Antibodies against erythrocyte Ca^{2+} -transport ATPase specifically inhibit the calmodulin-dependent fraction of the enzyme's activity

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Antibodies against purified Ca^{2+} -transport ATPase from human erythrocytes were raised in rabbits. Immunodiffusion experiments revealed that precipitating antibodies had been developed. The immunoglobulin fraction inhibited solely the calmodulin-dependent fraction of erythrocyte Ca^{2+} -transport ATPase activity, whereas the basal (in the absence of added calmodulin) activity of the enzyme was not significantly affected by the antibodies. The antibodies produced similar dose-response curves for the calmodulin- and the oleic acid-stimulated enzyme. However, the immunoglobulin fraction was considerably less effective in inhibiting Ca^{2+} -transport ATPase activated by limited proteolysis. The results obtained with our antibodies are compatible with the interpretation that at least one subpopulation of the antibodies attacks the enzyme at or close to the calmodulin-binding site of the ATPase. The antibodies also inhibited the calmodulin-regulated Ca^{2+} -transport ATPase from pig smooth-muscle plasma membrane, though with lower potency. However, the immunoglobulin fraction failed to suppress pig cardiac sarcoplasmic reticulum Ca^{2+} -transport ATPase activity in the concentration range investigated. In addition, the activity of phosphodiesterase from rat brain, another enzyme modulated by calmodulin, was not at all affected by the immunoglobulin fraction.

Ca^{2+} -transport ATPase of human erythrocytes is responsible for the low intracellular Ca^{2+} concentration, approx. $0.1 \mu\text{M}$ (Schatzmann, 1973; Lew *et al.*, 1982). The activity of this enzyme is modulated by direct interaction with the Ca^{2+} -receptor protein calmodulin, this being dependent on the cytoplasmic Ca^{2+} concentration (Scharff & Foder, 1978; Lynch & Cheung, 1979). This property is presumably shared by plasma-membrane Ca^{2+} -transport ATPases of all tissues (Carafoli & Zurini, 1982), whereas Ca^{2+} -transport ATPases of sarcoplasmic reticulum seem to be regulated indirectly by calmodulin via protein kinases (Le Peuch *et al.*, 1979; Campbell & MacLennan, 1982).

Isolation of large quantities (10–20 mg) of homogeneous Ca^{2+} -transport ATPase from erythrocytes has been accomplished by the combination of two efficient methods: (1) preparation of calmodulin-deficient erythrocyte membranes, exhibiting high specific activity of Ca^{2+} -transport ATPase, by a molecular filtration technique; and (2) a batch procedure using calmodulin–Sepharose 4B conju-

gate for purification of the enzyme (Gietzen & Kolandt, 1982). The availability of sufficient amounts of purified Ca^{2+} -transport ATPase enabled us to raise potent antibodies directed against this protein.

In the present paper the antibodies have been characterized with respect to their effects on the enzyme's function and on their cross-reactivity with Ca^{2+} -transport ATPases from other tissues. The immunoglobulin fraction was shown to inhibit solely the calmodulin-dependent fraction of the ATPase activity. These antibodies also inhibited the plasma-membrane Ca^{2+} -transport ATPase from pig smooth muscle, whereas the activity of Ca^{2+} -transport ATPase from pig cardiac sarcoplasmic reticulum was not blocked. Though obtained by a different approach, the latter finding confirms the results of previous studies (Verma *et al.*, 1982; Wuytack *et al.*, 1983) in that the enzyme from sarcoplasmic reticulum is immunologically dissimilar from that of plasma membranes. A preliminary report of this work has appeared (Kolandt & Gietzen, 1984).

Materials and methods

Preparation of proteins

Calmodulin was prepared from bovine brain as described by Kakiuchi *et al.* (1981). Calmodulin-sensitive phosphodiesterase from rat brain was partially purified by a method based on that of Wang & Desai (1977), with slight modifications as described by Gietzen *et al.* (1982). Homogeneous Ca^{2+} -transport ATPase from human erythrocytes was isolated by the method of Gietzen & Kolandt (1982). The purified Ca^{2+} -transport ATPase was eluted from the affinity gel in a detergent-lipid system containing Tween 20 (0.5 mg/ml), Triton X-100 (0.5 mg/ml) and phosphatidylcholine (0.5 mg/ml). Before use, the enzyme was concentrated by use of Amicon XM100 ultrafiltration membranes to a concentration of 0.4 mg/ml. Sarcoplasmic-reticulum vesicles enriched in Ca^{2+} -transport ATPase activity were prepared from pig heart by the procedure of Suko & Hasselbach (1976). Ca^{2+} -transport ATPase from pig smooth-muscle plasma membrane was purified as described previously and eluted from a calmodulin-Sephadex conjugate in a detergent-lipid system consisting of asolectin (0.5 mg/ml) and Triton X-100 (4 mg/ml) (Wuytack *et al.*, 1981).

Production of antiserum

Before starting the immunization, the rabbits (chinchilla) were bled several times to obtain sufficient amounts of control sera. Rabbits were immunized by subcutaneous injections of 200 μg of purified human erythrocyte Ca^{2+} -transport ATPase mixed with 0.5 ml of Freund's complete adjuvant. Booster injections of the same amount of antigen were administered at weekly intervals. After the fourth week, the rabbits received further injections of 100 μg each at intervals of 2 weeks. The rabbits were bled weekly starting 4 weeks after the initial injection. After clotting of the blood at room temperature, it was centrifuged at 12000 g for 20 min. Clear serum was stored in liquid N_2 .

Immunodiffusion experiments

Ouchterlony immunoprecipitation tests were done on 0.8% agarose (Pharmacia) gels containing 0.02% (w/v) NaN_3 , 0.05% (w/v) Triton X-100 and 25 mM-veronal buffer adjusted to pH 8.6. Before samples were placed into the wells, they were made 0.05% in Triton X-100. After filling of the sample wells with appropriate amounts of antigen and antiserum (or control serum), the plates were developed in a moist chamber for 2 days at room temperature. Before staining, the gels were soaked in 0.9% NaCl for 2 days at room temperature to remove unprecipitated protein (solution was changed twice) and soaked additionally for 2 h in

distilled water. The gels were then dried at 40°C, stained for 15 min with Coomassie Blue, destained in methanol/acetic acid (9:1, v/v) and photographed.

Isolation of immunoglobulins

The immunoglobulin fractions were prepared from antiserum or control serum by repeated $(\text{NH}_4)_2\text{SO}_4$ precipitations. For this purpose 0.6 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added per ml of serum with stirring and the solution kept for 30 min at room temperature. The pellet obtained after centrifugation (30 min at 1000 g) was dissolved in 0.9% NaCl. Two more precipitations with $(\text{NH}_4)_2\text{SO}_4$ were necessary for complete removal of albumin. The final pellet was again dissolved in 0.9% NaCl and exhaustively dialysed against 0.9% NaCl buffered with 5 mM-Mops (pH 7.4) until no more sulphate was detectable. Immunoglobulins were stored in small batches in liquid N_2 .

Assay of enzyme activities

ATPase and phosphodiesterase activities were determined at 37°C by measuring the rate of P_i liberation as reported by Stewart (1974), with slight modifications (Lanzetta *et al.*, 1979). Briefly, phosphodiesterase activity was assayed by coupling the phosphodiesterase reaction with the 5'-nucleotidase reaction and measuring the P_i produced within 30 min (Butcher & Sutherland, 1962; Wang & Desai, 1977). The assay mixture (final volume 1 ml) consisted of 25 mM-Hepes (pH 7.5), 3 mM-magnesium acetate, 1.2 mM-cyclic AMP and 0.1 mM- CaCl_2 . The reaction of the different Ca^{2+} -transport ATPases was followed discontinuously over various time periods depending on the specific activity of the respective enzyme. The assay medium for Ca^{2+} -transport ATPase contained, in a final incubation medium of 1 ml, 25 mM-Mops (pH 7.0), 100 mM-KCl, 0.25 mM-ouabain, 10 mM- NaN_3 , 1 mM-ATP, 2 mM- MgCl_2 and 36 μM - Ca^{2+} [as a 0.4 mM- Ca^{2+} / Mg^{2+} /EDTA buffer (Wolf, 1973)]. Ca^{2+} -free controls contained 0.4 mM- Mg^{2+} /EGTA instead of the Ca^{2+} / Mg^{2+} /EDTA buffer. Ca^{2+} -transport ATPase activity refers to the difference in activity obtained in the presence and absence of Ca^{2+} . If not otherwise stated, enzymes were first preincubated with immunoglobulin at 37°C for 30 min, followed by another 10 min at 37°C when an activator (oleic acid, calmodulin) was added. Then the reaction was started by the addition of the appropriate substrate (ATP, cyclic AMP). Whenever erythrocyte Ca^{2+} -transport ATPase was tested, disrupted erythrocyte membranes (100 μg of membrane protein/ml) were used as a source of enzyme.

Preparation of oleic acid dispersions

Oleic acid microdispersions were prepared by sonication (Branson Sonifier B12; approx. 2 min at setting 2) in a buffer containing 0.1 mM-EGTA and 5 mM-Mops (pH 7.0) under a stream of N_2 .

Controlled tryptic digestion

Ca^{2+} -transport ATPase was digested at 37°C by 0.2 mg of trypsin/mg of erythrocyte membrane protein. Proteolysis was performed in the assay medium and was terminated after 3 min by the addition of a 5-fold excess (w/w) of trypsin inhibitor (Gietzen *et al.*, 1982).

Results

Properties of antibodies against human erythrocyte Ca^{2+} -transport ATPase

After 10 injections of antigen, the production of precipitating antibodies against human erythrocyte Ca^{2+} -transport ATPase could be detected by applying the Ouchterlony immunodiffusion test. The antiserum showed a dose-dependent single precipitin line on immunodiffusion with purified Ca^{2+} -transport ATPase (Fig. 1a). Control serum failed to give a precipitin line under the same conditions (Fig. 1b).

The interaction of our antibodies with Ca^{2+} -transport ATPase of disrupted erythrocyte membranes inhibited the enzyme's activity, whereas control immunoglobulin did not show any effect. The inhibition of the calmodulin-stimulated enzyme by $50\text{ }\mu\text{g}$ of immunoglobulin/ml is dependent on the time of preincubation at 37°C . Preincubating Ca^{2+} -transport ATPase for 30 min with the antibodies resulted in 50% inhibition of the enzyme's activity (results not shown). With longer periods of preincubation, inhibition of the enzyme progressed only slowly.

Determination of the immunoreactive sites of Ca^{2+} -transport ATPase

In order to characterize the specificity of the antibodies and to determine which portions of the ATPase molecules were involved in the antigen-antibody reaction, we investigated the effect of the antibodies on calmodulin-stimulated and basal activity of erythrocyte Ca^{2+} -transport ATPase ('basal' Ca^{2+} -transport ATPase activity was defined as that activity determined in the absence of added calmodulin). Fig. 2 gives evidence that the antibodies specifically inhibited the calmodulin-dependent fraction of the ATPase activity, since basal activity of the enzyme was not significantly affected by immunoglobulin in the concentration range investigated. Half-maximal inhibition of the calmodulin-dependent fraction of Ca^{2+} -transport

Fig. 1. Ouchterlony immunodiffusion test. Central wells (a and b) contained purified Ca^{2+} -transport ATPase from human erythrocytes and surrounding wells of different dilutions of (a) antiserum and (b) control serum. Well numbers and dilutions are as follows: (1) 1; (2) 1:2; (3) 1:4; (4) 1:8; (5) 1:16; (6) 1:32.

ATPase activity was achieved by $50\text{ }\mu\text{g}$ of immunoglobulin/ml with preincubation at 37°C for 30 min (Fig. 2a). The same effect was obtained with a considerably lower concentration of immunoglobulin (approx. $18\text{ }\mu\text{g}/\text{ml}$) by preincubating the enzyme for 24 h at 4°C (Fig. 2b). In addition, Fig. 2(a) shows that control immunoglobulin tested over a wide concentration range had no effect on Ca^{2+} -transport ATPase activity.

Fig. 3 demonstrates the influence of the sequence of preincubation of the different components that participate in the inhibitory process (i.e. ATPase, calmodulin, antibodies). The inhibitory

Fig. 2. Concentration-dependent inhibition of Ca^{2+} -transport ATPase by immunoglobulin

Preincubation of erythrocyte membranes with the immunoglobulin fractions was performed (a) at 37°C for 30 min and (b) at 4°C for 24 h. The activity of the enzyme was assayed in the absence (■) and presence of 30 nM-calmodulin (○, ●). Control (preimmune) immunoglobulin (○); immunoglobulin (●, ■). Each point is the mean of four to six determinations.

effect of immunoglobulin was less pronounced when ATPase and calmodulin were preincubated before the attack of antibodies than when ATPase was preincubated first with antibodies, then with calmodulin. There was no detectable difference in the resulting ATPase activity if the ATPase was preincubated with calmodulin for 10 or 40 min at the applied Ca^{2+} and calmodulin concentrations. At high immunoglobulin concentrations, marked with an asterisk in Fig. 3, the difference between the inhibitory effects obtained with the two modes of preincubation was significant ($P < 0.01$, $n = 6$). To obtain further information about the binding sites of the antibodies on the Ca^{2+} -transport ATPase molecule, we investigated the influence of the calmodulin concentration on the inhibition of

Fig. 3. Influence of the mode of preincubation on the inhibitory potency of immunoglobulin

The sequence of preincubation (37°C) of the different components (ATPase; immunoglobulin; 30 nM-calmodulin) was varied. ●, ATPase and immunoglobulin were preincubated for 30 min; calmodulin was added, and the mixture was preincubated for 10 min more before assay. ○, ATPase plus calmodulin were preincubated for 10 min; immunoglobulin was added, and the mixture was preincubated for 30 min more before assay. At a given immunoglobulin concentration, points marked with an asterisk are significantly different from each other ($P < 0.01$, $n = 6$, Student's t test).

the enzyme caused by 50 μg of immunoglobulin/ml. Fig. 4 shows that increasing calmodulin concentrations almost completely reverse the inhibition produced by the antibodies.

Additional experiments were performed by testing the antibodies on human erythrocyte Ca^{2+} -transport ATPase that was stimulated by calmodulin, oleic acid or limited tryptic digestion. The antibodies were equipotent in antagonizing the activating effect of both calmodulin and oleic acid, since the respective dose-response curves are almost identical (Fig. 5). However, the antibodies were considerably less potent to inhibit the stimulation of the enzyme provoked by limited proteolysis (Fig. 5).

Immunological cross-reactivity

The immunological cross-reactivity of antibodies against human erythrocyte Ca^{2+} -transport ATPase with different Ca^{2+} -transport ATPases and calmodulin-sensitive phosphodiesterase was tested by using the enzymes' activity as test parameter. Anti-(human erythrocyte Ca^{2+} -transport ATPase) immunoglobulin also inhibited the

Fig. 4. Influence of the calmodulin concentration on the inhibitory effect of immunoglobulin

Membrane-bound erythrocyte Ca^{2+} -transport ATPase was first preincubated for 30 min at 37°C with immunoglobulin, followed by another 10 min with the respective calmodulin concentration before the enzyme reaction was started. The ordinate represents only the calmodulin-dependent fraction of the enzyme's activity. Each point is the mean of four determinations.

Fig. 5. Effect of immunoglobulin on erythrocyte Ca^{2+} -transport ATPase stimulated by calmodulin (●), oleic acid (▲) or limited proteolysis (■)

Before assay of ATPase activity, erythrocyte membranes were first preincubated with the immunoglobulin at 37°C for 30 min, followed by another 10 min with the activator (30 nM-calmodulin or 50 μM -oleic acid). In the case of stimulation of the ATPase by trypsin after proteolysis the samples were preincubated for 40 min at 37°C with immunoglobulin. Each point is the mean of four determinations.

activity of calmodulin-binding Ca^{2+} -transport ATPase from pig smooth-muscle plasma membrane, though with lower potency as compared with the inhibition of the respective enzyme from

Fig. 6. Effect of antibodies on different Ca^{2+} -transport ATPases and phosphodiesterase

Ca^{2+} -transport ATPases from human erythrocytes (●), pig cardiac sarcoplasmic reticulum (○) or pig smooth-muscle plasma membrane (□) and rat brain phosphodiesterase (Δ) were preincubated with the antibodies at 37°C for 30 min. Erythrocyte and smooth-muscle Ca^{2+} -transport ATPase and phosphodiesterase were additionally preincubated with calmodulin for 10 min at 37°C before the enzyme reaction was started. 100% activity refers to 0.08, 0.32, 4.5 or 0.9 $\mu\text{mol/min}$ per mg of protein for Ca^{2+} -transport ATPases from human erythrocytes, pig cardiac sarcoplasmic reticulum or pig smooth-muscle plasma membrane and rat brain phosphodiesterase respectively.

human erythrocytes (Fig. 6). The inhibition of this enzyme obtained with 100 and 200 μg of immunoglobulin/ml was statistically significant ($P < 0.01$, $n = 4$). However, Ca^{2+} -transport ATPase from pig cardiac sarcoplasmic reticulum was activated by 20% at higher concentrations of antibodies (Fig. 6), as was also the case when control immunoglobulin was used (results not shown). This indicates that the Ca^{2+} -transport ATPase from pig sarcoplasmic reticulum is immunologically dissimilar from plasma-membrane Ca^{2+} -transport ATPases such as the pig smooth-muscle or the erythrocyte enzyme. Since the results obtained thus far implied the interaction of the antibodies with the calmodulin-binding site of Ca^{2+} -transport ATPase, we investigated whether our antibodies would also antagonize the stimulation of rat brain phosphodiesterase by calmodulin. As shown in Fig. 6 even concentrations up to 1 mg of immunoglobulin/ml failed to affect phosphodiesterase activity.

Discussion

This study describes the production of potent and precipitating antibodies against human eryth-

rocyte Ca^{2+} -transport ATPase, these being characterized with respect to their effects on the enzyme's function and their cross-reactivity with different enzymes.

Our antibodies against Ca^{2+} -transport ATPase differ in two respects from those directed against the same enzyme described by Verma *et al.* (1982). The antibodies developed in our laboratory exhibited a considerably higher potency and inhibited specifically the calmodulin-dependent fraction of the ATPase activity. In contrast, Verma *et al.* (1982) reported that their antibodies were equipotent in inhibiting both the calmodulin-dependent and calmodulin-independent ATPase activities. Our results are compatible with the interpretation that at least one subpopulation of the antibodies attacks the enzyme at or close to the calmodulin-binding site of the Ca^{2+} -transport ATPase molecule, since: (1) only the calmodulin-dependent fraction of the enzyme's activity was blocked; (2) calmodulin had a protective effect when added to the ATPase before the attack of antibodies, which provided the condition for the formation of a calmodulin-ATPase complex first; and (3) the inhibitory effect of the antibodies on the enzyme could be antagonized by increasing calmodulin concentrations. The high calmodulin concentrations required for complete antagonism of the antibodies' effect can be explained by the assumption that, at the time the enzyme assay was started, the system may not have been in equilibrium.

It has been demonstrated with several enzymes that anionic amphiphiles and limited proteolysis mimic the effect of calmodulin (Wolff & Brostrom, 1976; Depaoli-Roach *et al.*, 1979; Taverna & Hanahan, 1980; Sarkadi *et al.*, 1980). Our antibodies proved to be equipotent in antagonizing the stimulation of Ca^{2+} -transport ATPase induced either by calmodulin or by oleic acid. This could mean that the binding sites for calmodulin and oleic acid are identical (cf. Gietzen *et al.*, 1982) or that the antibodies are able to prevent an identical conformational change of the ATPase induced by the two activators when assuming different binding sites. Limited proteolysis is thought to transform a calmodulin-dependent enzyme into its high-activity state by cleavage of an inhibitory peptide sequence on which the calmodulin-binding site may be located (Sarkadi *et al.*, 1980; Stieger & Schatzmann, 1981; Carafoli & Zurini, 1982). Since the antibodies also block the stimulation of Ca^{2+} -transport ATPase produced by mild proteolysis, although with significantly lower potency, at least two antigenic sites on the ATPase molecule can be assumed. One binding site of the antibodies may be located on the residual enzyme and the other on the peptide fragment, cleaved off by proteolysis, which is thought to carry the calmodulin-receptor

site. This experiment further strengthens our suggestion that at least one subpopulation of the antibodies binds at or close to the calmodulin-binding site.

Our antibodies against human erythrocyte Ca^{2+} -transport ATPase were shown to cross-react with the calmodulin-sensitive ATPase from pig smooth-muscle plasma membrane, but not with the ATPase from sarcoplasmic reticulum from pig heart muscle. The lower potency of the antibodies in inhibiting the activity of the enzyme from pig smooth muscle as compared with the ATPase from human erythrocytes may be attributed to tissue and species differences. These results, which indicate similarity between different plasma-membrane Ca^{2+} -transport ATPases but dissimilarity between ATPases from plasma membrane and sarcoplasmic reticulum, are in agreement with earlier reports in which different immunological approaches have been applied (Verma *et al.*, 1982; Wuytack *et al.*, 1983). Since the effect of antibodies on Ca^{2+} -transport ATPase implied interaction with the calmodulin-binding site, which might be similar for different calmodulin-dependent enzymes, we investigated the influence of antibodies on phosphodiesterase. However, the antibodies failed to show cross-reactivity with rat brain phosphodiesterase in that they did not inhibit the calmodulin-dependent activity of this enzyme. This result does not exclude the possibility of a similar calmodulin receptor of different enzymes, since our experiments do not allow us to distinguish whether the antibodies bind at or close to the calmodulin-binding site of Ca^{2+} -transport ATPase.

The antibodies described provide an opportunity to isolate the calmodulin receptor of erythrocyte Ca^{2+} -transport ATPase by using the antibodies as affinity ligands. Furthermore, it may be possible to use these antibodies to investigate further diseases such as cystic fibrosis, which display abnormal Ca^{2+} regulation (Horton *et al.*, 1970; Katz & Emery, 1981; Gietzen *et al.*, 1984). Quantification of the number of ATPase molecules per cell by using our antibodies would help to distinguish whether the decreased specific activity of the enzyme is dependent on a defect in the ATPase itself or whether the number of enzyme copies per cell is simply decreased in cystic-fibrosis patients.

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