization state, the structure of the membrane and the presence of ligands for the regulatory and catalytic sites. Thus the ecto-ATPase exhibits a low-activity state in the isolated preparations of T-tubules (Scheme 1A). The shift of the equilibrium towards the high-activity state is elicited by compounds and conditions (lectins and cross-linking reagents) that stabilize the oligomer (Scheme 1B). In contrast, compounds (detergents, alcohols and lipid-modifying enzymes) and conditions (temperature) that disturb the membrane (Scheme 1C) inhibit the ecto-ATPase, probably modifying the interactions between monomers established at the level of transmembrane domains or catalytic domains or both, and promoting the dissociation of the normal oligomerization state. Nevertheless, these compounds do not abolish the activation by Con A or cross-linkers unless the membrane is severely damaged. In contrast, the occupancy of the putative regulatory site (by ATP, UTP, p[NH]ppA or P_i) might or might not inhibit the ecto-ATPase catalytic activity but it completely prevents the stimulation of activity induced by cross-linking, suggesting that the conformational transition of the enzyme is blocked (Scheme 1D).

In summary, the results reported here show that the ecto-ATPase from chicken skeletal muscle T-tubules exhibits a very complex and finely tuned regulation mechanism, probably related to the need for processing widely variable amounts of extracellular ATP. The physiological significance of muscle ecto-ATPases has not yet been elucidated. However, it was recently reported that α -sarcoglycan, a component of the sarcoglycan complex of dystrophin-associated proteins, is a sarcolemmal ecto-ATPase; mutations in the α -sarcoglycan gene are known to cause specific forms of muscular dystrophies [34].

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